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Mechanisms of vasopressin hypersensitivity in septic shock

Dr Lucinda K Barrett MA MBBS MRCP

Thesis presented for the award of Doctor of Philosophy

University College London, March 2008



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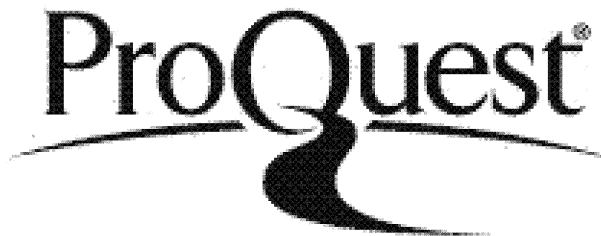
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Abstract

Patients in prolonged, catecholamine-refractory septic shock have plasma vasopressin levels inappropriately low for their hypotension, yet show enhanced responses to exogenously administered hormone. I hypothesised that altered vasopressin signalling within vascular smooth muscle is responsible for this heightened sensitivity. Both vasopressin and the catecholamine, norepinephrine vasoconstrict via sarcolemmal G protein-coupled receptors. Diversity in the calcium signalling pathways downstream of these receptors may explain the differential effect of sepsis on vascular reactivity to the two hormones.

To investigate this, I characterised a long-term fluid-resuscitated, rat model of faecal peritonitis, and examined *in-vivo* reactivity to these vasopressors. In subsequent *ex-vivo* studies performed on mesenteric resistance arteries taken from these animals, I compared concentration-response characteristics, calcium mobilisation pathways, and calcium-tension relationships for the two agonists, using wire myography and fluorescence microscopy. I also measured hormone levels in a cohort of septic and non-septic intensive care patients and undertook preliminary myography studies on human small mesenteric arteries.

In prolonged illness, vasopressin levels were not elevated in either the septic rats or in septic patients, despite hypotension and organ dysfunction. Pressor responses to norepinephrine, but not vasopressin, were diminished in septic

rats. This pattern of reactivity was mirrored *ex-vivo*, with decreased efficacy of norepinephrine, but increased potency of vasopressin. Differences were apparent in the calcium mobilisation pathways contributing to norepinephrine- and vasopressin-induced responses in septic vessels, with a greater reliance on store-operated calcium channels with vasopressin, compared to voltage-gated calcium channels with norepinephrine. The norepinephrine calcium-tension relationship was similar in sham and septic vessels but, for vasopressin, there was evidence of agonist-specific increased calcium sensitivity of the contractile apparatus in the septic tissues.

In conclusion, my long-term septic model was able to satisfactorily mimic the clinical scenario. I demonstrated increased vasoconstriction to vasopressin suggesting enhanced receptor coupling to calcium signalling. Vasopressin, but not norepinephrine, may be able to both effectively mobilise calcium in septic vascular smooth muscle and sensitise the contractile apparatus to its effect. In addition to providing insight into the phenomenon of vasopressin hypersensitivity in septic shock, this work supports modulation of calcium mobilisation channels and/or sensitisation pathways as a potential new therapeutic paradigm.

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Abbreviations

α_1R	α_1 adrenoceptor
ABG	arterial blood gas
ACh	acetylcholine
ACTH	adrenocorticotrophic hormone
ADH	antidiuretic hormone
ANS	autonomic nervous system
APACHE	Acute Physiology and Chronic Health Evaluation
cAMP	cyclic AMP
Ca^{2+}	calcium
cGMP	cyclic GMP
CIF	calcium influx factor
CLP	caecal ligation and puncture
CO	cardiac output
DAG	diacylglycerol
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
ICU	intensive care unit
iNOS	inducible nitric oxide synthase
IP_3	inositol triphosphate
K^+	potassium
K_{ATP}	ATP-sensitive potassium channel
K_{Ca}	calcium-activated potassium channel

K _{ir}	inward rectifier potassium channel
K _v	voltage-gated potassium channel
L-NAME	NG-nitro-L-arginine methyl ester
LPS	lipopolysaccharide
MAP	mean arterial pressure
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MLC ₂₀	20kDa regulatory light chain of myosin
NE	norepinephrine
NO	nitric oxide
NOS	nitric oxide synthase
OTR	oxytocin receptor
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PSS	physiological salt solution
PVN	paraventricular nucleus of hypothalamus
ROC	receptor-operated channel
ROK	Rho-associated kinase
SEM	standard error of mean
SERCA	sarcoendoplasmic reticulum Ca ²⁺ -ATPase
SOC	store-operated channel
SON	supraoptic nucleus of hypothalamus
SR	sarcoplasmic reticulum
TP	terlipressin

TRP	transient receptor potential
TXA ₂	thromboxane A ₂
UCLH	University College London Hospital
VGCC	voltage-gated calcium channel
VP	vasopressin
V ₁ R	vasopressin V ₁ receptor
V ₂ R	vasopressin V ₂ receptor
V ₃ R	vasopressin V ₃ receptor

Schema for Introductory Chapter

1.1 Overview of vascular smooth muscle physiology:

- Contractile mechanisms
- Intracellular calcium regulation
- Sarcolemmal potassium channels
- Interaction with the endothelium

1.2 Vasopressin:

- Physiology
- Cellular signalling
- Comparison of norepinephrine to vasopressin

1.3 Septic shock:

- The clinical problem
- Pathophysiology
- Vascular smooth muscle changes

1.4. Vasopressin in septic shock:

- Vasopressin levels
- Clinical use
- Experimental evidence for hypersensitivity
- Mechanisms underlying hypersensitivity

1.1 Overview of vascular smooth muscle physiology

1.1.1 Introduction

Resistance blood vessels are those chiefly involved in the regulation of arterial blood pressure and tissue blood flow. These are small arteries and arterioles across which the pressure drop is greatest, and where small adjustments in tone have a significant impact on systemic haemodynamics [Davis *et al.*, 1986]. The walls of the resistance vessels are composed of three layers: an outer adventitial layer containing sympathetic nerve endings, a medial layer containing smooth muscle cells, and an inner single layer of endothelial cells facing the vessel lumen. By virtue of the helical arrangement of the muscle cells, their contraction and relaxation produces changes in vessel diameter. As in skeletal muscle, thin (actin) and thick (myosin) filaments supported by the cytoskeleton comprise the cells' contractile apparatus.

Vascular smooth muscle cells are inherently less permeable to potassium (K^+) than those in cardiac muscle, but possess significant basal permeability to other monovalent ions (particularly chloride) [Hathaway *et al.*, 1991]. The electrochemical gradient of these ions supports a resting membrane potential of between -40 and -60mV [Hathaway *et al.*, 1991; Mulvany & Aalkjaer, 1990]. Membrane potential is not the only factor regulating vascular tone, as smooth muscle is subject to modulation by neural, humoral, myogenic, metabolic and endothelial factors. Neural regulation occurs predominantly via sympathetic

nerves releasing the catecholamine, norepinephrine (NE), though other vasoactive substances such as ATP may be co-released. Neurotransmitters and circulating vasoactive hormones (including vasopressin) mediate their cellular effects by binding to cell surface receptors on vascular smooth muscle, with the subsequent activation of G proteins triggering various second messenger signalling cascades.

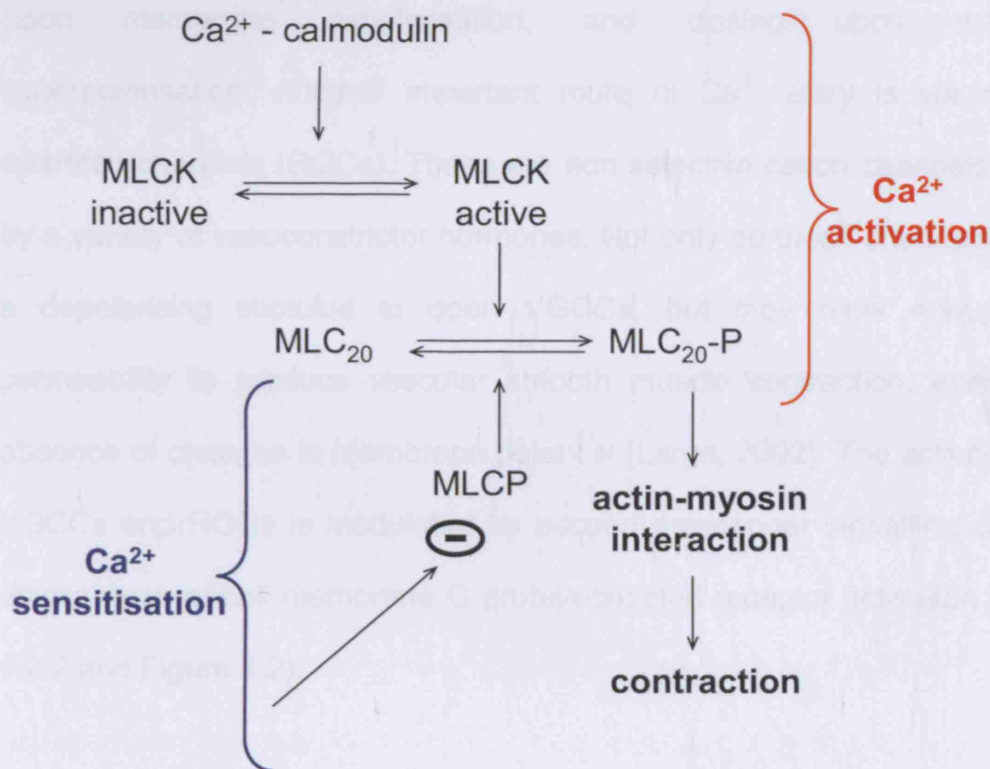
1.1.2 Contractile mechanisms

Intracellular free calcium (Ca^{2+}) concentration is the major determinant of smooth muscle contractility. Calcium binds to and activates the protein calmodulin, and the resultant Ca^{2+} -calmodulin complex activates myosin light chain kinase (MLCK). This kinase phosphorylates the 20kDa regulatory light chain of myosin (MLC_{20}) [Horowitz *et al.*, 1996]. This phosphorylation step is critical for the formation of actin-myosin cross-bridges (Figure 1.1). Contraction depends on cross-bridge cycling, whereby conformational changes in the myosin head exert a pulling force on the attached actin filament, followed by release and subsequent re-attachment. This process is catalysed by the phosphorylated myosin ATP-ase [Hathaway *et al.*, 1991]. Cross-bridge cycling continues until MLC_{20} is dephosphorylated by myosin light chain phosphatase (MLCP).

Modulation of MLCP activity can regulate vascular smooth muscle contractility through a seemingly Ca^{2+} -independent mechanism. A decrease in MLCP activity results in a greater phosphorylation of MLC_{20} for a given level of

intracellular Ca^{2+} , thus enhancing actin-myosin interaction. This process is commonly referred to as Ca^{2+} sensitisation of the myofilaments [Wier & Morgan, 2003] (Figure 1.1). Activation of plasma membrane (sarcolemmal) receptors by contractile agonists can mediate both Ca^{2+} activation and Ca^{2+} sensitisation. This will be described in more detail in subsequent sections.

Figure 1.1 Calcium activation and sensitisation in vascular smooth muscle



The actin-myosin interaction which underlies vascular smooth muscle contraction is enhanced by an increase in the activity of myosin light chain kinase (MLCK) (calcium activation), and/or by a decrease in the activity of myosin light chain phosphatase (MLCP) (calcium sensitisation).

MLC_{20} 20kDa regulatory light chain of myosin

$\text{MLC}_{20}\text{-P}$ phosphorylated 20kDa regulatory light chain of myosin

1.1.3 Intracellular calcium regulation

The resting smooth muscle cytoplasmic free Ca^{2+} concentration (approximately 100nM) is increased either by influx of extracellular Ca^{2+} across the plasma membrane or by release of Ca^{2+} from intracellular stores contained within the sarcoplasmic reticulum (SR). The best characterised pathway mediating Ca^{2+} influx is through sarcolemmal L-type voltage-gated Ca^{2+} channels (VGCCs), the activity of which is primarily governed by the cell membrane potential [Horowitz *et al.*, 1996]. Opening of these channels occurs upon membrane depolarisation, and closing upon membrane hyperpolarisation. Another important route of Ca^{2+} entry is via receptor-operated channels (ROCs). These are non-selective cation channels opened by a variety of vasoconstrictor hormones. Not only do these channels provide a depolarising stimulus to open VGCCs, but they have enough Ca^{2+} permeability to produce vascular smooth muscle contraction, even in the absence of changes in membrane potential [Large, 2002]. The activity of both VGCCs and ROCs is modulated by second messenger signalling cascades downstream of cell membrane G protein-coupled receptor activation (Section 1.2.2 and Figure 1.2).

Intracellular store depletion, triggered by inositol triphosphate (IP_3)-induced activation of Ca^{2+} release channels located on the SR, can provide yet another pathway for extracellular Ca^{2+} influx. Emptying of the store promotes opening of store-operated channels (SOCs) via a mechanism still incompletely understood, but that may involve a calcium influx factor (CIF) of

unknown chemical identity and/or protein kinase C (PKC)-mediated phosphorylation [Albert & Large, 2003]. IP_3 is predominantly generated following phospholipase C (PLC) activation by G protein-coupled agonist receptors on the plasma membrane (Section 1.2.2 and Figure 1.2). However, in contrast to skeletal muscle, the intracellular stores themselves play a relatively minor role in directly adding to the myoplasmic Ca^{2+} pool, particularly in small resistance arteries [Mulvany & Aalkjaer, 1990].

Vascular smooth muscle relaxation is usually accompanied by a decrease in intracellular Ca^{2+} to basal levels. Calcium may be extruded from the cells via the sarcolemmal $Na^+ : Ca^{2+}$ exchanger and Ca^{2+} -ATPase, or re-sequestered into intracellular stores via the sarcoendoplasmic reticulum Ca^{2+} -ATPase (SERCA) [Horowitz *et al.*, 1996]. The activity of the latter is increased by vasodilatory agonists through the phosphorylation of the SR protein, phospholamban [Hathaway *et al.*, 1991; Horowitz *et al.*, 1996].

1.1.4 Sarcolemmal potassium channels

Potassium flux across the sarcolemmal membrane is fundamental to the regulation of both vascular smooth muscle resting membrane potential and contractility. Increased channel opening permits K^+ efflux from the cell and results in membrane hyperpolarisation, thereby reducing VGCC activity and Ca^{2+} influx. Four main types of K^+ channel have now been identified in the vasculature: the calcium-activated (K_{Ca}), the ATP-sensitive (K_{ATP}), the voltage-gated K^+ (K_v) and the inward rectifier (K_{ir}) channel [Oliver & Landry, 2006]. Of

these, the most abundant is the K_{Ca} channel which provides negative feedback following elevation of intracellular Ca^{2+} to limit depolarisation and muscle contraction [Clapp & Tinker, 1998]. K_{ATP} channels seem to play a minor role in the regulation of resistance vessel tone in health, but may assume greater importance in various pathophysiological states [Clapp & Tinker, 1998]. These channels are activated by hypoxaemia, acidosis, and reduced levels of ATP, and can thus respond to metabolic demand by increasing local blood flow [Clapp & Tinker, 1998]. Furthermore, K_{ATP} channels are inhibited by vasoconstrictor agonists, opened by vasodilators, and can be pharmacologically activated by potassium channel opener drugs such as levcromakalim [Quayle *et al.*, 1997; Clapp & Tinker, 1998].

1.1.5 Interaction with the endothelium

The close apposition of endothelial and smooth muscle cells in the blood vessel wall allows functional interaction between these layers. The endothelium is responsible for synthesising several key vasoactive mediators, including nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarising factor (EDHF) and endothelin. The enzyme NO synthase (NOS) catalyses production of NO from L-arginine and molecular oxygen. The constitutive endothelial form of this enzyme (eNOS) is activated by shear stress exerted on the vessel lumen, or by circulating vasoactive mediators acting on endothelial cell surface receptors. Under the influence of inflammatory stimuli, inducible NOS (iNOS) can also be expressed in all layers of the blood vessel wall [Titheradge, 1999]. NO produced within the endothelium diffuses into the

smooth muscle cell layer where it activates soluble guanylyl cyclase to produce cyclic GMP (cGMP). NO mediates smooth muscle relaxation via a number of cGMP-dependent and independent pathways, acting to reduce intracellular Ca^{2+} , open K^+ channels, and activate MLCP [Titheradge, 1999; Landry & Oliver, 2001].

1.2 Vasopressin

Vasopressin (VP), or antidiuretic hormone (ADH), is essential for cardiovascular homeostasis. It acts via the kidney to regulate water reabsorption, on the vasculature to regulate smooth muscle tone, and as a central neurotransmitter, modulating brainstem autonomic function.

1.2.1 Vasopressin physiology

Vasopressin (arginine vasopressin) is a nonapeptide hormone synthesised primarily in the magnocellular neurones of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. Following synthesis, hormone precursors migrate along the axons of these neurones via the supraoptic-hypophyseal tract to the posterior pituitary gland, to be stored in neurosecretory vesicles [Holmes *et al.*, 2001]. Excitation of the magnocellular neurones triggers VP secretion into the circulation. Only 10-20% of the hormone within the posterior pituitary can be rapidly released. Further secretion occurs at a greatly reduced rate, even in the presence of sustained stimulation [Holmes *et al.*, 2001]. Vasopressin is rapidly metabolised by liver

and kidney vasopressinases and has a plasma half-life of between ten and 35 minutes [Holmes *et al.*, 2001].

Regulation of vasopressin release is complex. Under normal conditions, VP secretion is primarily regulated by changes in serum osmolarity (osmoregulation). This system is highly sensitive, such that a small (2%) increase in osmolarity is counteracted by a few pg/ml increase in VP, acting on the kidney to promote water reabsorption [Mutlu & Factor, 2004]. In contrast, baroregulation of vasopressin secretion plays a minimal role in health, with a greater than 10% drop in blood pressure needed to significantly elevate circulating hormone levels. In the setting of significant acute hypotension, however, an exponential increase in plasma VP is needed to help restore normotension [Mutlu & Factor, 2004].

Vasopressin release is also affected by other hormones. Of particular relevance are the interactions with catecholamines and the hypothalamic-pituitary-adrenal axis. Central adrenoceptors regulate VP release, but their activation can have inhibitory or stimulatory effects depending upon the catecholamine concentration and the receptor subtype involved [Leng *et al.*, 1999]. In general, catecholamines exert stimulatory effects at low doses via α_1 adrenoceptors but, at high doses, may inhibit VP release via α_2 and β receptors [Day *et al.*, 1985; Leng *et al.*, 1999]. Secretion of vasopressin stimulates release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, with consequent negative feedback of glucocorticoids on the posterior pituitary [Mutlu & Factor, 2004].

Other factors are important in critical illness. Hypoxaemia and acidosis stimulate carotid body chemoreceptors to increase VP release [Holmes *et al.*, 2001]. Furthermore, both endotoxin and cytokines enhance vasopressin production [Mutlu & Factor, 2004], whilst NO plays a mainly inhibitory neuromodulating role on its secretion [Reid, 1994].

1.2.2 Vasopressin cellular signalling

The actions of vasopressin are mediated via G protein-coupled receptors, classified by virtue of their location and second messenger pathways into V_1 (previously V_{1a}), V_2 and V_3 (previously V_{1b}) receptors [Birnbaumer, 2000]. In addition, VP has equal affinity with oxytocin for oxytocin receptors (OTRs), and may exert some of its actions via this route [Holmes *et al.*, 2003].

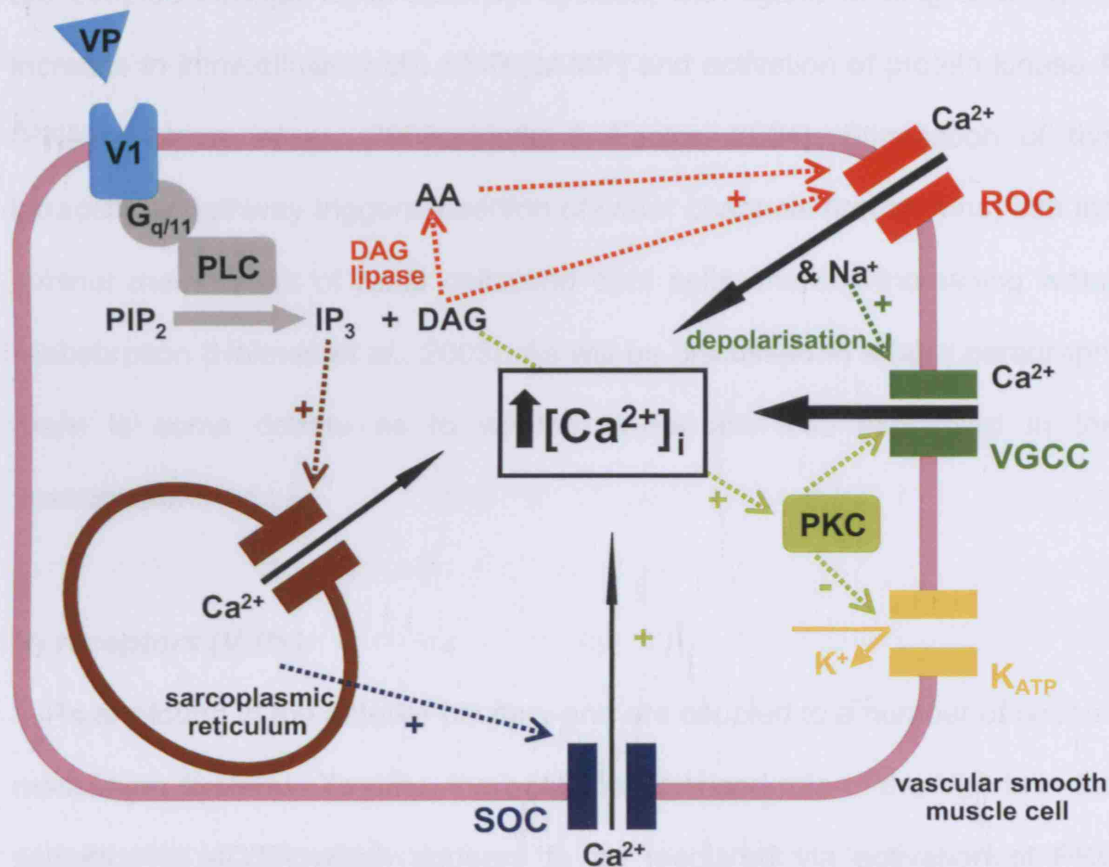
V₁ receptors (V₁Rs)

V_1 Rs are found mainly on vascular smooth muscle in the systemic, splanchnic, renal and coronary circulations, and their stimulation produces vasoconstriction. These receptors are coupled through $G_{q/11}$ to PLC which, when activated, produces the second-messengers IP_3 and diacylglycerol (DAG), the latter in turn activating PKC [Holmes *et al.*, 2003] (Figure 1.2). Some of the initial rise in intracellular Ca^{2+} is produced by the action of IP_3 on the SR, while a sustained rise is triggered by influx of extracellular Ca^{2+} [Ruegg *et al.*, 1989; Nakajima *et al.*, 1996]. As discussed above, the effect of Ca^{2+} is mediated through its binding to calmodulin and activation of MLCK, hence promoting muscle contraction (Figure 1.1).

The pathways leading to vasopressin-induced extracellular Ca^{2+} entry are shown in Figure 1.2. Store-operated, or capacitative, Ca^{2+} channels may play a minor role in comparison to the VGCCs and ROCs [Broad *et al.*, 1999; Katori *et al.*, 2001]. VGCCs are activated by depolarisation produced by cation influx through ROCs [Large, 2002] and/or chloride efflux through chloride channels [Van Renterghem & Lazdunski, 1993]. PKC can also trigger cell depolarisation via closure of K_{ATP} channels [Quayle *et al.*, 1997], and can open VGCCs directly through channel phosphorylation [Beech, 1997]. Opening of ROCs is a G protein-dependent event involving PLC, with a downstream mechanism that employs DAG and arachidonic acid, acting either alone or in combination [Broad *et al.*, 1999; Large, 2002]. In addition to its effects on Ca^{2+} influx, V_1R stimulation may sensitise the contractile apparatus to the effect of calcium via the inhibition of MLCP by PKC and/or Rho-associated kinase (ROK) [Bauer & Parekh, 2003] (Figure 1.1). ROK is the effector molecule activated by Rho A, a small G protein stimulated following a complex signal transduction cascade downstream of the V_1R [Bauer & Parekh, 2003; Somlyo & Somlyo, 2003]

V_1Rs are also found on platelets, renal collecting duct cells and in the brainstem [Holmes *et al.*, 2003]. The latter mediate vasopressinergic modulation of the autonomic nervous system (ANS) [Koshimizu *et al.*, 2006], and are responsible for a baroreflex-mediated decrease in heart rate that precludes a pressor effect when vasopressin acts on the vascular smooth muscle in healthy people [Peuler *et al.*, 1990].

Figure 1.2 Schematic showing the pathways of intracellular calcium elevation following V_1R stimulation in a vascular smooth muscle cell



VP	vasopressin	V_1	V_1 receptor
PLC	phospholipase C	AA	arachidonic acid
IP_3	inositol triphosphate	DAG	diacylglycerol
PIP_2	phosphatidylinositol bisphosphate	PKC	protein kinase C
ROC	receptor-operated channel		
VGCC	voltage-gated Ca^{2+} channel		
SOC	store-operated channel		
K_{ATP}	ATP-sensitive K^+ channel		

The weighting of the black solid arrows depicts the supposed relative contributions of the different pathways (see text).

V₂ receptors (V₂Rs)

V₂Rs mediate the antidiuretic actions of vasopressin within the kidney. They are coupled through G_s to adenylyl cyclase, with ligand binding causing an increase in intracellular cyclic AMP (cAMP) and activation of protein kinase A (PKA) [Holmes *et al.*, 2003; Mutlu & Factor, 2004]. Stimulation of this intracellular pathway triggers insertion of water channels (aquaporins) into the luminal membranes of renal collecting duct cells, thereby increasing water reabsorption [Holmes *et al.*, 2003]. As will be discussed in a later paragraph, there is some debate as to whether V₂Rs are also expressed in the vasculature.

V₃ receptors (V₃Rs)

V₃Rs are found in the anterior pituitary and are coupled to a number of second messenger systems. To date, the best characterised role of the V₃R is in the secretion of ACTH which appears to be mediated via activation of PKC [Holmes *et al.*, 2003].

Oxytocin receptors (OTRs)

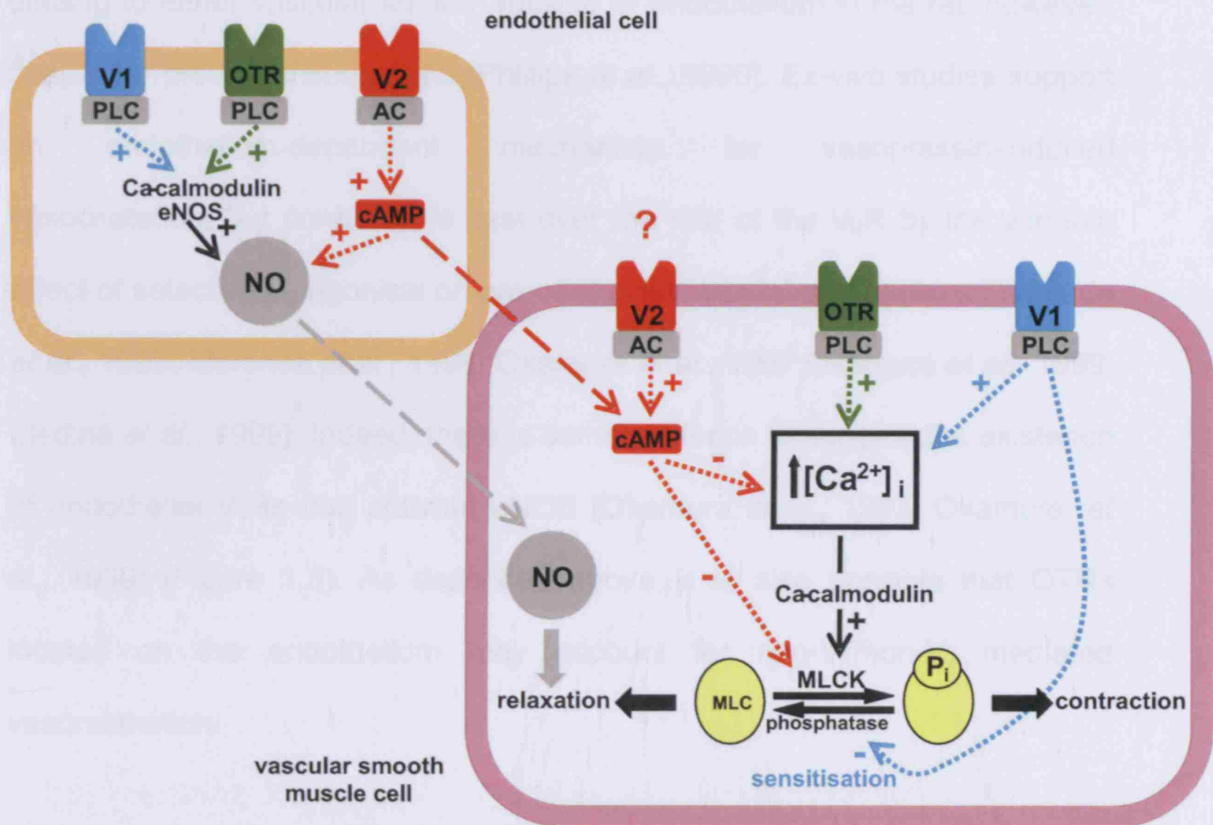
Like V₁Rs, OTRs are coupled to PLC, the metabolism of phosphoinositides and the consequent elevation of intracellular Ca²⁺ [Holmes *et al.*, 2003]. In myometrial and mammary myoepithelial cells, stimulation of OTRs produces smooth muscle contraction via MLCK [Holmes *et al.*, 2003]. This may also occur in vascular smooth muscle [Yazawa *et al.*, 1996; Stam *et al.*, 1998] (Figure 1.3). In addition, OTRs are highly expressed on vascular endothelium [Thibonnier *et al.*, 1999], where an increase in intracellular Ca²⁺ activates

constitutive NOS to release NO, thereby increasing smooth muscle cGMP to produce vasorelaxation [Holmes *et al.*, 2003] (Figure 1.3). The lack of pressor response observed with oxytocin infusions in obstetric practice may be consequent to the opposing effects of OTR stimulation on endothelial and smooth muscle cells.

It is well documented that vasopressin produces vasodilatation in some vascular beds, but the receptor subtype responsible is uncertain. This may also vary between blood vessels and be dependent upon the circulating hormone concentration [Holmes *et al.*, 2004]. There is additional uncertainty as to whether receptors mediating this effect are situated on endothelial and/or smooth muscle cells.

Stimulation of V₂Rs located on vascular smooth muscle may produce vasorelaxation via a cAMP-mediated drop in intracellular Ca²⁺ (Figure 1.3). Infusion of the selective V₂ agonist, DDAVP in anephric dogs elevated levels of plasma cAMP coincident with a fall in peripheral vascular tone, supporting the existence of extra-renal V₂Rs [Liard, 1992]. Alternatively, generation of cAMP in the endothelium would trigger eNOS activation and hence NO liberation (Figure 1.3). This is consistent with data from healthy human volunteers where high doses of vasopressin produced a decrease in forearm vascular resistance which appeared to be V₂R- and NO-dependent [Tagawa *et al.*, 1993; Tagawa *et al.*, 1995]. Interestingly, this vasodilatation was not seen in patients with nephrogenic diabetes insipidus secondary to a V₂R defect [van Lieburg *et al.*, 1995]. Real-time polymerase chain reaction

Figure 1.3 Interplay between an endothelial and a vascular smooth muscle cell showing the mechanisms by which vasopressin may produce vasoconstriction and/or vasodilatation



V1	<i>V₁ vasopressin receptor</i>	OTR	<i>oxytocin receptor</i>
V2	<i>V₂ vasopressin receptor</i>	PLC	<i>phospholipase C</i>
AC	<i>adenylate cyclase</i>	cAMP	<i>cyclic AMP</i>
eNOS	<i>endothelial nitric oxide synthase</i>	NO	<i>nitric oxide</i>
MLCK	<i>myosin light chain kinase</i>	MLC	<i>myosin light chain</i>
P _i	<i>phosphate</i>		

The experimental evidence for the existence of *V₂R*s on vascular smooth muscle cells is weak. Endothelial-derived NO causes vascular smooth muscle relaxation by a number of different pathways that are not detailed here.

(RT-PCR) has been used to demonstrate V₂R expression on cultured human lung endothelial cells and in heart, spleen and lung whole tissue specimens [Kaufmann *et al.*, 2003]. Use of a selective V₂R radioligand to demonstrate binding to either vascular smooth muscle or endothelium in the rat, however, has so far proved unsuccessful [Phillips *et al.*, 1990]. *Ex-vivo* studies support an endothelium-dependent mechanism for vasopressin-induced vasodilatation, but confusion is cast over the role of the V₂R by the variable effect of selective V₂ agonists on pre-contracted arterial preparations [Yamada *et al.*, 1993; Martinez *et al.*, 1994; Okamura *et al.*, 1997; Okamura *et al.*, 1999; Medina *et al.*, 1999]. Indeed, there is some evidence to suggest the existence of endothelial V₁R_s that activate eNOS [Okamura *et al.*, 1997; Okamura, *et al.*, 1999] (Figure 1.3). As described above, it is also possible that OTRs located on the endothelium may account for non-V₁/non-V₂ mediated vasorelaxation.

1.2.3 Comparison of norepinephrine to vasopressin

Norepinephrine is the major neurotransmitter released from sympathetic nerve endings within the blood vessel wall, and thus mediates ANS control of resistance vessel tone. NE also acts as a hormone, and is secreted into the circulation from the adrenal medulla under conditions of stress, including hypotension. Like VP, NE acts on vascular tissue via a number of G protein-coupled receptors. In the resistance vasculature, α_1 adrenoceptors (α_1 R_s) on the sarcolemmal membrane predominate [Mulvany & Aalkjaer, 1990; Guimaraes & Moura, 2001]. Similar to the V₁R, the α_1 R is coupled via G_{q/11}

proteins to PLC and the downstream signalling mechanisms described in Section 1.2.2 and Figure 1.2 [Wier & Morgan, 2003]. There may be differences, however, in the relative importance of the various Ca^{2+} mobilisation pathways utilised by the two vasoconstrictors (Section 1.4.4.1). Adrenoceptors are also present on the endothelium (α_2 and β subtypes) where stimulation produces vasodilatation via eNOS activation [Guimaraes & Moura, 2001].

1.3 Sepsis and septic shock

1.3.1 The clinical problem

Sepsis is defined as a systemic inflammatory response syndrome (≥ 2 of fever [or hypothermia], tachycardia, tachypnoea, and leucocytosis [or leucopenia]) in the presence of suspected or proven infection [Bone *et al.*, 1992]. The preceding infection can be due to any micro-organism and can originate at any site in the body. It is an important clinical problem, with a yearly incidence of 50-95 cases per 100,000, and accounting for 2% of all hospital admissions [Annane *et al.*, 2005]. In the UK, around of a quarter of patients nursed on an intensive care unit (ICU) fulfil the criteria for severe sepsis within the first 24 hours of their ICU admission [Harrison *et al.*, 2006]. Mortality in this setting is upward of 40% [Annane *et al.*, 2005]. The incidence of sepsis is still increasing, particularly as the population grows more elderly, and as more invasive surgery and immunosuppressive therapy are being performed.

The most severe complication of sepsis is septic shock, a condition characterised by organ dysfunction and hypotension not responding to adequate fluid resuscitation [Bone *et al.*, 1992]. In this scenario, vasopressor agents are used to elevate blood pressure from a critically low level in order to provide a perfusion pressure that is adequate to support organ blood flow. Inherent to this manoeuvre is the risk of excessive regional vasoconstriction and iatrogenic tissue damage [Holmes, 2005]. Controversy remains regarding the choice, dose and combination of drugs used in septic shock. Indeed, the optimal blood pressure for an individual patient is still unknown. A paucity of controlled clinical trials and outcome data means evidence-based practice is limited [Beale *et al.*, 2004; Dellinger *et al.*, 2008]. Currently, norepinephrine is the most frequently used pressor in patients who remain hypotensive despite fluid resuscitation and who are deemed to require blood pressure elevation.

Shock states are generally characterised by sympathetic and renin-angiotensin system activation. Thus, in severe haemorrhage or acute cardiac failure, there is profound peripheral vasoconstriction. In septic shock, however, there is a reduced ability of the vascular smooth muscle to contract (so-called vasodilatory shock) [Landry & Oliver, 2001]. In this scenario, hypotension is often refractory (hyporeactive) to vasopressor therapy with exogenous catecholamines. This results in a need for very high doses and the incumbent increased risk of adverse effects.

1.3.2 Pathophysiology

Sepsis results from complex interactions between the infecting pathogen and the immune and inflammatory cells of the host. Bacterial surface molecules (lipopolysaccharide [LPS] in Gram-negative species and peptidoglycan in Gram-positives) bind to pattern-recognition receptors (CD14 and toll-like receptors [TLRs]) on immune cells [Russell, 2006]. This activates the transcription factor nuclear factor kappa B (NF- κ B) which up-regulates the synthesis of immunomodulatory cytokines including tumour necrosis factor α (TNF α), interleukin-1 β (IL-1 β) and IL-10. These cytokines provoke further immune cell and endothelial activation, with increased production of prostaglandins, leukotrienes and NO [Russell, 2006]. Furthermore, a pro-coagulant state is induced, and hormonal and metabolic regulation is impaired. The consequent circulatory and bioenergetic changes are responsible for the most severe complications of sepsis: septic shock and multi-organ failure [Annane *et al.*, 2005; Russell, 2006].

1.3.2.1 Vascular smooth muscle changes

The pathogenesis of vasodilatory shock is multifactorial. Increased levels of NO play an important, but not exclusive, role. In animal models of sepsis, vascular hyporeactivity is attenuated both in iNOS knockout mice [Laubach *et al.*, 1995; Hollenberg *et al.*, 2000], and by pharmacological iNOS inhibition [Wray *et al.*, 1998; Hollenberg *et al.*, 1999; Strunk *et al.*, 2001]. Increased production of NO is found in septic patients, albeit at lower levels

[Thiemermann, 1997; Vallance & Chan, 2001], and is reported to be inversely correlated with mean arterial pressure and systemic vascular resistance [Annane *et al.*, 2000]. Compared to animals, induction of iNOS appears restricted in humans, occurring mainly in blood vessels at the very point of infection [Annane *et al.*, 2000]. The importance of either iNOS and/or up-regulated eNOS in human sepsis is supported, however, by the marked blood pressure rise following infusion of a non-specific NOS inhibitor in septic shock patients [Bakker *et al.*, 2004; Lopez *et al.*, 2004].

Increased NO impairs vascular smooth muscle contractility by lowering intracellular Ca^{2+} , reducing MLC phosphorylation, and opening K^+ channels (Section 1.1.5) [Landry & Oliver, 2001]. Both K_{Ca} and K_{ATP} channels appear to be targets of NO, with channel activity being stimulated through cGMP-dependent and independent mechanisms [Buckley *et al.*, 2006]. In vasodilatory shock, persistent K_{ATP} channel activation produces vascular smooth muscle hyperpolarisation [Clapp & Tinker, 1998; Buckley *et al.*, 2006], contributing to both hypotension and hyporesponsiveness to catecholamines [Sorrentino *et al.*, 1999; Chen *et al.*, 2000; O'Brien *et al.*, 2005]. In addition to the effect of elevated NO concentrations, increased K_{ATP} channel opening in septic shock may result from tissue hypoxia, acidosis, reduced ATP concentration, and changes in hormone levels (CGRP, adenosine and ANF) [Clapp & Tinker, 1998; Buckley *et al.*, 2006]. A further contributor to vasodilatory shock is the adrenoceptor desensitisation and down-regulation which results from high circulating endogenous catecholamine levels in combination with exogenous catecholamine treatment [Hwang *et al.*, 1994;

Saito *et al.*, 1995; Hotchkiss & Karl, 2003; Lin *et al.*, 2005]. This is compounded by the inhibition of α_1 R expression by NO and inflammatory cytokines [Bucher *et al.*, 2003].

1.4 Vasopressin in septic shock

1.4.1 Vasopressin levels

The finding that some patients with severe, refractory septic shock were exquisitely sensitive to the pressor effects of exogenous vasopressin led to the investigation of its endogenous profile [Landry *et al.*, 1997a]. In acute septic shock, an early rise in plasma levels is seen both in patients (c.20pg/ml) [Sharshar *et al.*, 2003a; Lin, *et al.*, 2005] and in animal models (up to several hundred pg/ml) [Wilson *et al.*, 1981b; Brackett *et al.*, 1985]. In human prolonged septic shock (≥ 24 hours), VP levels fall towards baseline unstimulated values (≤ 5 pg/ml) [Landry *et al.*, 1997a; Sharshar *et al.*, 2003a]. Hence, inappropriately low levels (i.e. a relative deficiency) of vasopressin may be another key component in the altered functional status of vascular smooth muscle. In support of this, endotoxic models have been used to demonstrate that V_1 R blockade exacerbates hypotension [Matsuoka & Wisner, 1997], and that survival is decreased in vasopressin-deficient Brattleboro rats [Brackett *et al.*, 1983].

The explanation for inappropriately low VP levels is unclear. Levels of vasopressinase, the enzyme responsible for vasopressin breakdown, are not

elevated in septic shock patients [Sharshar *et al.*, 2002]. Frequent hepatic and renal dysfunction in this clinical scenario makes the possibility of increased hormone breakdown even less likely. Depletion of neurohypophyseal stores, inhibition of hormone synthesis, or depressed release may therefore be responsible [Sharshar *et al.*, 2002]. All of the above may be a consequence of abnormal osmoregulation or baroregulation. Autonomic baroreflex dysfunction could explain the lack of relationship between blood pressure and VP levels in septic shock [Jochberger *et al.*, 2006b]. Furthermore, impaired VP release has been documented in patients with autonomic insufficiency [Zerbe *et al.*, 1983; Kaufmann *et al.*, 1991], a phenomenon well recognised in sepsis [Garrard *et al.*, 1993]. Elevated levels of NO may also contribute to autonomic dysfunction [Sharshar *et al.*, 2003b], and have direct inhibitory effects on VP secretion [Reid, 1994]. Indeed, sustained elevation of hormone levels following endotoxin challenge in mice was seen in iNOS knockouts and after pharmacological inhibition of NOS [Giusti-Paiva *et al.*, 2002; Giusti-Paiva *et al.*, 2005; Carnio *et al.*, 2005]. In addition, vasopressin levels may be depressed by the high levels of circulating catecholamines that result from both endogenous production and therapeutic administration [Day *et al.*, 1985; Leng *et al.*, 1999].

1.4.2 Clinical use of vasopressin in septic shock

In 1997, Landry and colleagues published a case series of five septic patients in whom low-dose vasopressin infusion produced dramatic pressor effects in the context of catecholamine-refractory hypotension [Landry *et al.*, 1997b].

The hormone doses administered would have produced no blood pressure rise in healthy individuals [Abboud *et al.*, 1990]. Since then, numerous clinical studies have reported similar findings with both VP and terlipressin (TP) in septic and other forms of vasodilatory shock [den Ouden & Meinders, 2005; Pesaturo *et al.*, 2006; Russell, 2007]. Terlipressin (1-triglycl-8-lysine-vasopressin) is the synthetic vasopressin analogue commercially available in the UK, and it has a more favourable pharmacological profile than endogenous arginine vasopressin, with a longer half-life and a two-fold greater V₁R:V₂R selectivity [Bernadich *et al.*, 1998; Pesaturo *et al.*, 2006]. Such clinical study results support the addition of these therapies to the intensive care armoury. Most trials, however, have been non-randomised, or have included only small numbers of patients. Study durations have been short and very few have examined outcome in terms of disease survival [den Ouden & Meinders, 2005]. Evidence is accumulating to suggest that, at low-doses, VP has neutral or beneficial effects on renal blood flow and urine output, and does not impair cardiac function [Luckner *et al.*, 05; Russell, 2007]. Despite this, concerns remain regarding potential deleterious effects on mesenteric and peripheral perfusion.

The Surviving Sepsis guidelines of 2004 indicated that vasopressin was unproven in septic shock and were neutral in their recommendation pending the result of the VAsopressin and Septic Shock Trial (VASST) [Dellinger *et al.*, 2004]. The results of this multicentre Canadian randomised trial were presented at the 2006 Congress of the European Society of Intensive Care Medicine. The authors reported that low-dose vasopressin (≤ 0.03 units/min)

combined with norepinephrine provided significant outcome benefit (28- and 90-day mortality) over NE alone in patients with low baseline NE requirements ($\leq 15\mu\text{g}/\text{min}$). However, patients requiring higher NE doses at baseline showed similar mortality rates with NE \pm VP. This finding will no doubt stimulate further use of VP in septic shock though debate will continue over what maximal dose is both effective and safe [Luckner *et al.*, 2007]. Whether TP has additional benefits to VP remains to be investigated, though unlike vasopressin, this agent does not appear to cause rebound hypotension upon cessation of treatment [O'Brien *et al.*, 2002a; Morelli *et al.*, 2004].

1.4.3 Experimental evidence for vasopressin hypersensitivity

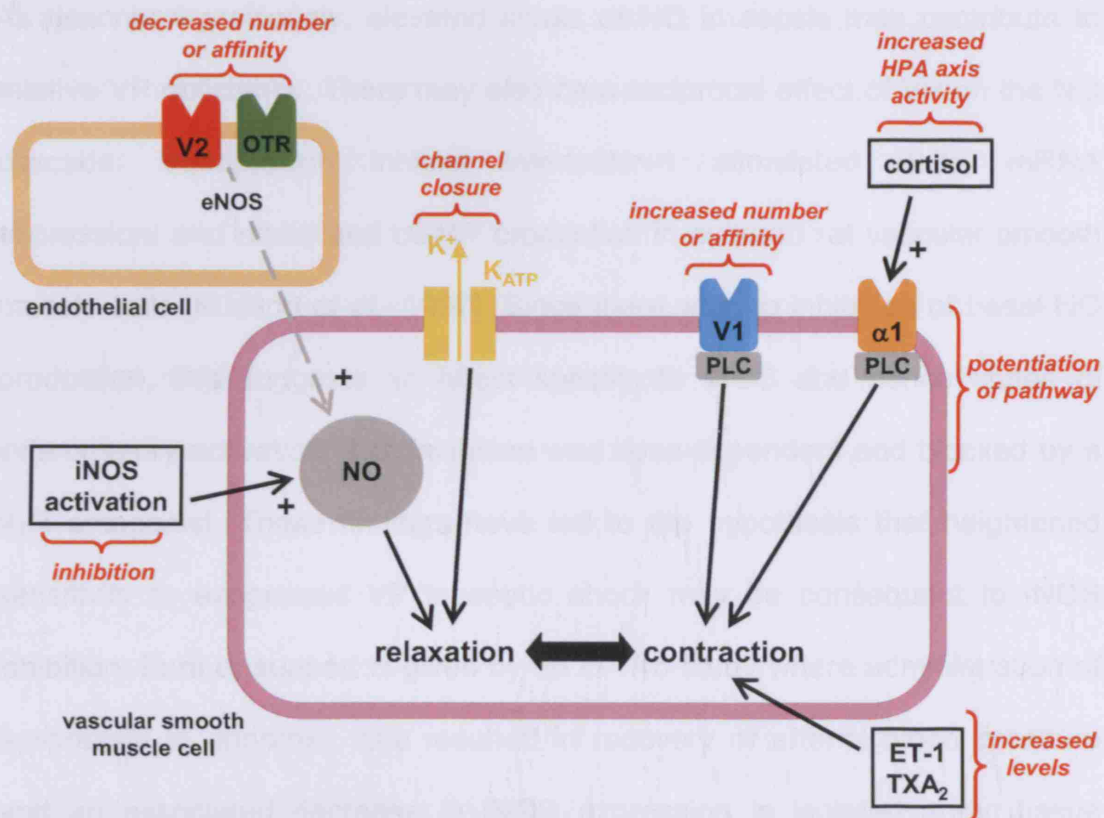
Heightened pressor responses to vasopressin have been found in several animal models of septic shock. An anaesthetised rat endotoxin model revealed a hypersensitivity of exposed cremaster muscle microvessels to topical VP, yet hyporeactivity to NE [Baker & Wilmoth, 1984; Baker *et al.*, 1990]. Similarly, in a fluid-resuscitated endotoxic rat model developed in our laboratory, there was a marked pressor response to TP, but a reduced response to NE [O'Brien *et al.*, 2002b]. The opposite pattern was seen in paired sham-operated controls. Hypersensitivity to TP has also been demonstrated in conscious ewes after 16 hours of endotoxaemia [Westphal *et al.*, 2003]. Other *in-vivo* models have, however, produced conflicting results [Hollenberg *et al.*, 1997; Albert *et al.*, 2004; Bennett *et al.*, 2004], most likely related to the wide variation in experimental design in terms of duration, insult, sepsis severity, and degree of fluid resuscitation.

Although *ex-vivo* reproduction of the vascular hyporeactivity to catecholamines is well described in septic models [Julou-Schaeffer *et al.*, 1990; Mitolo-Chieppa *et al.*, 1996; O'Brien *et al.*, 2001], there has been relatively little work examining vascular reactivity to vasopressin. In one study, VP showed increased potency to constrict isolated perfused mesenteric vascular beds from endotoxaemic compared with control rats [Tarpey *et al.*, 1998]. In contrast, a study using human gastroepiploic arteries showed attenuated contractile responses to VP after treatment with endotoxin, but significant enhancement of NE-induced contractions when co-administered to the same tissue [Hamu *et al.*, 1999]. Decreased sensitivity to both VP and NE was found in isolated rat mesenteric arteries pre-treated with an NO donor to simulate septic shock-like conditions [Leone & Boyle, 2006].

1.4.4 Mechanisms underlying vasopressin hypersensitivity

Despite increasing clinical use of vasopressin in septic shock, the mechanism underlying the observed heightened pressor response is unknown. Simple replacement of a relative hormone deficiency does not appear to be the answer, as VP treatment is associated with supra-physiological hormone levels, and blood pressure response does not correlate with the baseline vasopressin level [Dunser *et al.*, 2004a]. The potential mechanisms discussed below are summarised in Figure 1.4.

Figure 1.4 Schematic showing (in red) the potential mechanisms of hypersensitivity to vasopressin in septic shock at the level of the vascular smooth muscle cell



V1	V ₁ vasopressin receptor	V2	V ₂ vasopressin receptor
OTR	oxytocin receptor	α1	α ₁ adrenoceptor
PLC	phospholipase C	K _{ATP}	ATP-sensitive K ⁺ channel
NO	nitric oxide	ET-1	endothelin-1
TXA ₂	thromboxane A ₂		
eNOS	endothelial nitric oxide synthase		
iNOS	inducible nitric oxide synthase		
HPA	hypothalamic-pituitary-adrenal		

A further possibility not shown here is altered baroreflex sensitivity consequent to autonomic dysfunction.

1.4.4.1 Interaction with other factors contributing to vasodilatory shock (Section 1.3.2.1)

Nitric oxide

As described previously, elevated levels of NO in sepsis may contribute to relative VP deficiency. There may also be a reciprocal effect of VP on the NO cascade. Vasopressin inhibits interleukin-1 stimulated iNOS mRNA expression, and nitrite and cGMP production in cultured rat vascular smooth muscle cells [Kusano *et al.*, 1997]. Since there was no inhibition of basal NO production, this suggests an effect specific to iNOS and hence states of inflammatory activation. The inhibition was dose-dependent and blocked by a V₁R antagonist. These findings have led to the hypothesis that heightened sensitivity to exogenous VP in septic shock may be consequent to iNOS inhibition. Further support is given by an *in-vivo* study where administration of terlipressin to endotoxic rats resulted in recovery of arterial blood pressure and an associated decrease in iNOS expression in isolated aortic tissue [Moreau *et al.*, 2002]. However, no decrease in serum nitrite/nitrate concentrations was demonstrated in patients with vasodilatory shock after vasopressin infusion [Dunser *et al.*, 2004b].

ATP-sensitive potassium (K_{ATP}) channels

Inhibition of K_{ATP} channels in septic shock could help to restore normal vascular reactivity [Sorrentino *et al.*, 1999; O'Brien *et al.*, 2005; Singer *et al.*, 2005]. *In-vitro* work with insulin-secreting cells known to express the K_{ATP} channel demonstrated the ability of vasopressin to close these channels if

applied to the outside of the membrane [Martin *et al.*, 1989]. Of greater relevance to the cardiovascular system was the reproduction of this channel inhibition in cultured porcine vascular smooth muscle cells [Wakatsuki *et al.*, 1992] and in isolated cardiac myocytes [Tsuchiya *et al.*, 2002]. PKC is the likely mediator since the effects of VP on channel activity could be prevented by pre-treatment with the relatively selective PKC inhibitor, calphostin-C [Tsuchiya *et al.*, 2002]. It is unclear whether PKC acts by direct phosphorylation of the channel [Quinn *et al.*, 2003] or by increasing sarcolemmal ATP [Tsuchiya *et al.*, 2002]. Other mechanisms of K_{ATP} channel inhibition by VP are also possible. An increase in intracellular Ca²⁺ evoked by this agonist could activate the calcium-dependent phosphatase, calcineurin, and thus promote channel inhibition [Wilson *et al.*, 2000; Singer *et al.*, 2005]. In addition, calcineurin regulates gene transcription via the nuclear transcription factor NFAT; this, in turn, may down-regulate genes encoding K_{ATP} channel subunits, as has been shown for delayed rectifier potassium channels [Amberg *et al.*, 2004].

Catecholamine sensitivity

Clinical experience with vasopressin and terlipressin in patients with catecholamine-resistant septic shock suggests that these agents restore vascular reactivity to endogenous and exogenous catecholamines [Landry *et al.*, 1997b; O'Brien *et al.*, 2002a; Dunser *et al.*, 2003]. Potentiation of the vasoconstrictor actions of endogenous NE by physiological doses of exogenous VP was first reported forty years ago in a series of dog, cat and rat *in-vivo* experiments [Bartelstone & Nasmyth, 1965]. Parallel *ex-vivo* studies

with rat aortic strips suggested a direct vascular rather than a central mechanism of vasopressin action [Bartelstone & Nasmyth, 1965]. More recent studies with rat and human resistance arteries have confirmed this potentiation, both in normal vessels [Medina *et al.*, 1997; Noguera *et al.*, 1997; Hamu *et al.*, 1999], and in those exposed to endotoxin [Hamu *et al.*, 1999].

Several possible explanations may underlie this interaction. Norepinephrine vasoconstricts via α_1 adrenoceptors that, like V_1 Rs, are coupled via $G_{q/11}$ proteins to PLC. Despite this, NE-induced contractions appear more dependent on release of intracellular Ca^{2+} stores rather than influx of extracellular Ca^{2+} , whereas the opposite seems to apply for vasopressin [Cauvin *et al.*, 1988]. Thus the utilisation of different Ca^{2+} pathways may, at least in part, explain the synergism seen between the two agonists. In support of this, VP potentiation of adrenergic contraction in isolated rat mesenteric arteries was blocked both by a V_1 R antagonist [Noguera *et al.*, 1997; Hamu *et al.*, 1999] and by nifedipine, a blocker of VGCCs [Noguera *et al.*, 1997]. Alternatively, VP may act via PKC and/or ROK to inhibit myosin light chain phosphatase, thereby sensitising the contractile apparatus to the intracellular Ca^{2+} rise produced by α_1 R stimulation [Bauer & Parekh, 2003].

Cross-regulation may also occur at the level of the receptors. Work carried out in cell lines demonstrated the ability of V_2 R activation to non-reciprocally inhibit adrenoceptor internalisation [Klein *et al.*, 2001]. The proposed mechanism is via an inhibition of receptor internalisation by β arrestin. The



latter normally functions by binding to activated G protein-coupled receptors and promoting their removal from the cell membrane.

1.4.4.2 Changes in vasopressin receptor behaviour

As explained in Section 1.2.2 and Figure 1.3, the opposing effects of vasopressin on vascular tissue are consequent to the stimulation of the different VP receptor subtypes located on smooth muscle and/or endothelial cells. Differential changes in the regulation of these subtypes could therefore explain the pressor hypersensitivity seen in septic shock.

V₁ receptors

This subtype predominates on smooth muscle cells of the resistance vasculature and mediates vasoconstriction. In contrast to high norepinephrine levels and the resultant α_1 R changes, relatively low circulating concentrations of VP in prolonged septic shock would leave more V_1 Rs available for occupancy by exogenous hormone and so decrease the endogenous stimulus for receptor desensitisation [Birnbaumer, 2000; Landry & Oliver, 2001].

Another possibility is that V_1 Rs are up-regulated in sepsis though this has not yet been confirmed. NO and cytokine-mediated changes in cell membrane receptor function are recognised [Bucher *et al.*, 2002; Takakura *et al.*, 2002], and endotoxin may perturb the lipid bilayer of the plasma membrane sufficiently as to alter receptor function directly [Ghosh & Liu, 1983]. However, no change in either number or affinity of V_1 Rs was reported in cultured aortic

smooth muscle cells exposed to endotoxin for 24 hours [Burnier *et al.*, 1995]. Moreover, a decrease in V₁R gene expression was seen in liver, lung, kidney and heart tissue isolated from rats exposed to experimental endotoxaemia for up to 24 hours [Bucher *et al.*, 2002]. Notably, this sepsis model was not fluid resuscitated, and did not demonstrate hypersensitivity to *in-vivo* administration of a vasopressin agonist. V₁R number was also reduced in hepatic cells isolated from non-shocked rats who received a continuous, low-dose endotoxin infusion for 30 hours [Roth & Spitzer, 1987]. Further studies are required to examine changes in receptor binding in tissues from models more representative of human septic shock.

V₂ and oxytocin receptors

Mechanisms for vasorelaxation through both V₂Rs and OTRs have been demonstrated. Decreased activity of these receptor subtypes in the vasculature would enhance the contractile effects of vasopressin. Indeed, decreased expression of V₂Rs was found in rat kidney 24 hours after intraperitoneal endotoxin injection [Grinevich *et al.*, 2004]. Furthermore, V₂R recycling and resensitisation is slow compared to that of V₁Rs [Birnbaumer, 2000; Holmes *et al.*, 2003]. This may well be of relevance in the context of exogenous vasopressin administration, and could explain the observation that the rebound hypotension seen on cessation of VP treatment in septic shock does not occur with TP [O'Brien *et al.*, 2002a; Morelli *et al.*, 2004], due to its greater selectivity for V₁Rs over V₂Rs [Bernadich *et al.*, 1998].

1.4.4.3 Other possible mechanisms

Autonomic nervous system dysfunction

Vasopressin release is under the control of the ANS, with baro- and chemoreceptor afferents projecting to the brainstem, and efferents from the brainstem to the PVN and SON [Leng *et al.*, 1999]. By virtue of its neurotransmitter role, ANS output is also modulated by VP [Koshimizu *et al.*, 2006]. Therefore, the autonomic and vasopressinergic system abnormalities seen in sepsis may well be related. Further complexity is added by the apparent negative correlation between NO levels and sympathetic cardiovascular output [Li & Patel, 2003; Sharshar *et al.*, 2003b], as well as the known interactions between VP and NO described above. In patients who died from septic shock, iNOS expression was linked to apoptosis in the PVN and SON [Sharshar *et al.*, 2003b]. Primary autonomic failure is associated with hypersensitivity to vasopressin's pressor effects [Mohring *et al.*, 1980] as well as with abnormalities of its release [Kaufmann *et al.*, 1991]. Hypersensitivity has also been reported in dogs with baroreceptor denervation [Cowley *et al.*, 1974]. Moreover, cirrhotic patients show an abnormally prolonged blood pressure response to VP, and this has been ascribed to abnormal autonomic cardiovascular regulation [Moreau *et al.*, 1990]. Baroreflex impairment in septic shock is suggested by the failure of VP administration to elicit a marked bradycardia as is observed in normal individuals [Landry *et al.*, 1997a].

Interaction with other vasoconstrictors

Elevated levels of the endogenous vasoconstrictors endothelin-1 (ET-1) and thromboxane A₂ (TXA₂) are found in septic shock. These contribute to the observed circulatory disturbances and explain, in part, the heterogeneity in tone seen across different vascular beds in sepsis [Young, 2004]. Vasopressin may increase production of both these vasoconstrictors. In human platelets, V₁Rs are stimulated by micromolar concentrations of VP to activate not only PLC but also phospholipase A₂, resulting in arachidonic acid metabolism and TXA₂ production [Siess *et al.*, 1986]. In cultured endothelial cells, VP induces preproendothelin-1 mRNA expression and enhances cellular release of the mature peptide [Emori *et al.*, 1991; Imai *et al.*, 1992]. *In vivo* findings support the potential role of ET-1 in vasopressin hypersensitivity: an exaggerated blood pressure response to exogenous VP in spontaneously hypertensive rats was abolished by pre-treatment with the endothelin antagonist, bosentan [Balakrishnan *et al.*, 1997].

Interactions with the hypothalamic-pituitary-adrenal axis

Vasopressin stimulates ACTH and hence cortisol secretion [Mutlu & Factor, 2004]. Relative adrenal insufficiency is recognised in severe sepsis [Annane *et al.*, 2002], and “low dose” steroid replacement may provide outcome benefit in such patients, especially those with severe shock [Annane *et al.*, 2004]. One of the mechanisms by which steroid administration is thought to restore vascular sensitivity to catecholamines is via an increase in adrenoceptor gene expression [Saito *et al.*, 1995]. While a similar effect on vasopressin receptor expression is feasible [Sutherland *et al.*, 2006; Ertmer *et al.*, 2007], VP

replacement may actually increase cortisol levels, thus acting synergistically to restore reactivity. However, this contradicts the finding that septic patients with relative adrenal insufficiency were less likely to have a relative deficiency of vasopressin than those with normal adrenal function [Sharshar *et al.*, 2003a]. Furthermore, hypersensitivity to vasopressinergic agonists may still occur despite no haemodynamic response to corticosteroids [O'Brien *et al.*, 2002a].

1.5 Summary

Understanding the pathogenesis and pathophysiology of septic shock represents a major challenge. Reviewing the literature relevant to vasopressin hypersensitivity shows that this particular area is no exception. The relationship between relative deficiency of endogenous VP and heightened sensitivity to its exogenous administration is not straightforward. The explanation behind this complex vasopressinergic system dysfunction is likely to be multifactorial, and hence many possibilities exist for further investigation. Patient studies are limited by the critical nature of septic shock and the difficulty in obtaining vascular and other tissues. Therefore, an *in-vivo* model truly representative of prolonged, severe sepsis is required to evaluate temporal changes in vascular reactivity, endocrine and autonomic function, and to provide suitable tissue samples for subsequent *ex-vivo* studies.

In this thesis, I have taken such an approach to examine how altered vascular reactivity to VP in septic shock relates to underlying changes in receptors and

calcium signalling pathways in resistance blood vessels. The interactions between VP and NO, K⁺ channels, other vasoconstrictors and the hypothalamic-pituitary-adrenal axis described in this chapter provide many avenues for related future studies. Increased mechanistic insight thus obtained will allow more educated clinical use of vasopressin.

1.6 Hypothesis

Pressor hypersensitivity to vasopressin in septic shock is consequent to changes either at the level of the vasopressin receptors within the vasculature, and/or an alteration of intracellular calcium handling that occurs downstream of receptor activation. Any such changes must differ from those affecting the adrenoceptor-mediated response to norepinephrine to account for the differential effect of sepsis on vascular reactivity to the two hormones.

1.7 Aims

1. In a 72-hour, conscious, fluid-resuscitated rat model of faecal peritonitis, I plan to:
 - characterise the model in terms of clinical illness, blood pressure and organ dysfunction
 - measure temporal changes in endogenous plasma levels of NE and VP and correlate these with illness severity
 - measure *in-vivo* blood pressure responses to fixed intravenous doses of NE and VP
 - compare septic animals with sham-operated controls throughout.

2. In small mesenteric arteries obtained from septic and sham-control rats, I propose to measure isometric tension responses using wire myography to:
 - examine the concentration-response characteristics of NE and VP

- assess the contribution of different receptor subtypes to the VP contractile response through the use of selective VP receptor agonists and/or antagonists
 - examine the effect of endothelial removal and thus assess the influence of endothelial versus smooth muscle vasopressin receptors
 - assess the contribution of different Ca²⁺ mobilisation pathways to NE- and VP-induced contractions through manipulation of extracellular Ca²⁺ concentration and the use of specific channel inhibitors.
3. Using simultaneous wire myography and fluorescence microscopy, I will:
- examine the role of calcium sensitisation by measuring the calcium-tension relationships for NE and VP in septic and sham arteries.
4. I will see whether the findings made in my laboratory model can be replicated in patients and human resistance vessels by:
- measuring temporal changes in endogenous plasma levels of VP in septic and non-septic intensive care patients
 - performing wire-myography studies on human small mesenteric arteries from patients undergoing laparotomy for septic or non-septic pathology to parallel those described above.

Chapter 2: *In-vivo* rat model of faecal peritonitis

2.1 Introduction

The clinical heterogeneity and critical nature of septic shock places significant limitations on patient studies. Consequently, animal models have been extensively used to study the pathogenesis of sepsis and to generate preclinical data for new therapies. An ideal *in-vivo* model should closely mimic the human illness; this is extremely challenging because, in addition to the aforementioned characteristics, the septic disease process in humans is influenced by conventional medical interventions such as antibiotics, sedation, fluid and vasoactive drug resuscitation, as well as mechanical organ support.

The majority of animal work has been carried in endotoxic septic models involving administration of the Gram-negative bacterial cell surface molecule, lipopolysaccharide (LPS) [Baker *et al.*, 1990; Giusti-Paiva *et al.*, 2002; Westphal *et al.*, 2003; Albert *et al.*, 2004; Bennett *et al.*, 2004]. LPS is one important trigger for the cascade of inflammatory and immune changes seen in sepsis [Annane *et al.*, 2005]. Use of a single, purified mediator can produce a controlled, reproducible model, but this approach fails to replicate the complexity and prolonged nature of the inflammatory response characteristic of human infection [Annane *et al.*, 2005; Remick & Ward, 2005]. So-called “focus of infection” models, such as caecal ligation and puncture (CLP) or intra-peritoneal insertion of faecal material, produce a more clinically realistic insult and systemic response [Hollenberg *et al.*, 1997; Hollenberg, 2005;

Remick & Ward, 2005]. Regardless of the insult chosen, adequate fluid resuscitation is vital if the hyperdynamic circulation typical of septic patients is to be achieved [Hollenberg, 2005].

A 3-day rat model of faecal peritonitis has been established in our laboratory [Brealey *et al.*, 2004]. My initial aim was to use this model to examine temporal changes in endogenous vasopressin levels in a prolonged septic illness, and to replicate the enhanced pressor response to exogenous vasopressin seen in patients.

2.2 Materials and Methods

2.2.1 Animal preparation

Experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986. The model used has been granted Home Office approval (Licence Number: PPL 70/6143; Principal Licence Holder: M. Singer). Male Wistar rats (Charles River, Margate, Kent) of 250-275g weight were housed in the local animal unit 5 days prior to experimentation to allow acclimatisation to their new environment. Instrumentation was performed under isoflurane (Abbott Laboratories, Queenborough, Kent) anaesthesia maintained via a face mask. Internal jugular venous and carotid arterial lines (internal diameter 0.58mm, external diameter 0.96mm) were inserted and tunnelled subcutaneously to emerge at the nape of the neck. This differed from the femoral approach formerly described [Brealey *et al.*, 2004] because

in-vivo blood sampling requires the use of larger diameter cannulae. The lines were then mounted onto a swivel/tether system secured to the rat using silk sutures. This enabled the rat, on recovery from anaesthesia, to have unimpaired movement around the cage with free access to food and water. Both lines were flushed continuously with 0.15ml/h of heparinised saline (1:1000). Mean arterial pressure (MAP) was measured (P23XL transducers, Viggo-Spectramed, Oxnard, CA, USA) and recorded onto a pre-calibrated PowerLab system (ADInstruments, Sydney, Australia).

Twenty-four hours later, sepsis was induced by intraperitoneal injection of faecal slurry (0.625mg/100g body weight). This was prepared from the bowel contents of a rat from the same batch, suspended in normal saline and then filtered to remove fibrous material. Blood and other tissues were also taken from these naïve, un-instrumented animals. Fluid resuscitation was commenced after a further two hours via the internal jugular venous cannula. For the first day, 20ml/kg/h of a 1:1 solution of 6% hetastarch (EloHaes, Fresenius Kabi, Warrington, Cheshire) and 5% glucose was infused. This was reduced to 17.5ml/kg/h between 24 and 48 hours, and then to 10ml/kg/h from 48 to 72 hours. Sham-operated controls underwent the same initial operative procedure and fluid resuscitation regimens as the septic animals, but received no intraperitoneal injection to avoid accidental bowel perforation.

A clinical illness severity scoring system was devised previously for this model (Table 2.1). From 24 hours post-intraperitoneal injection, septic rats were

Table 2.1 Severity scoring system for the rat model of faecal peritonitis (as published in Brealey D *et al.*, *Am. J. Physiol Regul Integr Comp Physiol* 2004: R491-R497)

	Mild	Moderate	Severe
Appearance	Hunched Piloerection No bloating	Hunched Marked piloerection Bloated abdomen Sunken eyes	Marked piloerection Markedly bloated abdomen Conjunctival injection
Alertness	Alert Occasional interest in environment Moves freely	Depressed alertness Little interest in environment Moves with difficulty	Markedly depressed alertness No interest in environment No movement
Mean blood pressure mmHg	>90	75-90	<75

The rat needed to show at least 2 characteristics within the appearance and alertness categories to obtain a score for that category. The blood pressure recording was only used if the arterial line was patent and the trace was stable over a 10 minute period.

scored (2 observers, 1 blinded) as being mild, moderately or severely affected. Animals were sacrificed at fixed times, or at any point when a rat appeared in obvious distress. Overall mortality calculation took into account spontaneous deaths, and assumed that all rats appearing severely unwell at the time of sacrifice would not have survived to complete the experiment, but that all those classified as mild or moderate would have done so.

A 72 hour septic mortality of 40% with 0% sham mortality was achieved when this model was first established [Brealey *et al.*, 2004], but pilot studies for the currently reported set of experiments revealed significantly higher death rates in both the septic and sham groups. It was initially uncertain whether such changes in the model's mortality rate were a consequence of a change in animal phenotype, including altered fluid tolerance, and/or infection or vagal stimulation related to neck as opposed to groin line insertion. Advice was sought from a veterinary surgeon. As mortality was associated with the frequent observation of oedema, weight gain and apparent respiratory distress, this suggested fluid overload as the predominant problem. The excess mortality was rectified by a 50% decrease in the amount of fluid resuscitation given over each 24 hour period (i.e. 10ml/kg/h 0-24, 8.75ml/kg/h 24-48, 5ml/kg/h 48-72). All results presented in this thesis are from animals treated with the reduced fluid regimen.

2.2.2 Blood sampling

In-vivo blood sampling was performed at pre-determined time-points when line patency allowed. As a consequence of decreasing patency rates with increasing experimental duration, this technique was only reliable until 24 hours after the induction of sepsis. Up to 3ml of whole blood was aspirated from the indwelling arterial or venous cannula at any one time. Blood was also obtained by cardiac puncture under anaesthesia at the time of sacrifice. Blood was drawn into chilled EDTA di-potassium salt (Sigma-Aldrich, St. Louis, MO, USA) (1mg/ml blood) for norepinephrine (NE) measurement, and into EDTA plus aprotinin (Trasylol, Bayer, Newbury, Berks) (500KIU/ml blood) for vasopressin (VP) measurement. These samples were centrifuged at 1600g and 0°C for 15 minutes, and then the plasma supernatant decanted and frozen at -80°C. Serum for biochemical analysis (performed by the Clinical Chemistry Department, UCL Hospitals and The Doctors Laboratory, London) was obtained by the centrifugation of whole blood which had first been allowed to clot in plain tubes. In addition, rapid assessment of arterial blood gases (ABGs) was performed in some animals using 200µl heparinised samples in an ABL 300 analyzer (Radiometer, Copenhagen, Denmark).

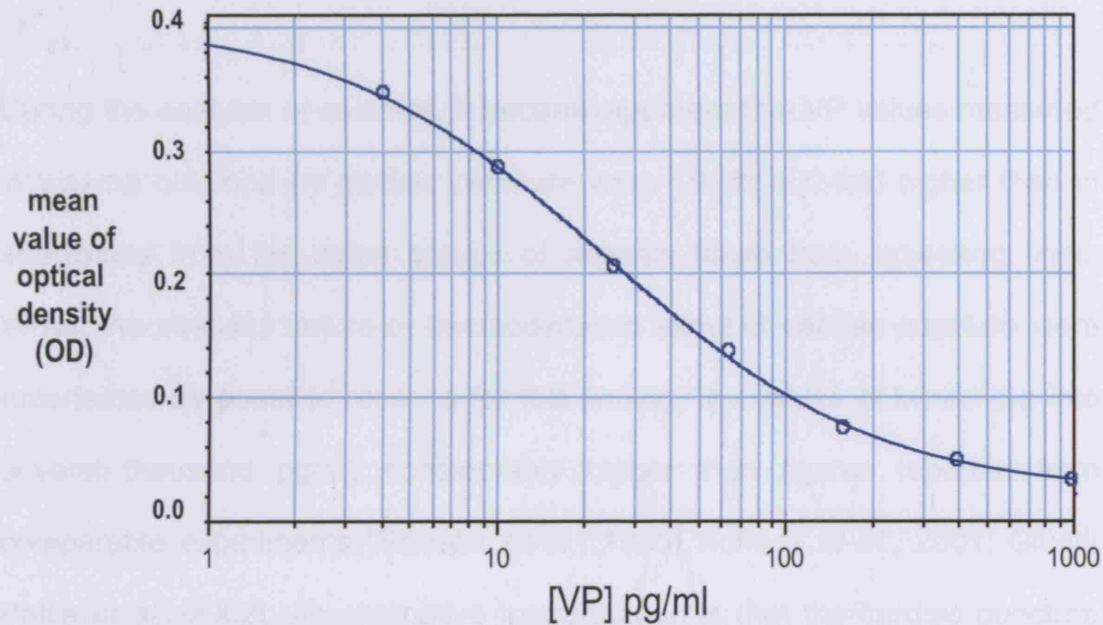
2.2.3 Vasopressin measurement

Frozen plasma samples containing EDTA plus aprotinin were first allowed to thaw. To avoid erroneous measurement of other proteins, an extraction procedure was performed. The samples were mixed with ice-cold acetone and

then centrifuged at 12000g for 20 minutes. The supernatant was transferred to a separate tube and vortexed with ice-cold petroleum ether. This mixture was then centrifuged for 10 minutes at 10000g. The top ether layer was carefully removed, and the remaining volatile liquid layer evaporated under vacuum at 45°C (Maxi dry plus, Heto, Jouan Nordic, Allerod, Denmark). The resulting solid sample was reconstituted in tris-buffered saline and VP quantified using an enzyme-linked immunosorbent assay (ELISA) technique (Assay Designs Inc, Ann Arbor, MI, USA). In brief, the extracted samples, a VP-alkaline phosphatase conjugate, and a rabbit polyclonal antibody to VP were combined in microtitre wells coated with goat anti-rabbit IgG. The wells were washed after overnight incubation, and p-nitrophenyl phosphate added as substrate. This reaction produced a yellow colouration, the intensity of which was inversely proportional to the amount of VP in the sample. This signal was quantified using a plate reader (Spectra Max Plus, Molecular Devices Corporation, Sunnyvale, CA, USA) which was set to measure absorbency at 405nm. Suitable analysis software (Soft Max Pro, Molecular Devices Corporation) produced a calibration curve (four-parameter logistic fit) from the optical density values of serially diluted standards, thus enabling determination of the sample VP concentrations.

Samples and standards were processed in duplicate throughout. Standard curves with an r^2 value approaching 1 were routinely obtained (Figure 2.1). To ensure efficiency of the extraction procedure, this was performed on serially diluted standards, and a VP recovery of $\geq 90\%$ was achieved. Further control experiments, in which samples were either spiked with a known amount of VP

Figure 2.1 Specimen standard curve (four-parameter logistic fit) obtained with vasopressin ELISA kit



	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>R²</u>
○ Std (Standards: Concentration vs MeanVal...	0.404	0.933	24.183	0.022	1

The "Soft Max Pro" programme (Molecular Devices Corporation, Sunnyvale, CA, USA) was used to determine the concentration in plasma samples from the optical density recordings made by the plate reader.

or serially diluted with buffer solution, confirmed the reliability of this technique. Samples taken simultaneously from arterial and venous lines in the same animal revealed there to be no significant difference in VP levels from these two sources.

During the analysis of samples, it became apparent that VP values measured in plasma obtained by cardiac puncture were 10- to 100-fold higher than in specimens from the same groups of animals taken from indwelling lines. Whilst the stressful nature or haemodynamic effect of cardiac puncture were entertained as possible reasons for this finding, the values obtained ran into several thousand pg/ml, considerably higher than figures reported from comparable experiments [Schaller *et al.*, 1985; Holmes *et al.*, 2001; Giusti-Paiva *et al.*, 2002]. An alternative interpretation is that the cardiac puncture results were artefactually elevated. This was supported by the finding that VP values taken simultaneously from an indwelling line and by cardiac puncture, both under anaesthetic, produced vastly different results.

A review of the literature and direct discussions with experts in this field has provided no definite explanation for this artefact. Intra-cardiac VP synthesis has been demonstrated in isolated, pressure-overloaded rat hearts, but resulting peptide levels in cardiac effluents were much lower than the values measured in my cardiac puncture samples [Hupf *et al.*, 1999]. Measurement of biochemical indices revealed frequent evidence of haemolysis in the blood taken by cardiac puncture. It is well known that VP is contained in platelets [Mutlu & Factor, 2004], and therefore blood cell damage could potentially

have caused erroneous elevation. There was no correlation, however, between plasma VP and serum potassium levels, suggesting that this is unlikely. Interestingly, analysis of serial aliquots of blood obtained by cardiac puncture from the same animal revealed a marked decrease in the apparent VP level with each millilitre of blood withdrawn. This points to a substance being released from the cardiac muscle or the surrounding tissue that may either be cross reacting with the VP assay or potentially degrading a component of the kit to produce a falsely high reading.

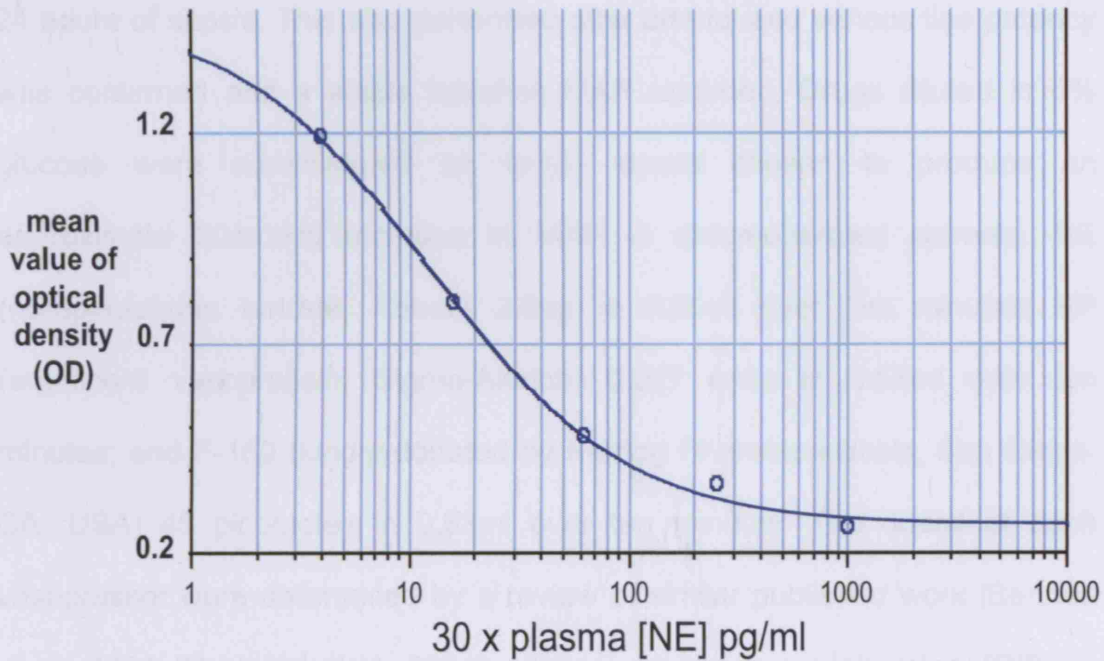
As a consequence of this difficulty, VP results from cardiac puncture blood samples have been excluded from the presented data. Unfortunately, as line patency was unreliable beyond the 24 hour time-point, it was only possible to obtain a few samples for VP assay beyond this stage of the model.

2.2.4 Norepinephrine measurement

Frozen EDTA plasma samples were allowed to thaw and then analysed using an ELISA technique (Labor Diagnostika Nord, Nordhorn, Germany). In brief, NE was first acylated to N-acylnorepinephrine in a microtitre plate. This compound was then eluted using 0.025M hydrochloric acid and transferred to microtitre wells containing solid phase bound NE. Rabbit NE anti-serum was then applied and the system allowed to equilibrate. After washing away antibody bound to liquid phase (sample-derived) NE, the antibody bound to the solid phase NE was detected using an anti-rabbit IgG-peroxidase conjugate with tetramethylbenzidine (TMB) as substrate. This reaction

produced a yellow colouration, the intensity of which was inversely proportional to the amount of NE in the plasma sample. This signal was quantified using a plate reader (Spectra Max Plus), which was set to measure absorbency at 450nm. Suitable analysis software (Soft Max Pro) produced a calibration curve (four-parameter logistic fit) from the optical density values of six kit standards, thus permitting determination of the sample NE concentrations. The kit used is designed for NE measurement in both plasma and urine samples, and since a larger sample volume is used for the former, the plasma results read from the standard curve must be divided by 30. Samples and standards were processed in duplicate throughout. Standard curves with an r^2 value approaching 1 were repeatedly obtained (Figure 2.2). Control experiments, in which samples were spiked with a known amount of NE or serially diluted with buffer solution, confirmed the reliability of the assay. Samples taken simultaneously from arterial and venous lines in the same animal revealed no difference in NE levels from these two sources. Values obtained were in the region of those reported in comparable experiments [Schaller *et al.*, 1985]. Unlike my experience with the VP ELISA, I found no obvious discrepancy between NE measurements from indwelling line and cardiac puncture samples.

Figure 2.2 Specimen standard curve (four parameter logistic fit) obtained with norepinephrine ELISA kit



	A	B	C	D	R^2
Std (Standards: Concentration vs MeanVal...	1.483	0.967	12.584	0.263	0.999

The "Soft Max Pro" programme was used to determine the concentration in plasma samples from the optical density recordings made by the plate reader. The kit used is designed for NE measurement in both plasma and urine samples; since a larger sample volume is used for the former, the plasma results read from the standard curve are divided by 30.

2.2.5 *In-vivo* drug administration

In-vivo blood pressure responses to intravenous vasopressors (NE, VP and the selective V₁R agonist, F-180 [Bernadich *et al.*, 1998]) were assessed after 24 hours of sepsis. This was performed after arterial and venous line patency was confirmed and a stable baseline MAP recorded. Drugs diluted in 5% glucose were administered as single doses chosen to produce an approximate 30mmHg increase in MAP in sham-operated animals: NE (norepinephrine tartrate, Abbott) 2.5µg in 0.25ml over five minutes; VP (arginine-8 vasopressin, Sigma-Aldrich) 0.017 units in 0.53ml over ten minutes; and F-180 (kindly donated by Ferring Pharmaceuticals, San Diego, CA, USA) 45 picomoles in 0.53ml over ten minutes. The doses of each vasopressor were determined by a review of similar published work [Bennett *et al.*, 2004; Westphal *et al.*, 2004]; past experience in our laboratory [O'Brien *et al.*, 2002b]; and a series of preliminary experiments. Each drug infusion was followed by continuous fluid administration to ensure all active compound had travelled through the line system. The plateau of the resultant blood pressure response was recorded. When more than one drug was infused, at least 30 minutes was allowed between drug infusions, and the order of administration varied.

2.2.6 Data and statistical analysis

The group sizes reported for blood pressure, biochemistry and hormone level measurements vary and decrease over time, reflecting loss of arterial line

patency and the significant mortality rate in septic animals. Six to eight animals per group were used in the *in-vivo* vasopressor response studies. This number was chosen on the basis of previous similar experiments conducted in our laboratory. A formal power calculation, from which the sample size needed to show statistically and clinically significant differences could have been determined, was not performed. *In-vivo* blood pressure, biochemistry and hormone level measurements are presented as mean \pm standard error (SEM) of n observations. They were compared between time-points and clinical groups using one-way ANOVA and post-hoc least-squares difference testing (SPSS 15.0, Chicago, IL, USA). In all analyses, a p value <0.05 was considered statistically significant.

2.3 Results

2.3.1 General features of the model

In the initial characterisation phase, 92 rats (313 ± 2 g body weight) were successfully instrumented and made uneventful post-operative recoveries. All appeared well at the time of intra-peritoneal injection (0h). Forty-two rats were used as sham-operated controls, while sepsis was induced in 50. A further 20 animals (8 control, 12 septic) were instrumented for the F-180 vasopressor experiments. With the adoption of the reduced fluid regimen as described in section 2.2.1, sham animals appeared outwardly normal for the duration of the experiment, continuing to eat and drink and maintaining an interest in their

environment. The septic animals began to show clinical features of illness (Table 2.1) from about 12 hours after the injection of faecal slurry.

Continuous blood pressure data collection was possible in 83% of animals up to the 24 hour time-point, and in 76% until the 48 hour stage. Despite this high proportion of reliable arterial line traces, aspiration of blood from the indwelling lines was possible in approximately 50% of rats at 24 hours, in only 20% at 48 hours, and in none at 72 hours. When this model was previously characterised, it was observed that septic animals surviving to 72 hours showed signs of recovery. This knowledge, in combination with the finding that the *in-vivo* blood sampling needed for accurate VP measurement was essentially impossible at this time point, led to the conclusion that I would not continue studies beyond 48 hours.

Blood pressure values of the septic rats were higher than those recorded when the model was first established, with MAPs rarely dropping below 90mmHg (Section 2.3.2). This may have been due to the change in arterial line position from femoral to internal carotid, though changes in animal phenotype could not be excluded. As the severity scoring system is partly dependent on blood pressure data (Table 2.1), the change in MAP range meant that increased reliance was placed on clinical features. As this assessment can be difficult and rather subjective, the decision was made to classify septic animals into two rather than three subgroups: mild/moderate and severe. By 24 hours, 64% were graded as mild/moderate and 18% as severe; 14% had died spontaneously by this stage, and no score was

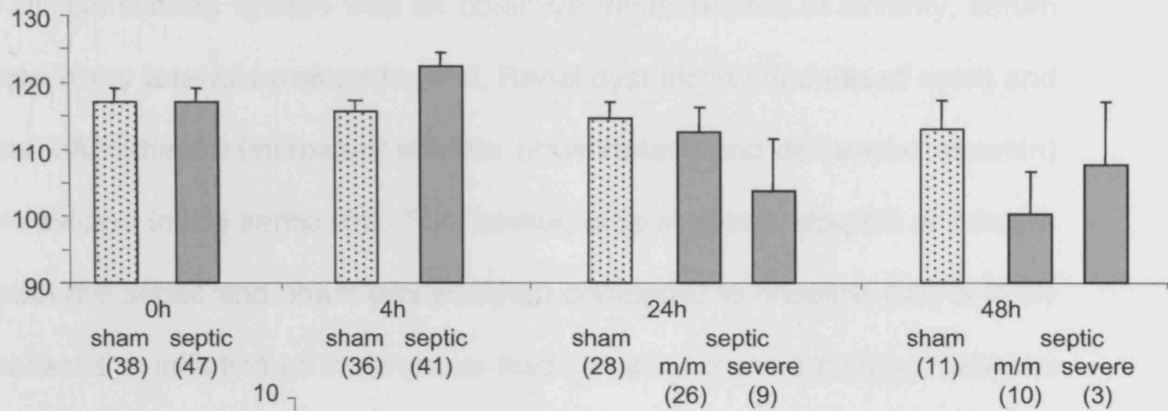
recorded for the remaining 2%. This gave a calculated 24 hour mortality of 32%. At 48 hours, 43% were deemed to be mild/moderate, 14% severely ill, and a further 43% had died. Assuming that all rats labelled as mild/moderate would have survived, and all those labelled severe would have not, the 48 hour mortality was 64%. During the harvesting of mesenteric tissue for later *ex-vivo* experiments, all septic rats showed evidence of peritoneal inflammation. This varied from small volume ascites and mild bowel oedema, in those only mildly affected, to gross bowel distension with purulent ascites and multiple adhesions in the most severely ill animals.

2.3.2 Blood pressure data

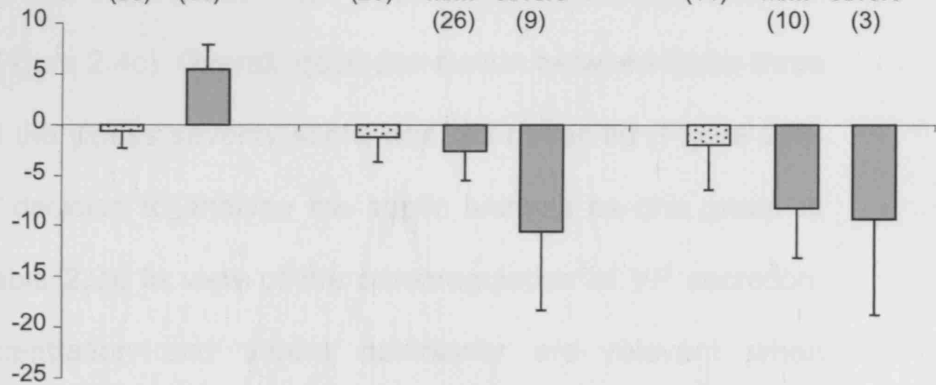
Figure 2.3a shows an overall trend of decreased MAPs in prolonged rodent sepsis when compared with sham-operated controls. The differences are emphasised graphically by the analysis of percentage change in MAP rather than absolute values (Figure 2.3b). Apparent from these figures are the small group sizes and large error bars for the severely septic rats. As a consequence of this and the aforementioned uncertainty in the clinical scoring system, I decided to pool together the septic groups at each time point (Figure 2.3c). Interestingly, MAPs were consistently higher in the septic rats four hours after intraperitoneal injection. There were significant drops in blood pressure in the septic rats at 24 and 48 hours when compared to baseline (0h) values. When sham-operated and septic groups were compared within these time-points, however, the numerically lower MAPs in the latter were not statistically significant.

Figure 2.3 Mean arterial pressures in the *in-vivo* faecal peritonitis model

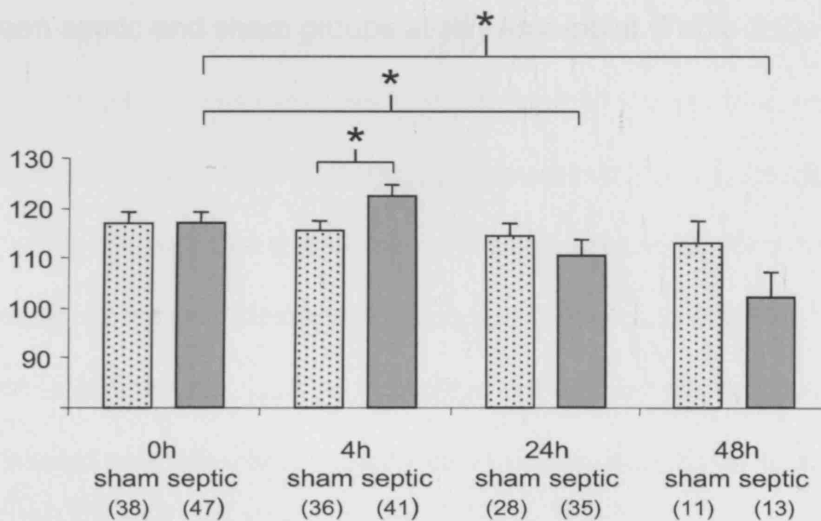
a) absolute MAP (mmHg)



b) % change MAP



c) absolute MAP (mmHg) – septic groups combined at 24 and 48 hours



Bars represent mean \pm SEM. Group sizes are shown in parentheses.

m/m = mild/moderate; 0h indicates samples taken immediately prior to injection of faecal slurry

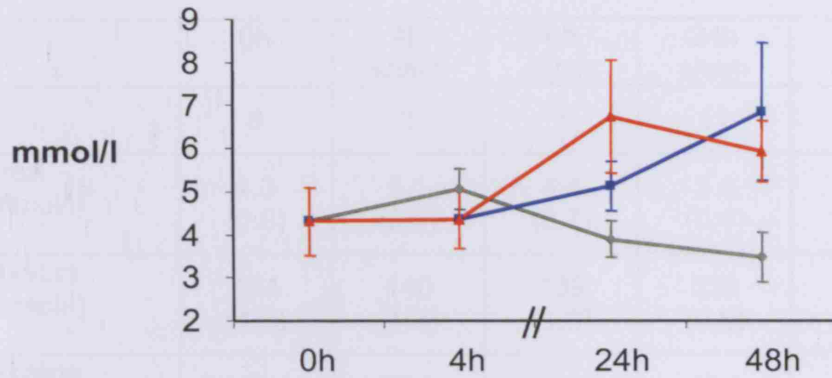
* $p < 0.05$ for comparisons between groups indicated by brackets

2.3.3 Serum biochemistry

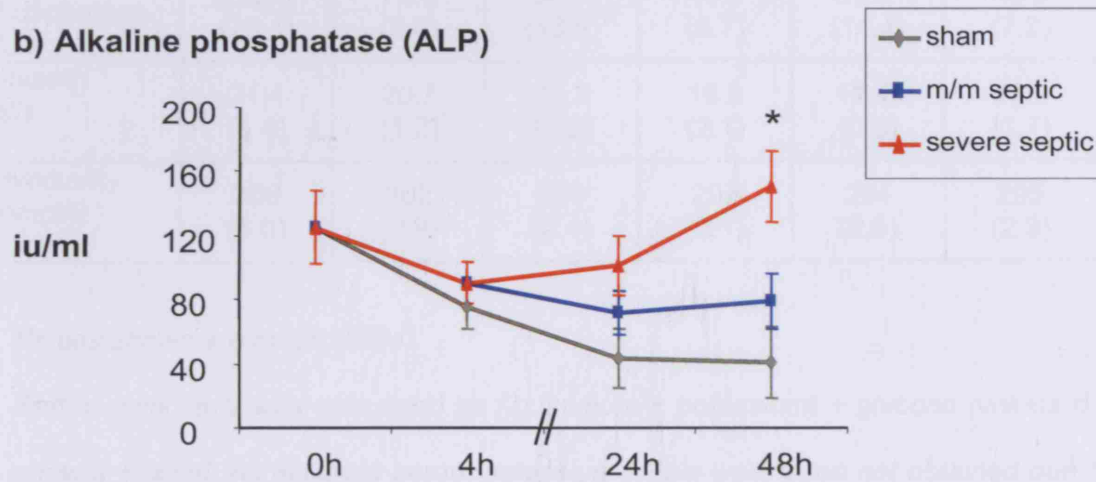
To assess the degree of organ dysfunction in the model, and to ascertain if the clinical scoring system was an objective measurement of severity, serum biochemistry analysis was performed. Renal dysfunction (increased urea) and acute inflammation (increased alkaline phosphatase and decreased albumin) were evident in the septic rats. The marked drop in serum albumin at 4 hours in both the septic and sham groups when compared to baseline (0h) is likely to reflect the initiation of intravenous fluids which occurred midway between these time-points (Figure 2.4c). Overall, good correlation between these three chosen indices and the illness severity score was not observed (Figure 2.4). This supported the decision to analyse the septic animals as one group at each time-point (Table 2.2). In view of the osmoregulation of VP secretion, both sodium concentration and serum osmolarity are relevant when interpreting circulating VP levels. These values did not vary significantly between septic and sham groups at any time-point (Table 2.2).

Figure 2.4 Serum biochemistry in the *in-vivo* faecal peritonitis model

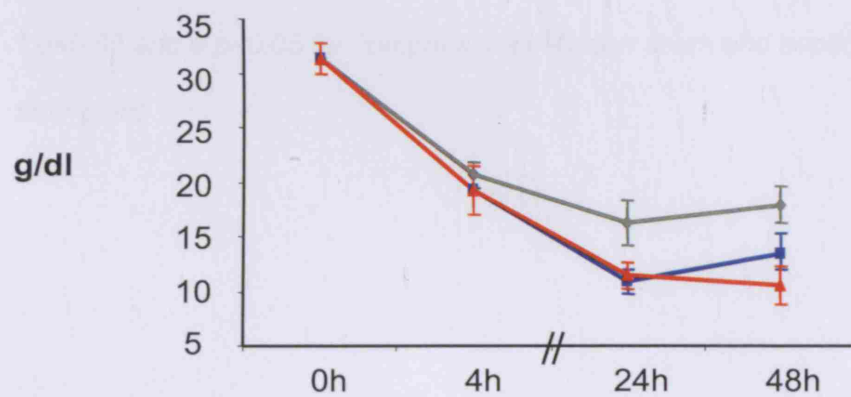
a) Urea



b) Alkaline phosphatase (ALP)



c) Albumin



Values shown are mean \pm SEM.

0h indicates samples taken immediately prior to injection of faecal slurry.

m/m = mild/moderate

* $p < 0.05$ for comparison of ALP between m/m and severe groups at 48h; this was the only statistically significant difference found between the two septic groups at any one time-point.

Analysis of the m/m and severe septic groups combined is shown in Table 2.2.

Table 2.2 Serum biochemistry in the *in-vivo* faecal peritonitis model

	0h	4h sham	4h septic	24h sham	24h septic	48h sham	48h septic
n	8	3	7	11	21	10	15
Urea (mmol/l)	4.3 (0.8)	5.1 (0.5)	4.4 (0.7)	3.9 (0.4)	5.8 # (0.7)	3.5 (0.6)	6.4* (0.9)
Sodium (mmol/l)	144 (1.3)	140 (1.3)	139 (1.0)	138 (0.8)	136 (1.3)	137 (0.9)	135 (1.8)
Alkaline phosphatase (IU/l)	125.0 (23.1)	75.3 (2.6)	90.7 (13.5)	43.1 (5.7)	84.3* (11.4)	40.9 (7.2)	112.3* (16.4)
Albumin (g/l)	31.4 (1.4)	20.7 (1.2)	19.3 (2.2)	16.3 (2.1)	11.1* (0.8)	17.9 (1.7)	12.1* (1.1)
Osmolarity (mmol/l)	308 (3.0)	302 (2.8)	299 (2.4)	297 (2.1)	294 (2.5)	295 (2.3)	292 (3.8)

Values shown are mean (SEM).

Serum osmolarity was calculated as $[2x(\text{sodium} + \text{potassium}) + \text{glucose (values not shown)} + \text{urea}]$. As accurate serum potassium levels were often not obtained due to sample haemolysis, a value of 4.0mmol/l was used for the purpose of this calculation.

* $p < 0.05$ and # $p = 0.05$ for comparisons between sham and septic groups at the same time-point

2.3.4 Arterial blood gas analysis

Blood gas analysis was performed on arterial samples from randomly selected septic and sham-operated rats 24 hours after the septic insult. Animals with faecal peritonitis had lower bicarbonate levels, a more negative base excess, and higher lactate levels, consistent with sepsis (Table 2.3). The unchanged pH but lower partial pressures of CO₂ in the septic rats may reflect successful compensation of metabolic acidosis by hyperventilation.

Table 2.3 Arterial blood gas results in the *in-vivo* faecal peritonitis model

	24h sham	24h septic
n	13	13
pH	7.48 (0.01)	7.49 (0.02)
pCO₂ (kPa)	4.64 (0.21)	3.94 (0.25) *
pO₂ (kPa)	14.65 (0.65)	12.52 (0.76) *
SBE (mmol/l)	2.15 (1.05)	-0.79 (1.03)
SBC (mmol/l)	26.7 (0.84)	24.74 (0.76)
Lactate (mmol/l)	1.31 (0.27)	3.11 (0.49) *

Values shown are mean (SEM).

pCO₂, partial pressure of carbon dioxide; pO₂, partial pressure of oxygen; SBE, standard base excess; SBC, standard bicarbonate

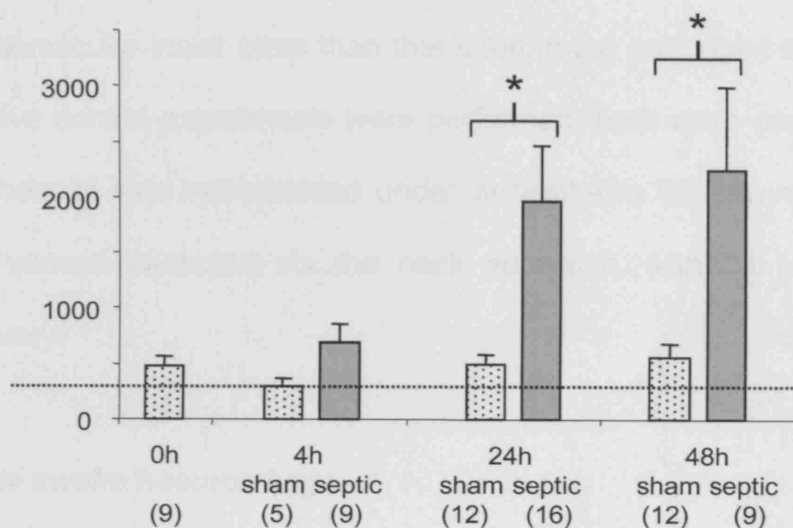
** p<0.05 when septic and sham groups compared (t tests used for each parameter)*

2.3.5 Vasopressin and norepinephrine levels

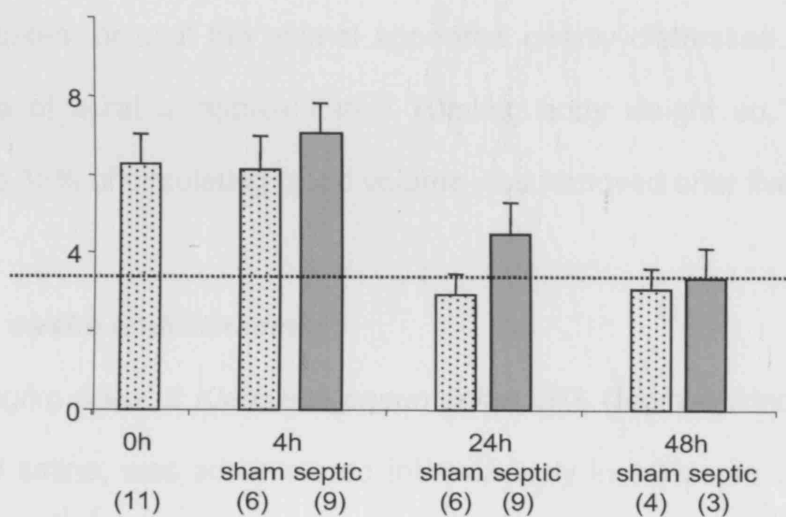
Figure 2.5 shows the plasma levels of NE and VP. As discussed in section 2.2.3, few VP samples were available at 48 hours. Baseline values in the unstressed rat are reported as approximately 300pg/ml for NE [Schaller *et al.*, 1985; Pajovic *et al.*, 2005], and 3.5pg/ml for VP [Giusti-Paiva *et al.*, 2002; Cisowska-Maciejewska & Ciosek, 2005]. I found NE levels were significantly elevated in septic rats at all time-points after 4 hours (Figure 2.5a). VP levels were highest in the first few hours of the experiment, but decreased at 24 and 48 hours (Figure 2.5b). No significant difference was seen between septic and sham-operated control rats at any one time-point despite clinical illness, a decrease in blood pressure (Figure 2.3), and evidence of a catecholamine stress response (Figure 2.5a) in the septic animals. Since other short-term animal studies of sepsis have found up to 40-fold rises in circulating VP [Brackett *et al.*, 1985; Schaller *et al.*, 1985; Giusti-Paiva *et al.*, 2002], I also measured VP levels at 12 hours following intra-peritoneal injection. The values (3.6 ± 0.8 pg/ml, n=5) were comparable to those found at 24 and 48 hours.

Figure 2.5 Plasma hormone levels in the *in-vivo* faecal peritonitis model

a) plasma norepinephrine (pg/ml)



b) plasma vasopressin (pg/ml)



Bars represent mean \pm SEM. Group sizes are shown in parentheses.

Dotted lines represent the normal values for rat reported in the literature for NE (300 pg/ml) and VP (3.5pg/ml).

* $p < 0.05$ for comparisons between sham and septic groups

2.3.5.1 Validation of vasopressin measurements

To ensure that the ELISA technique used was capable of detecting a VP rise, and to assess the magnitude of such a rise induced by a severe cardiovascular insult other than that seen in the prolonged sepsis model, two positive control experiments were performed. Both were carried out on small numbers of rats instrumented under anaesthesia for the insertion of arterial and venous cannulae via the neck approach, with 24 hours allowed for recovery.

Acute awake haemorrhage

With concurrent clinical observation and blood pressure monitoring, 1.5ml of blood for subsequent VP assay was aspirated from the arterial line at 15 minute intervals. This was continued until a maximum of five samples had been taken, or until the animal appeared overtly distressed. The circulating volume of a rat is approximately 70ml/kg body weight so, for a 300g rat, around 35% of circulating blood volume was removed after five samples.

Acute awake endotoxaemia

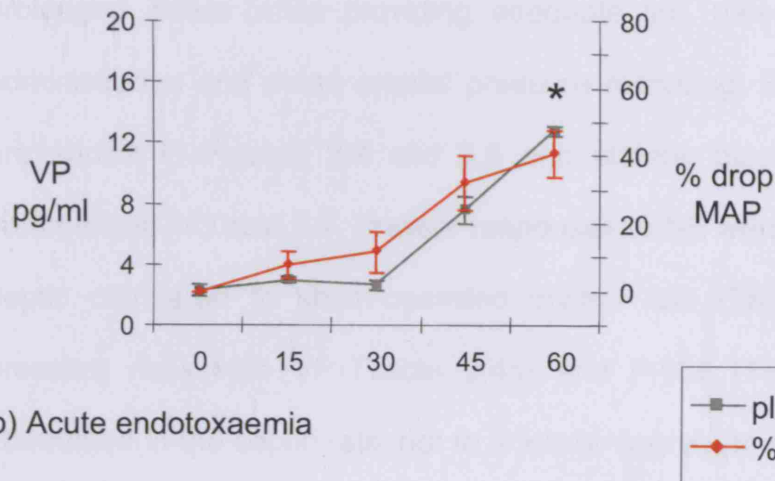
A 20mg/kg dose of *Klebsiella pneumoniae* LPS (Sigma-Aldrich), dissolved in normal saline, was administered intravenously in a volume of 1ml. Previous work in our laboratory found this dose to reliably produce severe acute endotoxic shock in Wistar rats. Immediately prior to injection, 0.75ml of blood was aspirated from the arterial line for subsequent VP assay. This was followed by a replacement intravenous hetastarch colloid bolus of the same

volume to minimise the haemodynamic effect of blood sampling. Blood samples were then taken in the same manner at 15 minute intervals until a maximum of five samples had been taken or until the animal appeared overtly distressed. Blood pressure was continuously monitored during the procedure.

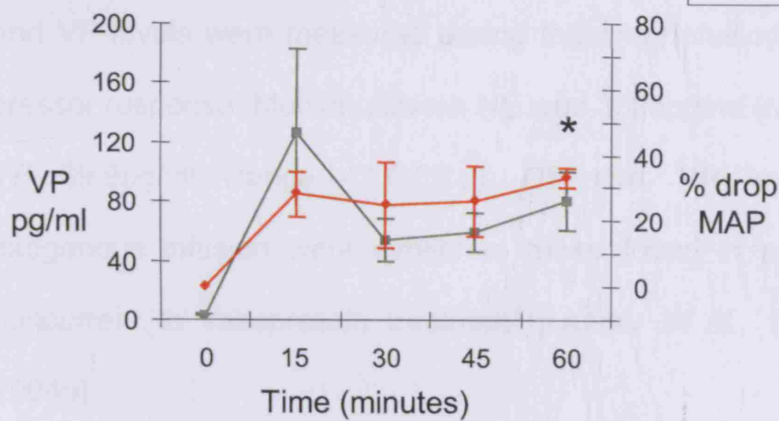
In both these experiments, a clear rise in VP levels was measured following the acute stressor insult (Figure 2.6). In the haemorrhage model, hormone levels were approximately tripled after 6ml (c.25% of circulating volume) of blood had been withdrawn (45 minute stage). This corresponded to a drop in MAP of approximately 33% (Figure 2.6a). Interestingly, the magnitude of increase in VP was much higher in the acute endotoxin model, with a maximal increase of more than 50-fold for a MAP change comparable to that seen in the haemorrhage study (Figure 2.6b).

Figure 2.6 Plasma vasopressin levels and changes in mean arterial pressure in acute shock models

a) Acute haemorrhage



b) Acute endotoxaemia



Values shown are mean \pm SEM; n=4 rats for each experiment.

Note different scales for the y axes (VP concentration) in the two experiments.

In study a), 1.5ml of arterial blood was taken at time 0 and this was repeated at 15 minute intervals thereafter. In study b), intravenous endotoxin was administered at time 0.

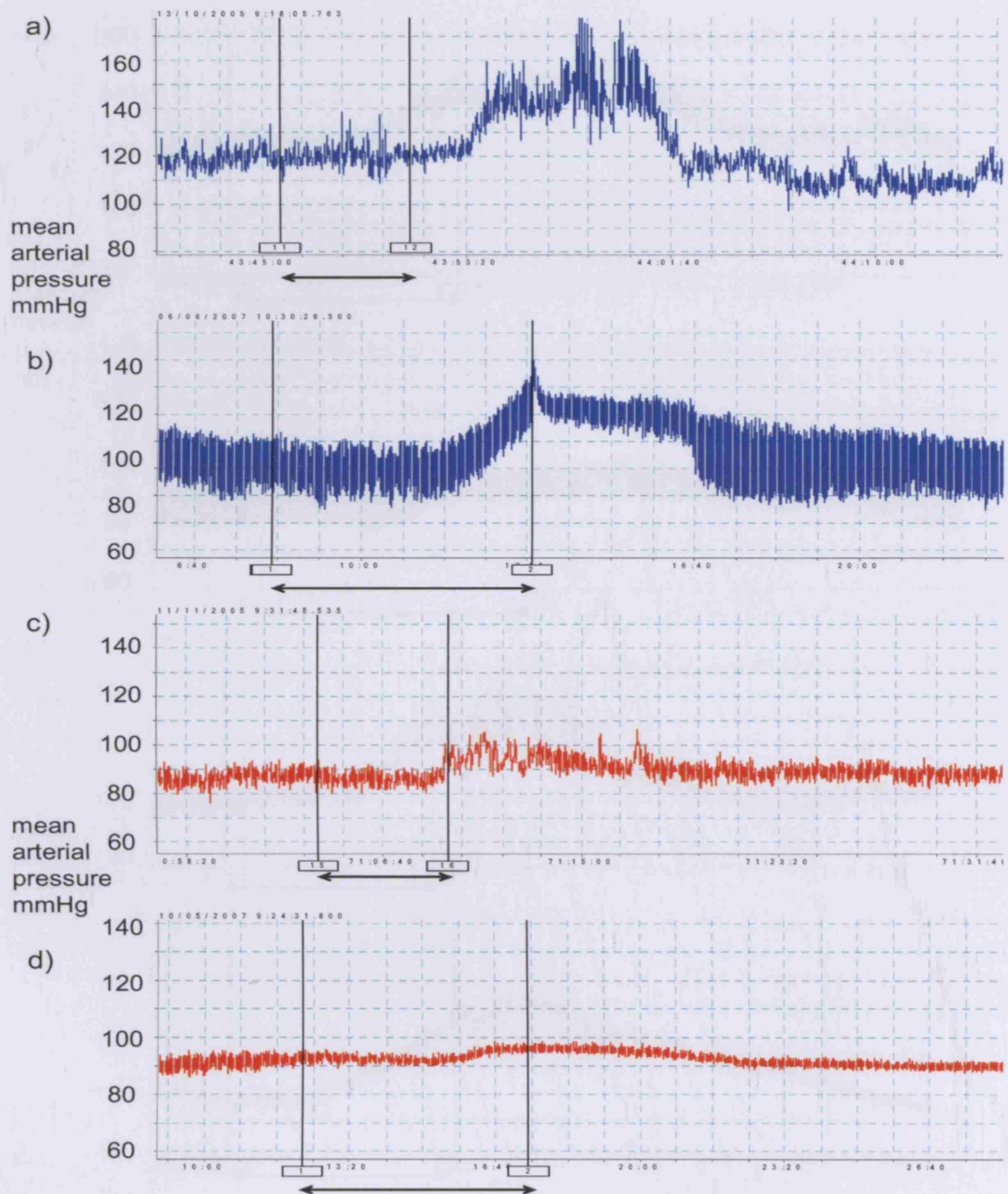
* $p < 0.05$ when time 60 VP and MAP values were compared with time 0 measurements in each experiment

2.3.6 *In-vivo* blood pressure responses

Intravenous infusions of vasopressors were given 24 hours post-induction of peritonitis. This time-point was chosen as it was considered to represent prolonged illness while providing adequate line patency for reliable drug administration and mean arterial pressure recording. Specimen MAP traces are shown in Figures 2.7 and 2.8 and plateau blood pressure rises are summarised in Table 2.4. Pressor responses to NE were markedly reduced in septic compared to sham-operated control rats (Table 2.4a). The blood pressure rises with VP (Table 2.4a) and F-180 (Table 2.4b) were also attenuated in the septic rats, but to a lesser degree. In three rats, plasma NE and VP levels were measured during the drug infusions at the time of peak pressor response. Median plasma NE was 3525pg/ml (range 2883-4250); and VP 69.9pg/ml (range 47.6-89.6). Of note, VP levels measured after exogenous infusion were similar to those found in patient samples taken concurrent to vasopressin treatment [Landry *et al.*, 1997b; Dunser *et al.*, 2004a].

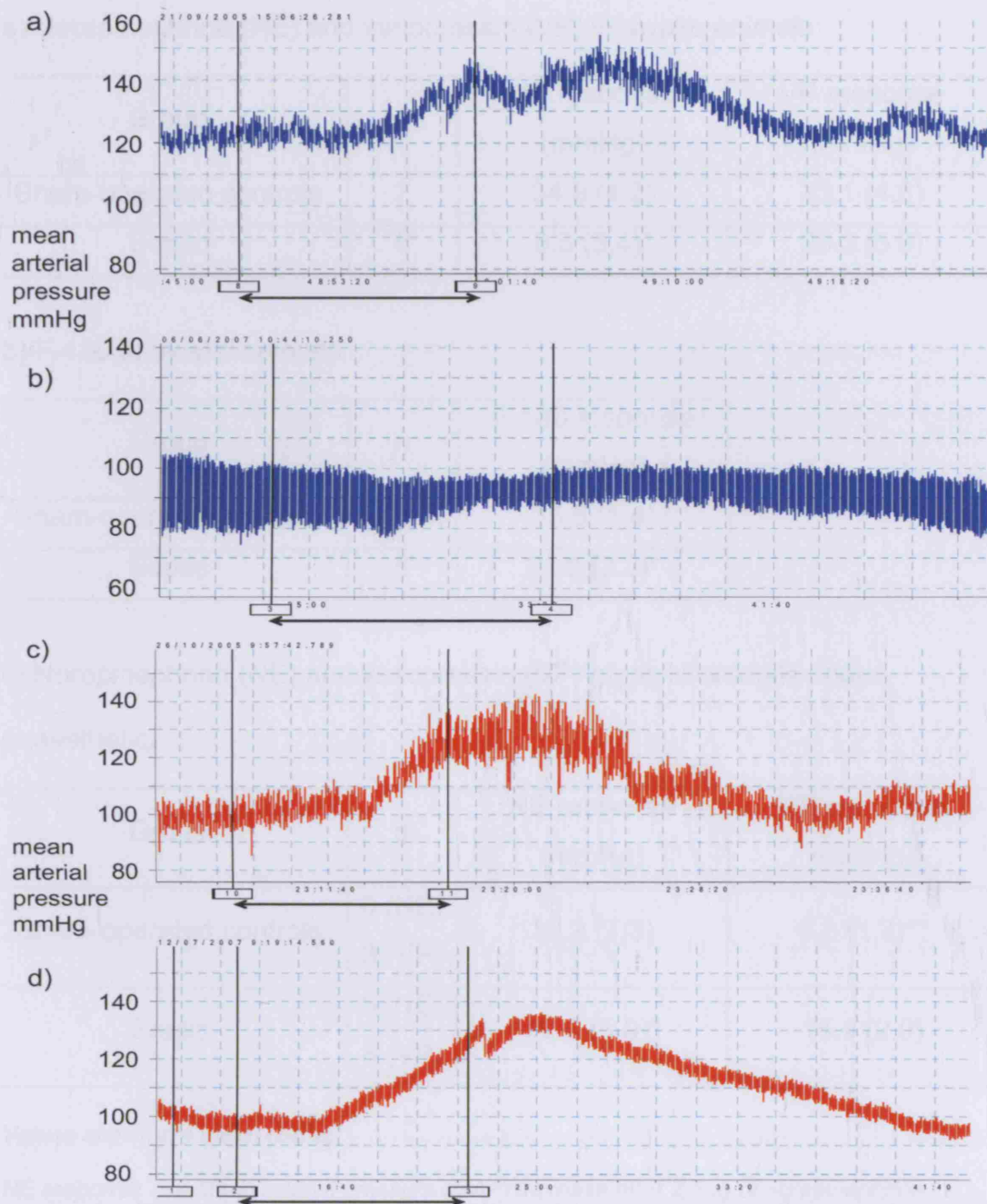
In an attempt to better simulate conditions seen in septic shock patients, I repeated the NE and VP in experiments in a separate cohort of septic and sham rats under anaesthesia (Table 2.4c). Prior to vasopressor administration, animals were transferred from their cages onto a warmed operating mat, allowing controlled inhalation of isoflurane via a face mask. To facilitate handling, the venous and arterial lines were detached from the tether system. As in the awake vasopressor experiments, a stable MAP was established prior to drug infusion. NE and VP were administered at the same

Figure 2.7 *In-vivo* blood pressure responses to norepinephrine



Typical blood pressure traces with infusion of $2.5\mu\text{g}$ norepinephrine over 5 min (\leftrightarrow indicates infusion period; note longer trace lengths & smaller x axis scales in a) & c):
 a) sham rat awake; b) sham under anaesthetic; c) septic rat awake; d) septic under anaesthetic

Figure 2.8 *In-vivo* blood pressure responses to vasopressin



Typical blood pressure traces with infusion of 0.017units arginine-vasopressin over 10 mins (\leftrightarrow indicates infusion period; note longer trace lengths & smaller x axis scales in a) & c); a) sham rat awake; b) sham under anaesthetic; c) septic rat awake; d) septic under anaesthetic

Table 2.4 *In-vivo* blood pressure responses to vasopressor infusions

a) Norepinephrine (NE) and vasopressin (VP) in awake animals

Group	n	NE response (mmHg)	VP response (mmHg)
Sham-operated controls	7	34.9 (4.2)	33.1 (4.6)
Septic	6	8.5 (3.4)*	20.3 (5.6)

b) F-180 in awake animals

Group	n	F-180 response (mmHg)
Sham-operated controls	6	35.5 (1.4)
Septic	6	21.0 (2.2)*

c) Norepinephrine (NE) and vasopressin (VP) given to animals under anaesthetic

Group	n	NE response (mmHg)	VP response (mmHg)
Sham-operated controls	6 (NE) 8 (VP)	39.2 (7.3)	6.3 (1.3)**
Septic	6 (NE) 8 (VP)	12.0 (5.0)*	15.4 (2.9)

Values shown are mean (SEM).

NE response = Mean arterial pressure (MAP) increase after 2.5µg of norepinephrine i.v.

VP response = MAP increase following 0.017 units of vasopressin i.v.

F-180 response = MAP increase following 45 picomoles of F-180 i.v.

* $p < 0.05$ when compared with response to same drug under same conditions in sham-operated controls; ** $p < 0.05$ when compared to response to same drug in awake sham rats

dose, rate and protocol as described in Section 2.2.5. In all of these rats, drug effect was observed more rapidly than in the awake experiments due to the shortened venous line length once detached from the tether (e.g. compare Figures 2.7a and b). No differences were seen between the awake and anaesthetised responses to NE in sham or septic animals, with a depressed response still obvious in the latter (Figure 2.7 and Table 2.4). In contrast, the VP response in sham-operated animals was significantly decreased under isoflurane anaesthesia. The septic rats showed numerically greater responses to vasopressin than the sham-controls under these conditions, but this finding did not reach statistical significance (Figure 2.8 and Table 2.4).

2.4 Discussion

2.4.1 General characteristics of the septic model

This rat model of faecal peritonitis aimed to mimic human septic illness in terms of type and duration of insult, and subsequent fluid resuscitation. The clinical observations, mortality rate and biochemical data presented suggest that this was largely achieved. The marked, prolonged catecholamine stress response is also consistent with critical illness. The relatively modest degree of hypotension is likely related to adequate volume loading and the preservation of cardiovascular reflexes in conscious animals.

When compared to the original characterisation of this model in our laboratory [Brealey *et al.*, 2004], a number of differences are apparent. Because of new

concerns over fluid overloading, as indicated by significant initial mortality in the sham-control group, all rats were given less aggressive fluid resuscitation. Despite maintenance of higher mean arterial pressures than previously described, mortality rates were higher in the septic animals (64% at 48 hours, compared to 40% at 72 hours [Brealey *et al.*, 2004]). I was also unable to demonstrate a good correlation between clinical severity score and the objective measures of illness (MAP and serum biochemistry), hence my decision to analyse the septic rats as a single, larger group. The explanation for these findings is unclear but change in animal phenotype is likely to be partly responsible. Although this method of induction of intra-abdominal sepsis is clinically realistic, a significant drawback of blind faecal slurry injection is the inability to accurately quantify and therefore administer a consistent bacterial insult.

On initial experiment planning, I had hoped that the carotid arterial cannulae would enable *in-vivo* blood sampling and continuous blood pressure monitoring for up to 72 hours after the septic insult. In the case of a non-patent arterial line, the intention was to obtain blood by cardiac puncture at the time of animal sacrifice. I found, however, that aspiration of blood from the indwelling lines became difficult at 48 hours and beyond, and, as discussed in Section 2.2.3, the VP measurements in cardiac puncture samples were erratic and were markedly higher than levels recorded from line-aspirated blood. Furthermore, the requirement for both arterial and venous line patency for *in-vivo* vasopressor administration meant that this could only realistically be performed in the early stages of the model (up to 24 hours). The relative

deficiency of vasopressin and hypersensitivity to its exogenous administration have been described as features of prolonged (>24 hours) septic shock in patients [Landry *et al.*, 1997a; Sharshar *et al.*, 2003a]. In aiming to reproduce these features in an animal model, a judgement must be made as to how this time course in generally elderly humans relates to experimental duration in a comparatively young rat. Previous work in our laboratory has shown that recovery from the faecal peritonitis insult is evident in surviving rats by 72 hours [Brealey *et al.*, 2004]. This represents a much shorter illness duration than is seen in adult patients, and would suggest that a state of “prolonged” sepsis is achieved by 24 hours. With this in mind, and in light of the encountered experimental difficulties, my *in-vivo* and *ex-vivo* studies focussed on rats at 24 hours after induction of sepsis.

2.4.2 Vasopressin levels

Baseline VP levels measured in this study were slightly higher than normal values reported in the literature [Giusti-Paiva *et al.*, 2002; Cisowska-Maciejewska & Ciosek, 2005]. We anticipated an early rise in VP levels in septic rats, but found no difference from paired sham-controls at any time-point investigated. The difficulty in reliable quantification of this hormone due to its instability and association with platelets is well acknowledged [Struck *et al.*, 2005]. Other investigators have used radioimmunoassay [Schaller *et al.*, 1985; Giusti-Paiva *et al.*, 2002] rather than ELISA [Lodha *et al.*, 2006] to measure vasopressin. I confirmed the ability of the ELISA to detect several-fold elevations in plasma VP in the acute endotoxic and hemorrhagic shock

experiments (Section 2.3.5), and from the samples analyzed following *in-vivo* VP infusion (Section 2.3.6). Moreover, the results of the former were found to be comparable with those reported in other short-term rat LPS models [Brackett *et al.*, 1985; Schaller *et al.*, 1985].

It is possible that an acute peak was missed in the septic rats by virtue of the short half-life of VP (10-35 minutes) [Holmes *et al.*, 2001], and the relatively long sampling time intervals employed. An alternative explanation is that the peritonitis insult was insufficiently severe to trigger significant vasopressin release. A blood pressure drop of at least 15% is required to evoke vasopressin release [Mutlu & Factor, 2004], a statement supported by the results of my acute haemorrhage experiments (Figure 2.6). Although this degree of hypotension was rarely seen in rats with faecal peritonitis, other stimuli of VP secretion, including endotoxin, cytokines and metabolic acidosis were presumably in operation [Holmes *et al.*, 2001; Mutlu & Factor, 2004]. Indeed, my short term study suggests that intravenous endotoxin produces a rise in hormone levels far greater than would be predicted from the associated hypotension (Figure 2.6). Moreover, the marked elevation in plasma norepinephrine in the septic animals is consistent with severe illness. Therefore, particularly at the 24 hour time point, the lack of elevation in VP levels in the septic animals could be deemed inappropriate, and this is consistent with the concept of relative vasopressin deficiency described in patients with prolonged septic shock [Landry *et al.*, 1997a; Sharshar *et al.*, 2003a].

As reviewed in Chapter 1 (Section 1.4.1), the explanation for the lack of rise in VP levels in prolonged sepsis is multifactorial. It is likely that increased NO, previously demonstrated in this model to peak between 24 and 48 hours [Brealey *et al.*, 2004], and high circulating NE levels act to inhibit hormone synthesis and/or release [Reid, 1994; Leng *et al.*, 1999; Giusti-Paiva *et al.*, 2002]. In support of this, immunohistochemical studies on rat brains from this peritonitis model have shown decreased VP staining in the hypothalamic supraoptic nucleus and the posterior pituitary of septic animals (T. Sharshar, University de Versailles, France, personal communication).

2.4.3 *In-vivo* blood pressure responses

Decreased pressor sensitivity to catecholamine infusion is a feature of prolonged septic shock [Paya *et al.*, 1993; Annane *et al.*, 1998; Landry & Oliver, 2001; Pickkers *et al.*, 2006]. In awake animals, I was unable to reproduce the hypersensitivity to VP reported in septic patients [Landry *et al.*, 1997b; Malay *et al.*, 1999; Patel *et al.*, 2002], although the effect of VP was relatively preserved compared to that seen with NE. One possible explanation could be simultaneous activation of both vasoconstrictor (V_1) and (non- V_1) vasodilatory VP receptors. However, I obtained similar results with the selective V_1 R agonist, F-180, suggesting that this is not the case. Contrary to findings in healthy human volunteers [Abboud *et al.*, 1990], I found that low-dose VP infusion produced a marked pressor effect in sham-operated control rats. Preliminary studies revealed the blood pressure response to VP in these animals increased several-fold across a narrow dose range, presenting an

additional difficulty in choosing an optimal dose to demonstrate a potential enhanced sensitivity in septic animals. The literature reports a considerable species variation in the ability to dissociate VP-induced changes in systemic vascular resistance (SVR) from changes in MAP, with a much steeper gradient of the blood pressure versus VP curve in rats than in humans [Cowley, 1982]. This knowledge, in combination with the recognition that vasopressin treatment is very rarely given to conscious septic patients, prompted the repetition of these experiments in anaesthetised rats.

Under anaesthesia, the decreased pressor effect of norepinephrine in septic compared to sham-control rats remained. With vasopressin, however, blood pressure responses were blunted in the sham-operated group yet maintained in the septic animals. As a formal sample-size calculation was not performed, it may be that the study was underpowered to detect a heightened septic versus sham VP response. Anaesthetic administration may alter vascular reactivity via disruption of autonomic reflexes. Furthermore, the changes in autonomic function observed in septic patients [Garrard *et al.*, 1993] are one plausible explanation for VP hypersensitivity (Section 1.4.4). It may be, therefore, that the usual concomitant administration of sedation in such patients exaggerates the increased pressor effect. Interpretation of the current findings in relation to the clinical situation is difficult however, in view of the aforementioned underlying species' differences in VP-baroreflex interactions [Cowley, 1982].

2.4.4 Summary

I have used this rat model of faecal peritonitis to demonstrate inappropriately low endogenous levels of vasopressin in prolonged sepsis. Despite only mild hypotension in the septic animals, the markedly decreased pressor responses to exogenous norepinephrine in this group support the presence of vasodilatory shock. Vasopressin responses were preserved in sepsis, and were numerically greater than those in sham-controls when the drug was administered to anaesthetised rats. These results validate the use of this model for further exploration of the mechanism of vasopressin hypersensitivity in septic shock.

Chapter 3: *Ex-vivo* wire myography studies in small arteries taken from septic and sham-operated rats

3.1 Introduction

The development of wire myography has enabled the *ex-vivo* investigation of the mechanical and pharmacological properties of the small arteries comprising the resistance vasculature [Mulvany & Aalkjaer, 1990]. Prior to this, the available organ bath methods precluded studies of vessels smaller than c.400 μ m internal diameter. Wire myography permits the relatively atraumatic mounting of vessel segments on two secured, small (40 μ m diameter) wires so that isometric tension measurements can be made under loading conditions simulating *in-vivo* pressures [Mulvany & Aalkjaer, 1990]. Much of the myograph work characterising the resistance vasculature of the rat has been performed on second and third order branches of the mesenteric artery [Mulvany & Aalkjaer, 1990; Buus *et al.*, 1994]. I therefore chose the wire myography approach to investigate the *ex-vivo* vascular reactivity of small mesenteric arteries dissected from the sham-operated and septic rats from the faecal peritonitis model. To formally learn this method, I attended a week's course held at the Department of Pharmacology of the University of Aarhus in Denmark. I was also able to benefit from the already considerable experience of this technique in our laboratory [Chauhan *et al.*, 2003; Orié *et al.*, 2006].

In this chapter, I have characterised the baseline reactivity of mesenteric vessels from sham-control and septic rats to norepinephrine and vasopressin. As demonstrated in the *in-vivo* peritonitis model (Section 2.3.6), I hoped to reveal differences in the impact of sepsis on vascular reactivity to the two vasoconstrictors. Furthermore, in view of the existence of several VP receptor subtypes in the vasculature (Section 1.2.2), I aimed to employ manoeuvres to dissect out the possible different components of this agonist's response. V_1 receptors are the major subtype producing vasoconstriction, and their contribution was assessed by determining the dose-response characteristics of the selective V_1 R agonist, F-180 [Bernadich *et al.*, 1998]. The role of endothelial receptors was investigated through the effects of its physical removal and the inhibition of eNOS. Finally, the possible V_2 R component of VP's action was explored through the use of a selective V_2 R antagonist, FE992082. For comparison with an alternative vascular bed, a small number of similar experiments were done using tail arteries.

3.2 Materials and Methods

3.2.1 Dissection and mounting of resistance vessels

For the initial *ex-vivo* studies investigating the baseline reactivity of vessels to NE and VP, arteries were taken from the cohort of rats described in Chapter 2. Subsequent experiments required additional animals that underwent the same experimental protocol as explained in Section 2.2.1.

Twenty-four hours after intra-peritoneal injection of faecal slurry (or no injection in sham-operated controls), and after at least one hour's recovery if vasoactive drugs had been administered, rats were deeply anaesthetised with isoflurane and then killed by cervical dislocation. A midline laparotomy was then performed to expose the mesenteric vascular bed. A segment of intestine with its proximal end about 10cm distal to the pylorus was identified. Using scissors, this section and its feeding vasculature, including part of the superior mesenteric artery, was carefully removed. A nick was made in the proximal end of the excised bowel to aid later identification. The specimen was then immediately placed into cold (4°C) physiological salt solution (PSS) of the following composition (mM): NaCl 112, KCl 5, CaCl₂ 1.8, MgCl₂ 1, NaHCO₃ 25, KH₂PO₄ 0.5, NaH₂PO₄ 0.5, and glucose 10, previously bubbled with carbogen gas (95% O₂, 5% CO₂). The fresh mesenteric tissue was transferred to a Petri dish containing a layer of agar to hold fixing pins and kept moist with fresh cold PSS. Having identified the proximal end of the segment, the tissue was pinned such that the mesenteric veins lay above the arteries. Under a light microscope, the adipose tissue surrounding the vessels was cut away. The veins were then removed, and second or third order mesenteric arterial branches (c.200µm internal diameter) dissected and cleaned of connective tissue.

To obtain tail arteries, the tough outer skin of the tail was scored proximally with scissors and then completely peeled away with forceps. The tail was sectioned, and the distal third immediately placed into cold PSS. This portion was pinned with the ventral side uppermost, and the artery identified in the groove running along the ventral surface. This was carefully lifted away, using

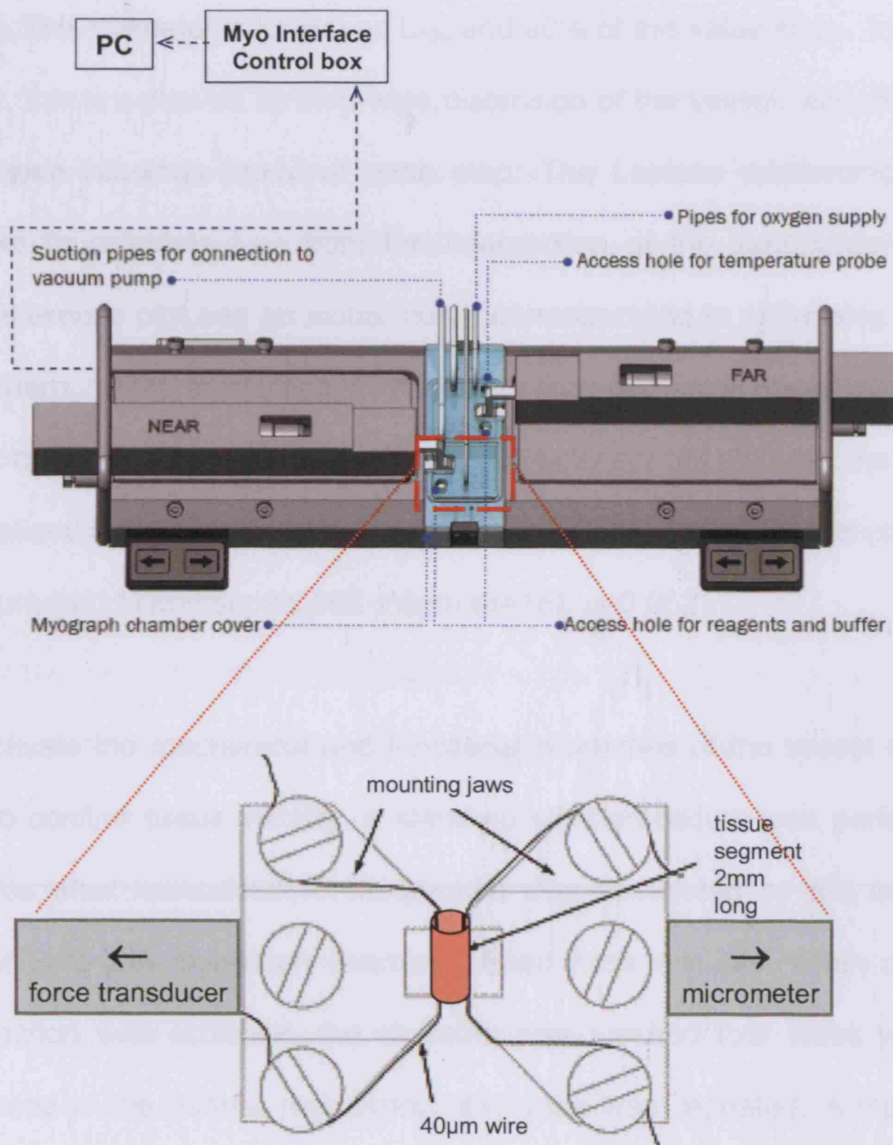
scissors to cut through the encasing connective tissue and small side branches. The arterial segments thus dissected were of approximately 500 μ m internal diameter.

Arterial segments of 2mm in length were then mounted in a small vessel, dual chamber wire myograph (model 510A, Danish Myo Technology, Aarhus, Denmark) for isometric tension recording. Each vessel segment was mounted on two 40 μ m stainless steel wires, one attached to a force transducer and one attached to a micrometer, via a pair of stainless steel jaws (Figure 3.1). Adjustments to and measurements from the transducer and micrometer were performed via an electronic controller (Myo Interface, Danish Myo Technology), the information from which was fed to a PC and visualised using appropriate software (Mydaq and Myodata, National Instruments Corporation, Austin, TX, USA). Two segments were mounted in one myograph at a time. The vessels were bathed in 10ml of PSS, heated to 37°C and bubbled continuously with carbogen. A plastic divider could be inserted between the two sets of jaws to form two separate chambers, each of 5ml volume.

3.2.2 Normalisation and vessel activation

Following equilibration for 30 minutes, an automated normalisation procedure was performed to determine the arterial lumen necessary for optimal force generation [Mulvany & Halpern, 1977]. It has been shown previously that for rat small mesenteric arteries, maximal force generation is achieved when a

Figure 3.1 Schematic of dual chamber small vessel wire myograph



Each set of jaws, known by convention as "NEAR" and "FAR", is attached on one side to a micrometer which measures jaw separation, and on the other to a force transducer which measures the force exerted by the arterial contraction on the mounting wires.

segment is stretched to 90% of the diameter expected had it been relaxed and exposed to a transmural pressure of 13.3kPa (100mmHg) [Buus *et al.*, 1994]. This diameter is known as L_{100} and 90% of this value as L_1 . In practical terms, this is achieved by step-wise distension of the vessel, with micrometer and force readings made at each step. The Laplace relationship is then applied to calculate L_{100} from the intersection of the exponential tension-circumference plot and an isobar curve corresponding to 100mmHg [Mulvany & Halpern, 1977]. In practice, L_1 values for mounted small mesenteric arteries were close to 200 μ m. In the initial characterisation studies, the average normalised diameter was $212 \pm 8\mu\text{m}$ (mean \pm SEM) for 29 vessels (sham: $228 \pm 14 \mu\text{m}$ (n=11) and septic: $202 \pm 8\mu\text{m}$ (n=18), $p=0.052$).

To activate the mechanical and functional properties of the vessel segments and to confirm tissue viability, a standard start procedure was performed 30 minutes after normalisation. Contraction was stimulated by the addition of 5 μ M NE to the myograph chamber. After three minutes, when a plateau contraction was achieved, the chamber was washed four times with PSS. Following a five minute rest period, the dose was repeated. A measure of viability was calculated to determine whether the isolation and mounting of a vessel had damaged the arterial wall. The following relationship was used, which corrects for differences in the length and diameter of vessel segments:

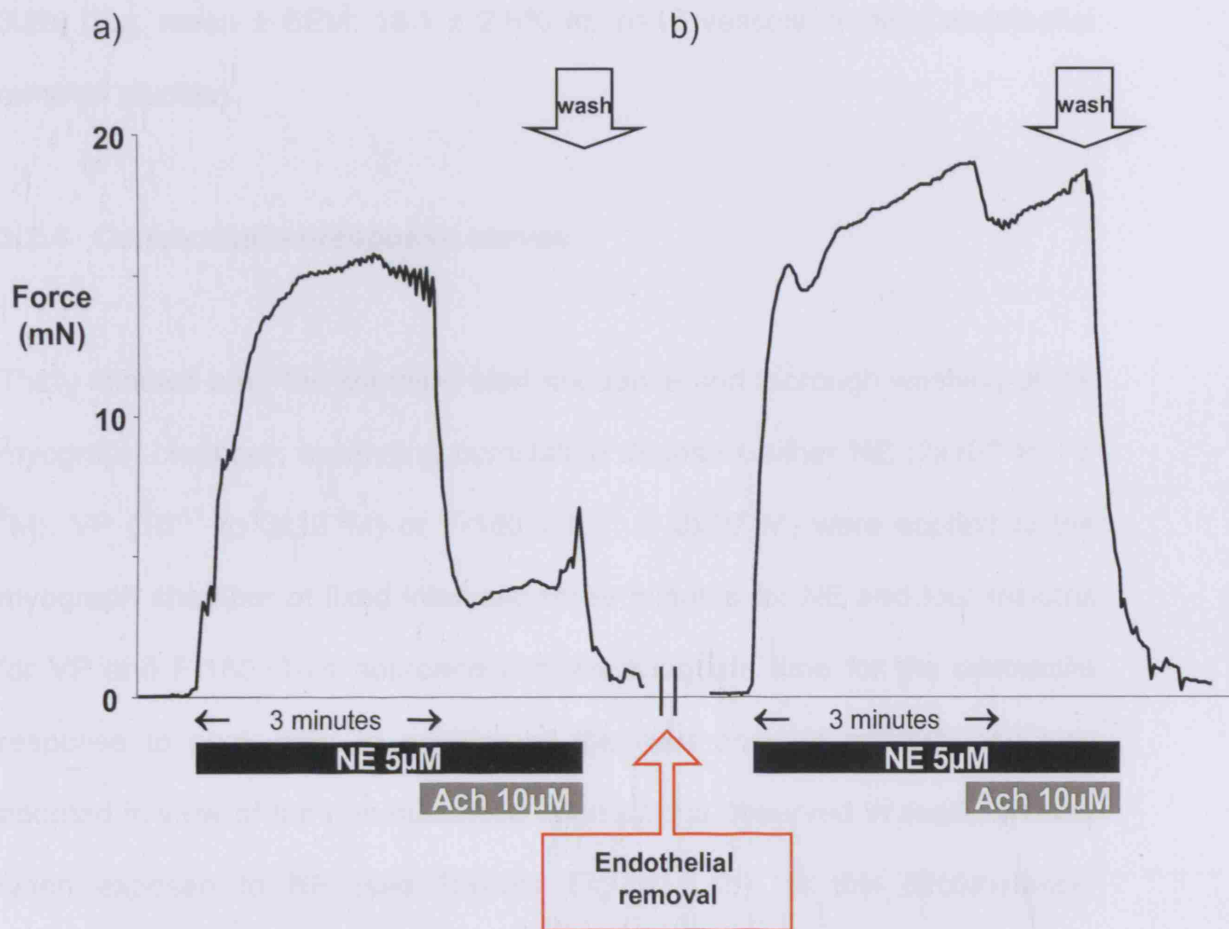
$$\text{Effective pressure (kPa) produced by contractile agonist} = \frac{2 \times \Delta \text{ wall tension (mN/mm)}}{L_1 \text{ (mm)}}$$

Exposure of rat mesenteric arteries usually produces a pressure response of >20kPa. By convention, arteries were considered non-viable if the effective pressure generated was <13.3kPa [Mulvany & Halpern, 1977]. Vessels removed from septic animals often showed non-sustained contractions when exposed to NE (see Results Figure 3.4). In this circumstance, calculations of effective pressure were performed using the peak values. On the third activation step, 10 μ M acetylcholine (ACh) was applied at the peak of the NE-induced contraction to test for endothelial function (Figure 3.2a). Endothelial integrity was confirmed if a \geq 50% relaxation was achieved; in practice an average relaxation of around 80% was observed (e.g. mean \pm SEM: 75.7 \pm 3.5% for n=29 vessels in initial characterisation studies).

3.2.3 Endothelial removal

In some experiments, the effect of endothelial removal on agonist-induced contractile responses was examined. In these studies, vessels were washed thoroughly after the standard start procedure. Following a 15 minute equilibration period, a force of 0.5mN was applied to the artery to be denuded by manually increasing the separation of the jaws by a few μ m. Under light microscopy, a thick human hair was inserted into the vessel lumen using fine forceps. The luminal arterial surface was then rubbed gently along its length for five minutes, taking care not to damage the underlying muscle layer. The vessel was then returned to its normalised diameter, washed with PSS and left to equilibrate for a further 15 minutes. The standard start procedure was repeated (Section 3.2.2). Endothelial removal was considered successful if

Figure 3.2 Wire myography trace showing testing of endothelial function in a small mesenteric artery from a sham-operated rat



Trace from a freshly mounted arterial segment contracted with 5µM norepinephrine (NE) before and after endothelial removal with a single thread of hair. After a plateau was reached, 10µM acetylcholine (ACh) was applied before being washed out as indicated by arrows. In b), a <25% relaxation response to ACh demonstrates adequate endothelial removal.

the contractile response to NE was $\geq 95\%$ of the force measurement previously recorded, and the relaxation produced by ACh was $\leq 25\%$ (Figure 3.2b) (e.g. mean \pm SEM: $18.1 \pm 2.6\%$ for $n=16$ vessels in initial endothelial removal studies).

3.2.4 Concentration-response curves

Thirty minutes after the standard start sequence and thorough washing of the myograph chamber, ascending cumulative doses of either NE (2×10^{-8} to 10^{-5} M), VP (10^{-11} to 3×10^{-8} M) or F-180 (10^{-10} to 3×10^{-7} M) were applied to the myograph chamber at fixed intervals: three minutes for NE and four minutes for VP and F-180. This approach allowed adequate time for the contractile response to peak prior to addition of the next dose of agonist, and was adopted in view of the non-sustained contractions observed in septic arteries when exposed to NE (see Results Figure 3.4b). In this circumstance, standardised recording of peak and sustained tension values was performed (see Results Figure 3.4a). When more than one concentration-response curve was performed, a 30 minute equilibration time was allowed between experiments, and the order in which agonists were applied varied.

The contribution of the V_2R to vasopressin responses was investigated by adding a V_2R antagonist (FE992082, 30nM) to the chamber 30 minutes before the addition of VP. In a series of experiments with sham arteries, eNOS was blocked by the addition of NG-nitro-L-arginine methyl ester (L-NAME, 100 μ M) to the bathing solution 30 minutes before the application of F-180.

3.2.5 Drugs and reagents

Norepinephrine (norepinephrine tartrate) was purchased from Abbott Laboratories. F-180 (V₁R agonist) and FE992082 (V₂R antagonist) were kindly donated by Ferring Pharmaceuticals. All other drugs and reagents were obtained from Sigma-Aldrich.

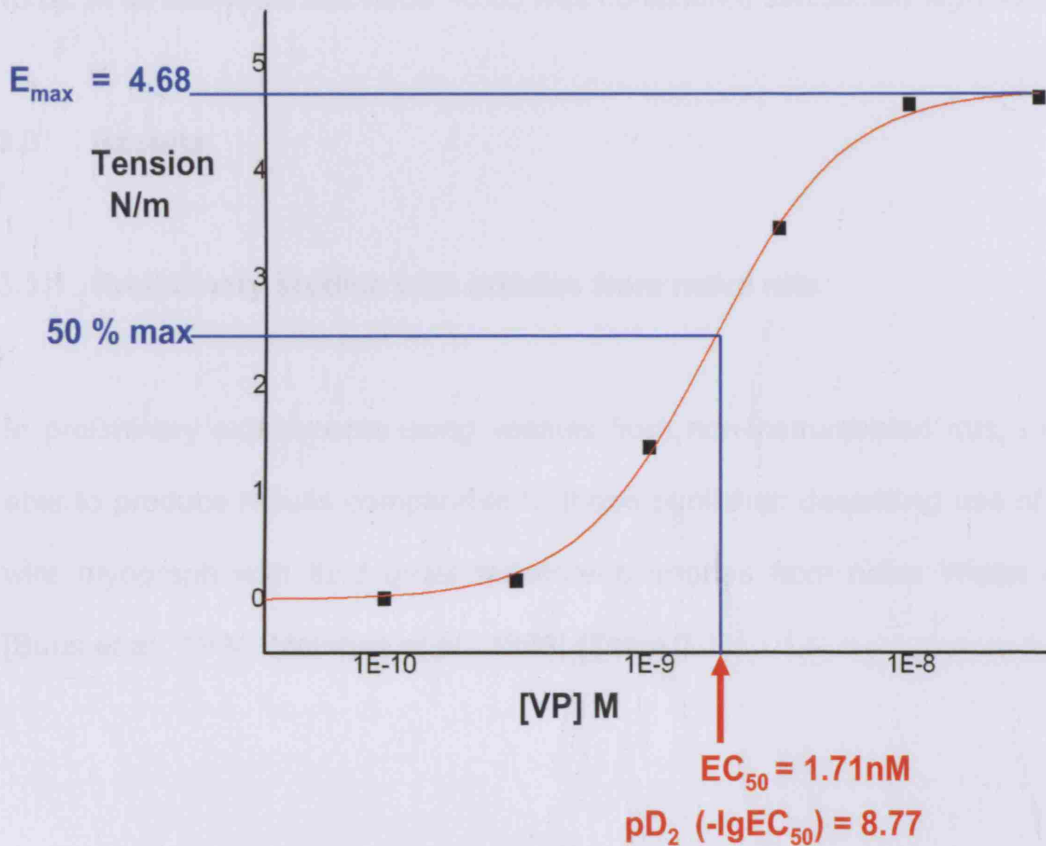
3.2.6 Data and statistical analysis

Unless otherwise stated, data are shown as mean ± SEM of n animals. Force measurements (mN) were corrected for vessel length to give tension values (N/m). Data were then analysed using GraphPad Prism software (GraphPad Software Inc, San Diego, CA, USA). Tension versus concentration data for individual experiments were plotted using a logistical fitting routine to produce a sigmoidal curve with a variable slope, according to the following equation:

$$y = \text{min} + \frac{\text{max-min}}{1 + 10^{(\log EC_{50}-x) \text{ Hill slope.}}}$$

These individual fits were used to obtain values for agonist sensitivity (or potency), expressed as the negative log of the concentration required to produce 50% of the maximum tension (pD₂ or -log EC₅₀), and agonist efficacy, expressed as the maximal tension (E_{max}) response (Figure 3.3). The combined mean concentration-response curves for sham-operated and septic groups were compared using two-way ANOVA for repeated measures with Bonferroni post-hoc analysis for multiple comparisons. The mean EC₅₀ and

Figure 3.3 A typical plot of tension against vasopressin concentration for a wire myograph experiment in a single vessel



Tension is plotted on a linear scale and concentration on a logarithmic scale. The data were fitted to a sigmoid curve with a variable slope using the logistical function in GraphPad Prism software. From this, the maximal response (E_{max}) and the concentration producing 50% of this response (EC_{50}) can be estimated. By convention, the EC_{50} is expressed as the negative log value, pD_2 . E_{max} is a measure of agonist efficacy and pD_2 is a measure of agonist sensitivity or potency.

E_{\max} values were compared between two animal groups using Student's t test (unpaired) or ANOVA when more than two groups were compared (i.e. for analysis of peak and sustained data in NE dose-response curves) (SPSS 15.0). In all analyses, a p value <0.05 was considered statistically significant.

3.3 Results

3.3.1 Preliminary studies with arteries from naïve rats

In preliminary experiments using vessels from non-instrumented rats, I was able to produce results comparable to those published describing use of the wire myograph with third order mesenteric arteries from naïve Wistar rats [Buus *et al.*, 1994; McIntyre *et al.*, 1998] (Table 3.1).

Table 3.1 Preliminary wire myography studies of small mesenteric arteries from naïve Wistar rats compared to published data

a) Preliminary studies

	Vessel diameter (µm)	Effective pressure (kPa)	Max Tension (N/m)	Potency of NE pD ₂	Potency of VP pD ₂
Mean	253	38.7	4.93	5.45	8.80
(SEM)	(9)	(1.70)	(0.31)	(0.04)	(0.07)
n (vessels)	16	16	16	16	8

b) Buus NH *et al.*, *Br. J. Pharmacol* 1994: 579-587

	Vessel diameter (µm)	Effective pressure (kPa)	Max Tension (N/m)	Potency of NE pD ₂	Potency of VP pD ₂
Mean	-	-	3.85	5.77	8.98
SEM			(0.25)	(0.05)	
n (vessels)	-	-	8	8	7

c) McIntyre CA *et al.*, *Br. J. Pharmacol* 1998: 1555-1560

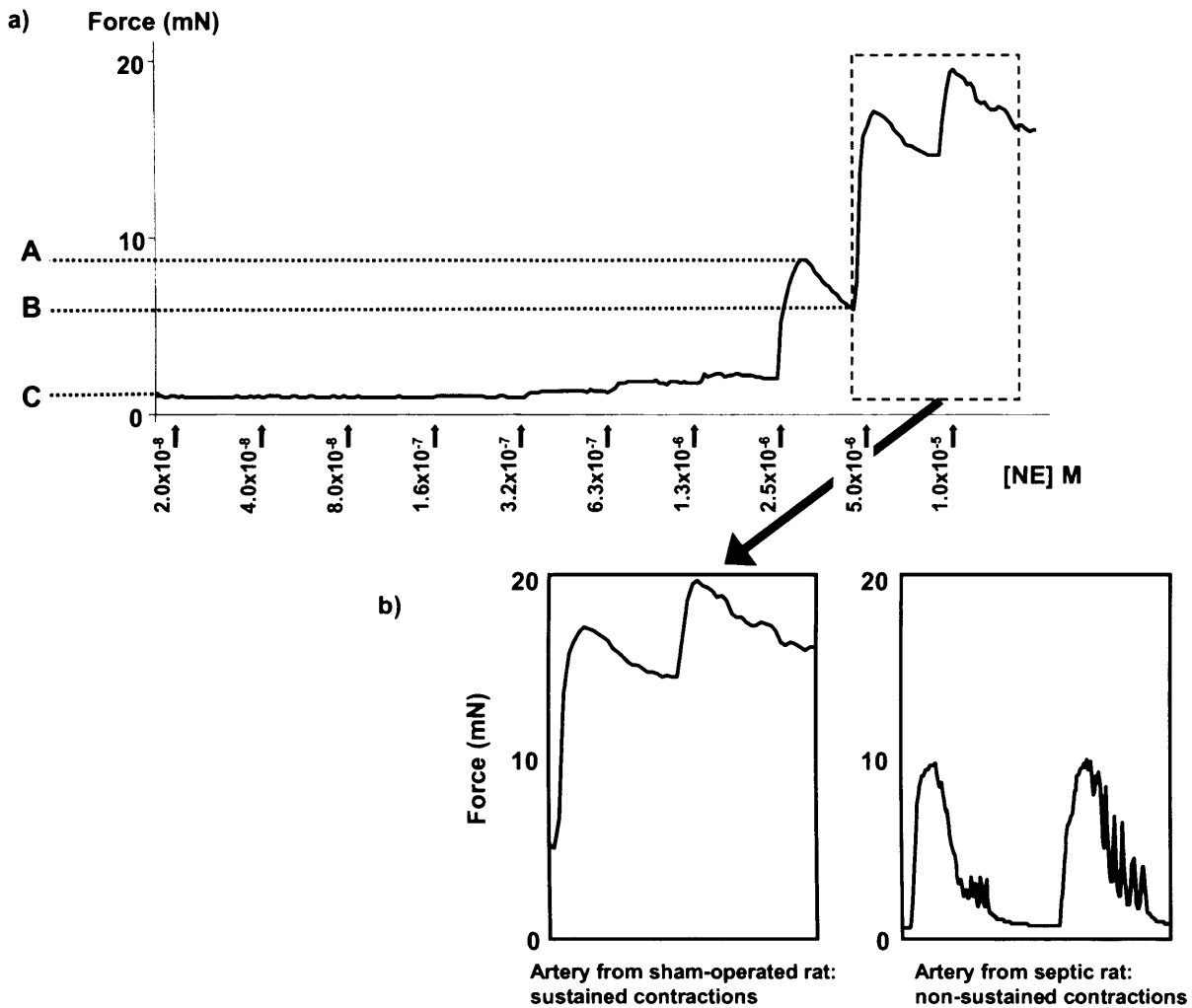
	Vessel diameter (µm)	Effective pressure (kPa)	Max Tension (N/m)	Potency of NE pD ₂	Potency of VP pD ₂
Mean	237	26.33	2.79	5.76	-
SEM	(6)	(1.78)	(0.28)	(0.08)	
n (vessels)	107	24	24	24	-

3.3.2 *Ex-vivo* reactivity of small mesenteric arteries to norepinephrine

Figure 3.4 shows a typical myograph recording from a norepinephrine dose-response experiment. The non-sustained nature of the contractions elicited by this agonist in arteries taken from septic animals (Figure 3.4b) necessitated measurement of both peak and sustained responses (Figure 3.4a).

Combined concentration-response curves of septic arteries were significantly right shifted ($p < 0.0001$) when compared to sham-control arteries (Figure 3.5a), this effect being more pronounced when sustained tensions were plotted (Figure 3.5b). The efficacy of NE, as measured by maximum contractile response (E_{max}), was significantly decreased in arteries taken from septic animals, and again this was more marked when comparing sustained tension values (Table 3.2). Such analyses also un-masked a trend towards a decreased potency of NE in septic vessels (Table 3.2), though this did not reach statistical significance.

Figure 3.4 Myograph recordings in response to cumulative addition of norepinephrine (NE)

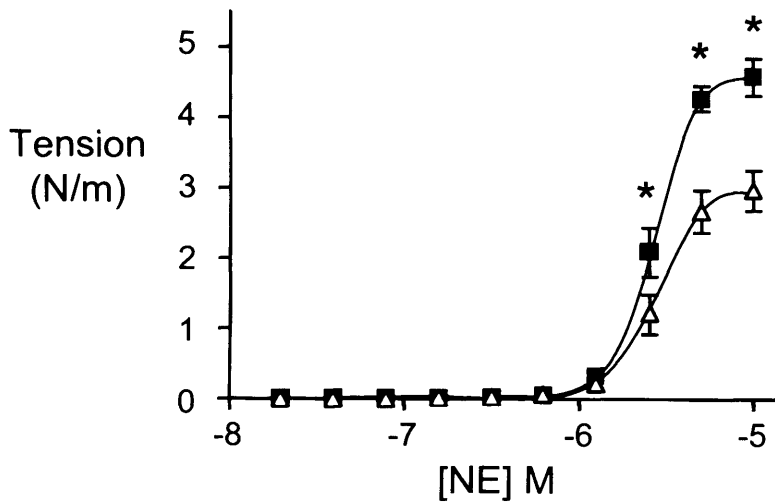


a) Complete force recording from a segment of small mesenteric artery from a sham-operated rat. Arrows indicate the addition of each NE dose (2×10^{-8} to $10^{-5} M$). Peak (A-C) and sustained (B-C) tension measurements were made for each dose step.

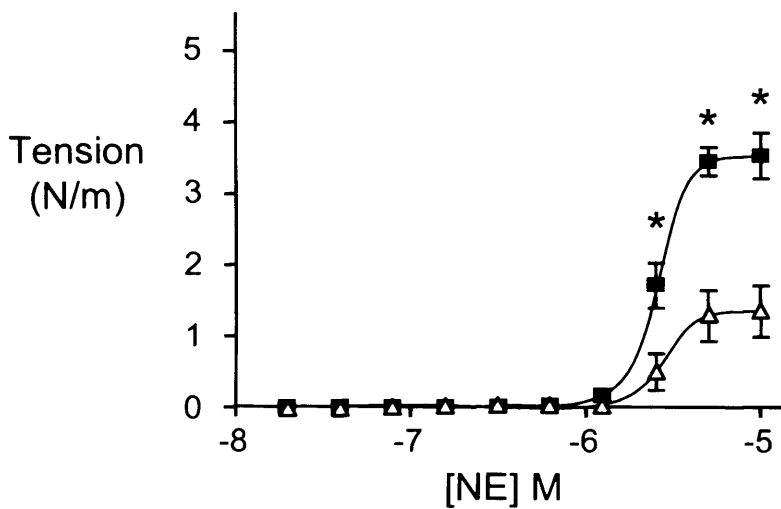
b) Close-up view comparing portions of tension recordings from a sham-operated and a septic rat artery, showing the typical smaller, non-sustained contractions observed in the latter.

Figure 3.5 *Ex-vivo* reactivity of small mesenteric arteries to norepinephrine (NE)

a)



b)



Mean concentration-response curves of a) peak and b) sustained tension obtained from arteries taken from sham-operated (squares, $n=9$) and septic (triangles, $n=12$) rats

* $p < 0.05$ for comparison between sham and septic arteries

Table 3.2 Ex-vivo reactivity to norepinephrine: E_{max} (maximal tension) and pD₂ (-logEC₅₀, potency) values

		E_{max}	pD₂
Sham-operated n=9	peak	4.69 (0.24)	5.60 (0.04)
	sustained	3.83 (0.24)	5.59 (0.05)
Septic n=12	peak	2.98* (0.28)	5.54 (0.04)
	sustained	1.57* (0.35)	5.36 (0.11)

Values shown are mean (SEM).

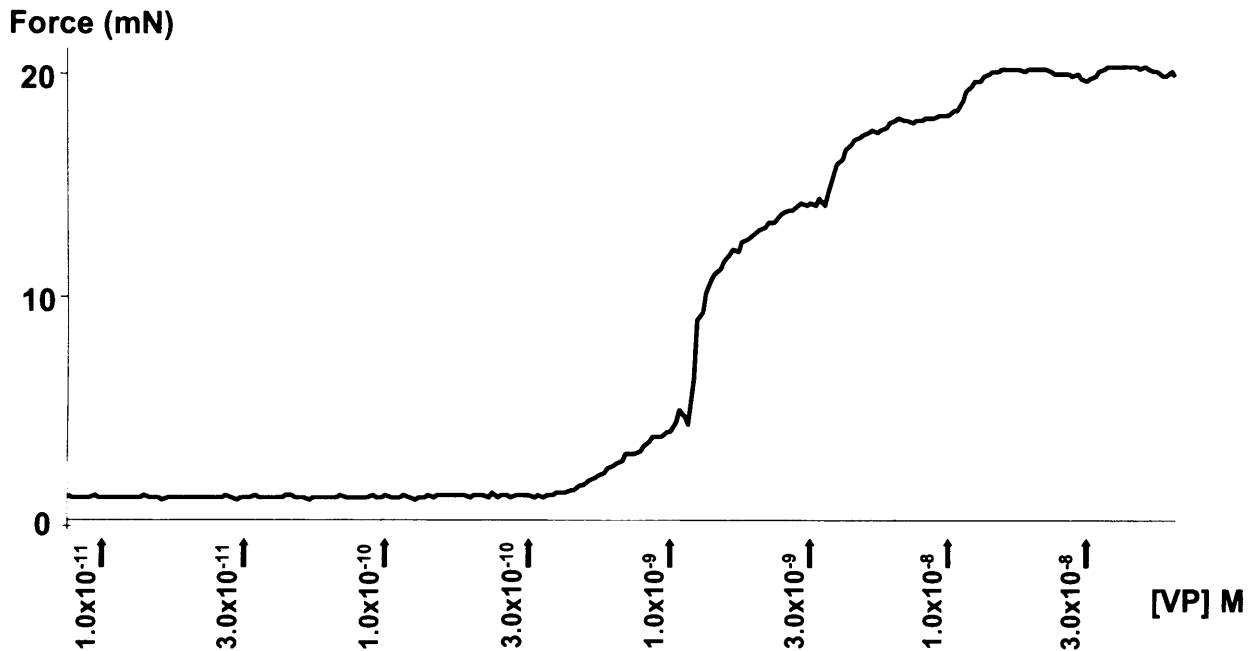
** p<0.05 for comparison between sham and septic groups*

3.3.3 *Ex-vivo* reactivity of small mesenteric arteries to vasopressin and the V₁R agonist, F-180

Figure 3.6 shows a typical myograph recording from a vasopressin dose-response experiment. In arteries from sham-operated and septic rats, contractions elicited by both VP and F-180 were maintained across the four minute dose interval employed.

In contrast to the pattern observed with NE, there was a significant leftward shift ($p=0.016$) of the VP concentration-response curves of septic compared to sham arteries, with minimal overall difference between peak and sustained measurements within groups (Figures 3.7a and b). VP efficacy (E_{max}) was not decreased in septic arteries, while pD_2 values were significantly increased (Table 3.3). Results obtained with the selective V₁R agonist, F-180 showed an exaggeration of the changes seen with VP; there was a half log unit leftward shift of the septic artery concentration-response curve ($p<0.0001$) (Figure 3.7c), and a significant increase in agonist efficacy and potency (Table 3.3). Again there were minimal differences between peak and sustained measurements within groups for this agonist (sustained data not shown).

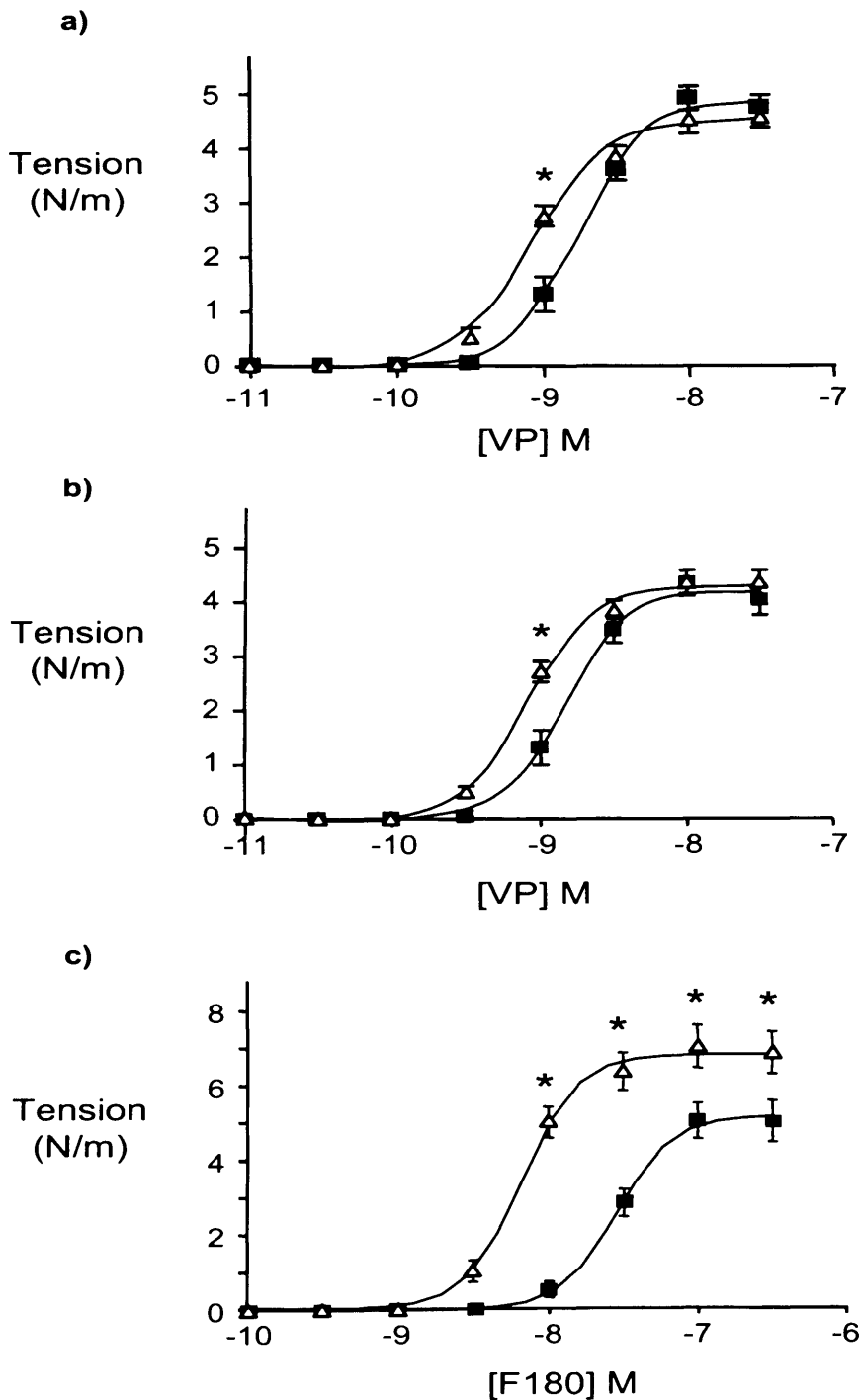
Figure 3.6 Myograph recording in response to cumulative addition of vasopressin (VP)



Complete force recording from a segment of small mesenteric artery from a septic rat. Arrows indicate the addition of each VP dose (10^{-11} to 3×10^{-8} M).

In contrast to contractions elicited by NE in septic arteries, the peak response to each concentration of VP was maintained across the four minute dose interval employed. Dose-response recordings from sham-operated control arteries were of a similar morphology (not shown).

Figure 3.7 Ex-vivo reactivity of small mesenteric arteries to vasopressin (VP) and F-180



Mean vasopressin concentration-response curves of a) peak and b) sustained tension obtained from arteries taken from sham-operated (squares, n=9) and septic (triangles, n=13) rats

c) Mean F-180 concentration-response curves of peak tension obtained from arteries taken from sham-operated (squares, n=6) and septic (triangles, n=6) rats

* p<0.05 for comparison between sham and septic arteries

Table 3.3 Ex-vivo reactivity to vasopressin and F-180: E_{max} (maximal tension) and pD₂ (-logEC₅₀, potency) values

	Vasopressin		F-180	
	E _{max}	pD ₂	E _{max}	pD ₂
Sham-operated n=9 (VP), 6 (F-180)	5.00 (0.21)	8.74 (0.05)	5.11 (0.54)	7.60 (0.07)
Septic n=13 (VP), 6 (F-180)	4.62 (0.23)	9.07* (0.04)	6.84 # (0.56)	8.18* (0.04)

Values shown are mean (SEM) from plots of peak tension responses.

** p<0.05 and #p=0.05 for comparison between sham and septic groups*

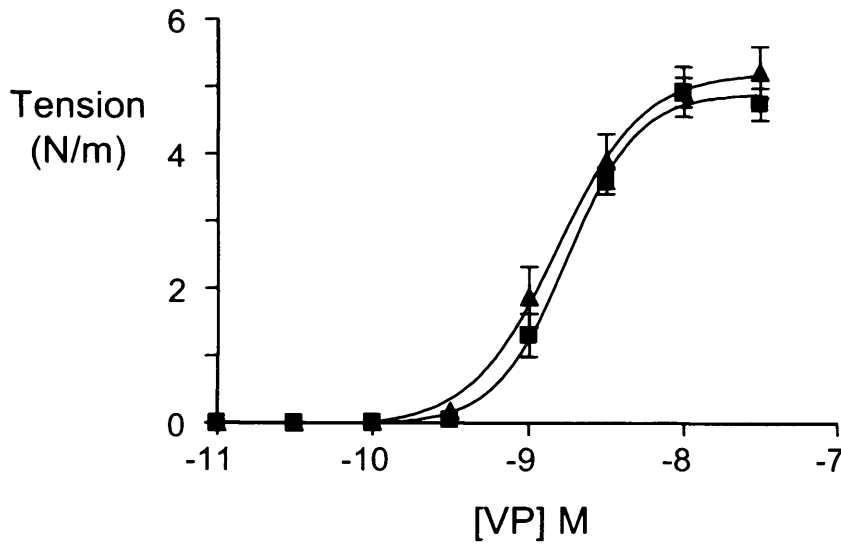
3.3.4 Effect of endothelial removal on vasopressin and F-180 responses

Removal of the endothelium from arteries taken from sham-operated rats did not alter reactivity to VP (Figure 3.8a and Table 3.4). However, arteries from septic animals became more sensitive to the effects of this agonist at concentrations below 3nM after endothelial rubbing (Figure 3.8b and Table 3.4).

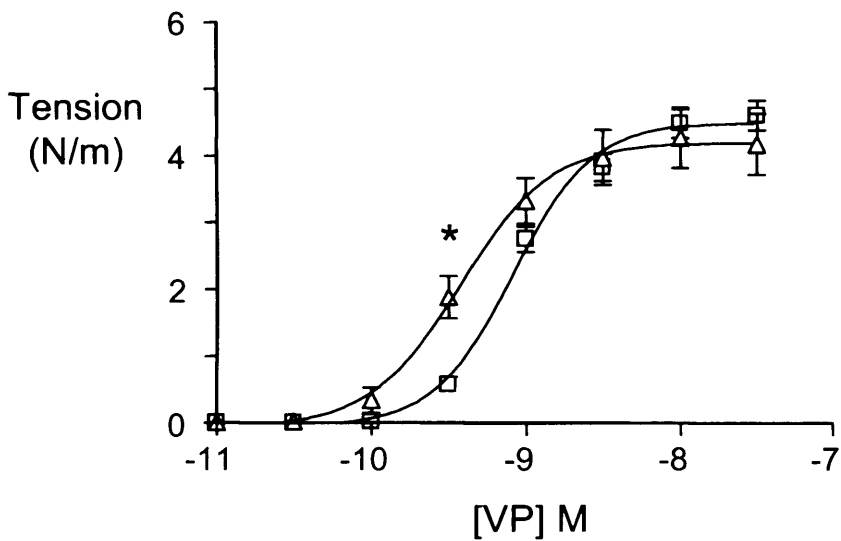
In contrast to VP responses in sham arteries, a dramatic leftward shift of the F-180 concentration response curve was observed in sham vessels denuded of endothelium ($p < 0.001$, Figure 3.9a). There was a clear increase in agonist sensitivity but only a trend to increased efficacy. In septic arteries, a small, but significant enhancement of F-180 potency was seen after endothelial removal (Figure 3.9b and Table 3.4).

Figure 3.8 Effect of endothelial removal on vasopressin (VP) concentration-response curves

a)



b)

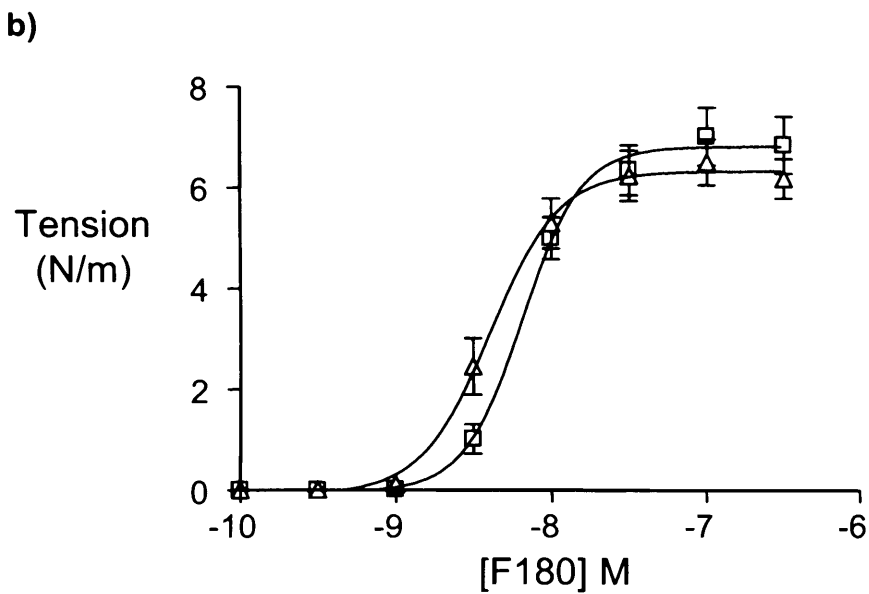
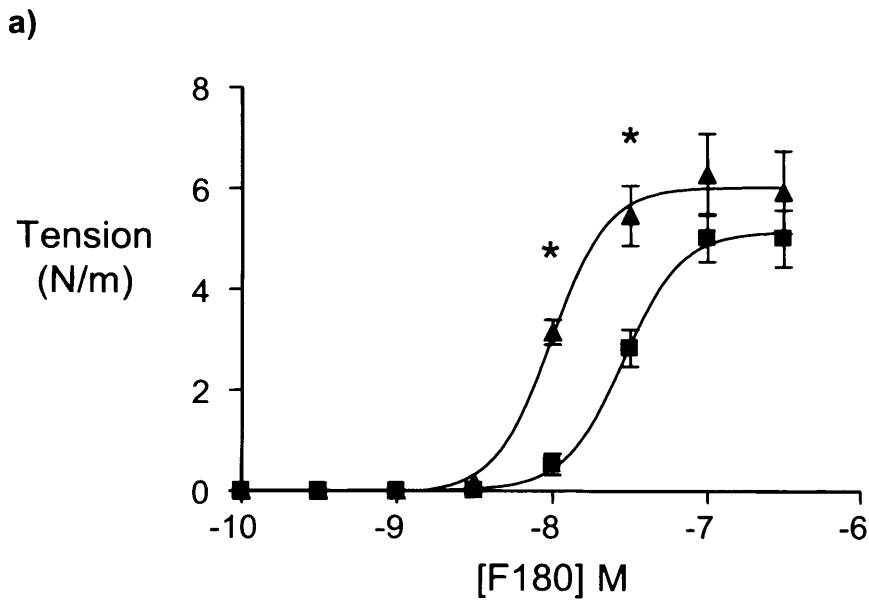


a) Mean concentration-response curves of peak tension obtained with arteries taken from **sham-operated** rats with intact (squares, n=9) and denuded endothelium (triangles, n=4).

b) Mean concentration-response curves of peak tension obtained with arteries taken from **septic rats**, with intact (squares, n=13) and denuded endothelium (triangles, n=4)

* $p < 0.05$ for comparison between vessels with intact and removed endothelium within sham-operated and septic groups

Figure 3.9 Effect of endothelial removal on F-180 concentration-response curves



a) Mean concentration-response curves of peak tension obtained with arteries taken from **sham-operated** rats with intact (squares, n=6) and denuded endothelium (triangles, n=6).

b) Mean concentration-response curves of peak tension obtained with arteries taken from **septic** rats, with intact (squares, n=6) and denuded endothelium (triangles, n=6)

* $p < 0.05$ for comparison between vessels with intact and removed endothelium within sham-operated and septic groups

Table 3.4 Effect of endothelial removal: E_{max} and pD₂ values

a) Sham-operated arteries

	Vasopressin		F-180	
	E _{max}	pD ₂	E _{max}	pD ₂
Endothelium intact n=9(VP), 6(F-180)	5.00 (0.21)	8.74 (0.05)	5.11 (0.54)	7.60 (0.07)
Endothelium removed n=4(VP), 6(F-180)	5.23 (0.38)	8.82 (0.08)	6.17 (0.77)	8.00* (0.06)

b) Septic arteries

	Vasopressin		F-180	
	E _{max}	pD ₂	E _{max}	pD ₂
Endothelium intact n=13(VP), 6(F-180)	4.62 (0.23)	9.07 (0.04)	6.84 (0.56)	8.18 (0.04)
Endothelium removed n=4(VP), 6(F-180)	4.29 (0.45)	9.43* (0.10)	6.30 (0.44)	8.38* (0.06)

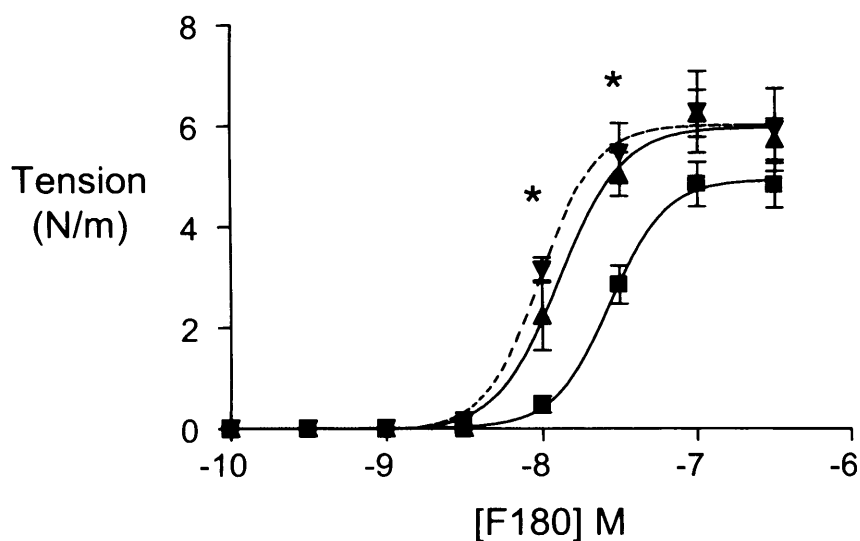
Values shown are mean (SEM) from plots of peak tension responses.

** p<0.05 for comparison between vessels with intact and removed endothelium within sham-operated and septic groups*

3.3.5 Effect of nitric oxide synthase inhibition on contractile responses to F-180

The leftward shift of the F-180 concentration-response curve induced by endothelial denudation in sham-operated rat arteries was similar to that observed with the application of the NOS inhibitor, L-NAME (100 μ M), 30 minutes prior to agonist exposure (Figure 3.10).

Figure 3.10 Effect of L-NAME on contractile responses to F-180 in arteries from sham-operated rats



Mean concentration-response curves of peak tension obtained with arteries taken from sham-operated rats, in the presence (upward triangles, $n=3$) or absence (squares, $n=9$) of NG-nitro-L-arginine methyl ester (L-NAME) 100 μ M. The contractile response to F-180 in endothelium denuded sham-operated arteries from Figure 3.9a (dotted line, downward triangles) is overlaid for visual comparison.

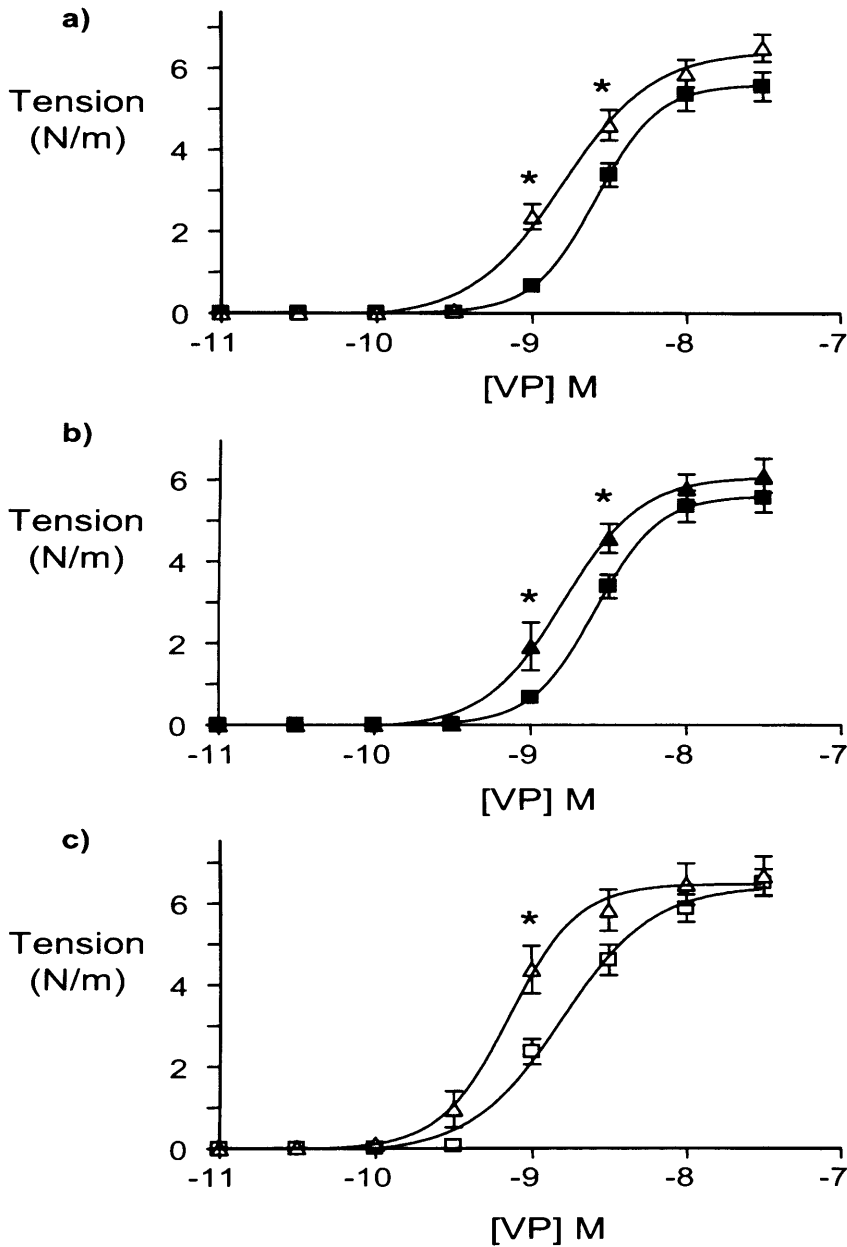
* $p < 0.05$ for comparison between endothelium-intact curves with and without L-NAME

3.3.6 Role of V₂ receptor in vasopressin responses in small mesenteric arteries

As these studies were performed at a later date and in a cohort of animals separate to those in which *ex-vivo* reactivity to VP was characterised (Section 3.3.3), I first reconfirmed the overall pattern of enhanced responses in arteries from septic rats (Figure 3.11a). Endothelial removal had a similar effect on dose-response curves obtained from septic arteries (Figures 3.8b and 3.11c). Unlike previous studies, however, in this batch of experiments the sham-operated concentration-response curves to VP were shifted to the left following endothelial removal (Figure 3.8a and 3.11b).

Pre-incubation of vessels with the V₂ receptor antagonist, FE992082 (30nM), produced no statistically significant changes in the *ex-vivo* reactivity to VP in sham or septic arteries whether the endothelium was intact or removed (Figures 3.12 and 3.13 and Table 3.5). However, a numerical increase in the VP sensitivity of endothelium-intact sham vessels in the presence of the antagonist was observed (pD_2 8.75 ± 0.10 vs. 8.57 ± 0.03, $p=0.098$).

Figure 3.11 Effect of endothelial removal on vasopressin (VP) concentration-response curves: V₂ receptor antagonist experimental cohort



a) Mean concentration-response curves of peak tension obtained from arteries taken from sham-operated (squares, n=8) and septic (triangles, n=6) rats

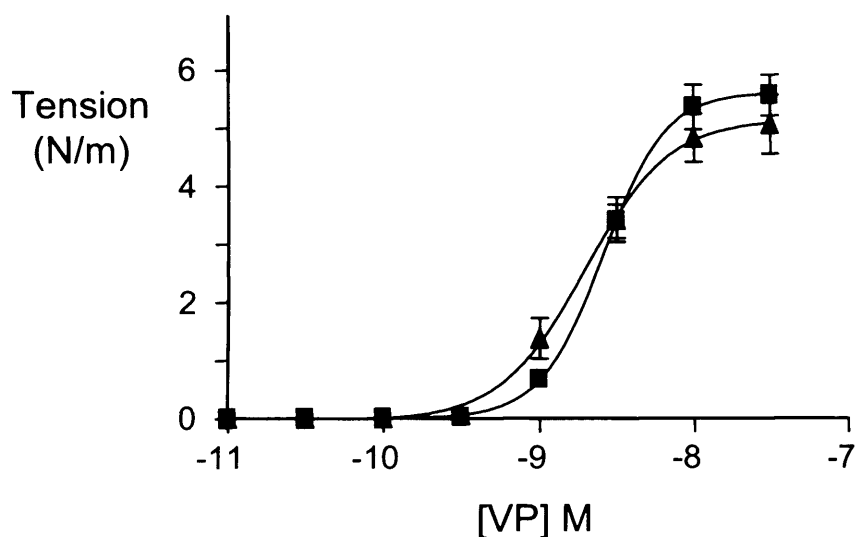
b) Mean concentration-response curves of peak tension obtained with arteries taken from **sham-operated** rats, with intact (squares, n=8) and denuded endothelium (triangles, n=6)

c) Mean concentration-response curves of peak tension obtained with arteries taken from **septic** rats, with intact (squares, n=6) and denuded endothelium (triangles, n=6)

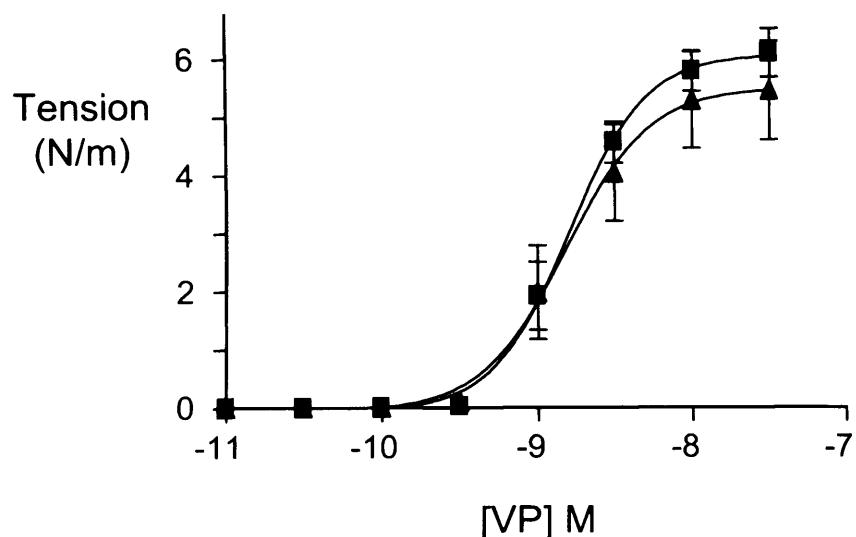
* p<0.05 for comparison between sham and septic vessels in a), and between vessels with intact and removed endothelium within sham-operated and septic groups in b) and c)

Figure 3.12 Effect of V₂ receptor antagonist (FE992082) on vasopressin (VP) concentration-response curves in sham-operated rat arteries

a) Endothelium intact



b) Endothelium removed

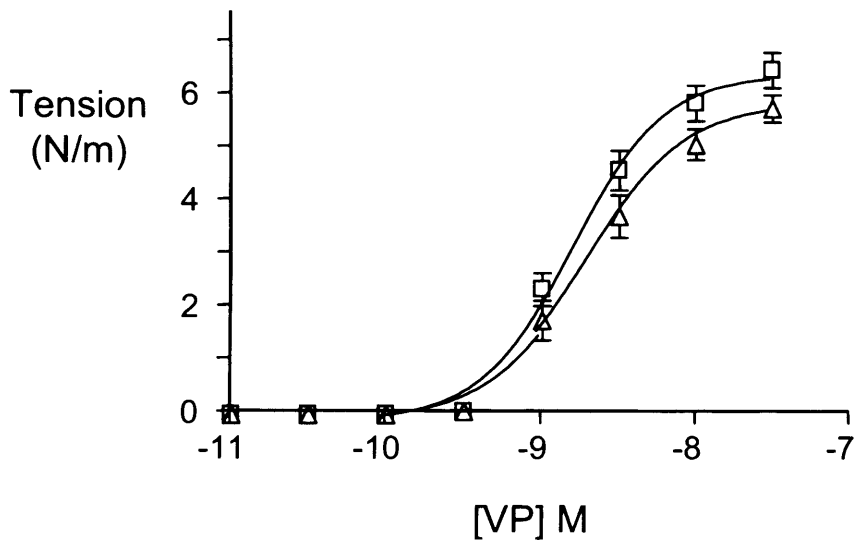


a) Mean concentration-response curves of peak tension obtained from arteries with intact endothelium in the presence (triangles, n=8) or absence (squares, n=8) of the V₂ receptor antagonist FE992082 (30nM)

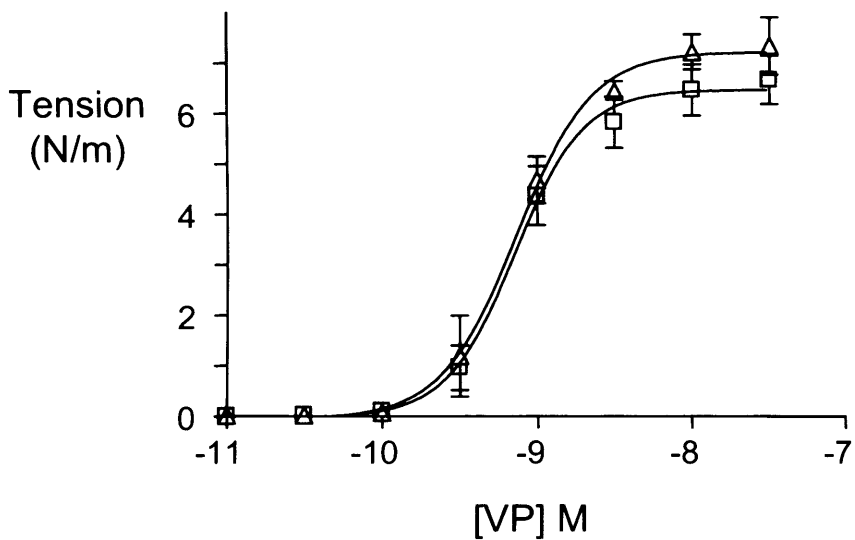
b) Mean concentration-response curves of peak tension obtained with arteries with denuded endothelium in the presence (triangles, n=6) or absence (squares, n=6) of FE992082 (30nM)

Figure 3.13 Effect of V₂ receptor antagonist (FE992082) on vasopressin (VP) concentration-response curves in septic rat arteries

a) Endothelium intact



b) Endothelium removed



a) Mean concentration-response curves of peak tension obtained from arteries with intact endothelium in the presence (triangles, n=7) or absence (squares, n=6) of the V₂ receptor antagonist FE992082 (30nM)

b) Mean concentration-response curves of peak tension obtained with arteries with denuded endothelium in the presence (triangles, n=6) or absence (squares, n=6) of FE992082 (30nM)

Table 3.5 Effect of V₂ receptor antagonist (FE992082) on E_{max} and pD₂ values

a) Sham-operated arteries:

	Endothelium intact		Endothelium removed	
	E +		E-	
	E _{max}	pD ₂	E _{max}	pD ₂
FE992082 - n=8(E+), 6(E-)	5.67 (0.35)	8.57 (0.03)	6.03 (0.38)	8.80 (0.08)
FE992082 + n=8(E+), 6(E-)	5.05 (0.50)	8.75 (0.10)	5.43 (0.79)	8.80 (0.10)

b) Septic arteries:

	Endothelium intact		Endothelium removed	
	E +		E-	
	E _{max}	pD ₂	E _{max}	pD ₂
FE992082 - n=6(E+), 6(E-)	6.45 (0.34)	8.78 (0.08)	6.67 (0.48)	9.14 (0.07)
FE992082 + n=7(E+), 6(E-)	5.85 (0.29)	8.69 (0.11)	7.35 (0.57)	9.16 (0.14)

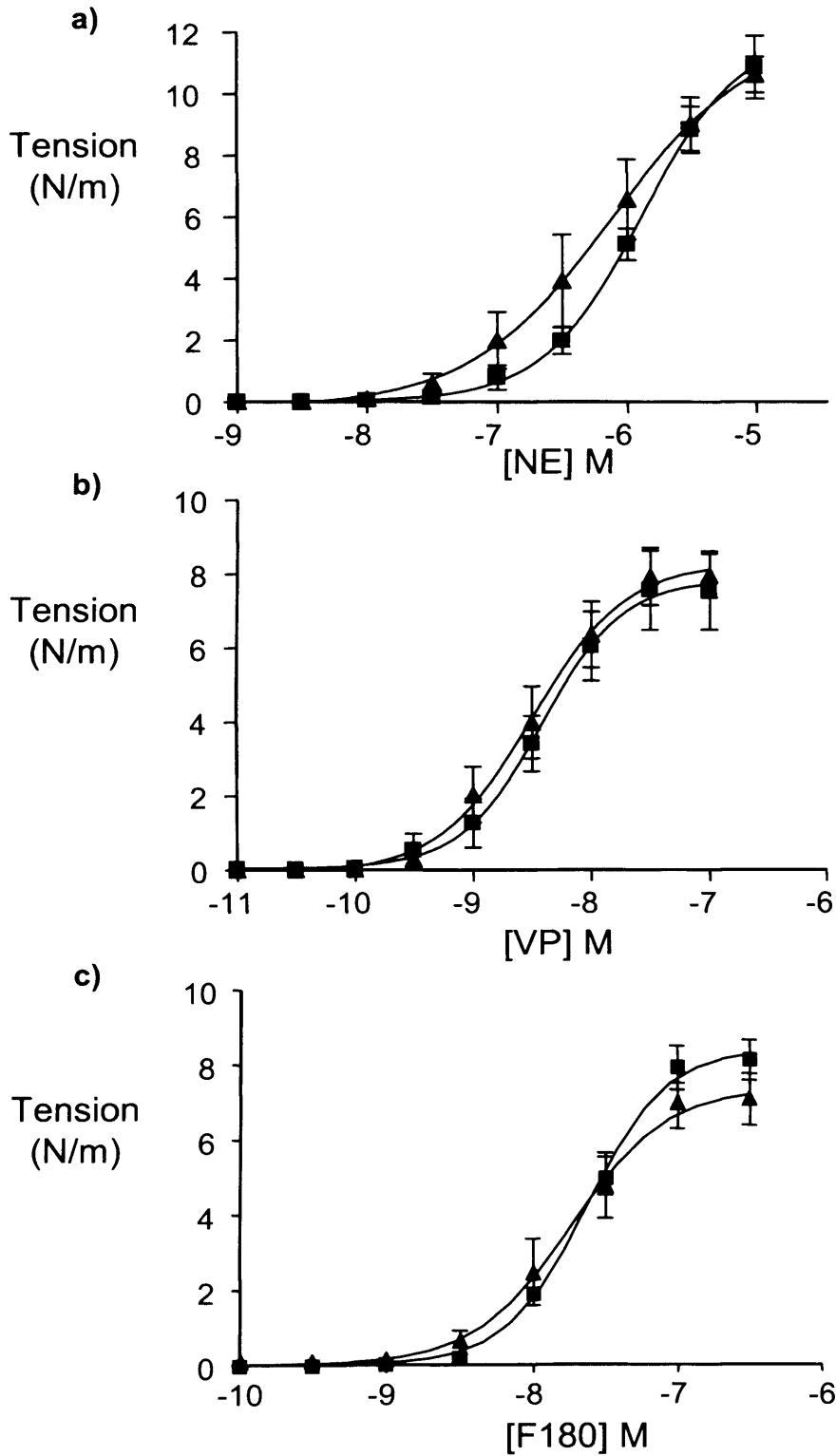
Values shown are mean (SEM) from plots of peak tension responses.

There were no statistically significant differences found between FE992082 present and FE992082 absent values when compared within endothelium intact or removed groups.

3.3.7 *Ex-vivo* reactivity of tail arteries from sham-operated and septic rats

To examine the reactivity of a vascular bed further away from the initial site of infection, contractile responses to NE, VP and F-180 were examined in tail arteries taken from septic and sham-operated rats. These vessels had larger normalised diameters than the small mesenteric arteries used in previous experiments (c.500µm c.f. 200µm). I found there to be no significant differences between the sham and septic concentration-response curves obtained with any of the three agonists used (Figure 3.14 and Table 3.6). Moreover, in contrast to observations made with small mesenteric arteries, contractile responses to NE were sustained in nature in both septic and sham-operated rat vessels.

Figure 3.14 *Ex-vivo* reactivity of tail arteries to norepinephrine (NE), vasopressin (VP) and F-180



Mean concentration-response curves of peak tension for a) norepinephrine, b) vasopressin and c) F-180 obtained from arteries taken from sham-operated (squares, n=5) and septic (triangles, n=5) rats

Table 3.6 Ex-vivo reactivity of tail arteries to norepinephrine, vasopressin and F-180: E_{max} (maximal tension) and pD₂ (-logEC₅₀, potency) values

	Norepinephrine		Vasopressin		F-180	
	E _{max}	pD ₂	E _{max}	pD ₂	E _{max}	pD ₂
Sham-operated n=5	12.03 (1.14)	5.89 (0.04)	7.76 (1.04)	8.45 (0.15)	9.23 (1.05)	7.63 (0.03)
Septic n=5	11.23 (0.55)	6.22 (0.25)	8.17 (0.57)	8.48 (0.14)	7.37 (0.71)	7.75 (0.17)

Values shown are mean (SEM) from plots of peak tension responses.

There were no statistically significant differences between sham-operated and septic arteries when compared for each agonist.

3.4 Discussion

I hypothesised that the heightened vascular reactivity to vasopressin observed in patients with septic shock was due to changes occurring within the resistance vasculature rather than through external influences from the autonomic nervous system. The method of wire myography described in this chapter permits direct investigation of the pharmacological properties of resistance arteries in an environment removed from the confounding effects of circulating vasoactive substances and neural inputs. Consistent with the *in-vivo* responses observed in anaesthetised rats (Sections 2.3.6 and 2.4.3), in arteries taken from septic animals after 24 hours of faecal peritonitis, I found decreased efficacy and potency of NE, but preserved efficacy and increased potency of VP. An exaggeration of the pattern of change of vascular reactivity to VP in sepsis was seen when dose-response studies were performed with the selective V₁R agonist, F-180. These results suggest that the V₁R vasoconstrictor pathway is up-regulated in mesenteric resistance arteries in this model of faecal peritonitis.

3.4.1 *Ex-vivo* vascular reactivity of small mesenteric arteries to norepinephrine

Hyporeactivity to catecholamines has been demonstrated in many *ex-vivo* and *in-vitro* animal models of sepsis [Baker & Wilmoth, 1984; Julou-Schaeffer *et al.*, 1990]. Rat mesenteric arteries are frequently used in such studies [Mitolo-Chieppa *et al.*, 1996; O'Brien *et al.*, 2001], although some have reported

decreased NE responses in such small vessels to be dependent on exogenous L-arginine, the substrate for nitric oxide synthase [Schneider *et al.*, 1992; Schneider *et al.*, 1994; Martinez *et al.*, 1996]. Hyporesponsiveness is likely to be multifactorial (Section 1.3.2), though down-regulation of α_1 R_s in response to high circulating catecholamine levels probably contributes [Hwang *et al.*, 2003].

In addition to decreased maximal tension responses, I observed a marked difference in the morphology of the NE-induced contractions in septic rat arteries. Despite continued presence of agonist, contractions were often transient in nature, whereas those produced in sham vessels were largely maintained (Figure 3.4). This phenomenon has been reported previously [Martinez *et al.*, 1996], and may indicate an alteration in calcium handling within the vascular smooth muscle. As described in Chapter 1 (Section 1.1.2), sustained vasoconstriction is dependent upon both elevation of intracellular Ca^{2+} (Ca^{2+} activation) and Ca^{2+} sensitisation [Horowitz *et al.*, 1996; Wier & Morgan, 2003], and these processes may be altered in sepsis (Chapters 4 and 5). An alternative hypothesis is that NE cannot produce a sustained effect due to its rapid breakdown. Superoxide anions generated in sepsis auto-oxidise catecholamines, and in an *in-vivo* rat endotoxin model, administration of a superoxide dismutase mimetic was shown to reverse hyporeactivity to norepinephrine [Macarthur *et al.*, 2000]. Whether a sufficient concentration of free radicals could be generated in the extracellular bathing fluid of the myograph chamber is unknown. Nevertheless, septic arteries tended to be normalised to smaller diameters than sham arteries (Section 3.2.2); this may

have been due to increased basal tone related to the production of superoxide anions [Hernanz *et al.*, 2003].

3.4.2 *Ex-vivo* vascular reactivity of small mesenteric arteries to vasopressin and the V₁receptor agonist, F-180

Although *ex-vivo* hypersensitivity to vasopressin has been found in other rodent septic models [Baker *et al.*, 1990; Tarpey *et al.*, 1998], the current work represents the first demonstration of a temporal association of normal endogenous VP levels with heightened vascular reactivity to exogenous hormone. Depressed VP reactivity has been reported in studies where endotoxin or an NO-donor have been used to simulate sepsis *in-vitro* [Hamu *et al.*, 1999; Leone & Boyle, 2006]. The discrepancy between these results and my data may relate to the ability of my model to more closely mimic the human condition. Interestingly, the point of maximal separation of the septic and sham-operated VP concentration-response curves (Figure 3.7a) corresponds to circulating plasma levels (c.100pg/ml) seen following treatment of septic patients with VP [Dunser *et al.*, 2004a].

Increased vasopressinergic reactivity in septic arteries was more pronounced when V₁R responses were selectively examined with F-180 (Figure 3.7c). Taken together, the VP and F-180 results suggest enhanced V₁R-mediated vasoconstriction. The wider separation of the septic and sham-operated F-180 concentration-response curves compared to that seen with VP suggests that VP also activates non-V₁R vasodilatory pathways, and that these may too be

up-regulated in sepsis. Enhanced V_1R -mediated contraction could be a consequence of increased receptor number or affinity, more efficient coupling to downstream second messengers, or alterations in Ca^{2+} handling mechanisms. Sustained contractile responses with VP and F-180 in septic arteries (Figure 3.6) imply adequate Ca^{2+} mobilisation and sensitisation. Whereas high circulating levels of catecholamines in sepsis might contribute to desensitisation of the α_1R , normal plasma VP concentrations provide no similar stimulus for V_1R down-regulation. However, two published studies have reported a decrease in V_1R gene expression in liver, lung, kidney and heart taken from rats exposed to endotoxin for up to 24 hours, although blood vessels were not examined [Roth & Spitzer, 1987; Bucher *et al.*, 2002].

3.4.3 Role of the endothelium in vasopressin and F-180 responses

The effect of endothelial removal on vasopressin reactivity was examined in two cohorts of sham-operated and septic rats. In both sets of experiments, I found that arteries from septic animals became more sensitive to vasopressin after endothelial removal (Figure 3.8b and Figure 3.11c). The results obtained with endothelium-denuded sham vessels were inconsistent, with no change in VP responses in the first set of studies (Figure 3.8a), but a small increase in sensitivity in the second set (Figure 3.11b). Animal numbers were greater in the later cohort, but there was no difference between the percentage relaxation responses to ACh post endothelial removal in the two experimental sets (data not shown).

The finding that disruption of the endothelium can alter vasopressinergic reactivity confirms that part of the agonist response in intact arteries is mediated via this cell layer. The net vasoconstrictor or vasodilatory effect of VP within a vascular bed is likely to reflect the balance of different receptor subtypes within the regional vasculature (Figure 1.3). There is considerable evidence to support vasopressin-mediated vasodilatation via receptors located on the surface of endothelial cells, although whether V₁Rs, V₂Rs, OTRs or multiple subtypes are involved is controversial [Martinez *et al.*, 1994; Tagawa *et al.*, 1995; Okamura *et al.*, 1997; Okamura *et al.*, 1999; Thibonnier *et al.*, 1999] (Section 1.2.2). The ability of vasopressin to increase thromboxane and endothelin synthesis means that endothelium-mediated vasoconstrictor actions are also feasible [Siess *et al.*, 1986; Imai *et al.*, 1992]. The increase in agonist sensitivity I found following endothelial rubbing implies net disruption of vasodilatory pathways. My results suggest that such pathways operate in both sham-operated and septic arteries, but possibly to a greater extent in the latter.

Further conclusions are difficult from the arginine-vasopressin studies as all receptor subtypes present would have been simultaneously activated by this agonist. Valuable information was gained from the comparable F-180 experiments, however. Endothelial removal in sham-operated arteries produced a profound increase in the sensitivity of this selective V₁R agonist (Figure 3.9a). This strongly supports the existence of this receptor subtype on the endothelium, which, under normal conditions, is activated to oppose the vasoconstrictor effect of V₁Rs on the smooth muscle layer. The similar increase in potency seen with the NOS inhibitor, L-NAME (Figure 3.10)

substantiates the hypothesis that the mechanism of vasorelaxation is via endothelial-derived NO, as previous studies have reported for vasopressin [Tagawa *et al.*, 1993; Okamura *et al.*, 1999]. In contrast to the results obtained with sham vessels, endothelial denudation of septic arteries produced only a small increase in F-180 potency. This difference could be explained by a specific dampening of the V₁R vasodilatory pathway in sepsis, or by the more pervasive effects on endothelial vasomotor control recognised in this clinical scenario [Scott *et al.*, 2002; Aird, 2003].

3.4.4 Contribution of the V₂ receptor to vasopressin responses

In-vitro studies have demonstrated V₂R expression on human endothelial cells [Kaufmann *et al.*, 2003], and infusion of the selective V₂R agonist, desmopressin to healthy human volunteers has been shown to produce forearm vessel dilatation [van Lieburg *et al.*, 1995]. With this evidence supporting the existence of vascular V₂Rs in mind, I investigated the role of this receptor subtype in our model. To this end, I was kindly given FE992082, a compound developed by Ferring Pharmaceuticals to selectively block the V₂R in the rat. Unlike F-180, the selectivity data for this peptide antagonist has not been published, but the dose used in my experiments (30nM) was chosen on the basis of the V₁R and V₂R K_i values (i.e. the concentration of antagonist required to produce a 50% decrease in receptor activity in the presence of a maximal concentration of agonist) provided by the manufacturer (65nM and 0.6nM, respectively).

Disappointingly, I found no statistically significant changes to vasopressin reactivity in arteries from sham-operated or septic rats, whether studied with or without an intact endothelium. The only hint that vasodilatory V₂Rs were present was given by the trend towards increased VP sensitivity in the presence of FE992082 when endothelium-intact sham vessels were studied. An endothelial location for these receptors is suggested by the disappearance of this trend once the endothelium was removed. The lack of a similar effect in septic arteries conflicts somewhat with the preceding discussion in which enhanced non-V₁R vasodilatory pathways in sepsis were proposed (Section 3.4.2).

These largely negative findings can be interpreted in several ways. A non-significant number of V₂Rs may be present in small mesenteric arteries of the rat. Indeed, most studies reporting the presence of vascular V₂Rs have been carried out in humans [Tagawa *et al.*, 1993; Tagawa *et al.*, 1995; Martinez *et al.*, 1994; Kaufmann *et al.*, 2003], and such binding sites could not be localised using a receptor selective radioligand in rat vessels [Phillips *et al.*, 1990]. It may be that oxytocin receptors account for non-V₁R-mediated relaxation rather than V₂Rs [Thibonnier *et al.*, 1999] (Figure 1.3). An alternative explanation is that an inadequate dose of the antagonist was used. A number of experiments were performed using three times the dose of FE992082 (100nM) (c.150 x K_i), but this produced no greater increase in VP sensitivity (data not shown). A further possibility is that the compound has inadequate selectivity for the V₂R, and that any V₂R-blocking effect promoting a contractile response is masked by a coincident V₁R-blocking effect. A recent

consultation with Ferring Pharmaceuticals revealed that this may well be the case.

This experience highlights the limitations of using supposedly selective receptor agonists or antagonists, and stresses the need for further studies in which vasopressin receptor mRNA and protein expression are directly quantified.

3.4.5 *Ex-vivo* reactivity of tail arteries to norepinephrine, vasopressin and F-180

To determine whether the patterns of *ex-vivo* vascular reactivity demonstrated in sham and septic rat arteries were evident outside the mesenteric circulation, I investigated the responses of tail arteries from the same groups of animals to NE, VP and F-180. Rat tail arteries have been used in the wire myograph for other pharmacological investigations in our laboratory [Orie *et al.*, 2006]. These vessels are easily accessible and are surrounded by little connective tissue, facilitating atraumatic dissection.

Neither hyporeactivity to NE nor hypersensitivity to VP or F-180 was observed in tail arteries from septic rats. Whilst this result raises questions as to the validity of our model, without broadening studies to other vascular beds it cannot be concluded that vasopressinergic hyper-reactivity is solely a characteristic of small mesenteric arteries. Separate investigations in our laboratory have demonstrated hyporeactivity to phenylephrine in aortic rings

taken from septic rats after 24 hours of faecal peritonitis (unpublished data), but VP reactivity is yet to be studied in these tissues. The proximity of small mesenteric arteries to the site of the infectious insult might mean that changes induced by the septic process are more apparent here. In addition, the particular ability of V₁R agonists to produce vasoconstriction of the mesenteric circulation is acknowledged, both in the treatment of portal hypertension and with regard to adverse side effects of their use in septic shock [Pesaturo *et al.*, 2006; Oliver & Landry, 2007].

The hypotension and altered *in-vivo* vasopressor responses in rats with faecal peritonitis (Chapter 2) are evidence of widespread, systemic circulatory changes consistent with septic shock. It should also be remembered that heterogeneity in tone across different vascular beds is well described in sepsis [Young, 2004]. Furthermore, tail arteries may be less representative of the resistance vasculature than small mesenteric vessels, by virtue of their larger internal diameters [Davis *et al.*, 1986].

3.4.6 Summary

In agreement with my *in-vivo* vascular reactivity studies, *ex-vivo* responses to norepinephrine were diminished in mesenteric resistance arteries taken from septic rats after 24 hours of faecal peritonitis. By contrast, vasopressinergic contractile responses were enhanced in septic vessels when compared to the sham-control group. This supports my hypothesis of an up-regulation of V₁R-mediated smooth muscle vasoconstrictor pathways in sepsis. This may be

due to changes occurring at the level of the receptor or further downstream. In addition, the existence of endothelial V₁R_s mediating vasodilatation via NO production is strongly suggested. This vasodilatory pathway appears depressed in sepsis.

Other receptors activated by vasopressin must be present in the mesenteric vascular bed to account for the differences observed between the VP and F-180 results. I was unable to demonstrate a role for the V₂R, however. This is an area that clearly requires further investigation.

Chapter 4: Investigation of the calcium mobilisation pathways contributing to norepinephrine- and vasopressin-induced contractions in small mesenteric arteries

4.1 Introduction

In Chapter 3, I described the differential effects of prolonged *in-vivo* sepsis on *ex-vivo* mesenteric arterial responses to norepinephrine and vasopressin. The increased sensitivity of septic arteries to vasopressin and the V₁R agonist, F-180 supports my hypothesis that the vascular smooth muscle V₁R-mediated contractile pathway is up-regulated in this disease process. While this may be a consequence of elevated plasmalemmal receptor number and/or affinity, an additional or alternative possibility is that receptor coupling to downstream calcium signalling is altered. Increased contractile responses could result from larger increases in intracellular Ca²⁺ concentration and/or heightened sensitivity of the contractile apparatus to Ca²⁺ (Ca²⁺ sensitisation) following receptor stimulation. While hyporeactivity to NE in sepsis has been attributed to down-regulation of vascular smooth muscle α₁Rs [Hwang *et al.*, 1994; Hotchkiss & Karl, 2003], my observation of non-sustained NE-induced contractile responses in arteries taken from septic rats (Figure 3.4) could also suggest impairment of adrenoceptor-coupled calcium mobilisation.

As reviewed in Chapter 1, both α₁Rs and V₁Rs are coupled through G_{q/11} to PLC, and the multiple pathways through which intracellular Ca²⁺ can be elevated following receptor stimulation are shown in Figure 1.2. Despite a

minor direct contribution to the myoplasmic Ca^{2+} pool in small arteries, emptying of the sarcoplasmic reticulum (SR) stores is thought to trigger a noteworthy Ca^{2+} influx via store-operated channels (SOCs) [Mulvany & Aalkjaer, 1990; Wier & Morgan, 2003]. There are two other types of sarcolemmal channel potentially mediating Ca^{2+} influx, namely the voltage-gated Ca^{2+} channel (VGCC), and the receptor-operated channel (ROC), a non-selective cation channel with significant permeability to Ca^{2+} . The idea that the various Ca^{2+} release and entry mechanisms contribute differentially to the contractile responses produced by agonists acting at different receptors is not new [Cauvin *et al.*, 1988; Large, 2002; Furutani *et al.*, 2002; Bauer & Parekh, 2003]. Moreover, the septic disease process may selectively impair and/or enhance Ca^{2+} mobilisation pathways [Steendijk, 2005]. For example, the opening of K_{ATP} channels in septic shock produces vascular smooth muscle hyperpolarisation, which would reduce the activity of VGCCs [Clapp & Tinker, 1998; Oliver & Landry, 2006]. In isolated vascular smooth muscle cells, nitric oxide (NO) appears to be involved in the regulation of both SOC and ROC Ca^{2+} currents [Moneer *et al.*, 2003]. Thus, supra-physiological NO levels in vasodilatory shock may impact on this regulation. Indeed, altered interactions between NO and calcium influx pathways have been shown to contribute to vascular dysfunction in animal models of pulmonary and portal hypertension [Atucha *et al.*, 2005; Jernigan *et al.*, 2006].

Through manipulation of the extracellular Ca^{2+} concentration and the use of drugs to differentially inhibit plasma membrane channels, the Ca^{2+} mobilisation profile activated by different receptors can be assessed. I used

such an approach in the wire myograph to dissect the pathways involved in mesenteric artery contractile responses to NE and VP. Responses were compared in vessels taken from rats with faecal peritonitis and from sham-operated controls.

4.2 Materials and Methods

4.2.1 Wire myography

Studies were performed on vessels taken from sham-operated and septic rats from the faecal peritonitis model described in Chapter 2. Twenty-four hours after intra-peritoneal injection of faecal slurry (or no injection in sham-operated controls), rats were deeply anaesthetised with isoflurane and then killed by cervical dislocation. Small mesenteric arteries were dissected as in Section 3.2.1. Arterial segments of 2mm in length were then mounted in an automatically operated dual chamber wire myograph (model 510A, Danish Myo Technology) for isometric tension recording (Figure 3.1). The vessels were bathed in 10ml of physiological salt solution (PSS), heated to 37°C and bubbled continuously with carbogen gas (95% O₂, 5% CO₂).

Thirty minutes after mounting, vessels were normalised to the physiologically realistic luminal diameters necessary for optimal force generation (Section 3.2.2). After a further 30 minutes' equilibration period, arteries were activated by three exposures to 5µM NE as described in Section 3.2.2. Effective pressure calculations were performed to confirm adequate vessel viability

before commencing the experimental protocols described below. The removal of several arteries from each animal allowed fresh arterial segments to be used for each experimental protocol.

4.2.2 Contribution to contraction of sarcoplasmic reticulum Ca^{2+} stores

The method employed is outlined in Protocol 1. Following activation, responses to E_{max} doses (“test doses”) of NE (10 μM) and VP (10nM) were measured. Extracellular Ca^{2+} was removed by exchanging normal PSS for Ca^{2+} -free PSS, in which CaCl_2 was replaced with 1mM of the Ca^{2+} chelator, EGTA. The subsequent contraction then produced by maximal agonist stimulation was deemed to be due solely to Ca^{2+} release from the SR (Figure 4.1). Percentage contribution of the SR to contractile responses in each vessel was calculated as follows:

$$\frac{\text{Peak tension response in } \text{Ca}^{2+}\text{-free PSS}}{\text{Test dose peak tension response in normal PSS}} \times 100.$$

Equilibration periods in normal PSS between agonist stimulations ensured adequate store refilling and recovery. High dose caffeine (20mM) was used to assess total intracellular store capacity.

4.2.3 Contribution to contraction of store-operated channels

The roles of SR stores and SOCs were assessed as part of the same experimental protocol. Contractions resulting from Ca^{2+} influx through SOCs

were measured when extracellular Ca^{2+} was returned following maximal agonist-mediated store depletion and then agonist wash-out (Figure 4.1) [Zhang *et al.*, 2002]. Percentage contribution of SOCs to contractile responses in each vessel was calculated as follows:

$$\frac{\text{Peak tension response on Ca}^{2+}\text{-add back}}{\text{Test dose peak tension response in normal PSS}} \times 100.$$

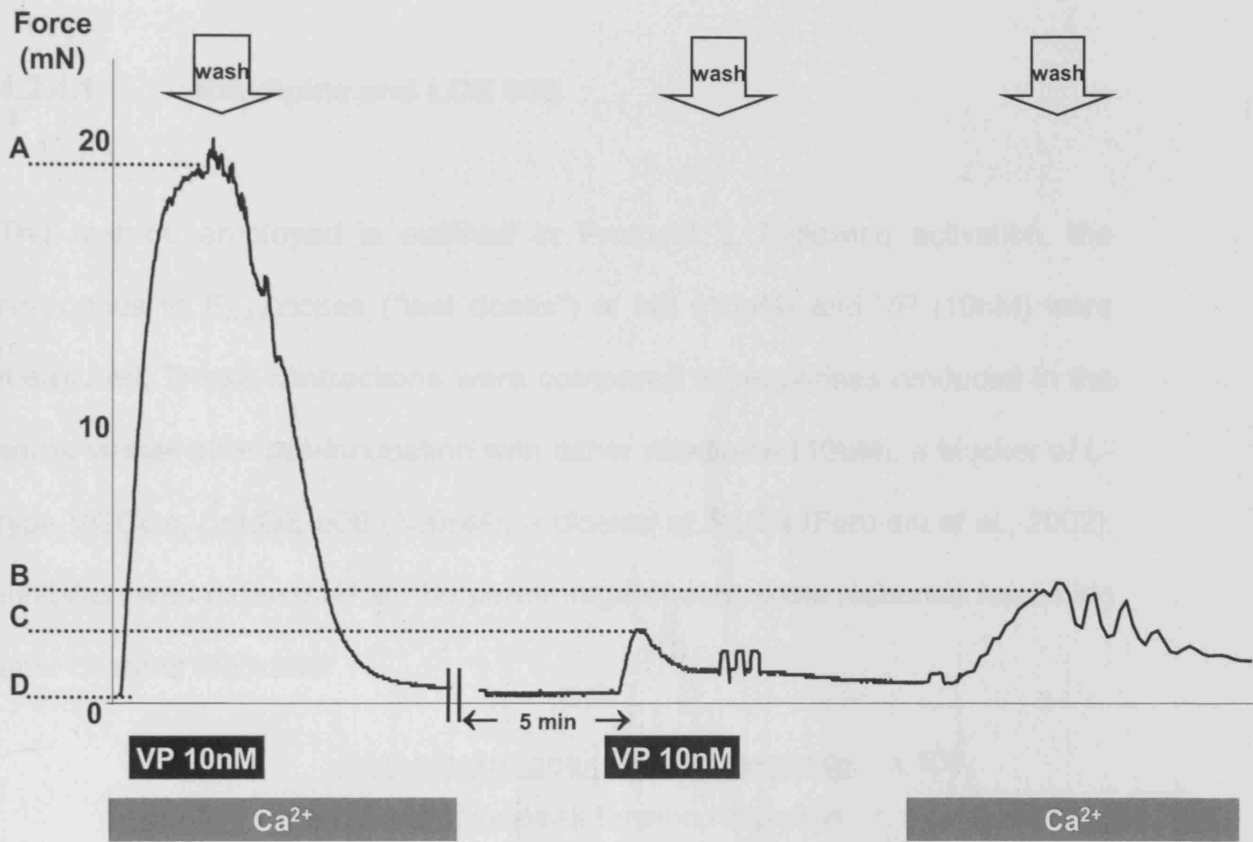
Protocol 1: Ca²⁺-free / add-back experiments

1. Normalisation
2. Activation NE 5µM x 3
3. **Test doses** **NE 10µM, VP 10nM**
4. Replace PSS with Ca²⁺ free PSS, leave 5 minutes
5. Caffeine 20mM
6. Wash x 3 with normal PSS and rest 10 minutes
7. Replace with Ca²⁺ free PSS, leave 5 minutes
8. **Agonist 1**, allow to plateau = **SR response**
9. Wash x 3 with Ca²⁺ free PSS, leave 5 minutes
10. Return Ca²⁺ as normal PSS, allow to plateau = **SOC response**
11. Wash x 3 with normal PSS and rest 10 minutes
12. Repeat 7-11 with **Agonist 2**
13. Repeat 7-11 with **Agonist 1 or 2** (to check reproducibility)
14. Repeat 7-11 with **Agonist 1 or 2** (whichever not used in 13)
15. Wash x 3 with normal PSS

NE, norepinephrine; VP, vasopressin; SR, sarcoplasmic reticulum; SOC, store-operated channel

The vessels are exposed to each agonist (i.e. NE or VP) twice in steps 8-14 at the test dose in a random order.

Figure 4.1 Ca^{2+} -free / add-back experimental protocol



A sample wire myograph force recording taken from an experiment performed on an artery from a sham-operated rat. The artery was first exposed to a test dose of vasopressin (VP) in the presence of extracellular calcium (Ca^{2+}) to allow measurement of the maximal contractile response (A-D).

Later in the experiment, after 5 minutes' incubation in Ca^{2+} -free solution, the same dose of agonist was applied. C-D represents contraction arising from Ca^{2+} released from intracellular stores. Agonist was then washed out, and after a further 5 minutes, Ca^{2+} returned to the bathing solution. B-D represents contraction arising from Ca^{2+} influx through store-operated channels.

4.2.4 Contribution to contraction of voltage-gated and receptor-operated channels

4.2.4.1 Nifedipine and LOE 908

The method employed is outlined in Protocol 2. Following activation, the responses to E_{\max} doses (“test doses”) of NE (10 μ M) and VP (10nM) were measured. These contractions were compared to responses produced in the same vessel after pre-incubation with either nifedipine (10 μ M), a blocker of L-type VGCCs, or LOE 908 (300nM), a blocker of ROCs [Furutani *et al.*, 2002]. Inhibition was expressed as the percentage of contractile response remaining after channel blockade:

$$\frac{\text{Post blocker peak tension response}}{\text{Test dose peak tension response}} \times 100.$$

Application of high concentrations of K⁺ produces vessel contraction resulting almost entirely from VGCC opening [Hathaway *et al.*, 1991]. Nifedipine was therefore used at a concentration found to abolish the response to 50mM K⁺ (Figure 4.2a). In preliminary concentration-response studies, LOE 908 partially inhibited the contraction produced by 50mM K⁺ at micromolar doses. No inhibition of potassium-induced contraction was seen at 300nM (Figure 4.2b), so this concentration was used in all subsequent experiments.

Protocol 2: Ca²⁺ channel blocker experiments

1. Normalisation
2. Activation NE 5µM x 3
3. **Test doses** **NE 10µM, VP 10nM**
4. Channel blocker added to one vessel (chamber divided)

 LOE 908 (300nM) or nifedipine (10µM)

 leave 10 minutes
5. **Agonist 1** to both vessels, allow to plateau
6. Wash x 3 with PSS, leave 5 minutes
7. Re-add same blocker to same vessel, leave 10 minutes
8. Repeat 5-7 with **Agonist 2**
9. Repeat 5-7 with **Agonist 1 or 2** (to check reproducibility)
10. Repeat 5-7 with **Agonist 1 or 2** (whichever not used in 9)
11. Wash x 3 with PSS

NE, norepinephrine; VP, vasopressin

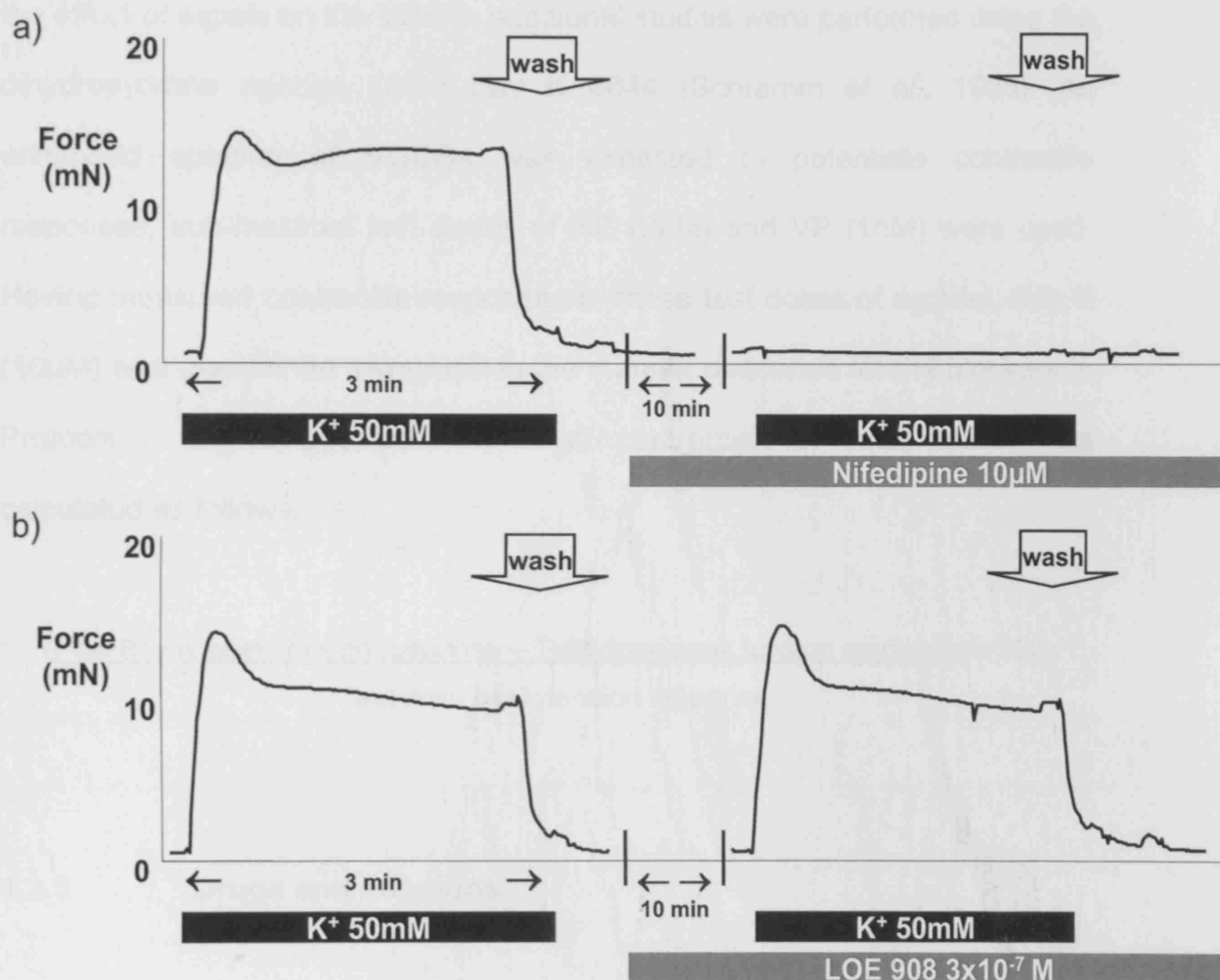
Two vessels from the same animal are mounted, and the myograph chamber divided.

Only one vessel is exposed to the blocker, the other acts as a control.

Only one blocker used in each experiment.

The vessels are exposed to each agonist (i.e. NE or VP) twice in steps 5-10 at the test dose in a random order.

Figure 4.2 Effect of (a) nifedipine and (b) LOE 908 on 50mM K⁺-induced contractile responses



Since high concentration K⁺-induced contractions result almost entirely from VGCC-mediated Ca²⁺ influx, doses of nifedipine (10µM) and LOE 908 (300nM) were chosen to produce complete (a) and negligible (b) inhibition of 50mM K⁺ responses, respectively.

4.2.4.2 Bay K 8644

To complement the experiments using nifedipine, and to further investigate the effect of sepsis on the VGCC, additional studies were performed using the dihydropyridine agonist, (S)(-) Bay K 8644 [Schramm *et al.*, 1985]. As enhanced opening of VGCCs was expected to potentiate contractile responses, sub-maximal test doses of NE (2 μ M) and VP (1nM) were used. Having measured contractile responses to these test doses of agonist, Bay K (10 μ M) was used in the myograph in the manner described for the blockers in Protocol 2. Percentage potentiation of contraction in each vessel was calculated as follows:

$$\frac{(\text{Post Bay K peak tension response} - \text{Test dose peak tension response})}{\text{Test dose peak tension response}} \times 100.$$

4.2.5 Drugs and solutions

Norepinephrine (norepinephrine tartrate) was purchased from Abbott Laboratories and LOE 908 was kindly donated by Boehringer-Ingelheim (Ingelheim, Germany). All other drugs and reagents were obtained from Sigma-Aldrich. Because of limited water solubility, nifedipine and Bay K 8644 were dissolved in dimethylsulfoxide (DMSO) (final concentration 0.1%). The presence of 0.1% DMSO alone in the myograph chamber was found to have no effect on vessel tone. Normal PSS was of the following composition (mM): NaCl 112, KCl 5, CaCl₂ 1.8, MgCl₂ 1, NaHCO₃ 25, KH₂PO₄ 0.5, NaH₂PO₄ 0.5,

and glucose 10. In high potassium PSS (KPSS), NaCl was replaced with an equimolar amount of KCl. To generate a solution containing 50mM K⁺, normal PSS was mixed (60:40) with KPSS. In Ca²⁺-free PSS, 1mM EGTA was added and CaCl₂ omitted.

4.2.6 Data and statistical analysis

Arteries from six sham-operated and six septic rats were studied in each set of experiments. Vessels from the same cohort of animals were used in the Ca²⁺ free / add-back and VGCC studies. Experiments using LOE 908 were performed at a later date using arteries taken from a separate batch of sham and septic rats. Peak force measurements (mN) were corrected for vessel length to give tension values (N/m). When agonist stimulations to the same vessel were repeated under similar conditions, the mean peak tension was calculated. Percentage contribution, inhibition and potentiation values for sham and septic groups are expressed as mean ± SEM. The means of the two groups were compared using Student's t test (unpaired). A p value <0.05 was considered statistically significant.

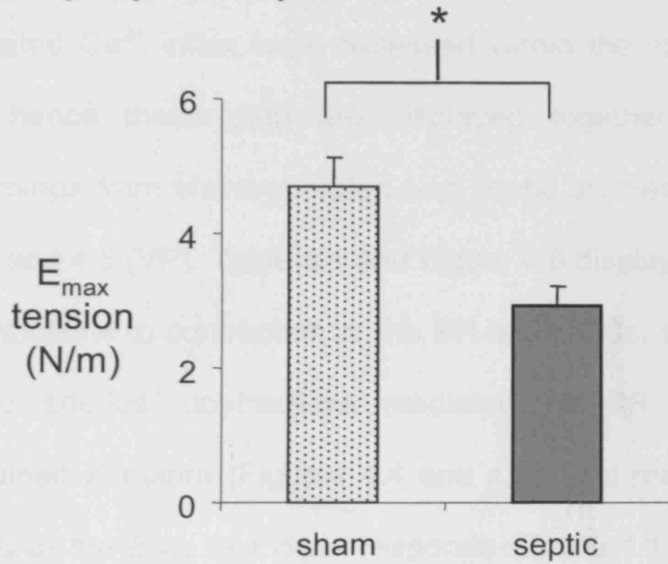
4.3 Results

4.3.1 E_{\max} responses to norepinephrine and vasopressin

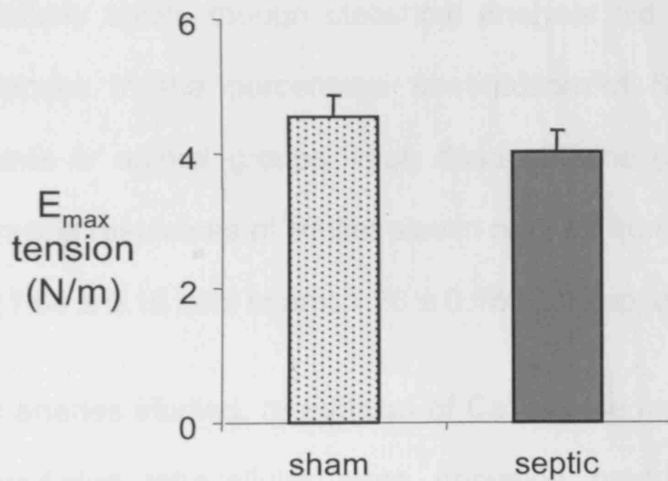
The peak tension responses produced by E_{\max} concentrations (test doses) of NE and VP (Figure 4.3) were similar to those reported for the *ex-vivo* vascular reactivity characterisation studies described in Chapter 3 (Tables 3.2 and 3.3). As shown previously, the E_{\max} induced by NE was significantly reduced in septic vessels, whereas VP-induced contractions were maintained.

Figure 4.3 Peak tension responses induced by E_{max} doses of contractile agonists in Ca^{2+} mobilisation studies

a) Norepinephrine 10 μ M



b) Vasopressin 10nM



Values shown are mean \pm SEM of $n = 12$ animals (results from Ca^{2+} free / add-back, nifedipine and LOE 908 studies combined)

* $p < 0.05$ for comparison between sham and septic groups

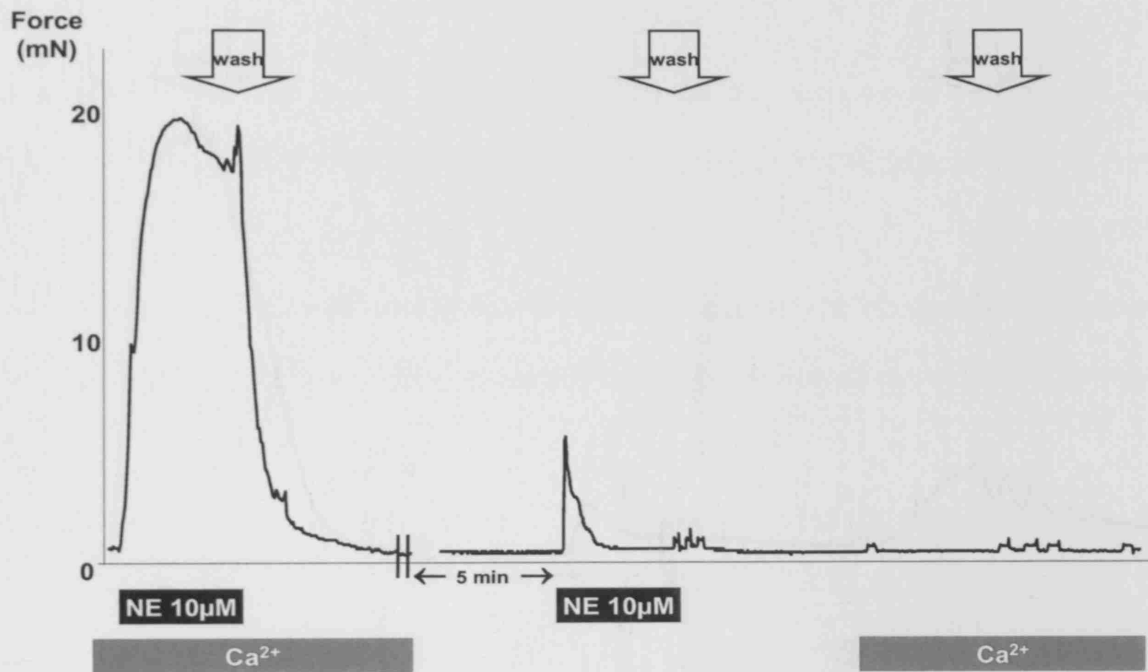
4.3.2 Contribution to contraction of sarcoplasmic reticulum Ca²⁺ stores and plasmalemmal store-operated channels

The contribution to contraction of both Ca²⁺ release from the SR and SOC-mediated Ca²⁺ influx were assessed within the same experimental protocol and hence these data are displayed together. Typical wire myograph recordings from sham-operated and septic arteries are shown in Figures 4.4 (NE) and 4.5 (VP). Table 4.1 and Figure 4.6 display the calculated percentage contributions to contraction of the SR and SOCs. In both sham-operated and septic arteries, contractions mediated via SR Ca²⁺ release were non-sustained in nature (Figures 4.4 and 4.5), and reached peak tension values $\leq 25\%$ of the E_{max} test dose response (Table 4.1). Contractile responses in septic arteries exposed to NE in the absence of extracellular Ca²⁺ were particularly small, though statistical analysis did not reveal any significant differences in the percentage contribution of SR Ca²⁺ release between agonists or animal groups. High dose caffeine (20mM) produced transient contractile responses of similar size in arteries from sham-operated and septic rats (1.04 ± 0.16 N/m sham; 1.26 ± 0.15 N/m septic).

In all arteries studied, re-addition of Ca²⁺ to the myograph chamber following NE-mediated intracellular store depletion produced negligible contractile responses. In contrast, Ca²⁺ influx via SOCs contributed significantly to VP-induced contractions, accounting for close to 20% in controls, and almost 50% of total tension in septic vessels.

Figure 4.4 Norepinephrine (NE)-induced contractile responses in Ca^{2+} -free / add-back experiments

a) typical wire myograph force recording from sham-operated artery



b) typical wire myograph force recording from septic artery

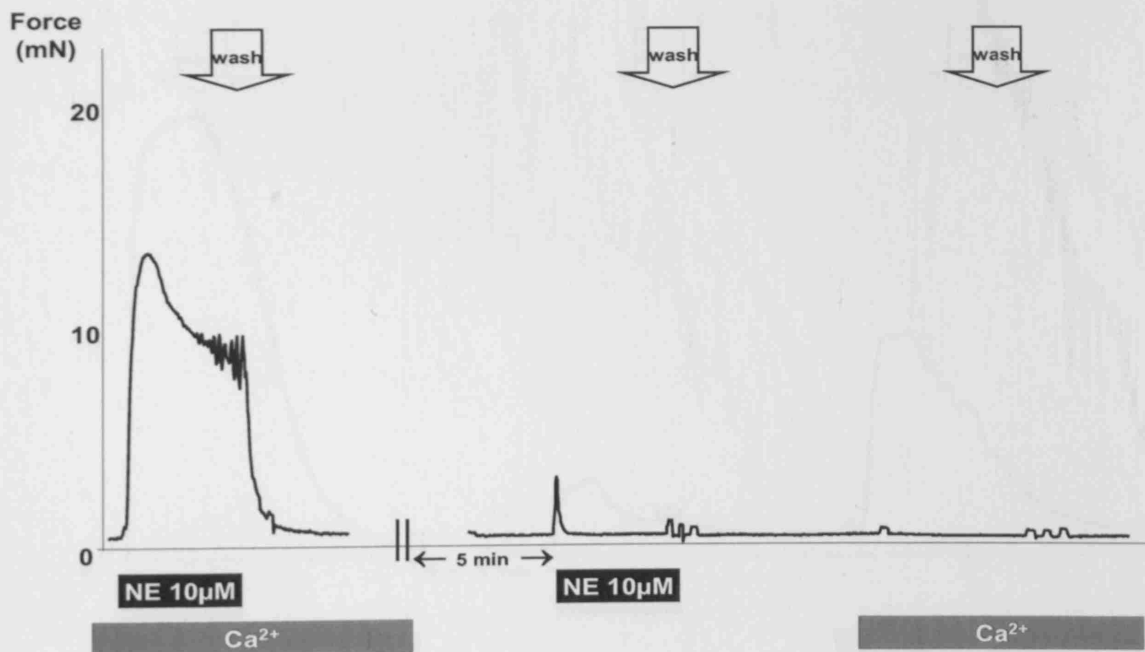
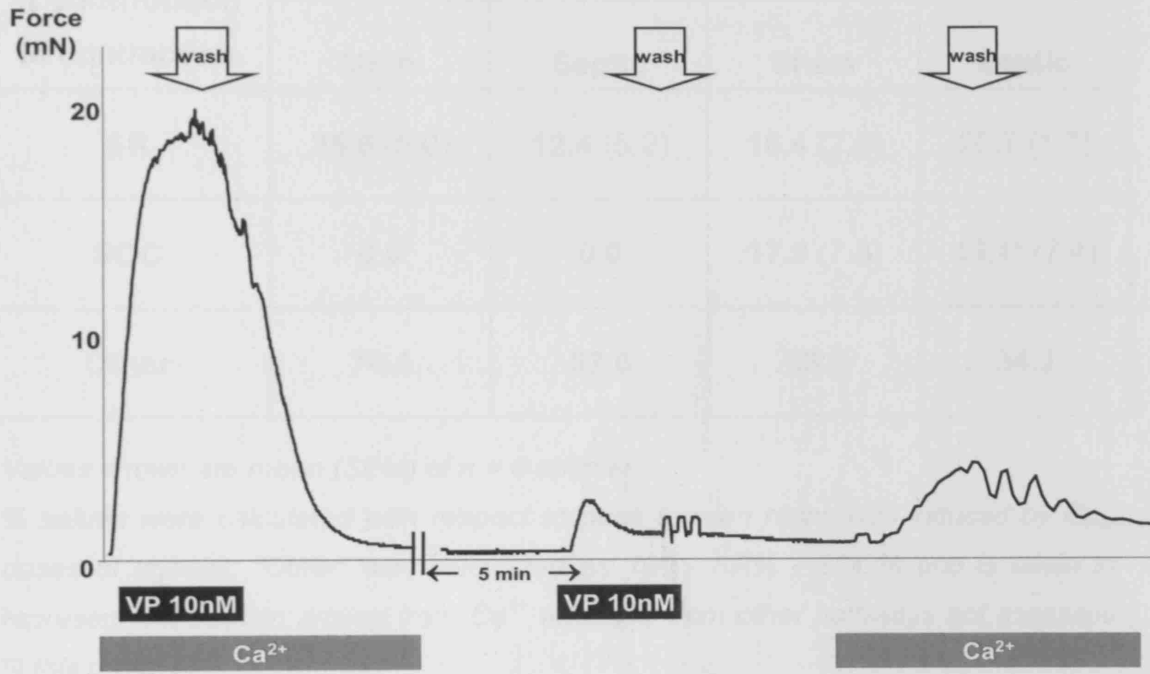


Figure 4.5 Vasopressin (VP)-induced contractile responses in Ca²⁺-free / add-back experiments

a) typical wire myograph force recording from sham-operated artery



b) typical wire myograph force recording from septic artery

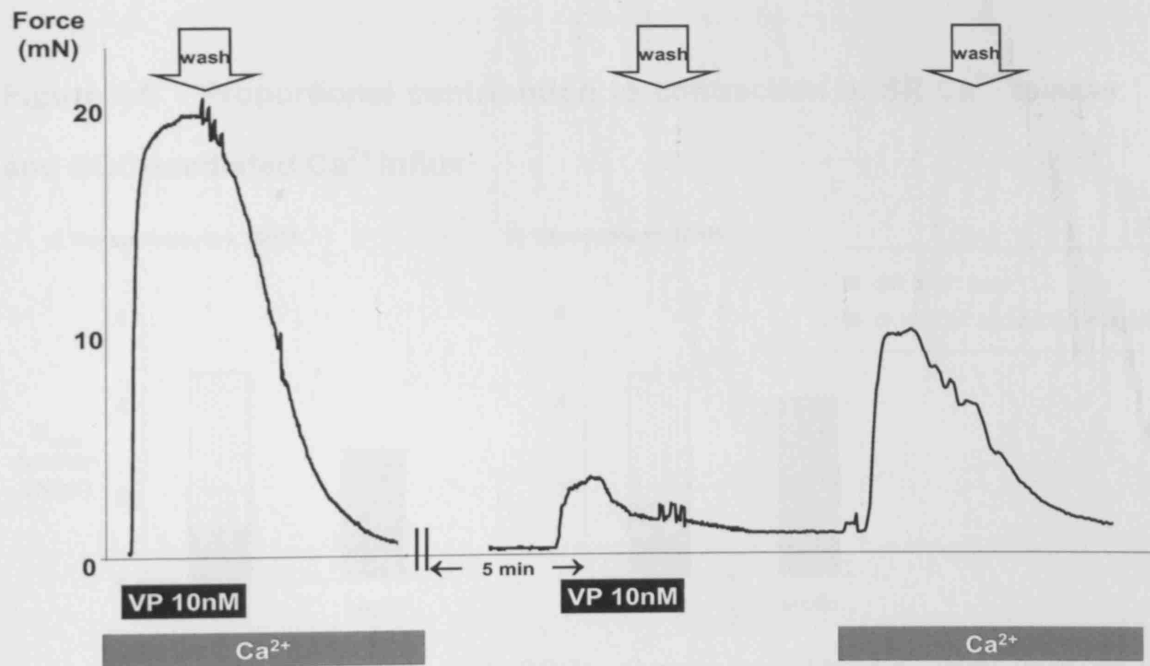


Table 4.1 Percentage contribution to contraction of SR Ca²⁺ release and SOC-mediated Ca²⁺ influx

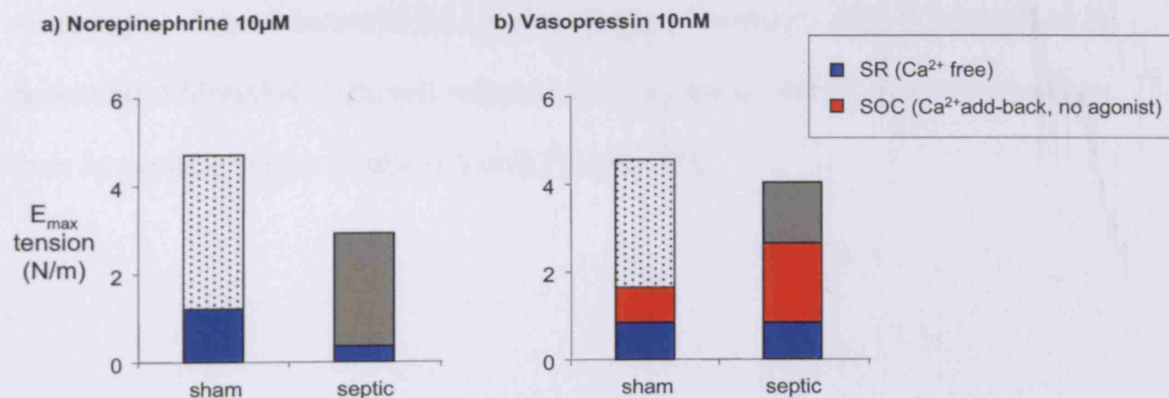
% contribution to contraction	Norepinephrine 10 μ M		Vasopressin 10nM	
	Sham	Septic	Sham	Septic
SR	25.6 (5.0)	12.4 (5.2)	18.4 (2.8)	20.7 (1.7)
SOC	0.0	0.0	17.9 (7.3)	45.1* (7.4)
Other	74.4	87.6	63.7	34.2

Values shown are mean (SEM) of n = 6 animals.

% values were calculated with respect to peak tension responses induced by E_{max} doses of agonist; "Other" was calculated as 100 - SR% - SOC% and is taken to represent contraction arising from Ca²⁺ released from other pathways not assessed in this protocol.

* p<0.05 for comparison between sham and septic groups

Figure 4.6 Proportional contribution to contraction of SR Ca²⁺ release and SOC-mediated Ca²⁺ influx



Percentage contributions SR and SOCs shown in Table 4.1 were used to proportionally divide the maximal agonist-induced contractile responses (Figure 4.3).

4.3.3 Contribution to contraction of voltage-gated Ca²⁺ channels

Addition of nifedipine (10 μ M) to the myograph chamber had no effect on basal vessel tone. Agonist stimulated contractions in both sham-operated and septic arteries were markedly inhibited, however, after 10 minutes' pre-incubation with the L-type VGCC blocker (Table 4.2 and Figure 4.7). NE-induced contraction was depressed to a greater extent than VP-induced responses. Least affected by nifedipine were the VP-mediated contractions in septic arteries; these were diminished by less than 50%, despite using a concentration that fully inhibited contractions to 50mM K⁺ (Figure 4.2a).

Unlike the consistent results obtained with nifedipine, use of the VGCC opener, Bay K 8644 (10 μ M) produced highly variable findings. Despite identical reagent preparation, addition of Bay K to the myograph increased basal vessel tone in sham-operated and septic arteries in some, but not all experiments. Furthermore, the potentiating effect of this compound on sub-maximal NE- and VP-induced contractions was erratic (Table 4.3), precluding meaningful data interpretation. Interestingly, however, Bay K reproducibly potentiated 50mM K⁺-induced responses to a greater extent in sham-operated than in septic arteries (Table 4.3 and Figure 4.8).

Table 4.2 Effect of nifedipine on agonist-induced contraction

	Norepinephrine 10 μ M		Vasopressin 10nM	
	Sham	Septic	Sham	Septic
% nifedipine insensitive	26.6 (3.2)	16.6 (6.7)	40.7* (1.8)	55.7# (1.2)
% nifedipine sensitive	73.4	83.4	59.3	44.3

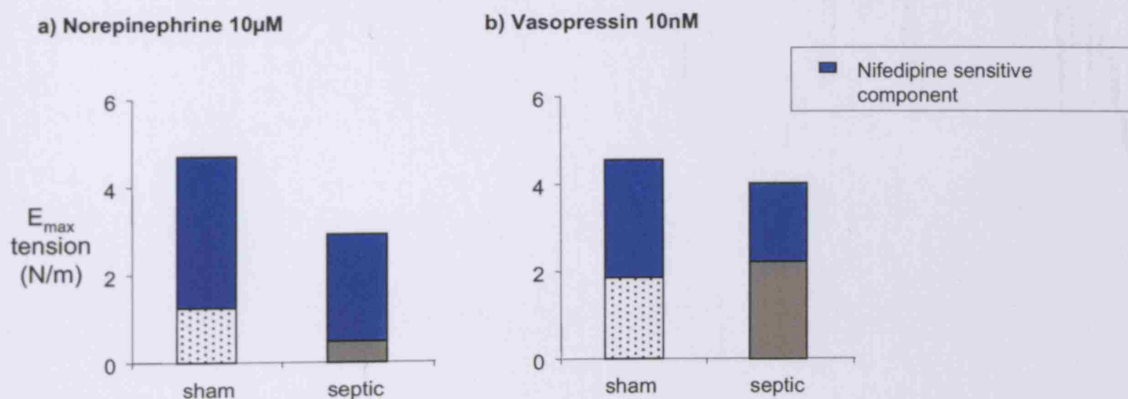
Values shown are mean (SEM) of $n = 6$ animals.

The nifedipine insensitive component of contraction was calculated as a percentage by dividing the peak agonist response in the presence of the Ca^{2+} channel blocker by the response obtained in the absence of blocker, and multiplying by 100. The nifedipine sensitive component was calculated as $100 -$ the nifedipine insensitive proportion.

* $p < 0.05$ for comparison between NE- and VP-induced responses in sham vessels

$p < 0.05$ for comparison between VP-induced responses in sham and septic vessels

Figure 4.7 Proportional contribution to contraction of nifedipine-sensitive VGCC-mediated Ca^{2+} influx



Nifedipine sensitive and insensitive percentages shown in Table 4.2 were used to proportionally divide the maximal agonist-induced contractile responses (Figure 4.3).

Table 4.3 Effect of Bay K on agonist-induced contraction

% potentiation of contraction	Sham	Septic
Norepinephrine 2μM	62.7 (33.3)	83.5 (51.4)
Vasopressin 1nM	31.8 (21.0)	38.8 (29.8)
K⁺ 50mM	22.8 (2.6)	5.0* (1.0)

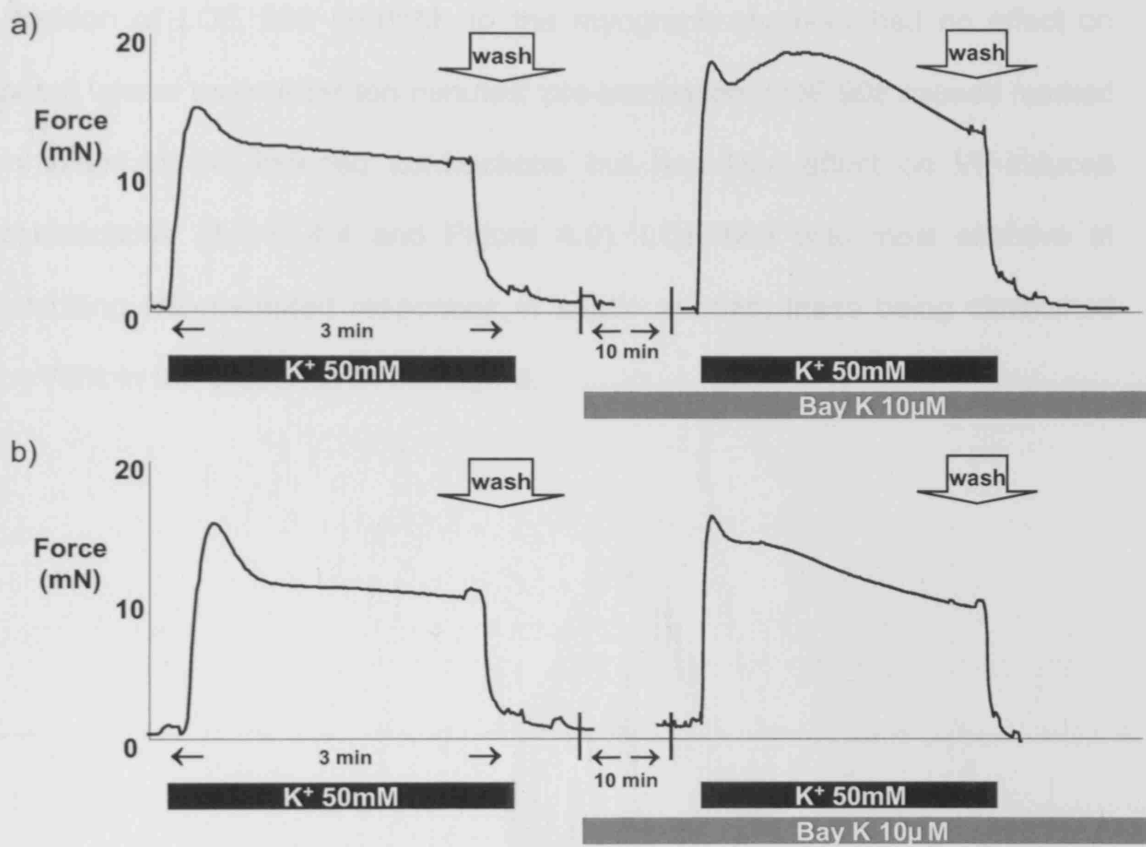
Values shown are mean (SEM) of n = 6 animals.

Bay K potentiation was calculated as follows:

$$\frac{(\text{Post Bay K peak tension response} - \text{Test dose peak tension response}) \times 100}{\text{Test dose peak tension response}}$$

** p<0.05 for comparison between sham and septic responses*

Figure 4.8 Effect of Bay K on 50mM K⁺-induced contractile responses



Typical wire myograph recordings showing the effect of pre-incubation with Bay K on 50mM K⁺-induced contractile responses in a) sham-operated and b) septic arteries. The VGCC opener was found to produce greater potentiation of contraction in the former.

4.3.4 Contribution to contraction of receptor-operated channels

Addition of LOE 908 (300nM) to the myograph chamber had no effect on basal vessel tone. After ten minutes' pre-incubation, LOE 908 caused marked inhibition of NE-induced contractions but had little effect on VP-induced contractions (Table 4.4 and Figure 4.9). LOE 908 was most effective at inhibiting NE-mediated responses in septic arteries, these being diminished by 75% in the presence of this agent.

Table 4.4 Effect of LOE 908 on agonist-induced contraction

	Norepinephrine 10 μ M		Vasopressin 10nM	
	Sham	Septic	Sham	Septic
% LOE 908 insensitive	61.1 (5.4)	24.3* (9.5)	94.2# (2.6)	91.4 (3.8)
% LOE 908 sensitive	38.9	75.7	5.8	8.6

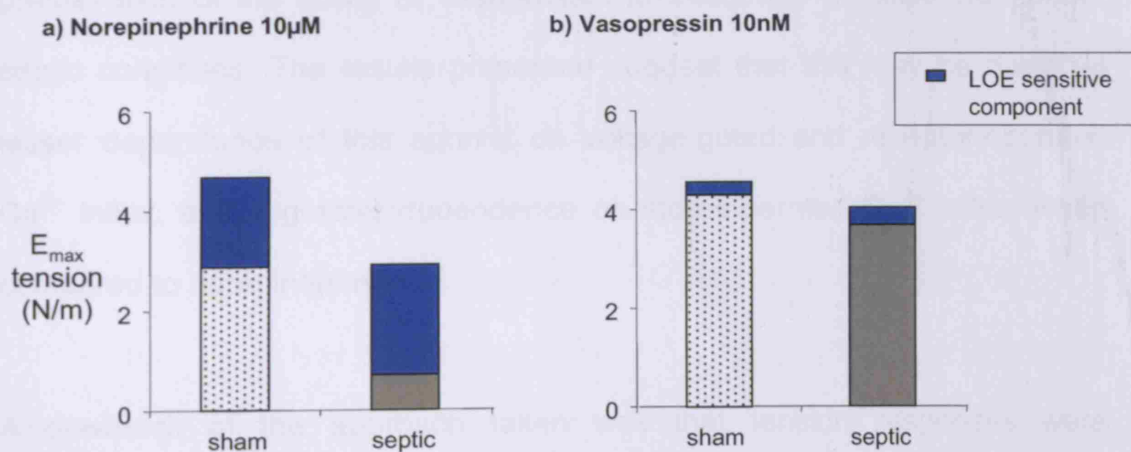
Values shown are mean (SEM) of $n = 6$ animals.

The LOE insensitive component of contraction was calculated as a percentage by dividing the peak agonist response in the presence of the Ca^{2+} channel blocker by the response obtained in the absence of blocker, and multiplying by 100. The LOE sensitive component is calculated as $100 -$ the LOE insensitive proportion.

* $p < 0.05$ for comparison between NE-induced responses in sham and septic vessels

$p < 0.05$ for comparison between NE- and VP-induced responses in sham vessels

Figure 4.9 Proportional contribution to contraction of LOE 908-sensitive ROC-mediated Ca^{2+} influx



LOE 908 sensitive and insensitive percentages shown in Table 4.4 were used to proportionally divide the maximal agonist-induced contractile responses (Figure 4.3).

4.4 Discussion

The aims of this section of my thesis were to compare the vascular smooth muscle Ca^{2+} mobilisation profiles utilised by norepinephrine and vasopressin, and to determine whether any differences exist that might account for the sepsis-induced changes in agonist vascular reactivity described in Chapter 3.

E_{max} doses of NE and VP were used to maximally activate receptors. Hence, these studies provide information most pertinent to the Ca^{2+} signalling mechanisms operating at the top of the agonist concentration-response curves. At this point, NE-induced contractile responses were significantly diminished, and were non-sustained in nature, in mesenteric arteries taken from septic rats (Figures 3.5 and 4.3). By contrast, VP-induced tensions were maintained, both in terms of magnitude and duration (Figures 3.7 and 4.3). I hypothesised that these changes in agonist efficacy reflect a selective preservation of the ability of vasopressin to effectively mobilise Ca^{2+} under septic conditions. The results presented suggest that this may be due to a lesser dependence of this agonist on voltage-gated and receptor-operated Ca^{2+} influx, and a greater dependence on store-operated Ca^{2+} influx, when compared to norepinephrine.

A drawback of the approach taken was that tension responses were measured as a surrogate of intracellular Ca^{2+} concentration. The relationship of Ca^{2+} concentration and tension is not linear, in part due to the phenomenon of Ca^{2+} sensitisation (Figure 1.1, and Chapter 5) [Somlyo & Somlyo, 2003]. In

addition, the Ca^{2+} influx via SOCs serves to refill intracellular stores as well contributing to activation of the myofilaments [Albert & Large, 2003]. Complex interactions exist between the various Ca^{2+} mobilisation mechanisms. For example, the non-selective cation current mediated by SOCs and ROCs can provide a depolarising stimulus to open VGCCs, while Ca^{2+} entry can produce further rises in intracellular Ca^{2+} by promoting its release from the SR, a process known as Ca^{2+} -induced Ca^{2+} release [Wier & Morgan, 2003]. Thus, attempts to isolate individual pathways can be problematic. Despite these considerations, the methodology I employed did reveal clear differences in the mechanisms by which norepinephrine and vasopressin receptors are coupled to Ca^{2+} signalling pathways. This offers an important contribution to our understanding of changes in vascular reactivity to different agonists during sepsis.

4.4.1 Contribution to contraction of sarcoplasmic reticulum Ca^{2+} stores

The small, non-sustained tensions produced by NE and VP in the absence of extracellular Ca^{2+} suggest that SR Ca^{2+} stores provide little direct contribution to agonist-stimulated contraction in rat small mesenteric arteries. The similar responses observed upon application of caffeine also imply a small releasable Ca^{2+} pool in these vessels. Measurements of Ca^{2+} signals in isolated aortic smooth muscle cells have demonstrated that a large initial peak in intracellular Ca^{2+} results from agonist-mediated SR store release [Ruegg *et al.*, 1989; Nakajima *et al.*, 1996; Furutani *et al.*, 2002]. A minor role for SR Ca^{2+} in tension development is supported, however, by the marked effect of

extracellular Ca^{2+} removal on contractile responses in several whole vessel preparations [Lagaud *et al.*, 1999; Katori *et al.*, 2001; Furutani *et al.*, 2002; Villalba *et al.*, 2007]. I found that in arteries taken from sham-operated rats, NE- and VP-induced responses were inhibited to a similar degree in Ca^{2+} -free solution. This conflicts with findings in larger vessels suggesting that NE-induced contractions are more dependent on SR Ca^{2+} release than those triggered by VP [Cauvin *et al.*, 1988; Bauer & Parekh, 2003].

An increased cytosolic Ca^{2+} concentration has been reported in vascular smooth muscle in sepsis, and this is attributed to abnormalities of intracellular Ca^{2+} storage [Martinez *et al.*, 1996]. This concept has been widely investigated in the context of sepsis-related myocardial dysfunction, and although there is evidence to suggest altered function of both SR Ca^{2+} release channels and the sarcoendoplasmic reticulum Ca^{2+} -ATPase (SERCA), study results have been inconsistent [Steendijk, 2005]. In my experiments, I found that caffeine produced equal contractile responses in sham-operated and septic arteries. This argues against significant impairment of SR store release in vascular smooth muscle during sepsis. Vasopressin produced Ca^{2+} -free tensions similar in magnitude to caffeine-induced responses in sham and septic vessels (c.1N/m). Norepinephrine-induced tensions, however, were notably smaller in septic tissues (Figure 4.6). This does hint at a possible defect in adrenoceptor coupling to SR Ca^{2+} release channels.

4.4.2 Contribution to contraction of store-operated channels

Depletion of SR Ca^{2+} triggers influx of extracellular Ca^{2+} through cation channels located at the plasma membrane. This influx not only replenishes intracellular stores, but also contributes directly to contraction [Albert & Large, 2003]. While signalling mechanisms coupling SR depletion to channel activation remain poorly understood, several gating mechanisms including a calcium influx factor (CIF), lysophospholipids and PKC appear involved in SOC activation in smooth muscle [Albert & Large, 2006]. With regard to the molecular identity of SOCs, increasing evidence suggests that the transient receptor potential (TRP) family of proteins are integral components [Albert & Large, 2006]. SOC-mediated Ca^{2+} currents have been demonstrated in vascular smooth muscle using both NE and VP as stimulators [Wier & Morgan, 2003; Moneer *et al.*, 2003; Brueggemann *et al.*, 2006], and shown to provide a significant contribution to the contractile response [Zhang *et al.*, 2002; Furutani *et al.*, 2002]. The results of my Ca^{2+} add-back experiments in rat small mesenteric arteries support this argument for vasopressin, but indicate a negligible role for SOCs with norepinephrine (Figures 4.4 and 4.6). The explanation for this unexpected finding is not clear. A possible defect in NE-induced SR Ca^{2+} release in septic arteries (Section 4.4.1), may impair any store depletion-triggered signal to sarcolemmal channels, but this does not account for the absence of SOC-mediated contraction in sham vessels. Alternatively, NE-activated Ca^{2+} entry through SOCs may be sequestered directly into a subcellular compartment that has limited access to the contractile machinery [Villalba *et al.*, 2007]. Another possibility is that SOCs

opened by NE are less Ca^{2+} permeable than those opened by VP. Influx of other cations might still be expected to produce contraction, however, secondary to cell depolarisation and VGCC activation [Villalba *et al.*, 2007].

In arteries obtained from septic rats, the contribution of SOCs to VP-induced contraction was significantly greater than in sham vessels. This implies decreased reliance on the other sarcolemmal Ca^{2+} channels, ROCs and VGCCs. If membrane hyperpolarisation in sepsis impairs the function of the latter [Clapp & Tinker, 1998; Oliver & Landry, 2006], vasopressin's ability to mobilise Ca^{2+} via SOCs could be one explanation to account for its preserved contractile efficacy. To date, no published work has reported enhanced SOC activity in sepsis. Of relevance, NO is important for the regulation of SOCs [Moneer *et al.*, 2003; Atucha *et al.*, 2005; Jernigan *et al.*, 2006]. Thus, iNOS activation in sepsis could impact on their activity.

4.4.3 Contribution to contraction of nifedipine-sensitive voltage-gated Ca^{2+} channels

Although both L-type and T-type (long-lasting and transient, respectively) voltage-gated calcium channels are described in smooth muscle, the role of the former in vasoconstriction is far better understood [Horowitz *et al.*, 1996]. An important role for Ca^{2+} influx through L-type VGCCs is suggested by the marked inhibition by nifedipine of NE- and VP-induced contractions in arteries from both sham-operated and septic animals. Consistent with the greater VP-mediated activation of SOCs discussed in the last section, responses to this

agonist were less affected than those to NE by VGCC blockade, particularly in septic vessels (Figure 4.7). The profound reduction of NE-mediated contraction in septic arteries following pre-incubation with nifedipine indicates that VGCCs can still be significantly activated at a maximal dose of agonist, despite the underlying membrane hyperpolarisation that is likely to be occurring in sepsis. Nevertheless, the decreased ability of Bay K 8644 to potentiate potassium-induced contractions in septic compared to sham arteries hints at an underlying dysfunction of these channels.

Several studies provide evidence for VGCC abnormalities in septic shock. Decreased L-type Ca^{2+} channel currents have been demonstrated in cardiac myocytes from endotoxic guinea pigs [Zhong *et al.*, 1997], and also in arteriolar smooth muscle cells harvested from rats in haemorrhagic shock [Zhao & Zhao, 2007]. A reduction in dihydropyridine binding sites in septic cardiac muscle has been found by some [Lew *et al.*, 1996] but not other investigators [Ives *et al.*, 1986]. *In-vivo* enhanced pressor responses to Bay K 8644 in endotoxic animals [Ives *et al.*, 1986; Preiser *et al.*, 1991] oppose a decrease in VGCC number. *Ex-vivo*, however, contractile responses to this agent were depressed in aortic rings from LPS treated rats [Biguad *et al.*, 1990]. Furthermore, Bay K 8644 was unable to reverse the impairment in endotoxic myocyte VGCC-mediated currents [Zhong *et al.*, 1997].

4.4.4 Contribution to contraction of receptor-operated channels

Like SOCs, receptor-operated channels mediate a non-selective cation current with variable permeability to Ca^{2+} , and are structurally comprised of TRP proteins. ROC activation, however, requires G protein-coupled receptor stimulation, with DAG acting as the key second messenger [Large, 2002; Albert & Large, 2006].

From the relative contributions of SR stores, SOCs and VGCCs deduced from the experiments discussed so far, little effect of ROC blockade on NE- and VP-induced contractions was expected. This was borne out in my findings with VP, but not with NE, where the ROC blocker, LOE 908 significantly inhibited contractions in all arteries studied. This suggests that ROC-mediated Ca^{2+} influx is important for NE- but not VP-induced contraction. However, if the percentages of the NE-induced contractions sensitive to nifedipine and LOE 908 are combined, values greater than 100% result (Tables 4.2 and 4.4). This must be attributed to some degree of overlap in the Ca^{2+} channel blockade produced by these agents. By virtue of the high dose used (10 μM), nifedipine may have been acting in a non-specific manner, thus the contribution from VGCCs could have been overestimated. Non-specific effects of LOE 908 are less likely as this compound has been shown to have good selectivity for ROCs over other types of plasma membrane Ca^{2+} channel even up to μM concentrations [Iwamuro *et al.*, 1999]. Despite this, I found that potassium-induced contractions were partially inhibited at this dose, suggesting blockade of VGCCs, and necessitating its use at a lower

concentration (300nM). As for SOCs, depolarisation consequent to cation influx via ROCs is a recognised mechanism of VGCC activation [Large, 2002; Wier & Morgan, 2003], and provides an alternative plausible explanation for the overlap in inhibition. NE responses were more sensitive to LOE 908 in septic than in sham vessels (Figure 4.9). This mirrors the pattern of effect of nifedipine on NE-induced contractions (Figure 4.7), and may therefore have been a consequence of the overlap in channel blockade.

The almost complete insensitivity of VP-induced responses to LOE 908 could be viewed as surprising, given that ROC-mediated Ca^{2+} influx appears as important as SOC-mediated Ca^{2+} influx for vasopressin-induced contractions in rat aorta, and to have a greater contribution to VP- than NE-stimulated responses [Furutani *et al.*, 2002]. Moreover, in cultured aortic smooth muscle cells, ROCs have been demonstrated as the predominant Ca^{2+} entry pathway following vasopressin stimulation [Broad *et al.*, 1999; Moneer *et al.*, 2003]. The discrepancy between my results and this published data may relate to the differences in type and size of vessel investigated [Large, 2002; Bauer & Parekh, 2003]. Furthermore, whether a pharmacological or electrophysiological approach is taken, definitive separation of SOC and ROC currents is difficult in view of their similar cation selectivity and molecular structure. Although non-selective cation influx via ROCs would be expected to trigger vasoconstriction via VGCC activation, it is also possible that VP- and NE-activated ROCs have different Ca^{2+} permeability and that by measuring the effects of these agonists on contraction rather than on ion currents the activity of the former could be overlooked.

4.4.5 Summary

My results clearly suggest that the Ca^{2+} mobilisation pathways stimulated by norepinephrine and vasopressin in small mesenteric arteries are not the same. In these resistance vessels, SOC-mediated Ca^{2+} influx is important for VP- but not NE-induced contraction. Moreover, VGCC-mediated, and probably ROC-mediated, Ca^{2+} influx provide a greater contribution to NE- than VP-stimulated responses. Sepsis was seen to have a marked effect on the pathways contributing to the contractile responses produced by vasopressin, with increased reliance on SOCs but decreased reliance on VGCCs. This may well be of significance in view of the VGCC dysfunction described in septic vascular smooth muscle. Little published work has focussed upon the effects of sepsis on store- or receptor-operated Ca^{2+} channels. However, endotoxin-treated knockout mice deficient in TRP vanilloid 1 (TRPV1), coding for a non-selective cation channel, showed enhanced hypotension and inflammation compared to wild-type controls, suggesting a potentially protective role for such channels in sepsis [Clark *et al.*, 2007].

Chapter 5: Investigation of the calcium-tension relationships of norepinephrine- and vasopressin-induced contractions in small mesenteric arteries

5.1 Introduction

Studies investigating calcium-tension relationships in vascular smooth muscle have revealed that the force generated at a given level of intracellular Ca^{2+} depends upon the type of excitatory stimulus applied. This led to the recognition of Ca^{2+} sensitisation, defined as agonist-induced muscle contraction in the absence of an increase in intracellular Ca^{2+} [Somlyo & Somlyo, 2003]. While the onset of contraction is dependent on Ca^{2+} activation of myosin light chain kinase, Ca^{2+} sensitisation is important in sustaining tension (Figure 1.1) [Horowitz *et al.*, 1996]. Although many mechanisms of Ca^{2+} sensitisation have been proposed in smooth muscle [Somlyo & Somlyo, 2003; Wier & Morgan, 2003], the best characterised is via inhibition of myosin light chain phosphatase (MLCP) following sarcolemmal G protein-coupled receptor stimulation (Figures 1.1 and 1.3) [Sward *et al.*, 2000; Hirano, 2007]. Work with small resistance arteries suggests the involvement of several kinases including PKC and ROK as second messenger molecules [Buus *et al.*, 98; Martinez *et al.*, 2000; Wier & Morgan, 2003]. These enzymes are activated downstream of V_1 and α_1 receptors, but appear to contribute differently to VP- and NE-induced contractile responses [Bauer & Parekh, 2003]. This may be relevant in septic shock, as vasodilatation is partly

attributed to decreased Ca^{2+} sensitivity of the vascular smooth muscle contractile apparatus [Landry & Oliver, 2001; Mansart *et al.*, 2006].

In Chapter 4, I demonstrated the ability of VP to mobilise Ca^{2+} more effectively than NE in arteries taken from septic animals. Such an effect may account for the *ex-vivo* results described in Chapter 3 where VP-induced E_{max} responses were preserved in septic arteries, whereas those to NE were markedly reduced. An intriguing possibility is that, in addition to maintaining Ca^{2+} activation of the contractile apparatus, the heightened potency of VP in septic vessels may also reflect increased Ca^{2+} sensitivity.

To investigate this, I simultaneously measured changes in tension and intracellular Ca^{2+} induced by NE and VP in small mesenteric arteries taken from sham-operated and septic rats. This involved combining wire myography with fluorescence microscopy, a method used in several published Ca^{2+} -tension relationship studies [Bian & Bukoski, 1995; Thomas *et al.*, 2005; Trautner *et al.*, 2006; Villalba *et al.*, 2007].

5.2 Materials and Methods

5.2.1 Wire myography

Studies were performed on vessels taken from sham-operated and septic rats from the faecal peritonitis model described in Chapter 2. Twenty-four hours after intra-peritoneal injection of faecal slurry (or no injection in sham-operated

controls), rats were deeply anaesthetised with isoflurane and then killed by cervical dislocation. Small mesenteric arteries were dissected as in Section 3.2.1. Arterial segments of 2mm in length were then mounted in an automatically operated dual chamber wire myograph (model 510A, Danish Myo Technology) for isometric tension recording (Figure 3.1). The vessels were bathed in 10ml of PSS, heated to 37°C and bubbled continuously with carbogen gas (95% O₂, 5% CO₂).

Thirty minutes after mounting, vessels were normalised to the physiologically realistic luminal diameters necessary for optimal force generation (Section 3.2.2). After a further 30 minutes' equilibration period, arteries were activated by three exposures to 5µM NE as described in Section 3.2.2. Effective pressure calculations were performed to confirm adequate vessel viability. The contractile response to high potassium PSS (KPSS) was also measured in each artery. This was taken to represent maximal agonist-independent tension response, and compared to later KPSS stimulations to exclude vessel damage during the course of the experiment.

5.2.2 Fura-2 loading and fluorescence microscopy

After activation with NE and KPSS, mounted arterial segments were loaded with 10µM of the cell-permeable dye, Fura-2 acetoxymethyl ester (Fura-2 AM, Invitrogen, Carlsbad, CA, USA). This ratiometric dye was chosen to minimise the effects of uneven dye loading, dye leakage, and photobleaching, as well as the problems associated with measuring Ca²⁺ in cells of unequal thickness.

Intracellular non-specific esterases hydrolyse the AM ester to formaldehyde and acetic acid, liberating the Ca^{2+} -sensitive indicator which, because of its poor lipid solubility, remains trapped inside the cell. After one hour's incubation of the dye at 37°C , the myograph chamber was washed, the carbogen gas switched off, and normal PSS exchanged for a HEPES- (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) buffered salt solution (pH 7.4 with NaOH). This allowed subsequent microscopy to be performed at a stable, physiological pH without the problem of image distortion due to bubbling of the bathing fluid with carbogen. Vessels were then re-stimulated with KPSS to ensure responses had not been affected by the solution change or dye loading.

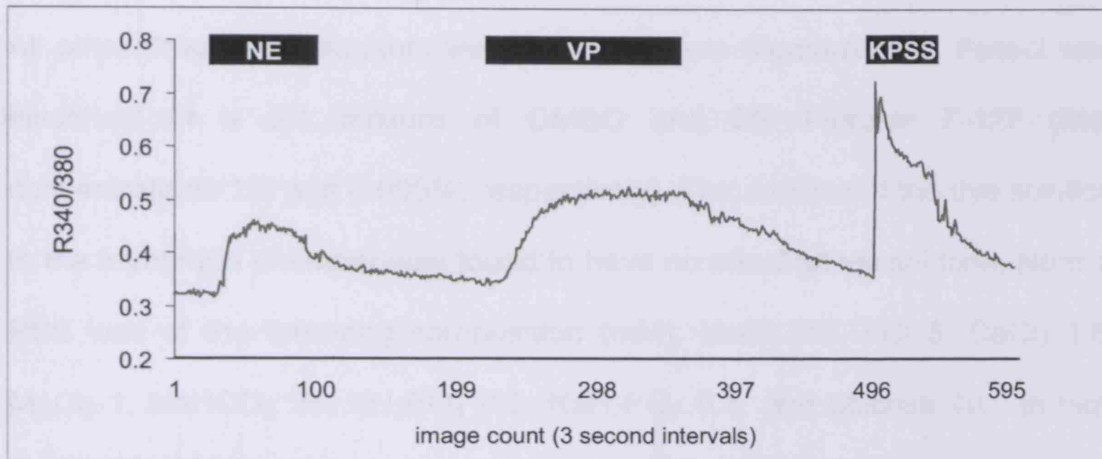
The myograph, which has glass windows covering the base of the chamber, was placed on the stage of an inverted Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan). Excitation wavelengths of 340 and 380nm were delivered to arteries at a frequency of 0.33Hz with a Polychrome IV monochromator Illuminator (TILL Photonics, Munich, Germany). The emitted light was passed through a 440nm long-pass filter and signals recorded by an Imago CCD camera (TILL Photonics). Digital images thus obtained were analysed with TILL vision software (TILL Photonics). Regions of interest (ROIs) were manually selected from still images and after background subtraction, the 340:380nm ratio ($R_{340/380}$) values were calculated. These values are proportional to Ca^{2+} concentration.

5.2.3 Simultaneous force and intracellular Ca^{2+} recording

To best assess any change in vascular smooth muscle Ca^{2+} sensitivity due to prolonged sepsis, earlier concentration-response studies (Chapter 3) were reviewed to determine doses at which sham-operated and septic artery curves were maximally separated: $5\mu\text{M}$ for NE (c. EC_{90}) (Figure 3.5) and 1nM for VP (c. EC_{50}) (Figure 3.7). Unexpectedly, however, preliminary studies using the HEPES-buffered solution revealed small rightward shifts of agonist potency when compared to experiments performed in normal PSS. In this bathing solution, the largest changes in contraction induced by sepsis were seen at $10\mu\text{M}$ NE and 3nM VP. These doses were therefore used in the fluorescence microscopy protocol. In each experiment, one vessel from a sham rat and one vessel from a septic rat were used. The kinetics of both force and Ca^{2+} fluorescence were simultaneously recorded before, during and after stimulation with single doses of NE and VP. Both contraction and intracellular Ca^{2+} changes were allowed to plateau before agonist washout. Only when these returned to baseline was the next agonist added. The order of NE and VP administration was varied, and each experiment was ended with a control stimulation with KPSS. Figure 5.1 shows some typical snapshot still images obtained using this protocol.

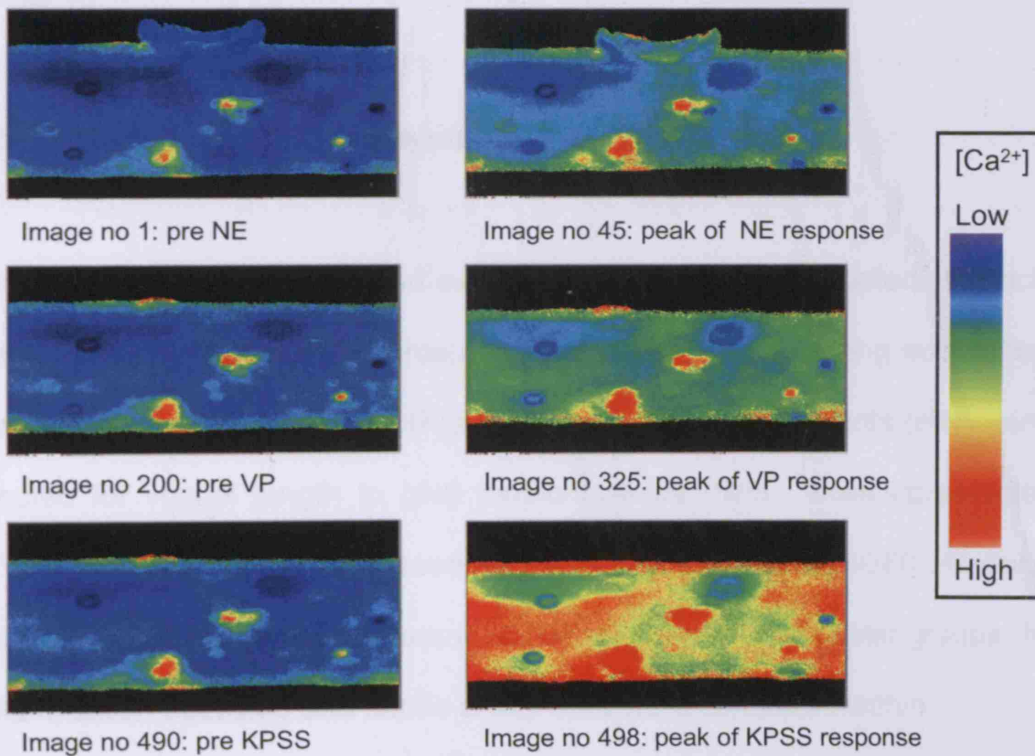
Figure 5.1 Fluorescence microscopy of small mesenteric arteries

a) Typical time-dependent changes in intracellular Ca^{2+} from a (septic) small mesenteric artery stimulated with $10\mu\text{M}$ norepinephrine (NE), 3nM vasopressin (VP) and 117.5mM K^+ (KPSS).



Ca^{2+} concentration is expressed as the background-corrected 340 to 380nm fluorescence ratio ($R_{340/380}$).

b) A selection of still images taken from the experiment shown above



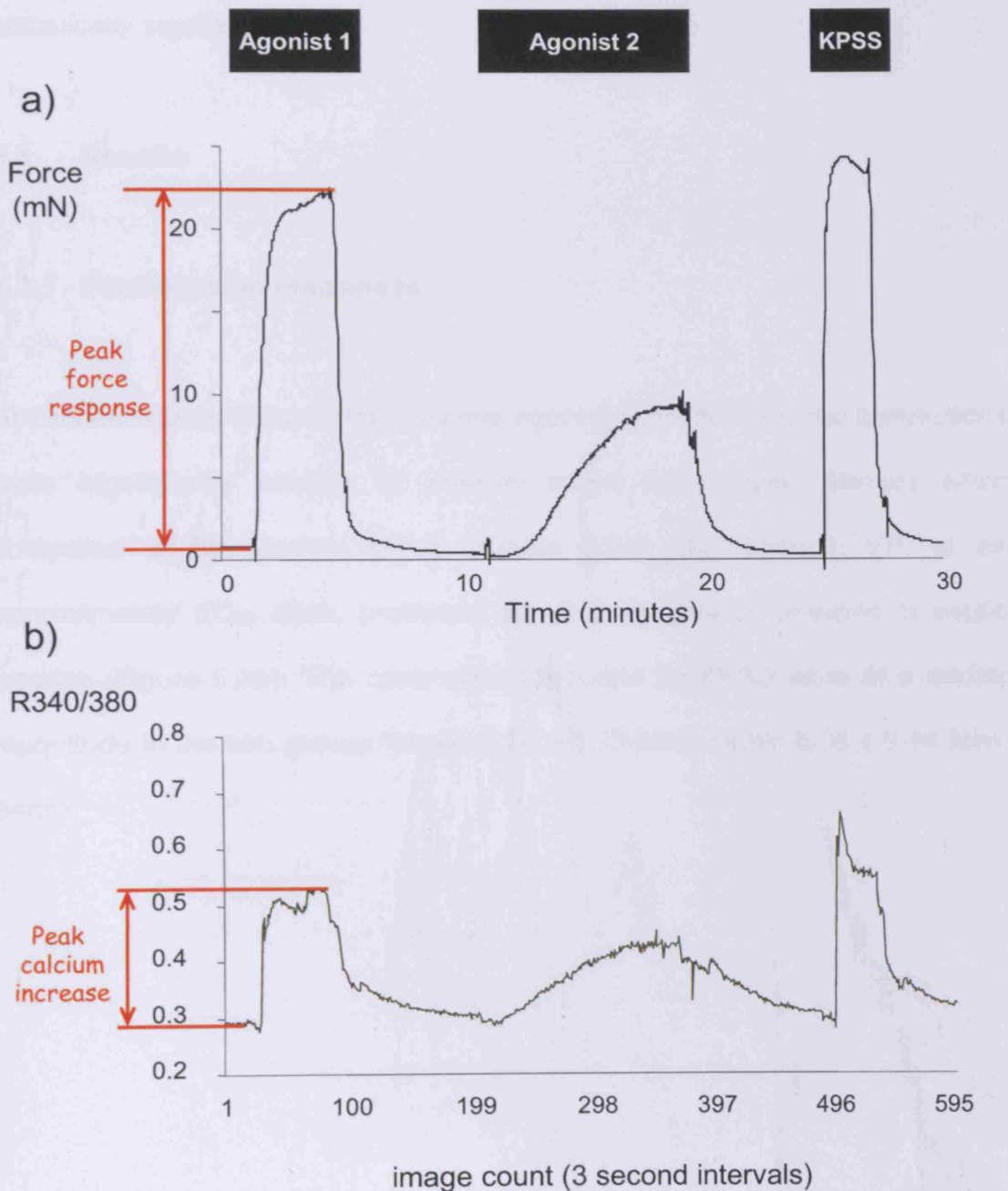
5.2.4 Drugs and solutions

Norepinephrine (norepinephrine tartrate) was purchased from Abbott Laboratories. Fura-2 AM and Pluronic F-127 were acquired from Invitrogen. All other drugs and reagents were obtained from Sigma-Aldrich. Fura-2 was dissolved in a 3:1 mixture of DMSO and 2% Pluronic F-127 (final concentrations 1% and 0.005%, respectively). The addition of the dye solution to the myograph chamber was found to have no effect on vessel tone. Normal PSS was of the following composition (mM): NaCl 112, KCl 5, CaCl₂ 1.8, MgCl₂ 1, NaHCO₃ 25, KH₂PO₄ 0.5, NaH₂PO₄ 0.5, and glucose 10. In high potassium PSS (KPSS), NaCl was replaced with an equimolar amount of KCl, to give a final K⁺ concentration of 117.5mM. HEPES-buffered PSS contained (mM): NaCl 135, KCl 5, NaOH 4.5, HEPES 10, CaCl₂ 1.8, MgCl₂ 1, and glucose 10.

5.2.5 Data and statistical analysis

Arteries from six sham-operated and six septic rats were studied. In each vessel, the peak increases in force and intracellular Ca²⁺ following application of each agonist were measured (Figure 5.2). Force measurements (mN) were corrected for vessel length to give tension values (N/m), while intracellular Ca²⁺ concentrations were expressed as 340:380nm ratios (R340/380). Results were combined to examine differences between sham and septic groups. In addition, sham-operated and septic artery data were compared within

Figure 5.2 Analysis of simultaneous wire myograph and fluorescence ratio recordings from a (sham-control) small mesenteric artery



For each vessel studied, measurements of peak force and peak Ca^{2+} increase following agonist application were measured from a) the myograph, and b) the fluorescence kinetic traces, respectively. Ca^{2+} concentration is expressed as the background-corrected 340 to 380nm fluorescence ratio (R340/380).

experiments (i.e. six sham-septic animal pairs). Pairs of means were compared using Student's t test (unpaired). A p value <0.05 was considered statistically significant.

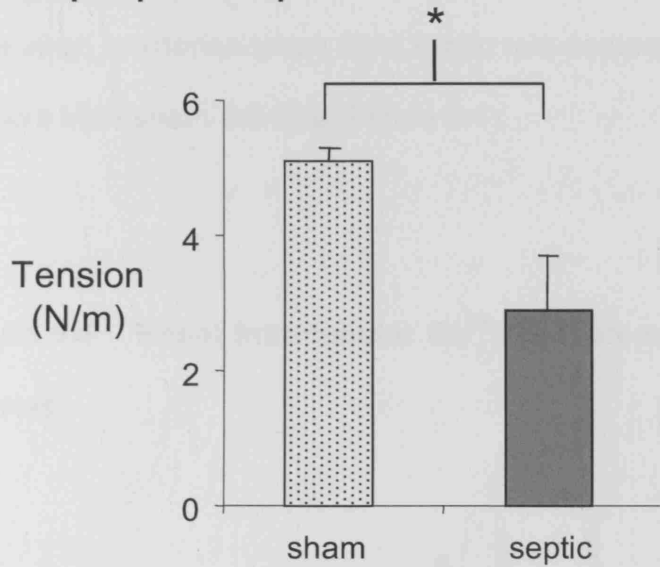
5.3 Results

5.3.1 Peak tension responses

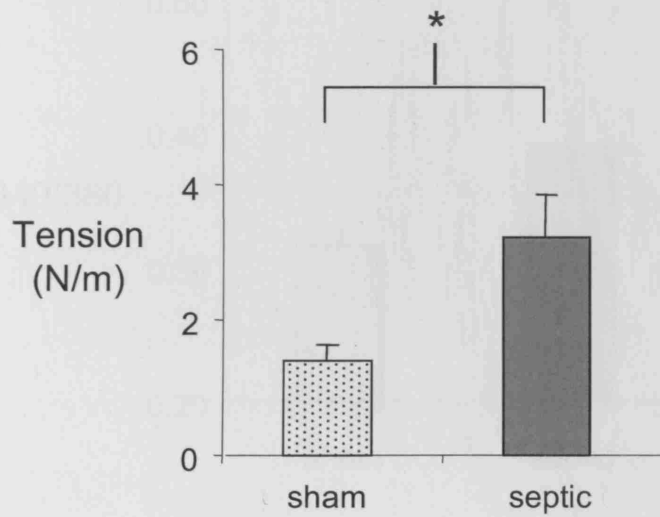
At concentrations close to the maximal agonist dose, NE-induced contractions were significantly smaller in arteries taken from septic animals when compared to the control group (Figure 5.3a). By contrast, VP, at an approximately EC₅₀ dose, produced significantly greater tensions in septic arteries (Figure 5.3b). The contractions induced by KPSS were of a similar magnitude in the two groups (sham 5.74 ± 0.33 N/m; septic 5.26 ± 0.84 N/m, p=ns).

Figure 5.3 Peak tension responses induced by single doses of contractile agonists in Ca²⁺ sensitisation studies

a) Norepinephrine 10 μ M



b) Vasopressin 3nM



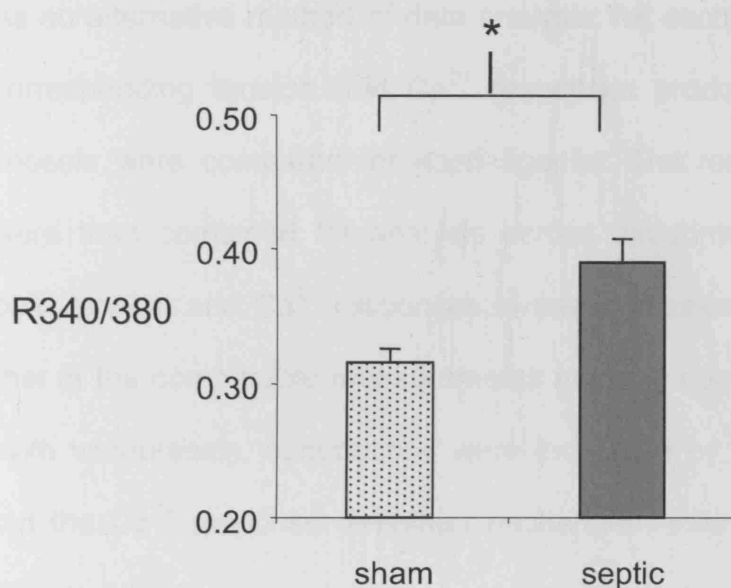
Values shown are mean \pm SEM of $n = 6$ animals.

* $p < 0.05$ for comparison between sham and septic groups

5.3.2 Baseline intracellular Ca²⁺

Measurements of baseline intracellular Ca²⁺ concentration were made before the contractile agonists were applied. Consistently higher 340:380nm ratios were seen in arteries taken from septic rats compared with those measured in vessels from sham animals (Figure 5.4).

Figure 5.4 Basal intracellular Ca²⁺ measurements in small mesenteric arteries



Values shown are mean \pm SEM of $n = 6$ animals.

Ca²⁺ concentration is expressed as the background-corrected 340 to 380nm fluorescence ratio (R340/380).

** $p < 0.05$ for comparison between sham and septic groups*

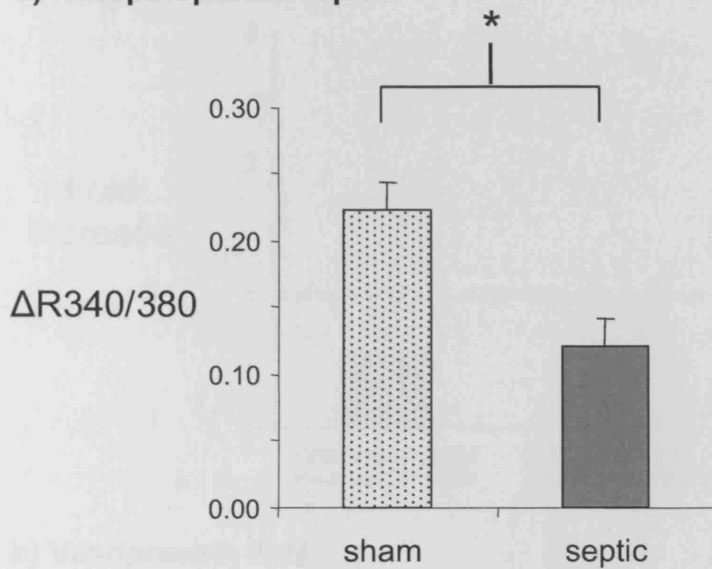
5.3.3 Agonist-induced increases in intracellular Ca^{2+}

In parallel with the findings for tension responses, NE-induced increases in intracellular Ca^{2+} in septic arteries were markedly smaller than those stimulated in vessels from sham-operated controls (Figures 5.3a and 5.5a). Despite enhanced contractile responses to vasopressin in septic vessels (Figure 5.3b), the concomitant elevation in intracellular Ca^{2+} induced by VP was not significantly greater in this group (Figure 5.5b). As noted for KPSS-induced tensions, high K^+ produced similar increases in Ca^{2+} in sham and septic arteries ($\Delta\text{R340/380}$: 0.34 ± 0.05 and 0.25 ± 0.04 , respectively, $p=\text{ns}$).

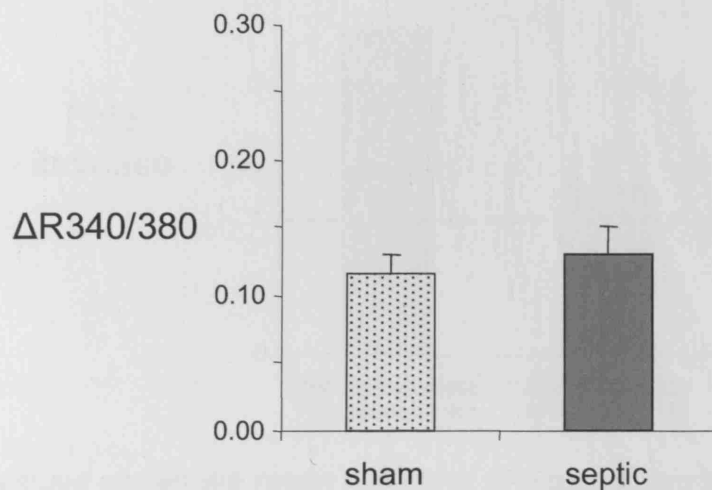
As an alternative method of data analysis, for each individual experiment the corresponding tension and Ca^{2+} responses produced in septic and sham vessels were compared for each agonist. The resulting septic:sham ratios were then combined for analysis across experiments. For norepinephrine, both tension and Ca^{2+} responses in septic arteries were approximately half that of the comparable measurements made in control vessels (Figure 5.6a). With vasopressin, contractions were increased by 2.5-fold in septic vessels but the Ca^{2+} response remained unchanged, thus giving a ratio close to 1 (Figure 5.6b).

Figure 5.5 Peak increases in intracellular Ca^{2+} induced by single doses of contractile agonists in Ca^{2+} sensitisation studies

a) Norepinephrine 10 μM



b) Vasopressin 3nM



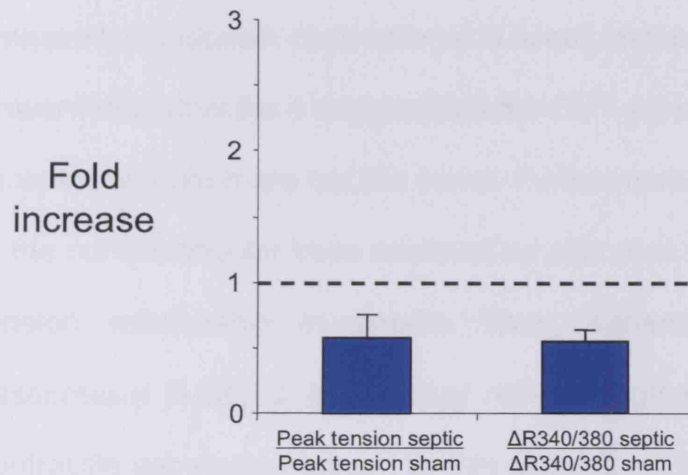
Values shown are mean \pm SEM of $n = 6$ animals.

$\Delta R_{340/380}$ is the fluorescence ratio 340:380nm calculated as the plateau agonist response minus that obtained pre-agonist exposure.

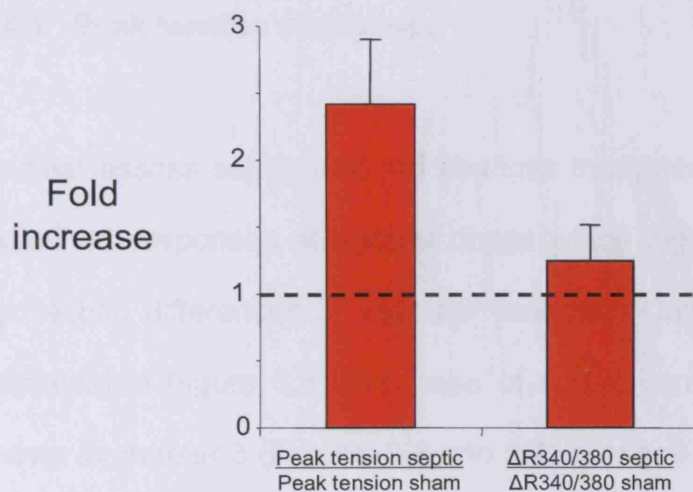
* $p < 0.05$ for comparison between sham and septic groups

Figure 5.6 Septic:sham ratios for individual agonist responses

a) Norepinephrine 10 μ M



b) Vasopressin 3nM



Values shown are mean \pm SEM of n=6 experiments.

The dotted lines represent equal responses in sham and septic arteries (fold increase = 1).

Ca²⁺ concentration is expressed as the background-corrected 340 to 380nm fluorescence ratio (R340/380). Δ R340/380 is the fluorescence ratio 340:380nm calculated as the plateau agonist response minus that obtained pre-agonist exposure.

5.4 Discussion

By combining wire myography and fluorescence microscopy, I was successful in examining the Ca^{2+} -tension relationships underlying norepinephrine- and vasopressin-induced contractions in small mesenteric arteries. The results presented in Chapter 4 suggest that the Ca^{2+} signals downstream of V_1 and α_1 receptor activation are not the same. Furthermore, the experiments described in the current chapter have revealed an alteration in the VP, but not NE, Ca^{2+} -tension relationship in sepsis. Thus, enhanced vascular reactivity to vasopressin in septic shock may reflect heightened Ca^{2+} sensitivity of the contractile apparatus induced by this agonist.

5.4.1 Peak tension responses

To best assess sepsis-induced changes in agonist-specific Ca^{2+} sensitivity, I examined responses at agonist doses which highlighted the maximal sham and septic differences in vascular reactivity. Comparing the tension values displayed in Figure 5.3 with those of earlier concentration-response curves shown in chapter 3 (Figures 3.5 and 3.7) confirms that this aim was achieved. In addition, the similar sized contractions to high-dose potassium in both sham and septic arteries imply that, despite differences in NE and VP reactivity, the contractile machinery is not damaged in my septic model.

5.4.2 Baseline intracellular Ca²⁺

Basal intracellular Ca²⁺ levels were higher in small resistance arteries taken from septic rats. This pattern has been previously reported in both mesenteric arteries and aortae taken from other rat models of sepsis induced by either endotoxin [Martinez *et al.*, 1996] or caecal ligation and puncture [Song *et al.*, 1993]. This elevation in cytosolic Ca²⁺ may reflect sepsis-related disturbances of one or more of the Ca²⁺ mobilisation pathways discussed in Chapter 4, or indicate a defect in re-uptake of Ca²⁺ back into the SR. In sepsis induced by CLP, ATP-dependent Ca²⁺ uptake into cardiac SR was unimpaired after nine hours, yet decreased at 18 hours [Wu *et al.*, 2001], an effect associated with reduced phosphorylation of phospholamban [Wu *et al.*, 2002]. Therefore, defects in the SERCA may contribute to contractile failure in the vasculature. Furthermore, increased basal intracellular Ca²⁺ may provoke metabolic dysfunction in vascular smooth muscle, as demonstrated in endotoxin-treated hepatocytes [Portoles *et al.*, 1991].

5.4.3 Agonist-specific Ca²⁺ sensitivity

Comparison of the peak tension and peak Ca²⁺ increases induced by NE and VP revealed obvious differences between the two agonists. This was borne out by analysis of data both across and within experiments. For norepinephrine, tension and Ca²⁺ responses were reduced in parallel when septic arteries were compared to sham-controls. This indicates no change in the Ca²⁺-tension relationship for this agonist. By contrast, vasopressin

produced a 2.5-fold greater rise in peak tension in septic arteries for a comparable peak change in Ca^{2+} to that induced in sham-operated vessels. Hence, there appears to be heightened Ca^{2+} sensitivity of the contractile apparatus to the effect of VP in sepsis.

As described in Chapter 1, NO synthesised following iNOS activation is a key mediator in the pathogenesis of vasodilatory shock (Section 1.3.2). Via cGMP, NO can activate MLCP, and thus reduce the Ca^{2+} sensitivity of the vascular smooth muscle contractile machinery [Landry & Oliver, 2001]. While this represents one of many mechanisms through which NO may promote vasodilatation, it is believed to contribute significantly to sepsis-induced vascular hyporeactivity [Martinez *et al.*, 1996; Mansart *et al.*, 2006]. The results currently presented suggest that enhanced signalling via the V_1 vasopressin receptor in sepsis can overcome any such Ca^{2+} desensitisation. The inability for high dose norepinephrine to produce a similar effect may be consequent to α_1 adrenoceptor down-regulation. An alternative explanation is that the two agonists utilise different Ca^{2+} sensitisation pathways that could be differentially affected in sepsis.

The predominant mechanism for agonist-stimulated alteration of Ca^{2+} sensitivity in vascular smooth muscle is believed to be via inhibition of MLCP [Hirano, 2007]. MLCP activity is reduced by phosphorylation of either the myosin phosphatase target subunit (MYPT) or the inhibitory protein CPI-17 [Hirano, 2007]. This phosphorylation is mediated by several kinases, including PKC and ROK, activated downstream of plasmalemmal G protein-coupled

receptors. The signalling mechanisms involved are complex and may involve $G_{12/13}$ as well as diacylglycerol liberated by PLC downstream of $G_{q/11}$ [Hirano, 2007]. Use of PKC and ROK inhibitors in *ex-vivo* rat studies suggested that Ca^{2+} sensitisation is at least as important as Ca^{2+} influx in the maintenance of NE- and VP-mediated contractile responses in normal vessels [Bauer & Parekh, 2003]. In addition, it was shown that PKC contributes more to NE- than VP-induced Ca^{2+} sensitisation of the contractile apparatus [Bauer & Parekh, 2003].

In mesenteric arteries taken from a rat model of severe haemorrhagic shock, vascular hyporeactivity and Ca^{2+} desensitisation was partially reversed by both PKC and ROK activators [Xu & Liu, 2005]. In the same model, it was later demonstrated that vasopressin could restore both *in-vivo* and *ex-vivo* responses to norepinephrine, an effect inhibited by HA-1077, a ROK antagonist [Yang *et al.*, 2006]. Potentiation by VP of NE reactivity has also been found in experimental septic shock [Hamu *et al.*, 1999], and is an observation frequently made when vasopressin is used in combination with norepinephrine in the treatment of septic patients [Landry *et al.*, 1997b; Dunser *et al.*, 2003]. Putting these data together, one could hypothesise that enhanced vascular reactivity to vasopressin in sepsis is, in part, mediated via ROK-mediated Ca^{2+} sensitisation.

Chapter 6: Patient studies

6.1 Introduction

Having demonstrated enhanced pressor sensitivity to exogenous vasopressin, Landry and colleagues proceeded to measure endogenous levels of the hormone in patients with catecholamine-refractory septic shock [Landry *et al.*, 1997a; Landry *et al.*, 1997b]. VP levels were found to be lower in these septic patients (c.3pg/ml) than in a group with comparable hypotension secondary to cardiogenic shock (c.20pg/ml), and so they labelled the former as having a “relative deficiency of vasopressin” [Landry *et al.*, 1997a]. It was hence postulated that low VP levels both contributed to the pathogenesis of vasodilatory shock and explained the heightened response to replacement therapy [Landry *et al.*, 1997a; Landry & Oliver, 2001]. Subsequent studies suggested that VP levels were elevated in the acute, early phase of septic shock, but that when the illness was prolonged, circulating concentrations of this hormone fell toward normal values despite continued hypotension [Sharshar *et al.*, 2003a; Leclerc *et al.*, 2003]. Similar observations were also made in patients with vasodilatory shock following cardiopulmonary bypass or prior to solid organ donation [Argenziano *et al.*, 1997; Chen *et al.*, 1999]. A more recently published, large cohort study, however, reported similarly elevated vasopressin levels (c.12pg/ml) across a mixed population of critically ill patients [Jochberger *et al.*, 2006b]. Vasopressin deficiency was rarely found, and a generally poor correlation between hormone levels and haemodynamic parameters was noted.

Ongoing at University College London Hospital (UCLH) is a prospective observational clinical study examining the temporal relationship of bioenergetic, metabolic, hormonal, and immune responses in critical illness. As one of the investigators, I have assayed vasopressin in blood samples taken from patients admitted to the ICU. These measurements could then be related to recorded clinical, haemodynamic and therapeutic data. Terlipressin (synthetic 1-triglycl-8-lysine-vasopressin) is occasionally given to septic shock patients at UCLH, so I was able to assess to what extent plasma VP concentrations were elevated by this treatment.

6.2 Materials and Methods

6.2.1 Clinical study protocol

Ethical approval for this study was obtained from the Joint UCL/UCLH Committees on the Ethics of Human Research (REC Ref No: 04/Q0505/60).

Patients were recruited into three groups:

Group 1: healthy control patients

These were non-ICU patients undergoing elective total hip replacement.

Group 2: non-septic, critically ill patients

These were patients requiring intensive care within 48 hours of admission to hospital from home, for a non-septic/non-inflammatory pathology. At enrolment, such patients must have received mechanical ventilation

commencing within 48 hours of admission to intensive care, should be likely to require mechanical ventilation for at least 48 hours, and must not fulfil the 2001 International Sepsis Definition Conference criteria for severe sepsis or septic shock [Levy *et al.*, 2003].

Group 3: patients with severe sepsis or septic shock

These were patients requiring intensive care within 48 hours of admission to hospital from home, with evidence of severe sepsis (organ dysfunction due to infection) or septic shock as defined by the 2001 International Sepsis Definition Conference criteria [Levy *et al.*, 2003].

Informed consent for inclusion was given by the patient where possible. In situations where the patient did not have mental competency, next-of-kin agreement was sought and retrospective consent obtained from the patient once he/she regained competency. The exclusion criteria applied to this study were as follows:

- age <18 years
- Child-Pugh Class C liver disease
- chronic dialysis-dependent renal failure
- hepatitis B or C infection
- immunosuppression (e.g. haematological malignancy, neutropenia, HIV infection)
- immunosuppressive drug therapy within past 6 months

- patient receiving oral or IV steroid therapy for greater than 1 week, within 6 months prior to ICU admission
- patient receiving thyroid hormone therapy prior to ICU admission
- next-of-kin declines agreement / patient declines consent.

Over a maximum period of two weeks, clinical data and blood samples were collected from the enrolled patients. For control patients (group 1) only one such set was obtained at the time of elective surgery. For patients in groups 2 and 3, data and samples were collected at six-hourly intervals for the first 48 hours following enrolment, 24 hourly for the next 48 hours, and on every third day thereafter. The clinical data recorded included Acute Physiology and Chronic Health Evaluation (APACHE) II scores, mean arterial pressure (MAP), cardiac output (CO), and inotrope administration.

6.2.2 Blood sample analysis

Blood was collected from indwelling arterial lines or by venepuncture into EDTA and aprotinin-containing (1mg/ml and 500KIU/ml blood, respectively) vacutainer tubes for vasopressin assay. After standing for 30 minutes to allow the serum samples to clot, tubes were centrifuged at 1600g and 0°C for 10 minutes. The supernatants were then decanted and frozen at -80°C. Plasma VP was assayed using a commercially available ELISA (Assay Designs Inc.) as described in Chapter 2 (Section 2.2.3). A control experiment confirmed one-to-one cross-reactivity for terlipressin (TP) in the VP ELISA.

6.2.3 Data and statistical analysis

Grouped data are expressed as mean \pm SEM. Patient clinical and hormonal indices were compared between multiple groups and sampling time-points using one-way ANOVA and post-hoc least-squares difference testing. Comparisons between critically ill and septic patients, or between survivors and non-survivors, were made with unpaired Student's t test. In all analyses, a p value <0.05 was considered statistically significant.

6.3 Results

6.3.1 Patient characteristics

Over a 30 month period, 39 patients were enrolled in the clinical study: 10 healthy controls, 7 non-septic, critically ill, and 22 with severe sepsis or septic shock (Table 6.1). Age and gender were well matched across the three groups and mean illness severity scores were similar for non-septic and septic patients. As expected, group 2 and 3 patients were hypotensive at baseline compared to the group 1 controls. Of relevance to vasopressin measurement, serum sodium values were normal in all groups at the time of study enrolment.

The mortality rate in the septic group was 45%. Of the 10 that died, five were male and five female. Sub-group analysis revealed that non-survivors and survivors were similar in terms of age but that the former tended to have higher baseline APACHE II scores (30.3 ± 2.6 vs. 24.5 ± 2.3 , $p=ns$).

Table 6.1 Characteristics of patients enrolled in the clinical study

Group	1 Healthy controls	2 Non-septic, critically ill	3 Severe sepsis or septic shock
Number enrolled survivor : non-survivor	10	7 4 : 3	22 12 : 10
Gender (M : F)	4 : 6	5 : 2	11 : 11
Age (years)	58.1 (3.6)	70.1 (5.4)	61.8 (3.7)
APACHE II score	N/A	30.7(3.0)	26.9 (1.8)
0h MAP (mmHg)	86.0 (2.4)	75.7 (8.7)	70.9 (1.9)*
0h Na (mmol/l)	139 (1.0)	142 (2.6)	141 (1.1)

Where applicable, values shown are mean (SEM).

0h, time of enrolment to study; MAP, mean arterial pressure; Na, serum sodium

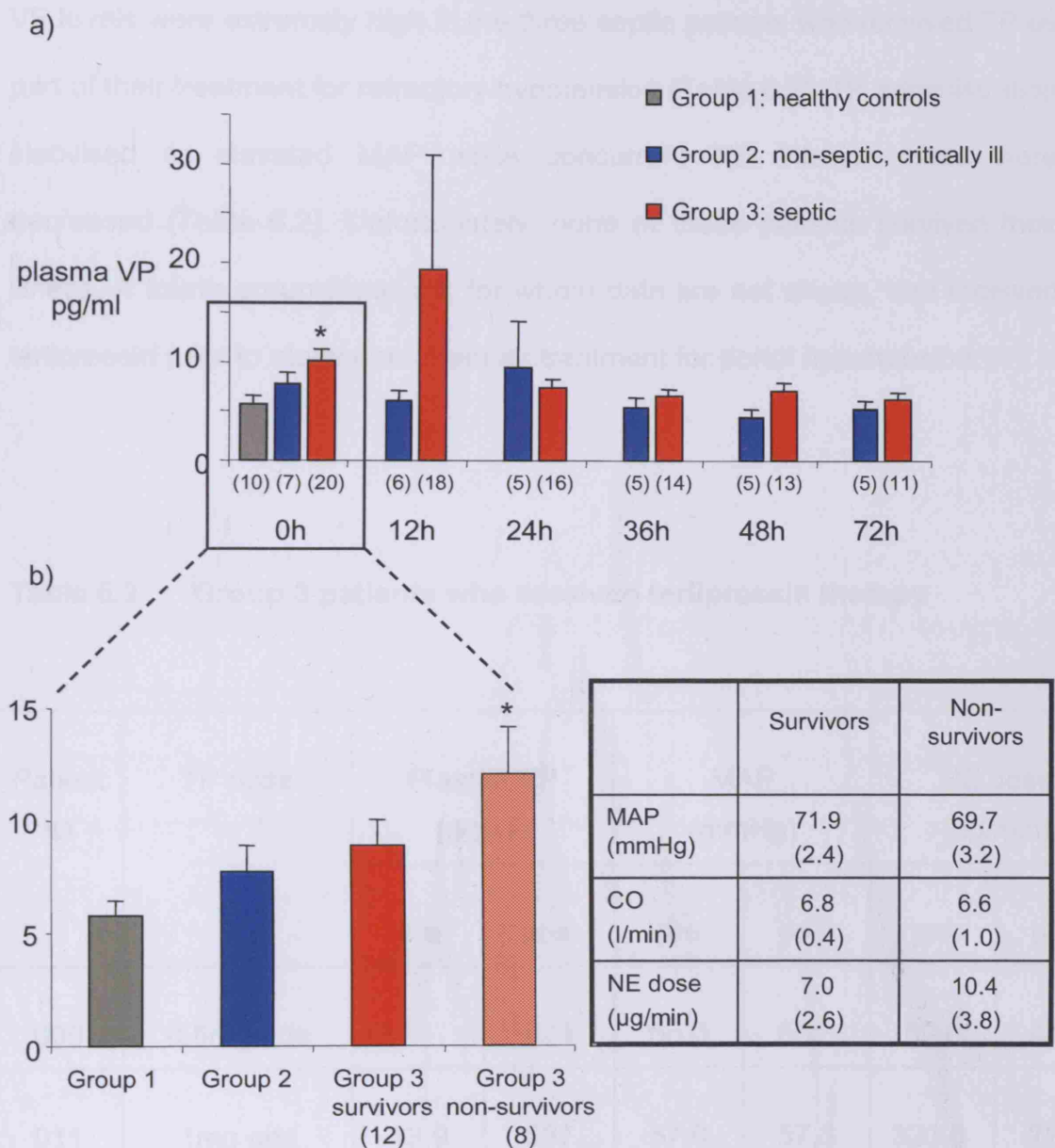
APACHE II = Acute Physiology and Chronic Health Evaluation score. This score is used as a predictor of mortality in ICU patients, with a higher score predicting greater risk of death.

** p<0.05 compared to group 1 controls*

6.3.2 Plasma vasopressin levels

Of the 22 patients in group 3, four received terlipressin at some stage during their illness. Because of the resulting very high hormone levels measured in the VP ELISA (Section 6.3.3), samples taken subsequent to TP treatment were excluded from this analysis. The healthy control patients in group 1 had normal VP levels averaging approximately 5pg/ml (Figure 6.1). Baseline hormone values were higher in septic than non-septic patients (group 3: 10.2 ± 1.1 vs. group 2: 7.8 ± 1.1 pg/ml, $p=ns$). When group 3 patients were subdivided into eventual survivors and non-survivors, it became apparent that the latter group accounted for the more elevated hormone levels (Figure 6.1b). Interestingly, these sub-groups were not clearly distinguishable in terms of blood pressure, cardiac output or inotrope requirement at this stage. Despite considerable variability, a trend towards an early (<24 hours) rise in VP followed by a fall back to control levels was observed in both septic and non-septic patients, although this was more pronounced in the former (Figure 6.1a).

Figure 6.1 Plasma vasopressin (VP) levels in patients



a) Temporal variation in VP levels over the 72 hour period following study enrolment; only one blood sample was taken from group 1 patients at 0h

b) Data from group 3 at 0h are further analysed with patients split into eventual survivors and non-survivors.

All values shown are mean \pm SEM with group sizes given in parentheses.

MAP, mean arterial pressure; CO, cardiac output; NE, norepinephrine

* $p < 0.05$ compared to group 1 controls at time of study enrolment

6.3.3 Patients receiving terlipressin treatment

VP levels were extremely high in the three septic patients who received TP as part of their treatment for refractory hypotension (Table 6.2). TP administration stabilised or elevated MAP while concurrent NE infusion rates were decreased (Table 6.2). Unfortunately, none of these patients survived their illness. A fourth group 3 patient, for whom data are not shown, had received terlipressin prior to study enrolment as treatment for portal hypertension.

Table 6.2 Group 3 patients who received terlipressin therapy

Patient ID	TP dose	Plasma VP (pg/ml)		MAP (mmHg)		NE dose (µg/min)	
		pre	post	pre	post	pre	post
009	0.5mg qds	-	521	55.0	55.3	80.0	41.4
011	1mg qds	13.9	637	57.0	57.3	320.0	72.0
040	1mg qds	7.8	960	74.0	82.7	13.3	2.0

TP, terlipressin; VP, vasopressin; MAP, mean arterial pressure; NE, norepinephrine
Pre-TP figures for VP, MAP and NE are the last set of values recorded prior to TP therapy; post-TP figures are the mean values recorded during 24 hours of TP therapy.

6.4 Discussion

6.4.1 Plasma vasopressin levels

The results described in this chapter are derived from a comparatively small patient cohort, but still provide a valuable contribution to the relevant literature. The growing body of work in this area now supports a complex vasopressinergic dysfunction, rather than a simple hormone deficiency in critical illness.

In line with the largest cohort of patients studied to date, I found that plasma VP levels were higher in early critical illness than in healthy control patients, and that values were generally poorly correlated with haemodynamic parameters in critically ill patients [Jochberger *et al.*, 2006b]. Few others have attempted serial VP measurements, but those who have report a biphasic response in adults but perhaps not in children with prolonged severe sepsis [Sharshar *et al.*, 2003a; Lodha *et al.*, 2006]. Despite considerable variability, a trend to an initial (<24 hours) rise in VP, followed by a fall towards control levels was observed in our group 3 patients. A similar, if less obvious pattern among the group 2, non-septic subjects contrasts Landry's initial findings in cardiogenic shock [Landry *et al.*, 1997a], but is in keeping with another study in patients evaluated after cardiac bypass surgery [Argenziano *et al.*, 1997]. Peak values in septic (group 3) subjects (mean c.10-20pg/ml), akin to those previously reported [Sharshar *et al.*, 2003a; Jochberger *et al.*, 2006b], are perhaps surprisingly low in comparison to findings in acute animal models

(Section 2.3.5) [Wilson *et al.*, 1981a; Brackett *et al.*, 1985; Schaller *et al.*, 1985]. As discussed for the faecal peritonitis rats in Chapter 3 (Section 2.4.2), this may relate to the choice of sampling time-points. Severe sepsis may have commenced several hours prior to study recruitment, making the “0h” measurements a somewhat artificial baseline. That said, VP levels were no higher in a patient cohort presenting to the emergency department at a very early stage in their septic illness [Lin *et al.*, 2005]. In our study, VP levels beyond 24 hours were not as low as those found previously in this clinical scenario [Landry *et al.*, 1997a; Patel *et al.*, 2002]. However, these values were probably still inappropriately low for the concomitant degree of hypotension. The osmo- and baro-regulatory mechanisms of VP secretion, reviewed in Chapter 1 (Section 1.2.1), operate with different sensitivities to alter hormone levels within very different concentration ranges [Mutlu & Factor, 2004]. This, in combination with the iatrogenic influence of intensive care, makes the determination of what is an appropriate vasopressin level in critical illness very difficult [Landry & Oliver, 2006]. The impact of sample timing and resuscitative treatments also complicates the possible interpretation of poor correlation between VP and MAP as evidence of baro-regulatory dysfunction [Jochberger *et al.*, 2006b; Sutherland *et al.*, 2006; Oliver & Landry, 2007].

Whether used to guide inotrope therapy, or as a possible marker of the development of vasodilatory shock, there is increasing call for accurate VP measurement in ICU patients [Oliver & Landry, 2007]. In view of the instability and platelet association of vasopressin and the incumbent difficulties in its

assay, a sandwich ELISA for copeptin, a stable peptide derived from the hormone precursor, was recently developed [Morgenthaler *et al.*, 2006]. Early studies suggest a good correlation between VP and copeptin levels [Struck *et al.*, 2005; Jochberger *et al.*, 2006a]. Inexplicably, however, the copeptin:VP ratio may vary between control and septic patients [Jochberger *et al.*, 2006c]. An alternative possibility is that, analogous to the management of adrenal insufficiency, dynamic tests of vasopressinergic system function are adopted in preference to static hormone measurements [Sharshar *et al.*, 2006].

The finding of higher baseline levels of plasma VP in eventual non-survivors has been reported in both septic [Leclerc *et al.*, 2003; Lodha *et al.*, 2006] and other critically ill patients [Boldt *et al.*, 1995]. Recently, similar observations have been made for copeptin levels [Muller *et al.*, 2007]. In our cohort, survivors and non-survivors could not be distinguished at the first sampling time-point in terms of haemodynamic parameters or NE dose requirements, suggesting a multifactorial explanation for the divergence in hormone levels.

6.4.2 Terlipressin treatment

Terlipressin was used to treat catecholamine refractory shock in three group 3 patients. Conclusions regarding the impact of this therapy, or comparisons with past studies, are difficult with such a small number of subjects. However, to the best of my knowledge, there are no published data concerning plasma VP levels during terlipressin treatment. It appears that six-hourly doses of 0.5-1mg achieve vastly supra-physiological circulating concentrations of VP. Few

investigators have measured hormone levels concurrent to arginine-vasopressin infusion, either in patients or animal models, although high, pharmacological levels have been reported by the majority [Landry *et al.*, 1997b; Dunser *et al.*, 2004a; Ertmer *et al.*, 2007]. This finding, along with the resistance vasculature changes investigated in this thesis, opposes the early hypothesis that heightened pressor responses to vasopressin simply reflect restoration of appropriate hormone levels. Indeed, the degree of blood pressure elevation seems not to be proportional to the plasma VP level prior to exogenous hormone infusion [Dunser *et al.*, 2004a].

Chapter 7: Summary and Discussion

7.1 Summary of results

7.1.1 *In-vivo* rat model of faecal peritonitis

This animal model of prolonged sepsis mimicked human illness in terms of insult type, duration, and fluid resuscitation. With respect to sham-operated rats, septic animals became clinically unwell with hypotension and biochemical evidence of organ dysfunction. After 24 hours, plasma norepinephrine levels were markedly elevated in this group but vasopressin levels were normal. Pressor responses to exogenous NE were significantly depressed in septic rats, whether assessed in anaesthetised or conscious states. In awake animals, blood pressure responses to VP and the selective V₁R agonist, F-180 were diminished in sepsis, but to a lesser extent than that seen for NE. On the other hand, when anaesthetised, VP-induced pressor responses were numerically greater in septic than in sham-operated rats, thus more closely imitating the response seen in septic patients. This finding did not reach statistical significance, however. This may be due to inadequate sample sizes producing an underpowered study.

7.1.2 Wire myography concentration-response curves

The efficacy of NE in contracting small mesenteric arteries removed from rats at the 24 hour time-point was significantly reduced in septic vessels compared to those from sham-operated controls. Furthermore, the tension responses

produced by NE in septic arteries were non-sustained in nature despite continued presence of the contractile agonist. By contrast, VP efficacy was preserved, and its potency in contracting arteries from septic rats was enhanced. Tension responses produced by VP were sustained in all vessels. The pattern of enhanced *ex-vivo* reactivity was exaggerated when the selective V₁ agonist, F-180 was used, with increased efficacy and potency observed in septic vessels. Hence, the vasoconstrictor pathways activated by NE and VP in these resistance vessels are differentially affected by the septic process.

Removal of the endothelium further enhanced the potency of the vasopressin agonists; this effect was more apparent in septic arteries for VP, but in sham arteries for F-180. The leftward shift in the sham F-180 concentration response curves produced by endothelial rubbing was reproduced by pre-incubation of endothelium-intact vessels with the non-selective NOS inhibitor, L-NAME. This suggests that vasodilatory V₁ receptors on the endothelium are coupled to NO production. Addition to the myograph of the V₂R antagonist, FE992082 had no significant effects on VP concentration-response curves in sham or septic arteries. This implies a negligible role for the V₂ receptor in this preparation.

When tail arteries were investigated, contractile responses to NE, VP and F-180 were similar regardless of whether the animals were septic or not. Thus, despite observing vascular hyporeactivity to NE *in-vivo*, such changes are not reflected *ex-vivo* in all arterial beds. The diameters of the tail artery segments

mounted were significantly greater than those of the small mesenteric arteries however, and therefore may not represent true resistance vessels.

7.1.3 Calcium mobilisation studies

Removal of extracellular Ca^{2+} markedly depressed maximal NE- and VP-stimulated contractions in small mesenteric arteries obtained from sham-operated and septic rats, suggesting a high degree of dependence on Ca^{2+} influx for both initiating and maintaining tension. Re-addition of Ca^{2+} to the myograph produced significant contractile responses following VP- but not NE-mediated intracellular store depletion. This implies Ca^{2+} influx via store-operated channels is more important for VP-induced vasoconstriction. Voltage-gated and receptor-operated channels appear to contribute more to NE-induced responses, as pre-incubation with either the L-type Ca^{2+} channel blocker, nifedipine or the ROC antagonist, LOE 908 had a greater inhibitory effect on contractions produced by this agonist. Sepsis had a clear effect on VP's utilisation of Ca^{2+} entry pathways, with an increased contribution of SOCs but a decreased contribution of VGCCs to contractions produced in septic vessels.

7.1.4 Calcium sensitisation studies

Baseline intracellular Ca^{2+} concentrations were consistently higher in mesenteric arteries taken from septic than sham-operated rats. This hints at dysregulation of calcium handling in septic vascular smooth muscle. NE-

induced elevation of intracellular Ca^{2+} was reduced by approximately 50% in septic compared to sham vessels, which paralleled the depressed tension responses to this agonist. By contrast, VP-induced Ca^{2+} changes were similar in sham and septic arteries despite a 2.5-fold increase in the magnitude of contractions induced in the latter. An enhanced contractile response for a comparable Ca^{2+} rise indicates a VP-mediated sensitisation of the myofilaments to the effect of calcium in septic vessels.

7.1.5 Patient studies

In the cohort of ICU patients studied, plasma vasopressin levels were elevated in early severe septic illness when compared to those in healthy controls. Within the septic group, those that went on to die had higher hormone levels at the time of study recruitment compared to those that went on to survive, despite having similar haemodynamic measurements. Beyond 24 hours, VP concentrations returned towards normal values, despite continued critical illness. In catecholamine-refractory septic shock, treatment with terlipressin produced supra-physiological circulating VP concentrations, suggesting that the drug is not simply working by restoring appropriate hormone levels.

7.2 Discussion and Future Investigations

Although successful in many respects in producing a clinically realistic animal model of sepsis, there are drawbacks to the faecal peritonitis method used.

Only mild hypotension was induced in septic animals, in the face of severe clinical illness and high mortality. Moreover, I was unable to demonstrate correlations between severity scores and blood pressure or biochemical indices. Whilst the biochemical measurements revealed organ dysfunction consistent with a systemic inflammatory response, this could have been further supported by comparing plasma cytokines and nitrite/nitrate levels in the septic and sham-control groups [Bernard *et al.*, 1998]. In addition, the septic insult produced by intra-peritoneal injection of faecal slurry could have been better quantified by determining its bacterial load and/or by assaying the consequent endotoxaemia [Bernard *et al.*, 1998].

Despite these limitations, in rats with faecal peritonitis, I demonstrated the pattern of endogenous VP and NE levels [Sharshar *et al.*, 2003a; Lin *et al.*, 2005] and the decreased catecholamine pressor response [Hotchkiss & Karl, 2003] characteristic of prolonged septic shock in patients. Blood pressure responses to norepinephrine were very similar in awake and anaesthetised animals. With vasopressin, however, anaesthesia diminished the sham-control, but not the septic pressor response. This suggests that the sedation of critically ill patients, a very common component of ICU care, might partly explain the therapeutic effect of vasopressin in this clinical setting. Although not investigated further in this thesis, the complex interactions between VP and the autonomic nervous system [Mohring *et al.*, 1980; Leng *et al.*, 1999; Koshimizu *et al.*, 2006], and the sepsis-related dysfunction of the latter [Garrard *et al.*, 1993; Sharshar *et al.*, 2003b], are likely to contribute to the

multifactorial mechanisms underlying vasopressin hypersensitivity (Section 1.4.4).

In an *ex-vivo* setting, away from any confounding neural influences, a pattern of sepsis-induced depressed vascular reactivity to NE yet enhanced reactivity to VP was seen. This suggests that changes in the direct effect of vasopressin on vascular smooth muscle in sepsis are an important contributor to hypersensitivity to this hormone. This finding also validates the use of small mesenteric arteries in the wire myograph for further exploration of this hypothesis.

Although only limited deductions regarding changes in receptor characteristics can be made from pharmacological concentration-response experiments, the obvious depression of maximal responses to NE in septic arteries does support a decrease in the number of sarcolemmal α_1 receptors. By contrast, the rise in F-180 E_{max} in septic vessels suggests that the V_1 receptor number is increased. The lack of change in VP E_{max} between septic and sham arteries can be reconciled by the existence of other, non- V_1 vasopressin receptors within the resistance vasculature, the expression of which may also be affected by the septic insult. This would also explain the greater degree of separation seen between sham and septic concentration-response curves for F-180 compared to VP.

From the endothelial removal studies, it can be surmised that receptors on this cell layer do contribute to vasopressin-stimulated responses. The lesser effect of endothelial denudation on F-180 responses in septic vessels implies

down-regulation of the NO-mediated V₁R vasodilatory pathway in sepsis. The accentuated leftward shift of the VP concentration-response curve in septic compared with sham vessels following endothelial removal however implies up-regulation of non-V₁R vasodilatory pathways. The V₂R is a likely candidate [Tagawa *et al.*, 1995; Kaufmann *et al.*, 2003], but unfortunately, little useful data were gleaned in this regard from the FE992082 experiments. The role of this receptor subtype would be better addressed in future investigations through the use of a more selective V₂R antagonist, or by assessing the direct vasodilatory effect of a selective V₂ agonist. In addition, a similar approach should be adopted with oxytocin receptor-selective agents, since evidence suggests that these receptors are also present in blood vessels [Yazawa *et al.*, 1996; Thibonnier *et al.*, 1999].

To complement these pharmacological studies, direct measurements of receptor characteristics are also needed. In-situ hybridisation, radioligand-binding, and nucleic acid amplification techniques have all been used to quantify vasopressin receptors in rat tissues [Roth & Spitzer, 1987; Phillips *et al.*, 1990; Bucher *et al.*, 2002; Grinevich *et al.*, 2004]. Although a sepsis-induced decrease in solid organ V₁R expression has been reported [Roth & Spitzer, 1987; Bucher *et al.*, 2002], these models did not show *in-vivo* enhanced vasopressinergic reactivity and the investigators did not examine the resistance vasculature.

Aside from any alteration in receptor number, modification of the coupling of α_1 Rs and V₁Rs to Ca²⁺ signalling mechanisms could account for the changes

in NE and VP vascular reactivity seen during sepsis. Non-sustained NE-induced contractions yet sustained VP-induced contractions in septic arteries suggest that the two agonists differentially activate Ca^{2+} entry and/or Ca^{2+} sensitisation pathways. This hypothesis is supported by my results.

Inference from the Ca^{2+} mobilisation studies was restricted due to the use of tension responses as a surrogate of intracellular Ca^{2+} concentration and the known complexity of interaction between the different pathways involved [Wier & Morgan, 2003]. Despite this, I have demonstrated that the Ca^{2+} mobilisation pathways stimulated by NE and VP in small mesenteric arteries are not the same. NE-stimulated tension responses depended primarily on VGCC- and/or ROC- mediated Ca^{2+} influx, whereas SOC-mediated influx was more important for VP-stimulated responses. Although VGCCs were still activated in septic vessels by high dose NE, sepsis-induced dysfunction of these channels [Zhong *et al.*, 1997; Zhao & Zhao, 2007] might significantly limit the Ca^{2+} rise produced by this agonist. Conversely, VP may be able to mobilise Ca^{2+} with unimpaired or even greater efficiency in sepsis if SOC activity is preserved (or even enhanced).

My findings seemingly contradict previous reports of significant contributions of the SOC to NE- and the ROC to VP-mediated contractions [Furutani *et al.*, 2002; Zhang *et al.*, 2002]. While this may simply relate to differences in vascular bed or vessel size, it would be pertinent to confirm my results by either refining or changing the methodology used. Other compounds reported to selectively block SOCs (e.g. 2-aminoethoxydiphenyl borate [2-APB]) or

both SOCs and ROCs (e.g. SK-96365) have been employed in similar studies [Furutani *et al.*, 2002; Villalba *et al.*, 2007]. Tension experiments could be performed to determine optimal concentrations for the various channel inhibitors which could then be used alone and in combination. In addition, maximal SOC activity in sham and septic vessels could be measured through the use of a SERCA inhibitor such as thapsigargin or cyclopiazonic acid (CPA) [Furutani *et al.*, 2002; Villalba *et al.*, 2007]. A complementary approach would be to quantify channel expression [Lew *et al.*, 1996; Jung *et al.*, 2002], although definitive separation of SOCs and ROCs remains complicated by the known similarities in their molecular structure [Albert & Large, 2006].

Sepsis-induced changes in intracellular calcium handling were clearly demonstrated in the Ca^{2+} sensitisation studies. VP, but not NE, sensitised the contractile apparatus to the effect of Ca^{2+} in septic arteries to generate a greater tension for a comparable increase in intracellular Ca^{2+} . This may result from VP-stimulated inhibition of MLCK via the ROK pathway [Yang *et al.*, 2006]. In future experiments, it would be very interesting to observe the effect of ROK antagonists, such as HA-1077, on the vasopressin Ca^{2+} -tension relationship. VP-mediated Ca^{2+} sensitisation might not only contribute to its enhanced pressor effect in septic patients, but may also explain why NE responsiveness is improved when the two drugs are given in combination [Dunser *et al.*, 2003]. The combined *in-vivo* and *ex-vivo* methods used in this thesis would be ideal for exploration of this theory.

To further validate all these findings, their successful translation across different vascular beds and, more importantly, into patient studies is necessary. It is uncertain why vascular reactivity was unaltered in tail arteries taken from rats with faecal peritonitis despite evidence of systemic septic illness. This should be addressed by repeating *ex-vivo* concentration-response experiments on other extra-abdominal resistance vessels, such as small renal or femoral arteries.

Serial hormone measurements in our cohort of severely septic patients revealed VP profiles similar to those reported previously in this clinical scenario [Sharshar *et al.*, 2003a; Jochberger *et al.*, 2006b]. Ideally, resistance vessels obtained from these subjects would have been used for wire myograph concentration-response and Ca^{2+} studies. Unfortunately, due to logistical and ethical constraints, I was unable to obtain tissue samples for vascular reactivity studies from any of these patients. Depressed NE reactivity in myograph-mounted human omental arteries from patients with intra-abdominal sepsis has been demonstrated previously [Stoclet *et al.*, 1999], and thus investigation of heightened VP reactivity in such a preparation is a realistic possibility.

7.3 Conclusions

The treatment of septic shock remains a major challenge in intensive care medicine. Although the first large, randomised trial comparing vasopressin to norepinephrine has shown non-inferiority rather than superiority [Russell *et*

al., 2008], further patient studies and continued clinical use of vasopressin are likely.

The work described in this thesis provides valuable insight into the sepsis-induced changes in vasopressin signalling in vascular smooth muscle. By virtue of its use as a comparator, valuable information concerning norepinephrine-mediated vasoconstriction has also been gained. The implied up-regulation of V_1 receptors in septic resistance vessels supports the development of more selective compounds for clinical use. Indeed, the greater V_1 selectivity of terlipressin may, in part, explain accumulating evidence that this drug may be preferable to vasopressin for the treatment of septic shock [Lange *et al.*, 2007].

Downstream of the V_1 receptor, vasopressin is able not only to effectively mobilise Ca^{2+} in septic vascular smooth muscle but also to sensitise the myofilaments to its effect. The different Ca^{2+} entry channels utilised by NE and the lack of a sensitising effect in sepsis likely contribute to the impaired pressor effect of this agent in prolonged septic shock. These findings thus promote modulation of Ca^{2+} sensitisation and/or Ca^{2+} mobilisation pathways as potential new therapeutic paradigms for the treatment of septic shock. Of relevance, recent work has demonstrated benefit of a cardiac muscle Ca^{2+} sensitising agent, levosimendan, in animal models of septic myocardial depression [Sorsa *et al.*, 2004; Barraud *et al.*, 2007].



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Publications

Original Article

Barrett LK, Orié NN, Taylor V, Stidwill R, Clapp LH, Singer M

Differential effects of vasopressin and norepinephrine on vascular reactivity in a long-term rodent model of sepsis

Critical Care Medicine 2007; 35:2337-2343

PMID: 17700487

Review Article

Barrett LK, Clapp LH, Singer M

Vasopressin: mechanisms of action on the vasculature in health and in septic shock

Critical Care Medicine 2007; 35:33-40

PMID: 17133186

Prize

The Intensive Care Society Research Gold Medal Award 2007

The mechanisms of vasopressin hypersensitivity in septic shock

Awarded on the basis of a written summary and oral presentation describing three years' work

Abstracts presented at meetings

European Society of Intensive Care Medicine Annual Congress 2007

Barrett LK, Taylor V, Clapp LH, Singer M

Can different calcium mobilisation pathways explain the changes in vascular sensitivity to norepinephrine and vasopressin in septic shock?

Intensive Care Medicine 2007; 33, Supplement 2: S34

Barrett LK, Patel S, Taylor V, Clapp LH, Singer M

Calcium sensitisation underlies enhanced vascular reactivity to vasopressin in septic shock

Intensive Care Medicine 2007; 33, Supplement 2: S34

British Pharmacological Society Annual Meeting 2006

Barrett LK, Taylor V, Singer M, Clapp LH

Enhanced vascular responses to vasopressin in a rat model of fecal peritonitis

Proceedings of the BPS (pA₂ online); Vol 4 Iss 2 abst 185P

European Society of Intensive Care Medicine Annual Congress 2006

Barrett LK, Taylor V, Stidwill R, Clapp LH, Singer M

Ex-vivo demonstration of enhanced vascular reactivity to vasopressin in prolonged septic shock

Intensive Care Medicine 2006; 32, Supplement 1: S76

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Vasopressin levels in septic shock: relationship to survival and effect of terlipressin treatment

Intensive Care Medicine 2006; 32, Supplement 1: S202

Medical Research Society, Academy of Medical Sciences and Royal College of Physicians Meeting for Clinical Scientists in Training 2006

Barrett LK, Clapp LH, Singer M

Vasopressin deficiency and hypersensitivity in septic shock [Abstract no. 91]

European Society of Intensive Care Medicine Annual Congress 2005

Barrett LK, Clapp LH, Singer M

Circulating vasopressin levels and vascular reactivity in prolonged septic shock

Intensive Care Medicine 2005; 31, Supplement 1: S218

