THE ROLE OF ENDOTHELIN-1 IN THE PULMONARY VASCULAR RESPONSE TO SEPSIS

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ABSTRACT

The clinical response to sepsis in man is characterised by systemic vascular dilation and hyporesponsiveness to pressor agents, together with elevated pulmonary vascular resistance and pulmonary artery pressure. Despite the production of copious amounts of nitric oxide in endotoxaemia, which contributes to the reduced responsiveness of systemic vessels, it remains unclear why pulmonary vessels do not exhibit this hyporesponsiveness. Endothelin-1 is a potent vasoconstrictor peptide produced by vascular endothelium, plasma concentrations of which are increased both in animal models of sepsis and in patients with septic shock. The role of endothelin-1 in the pulmonary vascular response to sepsis is unknown. This thesis addressed this question using a series of experiments performed on a rat model of endotoxaemia, using a variety of techniques including *in vivo* and *in vitro* bioassay, histological examination, RNase protection with gel electrophoresis, ELISA and other colourimetric determinations.

Endothelin-1 messenger RNA expression, as well as plasma arterial concentration, were increased by lipopolysaccharide treatment in this rat model. Isolated pulmonary artery from lipopolysaccharide-treated rats exhibited hyporesponsiveness to endothelin-1, which could be largely reversed by the prior administration of a nitric oxide synthase inhibitor. Evidence suggested that a component of the endothelin-1-induced contraction in isolated, intact pulmonary artery from lipopolysaccharide-treated rats was dependent on an endothelium-derived cyclooxygenase product. In the isolated whole pulmonary circulation, by contrast, there was hypersensitivity to exogenous endothelin-1 that was augmented by a nitric oxide synthase inhibitor, and could not be attenuated by

an inhibitor of cyclooxygenase. Despite both elevated circulating levels and local expression of endothelin-1, antagonism of endothelin-1 receptors (A and B) produced only small reductions in mean systemic and pulmonary arterial pressures *in vivo*.

These data confirm that endothelin-1 production is increased in an animal model of sepsis, but suggests that the local balance between nitric oxide, cyclooxygenase metabolites and functional effects of endothelin-receptor activation determines the pulmonary vascular response to exogenous endothelin-1 under these circumstances.

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ABBREVIATIONS

$A\text{-}aDo_2$	Alveolar-arterial oxygen gradient
ACh	Acetylcholine
AG	Aminoguanidine
AII	Angiotensin II
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BAL	Broncho-alveolar lavage
BH_4	Tetrahydrobiopterin
BK	Bradykinin
BSA	Bovine serum albumin
cAMP	Adenosine 3'5'-cyclic monophosphate
cGMP	Guanosine 3',5'-cyclic monophosphate
CO	Cardiac output
COX	Cyclo-oxygenase
dNPT	Deoxynucleotide triphosphate
DO_2	Oxygen delivery
DTT	Dithiothreitol
EC_{50}	Concentration of agonist required to induce half of the maximum effect
ECE	Endothelin-converting enzyme
EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxant factor
EDTA	Ethylene-diamine tetra-acetic acid
EFS	Electrical field stimulation
eNOS	Endothelial nitric oxide synthase
ET	Endothelin
ET_A	Endothelin-A receptor
ET_B	Endothelin-B receptor
FAD	Flavin adenosine dinucleotide
FMN	Flavin mononucleotide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GTN	Glyceryl trinitrate
HPV	Hypoxic pulmonary vasoconstriction
5-HT	5-hydroxytryptamine
IC_{50}	Concentration of antagonist required to elicit half maximal inhibition
IBPL	isolated, blood-perfused lung
IFN	Interferon gamma
IGF	Insulin-like growth factor
IL	Interleukin
im	Intramuscular
iNOS	Inducible nitric oxide synthase
ip	Intraperitoneal
IP_3	Inositol-1,3,5,-triphosphate
IPRL	isolated, perfused rat lung
iv	Intravenous
K^+_{ca}	Calcium-activated potassium channels
Krebs	Krebs-Henseleit solution
L-arg	L-arginine

L-NAME	N^{w}-nitro-L-arginine-methylester
L-NMMA	N^{G}-monomethyl-L-arginine
L-NNA	N^{G}-nitro-L-arginine
LAP	Left atrial pressure
LPS	Lipopolysaccharide / endotoxin
LT	Leukotriene
MAP	Mean arterial pressure
MB	Methylene blue
MDS	Myocardial depressant substances
MODS	Multiple organ dysfunction syndrome
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NAA	N^{G}-amino-L-arginine
NADPH	Nicotinamide adenine dinucleotide
NANC	Non-adrenergic-non-cholinergic
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	Nitric oxide metabolites
OER	Oxygen extraction ratio
PA	Pulmonary artery
PAF	Platelet activating factor
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phenylephrine
PEEP	Positive end-expiratory pressure
PGE_2	Prostaglandin E_2
PGF_{2a}	Prostaglandin F_{2a}
PGI_2	Prostacyclin
PHT	Pulmonary hypertension
Ppa	Pulmonary artery pressure
ppm	Parts per million
PPV	Positive pressure ventilation
PVR	Pulmonary vascular resistance
ROS	Reactive oxygen species
RT	Reverse transcription
SEM	Standard error of the mean
SIRS	Systemic inflammatory response syndrome
SOD	Superoxide dismutase
SNP	Sodium nitroprusside
SVR	Systemic vascular resistance
S6b	Sarafotoxin S6b
S6c	Sarafotoxin S6c
TCA	Trichloroacetic acid
TGF	Transforming growth factor beta
T_{max}	Maximum tension developed
TNF-α	Tumour necrosis factor alpha
TXA_2	Thromboxane
VSM	Vascular smooth muscle
V/Q	Ventilation/perfusion

CHAPTER 1

INTRODUCTION

1.1 SEPSIS AND ACUTE RESPIRATORY DISTRESS IN ADULTS

1.1.1 Historical Perspective

Sepsis and its associated syndromes represent a potentially devastating systemic inflammatory response estimated to occur in 1% of hospitalised patients, 10-20% of whom die (Bone, 1991). Mortality approaches 60-90% in those who develop septic shock, a figure that has changed little since intensive care was first developed, despite improvements in monitoring and supportive techniques. When invasive monitoring was first used in patients with septic shock, the characteristic pathological reduction in systemic vascular resistance was identified as the reason behind the failure of their central venous pressure and cardiac output to respond in a sustained manner to fluid resuscitation (Maclean et al. 1967). The majority of these patients also demonstrated a failure of multiple organ systems and metabolic derangement. Subsequent research has therefore been aimed initially at recognising and describing patterns of clinical sequelae resulting from "sepsis" and subsequently at understanding the pathophysiological mechanisms that underlie these manifestations.

In 1967 a group of 12 adult patients with respiratory failure, reduced pulmonary compliance and radiographic evidence of pulmonary oedema, following a variety of insults, were first described as having the acute respiratory distress syndrome (ARDS) (Ashbaugh et al. 1967). This term was used because

of the histological similarity between their lungs and those of babies with the infant respiratory distress syndrome. The description of respiratory distress in association with fluid accumulation in the lung was not new, however, having already been reported in some casualties of battle, giving rise to names such as "shock lung" and "wet lung" (Brewer et al. 1946). It has gradually become apparent that lung injury can occur in association with a wide variety of pulmonary and extra-pulmonary pathologies, both acute and chronic, and that there is a spectrum of clinical severity, at the extreme end of which lies ARDS (Bernard et al. 1994) **[Table 1.1]**. The identification of those insults that give rise most consistently to significant lung injury, and which patients tend to develop clinical manifestations has become the focus for intensive investigation.

1.1.2 Definitions of clinical sepsis syndromes

A constellation of features has been identified and used to classify the clinical and haematological responses of critically ill patients with sepsis **[Table 1.2]**. Such syndromes are associated with the positive identification of an infecting causative organism in less than 50% of cases (Balk and Bone, 1989). To avoid the requirement for an identifiable infection, for example in patients in whom the underlying aetiological condition is trauma, burns or pancreatitis, the term systemic inflammatory response syndrome (SIRS) is now used to define the resulting clinical state (Bone et al. 1992b). Established sepsis and SIRS represent the biggest cause of mortality in the critically ill (Vincent and Bihari, 1992), and up to 25% of such cases are complicated by ARDS. Lung injury in these patients is thus common: 35% of a population with sepsis having been shown to display mild to moderate lung injury, whilst 25% had severe lung injury or

fully-developed ARDS (Weinberg et al. 1984), with a mortality of between 50 and 75%. Lung injury rarely occurs in isolation, and ARDS is now considered to be a multisystem vascular disorder (Mizer et al. 1989; Bone et al. 1992a) characterised not only by impaired gas exchange, but also by a failure of oxygen uptake in the periphery, leading ultimately to multiple organ failure (MOF) (Griffiths and Evans, 1996). Thus, in one study respiratory failure accounted for only 16% of deaths, with the majority being attributable to MOF (Montgomery et al. 1985). This is therefore consistent with the hypothesis that lung injury represents only the pulmonary manifestation of a diverse systemic insult which also produces other overlapping clinical syndromes **[Figure 1.1]**. Moreover, it appears that the associated clinical conditions or risk factors and coexisting organ failures are the major determinants of survival (Bone et al. 1992a; Fowler et al. 1985). It is perhaps not surprising that sepsis occurs up to six times more commonly in patients with ARDS compared to other critically ill patients (Montgomery et al. 1985; Seidenfeld et al. 1986).

The current concept of sepsis is therefore one of a generalised inflammatory response leading to dysfunction and possibly failure of one or more organs which produces characteristic clinical syndromes regardless of the initiating factor **[Figure 1.2]**. Indeed, studies have failed to differentiate outcome and manifestations of sepsis based on the type of causative microorganism (Glauser et al. 1991).

ARDS is principally a clinical diagnosis, the criteria for which differ between centres (Macnaughton and Evans, 1992). Pulmonary endothelial damage can lead to mild respiratory impairment or the overwhelming alveolar oedema that is characteristic of ARDS. There are now several ways to approach the diagnosis of ARDS. Traditionally, there has been a requirement for bilateral diffuse pulmonary infiltrates on chest radiography; arterial hypoxaemia and a high gradient between

this value and the level of inspired oxygen (PaO_2:FiO_2); the exclusion of "cardiogenic" pulmonary oedema by a normal pulmonary artery occlusion pressure (PAOP); and a reduction in pulmonary compliance. The problems associated with making these measurements are numerous, as they are open to considerable subjective interpretation (Curzen et al. 1996). In addition, some criteria are necessarily rather arbitrary, such as the PaO_2:FiO_2 gradient, or the definition of a normal PAOP (Fowler et al. 1983). As a result of these difficulties, an attempt to modify the definition of ARDS has been made (Murray et al. 1988). Probably most important of all, a scoring system was introduced which allows for the inclusion of patients with a range of lung injury from mild to severe **[Table 1.3]**. The scoring system has several advantages. Firstly, it provides a tool by which the degree of injury can be assessed and reviewed, which is valuable for the study of the individual patient and of different patient populations. Secondly, it minimises the need for "cut-off" values for inclusion parameters such as the degree of hypoxaemia. Thirdly, it removes PAOP as part of the score since this, although frequently useful as adjunctive information in the clinical setting, is an unreliable guide to the origin of interstitial pulmonary fluid. Fourthly, as part of the definition there is a requirement to classify the stage which the condition has reached temporally. Finally, there is an attempt to summarise the disorders that are causing or contributing to the lung injury.

The problems surrounding the definition and classification of acute lung injury and ARDS have been recently reviewed by the American-European Consensus Conference on ARDS (Bernard et al. 1994). The conclusions of most relevance were as follows:

A) ARDS should be the abbreviation for the "ACUTE" Respiratory Distress Syndrome because the condition can be identified in children.

B) The term acute lung injury (ALI) was introduced to describe several degrees of pulmonary insult. ARDS was now to be used only for those patients at the most severe end of this spectrum. The arbitrary nature of "cut-off" values for the definition of ARDS was therefore recognised.

C) The clinical condition was to be classified by the description of the precipitating cause.

Such scoring systems, which allow the severity of the injury to be reliably estimated, have greatly increased the utility of clinical studies. Thus, using such scoring techniques, a multicentre study was able to identify patients with milder forms of lung injury who went on to develop full-blown ARDS (Bernard et al. 1994).

1.1.3. Pathogenesis of Sepsis and ARDS

1.1.3.1 **Endotoxin and other initiators**

The initial trigger for the complex cascade of inflammatory pathways that lead to the clinical sepsis syndromes is most frequently endotoxin or another comparable substance derived from yeasts, viruses, fungi or gram-positive bacteria (Bone, 1991). Endotoxin is derived from the lipopolysaccharides (LPS) that make up the outermost layer of the cell membrane of all gram-negative bacteria (Sonesson et al. 1994). LPS is composed of a polysaccharide portion bonded to the lipid A component, which is responsible for the endotoxic properties of LPS through its ability to interact with both cellular and humoral inflammatory pathways [**Figure 1.3**]. Endotoxin can be present in the host circulation either as a result of exogenous bacterial invasion, or by translocation of intestinal flora through the wall of the gastrointestinal tract (Meakins and Marshall, 1986). Endotoxaemia is

frequently found in patients with septic shock, whether or not blood cultures are positive for a specific pathogen (Danner et al. 1991). Indeed, several groups have been able to correlate the level of endotoxin in the blood with the development of ARDS and MODS in patients with sepsis (Manthous et al. 1993). It is unsurprising in view of the above that infusion of LPS reproduces the clinical features of sepsis, including acute lung injury, in animal models (Brigham and Meyrick, 1986). In addition, endothelial cell monolayers *in vitro* show dose-dependent damage, with increased permeability and eventually cellular destruction, in response to LPS (Read and Meyrick, 1994). The administration of a small bolus of LPS to healthy volunteers has been shown to produce haemodynamic features characteristic of sepsis (Moser et al. 1963), as well as evidence of acute lung injury (Suffredini et al. 1989; Taveira da Silva et al. 1993).

The weight of evidence supporting endotoxin as an initiator of the inflammatory response in sepsis has lead to the development of monoclonal anti-LPS antibodies directed against either core epitopes or lipid A in an attempt to neutralise LPS effects, thereby improving outcome in patients with Gram-negative infection. Despite several large clinical trials, however, such antibodies have so far failed to show any overall improvement in survival in these patients (Natanson et al. 1994).

1.1.3.2 **Cellular and humoral mediators of the inflammatory response**

Endotoxin has the ability to initiate an inflammatory response, the extent of which is dependent upon a self-amplifying cascade of events that incorporates humoral and cellular components, and which can occur even in the absence of any particular cell or mediator (Curzen et al. 1994). Early in the process there is the release of a wide range of pro-inflammatory mediators including histamine, bradykinin,

cytokines (including particularly tumour necrosis factor-α, TNF-α, and interleukins, ILs) and activation of peptide cascades (coagulation, complement and fibrinolysis). At this early stage of the inflammatory response, there is activation of neutrophils, endothelial cells, platelets and mononuclear cells. Subsequently, active, derived products such as reactive oxygen species (ROS), or nitric oxide (NO), products of arachidonic acid metabolism, endothelin-1 (ET-1), and proteinases are generated and contribute to local vasoactive and cytotoxic effects.

In the plasma, endotoxin binds to a 60-kDa acute phase glycoprotein known as endotoxin-binding protein (EBP). Animal studies have shown that macrophages and monocytes, which have specific surface proteins (CD14 family) that recognise this LPS-EBP complex can release cytokines including TNF-α, interleukins (IL-1,-6,-8) and platelet activating factor (PAF) as a result of this activation (Martin et al. 1994). In addition, endotoxin leads to the activation and attraction of neutrophils both directly and via the associated production of cytokines, especially TNF-α. TNF-α is a 17 kD peptide whose activation of neutrophils leads to them producing elastases (Smedly et al. 1986) as well as ROS such as superoxide and hydrogen peroxide (Tsujimoto et al. 1986). TNF-α also activates endothelial cells, and promotes their binding to neutrophils leading to endothelial damage and destruction (Gamble et al. 1985; Nathan, 1987). These are the early stages of an amplifying cascade of inflammatory events that ultimately can result in tissue damage and clinical "sepsis". Thus, injection of endotoxin causes TNF-α levels in the blood to rise acutely in both animals and humans (Michie et al. 1988), which in turn leads to the characteristic haemodynamic changes of sepsis such as hypotension and increased pulmonary capillary permeability. In fact, TNF-α levels are elevated in patients with sepsis, and probably correlate with prognosis (Damas et al. 1989; Calandra et al. 1990;

Waage et al. 1987). Furthermore, C3H/HeJ mice, which cannot synthesize TNF-α, are resistant to otherwise lethal doses of endotoxin (Beutler et al. 1986).

Interleukin-1 shares many of the properties of TNF-α, producing hypotension and pulmonary oedema in rabbits (Goldblum et al. 1987). IL-6 production is stimulated by other cytokines, acts by regulating lymphocyte function, and rises later in the inflammatory response (Kasid et al. 1989). IL-8 is a potent chemotactic agent for neutrophils (Van Zee et al. 1991), and its production by other cells such as endothelium ensures continuing local recruitment of neutrophils.

Platelet activation precipitates the release of numerous vasoactive, chemoattractant and endothelium-damaging substances. Endotoxin is a potent activator of the complement system, and also contributes to the initiation of both intrinsic and extrinsic coagulation pathways. Factor XII, in particular, initiates the intrinsic coagulation cascade as well as the contact system which generates bradykinin. Neutrophil-derived proteases and bradykinin simultaneously stimulate the fibrinolytic systems, and thus generate the potential for disseminated intravascular coagulopathy (Bone et al. 1976).

1.1.3.3 **The endothelium and its role in inflammation**

It has become apparent that the endothelium plays a central, pivotal role in the vascular response to sepsis. Until recently, it was perceived as a passive, metabolically inert barrier whose function was to contain blood and plasma. It is now known to be a metabolically and physiologically dynamic tissue with multiple functions (Petty and Pearson, 1989).

In order to maintain patency of blood vessels and the fluidity of the circulating surface for blood, endothelial cells synthesize and release various anticoagulant and antithrombotic substances, including thrombomodulin which

binds thrombin to lower its affinity for fibrinogen (Esmon, 1987). Tissue plasminogen activator (tPA) is also synthesised by the endothelium and activated by a variety of stimuli in the circulation (Van Hinsbergh, 1988) to initiate the production of plasmin. The endothelial cell surface is rich in heparin sulphates, which contribute to the inactivation of circulating thrombin (Marcum et al. 1984). Nevertheless, the synthesis and production of prostacyclin and endothelium-derived relaxant factor (s) (EDRF) is probably the primary component of its antithrombotic armoury, since both mediators are vascular smooth muscle dilators and potent inhibitors of platelet aggregation (Moncada et al. 1990; Radomski et al. 1991; Radomski et al. 1987a; Radomski et al. 1987b).

Pulmonary endothelial cells have a particularly wide range of metabolic functions, such as the uptake and degradation of vasoactive amines like noradrenaline and serotonin, and activate and deactivate others including angiotensin II, bradykinin and enkephalin (Said, 1982; Liu and Barnes, 1994). Pulmonary endothelial cells can also metabolise adenosine triphoshate (ATP) to adenosine.

As has been discussed, endothelial cells are capable of activation by endotoxin, TNF-α and other mediators of the early inflammatory response such as histamine. These cytokines are, however, also capable of inducing cell injury and stimulate ultrastructural changes within the endothelial cell leading to increased permeability (Phillips and Tsan, 1992), which correlate with the cytoarchitectural realignment of intracellular proteins that can be seen using electron microscopy. Once activated, endothelial cells are capable of releasing a variety of vasoactive and pro-inflammatory mediators, including specifically NO, prostanoids and ET-1, and also display cell surface proteins that play a critical role in the adherence and

subsequent migration of activated neutrophils as part of the so-called adhesion molecule cascade.

1.1.3.4 **Adhesion molecules**

Endothelial cell activation also facilitates the adherence and subsequent migration from blood to tissue of activated neutrophils, and an awareness as to the significance of this cellular interaction, mediated by a steadily expanding group of "adhesion molecules", is rapidly increasing. This adhesion molecule "cascade" provides the machinery for the neutrophil-endothelial cell interaction that results in neutrophil migration, and as such represents an attractive set of targets for guided immunotherapy. In fact, radiolabelling of neutrophils has demonstrated their rapid sequestration in the lungs after the onset of sepsis in both animals and humans (Hangen et al. 1990), and broncho-alveolar lavage fluid (BAL) from patients with ARDS is rich in neutrophils (Fowler et al. 1987; Weiland et al. 1986). The majority of this neutrophil migration occurs in post-capillary venules. Initially there is slowing of the white cells with margination, when they can be seen under light microscopy rolling along the vessel wall as a result of loose tethering to the underlying endothelial cells. Subsequently, the neutrophils become more adherent and they change from spherical to a flatter shape. This facilitates their slow migration between endothelial cells and then through the basement membrane into the interstitium. This process is now known to be mediated by interaction of specific cell adhesion molecules (CAM) on the surface of both the neutrophil and endothelial cell in a sequence known as the adhesion cascade (Albelda et al. 1994). Thus, the first phase of the process (slowing and rolling) depends upon the expression of a group of surface glycoprotein molecules collectively called selectins (Bevilacqua et al. 1991). Two

such molecules are expressed on the endothelial cell for this process: endothelial-leukocyte adhesion molecule-1 (E-selectin), and granulocyte-associated membrane protein (P-selectin). Endothelial E-selectin expression has been demonstrated in response to cytokines and LPS, and the kinetics of its production in cell cuture imply that it is protein synthesis-dependent. P-selectin is present in the granules of endothelial cells (and platelets), and its expression is stimulated by thrombin and histamine, thereby providing a mechanism by which neutrophil adhesion is initiated very early in the inflammatory response - even before protein synthetic pathways are active. Meanwhile, L-selectin is expressed on neutrophils and is shed from their cell surface during this phase. It has been reported recently that soluble L-selectin plasma levels were significantly lower in patients who progressed to ARDS compared to those who did not (Donnelly et al. 1994) amongst a population at risk of ARDS. Additionally, a significant correlation was identified between low L-selectin levels and requirement for ventilation, degree of respiratory failure and mortality.

The next stage in the neutrophil-endothelial cell interaction (firm adherence and migration) is dependent on the expression of another set of CAMs, known as integrins, on the neutrophil surface (Rouslahti, 1991). The most important of these are the CD11/CD18 complex, and increased expression of these molecules on activated neutrophils has been demonstrated in animal models both *in vivo* and *in vitro*. Ligands for these CAMs are present on the endothelial surface, and binding is promoted by cytokines including TNF-α, PAF and ILs. The intercellular adhesion molecules (ICAM-1 and -2) are also expressed constitutively by the endothelium, and therefore are available early on for neutrophil binding. In addition, the vascular adhesion molecule, VCAM-1, is expressed by the endothelium in response to the presence of endotoxin and

cytokines. The importance of CD11/CD18 molecules in patients with sepsis is gradually emerging; and recently an increase in the expression of CD11/CD18 on granulocytes from patients with ARDS has been demonstrated and appears to correlate with the hyperadhesiveness of these cells (Laurent et al. 1994). The actual migration of neutrophils is dependent upon a chemotactic gradient, and probably also upon the expression of platelet-endothelial cell adhesion molecule (PECAM-1) which is mainly situated at the endothelial cell junctions (Muller et al. 1993).

1.1.4 Pathophysiology of Sepsis and ARDS

Sepsis is characterised by dilatation and pharmacological hyporesponsiveness of the systemic vasculature, increased cardiac output (thus, the term "hyperdynamic") and frequently, elevated pulmonary vascular resistance (PVR) resulting in pulmonary hypertension. These changes can result in systemic hypotension with widespread hypoperfusion of tissues. In the clinical setting, treatment strategies concentrate upon fluid resuscitation, the administration of systemic vasopressors and positive inotropes, optimisation of oxygen delivery and antibiotics. Whilst these changes are easily recognisable clinical sequelae of sepsis, it is becoming increasingly clear that there is also considerable disturbance of the microcirculation which magnifies the problem of oxygen delivery and extraction (Sair et al. 1996), as well as contributing to the metabolic dysfunction of tissues which is sometimes manifest as hyperlactataemia.

Oxygen is the most supply-dependent substrate in the circulation, but stores relative to consumption are the lowest of any metabolite. This knowledge, combined with technology allowing invasive haemodynamic monitoring and

measurement of blood oxygen saturation, has led to the use of oxygen transport variables as indices of cardiovascular and metabolic function (Pallares and Evans, 1992). In critically ill patients with sepsis, there is thought to be an abnormal relationship between oxygen uptake (VO_2) and delivery (DO_2), resulting in a state of pathological "supply dependency" (Leach and Treacher, 1992). In normal subjects, VO_2 is independent of DO_2 at rest, provided it is maintained above a critical level. In some critically ill patients, VO_2 becomes DO_2-dependent so that the oxygen extraction ratio remains constant. Patients admitted to an intensive care unit who demonstrate this pattern of supply-dependency have a higher mortality than those who do not (Gutierrez and Pohil, 1986; Bihari et al. 1987). This tendency, and the apparent inability of the peripheral tissues to extract oxygen has led a number of investigators to advocate active manipulation of DO_2 to achieve pre-determined target levels of VO_2. It has subsequently been shown (Shoemaker et al. 1988; Bishop et al. 1993) that critically ill patients in whom supraphysiological targets for cardiac output (CO), DO_2 and VO_2 are attained have a lower incidence of MOF and mortality, although more recently, this theory has proved difficult to substantiate (Hayes et al. 1994). Pathophysiological mechanisms that may account for impaired peripheral oxygen uptake associated with sepsis include: loss of microvascular control with shunting, tissue oedema, compression of capillaries and microembolisation (Hunter et al. 1990; Harrison et al. 1992). One major problem with the strategy of increasing DO_2 in order to increase VO_2 is that regional differences in blood flow probably occur (Samsel et al. 1988; Gutierrez and Bismar, 1990). Hence the expected increase in VO_2 in areas with the greatest oxygen debt may be prevented by a disrupted microcirculation.

1.1.4.1 **Myocardial dysfunction**

Despite the measurable increase in cardiac output detectable in patients with sepsis, it has been clearly shown that such patients do have myocardial depression in the form of decreased biventricular ejection fraction (Kumar and Parrillo, 1995). Originally, myocardial hypoperfusion was proposed as the mechanism underlying this depression of function, but more recent studies have demonstrated preservation of both coronary blood flow (Cunnion et al. 1986) and myocardial high energy phosphates (Hotchkiss and Karl, 1992; Solomon et al. 1994). Attention has therefore turned to the theory that sepsis is associated with the generation of one or more circulating myocardial depressant substance(s) (MDS). Studies have suggested the presence of MDS activity in the serum of patients in the acute phase of septic shock that reduces *in vitro* myocyte contraction (Parillo et al. 1985; Reilly et al. 1989), the level of the MDS activity correlating with the degree of their myocardial depression. Interestingly, the sera from patients who either were classified as being "pre-septic" or in the recovery phase did not exhibit this effect, and there was a trend towards an association between MDS activity and increased mortality in patients with septic shock. MDS is known to be water soluble and heat labile, and exists in two fractions (0.5-5kD and >10kD) in filtration studies, but its precise identity remains unresolved. Whilst inflammatory mediators such as LPS and TNF-α are capable of reducing myocardial contraction under certain circumstances (Snell and Parrillo, 1991), none share the properties of MDS.

1.1.4.2 **Systemic vascular dysfunction**

The hallmark of the systemic vascular response is vasodilatation of veins and arteries with pooling of blood in capacitance vessels. The vessels themselves are

hyporesponsive to vasopressor agents such as catecholamines, despite a demonstrable elevation in endogenous catecholamine production (Chernow et al. 1982; Fink et al. 1985). This therefore mirrors the behaviour of isolated arteries *in vitro.* For example, in aortae removed from rats rendered endotoxaemic *in vivo*, contractile responses to noradrenaline, KCl and serotonin were all decreased (Wakabayashi et al. 1993). Unsurprisingly, the pressor response to exogenous catecholamines is also reduced in septic patients (Chernow et al. 1982), an effect that has been well-documented in animal models of sepsis (Auclair et al. 1982; Guc et al. 1990). This loss of peripheral tone causes the observed drop in SVR (and its accompanying rise in cardiac output) and usually results in systemic hypotension, which, if persistent despite vasoconstrictor therapy, is associated with a high mortality.

Microvascular changes, as discussed above, contribute to sub-optimal oxygen exchange at the tissue level through a number of mechanisms. A rise in microvascular permeability also causes leakage of plasma and small proteins which frequently results in the formation of interstitial oedema.

Certain vascular beds behave in a different fashion in sepsis. In particular, the mesenteric, renal and pulmonary vasculature often exhibit varying degrees of contraction. As a result of such mesenteric changes, increased permeability of the gut mucosa and a drop in the intraluminal pH of the gut occurs, recent evidence suggesting that these parameters may indicate the development of an inflammatory septic response (Sinclair et al. 1995). In the kidney, vasoconstriction can exacerbate the reduction in renal blood flow that occurs as a result of systemic hypotension and reduced intravascular volume.

1.1.4.3 Pulmonary vascular dysfunction

Clinical studies have emphasised the universal finding of elevated PVR in patient groups with respiratory failure (Zapol and Snider, 1977). In ARDS, PVR is increased, even after correction for arterial hypoxaemia, probably through a combination of increased vascular tone, and structural factors such as extrinsic compression (ie. oedema fluid, PEEP ventilation), thromboemboli or later remodelling of the vessel wall as part of the inflammatory response (Zapol et al. 1992; Leeman, 1991). The pulmonary hypertension (PHT) seen in ARDS contributes to pulmonary oedema formation (Bone et al. 1992a), and impaired right ventricular performance (Sibbald and Driedger, 1983) and is associated with increased mortality (Bernard et al. 1985).

Apart from the observed PHT, patients with ALI/ARDS also exhibit dysfunction of hypoxic pulmonary vasoconstriction (HPV) (Curzen et al. 1995). HPV was first described in 1946 (von Euler and Liljestrand, 1946) and is a physiological response through which blood flow is diverted away from hypoxic alveoli, with the beneficial result that perfusion (Q) and ventilation (V) remain "matched". If normal subjects breathe gas mixtures of low oxygen tension, increases in pulmonary artery pressure ensue (Motley et al. 1947). There is conflicting evidence regarding the primary site of HPV. Although pulmonary venoconstriction has been demonstrated during hypoxia (Morgan et al. 1968), animal experiments using rapid freezing histological assessment (Kato and Staub, 1966; Glazier and Murray, 1971), and direct micropuncture techniques (Nagasaka et al. 1984) suggest that small pulmonary arteries are the major site modulating increases in PVR. Nor has the mechanism by which hypoxia elicits local vasoconstriction been fully elucidated. Autonomic innervation is not required for HPV, since it persists in isolated lung preparations (Hauge, 1968; Hauge and

Melmon, 1968) and in human transplanted lungs (Robin et al. 1987). Furthermore, pharmacological intervention using adrenergic receptor blockade (Malik and Kidd, 1973), catecholamine depletion (Goldring et al. 1962) and sympathectomy (Fishman, 1961) does not modulate the response. This implies that HPV must be mediated via intrinsic mechanism(s). Most evidence suggests that this is an endothelium-dependent phenomenon, although interestingly cultured pulmonary artery vascular smooth muscle cells exhibit a contractile response to hypoxia, which is independent of any other cell type (Murray et al. 1990). HPV thus represents a beneficial homeostatic control mechanism which is disrupted in lung injury (Weir et al. 1976). This is of great clinical significance in ARDS, because the alveolar oedema and atelectasis that occur can induce enormous increases in physiological dead space. Investigations using the multiple inert gas technique (Dantzker et al. 1979) have demonstrated that the degree of intra-pulmonary shunting is large enough to account for the observed alveolar\arterial PO_2 gradient, even without invoking a reduction in diffusion capacity.

1.2 ENDOTHELIN-1 AND THE CARDIOVASCULAR SYSTEM

1.2.1 The Endothelin family of peptides

1.2.1.1 **Discovery**

It was demonstrated in 1982 that contraction of canine femoral artery *in vitro* during anoxia was greatly attenuated when the endothelium was removed (De Mey and Vanhoutte, 1982). In 1984, a substance in the supernatant of cultured endothelial cells was shown to be able to contract isolated bovine pulmonary artery (O'Brien and McMurtry, 1984), thus proving the existence of an endothelium-derived

contracting factor (EDCF), which was quickly shown to be a peptide by its proteinase-sensitivity. The EDCF was identified as a 21 amino acid peptide in the supernatant of cultured porcine aortic endothelial cells, and was termed endothelin (Yanagisawa et al. 1988). This peptide was found to produce sustained, potent contraction of vascular smooth muscle from a wide variety of different vessels and species.

1.2.1.2 **Endothelins -1,-2,-3**

Three similar, but distinct, ET-related genomic loci have now been identified which encode for three similar ET molecules (ET-1, ET-2, ET-3) (Inoue et al. 1989a). ET-1 is the only one that is produced in the endothelium, but is also found in human macrophages (Ehrenreich et al. 1990), renal epithelial cells (Shichiri et al. 1989), neurones and astrocytes in the central nervous system, hepatocytes, endometrium, Sertoli cells, and breast epithelial cells (Rubanyi and Polokoff, 1994). ET-1 is also produced by vascular smooth muscle under certain circumstances (Resink et al. 1990; Tokunaga et al. 1992). In particular, cultured vascular smooth muscle cells produce ET-1 in response to a variety of agonists including angiotensin II, transforming growth factor-β (TGFβ), arginine vasopressin and ET-1 itself (Hahn et al. 1990).

ET-2 has been detected in intestinal (Takahashi et al. 1990a), renal, myocardial, placental and uterine tissue. The cells from which it is derived have not been identified. ET-3 has been found in central nervous tissue, including neurones and astrocytes, as well as in kidney and gut tissue. The tissue distribution is reviewed in Rubanyi and Polokoff, 1994. Analysis by Northern blotting has failed to demonstrate expression of either ET-2 or ET-3 in either vascular smooth muscle or endothelium (Firth and Ratcliffe, 1992).

The search for ET production at tissue level, whilst not detailing the cell type of origin, demonstrates both ET-1 and ET-3 peptide (by Northern analysis) or mRNA (using RNase protection assay) in nearly all rat tissues studied including heart, lung, liver, kidney, spleen, small and large intestine, brain, salivary gland, stomach, testis, skeletal muscle and placenta (Firth and Ratcliffe, 1992; Onda et al. 1990). RNase protection assay for ET-2 mRNA, however, failed to demonstrate it in any tissues except large and small intestine, stomach, skeletal muscle and heart.

1.2.2 Biosynthesis of ET-1

1.2.2.1 **Molecular derivation of ET-1**

Each ET molecule is the product of a separate gene, and in man the genes for ET-1, ET-2 and ET-3 have been mapped to chromosomes 6 (Arinami et al. 1991; Hoehe et al. 1993), 1 (Arinami et al. 1991; Bloch et al. 1991) and 20 respectively (Arinami et al. 1991; Bloch et al. 1991). The human ET-1 gene comprises approximately 6.8kb incorporating five exons interrupted by 4 introns. The primary transcript is 2.3kb long and directs translation of the precursor peptide, pre-pro-ET-1, which contains 203 amino acids. The primary transcription site in endothelial cells was found to be 98 base pairs from a CAAT sequence and 31bp from a TATAA sequence, both of which are characteristic of promoter regions (Inoue et al. 1989a; Inoue et al. 1989b; Bloch et al. 1989). Various regulatory elements have been identified on the ET-1 gene, including the sequence CTGGGA, which is known to be an acute phase regulatory element, and another binding sequence for nuclear factor-1, a transcription factor (Gronostajski, 1987). In addition, there is a site that binds GATA-2, a regulatory protein associated with endothelial cell basal ET-1 production (Wilson et al. 1990).

Pre-pro-ET-1 is cleaved by a dibasic endopeptidase followed by a carboxypeptidase to a 39 amino acid prohormone called big ET-1, some of which is secreted and circulates in the plasma **[Figure 1.4]**. Whilst the pre-pro-peptide has no vasoactive property, big ET-1 is a vasoconstrictor, although about 140 times less potent than ET-1 itself (Kimura et al. 1989).

1.2.2.2 **The ECE enzymes**

Knowledge of the amino acid sequences of big ET-1 and ET-1 itself indicated that the final stage of ET-1 production requires a specific cleavage of the pro-peptide between Trp-21 and Val-22. The nature of the enzyme that performs this task, named endothelin-converting enzyme (ECE), has been the focus of intensive research. In animal experiments the conversion of exogenously administered big ET-1 to ET-1 was seen *in vivo* but not in the plasma *in vitro*. This activity could be inhibited by phosphoramidon. It appears that there are several tissue-specific types of ECE. Recently, the complementary DNA for a membrane-bound glycoprotein metallopeptidase ECE has been cloned (Xu et al. 1994). The cDNA of this "ECE-1" was successfully transfected into cultured cells that normally secrete only Big ET-1, and as a result of this the cells became able to secrete mature ET-1. In both endothelial cells and vascular smooth muscle cells, however, there appear to be both membrane-bound, phosphoramidon-sensitive ECE fractions, as well as cytosolic ECE activity, at least some of which is phosphoramidon-insensitive. For example, in the endothelial cell there are 3 fractions that have ECE activity. Two are intracellular: one is a metalloendopeptidase, sensitive to phosphoramidon and the other an aspartic protease, most active at pH 3.5 (Sawamura et al. 1990; Haynes and Webb, 1993). The most physiologically significant endothelial ECE is a membrane-bound metallendopeptidase which is most active at neutral pH and is

inhibited by phosphoramidon (Okada et al. 1990; Hisaki et al. 1991; Matsumura et al. 1991; Ikegawa et al. 1991), and classified as ECE-1. ECE-1 mRNA expression is widespread throughout rat, bovine, and human tissues (Rossi et al. 1996; Shimada et al. 1995). However, ECE-1 activity is missing in some cell types that produce ETs, such as neurones, initiating a search for a different ECE in such cells. Recently, a second ECE, ECE-2, has been described (Emoto and Yanagisawa, 1995), which, despite being membrane-bound and phosphoramidon-sensitive, differs from ECE-1 in several ways:

(a) ECE-2 has an acidic pH optimum.

(b) ECE-2 has 250-fold greater sensitivity to phosphoramidon than ECE-1.

The evidence suggests that ECE-2 acts as an intracellular processing enzyme that is responsible for the conversion of endogenously-synthesized big ET-1 at the Golgi network, where the vesicular fluid is acidic.

1.2.2.3 **Regulation of ET-1 synthesis**

ET-1 release is stimulated by a wide variety of local factors **[Figure 1.5]**. In addition, ET-1 can stimulate its own release in rat vascular smooth muscle (Hahn et al. 1990), thus potentially forming a positive autocrine feedback loop. The release of ET-1 from cultured endothelial cells is inhibited by atrial natriuretic peptide, nitroprusside and nitroglycerine, as well as NO and prostacyclin. The latter potentially form a negative feedback loop, which could have particular significance because of their release by ET-1 (*vide infra*).

1.2.2.4 **Release and clearance**

ET-1 is not apparently stored intracellularly in most tissues, although ET-1 immunoreactivity is present in granular form in the posterior pituitary (Yoshizawa et al. 1990). It was therefore thought until recently that almost all ET-1 must be synthesized *de novo,* especially since cycloheximidine, a protein synthesis inhibitor, can prevent its release by cultured endothelial cells (Schini et al. 1989) and intact aortic strips (Boulanger and Luscher, 1990). However, rapid increases in ET-1 release have recently been demonstrated under a variety of circumstances. In particular, the intravenous (*iv*) administration of TNF-α in rats causes a rise in plasma [ET-1] within minutes, which peaks by 30 minutes (Klemm et al. 1995), and in man a cold pressor test can lead to a significant elevation in [ET-1] within 2 minutes (Fyhrquist et al. 1990), as can upright tilt tests in patients who experience vasovagal syncope (Kaufmann et al. 1991). Finally, cultured rabbit endothelial cells are also capable of rapid ET-1 release when subjected to increased flow (Milner et al. 1990).

Animal studies have indicated that ET-1 is cleared from the circulation predominantly by the pulmonary, renal and splanchnic circulations. For example, the plasma half-life of a bolus of ^{125}I-labelled ET-1 was 40 seconds in one study in anaesthetised rats, with 82% uptake in the lungs and 10% in the kidneys (Sirvio et al. 1990). Bilateral nephrectomy in rats causes prolongation of plasma and tissue half-lives of ET-1 (Kohno et al. 1989), which helps to explain the elevated plasma [ET-1] seen in patients with renal dysfunction (Koyama et al. 1989; Saito et al. 1991). The half-life of ET-1 given *iv* in humans is estimated to be about 3.6 minutes (Weitzberg et al. 1991). Following low dose ET-1 infusion in man (4 pmol/kg/min over 20 minutes), approximately 50% of the total administered dose was removed

from the circulation in the lungs (Weitzberg, 1993), with 10% removed by the splanchnic and renal circulations together, and 5% by skeletal muscle.

1.2.3 Endothelin receptors

1.2.3.1 **Receptor sub-types and their distribution**

The cDNA of two ET receptors has so far been cloned and expressed (Arai et al. 1990; Sakurai et al. 1990; Sakurai et al. 1992). The genes for ET_A and ET_B are large, occupying 40 and 24 kb of DNA on chromosomes 4 and 13 respectively, and code for peptides that are members of the family of G-protein (guanine nucleotide-binding) receptors. There is about 63% homology between the two receptor subtypes (Rubanyi and Polokoff, 1994).

Endothelin-A (ET_A) receptors are predominantly situated on vascular smooth muscle and cardiac myocytes, but are not found on endothelial cells or hepatocytes (Hosoda et al. 1991), and have a rank order of selectivity ET-1>ET-2>ET-3, with the binding affinity for ET-1 being at least 10 times greater than for ET-3 (Arai et al. 1990). It is the ET_A receptor that mediates the direct vasoconstrictor activity of ET-1, although in some tissues ET_B receptors that are situated on the vascular smooth muscle can also mediate contraction **[Figure 1.5]**. ET-1 coupling with ET_A results in activation of phospholipase C, which in turn results in the formation of diacylglycerol and inositol 1,4,5-triphosphate (Takuwa et al. 1991; Resink et al. 1988; Kasuya et al. 1989). The resultant increase in intracellular calcium concentration ($[Ca^{2+}]i$) elicits contraction **[Figure 1.6]**, and both the elevation in $[Ca^{2+}]i$ and therefore contraction can persist even after ET-1 has left the receptor. The initial rapid rise in $[Ca^{2+}]i$ is due to inositol triphosphate-

mediated release of intracellular stores. By contrast, a subsequent plateau phase lasting up to 20 minutes can be prevented by the absence of extracellular calcium (Simonson et al. 1989). As a result of the increase in diacylglycerol and intracellular calcium, there is also activation of protein kinase C, and it is thought that this forms the secondary messenger system capable of stimulating cell mitogenesis and smooth muscle proliferation by increasing transcription of growth-promoting genes (Wang et al. 1992; Simonson and Herman, 1993). In addition, a protein kinase C inhibitor can abolish a component of the ET-1-induced contraction (Kodoma et al. 1989).

The mechanics of the interaction between ET-1 and the ET_A receptor have been elucidated by several groups. The binding of ET-1 to its receptors is extremely long-lived, with a dissociation half-life of over 30 hours in broken cell systems (Waggoner et al. 1992), and it is assumed that this is the main explanation for the sustained nature of ET-1-induced contractions. In experiments using ^{125}I-labelled ET-1 and cultured rat aortic myocytes (Marsault et al. 1993), it was estimated, using the calculated association constant, that after exposure of these cells to 25nM ET-1, 95% of the receptor sites available at the plasma membrane would be occupied. Following binding, ET-1 receptor complexes were rapidly internalised, but there was then a time-dependent recovery of binding sites. Importantly, it was deduced that the externalising receptors must have been recycled, because treatment with cycloheximidine did not prevent the process, thus making it unlikely that they were the result of new protein production. Furthermore, the externalised receptors were fully functional and able to take part in further ET-1-induced cell contraction (Marsault et al. 1993). It has also been demonstrated that ET-1-induced contractile responses can be slowly reversed both *in vitro* and *in*

vivo by BQ123 (ET_A receptor-mediated) or by a combined ET_A/ET_B receptor antagonist (Warner et al. 1994).

Endothelin-B (ET_B) receptors are predominantly endothelium-sited, although in some tissues they are also situated on vascular smooth muscle cells. The affinity of ET_B receptors is equal for all three ETs, and activation results in the same secondary messenger pathway as described for ET_A receptors. However, it appears that ET_B receptor stimulation produces inhibition of the formation of cyclic AMP, which is a feature of ET_A-mediated events. Whatever the exact secondary messenger mechanism, one of the principal end results of ET_B receptor activation on the vascular endothelium is the release of NO (Hirata and Emori, 1993), PGI_2 (Karaki et al. 1993a; de Nucci et al. 1988), and TXA_2 (Reynolds and Mok, 1989). It is this release of other vasoactive agents that equips ETs with the ability to produce vasodilatation (via NO and PGI_2) or "indirect" vasoconstriction (via TXA_2). Specifically, there is good evidence for the presence of ET_B receptors on rat pulmonary artery smooth muscle and endothelium (Eddahibi et al. 1993).

ET_B receptor mRNA has been demonstrated in lung, kidney, adrenal, cerebral cortex and cerebellum, as well as in endothelial cells in humans (Ogawa et al. 1991). Application of reverse transcriptase polymerase chain reaction assays for the mRNA coding for ET_A and ET_B receptors has also lead to confirmation of the expression of both types of receptor on the smooth muscle of human aorta, pulmonary, coronary, internal mammary arteries and saphenous veins (Davenport et al. 1995). Competition binding studies showed, however, that although both receptor sub-types were present, at least 85% of them were of the ET_A type. Similar methods were employed in order to confirm the same receptor distribution on the media of both human renal artery and vein (Maguire and Davenport, 1995; Maguire et al. 1994).

1.2.3.2 **Regulation and expression of ET receptors**

Local regulation of ET receptor expression may play an important role in the determination of tissue-specific, and condition-specific, effects of ETs. Perhaps the best example of this is the down-regulation of ET_B receptor mRNA in pulmonary hypertensive rats, despite an elevated ET-1 level in the plasma (Yorikane et al. 1993). The following factors have been shown to up-regulate expression of ET_A receptors in some tissues: hypoxia, cAMP, epidermal growth factor, basic fibroblast growth factor, oestrogen and cyclosporin. ET_A down-regulation occurs in response to ETs, angiotensin II, TGFβ, and platelet-derived growth factor. ET_B receptor up-regulation is known to be induced by angiotensin II and natriuretic hormone, and down-regulated by ET-1 and -3, catecholamines and cAMP.

The ability of ET-1 (and ET-3) itself to elicit down-regulation of both ET_A binding activity on vascular smooth muscle (Roubert et al. 1990) and ET_B mRNA on osteosarcoma cells (Sakurai et al. 1992) and endothelium (Clozel et al. 1993c) produces the potential for an important self-regulatory feedback loop. It has been demonstrated, for example, that in cells exhibiting ET_A receptors (mesangial cells) or ET_B receptors (endothelial cells), phosphoramidon treatment decreased the amount of ET-1 released but at the same time potentiated the ET-1-induced release of arachidonic acid from the mesangial cells, presumably because of the demonstrable increase in receptor-mediated binding of exogenous ligand (Clozel et al. 1993c).

1.2.3.3 **Receptor agonists and antagonists**

Investigation into the functional pharmacological role of ET-1 and its receptors has undergone rapid expansion with the discovery of, and in some cases, design of, receptor agonists and antagonists. The similarity between the structure of ETs and

various neurotoxins, including bee venom and α-scorpion toxin was noted in the original paper (Yanagisawa et al. 1988b). ET was then found to have close structural similarity to sarafotoxin S6b (S6b), a toxin in the venom of the snake, *Actractaspis engaddensis,* which is an ET_A receptor agonist. Subsequently, a large number of agonists and antagonists have become available, and these are listed in **Table 1.4**.

Of particular relevance to this thesis are the following agents: BQ123 is a peptide that acts as a selective ET_A receptor antagonist, with an IC_{50} of 7.3nM for ET_A receptors (aortic vascular smooth muscle cells) and 18μM for ET_B receptors (cerebellar cell membranes) (Ihara et al. 1992a). BQ788 is a selective ET_B receptor antagonist with an IC_{50} of 1.2nM for ^{125}I-labelled ET-1 binding to ET_B receptors (human Girardi heart cells) and 280nM for ET_A receptor binding on porcine coronary artery smooth muscle cells (Ishikawa et al. 1994). Bosentan (Ro 46-2005) is a non-peptide compound that potently inhibits binding of ET-1 at ET_A and ET_B receptors (IC_{50} 220nM for ET_A; 1μM for ET_B) (Clozel et al. 1993b; Breu et al. 1993; Clozel et al. 1993a; Clozel et al. 1994). Since it is non-peptide, it has been developed as an oral, as well as a parenteral, preparation. Finally, sarafotoxin S6c is a selective ET_B receptor agonist (Williams, Jr. et al. 1991a; Williams, Jr. et al. 1991b).

1.2.4 The role of ET-1 in cardiovascular physiology

1.2.4.1 **ET-1 as a modulator of systemic vascular tone**

ET-1 is the most potent endogenous vasoconstrictor known, its potency on a molar basis being 1000:1 compared to noradrenaline and 10:1 versus angiotensin II (Yanagisawa et al. 1988; Zuleica et al. 1989). As described above, this is achieved

in most tissues by the activation of ET_A receptors on vascular smooth muscle cells. In some systemic blood vessels, however, vascular smooth muscle-sited ET_B receptors also mediate contraction. This property is both tissue- and species-specific. For example, ET_B receptors mediate vasoconstriction in the rat kidney (Wellings et al. 1994b), rabbit jugular vein (White et al. 1993), saphenous vein (Sudjarwo et al. 1994) and pulmonary artery (LaDouceur et al. 1993) and human coronary arteries (Seo et al. 1994). There are therefore data demonstrating "direct" ET_B-mediated vasoconstriction both *in vitro* and *in vivo* (Clozel et al. 1992; Wellings et al. 1994a). The more universal distribution of ET_B receptors on the endothelium is responsible for the release of the vasodilators, NO and PGI_2, by ET-1 in systemic vessels. This property has been characterised in animal experiments *in vitro*, for example in cultured bovine endothelial cells (Filep et al. 1991a) and rat aorta (Karaki et al. 1993b; Karaki et al. 1993a), as well as *in vivo* in rats (de Nucci et al. 1988; Filep et al. 1993) and dogs (Filep et al. 1991b). The release of NO by ET-1 has also been demonstrated in human arteries and veins (Luscher et al. 1990). The combination of ET_B-mediated NO and PGI_2 release and ET_A-mediated direct vasoconstriction produces the characteristic pattern of haemodynamic response that follows a bolus *iv* injection of ET-1 into animals. This response consists of early and transient hypotension followed by a prolonged hypertensive phase resulting from widespread vasoconstriction (Yanagisawa et al. 1988), and this has been demonstrated in conscious (Bazil et al. 1992; Filep et al. 1994a), anaesthetised (Spokes et al. 1989; Cristol et al. 1993) and pithed rats (Guc et al. 1990). Unsurprisingly, the hypotensive phase of the response can be attenuated by inhibitors of NO synthesis (Filep et al. 1993; Gardiner et al. 1990b), and also mimicked by the administration of ET_B receptor agonists (Filep et al. 1994a; Cristol et al. 1993), even in the context of established ET_A receptor blockade. The pressor

phase can be largely attenuated by ET_A antagonists (Ihara et al. 1992b; Douglas et al. 1992; Cristol et al. 1993; McMurdo et al. 1993b), although in rats a portion of the ET-1-induced hypertensive response is spared even in the presence of BQ123 (McMurdo et al. 1993a; Filep et al. 1994a), and this, together with other evidence implies that there is, as mentioned, a contribution from non-ET_A receptors in this phase. The pressor response elicited by ETs or receptor agonists in anaesthetised rats has the following order of potency: Sx6b > ET-1 > Sx6c > ET-3 (Cristol et al. 1993). More detailed analysis of the haemodynamic response to *iv* ET-1 is available. In one early study, the sustained pressor response was attributable to increased total peripheral vascular resistance, produced predominantly by vasoconstriction in the renal, splenic, mesenteric, pancreatic beds, with no change in heart rate or cardiac output (MacLean et al. 1989). More recently, however, several groups have reported a tachycardia during the hypotensive phase, followed by bradycardia during the pressor phase of rats in response to *iv* ET-1 (Gardiner et al. 1994; Filep et al. 1994a). A continuous infusion of ET-1 in anaesthetised rats caused a large increase in systemic blood pressure, accompanied by increased total peripheral resistance and a reciprocal fall in cardiac output (Allcock et al. 1995). In the latter study, the vascular resistance of most organs was increased by the infusion, only brain and stomach escaping this effect. Interestingly, the ET-1-induced increase in vascular resistance was attenuated by the ET_A antagonist, BQ123, in kidney, skin, adrenal and caecum, but potentiated by the ET_B antagonist, BQ788, in the kidney, small and large intestines and mesentery. In addition, BQ788 also increased the effect of ET-1 on total peripheral resistance, therefore implying that overall the vasodilatory role of ET_B receptors predominates in the rat.

There is good evidence that ET_B-mediated release of the vasoconstrictor TXA_2 contributes to ET-1-induced contraction in rat aorta (Reynolds and Mok, 1989), coronary artery (Filep et al. 1994b), and renal artery (Wellings et al. 1994b). Another potentially important indirect mechanism by which ET-1 can influence vessel contraction is the potentiation of other vasoconstrictor agents such as noradrenaline and serotonin (Wong-Dusting et al. 1991; La and Rand, 1993; Yang et al. 1990). The presence of such vasoconstrictors correspondingly potentiates ET-1-induced vasoconstrictor responses.

In human vessels, the vascular effects of ET receptor activation appear to vary from tissue to tissue (Davenport and Maguire, 1994). Whilst forearm arteries and veins (Haynes et al. 1995), and internal mammary artery and vein (Seo et al. 1994) constrict in response to both ET_A and ET_B receptor stimulation, renal arteries (Maguire et al. 1994), and small omental arteries and veins (Riezebos et al. 1994) appear to do so only upon ET_A activation. Furthermore, in human epicardial coronary artery, internal mammary artery, aorta and saphenous vein, *in vitro* vasoconstriction by ET peptides is due to activation of smooth muscle ET_A receptors (Maguire and Davenport, 1995). In the human coronary artery the situation is even more complicated, in that although ET_A receptors mediate the ET-1-induced contractile response in distal epicardial coronary arteries, there is a contribution from ET_B receptors in proximal segments (Godfraind, 1993).

The effects of low dose infusions of ET-1 have been studied both invasively (Weitzberg, 1993), and non-invasively (Kiely et al. 1996b) in man. Intravenous infusion of ET-1 (4pmol/kg/min for 20 minutes) in human volunteers with full invasive monitoring, including Swan-Ganz catheters, decreased cardiac output by 14%, and increased mean systemic blood pressure by 7%, and systemic vascular

resistance, pulmonary artery pressure, pulmonary vascular resistance by 25%, 20% and 67% respectively (Weitzberg, 1993).

1.2.4.2 **The contribution of ET-1 to basal systemic vascular tone**

The evidence presented in 1.2.4.1 provides useful information about receptor populations and their behaviour, but does not answer the question: *does ET-1 contribute to basal systemic vascular tone?* Until recently, the main source of evidence with which to address this question was from studies whose primary aim was to modify the responses to exogenous ET peptides by the pretreatment of animals with an ET receptor antagonist. Certainly, no effect was seen on basal mean arterial pressure following the *iv* injection of BQ123 in conscious (Bazil et al. 1992; Filep et al. 1994a) or anaesthetised (McMurdo et al. 1993a) rats, or following the administration of the combined ET_A/ET_B antagonist, bosentan, in rats (Filep et al. 1994a) or anaesthetised dogs (Teerlink et al. 1995). However, in one study on anaesthetised rats, a dose of 1.6μmol/kg of BQ123 produced a drop in mean arterial pressure together with an increase in the vascular conductance of the carotid, coeliac, mesenteric, iliac and renal vessels, whilst a dose of 0.016μmol/kg BQ123 had no haemodynamic effect (Bigaud and Pelton, 1992). From knowledge of the mechanics of the coupling of ET-1 with ET_A receptors, it would be surprising if ET receptor antagonists were to displace endogenously-produced ET-1 from its receptors within a few minutes, although the latter study suggests that this may be a concentration-, as well as time-dependent phenomenon.

A more elegant approach to this investigation involves the prevention of ET-1 production. For example, infusion of phosphoramidon did produce a reduction in blood pressure in spontaneously hypertensive rats (McMahon et al. 1991).

The limited evidence available in humans to date suggests that, at least in the forearm, endogenous ET-1 contributes significantly to the maintenance of vascular tone. Thus, infusion of BQ123 leads to progressive dilatation, with an increase in forearm blood flow of 64% after 60 minutes. In addition, infusion of phosphoramidon induces a slow onset increase in forearm blood flow to 37% above baseline at 90 minutes (Haynes and Webb, 1994).

1.2.4.3 **ET-1 as a modulator of pulmonary vascular tone**

The mRNA coding for both ET_A and ET_B receptors can be detected in the smooth muscle of human pulmonary arteries (Davenport et al. 1995), as well as in the rat (Eddahibi et al. 1993) and many other species. ET-1 constricts isolated pulmonary artery rings of several species including man and rat (McKay et al. 1991; Hay et al. 1993). All three ET peptides cause a rise in PVR when administered to isolated perfused pulmonary circulations under basal conditions (Crawley et al. 1992), with a graded potency: ET-1>ET-2>ET-3. Pulmonary veins are more sensitive in their contractile response to ET-1 than pulmonary arteries (Toga et al. 1992). If the PVR in the isolated perfused rat lung is elevated by a different vasoconstrictor such as U46619, then both ET-1 and ET-3 induce vasodilatation (Crawley et al. 1992; Hasunuma et al. 1990). The contractile response of human (Maguire and Davenport, 1995; Buchan et al. 1994) pulmonary arteries, as well as other species, is mediated via ET_A receptors, and the vasodilatation by NO and PGI_2 derived from the endothelium in response to ET_B receptor stimulation. This release of NO, as well as prostanoids, including PGI_2 and TXA_2, has been conclusively demonstrated in the rat pulmonary circulation (Uhlig et al. 1995; Barnard et al. 1991; D'Orleans-Juste et al. 1992).

In the lung, the response of any vasoactive mediator to hypoxia assumes considerable clinical significance. Hypoxia is a potent stimulus for ET-1 production, the mRNA expression for pre-pro-ET-1 increasing significantly in cultured human endothelial cells, resulting in a large increase in ET-1 release (Kourembanas et al. 1991). Normobaric hypoxia also stimulates ET-1 gene expression in the rat (Elton et al. 1992). ET release has also been confirmed in response to hypoxia in cultured bovine endothelial cells (Hieda and Gomez-Sanchez, 1990) and in rat resistance vessels perfused *in vitro* (Rakugi et al. 1990). Importantly, it has also recently been shown that plasma [ET-1] increases significantly in normal humans after 30 minutes of hypoxaemia (SaO_2 75-80%) (Kiely et al. 1996a). Not only do ET-1 and ET-3 have both vasoconstrictor and vasodilator properties in the rat lung (Crawley et al. 1992; Hasunuma et al. 1990), they can reverse the HPV response in isolated lungs, an effect unchanged by indomethacin or glibenclamide but attenuated by L-NMMA. On the other hand, the hypoxia-induced contraction of isolated canine systemic and pulmonary artery rings *in vitro* was not influenced by pretreatment with the ET_A receptor antagonist, BQ-123, even though this agent at the same dose significantly antagonises exogenous ET-1-induced contractions (Douglas et al. 1993). This implies that ET-1 is not involved in HPV. Consequently, there is no clear evidence that ETs are responsible for mediating HPV, especially as the response time course is different for anoxia- and ET-induced contractions, the latter being slower in onset than the former (Vanhoutte et al. 1989). There is, however, evidence that hypoxia has other effects on ET-1-induced vasoreactivity. Thus, hypoxia not only stimulates ET production and release, but also alters the functional response to the peptides so that *in vitro* dose response curves to ETs-1 and -3 are altered, with an increased maximum pressor response, in mesenteric vessels (Douglas et al. 1991). Importantly, there is also evidence that ischaemia,

and then reperfusion, increases the maximum binding capacity for [^{125}I]-ET-1 in rat cardiac membranes, probably through externalisation of binding sites (Liu et al. 1990; Liu et al. 1989). In addition, the vasoconstrictor response to ET-1 was twice as great in perfused mesentery of pithed rats *in situ* when oxygen levels were reduced by lowering ventilation. This evidence implies that the functional role of ETs may be altered in hypoxia, which may be important in light of their ability to modulate the release and activity of other endothelium-derived vasoactive agents.

Endothelins are perfectly equipped to produce short- and long-term increases in PVR. Not only is ET-1 a potent and sustained direct vasoconstrictor of pulmonary vessels from main pulmonary arteries to veins, but the peptide is also mitogenic for pulmonary artery smooth muscle cells (Janakidevi et al. 1992), an effect that can be attenuated by an ET_A receptor antagonist (Zamora et al. 1993). It has also been shown to be capable of inducing chemotaxis and replication of pulmonary arterial fibroblasts in culture (Peacock et al. 1992). Thus, either directly or indirectly, ET-1 has the capability to induce potent vasoconstriction and local vascular changes that would contribute to remodelling of the pulmonary vasculature in a manner seen in PHT.

The original evidence implicating ET-1 in the pathogenesis of PHT came from descriptions of elevated circulating [ET-1] in patients with primary or secondary PHT. These, however, require careful interpretation, there being good evidence that there is substantial clearance of ET-1 from circulation through normal lung. Measurement of the arterial to venous [ET-1] ratio showed this to be less than 1 in normal individuals, significantly greater than 1 in those with primary PHT, and close to 1 in various types of secondary PHT (Zhang et al. 1991). The clearance of circulating ET-1 is probably reduced in secondary PHT, whilst the production (as assessed by ET-1 mRNA expression) is increased in patients with

primary PHT (Giaid et al. 1993) and in a rat model of idiopathic PHT (Stelzner et al. 1992). Interestingly, there are reports that the elevated [ET-1] in patients with PHT secondary to either valvular (Chang et al. 1993) or congenital (Ishikawa et al. 1995) heart disease can be reduced significantly by surgical correction of the condition.

Chronic hypoxia is a potent stimulus for increased ET-1 production in the lung (Li et al. 1994), and it has recently been demonstrated that simultaneous administration of a combined ET receptor antagonist to rats attenuates the pulmonary hypertension and right ventricular hypertrophy induced by chronic hypoxia (Eddahibi et al. 1995). There is understandably intense speculation as to the possible therapeutic (and perhaps prophylactic) benefit of ET receptor antagonists in PHT related to hypoxia. Indeed, it has already been shown that the ET_A receptor antagonist, BQ123, can attenuate ET-1-induced elevation of PVR (Bonvallet et al. 1993) and the ET-1-induced proliferation of human pulmonary artery smooth muscle cells (Zamora et al. 1993). The oral administration of bosentan has also been shown to prevent and reverse hypoxic PHT in rats (Chen et al. 1995).

Pulmonary hypertension is a manifestation of a heterogeneous collection of conditions, but the evidence suggests that ET-1 is more than an innocent bystander, and the race to apply ET antagonists as therapeutic agents, particularly in PHT that is secondary to hypoxia, is justifiably well underway.

1.2.4.4 **ET-1 as a modulator of cardiac function**

ET-1 is produced by cultured endocardial cells (Mebazaa et al. 1993) and by neonatal rat myocytes (Suzuki et al. 1993). In human heart, the presence of ET_A and ET_B receptor mRNA (by *in situ* hybridisation) and protein (by binding studies)

has been demonstrated on endocardium, atrial and ventricular myocytes and in conducting tissue (Molenaar et al. 1993).

ET-1 has positive inotropic activity in isolated adult rat ventricular myocytes (Kelly et al. 1990), isolated guinea pig atria (Ishikawa et al. 1988a) and in isolated, perfused heart preparations (Baydoun et al. 1989; Firth et al. 1990). In isolated, spontaneously beating atrial preparations, ET-1 induces a tachycardia (Ishikawa et al. 1988b), but, as already mentioned, it induces transient tachycardia followed by bradycardia *in vivo*. ET-1 prolongs the cardiac action potential (Watanabe et al. 1989), and has been found to be proarrhythmic when given intravenously or into the coronary circulation (Hom et al. 1992). In addition, basal ET-1 has been shown to regulate cardiac contractility in an isolated rat heart preparation, suggesting the possibility of a local modulatory role (McClellan et al. 1995).

In man, studies using low dose ET-1 infusions have demonstrated reductions in cardiac output (Kiely et al. 1996b; Weitzberg, 1993), which has been shown to be due to a combination of reduced heart rate, reduced stroke volume and increased SVR.

The constrictor effects of ET-1 on the coronary arterial tree have already been discussed. The potency of these, together with the associated increase in peripheral vascular resistance, probably largely explains the reduction in cardiac output that is observed *in vivo* upon administration of ET-1, despite its direct positive inotropic effect.

Much interest is currently focused upon the role of ET-1 in the pathophysiology of several common clinical cardiac conditions, including heart failure, cardiac hypertrophy, myocardial infarction and ischaemia, coronary atherosclerosis and systemic and pulmonary hypertension (Curzen and Kaddoura, 1996; Kaddoura and Curzen, 1996).

1.2.4.5 **Other properties of ET-1**

ET-1 constricts other types of smooth muscle, including bronchial, and has been shown to elicit bronchoconstriction in many different species (Barnes, 1994). Its precise role in the pathophysiology of asthma is still the subject of considerable investigation. ET-1 has been implicated in the pathophysiology of several conditions based upon local vascular smooth muscle contraction, such as coronary vasospasm (Prinzmetal's angina), Raynaud's disease, and the cerebral artery spasm that causes local ischaemia after subarachnoid haemorrhage (Curzen and Kaddoura, 1996).

ET-1 is also mitogenic for vascular smooth muscle cells (Janakidevi et al. 1992) and stimulates chemotaxis and proliferation of fibroblasts (Peacock et al. 1992), and is thus perfectly equipped to play a role in vascular remodelling in such conditions as pulmonary and systemic hypertension and even atherosclerosis (Curzen and Kaddoura, 1996). Evidence is accumulating that ET-1 is also involved in the pathophysiological mechanisms that produce myocardial hypertrophy (Kaddoura and Curzen, 1996) ET-1 has also been found to interact with the renin-angiotensin-aldosterone system, both at the level of the circulating hormone and at the tissue ACE level (Haynes and Webb, 1993). ET-1 is present in high concentrations throughout the central nervous system, and may be involved in regulation of the release of both anterior and posterior pituitary hormones.

1.3 NITRIC OXIDE

1.3.1 An endothelium-derived relaxant factor

In 1980 the vascular relaxation induced by acetylcholine was shown to be dependent on the presence of intact endothelium and to be mediated via the release of a non-prostanoid, labile vasodilator termed endothelium-derived relaxant factor (EDRF). Overwhelming evidence has since accumulated that the chemical and pharmacological properties of EDRF are shared by nitric oxide (NO) (Moncada et al. 1991). This can be summarised as follows:

- Both are chemically unstable with half-lives of 3-5 seconds under assay conditions.
- Both are inactivated by superoxide anion and generators of ROS, and both are stabilised by superoxide dismutase (SOD).
- Both have very high binding affinity for haem. As a consequence of this: (i) Both cause haem-dependent activation of soluble guanylyl cyclase (which can be inhibited by methylene blue) and (ii) both react rapidly with, and are inactivated by, haemoglobin to form the same NO-Hb product.
- Both produce guanosine 3',5'-cyclic monophosphate (cGMP)-mediated relaxation of intact vessels and vascular smooth muscle. This can be inhibited by methylene blue or haemoglobin, and potentiated by SOD to a similar degree.
- Both inhibit the aggregation and adhesion of platelets: this can be inhibited by ferrous ions and potentiated by SOD and cGMP phosphodiesterase inhibitors.
- Both are highly lipophilic.

NO itself is a small uncharged molecule with an unpaired electron, making it highly reactive, forming nitrite after 2-30 seconds. Thus, although NO diffuses rapidly across cell membranes, its biological activity depends upon its immediate synthesis and release, and therefore tends to be local.

1.3.2 Biosynthesis and regulation

Nitric oxide is synthesized *in vitro* from the semi-essential amino acid, L-arginine, by the membrane bound enzyme nitric oxide synthase (NOS) (Moncada et al. 1991). Three distinct NOS genes have been identified, and two forms of NOS exist in the blood vessel wall: a constitutive calcium- and calmodulin-dependent enzyme known as endothelial or eNOS, and an inducible calcium-independent enzyme termed inducible or iNOS. Both forms exist in the vascular endothelium (Knowles and Moncada, 1994), but iNOS has also been found in vascular smooth muscle, cardiac myocytes, macrophages and hepatocytes. The third sub-type is a constitutive NOS found in neuronal tissue (nNOS), including non-adrenergic, non-cholinergic (NANC) nerves. The properties of the vascular NOS enzymes are summarised in **Table 1.5**, and the list of factors known to be able to influence NO production in **Figure 1.8**. An elevation in intracellular calcium concentration is the essential stimulus to the increase in NOS activity initiated by receptor-dependent agonists, such as acetylcholine and bradykinin, or receptor-independent agents such as calcium ionophores. Following binding of an endothelium-dependent vasodilator agonist to its receptor, there is a transient rise in inositol 1,3,5-triphosphate which in turn causes both a release of calcium from intracellular stores and a more sustained transmembrane influx of calcium from the extracellular space (Busse et al. 1993).

A range of structural analogues to L-arginine are able to competitively inhibit NOS, including N^G-monomethyl-L-arginine (L-NMMA) (Moncada and Higgs, 1991) and N^w-nitro-L-arginine-methylester (L-NAME). These compounds have proved invaluable as tools with which to dissect out the role of NO in vascular reactivity.

1.3.3 NO as a regulator of cardiovascular physiology

Nitric oxide works by activating soluble guanylyl cyclase after binding to its haem moiety, which in turn causes an increase in intracellular cyclic guanosine monophosphate (cGMP) resulting in vascular smooth muscle relaxation (Rapoport and Murad, 1993; Ignarro et al. 1993). This cellular mechanism can be demonstrated experimentally. Thus, L-arginine administration has been shown to produce vasodilatation in lambs, and this effect can be blocked by the guanylyl cyclase inhibitor methylene blue or by L-NMMA (Fineman et al. 1991a; Fineman et al. 1991b). This vasodilatation can, however, be augmented by a cGMP phosphodiesterase inhibitor, whose ability to raise intracellular cGMP levels has also been shown to produce vasodilatation in other models. The measurement of cGMP levels in tissue can be used as an indicator of the degree of NOS activity, and has become a useful tool in experimental studies of enzymic activity.

1.3.3.1 **NO in the systemic circulation**

NOS inhibitors produce increases in mean arterial blood pressure and decreases in regional blood flow in both animals and man (Rees et al. 1989; Gardiner et al. 1990a; Vallance et al. 1989), implying that there is a continuous basal release of NO which contributes to vascular tone. The size of the pressor response induced

by *iv* L-NMMA or L-NAME in animal models seems to be dependent on basal vascular tone, but is L-arginine-reversible. The contribution of basal NO release to physiological flow is therefore significant, and has been shown to regulate flow in several organs such as brain, kidney, heart, lung, and GI tract (Lowenstein et al. 1994). It also plays an important role in maintaining flow by its anti-aggregatory effects on platelets. The range of stimuli that increase local NO release via eNOS, from shear stress to hypoxia, ensure that there is a rapid adjustment in vessel diameter. The basal release of NO also appears to influence vascular responsiveness to vasodilators and vasoconstrictors (Martin et al. 1986).

1.3.3.2 NO in the pulmonary circulation

Endogenous NO has been shown to be important in the maintenance of basal tone in the pulmonary circulation of several species, including rat (Barnard et al. 1993) and man (Stamler et al. 1994). Thus, infusion of low dose L-NMMA into healthy humans causes an approximately 40% increase in PVR and a 60% increase in SVR. Endothelium-dependent vasodilatation has been demonstrated in isolated human pulmonary arteries (Greenberg et al. 1987).

There is conflicting evidence for the contribution of NO to HPV. A reduction in NO production or a decrease in its target receptor sensitivity could theoretically result in vasoconstriction during hypoxia. Certainly, endothelium-dependent relaxation is impaired by hypoxia in rabbit and rat pulmonary artery rings (Johns et al. 1989; Rodman et al. 1990). In addition, the contraction induced by hypoxia in isolated human lobar arteries is potentiated by endothelial removal (Ohe et al. 1987). In isolated, perfused lung preparations, inhibition of NO release under hypoxic conditions with methylene blue (Mazmanian et al. 1989) or L-NMMA

(Archer et al. 1989) potentiates the pressor response, suggesting that NO release may rise during HPV. It is therefore tempting to speculate that alterations in NO production in the most hypoxic areas of lung could disrupt clinically appropriate blood diversion.

In sepsis, the induction of iNOS would be expected to reduce local HPV. However, recent evidence on pulmonary artery and aortic rings *in vitro* from lipopolysaccharide-treated rats suggests that not only does hypoxia inhibit cNOS, thus producing rapid vasoconstriction, but that it also inhibits LPS-induced iNOS production (Zelenkov et al. 1993).

1.4 MEDIATORS OF CARDIOVASCULAR DYSFUNCTION IN SIRS AND ARDS

[Figure 1.7]

1.4.1 The role of NO

1.4.1.1 Vascular and myocardial overproduction

Suprabasal release of NO is known to occur in response to a diverse range of stimuli including endotoxin, cytokines, hypoxia, histamine, thrombin, bradykinin, calcium ionophore, endothelin and substance P **[Figure 1.8].** As a result, an increase of NO release during the inflammatory response to sepsis is inevitable. Patients and animals with septic shock lose peripheral vascular tone, and the responsiveness of systemic vessels to constrictor agents such as catecholamines is diminished, both *in vitro* and *in vivo*. The incubation of bovine aortic endothelial cells with LPS causes rapid release of an NO-like factor. Futhermore, the levels of NO metabolites are significantly elevated in patients

with septic shock (Ochoa et al. 1991), and the administration of NOS inhibitors in these patients (Petros et al. 1991), or in animal models of sepsis (Klabunde and Ritger, 1991), can produce reproducible elevations in SVR where other vasoconstrictors are ineffective. In fact, huge quantities of NO are produced in sepsis. Endotoxin leads to the induction of the calcium-independent NOS (iNOS) in both the endothelium (Busse et al. 1993) and the vascular smooth muscle (Fleming et al. 1991), as well as in the myocardium where the increase in NO production has been shown to reduce contractility (Brady et al. 1992). Other cytokines (TNF, IL-1 and -2) also stimulate iNOS activity in vessel walls.

The time course of increased NO release in sepsis is the subject of considerable speculation. In isolated, endotoxin-treated rat main pulmonary arteries, NOS inhibitors reverse the vascular hyporesponsiveness to phenylephrine (Griffiths et al. 1995a), and there is no doubt that in established sepsis NO is largely reponsible for this reduced reactivity. NO-mediated hyporeactivity to noradrenaline starts within 60 minutes in a rat model of sepsis *in vivo* (Szabo et al. 1993), and may therefore be too rapid to be explained by the induction of iNOS. It therefore seems likely that early increases in NO release are explained by an elevation in NO production by endothelial cNOS, but this remains controversial, and in fact in one study, although the administration of L-NAME after one hour in endotoxin-treated pithed rats did elevate blood pressure and increase vascular responsiveness to catecholamines, this was to no greater degree than in saline-treated animals (Guc et al. 1992). This suggests that early hyporesponsiveness is caused not by NO, but by some other factor(s). However, it is clear that from about three hours after the endotoxic insult, there is massive NO production as a result of endothelial and vascular smooth muscle iNOS activity. The endothelium appears to be required for maximal NO response, so

that removal of the endothelium caused a significant delay in the onset of vascular responsiveness (6 compared with 4 hours) and reduced the sensitivity of rat aorta to LPS *in vitro* (Fleming et al. 1993). The technique of reverse transcription polymerase chain reaction (rt-PCR) has allowed the demonstration of the widespread tissue expression of iNOS mRNA in a rat model at 4 hours post LPS injection, compared with undetectable expression in control animals (Liu et al. 1993). In rat pulmonary artery this rise in iNOS mRNA expression could be attenuated by pretreatment with dexamethasone 30 minutes before the LPS challenge (Griffiths et al. 1995a). Furthermore, preliminary evidence suggests that there is differential regulation of the constitutive and inducible NOS mRNAs in rats treated with LPS *in vivo*, such that whilst there is greatly increased expression of iNOS mRNA in rat heart, lung and aorta 4 hours after intraperitoneal LPS administration, the expression of endothelial NOS mRNA was simultaneously down-regulated when compared with levels from control animals (Liu et al. 1995).

1.4.1.2 **NOS inhibitors as therapeutic agents in sepsis**

As has already been mentioned, *iv* L-NMMA was shown to (temporarily) correct systemic hypotension in two patients with septic shock (Petros et al. 1991). A second L-arginine analogue that non-specifically blocks NOS, N^G-nitro-L-arginine (L-NNA), has also been given to critically ill patients with sepsis (Lorente et al. 1993), but caused a significant increase in both SVR and PVR as well as in mean arterial pressure, but with an accompanying reduction in cardiac index. Further clinical trials of NOS inhibition are underway. The main theoretical objection to the administration of non-specific NOS inhibitors is that NO is known to contribute to basal vascular tone, and in some organ beds (notably renal,

mesenteric and pulmonary) there is frequently an overall reduction in regional blood flow in sepsis which could be amplified by NOS inhibition (Griffiths et al. 1994).

A gradual improvement in the understanding of the role of iNOS in sepsis has fuelled investigations into the possibility of the selective blockade of this enzyme in sepsis (Griffiths et al. 1994). The theoretical attraction of this as a therapeutic tool is obvious. In comparative studies between L-NMMA and aminoguanidine, the latter was found to be at least seven times more potent at inhibiting iNOS, whereas L-NMMA was 15 times more potent at inhibiting the constitutive enzyme than aminoguanidine. Aminoguanidine produces a dose-dependent increase in phenylephrine-induced tension in intact and endothelium-denuded pulmonary artery rings from endotoxin-treated rats, but it has no effect on controls (Griffiths et al. 1993). The aminoguanidine-precipitated contraction in the endothelium-denuded vessels was abolished by L-arginine or L-NMMA pretreatment, thus suggesting that its mechanism of action involves the L-arginine/NO axis.

1.4.2 **The role of ET-1**

Taking into account the factors known to stimulate ET production (*vide supra*), it is inevitable the inflammatory response associated with sepsis will augment release. ETs seem to be important mediators of vascular tonic responses under physiological conditions, since they are released in response to a variety of local factors including hypoxia. Their potent effects on vascular tone are likely to play an important role in the widespread changes associated with endotoxaemia.

1.4.2.1 **Animal studies**

ET release in response to endotoxin has been confirmed by radioimmunoassay *in vitro* and *in vivo* (Sugiura et al. 1989), and in endothelial cell cultures in response to TNF-α, interferon gamma, IL-1, TGF-β (Kanse et al. 1991a; Yoshizumi et al. 1990) and free radical species (Prasad et al. 1991). ET levels increase during endotoxaemia in many animal models, including mouse (Takahashi et al. 1990b), sheep (Morel et al. 1991), dog (Nakamura et al. 1991), pig (Weitzberg et al. 1995), piglet (Myhre et al. 1993), and rat (Morise et al. 1994; Lundblad et al. 1995; Hohlfeld et al. 1995; Lundblad and Giercksy, 1995). The rate and duration of elevation in [ET-1] in these models clearly depends upon the dose of endotoxin, and the route and method of administration. However, these studies tend to reinforce the hypothesis that ET-1 is not stored and therefore a rise in [ET-1] is not seen before 30 minutes. Indeed, expression of pre-pro-ET-1 mRNA in cultured rat pulmonary artery endothelial cells is elevated within one hour of exposure to LPS or interleukins 1-α or 1-β or TNF-α, followed by a rapid decline to control levels after 4 hours (Golden et al. 1995). These cells clearly have a capacity for a rapid transcriptional response to such mediators, and as a corresponding increase in ET-1 peptide release was detectable 6 hours after exposure to these mediators, an increase in the translational phase of ET-1 production is also possible. By contrast, it has been demonstrated in rats that *iv* TNF-α induces a very early rise in plasma [ET-1] which peaks at 30 minutes (Klemm et al. 1995). The speed of this elevation is difficult to explain in the context of previous evidence suggesting that ET-1 is not stored by the endothelium. When rats were treated with *iv* LPS, rather than TNF-α, the rise in [ET-1] was slower, and did not peak until 60 minutes, mirroring the rise in [TNF-α] (Hohlfeld et al. 1995). Another interesting finding of these studies was that upon removal of the hearts of the rats treated with TNF-α or

LPS into an *ex vivo* preparation, a profound coronary vasoconstriction developed that was not present *in vivo*. This coronary vasoconstriction could be completely prevented by pretreatment of the rats with the ET_A antagonist, FR 139317, or with anti-TNF-α antibody. It is difficult to explain this slow onset, *ex vivo*, ET-1-induced coronary vasoconstriction without speculating that the tendency for ET-1 to induce the contraction is counterbalanced *in vivo* by a vasodilator. This represents an important insight into the way in which such an apparently potent vasoactive agent as ET-1 can be present at high concentrations and yet not produce generalised vasoconstriction.

The role played by the ETs in the inflammatory response to sepsis is not yet clear. The release, and subsequent high circulating levels of such a potent vasoconstrictor would be expected to antagonize the observed systemic vasodilator response, and it is possible that interaction between ETs and other vasoactive mediators, particularly NO, explains this paradox. The effect of ET-1 is likely to be tissue- and species-specific. For example, in rats rendered endotoxaemic by intraperitoneal LPS 6 hours earlier, there was no difference in the vasoconstrictor response of perfused mesentery to ET-1 (or U46619, phenylephrine, 5-hydroxytryptamine) compared with controls (Mitchell et al. 1993). This is an unexpected result in view of the increase in local NO derived from iNOS, which could be expected to have induced hyporesponsiveness to these vasoconstrictors. In a second study (Morise et al. 1994), the rise in plasma creatinine and blood urea, and the fall in urine production were shown to correlate with the elevation in plasma [ET-1] in a rat model of septic shock.

1.4.2.2 **Human studies**

Plasma [ET-1] has been found to be elevated in critically ill patients with sepsis, and also to correlate with the degree of depression of cardiac output (Pittet et al. 1991), creatinine clearance (Voerman et al. 1992), as well as indices of overall illness severity and outcome, such as the APACHE (Acute Physiology and Chronic Health Evaluation) II (Pittet et al. 1991; Voerman et al. 1992), and organ failure (Sanai et al. 1996) scores.

More specifically, plasma [ET-1] has been shown to be elevated in patients with acute lung injury and ARDS (Langleben et al. 1993; Sanai et al. 1996; Druml et al. 1993). In one group, [ET-1] correlated positively with oxygen consumption, and negatively with PaO_2:FiO_2 ratio (Sanai et al. 1996). Interestingly, this study failed to show a significant correlation between [ET-1] and PVR, which the authors took as evidence that ET-1 has greater effects on microvascular permeability than on pulmonary vasoconstriction. In another study, the systemic arterial and venous plasma levels of ET-1 were compared in patients with lung injury and PHT and a control group (Langleben et al. 1993). Initial systemic and venous [ET-1] were elevated compared to controls, demonstrating increased ET-1 release, and the systemic/venous ET-1 ratio was significantly higher, which implies that net pulmonary clearance was also reduced. In addition, as patients improved clinically, mean [ET-1], arterial/venous [ET-1] ratio, and pulmonary arterial pressure fell significantly compared with those patients showing clinical deterioration. The pattern of increased ET-1 formation and release combined with reduced pulmonary clearance was confirmed by the third study (Druml et al. 1993). Apart from raising the clinical suspicion that ET-1 could be playing a role in the pathophysiology of acute lung injury, these results underline the reservations raised earlier about

interpreting [ET-1], which clearly represent a balance between production and renal and pulmonary clearance.

1.4.2.3 **Manipulation of ET-1 in models of sepsis**

The demonstration of an association between elevated plasma [ET-1] and sepsis in animal models and in critically ill patients has lead inevitably to the investigation of ways to interfere with the effects of ET-1 in these circumstances. Proposed interventions have been both specific and non-specific for ET-1. For example, the COX inhibitor, diclofenac, produced a delay in the rise of [ET-1] in a pig model of endotoxic shock, which was due to reduced ET-1 production, as well as attenuation of the pulmonary hypertensive response (Weitzberg et al. 1995). In a study of rats rendered septic by caecal perforation, pentoxifylline reduced blood concentrations of TNF-α, IL-6 and ET-1, and improved survival (Lundblad et al. 1995). It seems likely that the effect on [ET-1] was secondary to the effect on TNF-α. In another caecal perforation rat model of sepsis (Lundblad and Giercksy, 1995), administration of a monoclonal anti-endotoxin antibody reduced mortality, as well as concentrations of endotoxin, TNF-α and ET-1, but interestingly not of big ET-1. The lack of effect on big ET-1 concentration suggests that the antibody could have reduced release of ET-1 itself more than release of big ET-1, as well as peripheral conversion of big ET-1.

More specific anti-ET-1 measures have recently been reported. In the study discussed above (Morise et al. 1994) correlating reduction in renal function with elevated plasma [ET-1] in rats with endotoxaemia, the administration of 4 doses of an anti-ET-1 monoclonal antibody at the peak plasma [ET-1] (3 hours) caused a significant reduction in plasma creatinine and urea concentrations, as well as increased urine volume. It was concluded that the increase in endogenous ET-1

induced by sepsis in that model played an important role in renal dysfunction. Furthermore, in conscious rats undergoing LPS infusion over 24 hours, a non-selective ET receptor antagonist, SB 209670, enhanced the fall in mean arterial blood pressure, and converted the mesenteric vasoconstriction seen with LPS infusion alone to significant vasodilatation (Gardiner et al. 1995). These results suggest that ET-1 may oppose the fall in systemic arterial pressure that is observed in this model by attenuating vasodilatation in beds such as the mesentery. In another study, the combined ET receptor antagonist, bosentan, was given *iv* to pigs 30 minutes before an endotoxin infusion, and was found to attenuate the secondary phase of elevation in PVR seen with endotoxin alone (Lundberg et al. 1995).

1.4.3 Other mediators

1.4.3.1 **Products of arachidonic acid metabolism**

Arachidonic acid is the precursor of a variety of vasoactive and inflammatory mediators implicated in the pathogenesis of sepsis. The first step in arachidonic acid metabolism is its liberation from membrane-bound phospholipids, usually by the actions of phospholipase A_2. Once free in the cell, arachidonic acid is metabolised by various oxygenase enzymes, such as cyclo-oxygenase (COX) to form prostaglandins (PG), thromboxanes (TX) and prostacyclin (PGI_2) or lipoxygenase (LO) to form leukotrienes (LT). COX is now known to exist in two isoforms, a constitutive COX-1 and an inducible COX-2 (Mitchell et al. 1995a). It is thought that COX-2 predominates at sites of inflammation, including the lungs of rats 6 hours after LPS-challenge (Swierkosz et al. 1995). Thus, COX-2 may represent the main source of prostanoids released in the lung during septic

shock. The identification of COX-2 as a major inflammatory enzyme has led to the development of nonsteroidal anti-inflammatory drugs (NSAIDs) to specifically inhibit this isoform (Masferrer et al. 1994).

1.4.3.2 **Arachidonic acid metabolites in sepsis**

Cytokines stimulate prostanoid release both *in vivo* and *in vitro* due to induction of COX-2 (Mitchell et al. 1995b). During the inflammatory response, the generation of prostanoids and TXA_2 occurs in inflammatory cells such as macrophages as well as in the endothelium. The release of PGI_2 and TXA_2 is controlled by individual cell types. Thus, endothelial cells form mainly PGI_2 whereas platelets and to a lesser extent, eosinophils and neutrophils, are the main sources of TXB_2. COX-2 induction and accompanying increases in PGI_2 have been demonstrated in: humans treated with IL-1 (Hla and Neilson, 1992), bovine endothelial cells treated with LPS (Akarasereenont et al. 1994) and in intact human vessels (Waldman and Murad, 1987) as well as in lung homogenates and aortic smooth muscle from rats treated with LPS (Swierkosz et al. 1995). The increased release of PGI_2 following COX-2 induction may therefore attenuate PHT associated with sepsis. Thus, the balance between the local production of vasodilator (PGI_2) and vasoconstrictor (TXA_2 and endoperoxides) agents in this way undoubtedly contributes to vascular tone in that area (Petrak et al. 1989). The induction of COX-2 occurs from about 6 hours, and it is at about this time point that PGI_2 is released from cultured endothelial cells in response to endotoxin (Akarasereenont et al. 1994). More specifically, IL-1, known to be one of the principal mediators that amplifies the early inflammatory response in sepsis, induces PGI_2 production from human endothelial cells (Hla and

Neilson, 1992). Both IL-1 and TNF-α induce COX-2 mRNA expression in human endothelial cells.

TXA_2 is a vasoconstrictor, and its inhibition has been shown to diminish early PHT, but not the increased vascular permeability, in an experimental model of endotoxin-induced sepsis (Winn et al. 1983). Clinical studies of patients with ARDS have demonstrated increased serum levels of TXA_2 (Leeman et al. 1985) and leukotrienes in BAL fluid (Matthay et al. 1984). The pulmonary production of COX products is suggested by the finding in endotoxaemic sheep that their concentrations in lung lymph exceed plasma levels (Ogletree et al. 1986). Several animal studies of sepsis have successfully attenuated the early changes in pulmonary haemodynamics using COX inhibitors or TXA_2 receptor antagonists with a corresponding improvement in survival (Ahmed et al. 1986; Harlan et al. 1983). Trials of COX inhibitors in these patients are currently underway in the USA.

1.4.3.3 **Arachidonic acid metabolites and HPV**

The relative importance of arachidonic acid derivatives in the vasomotor response to hypoxia appears to vary between species and vascular preparation. However, several lines of evidence illustrate a role for COX-metabolites in these responses. For example, prostanoid inhibition by indomethacin augments HPV (Hamacher and Schaberg, 1994), implying a modulation by dilator prostanoids. Similarly, infusions of arachidonic acid decreased HPV and increased PGI_2 formation by canine pulmonary arteries (Gerber et al. 1980). By contrast, a different NSAID, flurbiprofen, increased the contraction induced by hypoxia in isolated human pulmonary arteries (Demiryurek et al. 1993). Moreover, the HPV observed in distal and proximal porcine pulmonary arteries were differentially

affected by indomethacin (Kovitz et al. 1993). TXA_2 and the vasoconstrictor LTC_4, have also been implicated in vascular responses to hypoxia. Indeed, LTC_4 is released in the rat lung after exposure to hypoxia (Morganroth et al. 1984b) and the consequent vasoconstriction can be inhibited by both LT antagonists and LO inhibitors (Morganroth et al. 1984a). In addition, both LTC_4 and TXB_2 are elevated in human subjects exposed to high altitudes (Richalet et al. 1991).

1.4 AIMS OF THE THESIS

The overall objective of this thesis was to investigate the role of ET-1 in the pathophysiology of the vascular inflammatory response to sepsis, with particular emphasis on the pulmonary circulation. Two distinct experimental strategies were applied. Firstly, the production and release of *endogenous* ET-1 were assessed, and wherever possible the vasoactivity of this ET-1 was elucidated using ET receptor antagonists. Secondly, the effect of the inflammatory response to endotoxin challenge on ET-1-mediated vascular reactions was investigated by the administration of *exogenous* ET-1. Again, the work was focused on the pulmonary circulation, which behaves in a different manner to the hyporesponsive vasodilatation seen in the systemic circulation. These investigations were undertaken by addressing the following specific questions:

1. Is endogenous ET-1 production increased in tissues and blood vessels in an experimental model of sepsis?
2. Does ET-1 contribute to vascular tone in endotoxaemia?
3. Is the pulmonary circulation hypo-or hyper-responsive to ET-1 in endotoxaemia?
4. Does ET-1 interact with other vasoactive and inflammatory mediators, particularly NO?

Table 1.1 **Clinical conditions associated with the development of ARDS**

Respiratory	Non-respiratory
Pneumonia (Bacterial/Viral/Fungal)	Sepsis syndrome
Aspiration of gastric contents	Major Trauma/Shock
Pulmonary contusion	Massive burns
Post-pneumonectomy	DIC
Inhalation of smoke or toxins	Transfusion reactions
Near-drowning	Fat embolism
Thoracic irradiation	Pregnancy-associated (eg. amniotic fluid embolism)
Oxygen toxicity	Pancreatitis
Ischaemia-reperfusion	Drug/Toxin reactions (eg.paraquat, heroin)
Vasculitis (eg. Goodpasture's)	Post-CP bypass
	Head injury/ raised ICP
	Tumour Lysis syndrome

Abbreviations:

DIC - disseminated intravascular coagulopathy
CP - cardiopulmonary
ICP - intracranial pressure.

Table 1.2 **Definitions of Sepsis and Septic Shock**

SEPSIS *"The systemic response to infection".*

Includes two or more of the following:

*Temperature >38°C or <36°C

*Heart rate >90 /min

*Respiratory rate >20 /min or $PaCO_2$ <4.3 kPa

*White cell count >12,000 /mm^3 <4,000 /mm^3 ,

or >10% band (immature) forms

SEPSIS SYNDROME *"Sepsis with evidence of altered organ perfusion."*

Altered organ perfusion includes one or more of the following:

*PaO_2/FiO_2 ≤280 (without other cardiopulmonary disease)

*Elevated lactate level (> upper limit of normal for the lab.)

*Oliguria <0.5 ml/kg body weight

SYSTEMIC INFLAMMATORY RESPONSE SYNDROME *"The response to a variety of severe clinical insults (not necessarily infective), which is indistinguishable from sepsis."*

SEPTIC SHOCK *"Sepsis with hypotension (sustained decrease in systolic blood pressure <90 mmHg, or drop >40 mmHg, for at least 1 hr) despite adequate fluid resuscitation, in the presence of perfusion abnormalities that may include, but are not limited to lactic acidosis, oliguria or an acute alteration in mental status."* Patients who are on inotropic or vasopressor agents may not be hypotensive at the time that perfusion abnormalities are measured.

Definitions from Chest 1992 **101**:1644-1655.

Table 1.3 **Components of the lung injury score**

(from Murray et al, Am Rev Respir Dis 1988;138:720-723)

Chest Xray Score	
No alveolar consolidation	0
Alveolar consolidation confined to 1 quadrant	1
" " " " 2 quadrants	2
" " " " 3 quadrants	3
" " " " 4 quadrants	4

Hypoxaemia score		
PaO_2:FiO_2	≥300	0
PaO_2:FiO_2	225-299	1
PaO_2:FiO_2	175-224	2
PaO_2:FiO_2	100-174	3
PaO_2:FiO_2	<100	4

PEEP (positive end-exspiratory pressure) score	
(if ventilated)	
≤5 cmH_2O	0
6-8 "	1
9-11 "	2
12-14 "	3
≥15 "	4

Pulmonary compliance score	
≥80 ml/cmH_2O	0
60-79 "	1
40-59 "	2
20-39 "	3
≤19 "	4

The final value is obtained by dividing the score sum by the number of components used:

No lung injury	0
Mild-moderate lung injury	0.1-2.5
ARDS	>2.5

Table 1.4 Endothelin receptor agonists and antagonists

	Agonist	Antagonist
ET_A	ET-1>ET-2>>ET-3 Sarafotoxin S6b	BQ123 FR139317 BQ153 BMS 18496/7
ET_B	ET-3>>ET-1 Sarafotoxin S6c BQ3020 IRL1620 BMS 18496/7	BQ788 IRL1038 IRL1620 $[Ala^{1,3,11,15}]$ET-1
Combined ET_A/ET_B	ET-1	Bosentan (Ro47-0203) PD142893 PD145065 SB209670

Table 1.5 **Vascular isoforms of Nitric Oxide Synthase**

NOS isoforms	eNOS	iNOS
Response in sepsis	Constitutive Immediate NO synthesis	Induced by LPS & cytokines Massive NO production after 2-6 hours
Location	Endothelial cell Membrane-bound	Mainly smooth muscle Cytosolic
Regulation	Oestrogens, shear stress & exercise increase activity	Induction prevented by corticosteroids & inhibitors of protein synthesis
Activation	Calcium-dependent	Calcium-independent
Non-selective inhibitors	L-arginine analogues (e.g. N^G-monomethyl L-arginine)	
Selective inhibitors	None known	Aminoguanidine L-canavanine

Abbreviations:

NO	Nitric oxide.
NOS	Nitric oxide synthase: "e" - endothelial; "i" - inducible
LPS	Lipopolysaccharide

Figure 1.1 **The overlapping clinical syndromes associated with sepsis**

Abbreviations: SIRS systemic inflammatory response syndrome
MOF multiple organ failure
ARDS acute respiratory distress syndrome

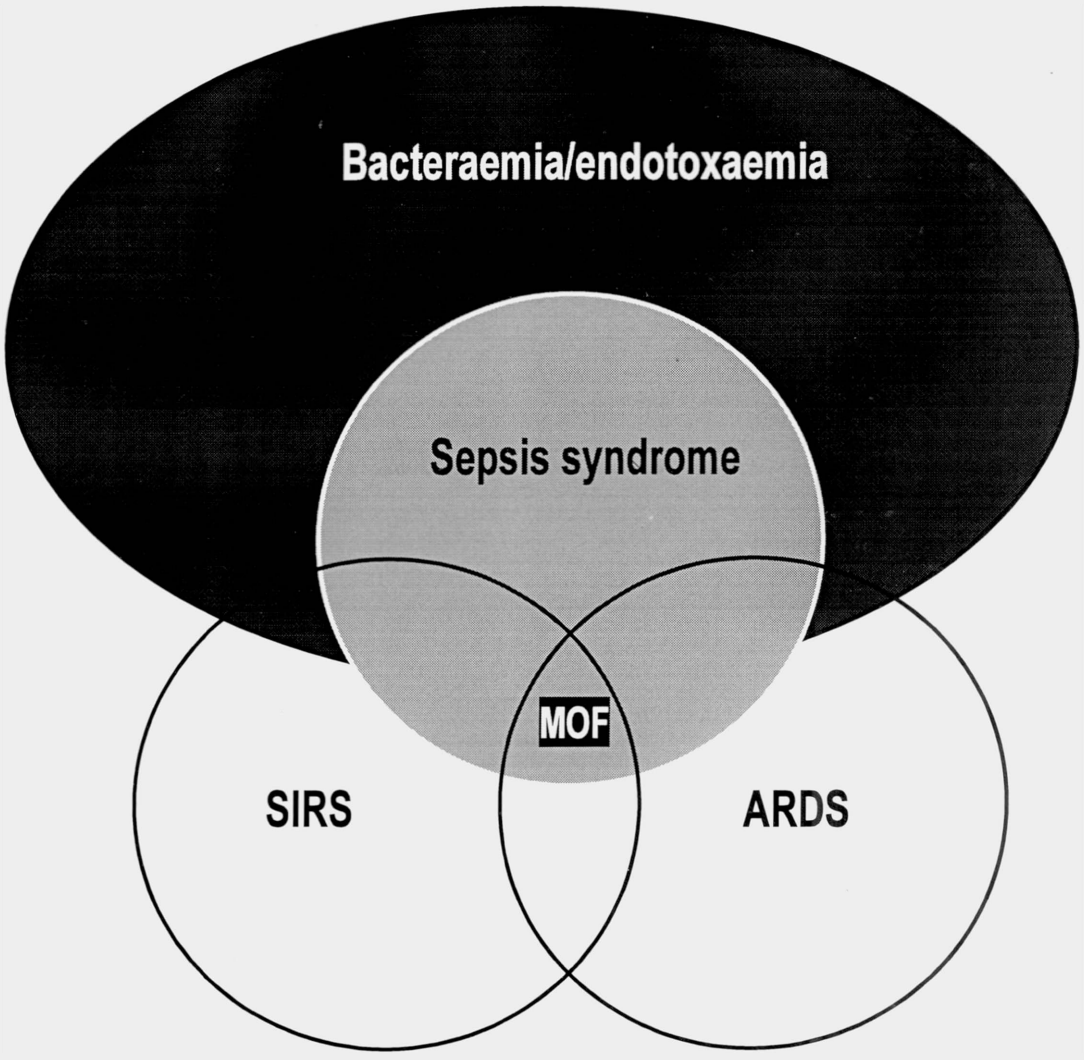


Figure 1.2 **Acute lung injury and sepsis: the spectrum of disease**

MOF multiple organ failure
ARDS acute respiratory distress syndrome

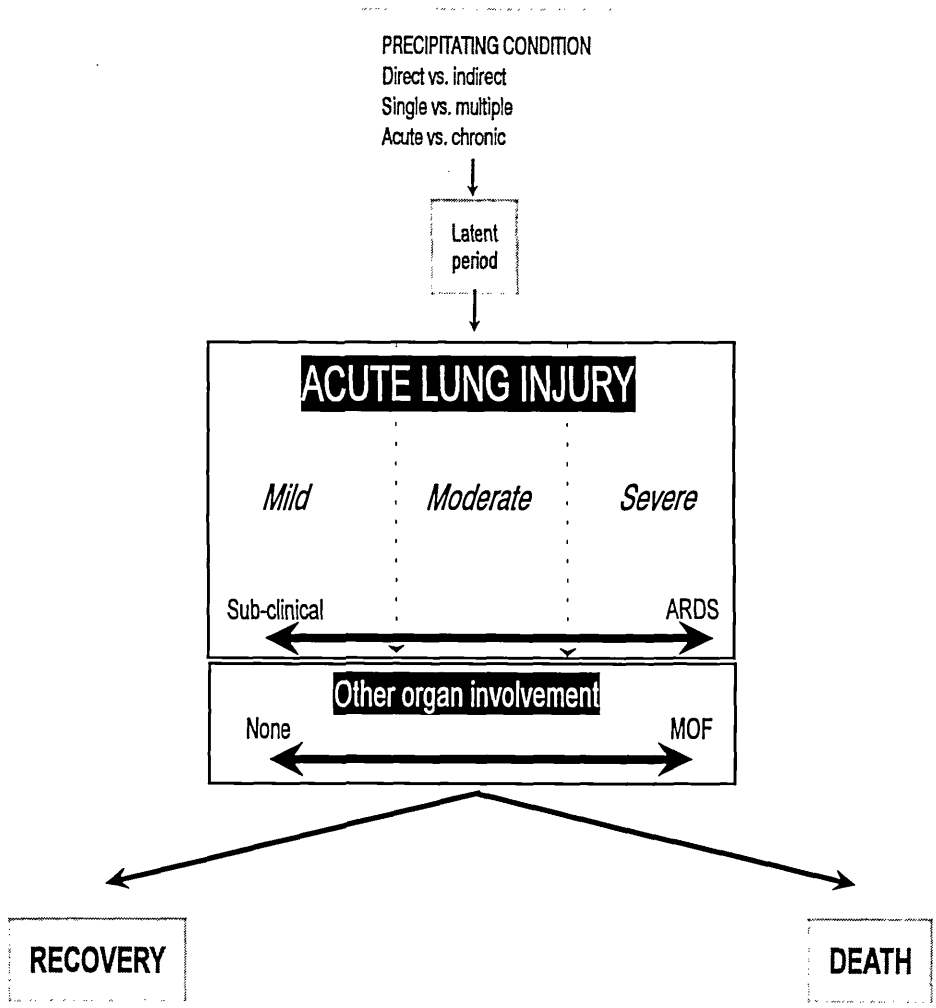


Figure 1.3 **Cellular and humoral inflammatory pathways activated by endotoxin**

Abbreviations: MDS myocardial depressant substance
DIC disseminated intravascular coagulopathy

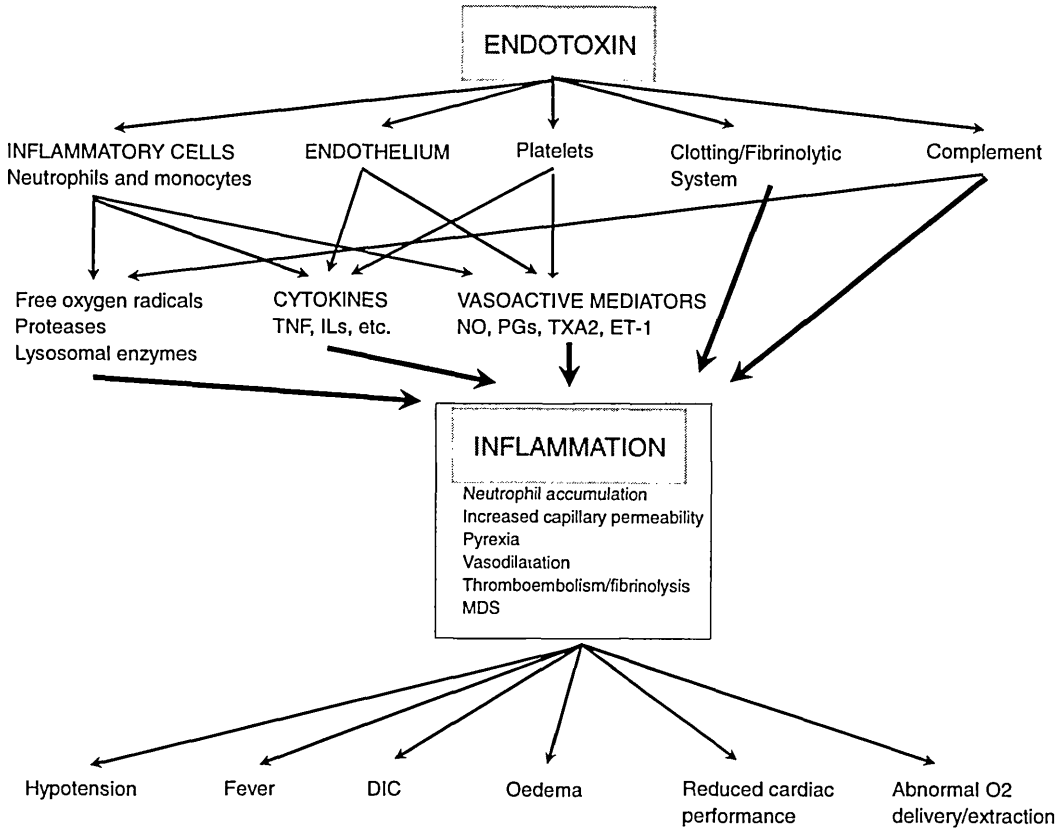


Figure 1.4 The biosynthetic pathway for ET-1

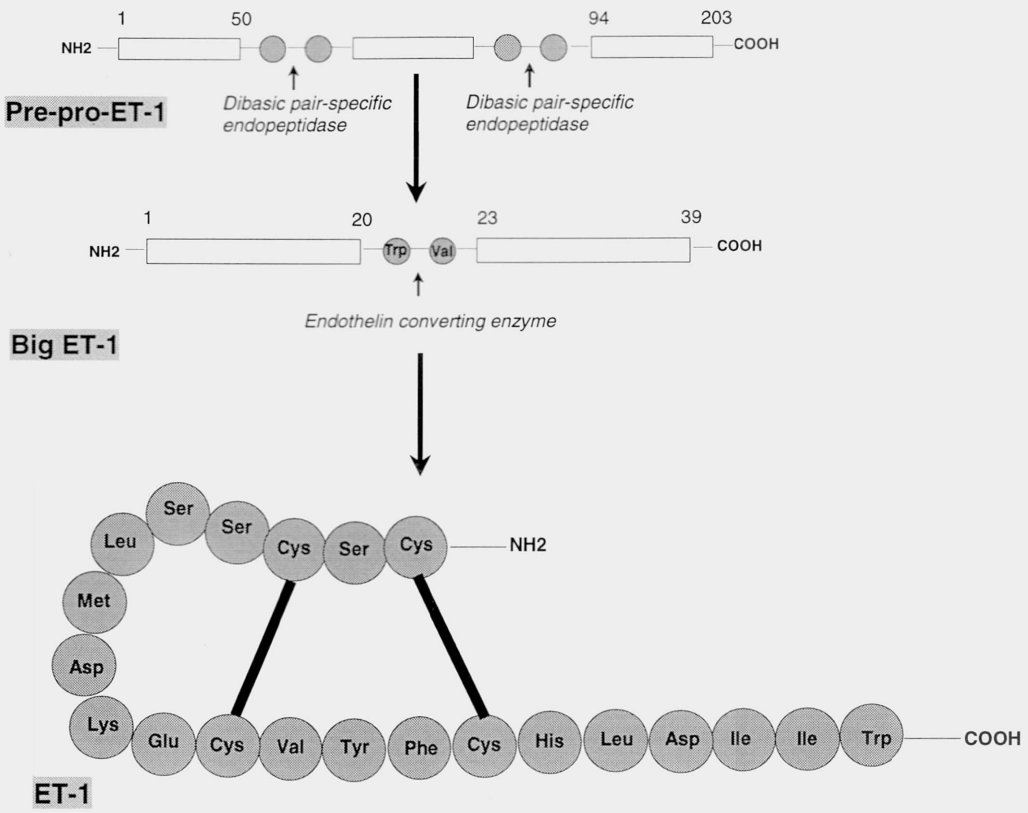


Figure 1.5 **ET-1: release and receptors**

Abbreviations:

TNF	tumour necrosis factor
AII	angiotensin II
PKC	phosphokinase C
PLC	phospholipase C
$[Ca^{2+}]$	intracellular free calcium

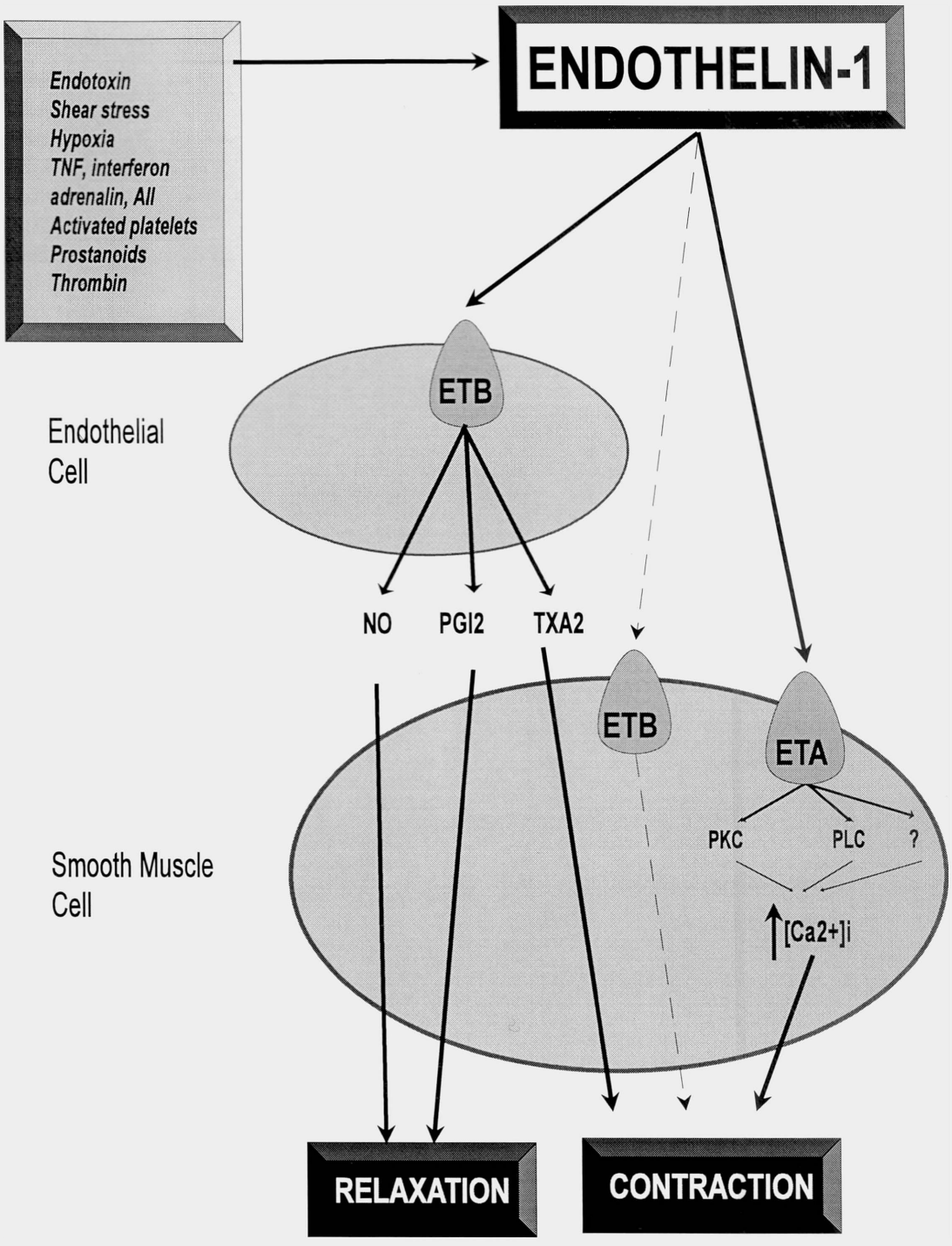


Figure 1.6 ET-1: secondary messenger systems

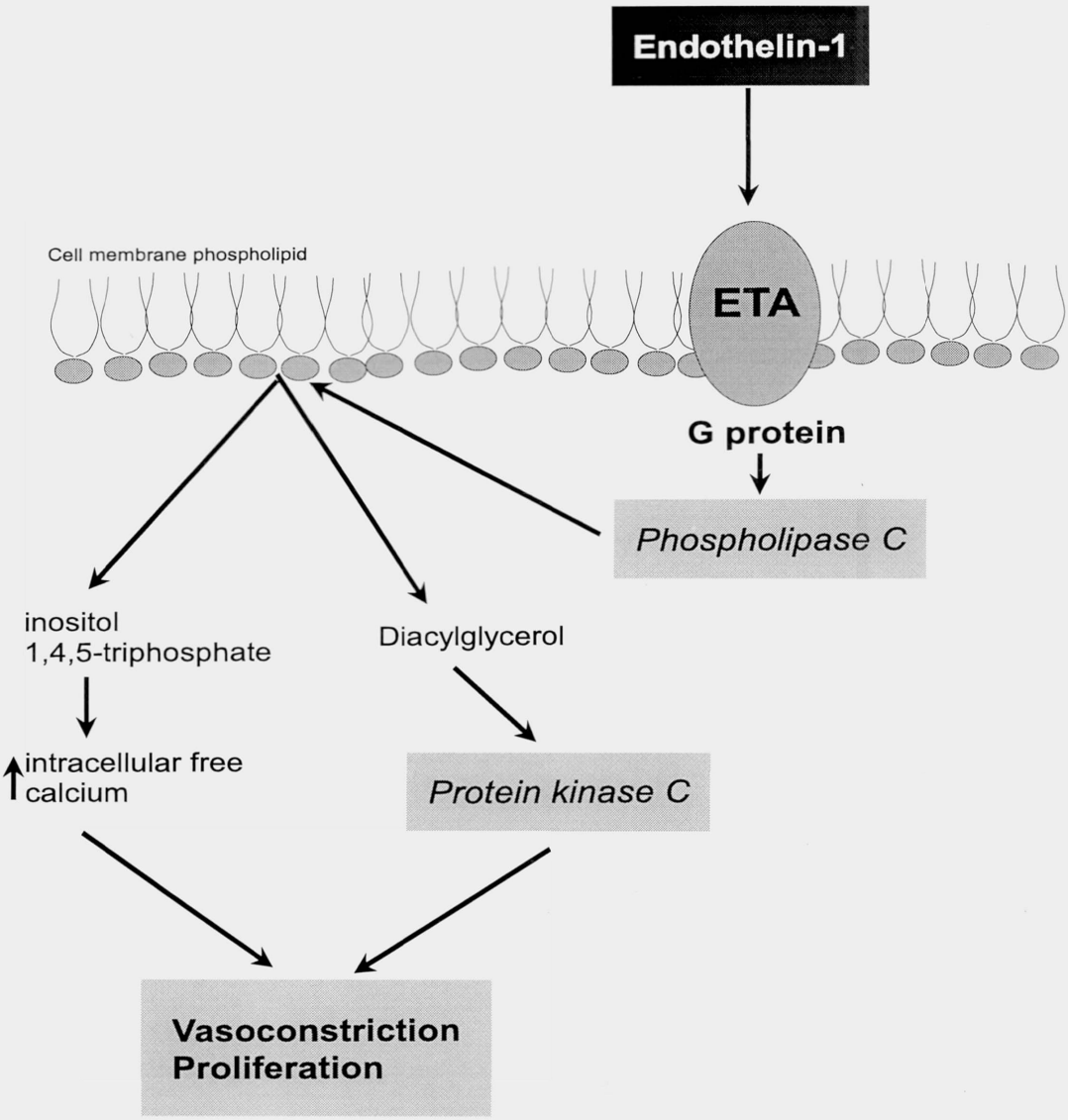
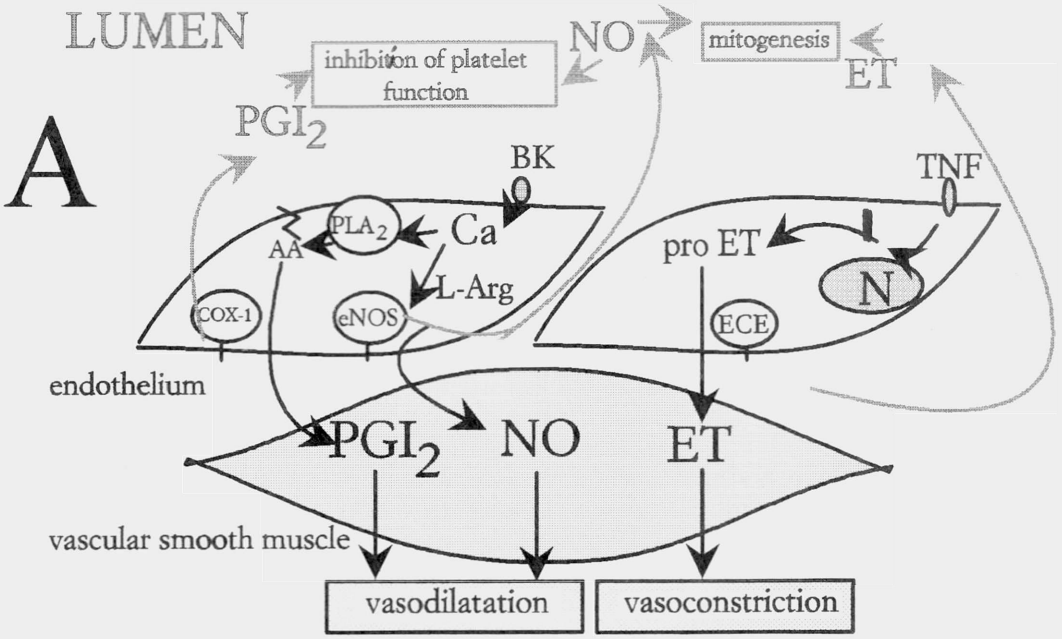


Figure 1.7 **The vascular response in sepsis**

Mediator release normally (A), and in endotoxaemia (B)

New abbreviations:

BK	bradykinin
L-arg	L-arginine
N	nucleus
PLA_2	phospholipase A_2



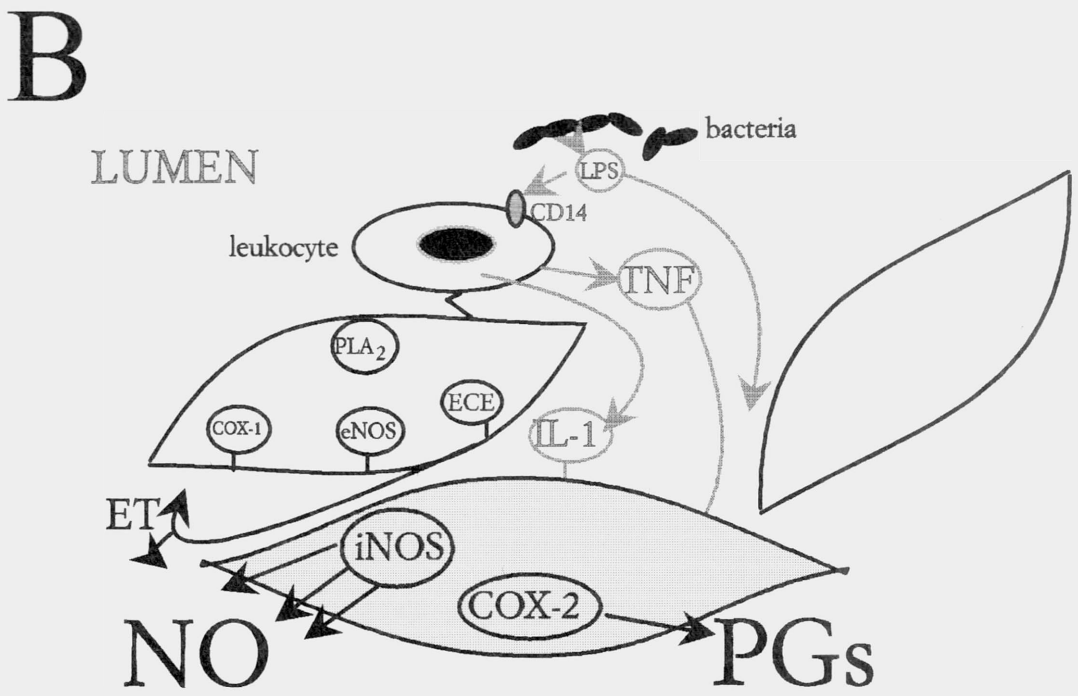
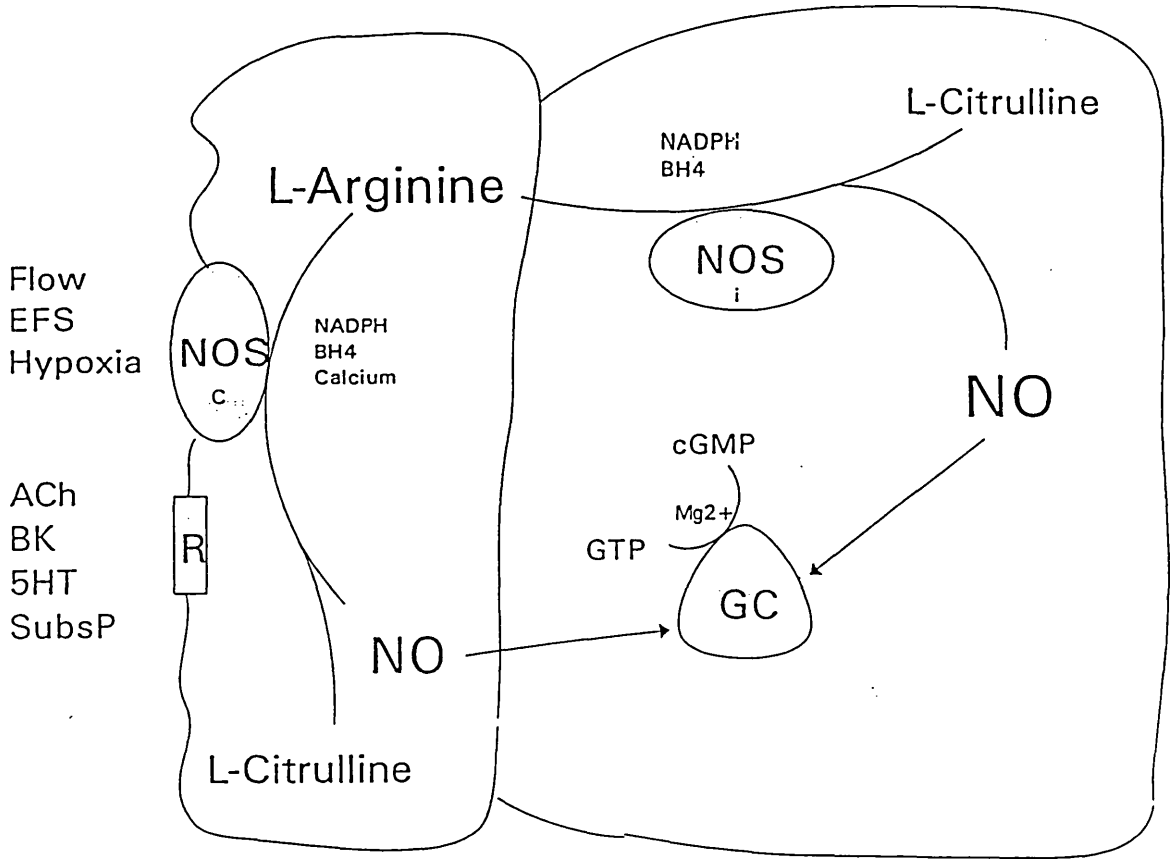


Figure 1.8 **Vascular nitric oxide synthase isoforms in sepsis**

iNOS activity in both vascular smooth muscle and endothelium produces copious amounts of NO in endotoxaemia. Preliminary results suggest that cNOS activity may be depressed in established sepsis.

Abbreviations:

EFS	electrical field stimulation
ACh	acetylcholine
BK	bradykinin
5HT	5-hydroxytryptamine
SubsP	substance P
BH4	tetrahydrobiopterin
GC	guanylyl cyclase



CHAPTER 2

METHODS AND MATERIALS

2.1. CHARACTERISATION OF THE LPS-TREATED RAT MODEL

2.1.1 Animals

Male Wistar rats weighing 275-300g (Charles Rivers UK, Ltd) were housed in steel bar cages in a humidity-controlled environment at 21°C. Food (Special Diet Services Ltd, Essex) and water were available *ad libitum*. All the studies were performed with the approval of the Home Office (UK) licensing authority, and the care and handling were in accordance to Home Office (UK) guidelines.

2.1.2 Pulmonary histology

The isolated perfused lung (*vide infra*) was prepared using sham (saline, 1ml/kg, by intraperitoneal (*ip*) injection) and LPS-treated (*Salmonella enteritidis* LPS, 20mg/kg, *ip*) rats (n=3 in each group). The pulmonary circulation was perfused with 4% bovine serum albumin (BSA) in Krebs alone at 18ml/minute until no further blood was seen being washed out in the effluent from the left atrial cannula, which required approximately 75ml of BSA per rat. The perfusate was then changed for formal saline, with which the circulation was perfused for 5 minutes whilst normoxic ventilation was continued as before. The lungs were then dissected free and stored in formal saline until they were processed for histological examination. Longitudinal

sections of the right and left lower lobes were embedded in paraffin and 5mm sections were stained with haematoxylin and eosin.

2.1.2.1 **Light microscopy**

Three blocks of lung tissue from sham and LPS treated rats were examined under oil emersion light microscopy (magnification x40). Neutrophils were counted by a single observer in ten high power fields from each block containing roughly equivalent areas of lung parenchyma, using coded slides in a blinded manner.

2.1.2.2 **Electron microscopy**

For EM studies, fixed tissues were rinsed in 0.05% cacodylate buffer and post-fixed in 1% osmium tetroxide calcium cacodylate buffer. After dehydration in a graded series of methanols, the tissue was embedded in epoxy resin (Araldite), cut into ultra-thin sections and stained with uranyl acetate and lead citrate.

2.1.3 <u>Levels of NO metabolites in the blood</u>

Rats were sedated using Hypnorm 0.2ml/kg (fentanyl citrate 0.315mg/ml, fluanisone 10mg/ml). The aorta was then exposed and blood removed from it via a 21G needle. Blood was collected and immediately centrifuged at 3000rpm for 10 minutes. The plasma was then removed and stored at -80°C until the assay was performed.

1-10μM dilutions of sodium nitrate and sodium nitrite were prepared in HPLC water (Rathburn Chemicals, Scotland). For each sample, duplicate 10μL aliquots of plasma or standard solution were added to 96 well plates, and 75μL of HPLC water added. 10μL of a solution containing 500μL of 3 units/ml *Aspergillus* sp. nitrate

reductase (Boehringer, Mannheim, Germany), 500μL of 1.5mM NADPH (Sigma) and 500μL of 150μM FAD (Sigma) was added to each well, and then incubated at 37°C for 15min. This gave a final concentration in each well of 0.1 units/ml nitrate reductase, 50μM NADPH and 5μM FAD. 10μL of a mixture of 100μM sodium pyruvate with lactate dehydrogenase (LDH, 100 units/ml) was then added to each well and incubated at 37°C for 5min. The LDH oxidizes remaining NADPH (which itself has optical absorbance at 550nm). 100μL of Griess reagent (1% sulphanilimide, 0.1% napthylethylenediamide in 5% phosphoric acid) was then added to each well, giving a final volume of 200μL, and the optical absorbance at 550nm read on a Titertek Multiscan II plate reader. Standard curves were constructed for nitrate and nitrite levels, and concentrations in each plasma sample calculated from the nitrite standard curve using the computer programme (Graph PAD Inplot, Graph PAD Software, San Diego, California, USA). Where the absorbance values were above those of the top of the standard curve, serial dilutions were made until the values were in this range.

2.2 DETECTION OF MESSENGER RNA FOR PRE-PRO-ET-1

2.2.1 Extraction of mRNA

Each sample of tissue was homogenized in RNAzol B (2ml/100mg tissue) using a polytron tissue homogenizer (Polytron, Brinkmann instruments, New York, USA) and total RNA extracted by a modification of the single step guanidinium thiocyanate/phenol/chloroform method of Chomczynski and Sacchi, 1987. Chloroform (100 ml/ml homogenate) was then added to each sample which was

vigorously agitated for 15 secs and allowed to stand on ice for 5 mins. Samples were centrifuged at 50G for 15 mins, thus extracting residual phenol. The aqueous layer was removed, added to an equal volume of isopropanol, and placed at 4°C for 30 mins, in order to allow precipitation of the RNA. Following centrifugation at 50G for 15 mins at 4°C, the RNA pellet was washed in 70% ethanol, and stored in ethanol at -70°C until the assay.

2.2.2 Quantitative ribonuclease protection assays for pre-pro-ET-1 and GAPDH mRNA

2.2.2.1 Templates for antisense riboprobes to pre-pro-ET-1 and GAPDH mRNAs

Standard polymerase chain reaction and cloning techniques were used to subclone a fragment of the gene for rat ET-1, producing a 0.8kb fragment containing 154 base pairs (bp) of exon 2 coding sequence for ET-1 and adjacent 5' intron, into the HindII/EcoR1 sites of riboprobe vector pAM19 (Amersham International, Amersham, UK), as previously described (Firth and Ratcliffe, 1992). Sequencing of the coding region by the deoxy-chain termination method (Sequenase; United States Biochemical Corporation, Cleveland, USA) confirmed the identity of ET-1. The EcoR1 cloning site was used to linearise the riboprobe template.

For analysis of constitutive glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels in the tissue experiments, a 316 bp fragment of the rat GAPDH gene derived from exons 5-8 (Tso et al, 1985), inserted into the Sac1/BamH1 sites of the pTRIPLEscipt transcription vector was used (Ambion, AMS Biotechnology, Whitney, UK). This plasmid was linearised by digestion with Hind III. In the pulmonary artery and aorta experiments, however, a 136 bp

fragment of the rat GAPDH gene was used to prepare a riboprobe protecting 136 nt of GAPDH mRNA, because we felt that this improved clarity of the ET-1 mRNA protected segments (see Chapter 3.2).

2.2.2.2 **Quantitative RNase protection assays**

(The principal features of this assay are illustrated in **Figure 2.1**).

Continuously labelled antisense RNA transcripts to ET-1 were generated by *in vitro* transcription of the linearised DNA template described above, using SP6 DNA-dependent RNA polymerase (Amersham International, Amersham, UK) and a [α^{32}P]GTP (specific activity 410 Ci/mmols, Amersham International). This produced a riboprobe protecting 154 nucleotides (nt) of pre-pro-ET-1 mRNA. This riboprobe has previously been shown to be specific for pre-pro-ET-1 mRNA and does not cross react with pre-pro-ET-2 or -ET-3 mRNA (Firth and Ratcliffe, 1992). This process, employing T7 RNA polymerase (Amersham International), was also used to produce a probe protecting 316nt (or 136nt in the PA and aorta experiments) of rat GAPDH mRNA. For analysis of mRNA, precipitated total RNA was dissolved in an aliquot hybridisation buffer (80% formamide, 40 mmols/L PIPES, 400 mmols/L NaCl, 1 mmol/L EDTA, pH 8) and RNA concentration determined by absorbance measurements at 260 nm using a spectrophotometer (model 2600, Guildford Instruments, Oberlin, USA). Concentrations were adjusted to yield 50µl samples containing 20mg of total RNA. After denaturation at 90°C for 15 mins, hybridization was performed overnight at 60°C with 2.5 x 10^5 counts/min of the ET-1 probe and 2.5 x 10^5 counts/min of the GAPDH probe. After hybridization, ribonuclease (RNase) digestion was carried out for 30 mins at 37°C by the addition of 350ml of a solution containing 14g/ml of RNase A (Boehringer Manheim, Lewis, UK), 2mgs/ml

of RNase T1 (Boheringer Manheim), 10mol/L Tris, pH 7.5, 5mmol/L EDTA and 300mmol/L NaCl. The reaction was terminated by the addition of 60mg/L of proteinase K (1mg/ml) with 3% SDA and further incubation at 37°C for 30 mins. Phenol-chloroform and chloroform extractions were performed and the RNA fragments precipitated with absolute alcohol, dissolved in 7ml of 80% formamide running buffer and electrophoresed on a denaturing 8% polyacrylamide gel. The gels were then dried and subjected to autoradiography at -70°C, and quantitated using laser densitometry (Howtech, Hudson, New Hampshire, USA) linked to a computer analysis system (PDI, Huntington Station, New York, USA). Results were expressed as ET-1 mRNA:GAPDH mRNA ratios.

2.3 MEASUREMENT OF ARTERIAL PLASMA ET-1 LEVELS

2.3.1 Sample extraction and preparation

Rats were sedated using Hypnorm 0.2ml/kg (fentanyl citrate 0.315mg/ml, fluanisone 10mg/ml). The aorta was then exposed and 4.5ml of blood removed from it via a 21G needle. Blood was collected into an EDTA bottle, and transferred on ice to a centrifuge and spun at 3000rpm for 10 minutes.

2.3.2 Description of the assay

Plasma [ET-1] was measured using a Parameter ELISA kit (R&D Systems Europe, Abingdon, Oxon, UK). Samples were extracted from plasma in a solution of acetone, HCL and water (40:1:5), and centrifuged for 20 minutes at 3000 rpm at 2°C. The ELISA is based upon a sandwich immunoassay for plasma ET-1 with an

anti-ET-1 antibody coated on the wells, and another anti-ET-1 antibody conjugated to horseradish peroxidase which was added after the extracted plasma samples. Upon its addition, the enzyme substrate produced a coloured product quantified photometrically using a microtiter plate reader set at 450nm with a correction wavelength of 620nm. Concentrations in each plasma sample were calculated, using a linear regression programme (Graph PAD Inplot, Graph PAD Software, San Diego, California, USA), from the standard curve that was obtained simultaneously using dilutions of the standard supplied in the kit. Each sample was performed in duplicate in order to improve accuracy. Manufacturer's claimed cross-reactivity for the system was as follows: big ET-1 <1%; ET-2 =45%; ET-3 =14%.

2.4 STUDIES OF ISOLATED PULMONARY ARTERY *IN VITRO*

2.4.1 Preparation of tissues

Male Wistar rats were treated with either *Salmonella enteritidis* endotoxin (20mg/kg i.p.) or saline vehicle (sham:3ml/kg) 4 hours prior to sacrifice by cervical dislocation. The heart and lungs were removed through a median sternotomy and placed in Krebs-Henseleit (Krebs) solution consisting of (in mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5, glucose 5.6. The Krebs was continually gassed with 95% O_2, 5% CO_2. The main left and right pulmonary arteries were dissected free of their surrounding tissue, and cut into rings 2mm long, care being taken not to traumatise the intimal surface. In one set of experiments, the thoracic aorta was dissected out and cut into similar rings.

Where appropriate, half of the artery rings had their endothelium removed by gentle abrasion using a roughened needle. Successful denudation was confirmed by failure to relax to acetylcholine (10^{-4}M) after contraction with phenylephrine (10^{-5}M).

2.4.2 Experimental procedure

Each artery ring was mounted over a pair of rigid Tungsten wires, one of which was fixed, and one attached to a force transducer (FT.03 Grass Instruments, Quincy, USA). Changes in isometric force were recorded on a polygraph multichannel recorder (Grass Model 7, Quincy, USA). A resting tension of 500mg was applied to the PA rings (or 1g for thoracic aorta) and then they were lowered into, and suspended in, 2ml organ baths containing oxygenated Krebs solution at 37^0C **[Figure 2.2]**.

2.4.3 Measurement of responses

After 15 minutes equilibration the rings were submaximally contracted with KCl (40mM) and relaxed to a uniform baseline tension of 500mg by repeated washing with KH solution. Rings were left to equilibrate in the bath for a total of 45 minutes and washed every 20 minutes.

Contractile responses of the rings to cumulatively increasing concentrations of different agonists were assessed, and the results expressed in actual tension generated in grams. In some cases relaxation experiments were performed after rings had been preconstricted, and the relaxation was then expressed as a percentage of the peak tension. The individual concentration-response curves

were fitted, and EC_{50} concentrations estimated, using a computer programme (Graph PAD InPlot, GraphPad Software, San Diego, CA, USA). Each bath contains 2ml, so that the final bath concentration for each agent used was achieved by adding 20μL of a solution 100fold more concentrated than the desired final bath concentration.

2.4.4 Length-tension relationships

A resting tension of 500mg is optimal for rat pulmonary artery rings from untreated animals (Crawley, 1992). In order to test the hypothesis that the optimal resting tension is not altered by endothelial-removal and by LPS treatment of the donor rat, the following experiments were performed. Main PA rings from sham and LPS-treated rats were contracted from a resting tension of 500mg by phenylephrine (PE: 1mM), relaxed by washing and left to equilibrate for 15 minutes. PE-induced contraction was repeated twice at a resting tension of 250 or 750mg, and then once more at 500mg. A 15 minute period of equilibration was permitted between changes in tension and each contraction. PE-induced contractions were compared at two resting tensions in the same PA rings and the results compared using a paired Student's t-test. Changing resting tension did not significantly alter contraction in any of the experimental groups **[Figure 2.3]**, thus justifying the continued use of the 500mg baseline tension in all ring groups.

2.5 ISOLATED HEART-LUNG EXPERIMENTS

2.5.1 Animals and anaesthesia

Male Wistar rats (275-300g) were treated with either lipopolysaccharide (LPS; 20mg/kg; intraperitoneal (*ip*)) or saline (3ml/kg) 4 hours prior to anaesthesia with *ip* pentobarbital sodium (Sagatal; 60mg/kg). Additional anaesthetic was given as required until there was complete absence of withdrawal response to foot pad compression.

2.5.2 Tissue preparation

Anaesthetised animals were placed on a plastic tray suspended over a water bath that was kept at 38°C and the trachea exposed through a median neck incision. They were ventilated via a tracheotomy with normoxic gas (21% O_2, 5% CO_2, balanced N_2) using a fixed volume small animal pump (Harvard, Kent, UK) with a tidal volume of 2.5ml and a frequency of 60/min. These parameters maintained the partial pressure of CO_2 in arterial blood ($PaCO_2$) within the physiological range. The animal was heparinised (30mg/kg) via a 21G needle in the aorta through which it was then exsanguinated, and the blood placed in a reservoir suspended in the heated water bath. LPS-treated rats were injected with heparin made up to a volume of 3ml with Krebs solution containing 4% bovine serum albumin (BSA) in order to compensate for smaller volume of blood obtained from this group during exsanguination. The lungs were exposed by careful bilateral thoracotomy followed by removal of the sternum. A purse string suture was placed around the left atrial appendage and used to secure a cannula

inserted through an atriotomy. This cannula drained freely into the reservoir, giving a measured left atrial pressure of zero. A second cannula was inserted into the main pulmonary artery via an incision in the right ventricular outflow tract, and secured in place by two silk ties running behind both the pulmonary trunk and ascending aorta. The perfusion circuit was established using silicone tubing (1.6mm internal diameter, Watson Marlow, Falmouth, Kent, UK) connecting the reservoir to the PA line via a roller pump (model 302S, Watson Marlow, Falmouth, Kent, UK). Also included in the circuit were a bubble trap, and a side-arm transducer (Bio Medical Systems Ltd., Strathclyde, UK) connected to a recorder (Multitrace 2, Ormed Ltd, Welwyn Garden City, Herts., UK), allowing continuous measurement of PA pressure (Ppa) **[Figure 2.4]**. The reservoir volume was topped up with Krebs solution containing 4% BSA in order to achieve a consistent circulating volume of 20ml. A flow rate of 20ml/min was employed in order to achieve starting Ppa within the range observed *in vivo*. Blood gas tensions were measured at the start of the experiment and the pH was adjusted into the physiological range by the addition of small volumes of 8.4% sodium bicarbonate.

The isolated, blood-perfused, ventilated *in situ* preparation of rat lungs in this way is a modification of the methods previously described (Messent et al. 1996).

2.5.3 Properties of the preparation

2.5.3.1 **Pressure-Flow relationship**

At the beginning of this set of experiments, the pump flow rate was calibrated, and then preliminary measurements were made to correlate the flow rate with the measured pressure. The results of these experiments are shown in **Figure 2.5**.

2.5.3.2 **Relationship of measured Ppa to PVR**

The pulmonary artery pressure (Ppa) was continuously monitored via the side-arm of the PA line. The transduced pressure trace was set to represent the mean of the pressure waveform. The pulmonary vascular resistance (PVR) is the difference between the inflow (ie. Ppa) and outflow (ie. left atrial pressure (LAP)) pressures divided by the pulmonary blood flow (I), which was 20ml/min:

$$PVR = (Ppa-LAP) / I$$

The LAP in this system was found to remain constant at 0mmHg. Thus changes in Ppa directly reflected changes in PVR.

2.5.3.3 **Differences between sham and LPS groups**

The starting Ppa was found to be consistently higher in the LPS-treated rats compared to the sham. In addition, it was established during preliminary studies using this model that the haemoglobin concentration in sham rats (n=41) was significantly higher than in the LPS group (n=32) (6.35±0.09 g/dl vs. 5.93±0.12 g/dl, respectively; $p = 0.007$). It was felt that this may be linked to the lower blood volumes obtained in the LPS rats compared to the sham, and this led to the

inclusion of an extra "cross-transfusion" experiment, described below, to validate the model.

2.5.4 Wet:dry lung weight ratio

Evidence of increased alveolar-capillary membrane permeability was sought by lung wet:dry weight ratio. At the end of experiments, the middle and lower lobes of the right lung were removed, blotted dry, and then weighed (wet weight). The samples were then freeze-dried (Edwards Micromodulyo, Edwards High Vacuum International, Crawley,UK) for 24 hours to a constant weight (dry weight).

2.5.5 Amplitude index

Further "on-line" assessment of changes in oedema accumulation in the lung was made by continuous measurement of the amplitude of movement of the lung during the ventilation cycle. A balanced arm, attached to an isometric force transducer, was rested gently on the surface of the right upper lobe so that at all times in the ventilation cycle it maintained contact. The transducer was connected to a recorder (Multitrace 2, Ormed Ltd, Welwyn Garden City, Herts., UK) so that a continuous recording of the amplitude of lung movement was obtained. Accumulation of significant amounts of oedema fluid reduced lung compliance, and this was reflected by reduced amplitude of movement of the side arm. The amplitude index was then taken as the amplitude at a particular Ppa expressed as a percentage of the starting amplitude.

2.6 MEASUREMENT OF SYSTEMIC AND PULMONARY ARTERY PRESSURES *IN VIVO*

2.6.1 Animals and anaesthesia

Male Wistar rats (300g) were used for all the experiments. Each animal was anaesthetised with an initial *ip* injection of pentobarbital sodium (Sagatal,60mg/kg), since in preliminary studies this agent was found to provide optimal anaesthesia with haemodynamic stability. Additional anaesthetic was given by further *ip* injection as required until full anaesthesia was achieved, as assessed by absence of withdrawal response to foot pad compression.

2.6.2 Preparation of the model

Four hours prior to anaesthesia, rats were treated with either LPS (20mg/kg ip) or saline vehicle (sham 3ml/kg). During the intervening period, they had free access to water and chow *ad libitum*. Animals were kept at 37°C using a heated blanket connected via a thermostat to a rectal probe (Harvard Homeothermic Blanket Control Unit; Harvard Apparatus Ltd., Edenbridge, Kent, UK). A tracheostomy was performed via a midline neck incision, to facilitate spontaneous respiration and the left femoral vein cannulated using heparinized polyethylene tubing (0.75mm external diameter, size 3FG, Portex Ltd, Hythe, UK) for intravenous drug administration. The left carotid artery was dissected free from the accompanying vagus nerve, and cannulated using heparinised polyethylene tubing (0.50mm internal diameter, 1.00mm external diameter; Portex Ltd, Hythe, UK) for measurement of mean systemic arterial pressure (MAP). The right external jugular

vein was cannulated using heparinised vinyl tubing (0.58mm internal diameter, 0.96mm external diameter; International Market Supply, Cheshire, UK) which had been precurved in boiling water in a modification of the method originally described by Po and Wenli (1984), and advanced through the right atrium, right ventricle and into the pulmonary artery, its position being determined by the monitored pressure waveform **[Figure 2.6].** Post mortem examination was also performed to confirm correct positioning in the first 10 animals. Pulmonary artery (Ppa) and systemic arterial (MAP) pressures were monitored by transducing the respective cannulae, zeroed at right atrial level, into a two channel pressure recorder (Marquette Electronics Series 7010 Monitor; Marquette Electronics Inc., Milwaukee, WI, USA) with continuous hard copy printout (Marquette Electronics Direct Digital Writer Series 7100). The patency of both cannulae was ensured by heparinized saline flush. After the pulmonary artery catheter was sited, 0.4mls of arterial blood was removed via the left carotid artery cannula for blood gas analysis.

An equilibration period of 15 minutes was allowed before any drug administration. Each drug was given as an intravenous bolus injection over 3-5 seconds via the femoral vein. Distilled water, in the same volumes, was used as the control because ET-1 and S6c were dissolved in this.

Mean arterial pressure values (MAP & Ppa) were obtained from the continuous printout of the actual pressures using the formula:

$$MP = DP + 1/3PP$$

where MP is mean arterial pressure, DP is diastolic pressure and PP is pulse pressure. Results are expressed as absolute changes in mean arterial pressure at 10 minutes from baseline values in mmHg.

2.6.3 Termination of the experiment

Once all haemodynamic data were obtained, the animals were given an *iv* overdose of Sagatal.

2.7 STATISTICAL ANALYSIS

Data are all expressed as mean ± standard error of the mean (SEM) of the given number of observations. To test the null hypothesis that no difference existed between the means of two sets of data, Student's *t* test was used for paired or unpaired data. The use of the *t* test was acceptable as long as the data were normally distributed and there was no significant difference in their standard deviations. Where data were normally distributed, but there was a difference between their standard deviations, an alternative (Welch) *t* test was used. A one way analysis of variance (ANOVA) followed by Tukey-Kramer post tests for multiple comparisons was used for stepward comparison of several groups' non-parametric data. This reduces the likelihood of finding a significant difference by chance, which is otherwise higher with multiple group comparisons.

p values of less than 0.05 were considered as significant for all tests.

2.8 DRUGS AND CHEMICALS

The following drugs, diluted and stored according to the manufacturer's recommendations were used: acetylcholine chloride, angiotensin II, bovine serum albumin, indomethacin, lipopolysaccharide from *Salmonella enteritidis* (code number L6011), L-phenylephrine hydrochloride, N^{w}-nitro-L-arginine-methylester, N^{G}-monomethyl-L-arginine acetate, sodium nitroprusside, from Sigma, Poole, Dorset, UK; bosentan was a gift from M.Clozel, Hoffman La-Roche, Basel, Switzerland; BQ123 from Scientific Marketing Associates, Barnet, Herts, UK; BQ788 was a gift from Ciba-Geigy Ltd., Takarazuka, Japan; dexamethasone from David Bull, Warwick, UK; ET-1 (human, porcine) from Novabiochem, Calbiochem-Novabiochem UK, Ltd, Nottingham, UK; ethylene-diamine-tetra-acetic acid from BDH Chemicals Ltd, Dagenham, Essex, UK; hypnorm (fentanyl 0.315 mg/ml and fluanisone 10 mg/ml) from Janssen, Wantage, UK; ICI 192605 was a gift from Zeneca Pharmaceuticals, Cheshire, UK; Parameter Elisa kit was a gift from R&D Systems Europe, Abingdon, UK; RNases A and T1 from Boehringer Manheim, Lewis, UK; RNAzol B from Biotecx Laboratories Inc, Biogenesis Ltd, Poole, UK; Sagatal from Rhone Merieux, Harlow, Essex, UK; sarafotoxin 6c from Bachem California, CA, USA; sodium heparin from Leo Laboratories Ltd, Princes Risborough, UK; [α^{32} P]GTP (specific activity 410 Ci/mmol), SP6 DNA-dependent RNA polymerase from Amersham International, Amersham, UK; U46619 from Cascade Biochem Ltd, Reading, UK.

Figure 2.1 **Principles of the RNase protection assay**

Abbreviations: bp base pairs
nt nucleotides

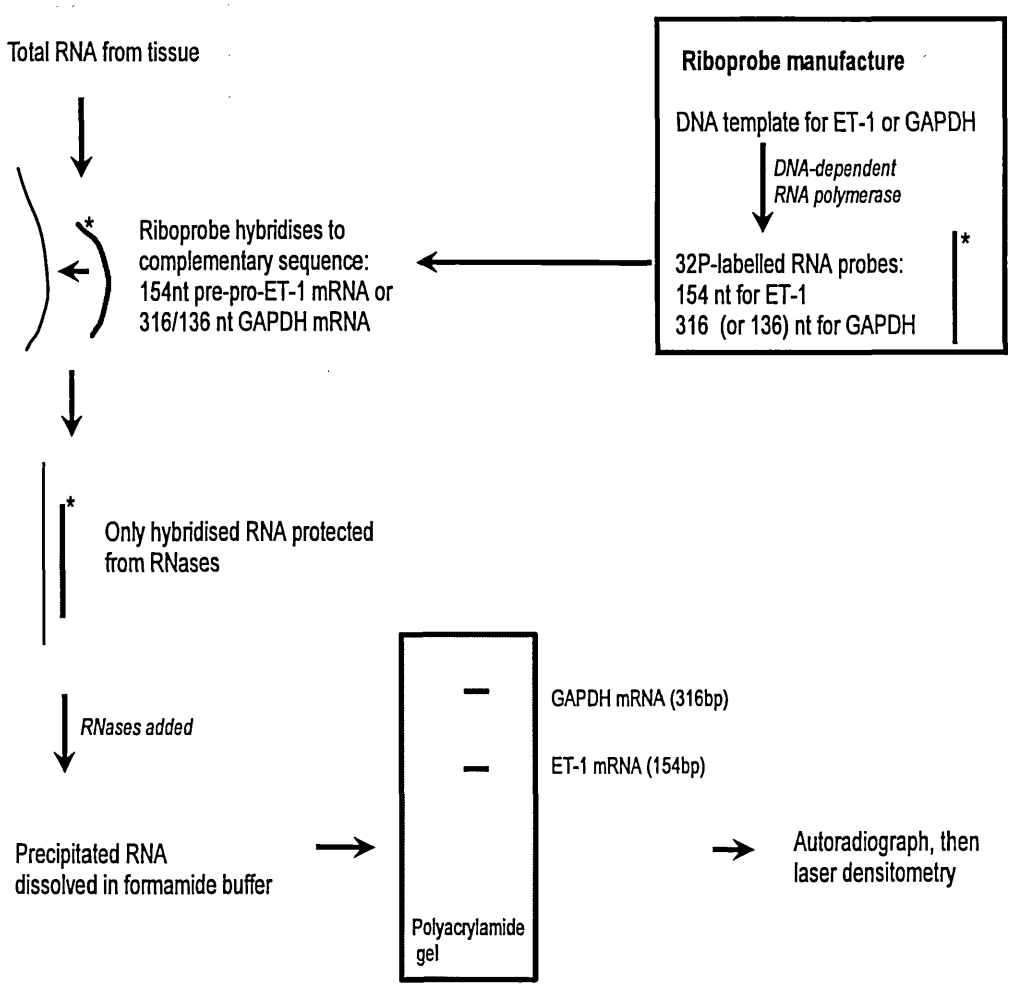


Figure 2.2 **Equipment used in the organ bath studies**

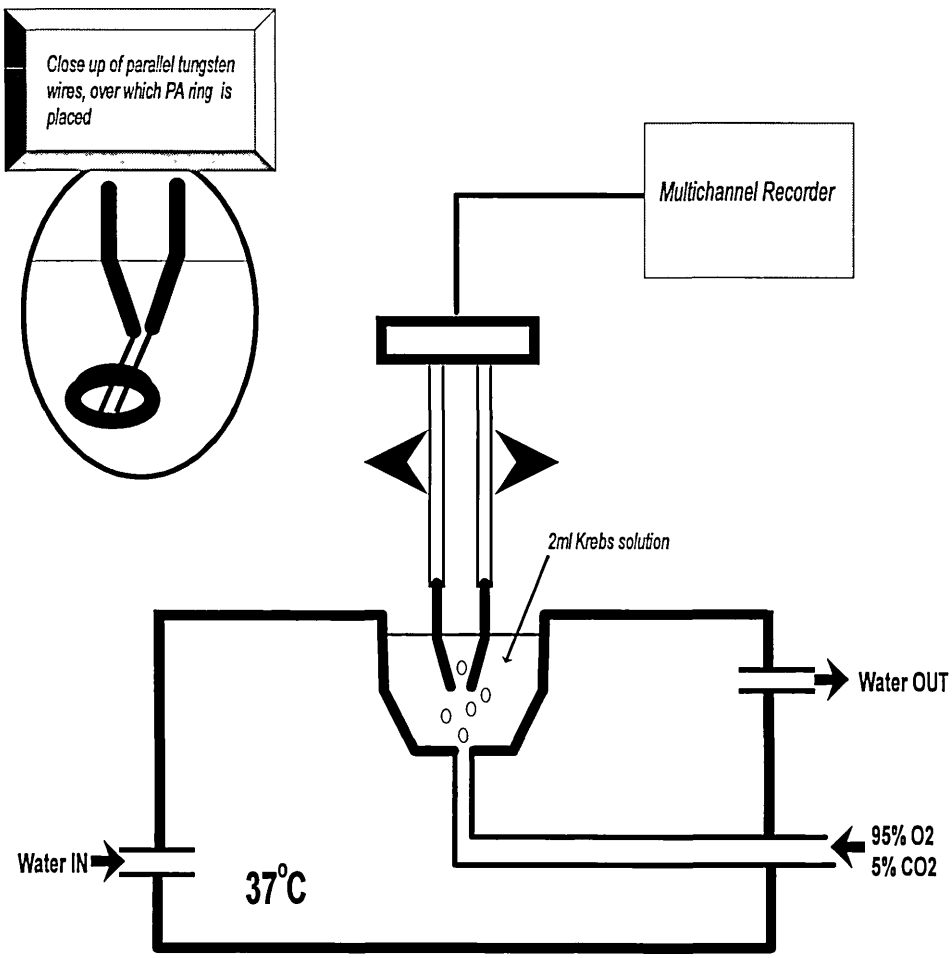


Figure 2.3 **Length-tension relationships in pulmonary artery rings from sham and LPS-treated rats**

The contraction of PA rings (n=8 in each group) was assessed to 10^{-5}M phenylephrine first at baseline tension of 500mg, then followed by a "test" tension of either 250mg or 750mg, after 20min equilibration. Open bars are intact rings, shaded bars are endothelium-denuded. Groups labelled **(a)** are sham, **(b)** are LPS-treated. No significant difference is seen, thus justifying the baseline tension of 500mg throughout.

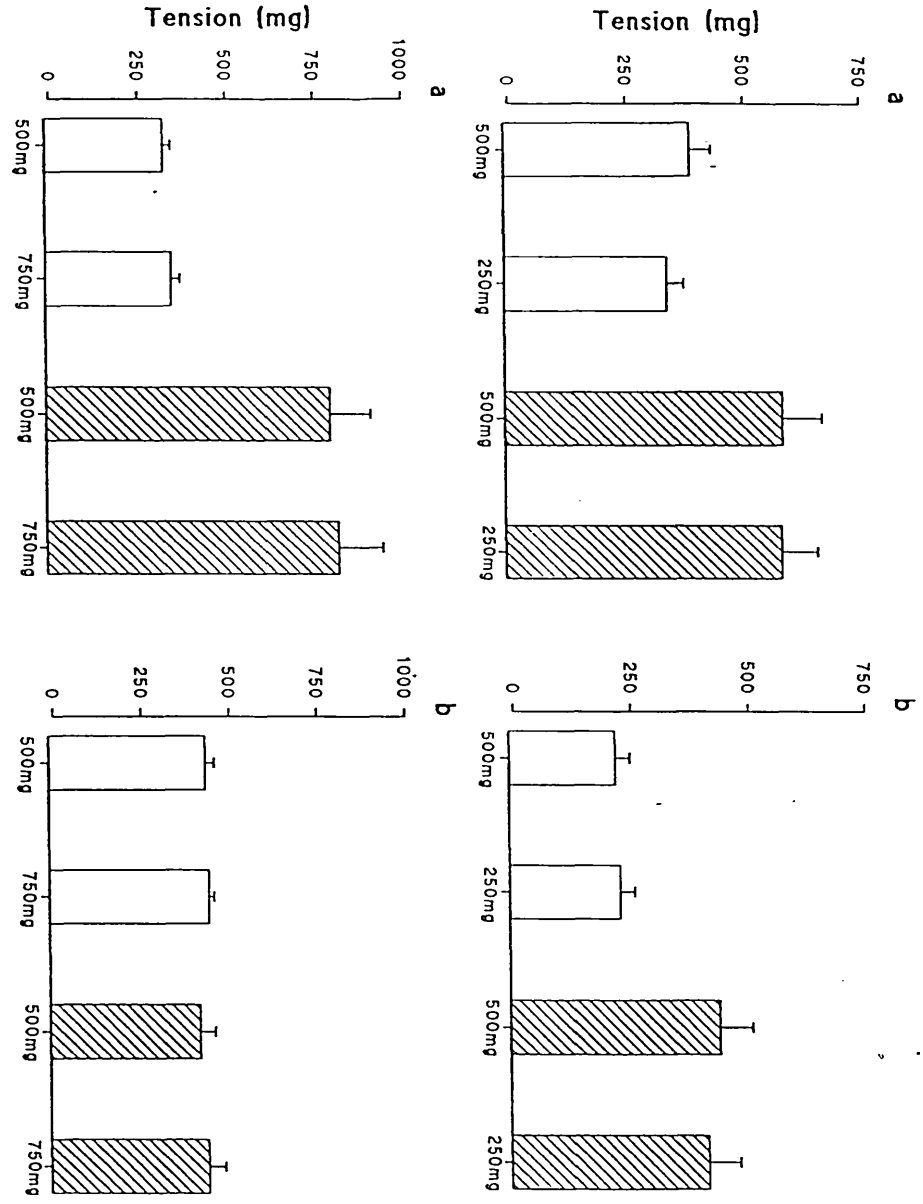
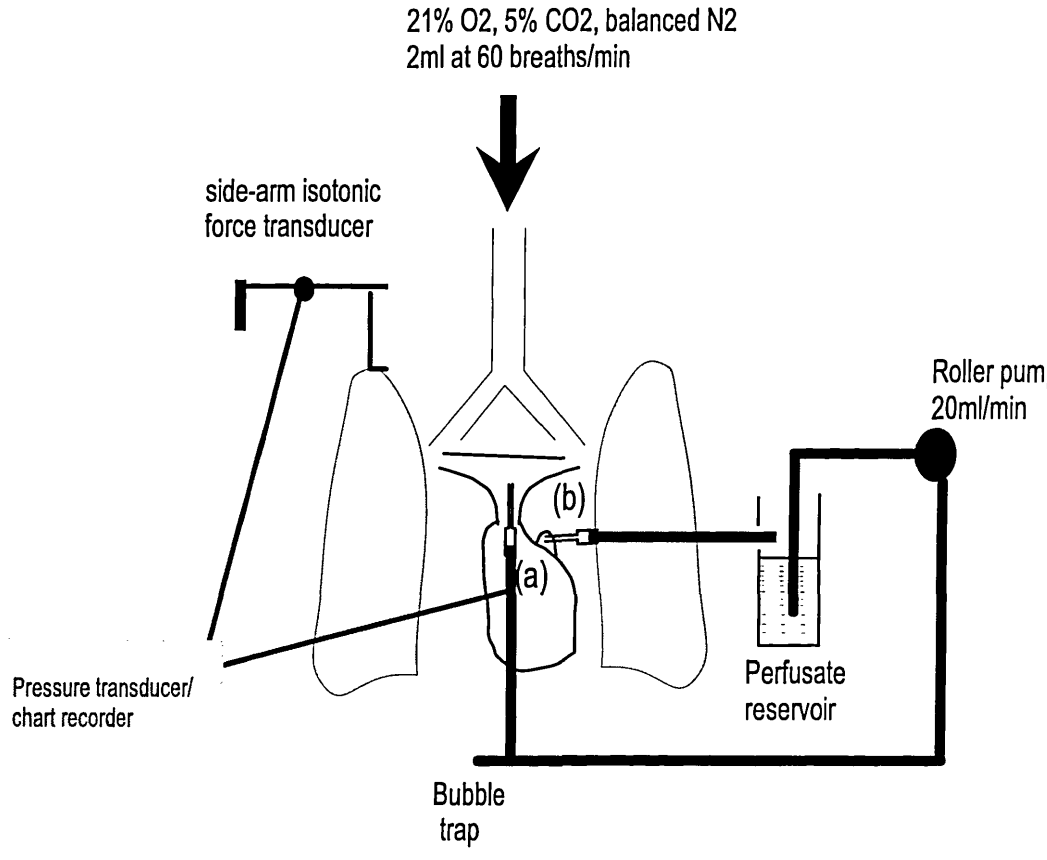


Figure 2.4 **The isolated, blood-perfused lung circuit**



(a) line inserted into main PA via RVOT
(b) line inserted into left atrial appendage

Figure 2.5 **Pressure-flow relationship of the IBPL**

The pump flow rate was first calibrated, and then the relationship between these flow rates and the resultant pulmonary artery pressure (Ppa) was measured. Results are expressed as mean ± SEM for 11 observations.

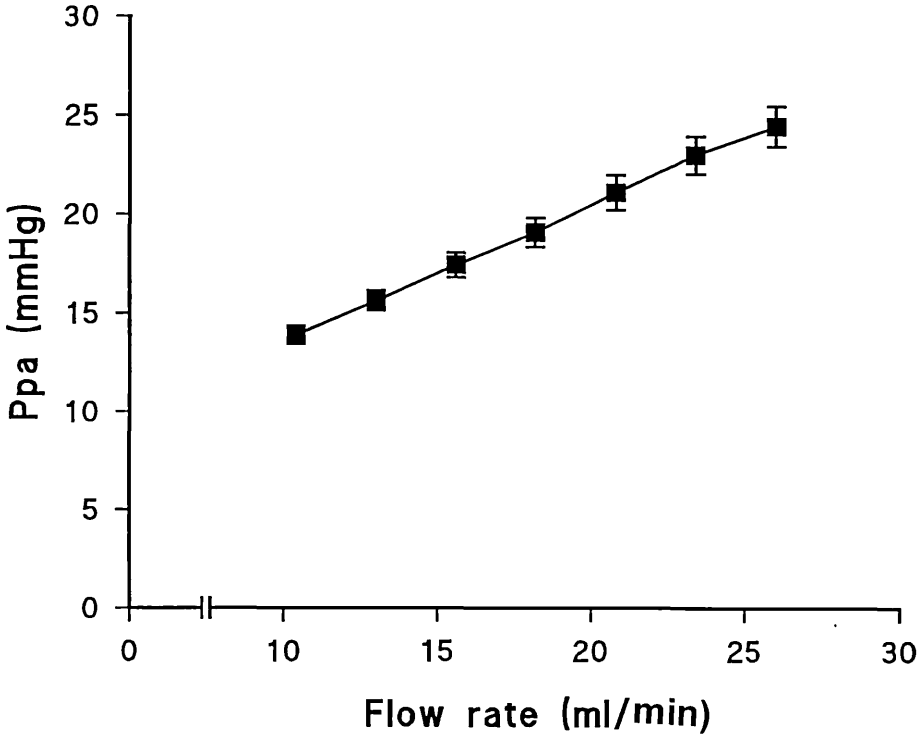
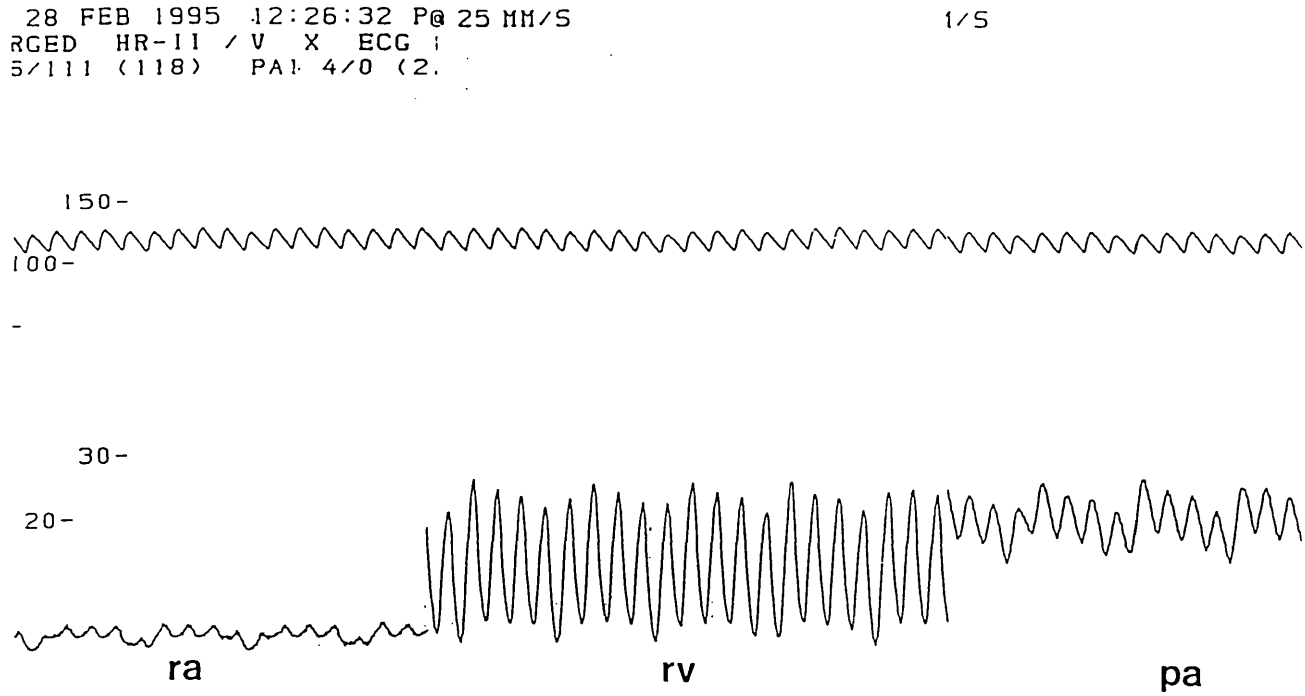


Figure 2.6 **Characteristic pressure waveform in passage through the right heart**

Right atrium (**ra**)
Right ventricle (**rv**)
Pulmonary artery (**pa**).



CHAPTER 3

EVIDENCE THAT INTRAPERITONEAL LPS TREATMENT IS A MODEL OF SEPTIC INFLAMMATION IN THE RAT AND STIMULATES PRODUCTION OF ET-1

3.1 INTRODUCTION

The administration of LPS by intraperitoneal (*ip*) injection at a dose of 20mg/kg has been used for many years by this and other groups in order to induce endotoxaemia and thereby an inflammatory septic response. Using this method, but with a smaller dose of LPS (2mg/kg, *ip*), it was shown that there is recruitment of neutrophils into the interstitial space of the lung (Rinaldo et al. 1984). Using the same dose of 20mg/kg, *ip,* it has been established that there is, by 4 hours, an overproduction of nitric oxide by iNOS in the aorta (Julou-Schaeffer et al. 1990) and pulmonary artery (Griffiths et al. 1995a). It has also been clearly demonstrated that there is a large increase in expression of iNOS mRNA in lung, liver, spleen, skeletal muscle (Liu et al. 1993) and pulmonary artery (Griffiths et al. 1995a) at 4 hours in this model. In addition, it has been established that plasma [ET-1] is elevated in several animal models of sepsis, as well as in patients who are critically ill (section 1.4.2). These data are not unexpected in view of the accumulated evidence that ET-1 release occurs in response to endotoxin and cytokines in endothelial cell culture (Golden et al. 1995; Kanse et al. 1991a). It has also been shown that ET-1 mRNA is detectable in all tissues assessed in untreated rats (Firth and Ratcliffe, 1992). The last experiments were performed using RNase protection assay, which is at least 10

times more sensitive than Northern analysis (Melton et al. 1984), and allows the simultaneous probing of samples for multiple RNA species. This group developed a riboprobe for pre-pro-ET-1 mRNA that does not cross react with those for ET-2 or -3.

The aim of these experiments was firstly to establish that *ip* LPS in this model produces inflammation in the lungs of rats, and a rise in circulating NO metabolites, thus validating the model as one of endotoxin-induced septic inflammation. More specifically, an increase in ET-1 mRNA expression was sought in heart, lung, kidney, skeletal muscle, and particularly in the vasculature (thoracic aorta and main pulmonary artery) in animals treated with LPS, and if present, a correlation was determined between the molecular up-regulation and plasma [ET-1].

3.2 EXPERIMENTAL PROTOCOLS

3.2.1 To demonstrate a pulmonary inflammatory response

Rats were either treated with *ip* LPS (20mg/kg) or saline, and 4 hours later their lungs prepared for histological examination, using both light and electron microscopy.

3.2.2 Measurement of plasma nitrate and nitrite levels

Using the assay technique described (Chapter 2.2), levels of nitrite were measured in rats that were either untreated or treated with LPS (20mg/kg, *ip*)

0.5, 1, 2, 4 or 6 hours before sacrifice, during which time they had free access to water and chow.

3.2.3 To investigate the effects of LPS treatment on tissue expression of pre-pro-ET-1 mRNA

3.2.3.1 **Tissue expression (Heart, lung, skeletal muscle and kidney)**

Twenty seven rats were divided into 3 experimental groups:

(a) Rats were treated with LPS (20mg/kg, *ip*) or (b) saline (3ml/kg, *ip*) either 1 or 6 hours prior to sacrifice. In group (c) dexamethasone (3mg/kg, *ip*) was given 0.5 hour prior to the LPS. Sample autoradiographs are shown, and then results are expressed as the mean and SEM for each group (n=3).

3.2.3.2 **Vascular expression (Pulmonary artery and aorta)**

In a separate experiment, the same treatment groups (a), (b) and (c) were used, and examined at 1 and 6 hour time points. However, these experiments were performed, as described, with a riboprobe protecting 136nt of pre-pro-ET-1 mRNA, rather than 316nt as was the case for the other tissues.

3.2.4 To investigate the effects of LPS treatment on arterial plasma concentration of ET-1

Using the assay technique described in Chapter 2.3, ET-1 levels were measured in rats that were either untreated or treated with LPS (20mg/kg, *ip*) 0.5, 1, 2, 4 or 6 hours before sacrifice, during which time they had free access to water and chow.

3.3 RESULTS

3.3.1 Effects of LPS treatment on pulmonary histology

Lung from LPS-treated rats contained significantly more neutrophils per microscope field than sham rats (15.9±0.6 and 2.8±0.3 neutrophils, respectively for 30 representative fields; p <0.0001) **[Figure 3.1a & b]**. This increase in neutrophil population was confined to the interstitium, with neither inflammatory cells, nor oedema fluid being seen in the air spaces in either group. Electron microscopy confirmed the presence of neutrophils in sections from the LPS rats, but did not show structural damage to the vascular endothelium **[Figure 3.2]**.

3.3.2 Time course for nitrate and nitrite levels in the blood following LPS treatment

Nitrite plasma concentrations were significantly greater than control levels only in the 4h and 6h groups **[Figure 3.3a]**.

3.3.3 Time course for the expression of pre-pro-ET-1 mRNA following LPS treatment

3.3.3.1 Heart, lung, skeletal muscle and kidney

In the control group there were tissue-specific differences in the expression of pre-pro-ET-1 mRNA, the highest level of expression being in the lung, and the

lowest in skeletal muscle **[Figure 3.4]**. By contrast, the expression of GAPDH mRNA was greatest in skeletal muscle, and lowest in kidney **[Figure 3.4]**.

No differences in expression of either type of mRNA were seen between control or LPS groups at 1 hour **[Figure 3.5a]**. At 6 hours, however, the pre-pro-ET-1 mRNA: GAPDH mRNA ratio was significantly elevated in both heart (0.36±0.01 vs. 0.20±0.02 for endotoxin vs. control, respectively; n=3; p <0.05) and in lung (3.03±0.20 vs. 1.73±0.05 for endotoxin vs. control, respectively; n=3; p <0.05), but not in kidney or skeletal muscle **[Figure 3.5b]**.

Dexamethasone pretreatment only caused a significant attenuation in pre-pro-ET-1 mRNA:GAPDH mRNA ratio expression compared to the endotoxin group in the heart at 6 hours (0.29±0.01 vs. 0.36±0.01 for dexamethasone vs. endotoxin, respectively; n=3; p <0.05). There was, however, a non-significant trend for an attenuation by dexamethasone pretreatment in heart at 1 hour, as well as in lung and kidney at 6 hours.

3.3.3.2 **Pulmonary artery and aorta**

There was an increase in pre-pro-ET-1 mRNA expression, as assessed by the pre-pro-ET-1 mRNA:GAPDH mRNA ratio, in pulmonary artery and aorta at both 1 and 6 hours **[Figures 3.6 & 3.7]**, achieving statistical significance at 1 hour for pulmonary artery, and at both 1 and 6 hours in aorta. Dexamethasone pretreatment did not significantly suppress this increase in either vessel type.

3.3.4 Time course for the rise in arterial plasma [ET-1] in response to LPS treatment

Untreated animals had a mean arterial ET-1 concentration of 0.79±0.31 pg/ml. ET-1 concentration increased progressively from 0.5 to 6 hours post LPS injection ($r = 0.87$; $p < 0.0001$) **[Figure 3.3b]**, and had not reached a plateau by 6h.

3.4 DISCUSSION

Administration of *ip* LPS (20mg/kg) to rats caused a marked infiltration of neutrophils into the interstitial space of the lung, a histological finding previously described (Rinaldo et al. 1984). In itself, this confirms that a significant inflammatory reaction is underway in the lungs at the time of histological assessment (4 hours). In addition, plasma levels of NO metabolites were significantly elevated only from 4h post LPS, in line with previous molecular data (Liu et al. 1993; Griffiths et al. 1995a). The lack of any structural damage to endothelial cells in the pulmonary circulation was unexpected, since it is known that in other experimental models, endotoxin and cytokines can cause ultrastructural changes in these cells, which are thought to underlie the increased microvascular permeability that characterises clinical and experimental sepsis. However, the current hypothesis regarding the role of the endothelium in the vascular inflammatory response to sepsis (Curzen et al. 1994) incorporates endothelial dysfunction at an earlier stage than structural damage, and evidence was later sought to support this hypothesis (*vide infra*).

Using RNase protection assays, differential tissue expression of pre-pro-ET-1 mRNA in rat endotoxaemia was demonstrated in this model, with significantly increased expression in lung and heart 6h post LPS, but with no increase in skeletal muscle or kidney. None of the first group of tissues showed significant elevation in pre-pro-ET-1 mRNA expression at 1 hour. By contrast, in the study on vascular tissue, an identical model was used to demonstrate that the expression of pre-pro-ET-1 mRNA in pulmonary artery and aorta is significantly increased 1h after *ip* LPS. Interestingly, whilst the elevation in pre-pro-ET-1 mRNA was only significant in pulmonary artery after 1h, the ratio of pre-

pro-ET-1 mRNA:GAPDH mRNA was significantly increased at both 1 and 6 hours in aorta. It is therefore possible that the maximum response of pulmonary artery to endotoxin occurs earlier than in aorta, heart and lung. Dexamethasone pretreatment did not lead to suppression of the increase in vascular pre-pro-ET-1 mRNA expression, although it did suppress the increased ET-1 message in heart at 6h. These data suggest that steroid pretreatment does not selectively influence ET-1 production at the transcription phase. This is in marked contrast to previous findings of vascular and tissue expression of iNOS mRNA following treatment of rats by *ip* LPS (Liu et al. 1993; Griffiths et al. 1995a), which demonstrated that following dexamethasone treatment there was complete attenuation of the increase in iNOS mRNA in both pulmonary artery and aorta, as well as in all other tissues studied, using the same protocol. Therefore, the data on tissue and vascular pre-pro-ET-1 mRNA expression from these experiments do not support the hypothesis that transcription for this peptide is a steroid-sensitive process, similar to that of iNOS. It is more likely that the small effect that was seen in response to dexamethasone was non-specific and related to its other anti-inflammatory properties. It is conceivable, however, that dexamethasone could have had a more profound effect on expression of pre-pro-ET-1 mRNA if it had been given more than 30 minutes prior to the LPS injection. In fact, dexamethasone and cortisol have actually been shown to induce ET-1 release from cultured vascular smooth muscle cells of both rat and rabbit aorta, although no such response was observed in cultured endothelial cells (Kanse et al. 1991b). It is therefore possible that the increase in ET-1 mRNA expression in some dexamethasone groups may reflect a genuine increase in ET-1 production.

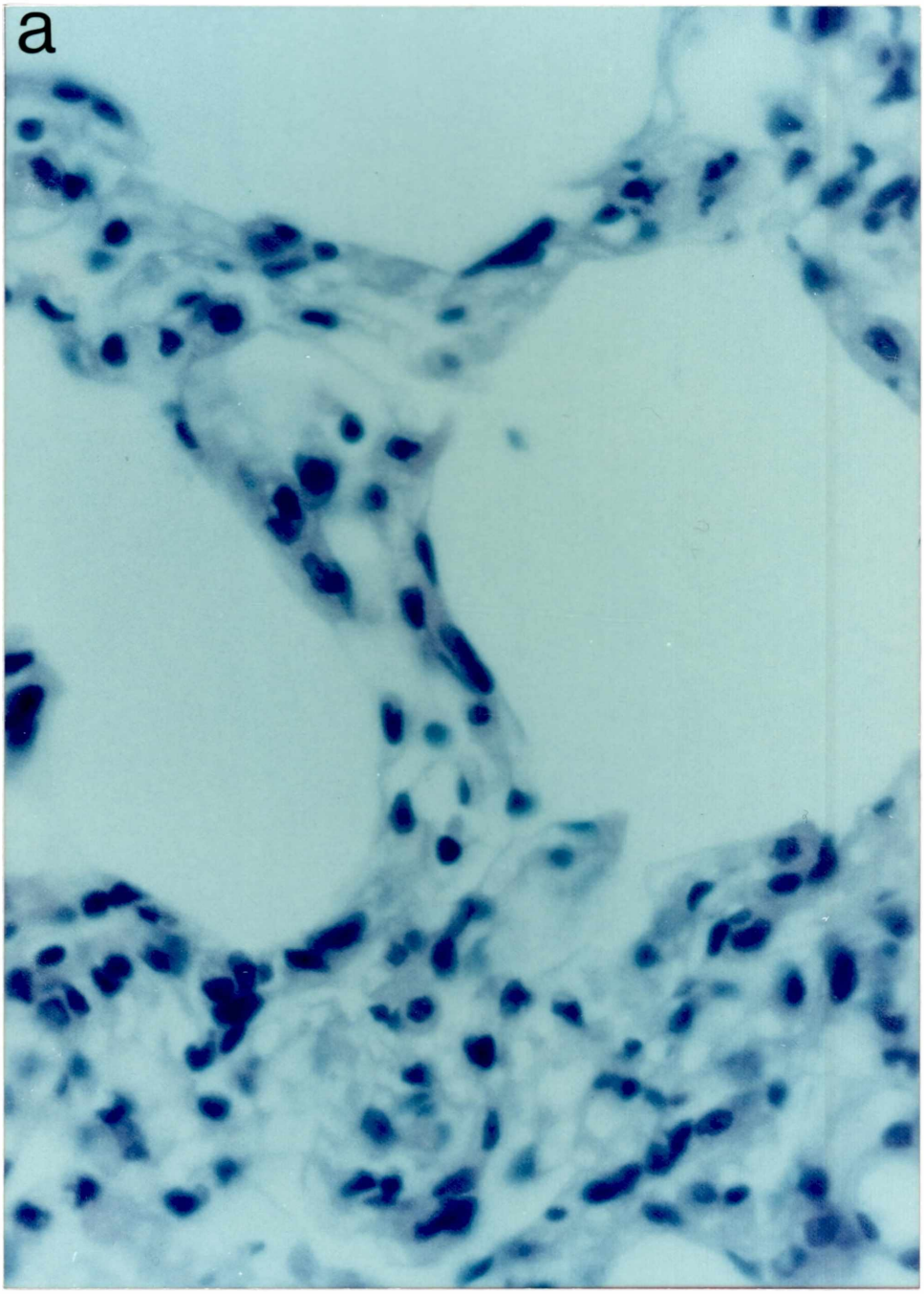
The methods employed do not permit confirmation of the precise origin of the pre-pro-ET-1 mRNA, since ET-1 production has been demonstrated both in endothelium and in vascular smooth muscle (Kanse et al. 1991c) in the rat. Specifically, it has been shown recently that expression of pre-pro-ET-1 mRNA in cultured rat pulmonary artery endothelial cells is elevated within one hour of exposure to LPS or IL1-α or 1-β, or TNFα (Golden et al. 1995). In these experiments, ET-1 mRNA expression was still significantly elevated 6 hours post LPS treatment and [ET-1] in arterial plasma had not yet reached a plateau at this timepoint, but this may reflect the self-amplifying nature of the inflammatory response to this endotoxic insult *in vivo*. It was particularly interesting to find that plasma ET-1 concentration had already begun to rise by 30 minutes post LPS (although the increase did not achieve statistical significance compared to control levels until 4 hour post LPS), because this pattern of change in ET-1 concentration is consistent with data from the experiments on vascular mRNA expression. Clearly, the rate with which ET-1 is produced and released into the circulation is dependent upon the way in which it is stimulated. Thus, plasma levels of ET-1 have been shown previously to peak within an hour of challenge with an intravenous bolus of TNF (Klemm et al. 1995), but the process is known to be slower when the primary stimulant is LPS (Hohlfeld et al. 1995), even when given intravenously. There remains doubt, however, about the origin of this early increase in plasma ET-1, since the posterior pituitary appears to be the only tissue capable of storing ET-1.

These experiments confirm that this model produces an acute inflammatory response in the lung, and stimulates production of both NO and ET-1. In particular, they provide evidence for an increase in tissue and vascular pre-pro-ET-1 mRNA expression, and a concurrent steady increase in arterial plasma

[ET-1]. The data suggest that vascular ET-1 production occurs earlier in response to this LPS challenge than in the other tissues studied. The model is therefore suitable for the assessment of responses of the pulmonary vasculature to endogenous and exogenous ET-1 under endotoxaemic conditions.

Figure 3.1 **Effects of LPS treatment on pulmonary histology: light microscopy**

One high power (X100) field showing a haemotoxylin and eosin stain of rat lung 4 hours after *ip* injection of (**a**) saline or (**b**; over page) LPS. Many neutrophils (marked **n**) are seen in the interstitial space of the LPS-treated rat lung.



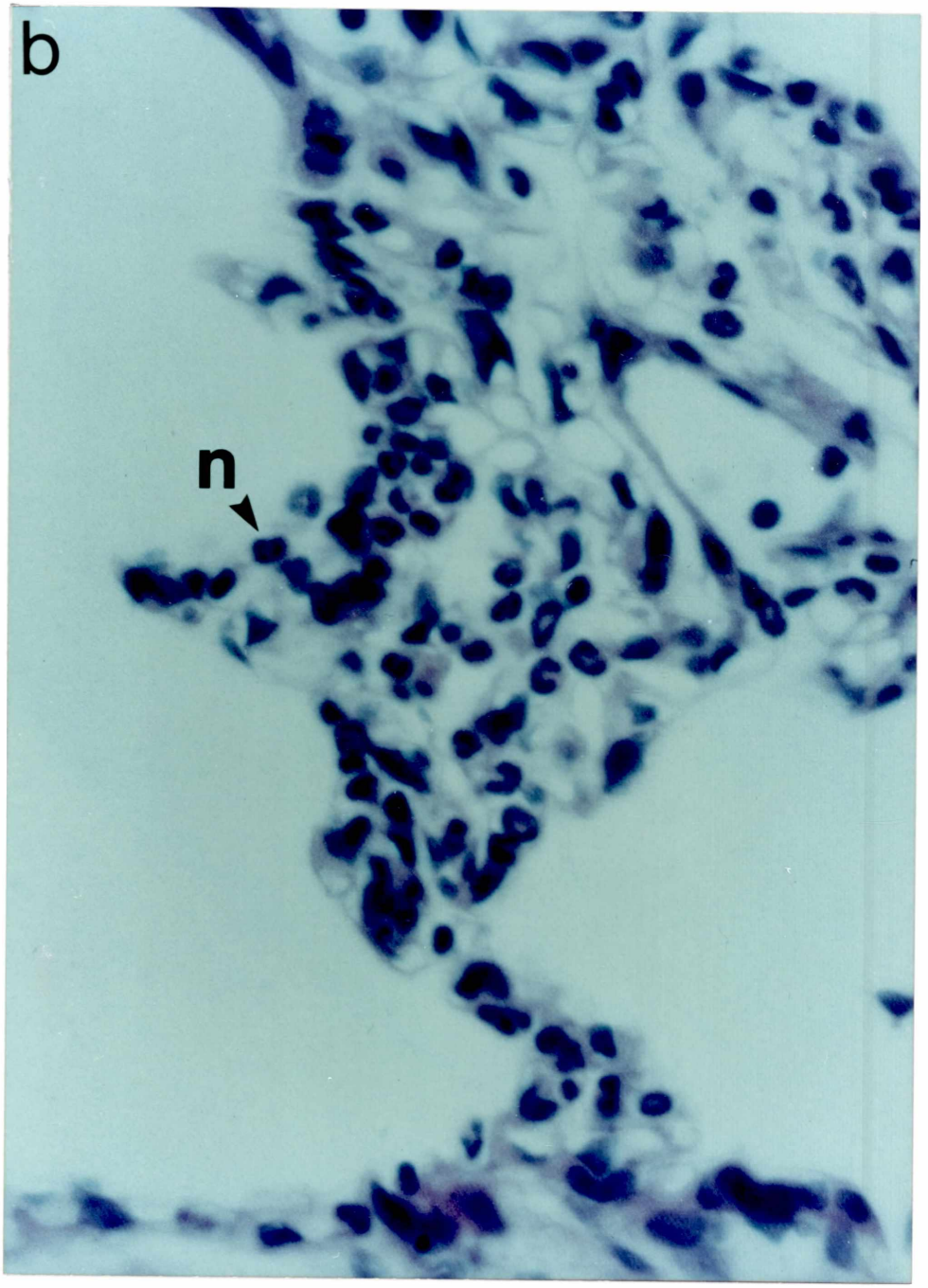

b
n

Figure 3.2 **Effects of LPS treatment on pulmonary histology: electron microscopy**

Electron micrograph of lung from a rat treated with LPS 4h prior to sacrifice. Neutrophils (**n**) are present in the capillary lumen, alveolar air spaces and interstitium. However, there is no evidence of structural disruption of the endothelial cell (**arrow**). Magnification is X6642; bar=3μm.

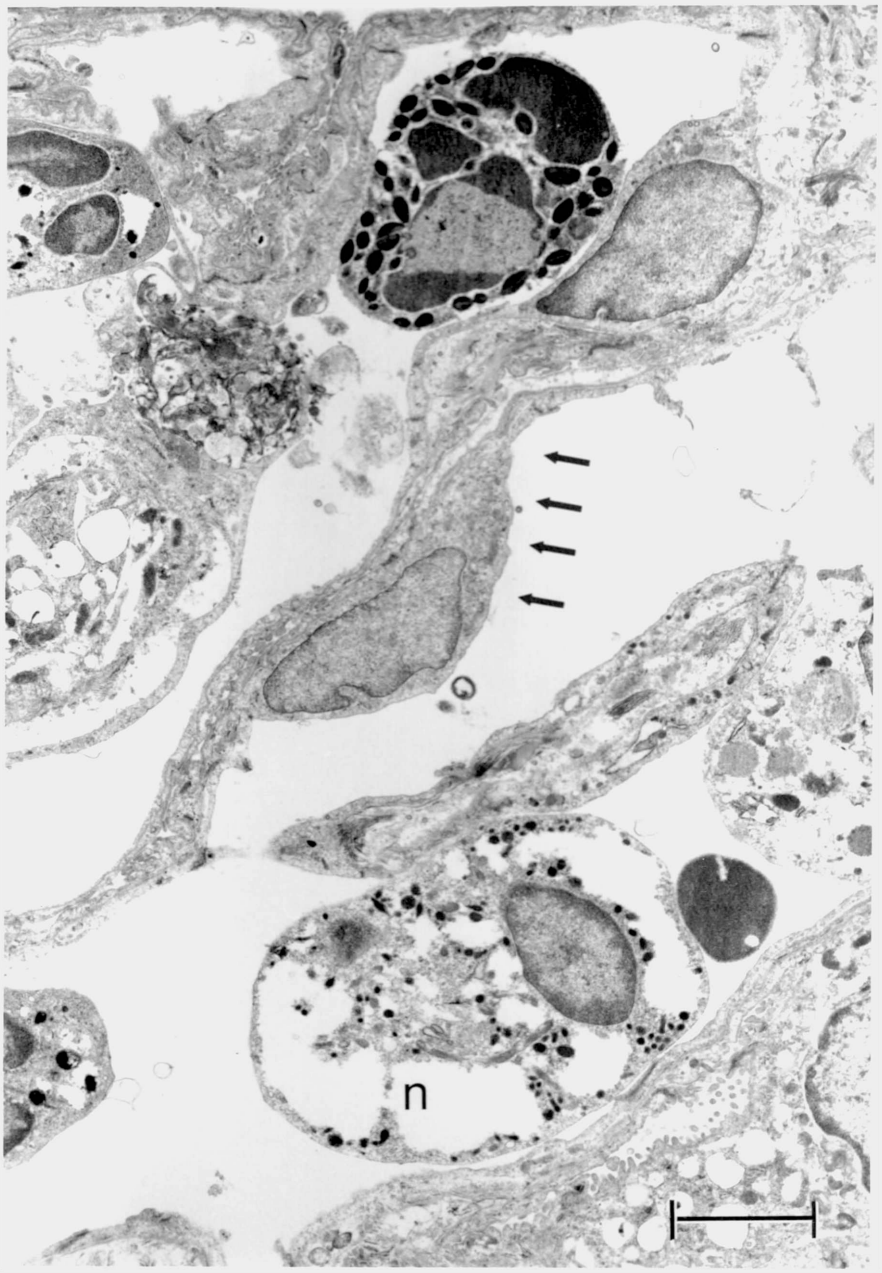
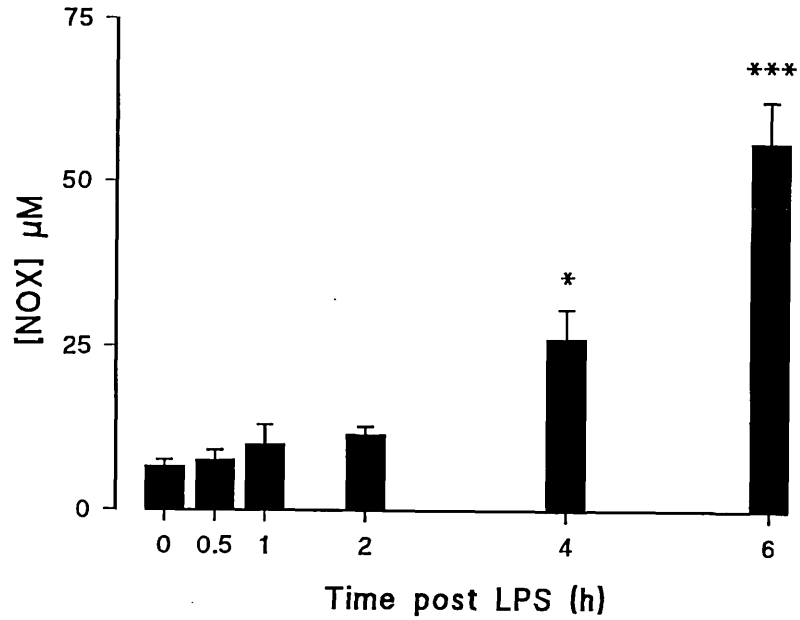


Figure 3.3 **Time course for arterial plasma levels of nitrite and endothelin-1 after LPS treatment**

Levels of nitrite (NOX) and endothelin-1 (ET-1) were measured in the arterial plasma of rats at various time points after *ip* LPS (20mg/kg). At each time point n=3 for nitrite and n≥4 for ET-1. Results are shown as mean±SEM, and statistical analysis was performed by ANOVA with Tukey-Kramer post tests. *p* values vs. control: * <0.05; ** <0.01; *** <0.001.



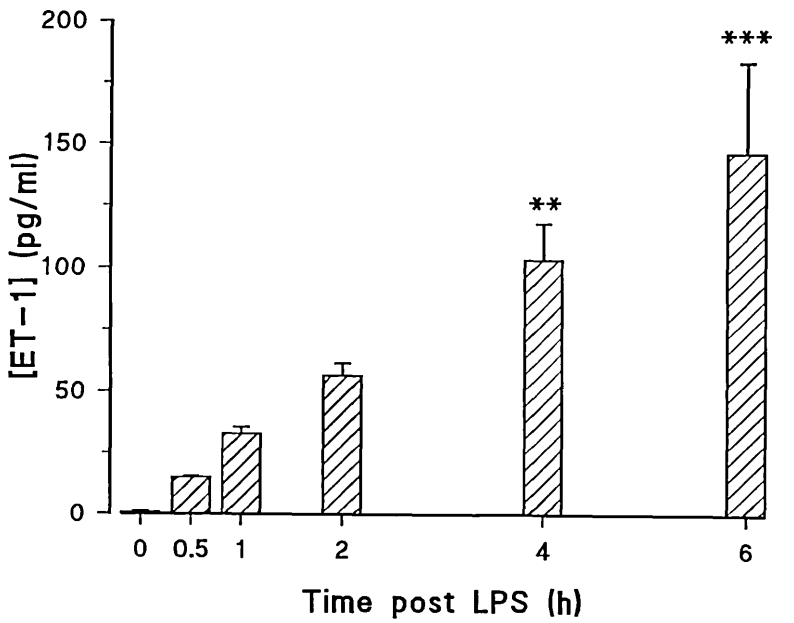


Figure 3.4 **Autoradiograph showing ET-1 mRNA and GAPDH mRNA expression in tissues**

For legend see opposite page.

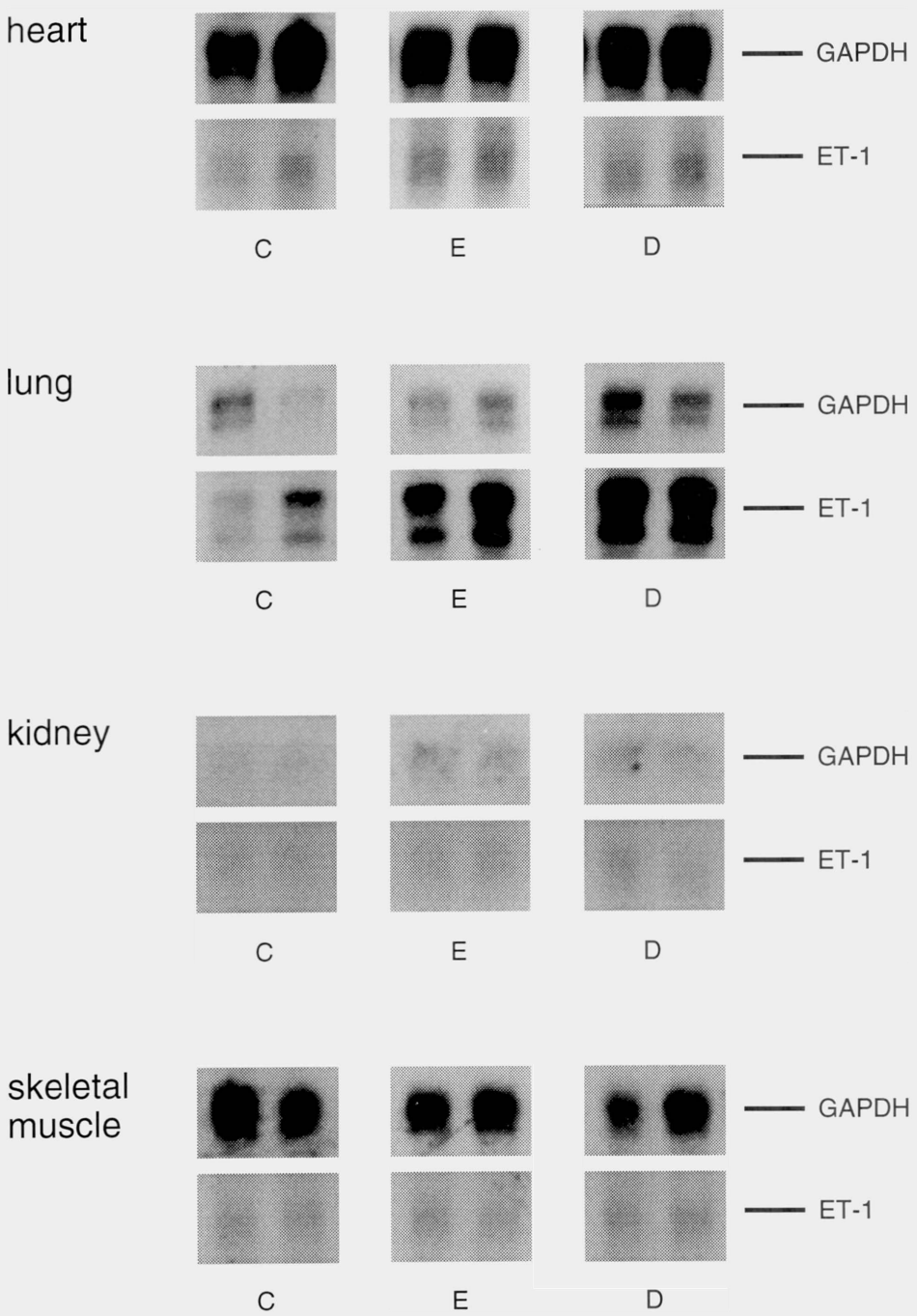
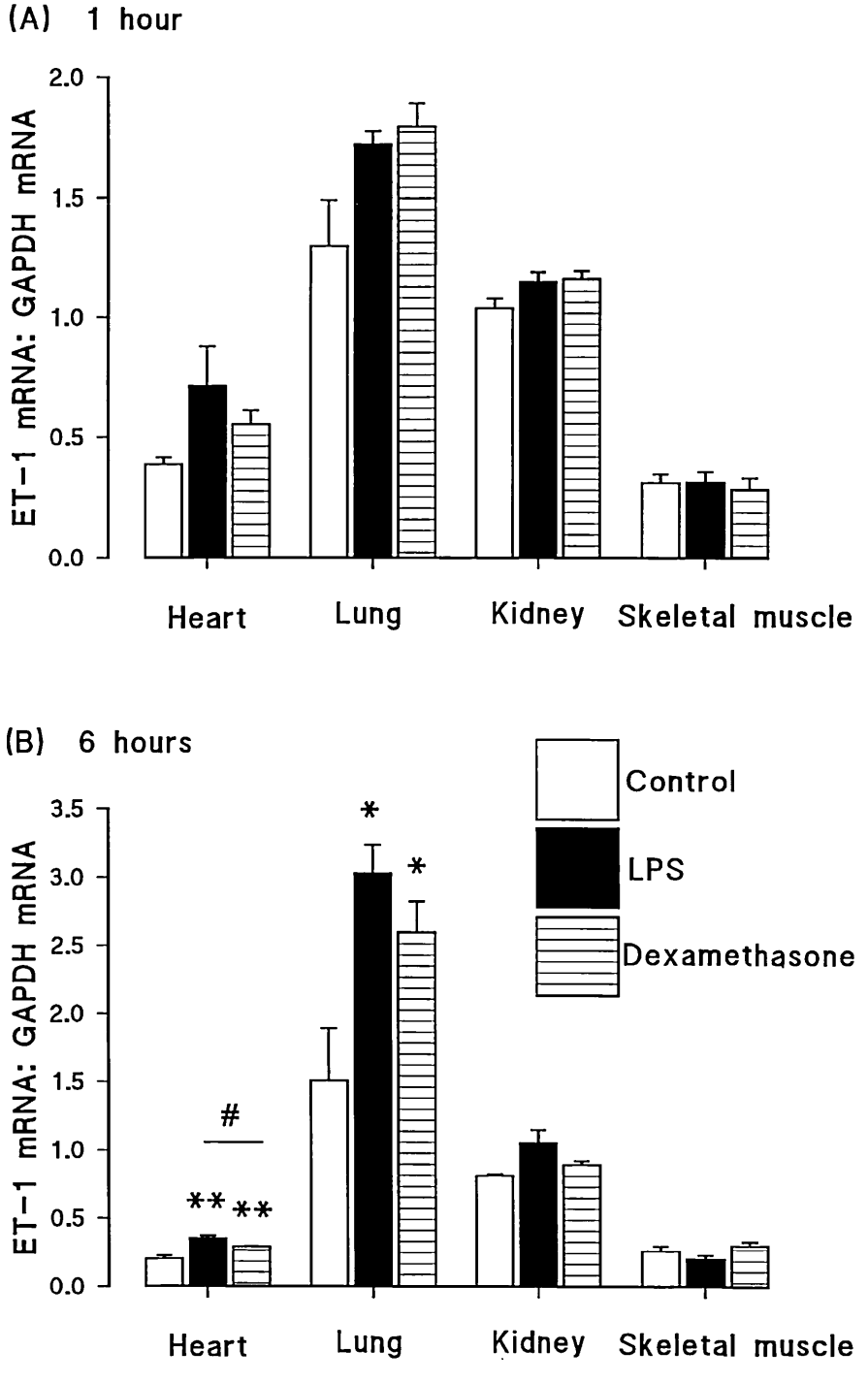


Figure 3.5 **Tissue ET-1 mRNA: GAPDH mRNA ratios after LPS**

Bar graphs show changes in the tissue expression of pre-pro-ET-1 mRNA at 1 or 6 hours in controls or following endotoxin/LPS, with or without dexamethasone pretreatment. Results are as mean±SEM for n=3 in each group. *p* values *vs.* controls: * <0.05; ** <0.01. *p* value for dex *vs.* LPS: # <0.05.

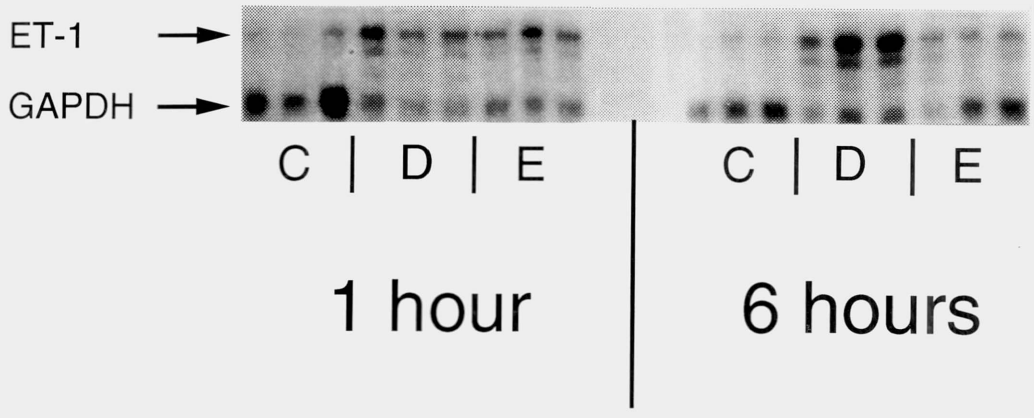


Representative autoradiographs of RNase protection assays showing expression of pre-pro-ET-1 mRNA and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNA in control vessels (**C**), and the effects of endotoxin/LPS (**E**) and dexamethasone plus LPS (**D**) pretreatment at 1 and 6 hours. Figure (**A**) is aorta, (**B**) pulmonary artery. Protected mRNA fragments were 154 and 136 base pairs for pre-pro-ET-1 mRNA for GAPDH mRNA respectively. Three lanes are shown for each group, and each lane represents the mRNA from single rat.

Figure 3.6 **Autoradiograph showing ET-1 mRNA and GAPDH mRNA expression in PA and aorta**

(legend on opposite page)

A



B

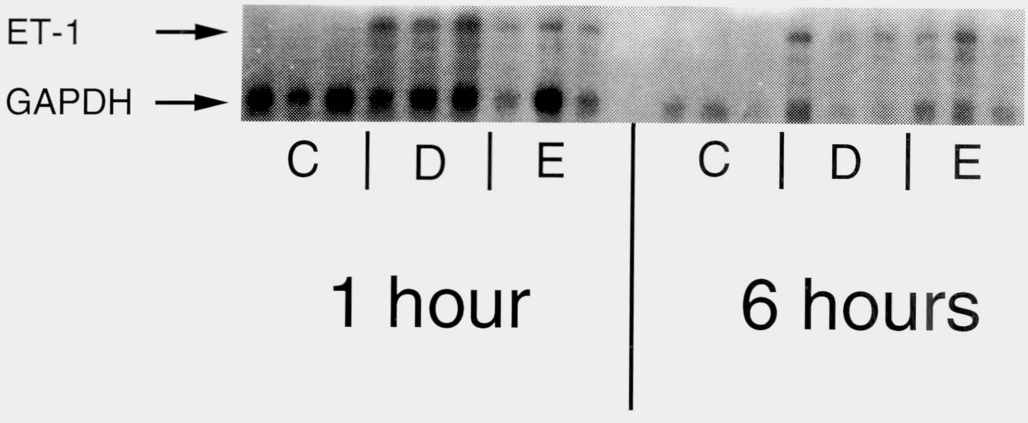
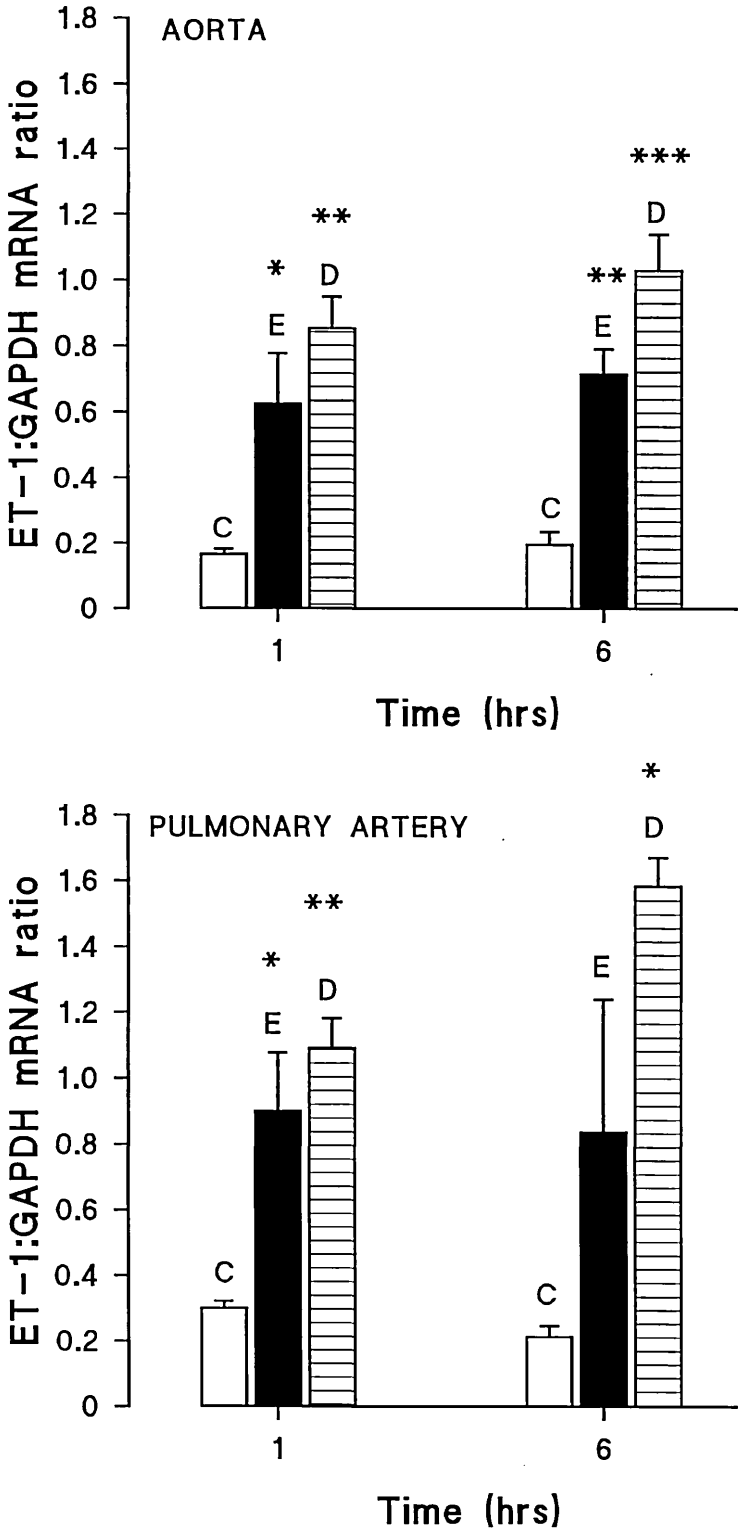


Figure 3.7 **Vascular ET-1 mRNA: GAPDH mRNA ratios after LPS**

Bar graphs show changes in the vascular expression of pre-pro-ET-1 mRNA at 1 or 6 hours in controls or following endotoxin/LPS, with or without dexamethasone pretreatment. Results are as mean±SEM for n=3 in each group. *p* values *vs.* controls: * <0.05; ** <0.01; *** <0.001.



CHAPTER 4

CHARACTERISATION OF CONTRACTILE RESPONSES TO ET-1 IN ISOLATED PULMONARY ARTERY FROM SHAM OR LPS-TREATED RATS

4.1 INTRODUCTION

A characteristic feature of acute lung injury is an elevation in PVR leading to pulmonary hypertension (Zapol and Snider, 1977). This elevated PVR is multifactorial in its aetiology (Fox and McCormack, 1992), but one of the most important underlying mechanisms is likely to be vasoconstriction. The increased pulmonary arterial and "whole lung" expression of pre-pro-ET-1 mRNA and elevated plasma [ET-1] raise the possibility that this peptide influences pulmonary vascular tone in endotoxaemia. The use of isolated pulmonary artery (PA) rings to investigate the properties of a vasoactive agent is valuable because it offers a discrete model in which to study the interaction between the endothelium and vascular smooth muscle in the response of the vessel. This information is important, but is limited by the fact that the properties of isolated large artery rings will not necessarily reflect the behaviour of the whole pulmonary circulation.

It has been established previously that isolated thoracic aortic rings from rats treated *in vivo* with LPS exhibit hyporesponsiveness to vasoconstrictors such as catecholamines and potassium chloride (KCl) (Wakabayashi et al. 1990; Wakabayashi et al. 1993). Similar hyporesponsiveness to both phenylephrine (PE) and KCl has been demonstrated in isolated PA rings from LPS-treated rats

(Brison and Pitts, 1989; Griffiths et al. 1995a), which is to a great extent reversed by pretreatment with inhibitors of iNOS. In addition, in PA rings from sham rats, the contractile responses to these agents is increased by removal of the endothelium. Previously reported data on the properties of ET-1 in isolated rat aortic and PA rings has established that ET-1 is a more potent constrictor of PA than aortic rings, and also that removal of the endothelium in aorta increased the relative efficacy of ET-1 compared to endothelium-intact rings (Rodman et al. 1989). This increase in ET-1-induced contractility of rat aortic rings following endothelial removal has also been demonstrated by several other groups (Topouzis et al. 1991; Mehta et al. 1992), and generally has been attributed to the loss of an endothelium-derived vasodilatory agent, either NO (Topouzis et al. 1991) or prostacyclin. It has also been reported, however, that pretreatment of rat aortic rings with a selective inhibitor of thromboxane A_2/prostaglandin H_2 (TP) receptors, causes a rightward shift in the dose-response curve and a reduction in the maximal response to ET-1 (Reynolds and Mok, 1989). This effect is due to an ET-1 stimulated, indomethacin-sensitive formation of TXA_2 in rings of rat thoracic aorta. The potential for the release of vasoactive eicosanoids by ET-1 in such preparations is thus established. As well as the release of NO and PGI_2 from the endothelium as a consequence of ET_B receptor activation, it has also been shown that ET-1 can activate phospholipase A_2 in cultured rat aortic vascular smooth muscle cells (Resink et al. 1989), thereby leading to the liberation of arachidonic acid.

In this set of experiments, there were 2 main objectives. The first was to establish whether the endothelium modulates the contractile response of isolated PA rings from sham and LPS-treated rats to ET-1. These experimental protocols were also performed on PE-induced contractile responses, so that a comparison

could be made. The second aim was to test the hypothesis that *in vivo* LPS treatment would induce hyperresponsiveness of PA *in vitro* to ET-1-induced vessel contraction.

4.2 EXPERIMENTAL PROTOCOLS

4.2.1 To study the contractile responses to PE and ET-1 in isolated PA rings from sham rats: effects of endothelial denudation

Concentration-contraction responses to PE and ET-1 were studied in PA rings, both intact and endothelium-denuded, from sham rats. Contractile responses were recorded in each ring to increasing concentrations of either PE (10^{-9} to 10^{-5}M) or ET-1 (10^{-11} to 10^{-6}M) in a cumulative fashion. Results are expressed as tension generated in grams, and as the concentration of an agonist required to induce half of maximal contraction (EC_{50}).

4.2.2 To study the effect of *in vivo* LPS treatment on contractile responses of intact and endothelium-denuded PA rings to PE and ET-1

Concentration-contraction response curves to PE and ET-1 were constructed in intact and endothelium-denuded PA rings from rats pretreated 4 hours prior to sacrifice with LPS (20mg/kg). This allowed a comparison to be made between responses of intact and denuded vessels from sham and LPS-treated animals.

4.2.3 To investigate the role of COX-derived products in ET-1-induced contractile responses

Intact and endothelium-denuded vessels from sham and LPS-pretreated rats were studied. In each experiment, half the rings were pretreated with active drug, and the other half with the solvent\buffer for that drug at the same dilution. Cumulative concentration-contraction responses to ET-1 were then performed as above.

i) *Indomethacin*

Indomethacin was dissolved in buffer solution (0.02M KH_2PO_4, 0.12M Na_2HPO_4) to form a stock solution of 10^{-3}M. Half of the rings were pretreated with 10^{-5}M solution 30 minutes before ET-1 concentration response was performed.

ii) *ICI 192605*

ICI 192605, a thromboxane receptor antagonist, was dissolved in ethanol and diluted in distilled water to 10^{-3}M stock solution, from which 20μL was added to half the baths 20 minutes before the ET-1 was added, giving a final bath concentration of 10^{-5}M. The other half of the baths were treated with the equivalent dilution of ethanol. This dose of ICI 192605 was chosen both with reference to previous data on rat arterial tissue *in vitro* (10^{-6}M) (Jessup et al. 1988) and from preliminary experiments on rat PA, using the thromboxane mimetic, U46619, which confirmed a large shift to the right for the concentration-response curve by 10^{-5}M ICI 192605 **[Figure 4.1]**.

4.2.4 To study the effect of ET_B receptor antagonism on ET-1-induced contraction of PA rings

BQ788, a recently described selective ET_B receptor antagonist (Ishikawa et al. 1994), was dissolved in ethanol and diluted in distilled water to a 10^{-4}M stock solution. From this two sets of experiments were performed following pretreatment of half the baths with final concentrations of either 10^{-8}M or 10^{-6}M BQ788 added 20 minutes before the ET-1 concentration-contraction response. In each case, the same dilution of ethanol was added simultaneously to the other half of the baths as controls. Previous investigation of this agent has demonstrated antagonism of contraction to the ET_B receptor agonist, BQ3020, in rabbit pulmonary artery *in vitro* with a PA_2 value of 8.4. In addition, the IC_{50} of BQ788 for ^{125}I-ET-1 binding to ET_B receptors (human Girardi heart cells) was 1.2nM whereas it was 280nM for ET_A receptor binding (porcine coronary artery smooth muscle cells) (Ishikawa et al. 1994).

4.2.5 To study the contribution of ET_A receptor activation to ET-1-induced contraction of PA rings from sham or LPS-treated rats

Intact and endothelium-denuded PA rings from sham and LPS-pretreated rats were studied. In each experiment, half the rings were pretreated with BQ123, a selective ET_A receptor antagonist (Ihara et al. 1992a) (either 10^{-6} or 10^{-5} M) and the other half with the same volume of distilled water 10 minutes before the ET-1 concentration-contraction response was initiated.

4.3 RESULTS

4.3.1 Concentration-contraction responses of intact and endothelium-denuded PA rings from sham rats to PE and ET-1

PE and ET-1 both induced contraction in all PA rings tested in a concentration-dependent fashion. Endothelial removal caused a significant shift to the left of the concentration-contraction response to PE compared to intact rings (EC_{50} values 7.52±.0.26 vs. 6.50±0.16, respectively; both n=6; p = 0.03), but there was no difference in the maximum contraction (T_{max}) achieved **[Table 4.1; Figure 4.2]**. In the ET-1 groups, endothelial denudation also caused an increase in the EC_{50} value (8.67±0.05 vs. 7.82±0.17 for endothelium-denuded (n=6) and intact (n=8) rings respectively; $p < 0.001$), but in addition the T_{max} was less than in intact vessels **[Table 4.2; Figure 4.3]**.

4.3.2 The effect of *in vivo* LPS treatment on contractile responses of intact and endothelium-denuded PA rings to PE and ET-1

In all cases, PE- and ET-1-induced contraction of intact and endothelium-denuded rings were diminished by pretreatment with LPS **[Figures 4.4 & 4.5; Tables 4.1 & 4.2]**. In both PE and ET-1 responses, hyporesponsiveness occurred at lower concentrations in endothelium-denuded groups than in intact vessels, although in neither case did this result in a significant difference in EC_{50}. In rings from LPS-treated animals, the contraction of intact vessels to either PE or ET-1 was significantly greater than that of endothelium-denuded vessels **[Figures 4.4a & 4.5a]**.

4.3.3 Effects of indomethacin or ICI 192605 pretreatment on ET-1 induced contraction of PA rings

i) *Indomethacin*

Indomethacin pretreatment significantly reduced the ET-1-induced contraction in intact vessels from endotoxin-treated rats (T_{max} 0.32±0.04 in controls vs. 0.12±0.03 in the indomethacin group; $p < 0.001$) **[Figure 4.6]**, but had no effect on endothelium-denuded vessels, nor in any sham rings **[Table 4.3]**. Interestingly, the maximal contraction in the buffer-pretreated control intact ring groups (ie E+ LPS- & E+ LPS+) was considerably smaller than in the equivalent groups that had no pretreatment at all, although it should be noted that these groups were studied in different experiments.

ii) *ICI 192605*

The EC_{50} and T_{max} values for these vessels, and for vessels with no endothelium are shown in **Table 4.4**. ICI 192605 pretreatment significantly reduced the ET-1-induced contraction in intact vessels from LPS-treated rats **[Figure 4.7]**, but only over the concentration range 10^{-8}M to 10^{-7}M ET-1. There was no effect on vessels from any other group. This effect was smaller than that observed following indomethacin treatment.

4.3.4 Effects of BQ788 pretreatment on ET-1-induced contraction

The EC_{50} and T_{max} values for these vessels, and for those denuded of endothelium are shown in **Table 4.5**. BQ788 pretreatment at 10^{-8}M significantly reduced the ET-1-induced contraction in intact vessels from LPS-treated rats over the concentration range of ET-1: 3 X 10^{-7}M to 3 X 10^{-6}M ($p < 0.05$ for control vs BQ788), but the difference in T_{max} values did not reach statistical significance ($p = 0.055$ for control vs BQ788) **[Figure 4.8a]**. There was no difference in EC_{50} values between these groups. This dose of BQ788 had no significant effect on vessels in any other group. The magnitude of this reduction in ET-1-induced contractile response was, however, smaller than that observed following indomethacin treatment. Pretreatment of the intact rings from LPS-treated rats with 10^{-6}M BQ788 resulted in a shift of the concentration-contraction response curve to the right, so that the EC_{50} in the treatment group was significantly different compared to controls (8.23±0.06 vs.8.63±0.12 respectively; $p = 0.008$) **[Figure 4.8b]**.

4.3.5 Effects of BQ123 pretreatment on ET-1-induced contraction

Pretreatment of rings with BQ123 produced significant attenuation of the ET-1-induced contraction of all groups **[Figures 4.9]**. The degree of this attenuation was reflected in the EC_{50} values in the groups pretreated with 10^{-6} M BQ123 **[Tables 4.6 & 4.7]**. The relative change in EC_{50} was similar in all vessel types. Those rings pretreated by 10^{-5} M BQ123 demonstrated even greater shifts to the right of their contractile responses. These vessels did not reach a plateau of maximum contraction at the highest concentration of ET-1 (10^{-6} M) administered, and so no EC_{50} data are available. In all these vessel types, except the intact

LPS-treated group, however, the maximum contraction obtained was not significantly altered by BQ123 **[Figure 4.9c]**. In the intact LPS-treated rings, the maximum response to ET-1 (10^{-6}M) was reduced by BQ123 ($p < 0.001$).

4.4 DISCUSSION

This study demonstrates that ET-1-evoked contraction of isolated rat PA is, in a similar manner to phenylephrine and KCl, subject to LPS-induced hyporesponsiveness. It has also shown that this contractile response in rings from LPS-treated animals is modified by the endothelium, since contraction to ET-1 in these circumstances was only maximal when the endothelium was present. The results from the indomethacin experiment suggest that the difference in contraction in the intact rings, compared to the endothelium-denuded rings, from LPS-pretreated rats depends partially upon the release of an endothelium-derived, COX-dependent constrictor agent.

The findings in untreated rats were unexpected. In these experiments, ET-1-evoked contraction was modified in a dose-dependent fashion by the endothelium, such that there was greater contraction in the denuded rings at lower ET-1 concentrations, although T_{max} was greater in intact rings. This contradicts some previous experimental data in isolated rat aortic rings (Mehta et al. 1992), although the relative shape of the ET-1 concentration-contraction curves in the endothelium-intact and -denuded rings is very similar to those described (Topouzis et al. 1991) in isolated rat aorta; a similarity reflected in comparable differences in the EC_{50} values for the two groups. In fact, the maximum concentration of ET-1 used in that study was only 10^{-7}M (Topouzis et al. 1991), and at this point the intact rings had a greater T_{max} (although this did not reach statistical significance). We were unable to demonstrate any significant modulation of this difference between intact and denuded rings from sham animals in our experiments using indomethacin, ICI 192605 or BQ788. It is possible that the increased sensitivity of denuded rings to ET-1 at lower concentrations reflects removal of a COX-

independent vasodilator such as NO. If this is the case, however, there is no evidence from these experiments that such an agent is released as a result of ET_B receptor activation (see Chapter 5). It is difficult to explain the greater maximal contraction of the intact rings to ET-1, which was not a feature of the equivalent PE groups.

ET-1-induced (and PE-induced) contraction of rings, whether intact or denuded, was attenuated by LPS pretreatment, thus disproving the hypothesis that the PA rings would exhibit hypersensitivity to ET-1. This result has not been described previously for ET-1, but is not surprising in view of the general hyporesponsiveness demonstrated to other potent vasoconstrictors such as PE, in clinical and experimental sepsis. The contractile response to ET-1 of the intact rings from LPS-treated rats was greater than in the endothelium-denuded vessels. Pretreatment with indomethacin induced a substantial reduction in the contraction of the intact, LPS group to ET-1, implying that inhibition of COX-2, but not COX-1, prevents ET-1-induced release of a vasoconstrictor eicosanoid from the endothelium in these circumstances. Previous studies (Simonson and Dunn, 1992; Resink et al. 1989) have shown that ET-1 can activate phospholipase A_2 in endothelium and vascular smooth muscle and as a result is capable of increasing production of arachidonic acid metabolites. In aortic rings from untreated rats (Reynolds and Mok, 1989), for example, a component of the ET-1-induced contraction could be removed by pretreatment with either indomethacin or the thromboxane receptor antagonist, SQ29548. It has also been shown, using the same pharmacological interventions, that a portion of the coronary arterial vasoconstriction resulting from intravenous ET-1 infusion in rats is mediated by constrictor prostanoids, including TXA_2 (Filep et al. 1994b). However, the current literature is unclear regarding ET-induced eicosanoid release in rats. Thus,

measurement of the primary metabolites of PGI_2 and TXA_2 in an isolated lung preparation in Wistar rats demonstrated that both are released by exogenous ET-1, although PGI_2 release was significantly greater (de Nucci et al. 1988). Consequently, previous experiments in untreated rat models demonstrate that ET-1 has the ability to activate the intracellular machinery for arachidonic acid metabolism, thereby leading to actual increases in PGI_2 and TXA_2 production. Prostacyclin itself is capable of acting as a vasoconstrictor, particularly when present in large quantities, and a change in the amount of PGI_2 represents an alternative mechanism by which ET-1 could achieve endothelium-dependent contraction. There was only a significant change with indomethacin pretreatment in the vasoconstrictor effect of ET-1 in LPS-pretreated PAs with intact endothelium. The results in this group are similar to those found in the aortic rings of spontaneously hypertensive rats (SHR) (Taddei and Vanhoutte, 1993), in which ET-1-induced contraction was reduced by endothelial removal. In addition, indomethacin, a TXA_2 receptor antagonist and a TX synthase inhibitor all reduced the maximal contraction to ET-1 in the intact, but not the denuded rings. The similarity of these results in SHRs with the data from intact LPS-treated rings in the current experiments may well reflect the existence of endothelial dysfunction in both groups. The vascular response to sepsis is known to involve endothelial damage and dysfunction, and recent evidence also suggests that endothelial dysfunction occurs in hypertension.

However, the disparity between the size of the reduction in ET-1-induced contraction in the intact rings from LPS-pretreated rats with indomethacin pretreatment compared to ICI 192605 pretreatment suggests that the latter agent did not completely antagonize the underlying process, despite the demonstrable effectiveness, in U46619 experiments, of this dose of ICI 192605 as a TP receptor

antagonist. There are two possible explanations for this discrepancy. Either the ET-1-released vasoconstrictor is TXA_2 and the receptor antagonist cannot adequately block the receptors at which it is working on the vascular smooth muscle, or the agent causing the vasoconstriction is only partially dependent on TP receptor activation.

The results of the experiments using the ET_B receptor antagonist BQ788, imply that activation of the endothelium via this mechanism may contribute to the release of the constrictor eicosanoid. Only two ET receptors have had their cDNA cloned and expressed so far. Of these, ET_A occurs on vascular smooth muscle and is relatively selective for ET-1. Evidence from pharmacological intervention using ET_A receptor antagonists both *in vitro* and *in vivo* suggests that ET_A receptor activation results in contraction of vascular smooth muscle (Ihara et al. 1992b), although it has also recently been shown that PGI_2 release can be blocked in rat lung by the ET_A receptor antagonist, BQ123 (D'Orleans-Juste et al. 1992). ET_B receptors are predominantly endothelial, and have equal sensitivity to all ETs. Stimulation of endothelial cell cultures by ET or ET_B agonists (Hirata and Emori, 1993; Hirata et al. 1993) has been shown to stimulate NO release. In rat studies of local hemodynamic profiles, results imply that in some organ beds, notably the kidney (Cristol et al. 1993; Wellings et al. 1994b), the vasoconstrictor response to ET-1 is mediated by ET_B stimulation, probably as a result of TXA_2 release. The fact that ET_B receptor blockade only had a significant effect in the LPS-pretreated, intact group may reflect an alteration in functional activity of the endothelial cell and/or its ET_B receptor population. The degree of attenuation in ET-1-induced responses of all the tested groups following the higher concentration of BQ123 makes it unlikely that there is a simple ET_B-mediated release of a constrictor such as TXA_2. If this was the case, it would be expected that however efficient was the

antagonism of ET_A receptors, there would still be some component of ET-1-induced contraction spared, which was due to ET_B-mediated effects. No evidence of this was found. The true underlying mechanism for the indomethacin-sensitive component of ET-1-induced contraction in intact rings from LPS-treated rats thus remains elusive following these experiments, but the results suggest that it is mediated predominantly by ET_A receptors. These data have not explored the interaction of NO with ET-1 in PA from endotoxaemic rats, and in particular the release of NO by ET_B receptor activation. This is examined in detail in the next chapter.

Table 4.1: **Response to phenylephrine of pulmonary artery rings from sham or endotoxin-pretreated rats**

Ring	Number	T_{max} (gram)	EC_{50} (-log [M])
E+ LPS-	n=8	0.33±0.36	6.50±0.15
E- LPS-	n=6	0.37±0.32	7.52±0.26 •
E+ LPS+	n=8	0.22±0.04 ¶	6.25±0.09
E- LPS+	n=7	0.23±0.05 #	7.02±0.16 Φ

Results are expressed as mean ± SEM.
E+ = intact.
E- = endothelium-denuded.
LPS- = sham.
LPS+ = endotoxin-treated.
Statistical analysis to compare these data was made using ANOVA followed by Tukey-Kramer post tests.

Tmax values all non-significant differences except:
¶ E+LPS+ vs. E+ LPS- $p < 0.05$
# E-LPS+ vs. E-LPS- $p < 0.05$
EC_{50} values all non-significant differences except:
• E-LPS- vs. E+LPS- $p < 0.01$
Φ E-LPS+ vs E+ LPS+ $p < 0.001$

Table 4.2: Response to ET-1 of pulmonary artery rings from sham or endotoxin-pretreated rats

Ring	Number	T_{max} (gram)	EC_{50} (-log [M])
E+ LPS-	n=8	0.82±0.08	7.82±0.17
E- LPS-	n=6	0.53±0.08 *	8.67±0.05 •
E+ LPS+	n=8	0.53±0.04 ¶	7.95±0.18
E- LPS+	n=7	0.26±0.05 # §	8.19±0.07

E+ = intact.
E- = endothelium-denuded.
LPS- = sham.
LPS+ = endotoxin-treated.

Statistical analysis to compare these data was made using ANOVA followed by Tukey-Kramer test for multiple comparisons.
Tmax values all non-significant differences except:
* E-LPS- vs. E+LPS- $p < 0.05$
¶ E+LPS+ vs. E+ LPS- $p < 0.05$
# E-LPS+ vs. E-LPS- $p < 0.05$
§ E-LPS+ vs. E+ LPS+ $p < 0.05$

EC_{50} values all non-significant differences except:
• E-LPS- vs. E+LPS- $p < 0.001$

Table 4.3 **Response of pulmonary artery rings to ET-1 with and without indomethacin pretreatment.**

Ring	T_{max} (gram)	EC_{50} (-log [M])
E+ LPS-	Control 0.64±0.15 (n=8)	8.30±0.18
	Indo 0.69±0.09 (n=8)	8.15±0.19
E- LPS-	Control 0.58±0.15 (n=6)	8.55±0.16
	Indo 0.44±0.07 (n=7)	8.30±0.13
E+ LPS+	Control 0.32±0.04 (n=6)	8.03±0.05
	Indo 0.12±0.03 *** (n=8)	7.83±0.11
E- LPS+	Control 0.29±0.04 (n=7)	8.29±0.08
	Indo 0.25±0.07 (n=8)	7.87±0.24

T_{max} and EC_{50} values for indomethacin group versus its control in each ring category showed no significant differences, except *** where $p < 0.001$.

Table 4.4: Response of pulmonary artery rings to ET-1 with and without ICI 192605 pretreatment.

Ring	T_{max} (gram)	EC_{50} (-log [M])
E+ LPS-	Control 0.98±0.07 (n=6)	7.88±0.13
	BQ192605 0.80±0.08 (n=8)	7.94±0.08
E- LPS-	Control 0.40±0.06 (n=6)	8.19±0.06
	BQ192605 0.33±0.08 (n=6)	7.99±0.10
E+ LPS+	Control 0.65±0.07 (n=12)	8.08±0.04
	BQ192605 0.48±0.05 (n=12)	8.00±0.06
E- LPS+	Control 0.26±0.09 (n=7)	7.64±0.27
	BQ192605 0.29±0.04 (n=8)	8.08±0.03

Comparisons of T_{max} and EC_{50} values between ICI 192605 and control for each category of ring were not statistically different by unpaired *t*-test.

Table 4.5: Response of pulmonary artery rings to ET-1 with and without 10^{-8}M BQ788 pretreatment

Ring	T_{max} (gram)	EC_{50} (-log [M])
E+ LPS-	Control 0.62±0.08 (n=7)	8.05±0.07
	BQ788 0.73±0.09 (n=8)	8.07±0.08
E- LPS-	Control 0.29±0.06 (n=9)	8.20±0.11
	BQ788 0.37±0.05 (n=10)	8.24±0.10
E+ LPS+	Control 0.48±0.05 (n=10)	8.03±0.08
	BQ788 0.34±0.04 (n=10)	7.94±0.07
E- LPS+	Control 0.30±0.05 (n=6)	7.67±0.10
	BQ788 0.24±0.03 (n=7)	7.94±0.07

Comparison between T_{max} and EC_{50} values for BQ788 in each ring category versus its control revealed no statistically significant results.

Table 4.6. **Maximum contraction (Tmax) of PA rings with and without BQ123**

	Control	10^{-6} M BQ123	10^{-5} M BQ123
E+ LPS-	0.69 ±0.06 (n=13)	0.48 ± 0.05 (n=6)	0.53 ± 0.08 (n=6)
E- LPS-	0.44 ±0.05 $^{\propto\propto}$ (n=14)	0.25 ± 0.05 (n=6)	0.30 ± 0.07 (n=6)
E+ LPS+	0.56 ± 0.05 (n=14)	0.41 ± 0.04 (n=6)	0.18 ± 0.04 *** (n=6)
E- LPS+	0.27 ± 0.04 $^{\sigma\sigma}$ (n=13)	0.15 ± 0.05 (n=6)	0.19 ± 0.06 (n=6)

Statistical evaluation: (multiple comparisons by ANOVA followed by Tukey-Kramer.)

*** : $p < 0.001$ for control vs. 10^{-5} M BQ123 groups of E+ lipopolysaccharide+

$\propto\propto$: $p < 0.01$ for E+ lipopolysaccharide- vs. E- lipopolysaccharide-

$\sigma\sigma$: $p < 0.01$ for E+ lipopolysaccharide+ vs. E- lipopolysaccharide+

Table 4.7. EC_{50} of PA rings with an without BQ123

	Control	10^{-6} M BQ123	10^{-5} M BQ123
E+ LPS-	7.80 ±0.13 (n=13)	6.92 ±0.17 ** (n=4)	NA
E- LPS-	8.05 ±0.21 (n=14)	6.01 ±0.64 *** (n=5)	NA
E+ LPS+	7.99 ±0.09 (n=14)	6.67 ±0.07 *** (n=6)	NA
E- LPS+	8.07 ±0.06 (n=13)	7.06 ±0.05 *** (n=3)	NA

EC_{50} values could only be calculated for those rings in which the ET-1 concentration-contraction response produced a sigmoid type curve with a plateau . The numbers in the 10^{-6} M BQ123 groups are therefore smaller than expected since not all the rings contributed to this overall EC_{50} value.

For each type of ring the control EC_{50} was compared with that of the value for the 10^{-6} M BQ123 group using an unpaired Student's *t* test.

p values: ** <0.01; *** < 0.001.

Figure 4.1 **Contraction of PA rings to U46619 with and without 10^{-5}M ICI192605**

Contraction induced by the TP receptor agonist, U46619 (alone shown as squares) in intact PA rings from untreated rats, is significantly attenuated by pretreatment with 10^{-5}M of the antagonist, ICI192605 (stars).
Results are expressed as mean ± SEM of 4 observations. *** $p<0.001$.

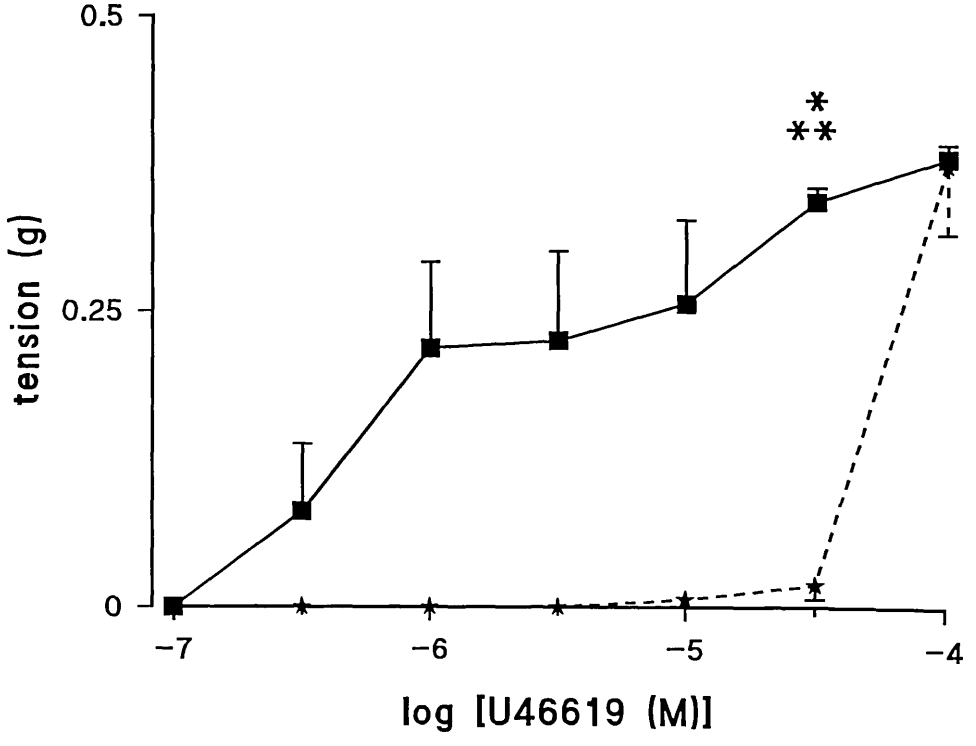


Figure 4.2 **Effects of endothelial denudation on phenylephrine-induced contraction of PA rings from sham rats**

Phenylephrine-induced (PE) contraction of intact (closed circles) or endothelium-denuded (open circles) rings from sham rats. Results are expressed as mean ± SEM of at least n=6. * $p<0.05$; ** $p<0.01$.

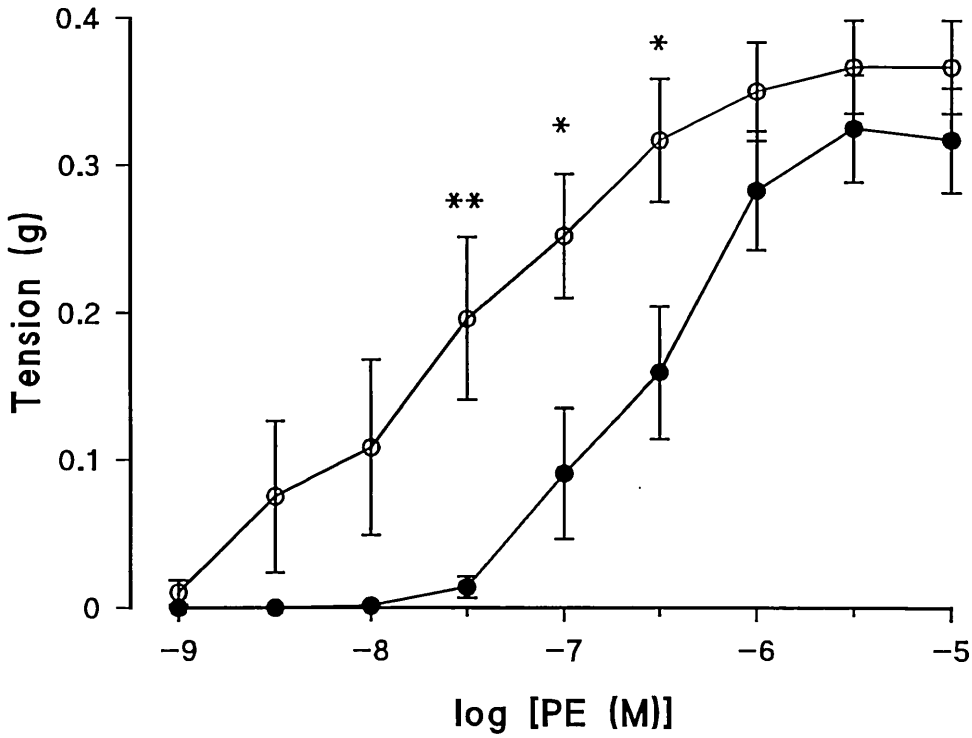
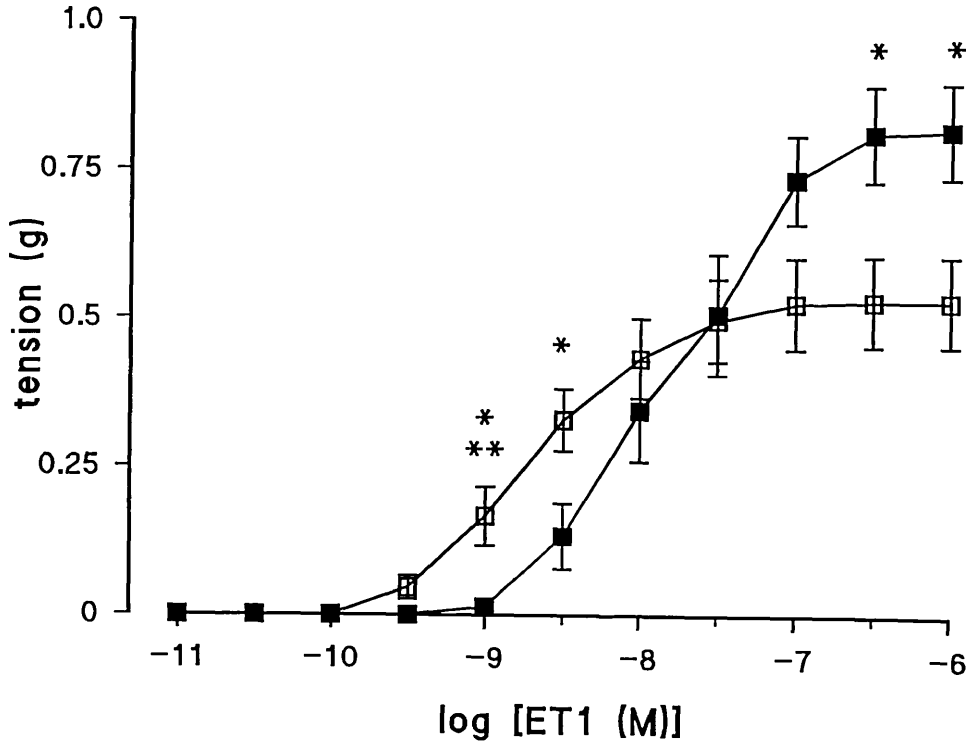


Figure 4.3 **Effects of endothelial denudation on ET-1-induced contraction of PA rings from sham rats**

ET-1-induced contraction of intact (closed squares) or endothelium-denuded (open squares) rings from sham rats. Results are expressed as mean ± SEM of at least 6 observations. * $p<0.05$; *** $p<0.001$.



UPPER PANEL

Phenylephrine-induced contraction of intact PA rings from sham (circles) or LPS-treated (triangles) rats.

MIDDLE PANEL

Phenylephrine-induced contraction of endothelium-denuded PA rings from sham (open circles) or LPS-treated (open triangles).

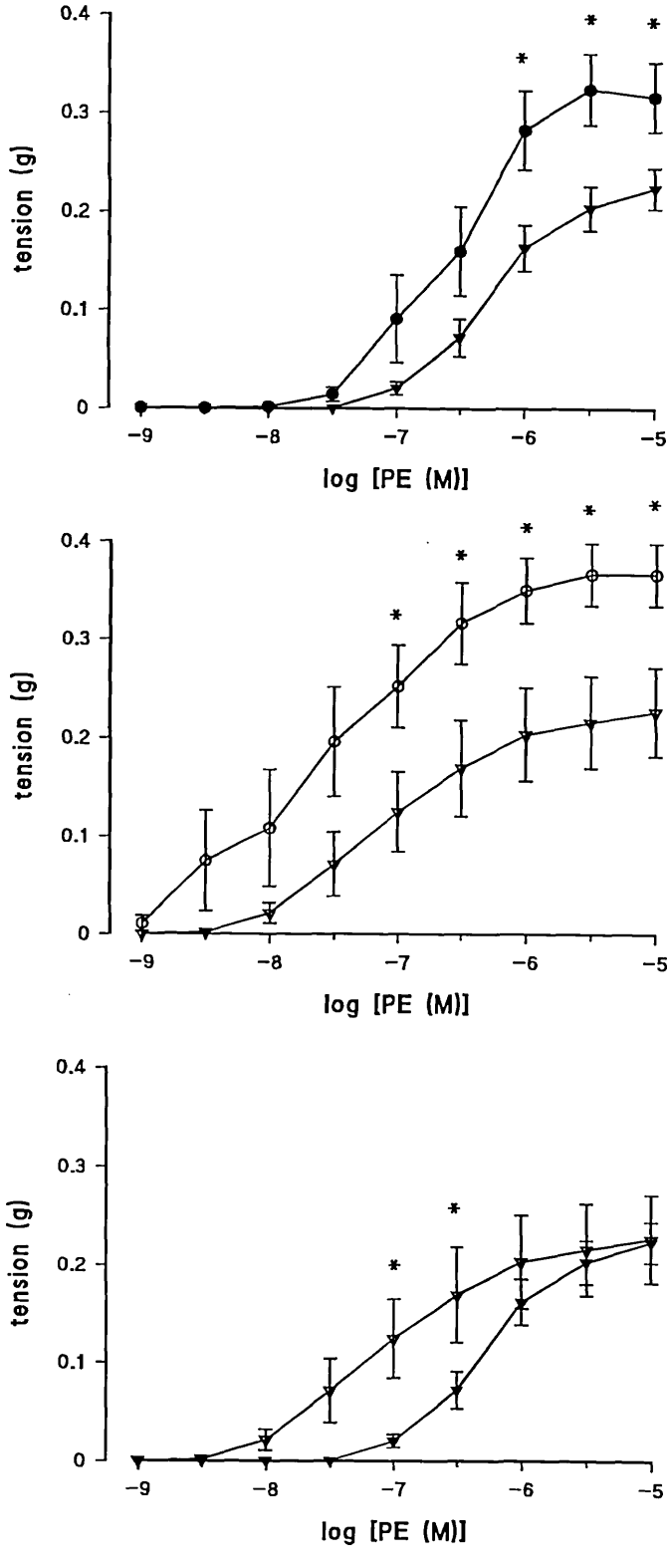
LOWER PANEL

Phenylephrine-induced contraction of endothelium-denuded (open triangles) and intact (closed triangles) PA rings from LPS-treated rats.

All groups contain $n \geq 6$. All results are mean±SEM. * $p < 0.05$.

Figure 4.4 **Phenylephrine-induced contraction of PA rings: effects of LPS treatment**

(Legend on opposite page)



UPPER PANEL

ET-1-induced contraction of intact PA rings from sham (squares; n=8) or LPS-treated (triangles; n=8) rats.

MIDDLE PANEL

ET-1-induced contraction of endothelium-denuded PA rings from sham (open squares; n=6) or LPS-treated (open triangles; n=7).

LOWER PANEL

ET-1-induced contraction of endothelium-denuded (open triangles; n=7) and intact (closed triangles; n=8) PA rings from LPS-treated rats.

All results are mean±SEM. * $p < 0.05$.

Figure 4.5 **ET-1-induced contraction of PA rings: effects of LPS treatment**

(Legend on opposite page)

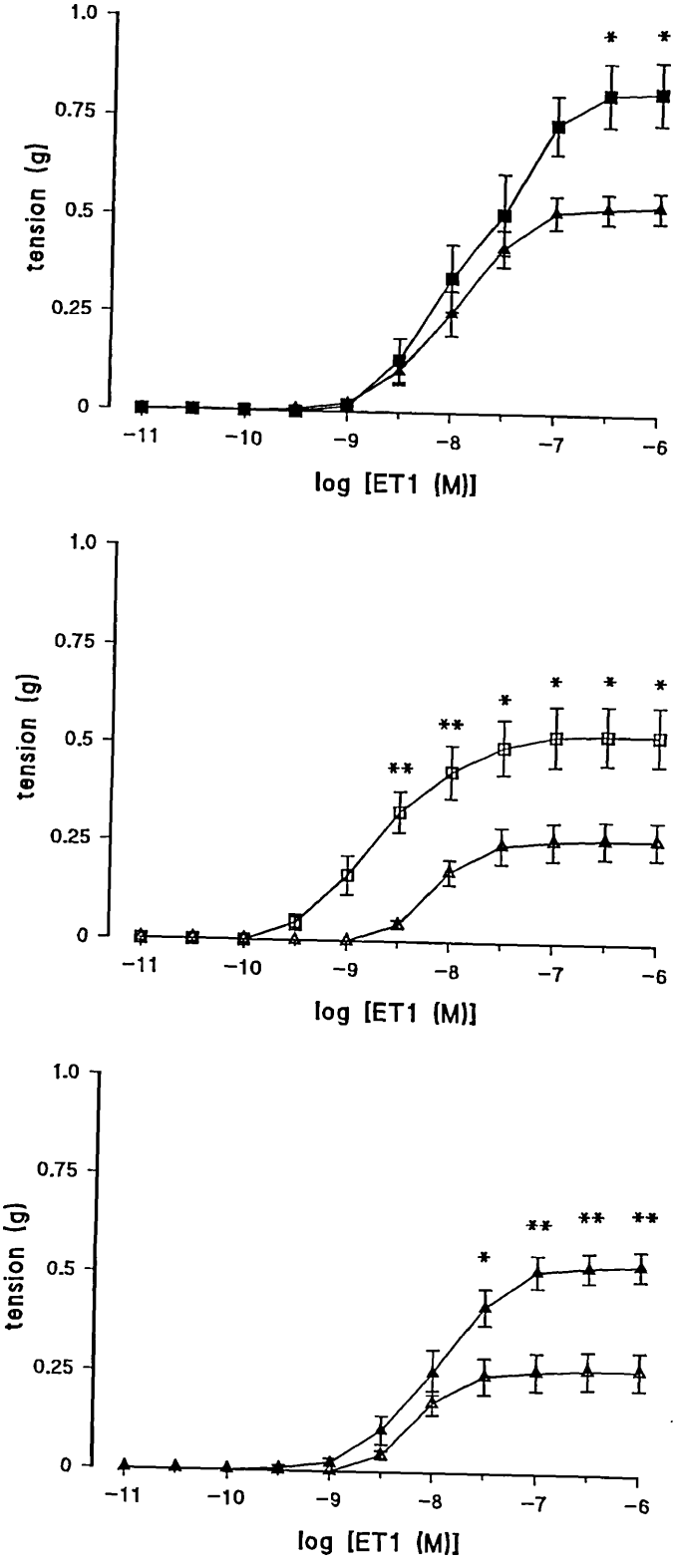


Figure 4.6 **Effect of 10^{-5}M indomethacin on ET-1-induced contraction of intact PA rings from LPS-treated rats**

Contraction to ET-1 in the presence of either 10^{-5}M indomethacin (broken line; n=8) or buffer solution (continuous line; n=6). Results are expressed as mean ± SEM.
** p<0.01; *** p<0.001.

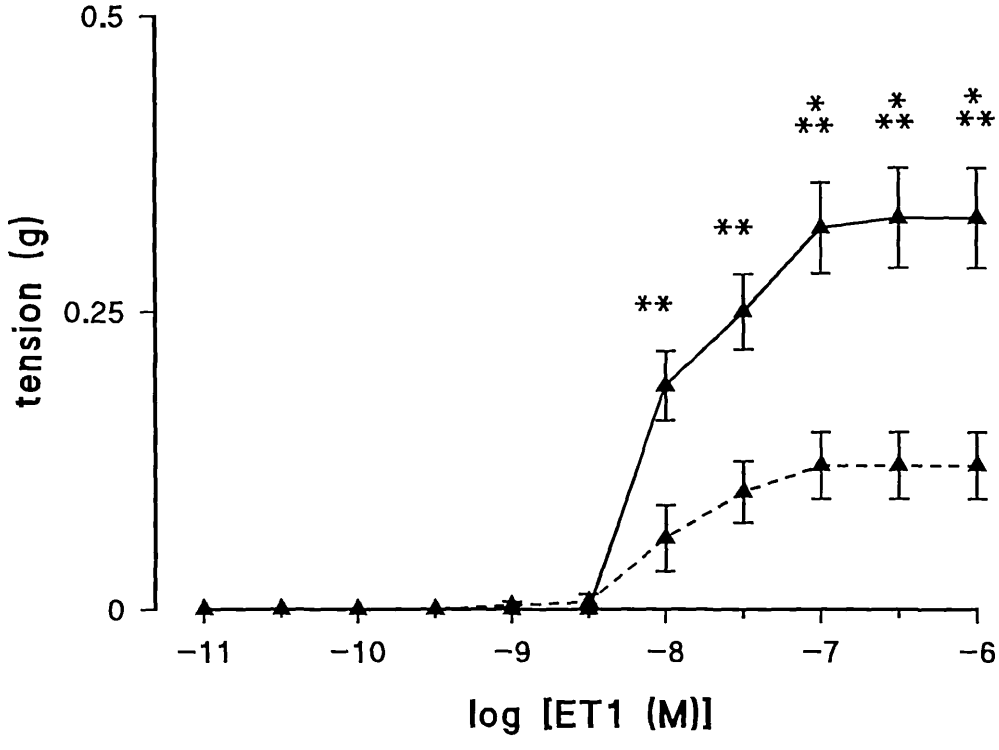


Figure 4.7 **Effect of 10^{-5}M ICI192605 on ET-1-induced contraction of intact PA rings from LPS-treated rats**

Contraction to ET-1 in the presence of either 10^{-5}M ICI192605 (broken line; n=12) or vehicle (continuous line; n=12). Results are expressed as mean ± SEM. * $p<0.05$.

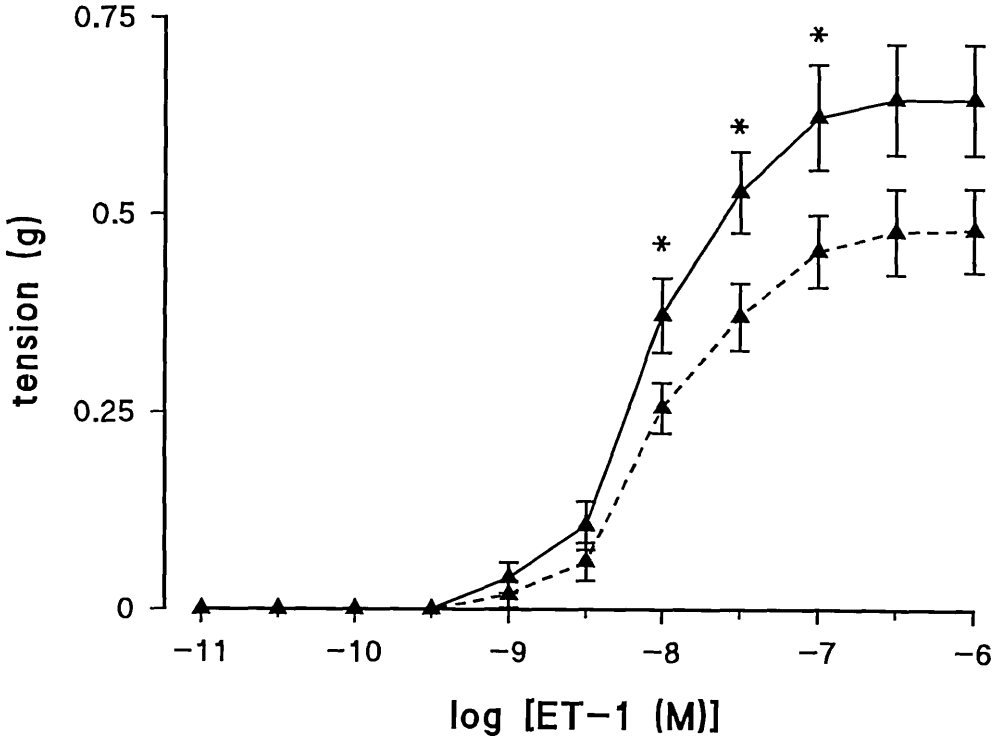
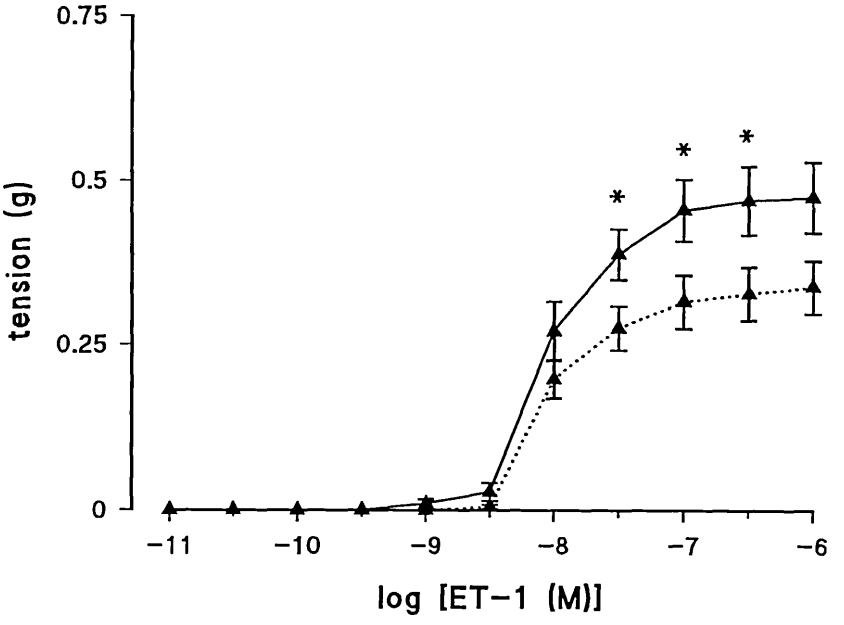


Figure 4.8 **Effect of BQ 788 on ET-1-induced contraction of intact PA rings from LPS-treated rats**

Contraction to ET-1 in the presence of either:
UPPER PANEL 10^{-8}M BQ788 (interrupted connect) or solvent (solid connect), both n=10 or LOWER PANEL 10^{-6}M BQ788 (interrupted connect) or solvent (solid connect), both n=6. *p* values: * <0.05.



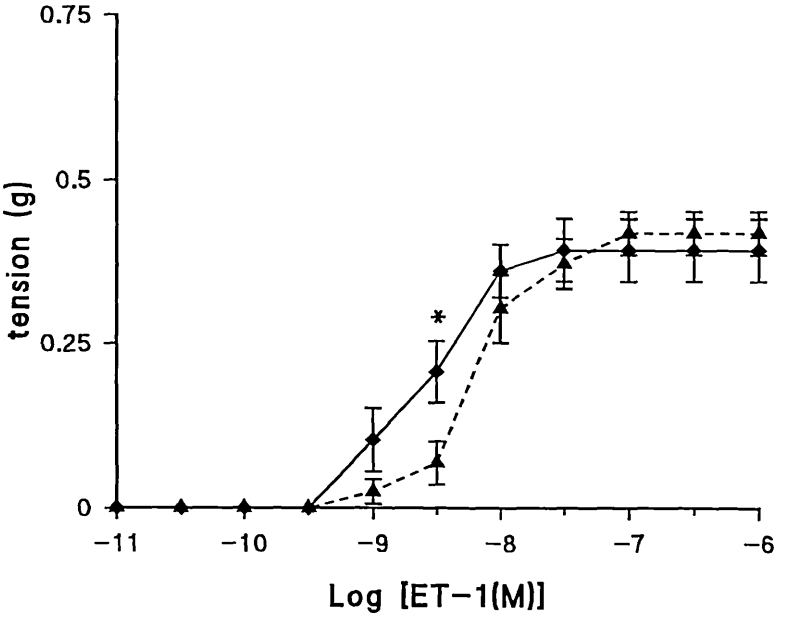
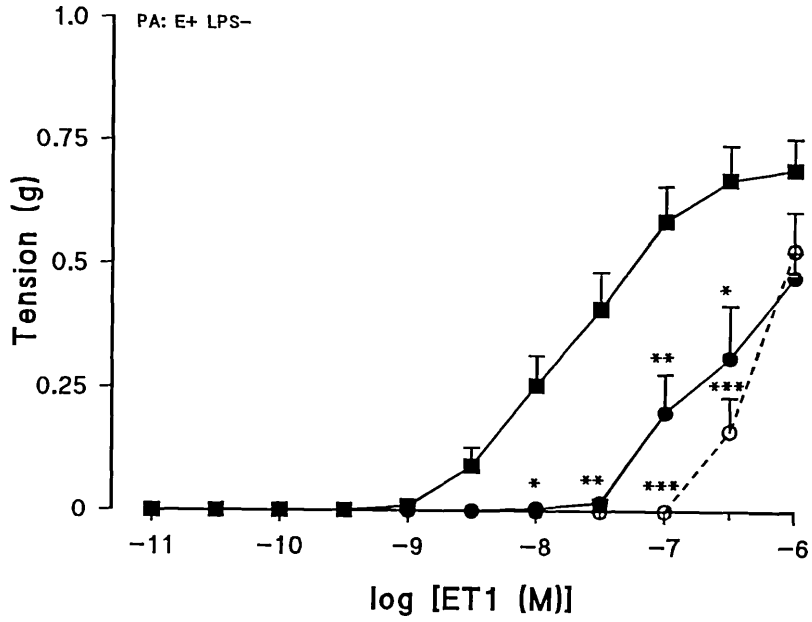


Figure 4.9 a&b **Contraction to ET-1 in intact PA rings: effects of BQ123**

Contraction of intact PA rings from sham rats (**a** UPPER PANEL;squares;n=13) or from LPS-treated rats (**b** LOWER PANEL;triangles; n=14) following either 10^{-6}M (solid circles; n=6) or 10^{-5}M (open circles;n=6) BQ123. Results are mean±SEM. Statistical analysis was by ANOVA followed by Tukey-Kramer post tests. *p* values vs. control: * <0.05; ** <0.01; *** <0.001. *p* between BQ123 groups: Δ <0.05.



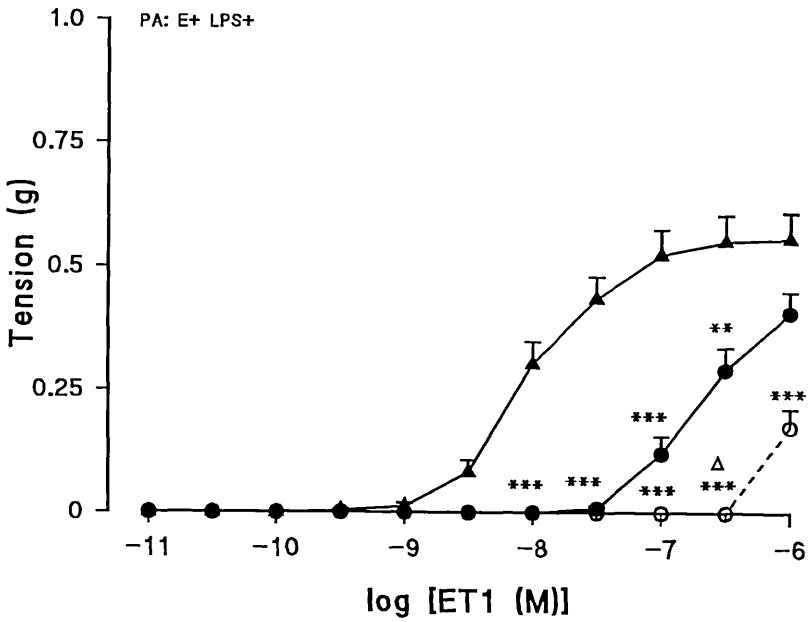
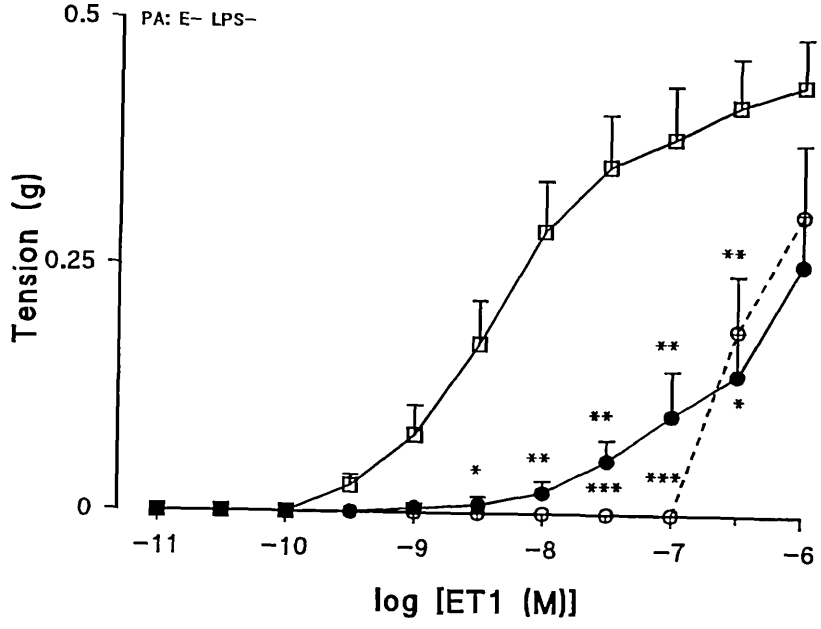
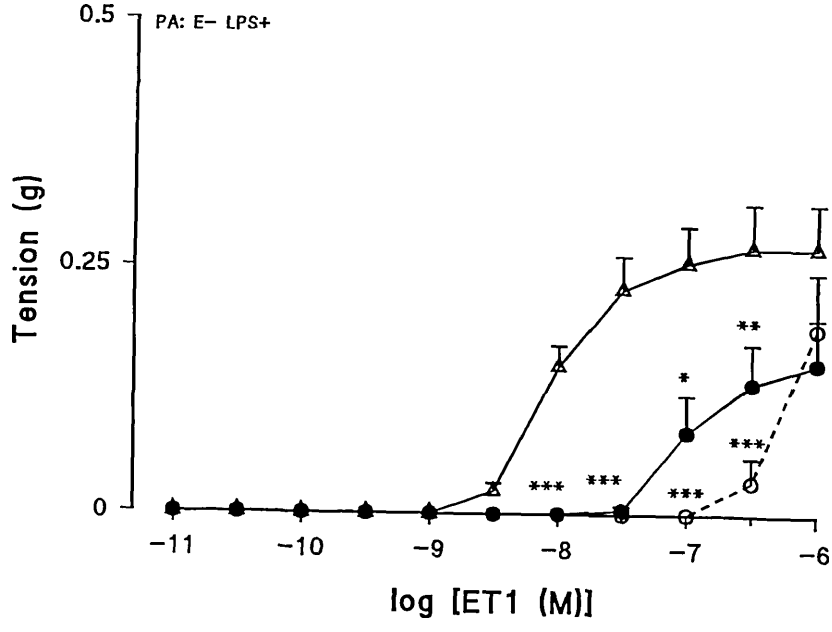


Figure 4.9 c&d **Contraction to ET-1 in endothelium-denuded PA rings: effects of BQ123**

Contraction of endothelium-denuded PA rings from sham rats (**c** UPPER PANEL;squares;n=14) or from LPS-treated rats (**d** LOWER PANEL; triangles;n=13) following either 10^{-6}M (solid circles;n=6) or 10^{-5}M (open circles;n=6) BQ123. Results are mean±SEM. Statistical analysis was by ANOVA followed by Tukey-Kramer post tests. *p* values vs. control: * <0.05; ** <0.01; *** <0.001.





CHAPTER 5

CHARACTERISATION OF THE ROLE OF ET_B RECEPTOR ACTIVATION IN ISOLATED PULMONARY ARTERY RINGS FROM SHAM OR LPS-TREATED RATS: NO RELEASE AND INTERACTION

5.1 INTRODUCTION

The ET_B receptor has equal affinity for all three ET molecules, and is situated mainly on the endothelium. It has also been found to be sited on vascular smooth muscle in several species, and specifically, there is evidence for the presence of ET_B receptors on rat PA smooth muscle, as well as on the endothelium (Eddahibi et al. 1993). In Chapter 4, the contractile responses to exogenous ET-1 in isolated PA rings were studied, and hyporeactivity was demonstrated in rings from LPS-treated rats. This was potentiated by removal of the endothelium or by COX blockade, but only by a much smaller degree by ET_B receptor antagonism. In addition, experiments employing the selective ET_A antagonist, BQ123, produced large shifts to the right in the ET-1 concentration-contraction curves, including rings from LPS-treated rats, regardless of the integrity of the endothelium. These results suggest that ET_A receptors mediate endothelium-dependent constriction in PA rings from LPS-treated rats. However, it is established from experiments *in vitro* (Hirata and Emori, 1993; Hirata et al. 1993; Warner et al. 1989) and *in vivo* (Clozel et al. 1992; de Nucci et al. 1988) that ET-1 releases NO, as well as PGI_2, from the endothelium via the activation of ET_B

receptors, by which mechanism ET-1 has vasodilator properties, both in the systemic (Filep et al. 1993; Gardiner et al. 1990b) and pulmonary circulation (Hasunuma et al. 1990). In addition, ET-3 can behave as a vasodilator by this mechanism in the isolated rat pulmonary circulation under conditions of elevated tone (Crawley et al. 1992). It has also been demonstrated that the hyporesponsiveness of both isolated systemic artery (Hollenberg et al. 1992; Fleming et al. 1993; Umans et al. 1993) and PA (Griffiths et al. 1993; Griffiths et al. 1995a) rings from rats rendered endotoxaemic *in vivo* can be reversed by NOS inhibitors.

In the experiments presented in this chapter, the role of the ET_B receptors in these vessels was therefore explored in an attempt to test the hypothesis that the interaction between NO and ET-1 modulates the contractile response to the latter. Dilator responses to the ET_B receptor agonist, sarafotoxin S6c, were compared with those of the endothelium-dependent vasodilator, acetylcholine (ACh), whose elicited response has previously been reported not to vary in PA rings from sham or LPS-treated rats (Griffiths et al. 1993). The aims were therefore threefold. Firstly, to confirm that ET_B receptor activation does not result in constriction of PA from control or LPS-treated rats. Secondly, to assess the modulatory effect of NO on ET-1-induced contractile responses. Finally, to assess the effect of ET_B-mediated NO release in preconstricted rings from control or LPS-treated rats, and compare these effects with ACh-mediated NO release.

5.2 EXPERIMENTAL PROTOCOLS

5.2.1 To determine the effects of ET_B receptor activation on isolated PA rings at baseline tension

Contractile responses of intact PA rings from sham and endotoxin-treated rats (n=4) to cumulatively increasing concentrations of sarafotoxin S6c (10^{-11} to 10^{-6} M) were studied. The sarafotoxin S6c was dissolved in distilled water to a stock solution of 10^{-3} M and then serially diluted. Each final bath concentration was achieved as stated above for ET-1.

5.2.2 To determine the effects of NOS inhibition on ET-1-induced contraction of pulmonary artery rings

Intact PA rings from either sham or LPS-treated rats were prepared as above. Following equilibration, half were pretreated with 10^{-4}M N^{w}-nitro-L-arginine-methylester (L-NAME), and the other half with distilled water (vehicle) 15 minutes before the cumulative concentration-contraction response to ET-1 was studied.

5.2.3 To determine the effects of ET_B receptor activation, and acetylcholine, in preconstricted pulmonary artery rings and the effect of NOS inhibition

Intact rings from sham or LPS-treated rats were prepared and equilibrated as described above. All rings were preconstricted with 10^{-6}M U46619, a TXA_2 mimetic, which had previously been found to produce rapid and sustained

contraction of this tissue. Once this U46619-induced contraction reached maximum, three doses of sarafotoxin S6c (10^{-7}, $3X10^{-7}$, 10^{-6}M), or the same volume of distilled water, were given in cumulative fashion, and the effect on tension measured and expressed as a percentage of the tone induced by U46619. A third of the rings were pretreated with L-NAME (10^{-4}M) 15 minutes before U46619 was added. In order to independently quantify the ability of endothelium to produce NO-mediated vasorelaxation, ACh (10^{-4}M) was given after the final dose of sarafotoxin S6c or distilled water.

5.3 RESULTS

5.3.1 The effect of sarafotoxin S6c on PA rings at baseline tension

Sarafotoxin S6c (10^{-11} to 10^{-6} M) failed to induce any change in tension in intact PA rings from either sham or endotoxin-treated rats.

5.3.2 Effects of L-NAME pretreatment on ET-1-induced contraction of pulmonary artery rings

In the groups pretreated with distilled water, the maximum contraction (T_{max}) to ET-1 was significantly lower in rings from LPS-treated animals (n=5; 0.32±0.04g) compared with sham (n=6; 0.51±0.04g); $p < 0.01$ **[Table 5.1]**. L-NAME (10^{-4}M) pretreatment significantly increased the T_{max} to ET-1 in both sham (n=6; 0.70±0.06g) **[Figure 5.1a]**, and LPS rats (n=5; 0.51±0.06g) **[Figure 5.1b]**. It is notable that T_{max} of the L-NAME-pretreated LPS rings was not significantly different from that of the sham vessels pretreated with DH_2O **[Table 5.1]**. There was no significant difference between any of the EC_{50} values in all 4 groups **[Table 5.1]**.

L-NAME itself caused a significantly greater rise in baseline tension in LPS rings than in controls (0.08±0.02g *vs* 0.01±0.01g; p <0.01).

5.3.3 Dilator effects of sarafotoxin S6c and ACh in pulmonary artery rings preconstricted with U46619, and modulation by L-NAME pretreatment

In rings from sham rats, a transient vasodilator response was seen in response to the initial concentration of sarafotoxin S6c (10^{-7}M) in 6 of the 10 rings tested. A typical example of this response is shown in **Figure 5.2**. The response was completely absent in the remaining 4 rings. If the data from all 10 rings are pooled, the effect was not significantly different from the distilled water control (p=0.06), but if the rings that responded are taken separately, a highly significant difference emerged between this group and the controls or the L-NAME treated rings **[Figure 5.3a]**. The S6c-induced relaxation was therefore characterised in the rats that did respond. The dilatation was transient (2-3min) and exhibited tachyphylaxis so that no further dilatation was elicited by the two subsequent doses of sarafotoxin **[Figure 5.2].** In tissues from animals that responded to S6c in this fashion, L-NAME pretreatment significantly reduced the observed dilatation **[Figures 5.2 & 5.3]**. In contrast to the sham animals, none of the rings from LPS-treated rats responded to S6c **[Figures 5.2 & 5.3]**, and the change in tension (expressed as a percentage of the tension at which the S6c was given) observed in response to 10^{-7} M S6c was significantly different between sham and LPS groups (65.67±6.24% vs. 99.57±2.86% respectively; p=0.003).

ACh elicited vasodilatation in all rings tested, and this response was significantly attenuated in rings pretreated with L-NAME **[Figures 5.2 & 5.4]**. The magnitude of the dilator response was smaller in the LPS group compared with equivalent rings from sham rats, although this difference did not reach statistical significance.

5.4 DISCUSSION

In these experiments, the interaction between ET-1-induced contraction and NO was explored. As before, the maximum contraction induced by ET-1 was significantly reduced in vessels from animals treated with LPS. It is now accepted that iNOS is expressed in various organs of the rat after LPS (Liu et al. 1993), and specifically it has been shown recently that iNOS is induced in rat PA in this model of sepsis (Griffiths et al. 1995a). In the present study, the NOS inhibitor, L-NAME, significantly potentiated ET-1-induced contraction in both sham and LPS rings. Interestingly, this effect reversed the hyporesponsiveness to ET-1 observed in tissues from LPS-treated animals, thus demonstrating that the induction of NOS in this tissue modulates ET-1-induced constrictor tone. These data have important implications. L-NAME is a non-specific NOS inhibitor. In rings from controls, the L-NAME-mediated increase in ET-1-induced T_{max} was of the same order of magnitude as in rings from LPS rats. The contribution of cNOS-derived NO to ET-1-induced vascular tone in these experiments appears to be as significant as the contribution of iNOS-derived NO is to rings from LPS-treated rats. This is surprising, because it might be expected that the response to non-specific NOS inhibition in vessels from LPS rats would be greater than in those from control animals, through inhibition of both cNOS- and iNOS-derived NO. However, there is preliminary evidence that cNOS activity is down-regulated in these tissues as iNOS activity increases (Liu et al. 1995), and these data would be consistent with that hypothesis. Furthermore, in both types of PA ring, NOS inhibition caused an increase in baseline tension, although this effect was much greater in rings from endotoxaemic rats. This implies that the relative contribution of iNOS to basal tone is greater in the LPS rings, and that the

modulation of ET-1-induced increases in tension in control rings by cNOS-derived NO must result from ET_B receptor activation, rather than as a result of continuous basal production of NO. These L-NAME effects are in contrast to the results of endothelial removal on ET-1-induced contraction seen in Chapter 4, which decreased T_{max} in both sham and LPS-treated groups. This implies that both endothelial cNOS and vascular smooth muscle iNOS contribute to the NO-dependent "brake" on ET-1-induced contraction. This also confirms that maximal ET-1-induced increases in tension are dependent on the presence of the endothelium in an NO-independent fashion.

The ET_B agonist, sarafotoxin S6c, had no direct constrictor action on PA from sham or LPS-treated animals. Furthermore, even when tissues were preconstricted with U46619, S6c caused no constriction of tissues from any of the groups tested. These observations show that no component of ET-1-induced contraction of any of the PA rings tested, including the endothelium-derived vasoconstrictor released by ET-1 in PA rings from LPS-treated rats, is the result of ET_B receptor activation. Indeed, endothelium-intact PA from sham rats, when constricted by U46619, relaxed in the presence of 10^{-7} M S6c. The S6c-induced relaxation was transient, tachyphylactic and occurred in only 6 out of 10 rings tested. By contrast, the dilator action of ACh in the same tissues was longer-lasting and present in tissues from all animals. L-NAME significantly inhibited the relaxation induced by both S6c and ACh, suggesting that both drugs act via the release of NO from the endothelium. Interestingly, none of the tissues prepared from LPS-treated rats relaxed to S6c, and their responses to ACh were attenuated, although this did not reach significance. These observations suggest that endothelial release of NO is reduced after LPS and is consistent with the decrease in eNOS mRNA seen in this tissue under the same conditions (Liu et

al. 1995). Previous studies using isolated rat PA rings have not shown a reduction in ACh-mediated relaxation in vessels from LPS-treated animals (Griffiths et al. 1993), but this has been seen in rabbit aortic rings (Umans et al. 1993). The loss of S6c-induced dilatation, and reduction in ACh effects, could be an early indication of LPS-mediated endothelial dysfunction, despite the absence of structural changes on electron microscopy. It is unclear why only 6 out of 10 sham rings responded to S6c, although this may be related to tachyphylaxis. The dilator effects of S6c are short-lived and only occur at the lowest concentration of drug used, and it is possible that the 4 non-responders were subject to higher levels of circulating ET *in vivo*. If this were the case, it could be that in these animals, ET_B receptors on PA endothelium were down regulated *in vivo*, in a similar way to the down-regulation of ET_B receptors that has been demonstrated under other pathophysiological conditions (Yorikane et al. 1993).

This study clearly demonstrates that the vascular actions of ET-1 in the rat PA are modified following LPS and that NO plays an active role in these modifications. All of the constrictor actions of ET-1, including those mediated by the endothelium, are achieved via activation of ET_A receptors. The functional effect of ET_B activation is, where present, vasodilatation, thus antagonising ET_A-mediated contraction. The finding that the endothelium modulates both the constrictor and dilator effects of ET-1 in control and endotoxaemic rat PA illustrates the importance of the role of endothelial cells in the vascular responses to sepsis.

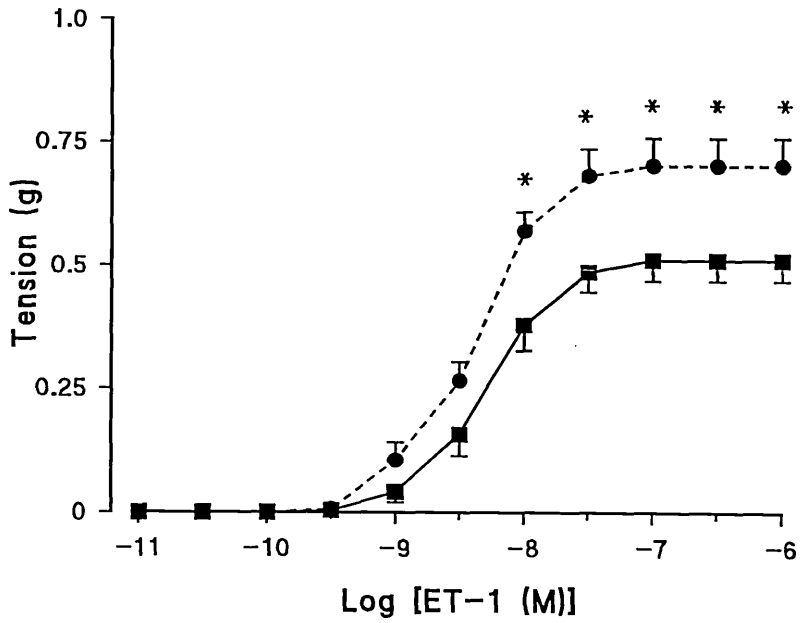
Table 5.1 **Response of pulmonary artery rings to ET-1 with and without L-NAME pretreatment.**

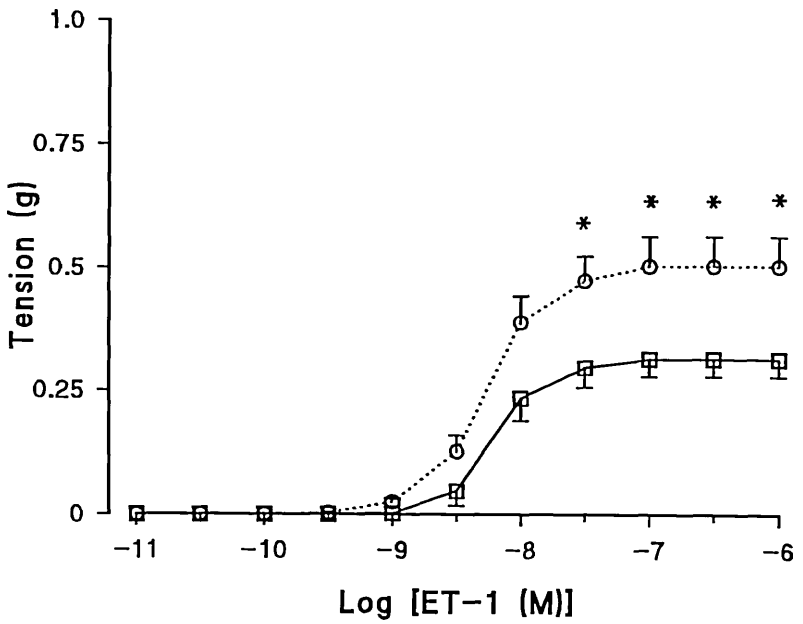
Ring	type	n	Tmax (g)	EC_{50}
LPS-	DH_2O	6	0.51±0.04	8.30±0.08
LPS-	L-NAME	6	0.70±0.06	8.42±0.06
LPS+	DH_2O	5	0.32±0.04	8.17±0.08
LPS+	L-NAME	5	0.51±0.06	8.26±0.07

There were no statistically significant differences bewteen the EC_{50} values.
For differences between T_{max} values,see Figure 5.1.
Note the similarity between T_{max} for the sham control rings and the LPS, L-NAME treated group.

Figure 5.1 **ET-1-induced contraction of PA rings: effects of N^{w}-nitro-L-arginine methylester (L-NAME)**

ET-1-induced contraction of intact PA rings from sham (UPPER PANEL; solid symbols) or LPS-treated (LOWER PANEL; open symbols) rats in the presence of 10^{-4}M L-NAME (circles) or vehicle (squares). Results are mean±SEM for n=6 in all groups. *p* values: * <0.05.





These figures are actual traces, showing continuous transduced tension, starting from baseline before any drugs are added.

UPPER PANEL

Trace showing typical dilator response to 10^{-7}M sarafotoxin S6c (**S**) in a PA ring from a rat that was constricted with 10^{-6}M U46619 (**U**), a thromboxane mimetic. A discrete, short-lived relaxation is seen after this dose of S6c which is not repeated with subsequent doses (marked by |). By contrast, acetylcholine (**A**) (10^{-4}M) elicits a larger, longer-lasting dilatation.

MIDDLE PANEL

Trace of a PA ring from a sham rat showing the effect of pretreatment with 10^{-4}M N^{w}-nitro-L-arginine-methylester (L-NAME) 15 minutes before the U46619. Dilator responses to both the S6c and acetylcholine are attenuated.

LOWER PANEL

Typical trace of a PA ring from a LPS-treated rat that is constricted with 10^{-6}M U46619. There is negligible dilatation in response to S6c, but the acetylcholine response is preserved.

Figure 5.2 **Typical responses of preconstricted PA rings to sarafotoxin S6c**

(see opposite page for legend)

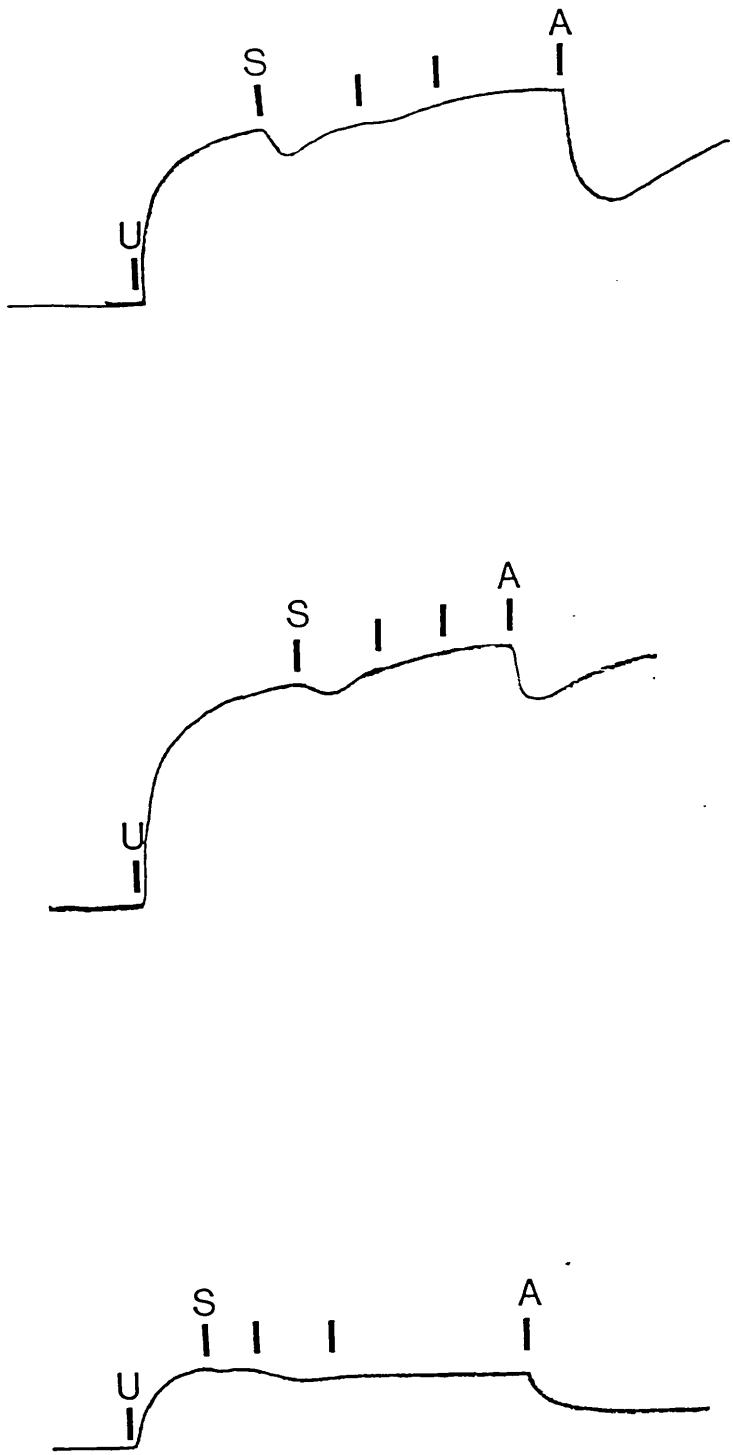


Figure 5.3 **Sarafotoxin S6c-induced dilation of preconstricted PA rings: effects of LPS and N^{w}-nitro-L-arginine methylester (L-NAME)**

Pooled data showing responses of 10^{-6}M U46619-preconstricted PA rings from sham (UPPER PANEL) or LPS-treated (LOWER PANEL) rats to sarafotoxin S6c alone (open bars), distilled water (solid bars) or sarafotoxin S6c after 10^{-4}M L-NAME (hatched bars). Results are mean±SEM for n=6, and represent the percentage of the contraction induced by U46619. Statistical analysis between all groups was performed using ANOVA with Tukey-Kramer post tests. *p* values vs. distilled water: * <0.05; ** <0.01.

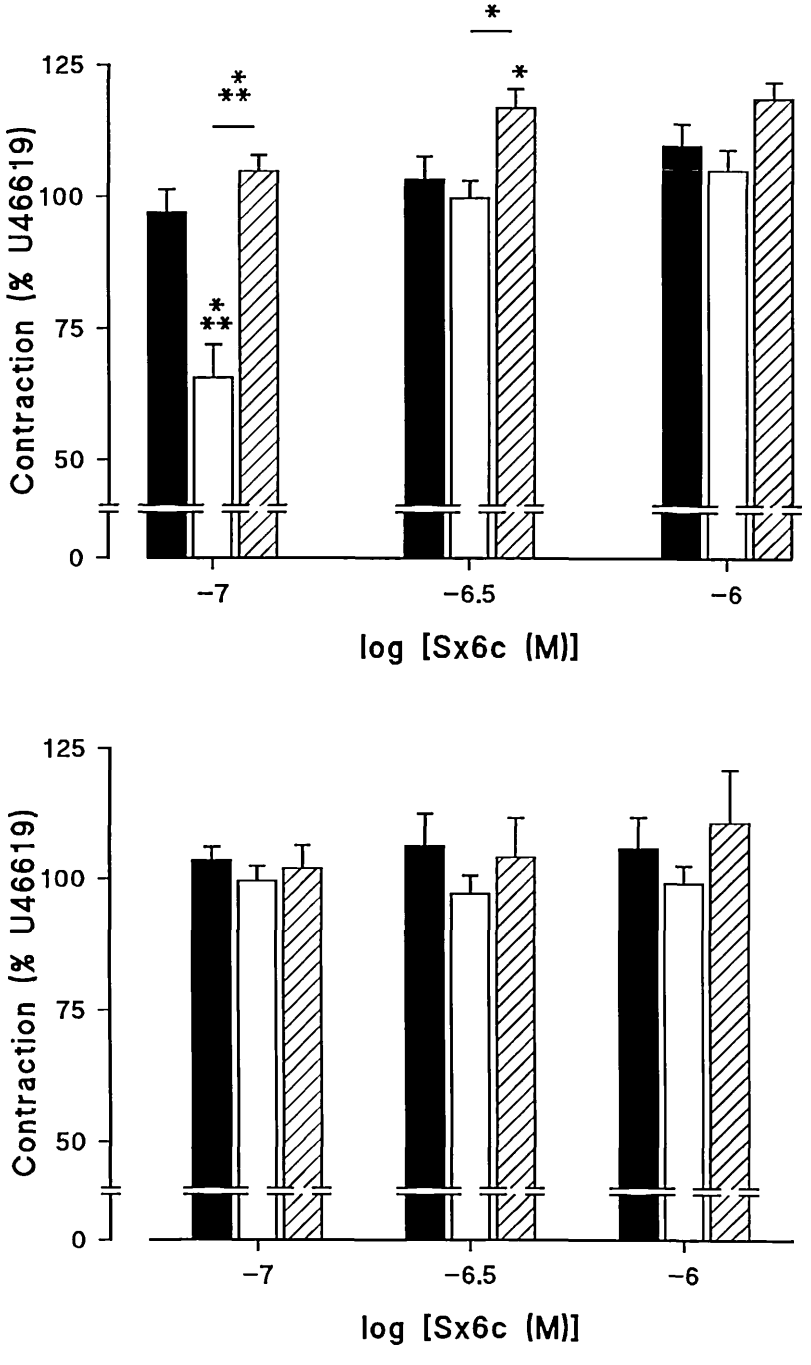
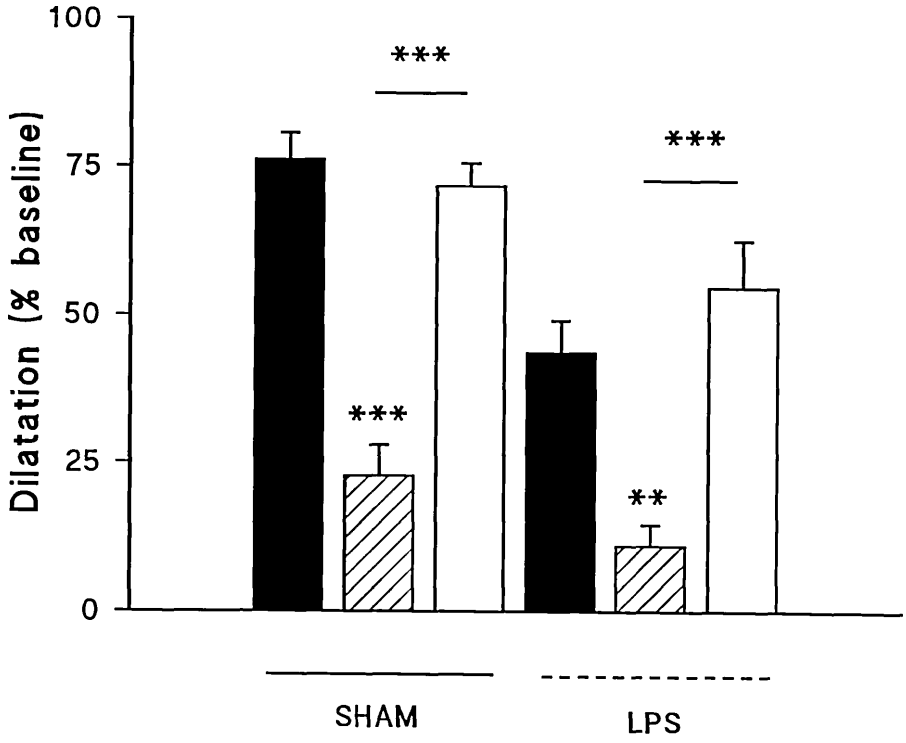


Figure 5.4 **Acetylcholine-induced dilator responses of preconstricted PA rings from sham and LPS-treated rats**

Dilator responses of rings preconstricted with 10^{-6}M U46619 to 10^{-4}M acetylcholine after 3 doses of sarafotoxin S6c (open), distilled water (solid) or S6c after pretreatment with 10^{-4}M L-NAME. Results are expressed as dilatation as a percentage of the baseline tension. All groups (n=6) were compared using ANOVA with Tukey-Kramer post tests. ** $p<0.01$; *** $p<0.001$.



CHAPTER 6

CHARACTERISATION OF THE EFFECTS OF ET_A AND ET_B RECEPTOR ACTIVATION IN THE ISOLATED, BLOOD-PERFUSED PULMONARY CIRCULATION OF SHAM OR LPS-TREATED RATS

6.1 INTRODUCTION

Sepsis is characterized by systemic vasodilatation and hyporesponsiveness to vasoconstrictors such as catecholamines (Wakabayashi et al. 1993), attributed, at least in part, to the release of endogenous vasodilator mediators. In sepsis, LPS-derived endotoxin stimulates the induction of NO synthase (iNOS) (Liu et al. 1993; Griffiths et al. 1995a) resulting in the release of copious amounts of NO. Thus, NOS inhibitors induce a demonstrable attenuation in the fall in blood pressure both in animal models of sepsis and in critically ill patients (Griffiths et al. 1994).

Sepsis is also associated with a reduction in regional blood flow to the lung, kidney and mesentery. An elevation in PVR has also been detected in such circumstances, leading to secondary right heart failure (Zapol and Snider, 1977; Sibbald and Driedger, 1983). Furthermore, both molecular and biochemical evidence has been presented that iNOS is expressed in the PA and lungs of rats treated with LPS (Liu et al. 1993; Griffiths et al. 1995a). Thus, despite a probable increase in local NO production in such patients, pulmonary vasoconstriction occurs. This suggests that, in parallel with local NO release, there may be increased production and/or availability of vasoconstrictor agents. In Chapter 3, it

was demonstrated that pre-pro-ET-1 mRNA expression is increased in whole lung tissue and PA of rats treated *in vivo* with LPS. Although ET_B receptors have been demonstrated on rat PA vascular smooth muscle (Eddahibi et al. 1993), there is no evidence that these receptors contribute directly to ET-1-induced PA contraction [Chapter 5]. In Chapter 4, ET-1-induced contraction of isolated rat PA was shown to be attenuated by LPS treatment *in vivo*, but the precise interaction between local vasodilators (NO, PGI_2) and vasoconstrictors (ET-1, TXA_2) in the pulmonary circulation as an intact unit is as yet unknown. To investigate this, the isolated blood-perfused lung (IBPL) model has several advantages over studies on isolated main PA. Firstly, the vasoactive agents to be assessed are presented in a controlled manner to the whole pulmonary circulation in correct sequence. Clearly this is important, because the responses of different parts of the vascular bed may vary, and one of the reasons for that variation may be the behaviour of "up-" or "downstream" components, acting either directly or via other mechanisms, such as metabolism of the agent given. For example, previous investigations on isolated perfused rat lung (IPRL) have suggested that whilst ET-1 itself causes constriction of both pre- and post-capillary pulmonary vessels, ET_B receptor activation alone causes predominantly post-capillary constriction (Uhlig et al. 1995). A second important advantage of the IPRL is the ability to adjust the rate of perfusate flow so that the basal PA pressure (Ppa) achieved is comparable to those found *in vivo*.

Apart from the differential effects of ET_A and ET_B receptor activation in the rat IPRL preparation, however, induction of oedema formation has also been demonstrated in the lung by ET-1. Some authors have suggested (Rodman et al. 1992) that this is purely due to hydrostatic forces created as a result of venoconstriction, so that a rise in post-capillary pressure induces increased

capillary fluid leakage. In fact, there is evidence that oedema formation depends upon the nature of the perfusate: ET-1 was found to cause oedema in salt-solution but not in blood-perfused lungs. Both of these factors are predictably important according to the Starling equation:

$$\text{Net fluid shift} \propto [(\Delta AVP) + (iOP)] - [(iP) + (pOP)]$$

where: ΔAVP= arterio-venous pressure difference; iOP= interstitial oncotic pressure; iP= interstitial hydrostatic pressure; pOP= plasma oncotic pressure.

The hypothesis was that, in contrast to isolated PA, the pulmonary circulation of LPS-treated rats would exhibit hyperresponsiveness to ET-1 when compared to controls, a finding that, if proven, would improve our understanding of the mechanisms that contribute to the PHT that is associated with acute lung injury.

There were 4 main aims for this group of experiments.

(1) In order to validate the model, experiments were performed in which the IBPL preparation from both LPS-treated and control rats were perfused at a constant rate for 45 minutes, and the Ppa and tendency to oedema formation were measured. In addition, since it was found in preliminary experiments that the starting Ppa was higher in the lungs from LPS-treated animals, an attempt was made to control for differences between preparations by "cross-transfusion" studies, in which the lungs from control rats were perfused with blood from LPS-treated animals, and *vice versa*.

(2) To assess the vascular effects of ET_A and ET_B receptor activation, as well as oedema formation, in the isolated, blood-perfused pulmonary circulation of rats treated with LPS *in vivo*, or controls. As well as ET-1, the selective ET_A receptor antagonist, BQ123, the selective ET_B receptor agonist, sarafotoxin S6c, and the

non-selective ET_A/ET_B receptor antagonist, bosentan, were used in these experiments.

(3) To determine whether the ET-1-induced vascular changes were modulated by NO, prostanoids, or TXA_2 by pretreatment of the preparation with the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA), the cyclooxygenase inhibitor, indomethacin, and the TP receptor antagonist, ICI 192605.

(4) To study the effects of the TXA_2 mimetic, U46619, on the preparation in order to establish whether LPS-induced differences in ET-1-induced effects were specific.

6.2 EXPERIMENTAL PROTOCOLS

6.2.1 Validation of the model

The following experiments were performed in order to validate the model and/or control for some of the interventions made later.

i) *Perfusion only*

Rats were prepared as described above. The system was perfused for 45 minutes to monitor Ppa and establish that there was no spontaneous change in either sham or LPS-treated animals. In addition, the middle and lower lobes of the right lung were removed and wet:dry ratio assessed as described below.

ii) *Cross-transfusion*

In order to establish whether the effects of LPS on Ppa were due to (a) small differences in blood volume, (b) blood-borne mediator(s) or (c) the pulmonary vasculature itself, cross-transfusion experiments were performed. In these, the lungs from exsanguinated LPS-treated rats were perfused with the blood from sham rats, and *vice versa*.

6.2.2 To assess the effects of the vasoconstrictors ET-1 and U46619 on Ppa in sham and LPS-treated rats

i) *Effects of ET-1 on Ppa*

Four concentrations of ET-1 (10^{-9} to $3x10^{-8}$M) were added to the reservoir in a cumulative fashion. Each dose was added after the rise in Ppa from the previous concentration had achieved a plateau. The experiment was terminated when (a)

the Ppa plateaued after the final dose, or (b) Ppa reached 60mmHg, or (c) 45 minutes elapsed since the final dose and neither (a) or (b) had been achieved.

ii) *Effects of U46619 on Ppa*

Following equilibration, Ppa response was assessed to cumulative concentrations of the TXA_2 mimetic, U46619, over the range 10^{-9}M to $3x10^{-6}$M in both sham and LPS-treated rats.

6.2.3 Effects of NOS inhibition on ET-1-induced Ppa responses in sham and LPS-treated rats

In separate experiments, the ET-1 cumulative concentration response was studied in sham and LPS-treated rats 15 minutes after pretreatment with either L-NMMA (10^{-5} or 10^{-3}M), or distilled water.

6.2.4 The effects of COX inhibition and thromboxane receptor antagonism on ET-1-induced Ppa responses in sham or LPS-treated rats

Cumulative concentration-dependent responses to ET-1 were studied in sham and LPS-treated rats 15 minutes after pretreatment with either:

I) 10^{-5}M indomethacin or diluted indomethacin buffer (0.02M KH_2PO_4, 0.12M Na_2HPO_4)

ii) 10^{-5}M ICI 192605, a TP receptor antagonist (Jessup et al. 1988), or diluted ethanol vehicle.

6.2.5 Effects of selective ET_B receptor activation on Ppa responses in sham and LPS-treated rats

Three concentrations (10^{-9} to 10^{-8}M) of sarafotoxin S6c were added to the reservoir in a cumulative manner, and the Ppa response assessed. Each dose was added at the plateau of the response to the previous dose. The nature of the response differed between ET-1 and S6c, so that the final dose of S6c caused a rapid rise in Ppa that did not plateau. Because of this, these experiments were terminated (arbitrarily) when the Ppa reached 50mmHg.

6.2.6 Effects of an ET_A receptor antagonist on ET-1- and S6c-induced Ppa responses in sham and LPS-treated rats

The following were added 15 minutes prior to the first dose of ET-1 or S6c:

i) 10^{-5}M BQ123

ii) distilled water

6.2.7 Effects of combined ET_A/ET_B antagonism on S6c-induced Ppa responses in sham and LPS-treated rats

Bosentan (10^{-5}M), or distilled water, were added 15 minutes before the first dose of S6c.

6.3 RESULTS

6.3.1 General properties

A trend was observed for the starting Ppa (ie. after equilibration, before any intervention) to be higher in LPS-treated rats. Pooling all the baseline Ppa data, a highly significant difference was confirmed (19.48±0.28 mmHg in sham, n=84; vs 22.60±0.28 in the LPS group, n=81; $p<0.0001$). The starting Ppa in the LPS-treated rats perfused with blood from sham animals was also higher than that of the sham rats perfused with blood from LPS rats (23.60± 1.03, n=5; and 18.40±1.08, n=5, respectively, $p=0.008$).

(i) *Perfusion only*

In both sham and LPS groups, Ppa remained stable for the full 45 minutes post equilibration. In no preparation did the Ppa vary from baseline by more than 2mmHg. Wet:dry lung weight ratios in the sham and LPS groups were 5.60±0.15 (n=6) and 5.91±0.38 (n=5) respectively, ($p= 0.44$). After 45 min perfusion, the amplitude index for sham rats was 88.67±8.27%, and for LPS rats 86.40±8.42%; $p=0.85$.

6.3.2 Effects of ET-1 and U46619 on Ppa in sham or LPS-treated rats

ET-1 caused an increase in Ppa in all rats tested, the response to ET-1 in LPS rats being greater. This difference was particularly obvious for the final concentration of ET-1 ($3x10^{-8}M$), when the rate of rise in Ppa was also more rapid in the LPS rats **[Figure 6.1]**. Ppa following this concentration of ET-1 was

therefore measured at three time points (1,5,10 minutes) as well as the peak pressure **[Figure 6.2]**. However, no significant differences were observed in the wet:dry lung weight ratios **[Table 6.1]** or in the amplitude indices in sham versus LPS-treated rats **[Figure 6.3]**. U46619 caused similar concentration-dependent increases in Ppa in both sham and LPS groups **[Figure 6.4]**.

6.3.3 Effects of L-NMMA pretreatment on ET-1-induced Ppa responses

L-NMMA at 10^{-3}M, but not at 10^{-5}M, caused an increase in baseline Ppa in sham rats (0.86±0.40mmHg; n=7), and a larger increase in Ppa from the starting value in the LPS rats (5.50±1.80mmHg; n=6; p=0.02). Further, in both groups, this concentration of L-NMMA, but not 10^{-5}M, resulted in significant potentiation of the ET-1-induced Ppa responses **[Figure 6.5]**.

6.3.4 Effects of indomethacin or ICI 192605 pretreatment on ET-1-induced Ppa changes

Neither ICI 192605 (10^{-5}M) nor indomethacin (10^{-5}M) had a significant effect on the contractile responses of ET-1 in either sham or LPS-treated rats **[Figure 6.6]**.

6.3.5 Effect of Sarafotoxin S6c (S6c) on Ppa responses

S6c caused a concentration-dependent increase in Ppa in both sham and LPS-treated rats. Similar to the effects of ET-1, S6c was more potent in the LPS

group **[Figure 6.7c]**, and increased Ppa more potently, on a molar basis, than ET-1 in both groups **[Figure 6.7a & b]**. Following the final concentration of S6c, pulmonary oedema rapidly developed, reflected by an increase in wet:dry lung weight ratios **[Table 6.1]**, and decreased amplitude index **[Figure 6.3]**. In fact, although there is a clear trend suggesting that the S6c-induced wet:dry weight ratios are higher than those of the equivalent ET-1 groups, neither of these differences achieved significance. However, the parallel decrease in amplitude index induced by S6c was significantly different from that in the ET-1 groups. Typical traces obtained using this technique are shown in **Figure 6.8**. Finally, the wet:dry ratio in sham rats treated with S6c was significantly higher than for LPS-treated animals.

6.3.6 Effect of BQ123 pretreatment on ET-1- and S6c-induced Ppa responses

(i) Pretreatment with 10^{-5}M BQ123 caused significant attenuations in ET-1-induced responses in both sham and LPS groups **[Figure 6.9]**. However, BQ123 treatment had no effect on the wet:dry lung weight ratios in either sham or LPS-treated rats **[Table 6.1]**.

(ii) Pretreatment with 10^{-5}M BQ123 had no effect on S6c-induced responses in either group.

6.3.7 Effect of bosentan on S6c-induced responses

Bosentan (10^{-5}M) did not significantly influence starting Ppa in either sham or LPS animals. However, bosentan significantly attenuated the effects of S6c on

Ppa in the LPS-treated rats, but not in the sham group **[Figure 6.10]**. Bosentan pretreatment did not significantly alter the wet:dry weight ratios in sham or LPS groups compared to those treated only with S6c **[Table 6.1]**, but again the sham bosentan rats had significantly higher wet:dry ratio than the LPS bosentan animals (p=0.01).

6.3.8 Effects of cross-transfusing blood on responses to ET-1 in sham and LPS-treated rats

The starting Ppa was higher in the LPS animals (with sham blood) than in sham animals (with LPS blood). In addition, the Ppa responses in the LPS animal group were more sensitive than those of the sham rats **[Figure 6.11]**. The wet:dry weight ratios and amplitude indices were no different compared to the conventional ET-1 groups.

6.4 DISCUSSION

Sepsis syndromes in man are commonly associated with PHT, which can contribute to hypoxaemia and right heart failure. The cardiovascular changes occurring under these conditions have become associated with a shift in the ratio of locally produced and circulating levels of vasodilator and vasoconstrictor agents. In particular, NO, and ET-1, have been implicated as being important determinants of vascular function in sepsis. Evidence has accumulated to suggest that there is increased NO production in the pulmonary circulation and lung during endotoxaemia in rats, which contributes to the hyporesponsiveness of this circulation to catecholamines. By contrast, the effects of endotoxaemia on the response of the pulmonary circulation to ET-1, and the modulation of these responses by NO, are unclear. In this study, the effects of LPS-induced endotoxaemia on the Ppa and oedema responses to ET-1 in IBPL were therefore investigated.

The isolated lung model has been employed by others in this context. These investigations have suggested that whilst ET-1 itself causes constriction of both pre- and post-capillary pulmonary vessels, ET_B receptor activation alone causes predominantly post-capillary constriction (Uhlig et al. 1995). However, the induction of oedema formation in the lung by ET-1, as well as hydrostatic forces, appears to depend upon the perfusate: thus, ET-1 was found to cause oedema in salt solution but not in blood-perfused lungs (Rodman et al. 1992). In addition to uncertainty regarding the "direct" vascular effects of ET-1 in the pulmonary bed, it is also unclear what are the effects of the prostanoids PGI_2, $PGF_{2\alpha}$, and TxA_2 or NO that have been shown to be released by ET-1 in the rat (Reynolds

and Mok, 1989; D'Orleans-Juste et al. 1992; de Nucci et al. 1988; Topouzis et al. 1991; D'Orleans-Juste et al. 1993; Uhlig et al. 1995).

Consistent with the PHT often seen clinically in sepsis, there was a significant difference in basal Ppa, and therefore PVR, in the LPS-treated rats. Moreover, this increase was not blocked by BQ123 or bosentan, suggesting that circulating ET-1 did not contribute directly to it. Similarly, neither the COX inhibitor, indomethacin, nor the TP receptor antagonist, ICI192605, had an effect on basal Ppa. Furthermore, basal Ppa remained elevated in LPS-treated rats perfused with blood from sham rats, suggesting that the increase in Ppa after LPS treatment is due to vascular inflammatory processes stimulated in response to LPS, such as neutrophil accumulation and thromboembolism, as opposed to an elevation in circulating vasoconstrictors. In addition to increased basal Ppa, the pulmonary circulation from LPS-treated rats was more sensitive to the constrictor actions of both ET-1 and S6c. These observations are consistent with the increased sensitivity to angiotensin II observed in this model previously under the same conditions (Griffiths et al. 1995b). By contrast, the constrictor actions of the TXA_2 mimetic, U46619, were unaltered in the lungs of LPS-treated animals. This implies that the increase in responsiveness to ET-1 and S6c seen in this study was due to up-regulation of ET receptor sub-types rather than to non-specific alterations in the response of the vascular smooth muscle to vasoconstrictors. Alternatively, an increase in the sensitivity to some vasoconstrictors could be the result of a regional decrease in the facility of the pulmonary vasculature to release vasodilators, especially NO. Indeed, our group has shown that messenger RNA for eNOS is reduced in the rat lung after LPS *in vivo* (Liu et al. 1995), and that the functional dilator response to ACh is greatly

attenuated under similar conditions in the IBPL preparation (Griffiths et al. 1995b). The results from experiments in Chapter 5 also suggest a reduction in ET_B-mediated NO release from isolated PA rings derived from LPS-treated rats. Paradoxically, the administration of LPS to rats causes iNOS induction in various cells, including vascular smooth muscle (Fleming et al. 1991), and specifically an increase in iNOS mRNA in the PA (Griffiths et al. 1995a). This induction of iNOS causes the hyporesponsiveness to vasoconstrictors in isolated main PA (Griffiths et al. 1995a), and presumably the local balance between these two sources of NO production determines both local and regional pulmonary vascular tone and responsiveness to other vasoactive agents. In this study, the non-specific NOS inhibitor, L-NMMA, increased basal Ppa in both sham and LPS-treated rats, but to a much greater extent in the latter group. In addition, L-NMMA potentiated the constrictor actions of ET-1 in both groups of animals. Interestingly, in the presence of L-NMMA, the constrictor effects of ET-1 remained greater in the LPS-treated than in control groups. These observations suggest that, in the pulmonary circulation, the expression of iNOS after LPS only partially compensates for an increased sensitivity to ET-1 and/or a reduction in endothelial NOS activity. These results may have important implications for the currently proposed policy to treat septic patients with NOS inhibitors (Griffiths et al. 1994).

By contrast to the significant changes seen with NOS inhibition, no contribution to the ET-1-induced Ppa changes from COX-derived products, especially TXA_2, could be demonstrated. Other groups have clearly demonstrated release of PGI_2, $PGF_{2\alpha}$, and TXA_2 in IPRL models (Barnard et al. 1991; Uhlig et al. 1995). It is possible that in this preparation there is a balanced

release of these COX products so that their removal has little effect. These results imply that COX products do not play an important role in ET-1-mediated constrictor effects, as long as the dose of indomethacin chosen was adequate to inhibit COX activity.

Sarafotoxin S6c was more potent, on a molar basis, at inducing rises in Ppa than ET-1. At the final concentration of S6c (10^{-8}M), Ppa rose sharply, without reaching a maximum response, and the lungs became obviously oedematous. This oedema formation was reflected by a trend towards an increase in wet:dry lung weight ratio, and a significant decrease in amplitude index. The latter technique was set up because of the poor sensitivity of wet:dry ratios in reflecting changes in lung oedema formation that were obvious to the naked eye. Using this measurement, the rise in Ppa in response to S6c, but not ET-1, was clearly associated with oedema formation. It is possible that, as suggested by other groups, the oedema formation simply reflects raised hydrostatic pressure associated with venoconstriction in response to ET_B receptor activation. This would not explain, however, the increased sensitivity of Ppa rise to S6c compared to ET-1. Since ET-1 acts on both ET_A and ET_B receptors, a component of the ET-1-induced effects on this model should be as a result of the same ET_B-mediated actions as seen with the ET_B agonist. This theory was tested by pretreatment with BQ123. This effectively attenuated ET-1-induced increases in Ppa, regardless of endotoxaemic status, and no component of the response (that could have been attributable to ET_B receptor activity) was spared. It is possible that ET-1 is metabolised so efficiently as it passes from one side of the capillary to the other that it is not available on the post-capillary side to stimulate the ET_B receptors there. However, the uptake and metabolism of

radiolabelled ET-1 has previously been studied in the isolated perfused rat lung (Westcott et al. 1990). When a low concentration of ^{125}I-labelled ET-1 ($4x10^{-12}$M) was given, over 90% of the label was removed by 5 minutes, but when $5x10^{-9}$M concentration was given, 73% of the label remained in the perfusate. Subsequent metabolism of the radiolabel was slow, more than 85% of the ET being intact 30 minutes after uptake. Autoradiography indicated that it was primarily located in the alveolar wall rather than in larger pulmonary vessels. It therefore seems unlikely that the ET-1 in these experiments was inactivated or removed from the circulation in high enough amounts to prevent its access to post-capillary ET_B receptors. Nor does it seem possible, in the light of the BQ123 data, that ET_A-mediated activity is protective or antagonises ET-1-activated ET_B-induced oedema formation. It therefore became important to exclude possible non-ET_B-mediated effects of S6c. In fact, BQ123 had no effect on the S6c changes, whilst bosentan, even at a dose that has previously been shown to antagonize ET-1 activity both *in vitro* (Clozel et al. 1994) and in the isolated lung (Uhlig et al. 1995), only caused modest attenuation of the S6c-induced Ppa changes. If the ET_B agonist caused oedema formation purely through venoconstriction, a discrepancy is apparent between the significantly reduced wet:dry ratio in the LPS S6c rats and the slight increase in the Ppa response detected in this group. It seems likely that the ET_B-mediated oedema formation is due to more than this simple hydrostatic mechanism, and on that basis, it is impossible to exclude an effect dependent upon a non ET_A-non ET_B receptor.

In summary, ET-1 induces vasoconstriction, with resultant increases in Ppa, but without statistically significant oedema formation in the IBPL, despite a clear trend in the wet: dry weight ratios. The sensitivity to ET-1 is increased in

rats pretreated *in vivo* with endotoxin. Both sham and LPS-treated preparations exhibited potentiation of this effect in the presence of 10^{-3}M L-NMMA, although this agent caused a significant rise in baseline Ppa only in the LPS rats. The pulmonary circulation is more sensitive to S6c at equivalent concentrations, and this ET_B-mediated activity includes oedema formation that is unexpectedly less in LPS lungs than in sham ones. The S6c-mediated effects on oedema are not explained by the simple theory of a post-capillary increase in hydrostatic pressure.

The elevated basal Ppa, and the increased sensitivity of the pulmonary vasculature to ET-1 is consistent with the PHT seen in patients with septic shock, and suggest a functional up-regulation of both ET_A and ET_B receptors in the pulmonary circulation in septic conditions. These experiments may also have important implications for the use of non-specific NOS inhibitors in patients with acute lung injury/sepsis, since the increased sensitivity of LPS-treated lungs to ET-1 after NOS inhibition suggests that patients with sepsis may experience further pulmonary vasocontriction after such treatments.

Table 6.1 Lung wet:dry weight ratios

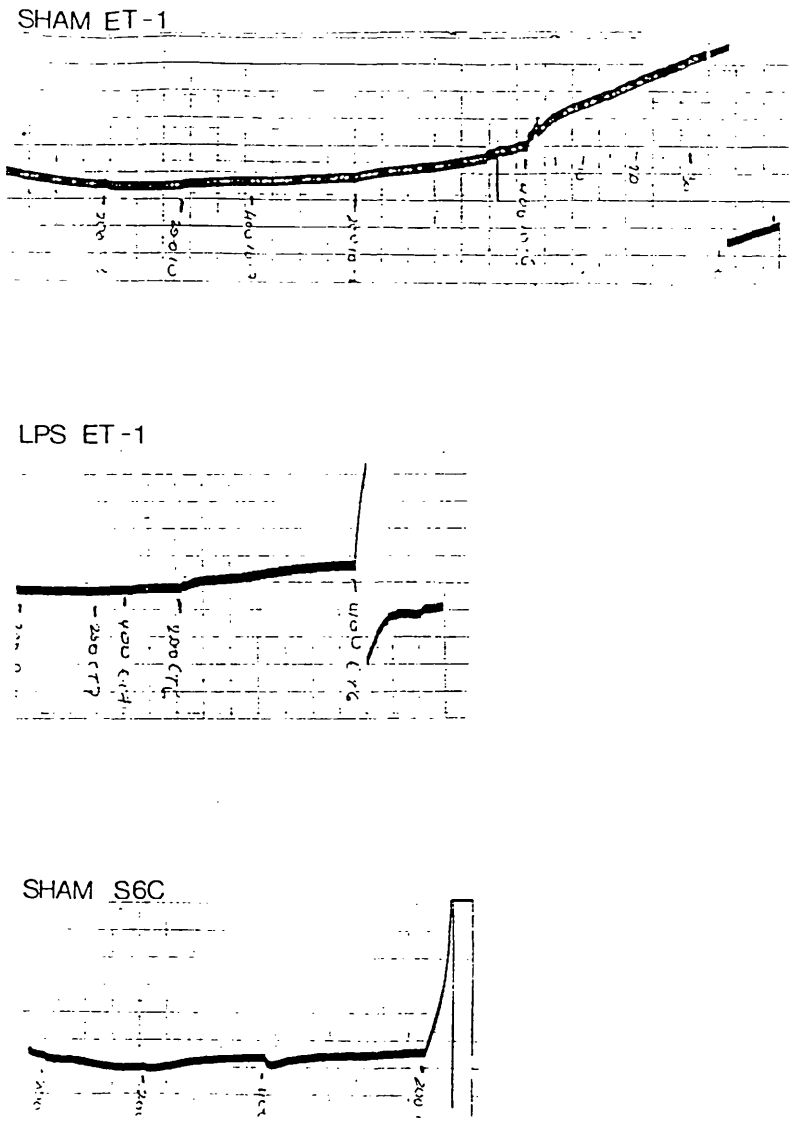
	Group	n	Mean±SEM
S	Perfusion only	6	5.60±0.14
H	ET-1 alone	6	7.49±1.11
A	S6c alone	6	9.26±0.41
M	ET + BQ123	6	8.85±1.70
	S6c + BQ123	5	9.23±1.32
	S6c + Bosentan	5	8.72±0.77
	sham rat with LPS blood-ET-1 alone	5	8.06±1.33
L	Perfusion only	5	5.91±0.38
P	ET-1 alone	6	6.39±0.50
S	S6c alone	6	7.12±0.66
	ET + BQ123	5	6.08±0.12
	S6c + BQ123	6	8.11±1.12
	S6c + Bosentan	6	6.43±0.25
	LPS rat with sham blood-ET-1 alone	5	6.89±0.38

Statistical analysis

When a direct comparison between 2 groups was sought, it was made using Student's *t* test, producing the following results:

	p value
Sham ET-1 *vs* Sham perfusion only	0.05
Sham S6c *vs* Sham perfusion only	<0.0001
Sham S6c *vs* LPS S6c	0.02
Sham (S6c + Bosentan) *vs* LPS (S6c + Bosentan)	0.01

Figure 6.1 **Typical pulmonary artery pressure responses to ET-1 and sarafotoxin S6c in the isolated, blood-perfused lung**



Each trace represents the hard copy of the continuously monitored Ppa in an individual animal (at 70% of the original size). Note the difference in the rate of rise in Ppa at the final concentration of ET-1 in the sham and LPS-treated rats. This is reflected in the way the results for groups of rats are displayed graphically.

Figure 6.2 **ET-1-induced increases in pulmonary artery pressure in sham and LPS-treated rats**

Starting pulmonary artery pressure (Ppa) and effect of 4 concentrations of ET-1 in sham (solid circles; n=6) or LPS-treated (open circles; n=6) rats. Results are expressed as mean±SEM. * $p<0.05$.

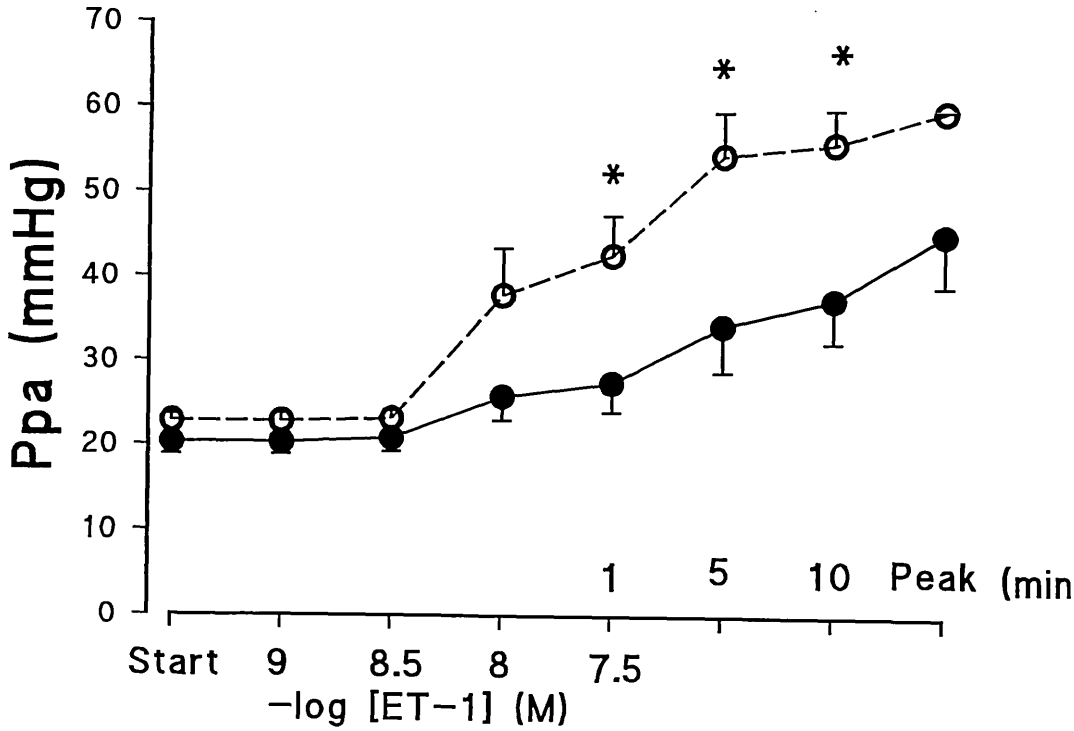


Figure 6.3 **Amplitude indices of sham or LPS-treated rats during treatment with either ET-1 or sarafotoxin S6c**

The amplitude index is derived from the movement of the right upper lobe of the lung during the ventilatory cycle. The index is proportional to the pulmonary compliance, and therefore goes down when the lung accumulates oedema fluid. Statistical analysis was by Student's *t* test. *p* values: * <0.05.

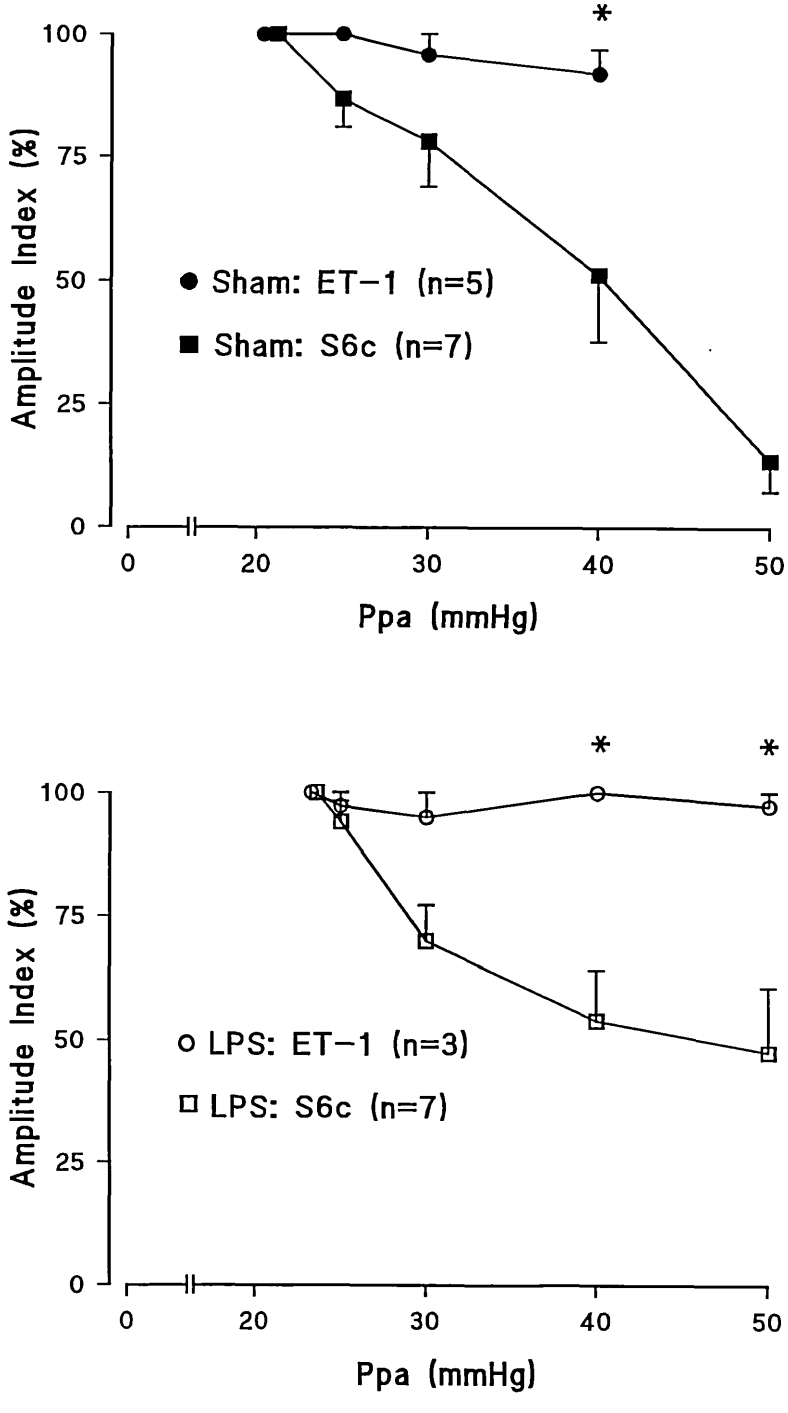


Figure 6.4 **U46619-induced increases in pulmonary artery pressure in sham and LPS-treated rats**

Starting pulmonary artery pressure (Ppa) and effect of increasing concentrations of U46619 in sham (solid squares; n=6) or LPS-treated (open squares; n=6) rats. Results are expressed as mean±SEM.

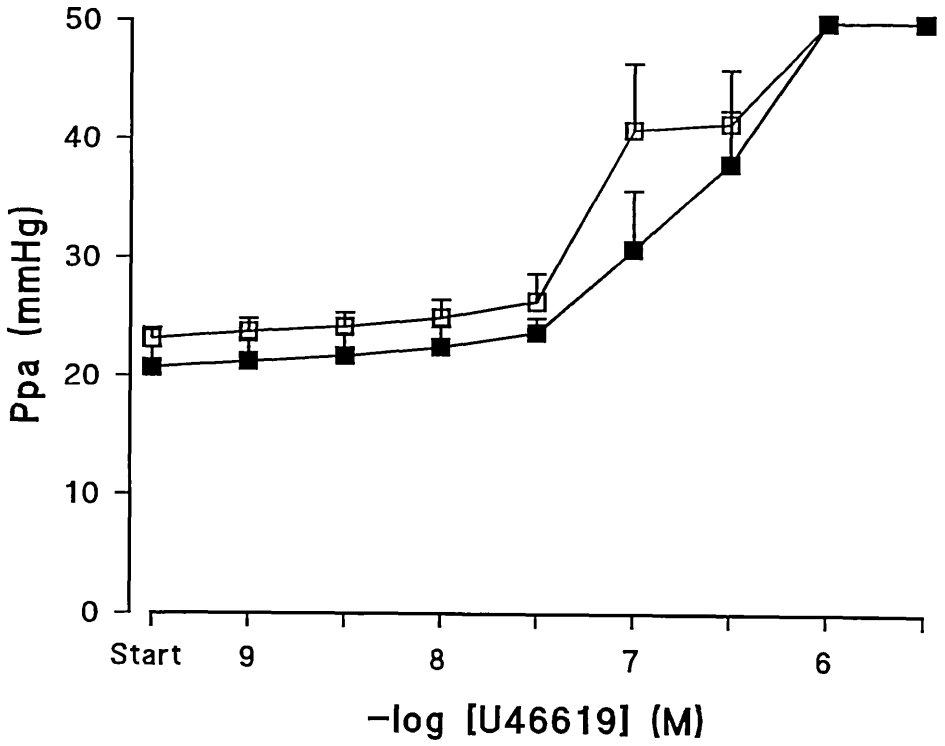


Figure 6.5 **Pulmonary artery responses to ET-1: effects of N^G-monomethyl-l-arginine (L-NMMA)**

Starting pulmonary artery pressure (Ppa) and effect of increasing concentrations of ET-1 following pretreatment with either (i) 10^{-3}M L-NMMA (solid triangles, interrupted line; n=6) (ii) 10^{-5}M L-NMMA (solid triangles, continuous connect; n=5) or (iii) distilled water in sham (UPPER PANEL; solid circles; n=8) or LPS-treated (LOWER PANEL; open circles; n=6) rats.
p values vs. vehicle: * <0.05; ** <0.01; *** <0.001.

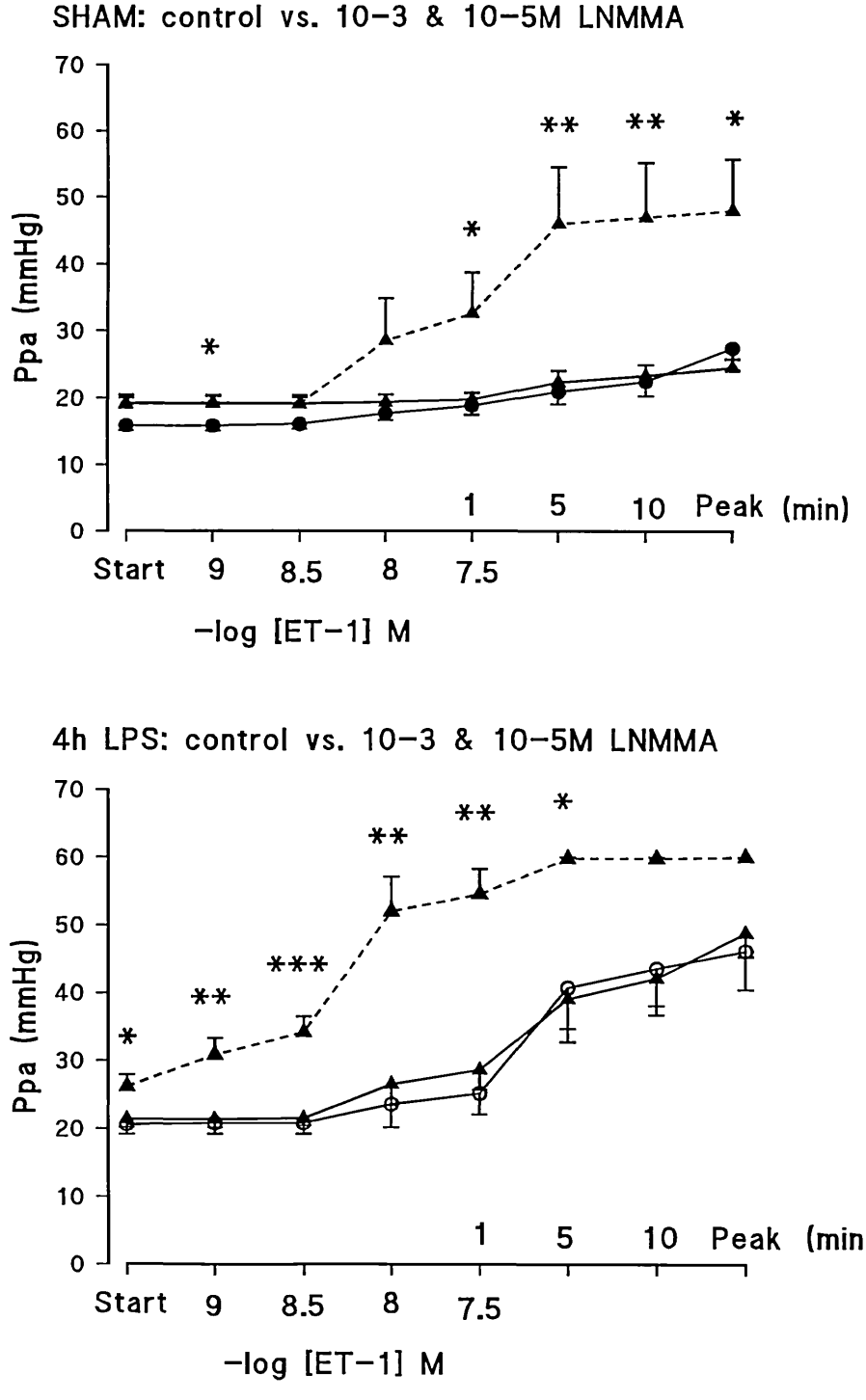
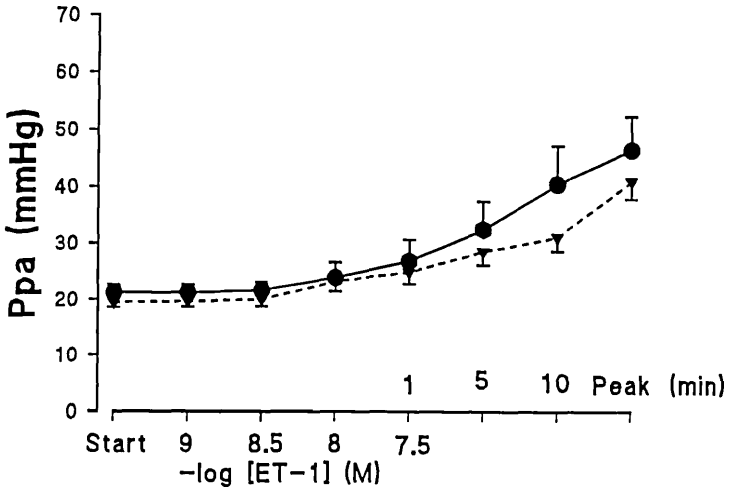


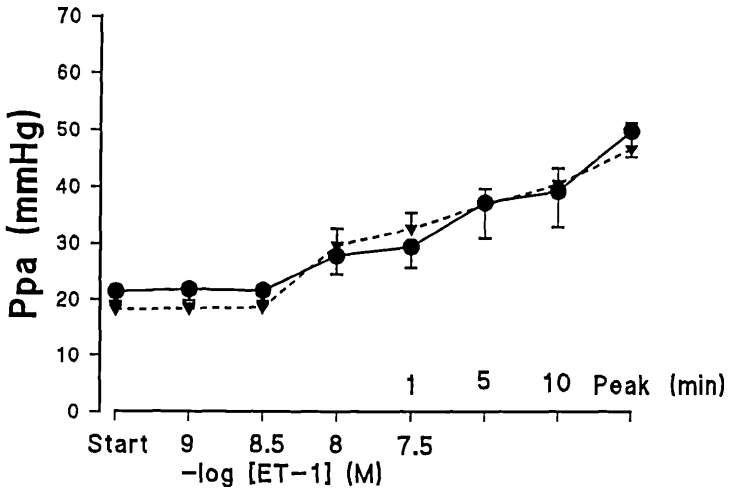
Figure 6.6 **Pulmonary artery pressure responses to ET-1: effects of ICI192605 or indomethacin**

Effects of ET-1 on Ppa 15 minutes following pretreatment with either 10^{-5}M ICI 192605 (triangles) (LEFT PANELS) or 10^{-5}M indomethacin (triangles) (RIGHT PANELS) or their vehicles in sham (solid circles; UPPER PANELS) or LPS-treated (open circles; LOWER PANELS) rats. For all groups, n≥5. Results are mean±SEM. Comparison was made in each case between vehicle and drug by unpaired *t* test. No statistically significant differences were detected.

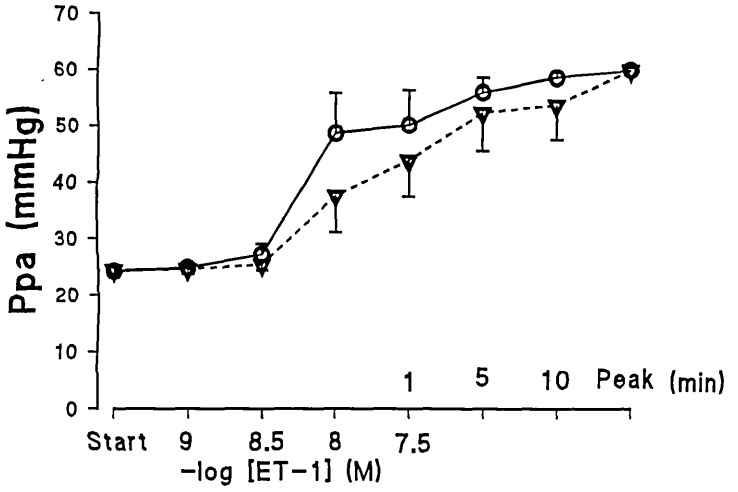
Sham: ICI192605 vs. vehicle



Sham: Indomethacin vs. vehicle



LPS: ICI192 vs. vehicle



LPS: Indomethacin vs. buffer

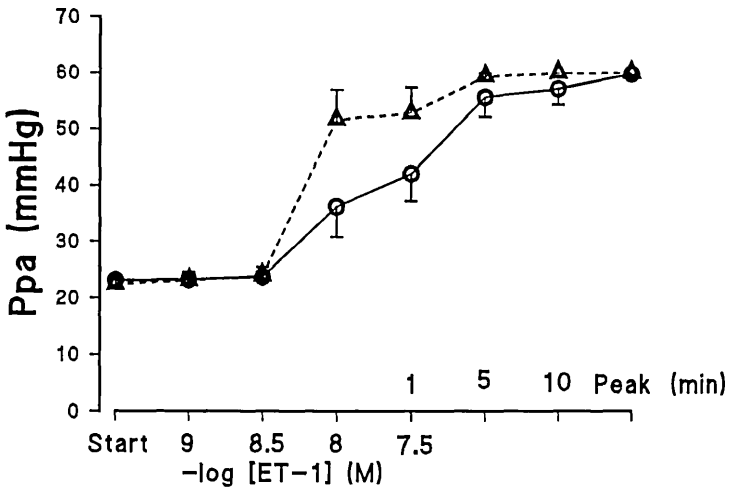


Figure 6.7 **Pulmonary artery pressure responses to sarafotoxin S6c in sham or LPS-treated rats**

UPPER & MIDDLE PANEL

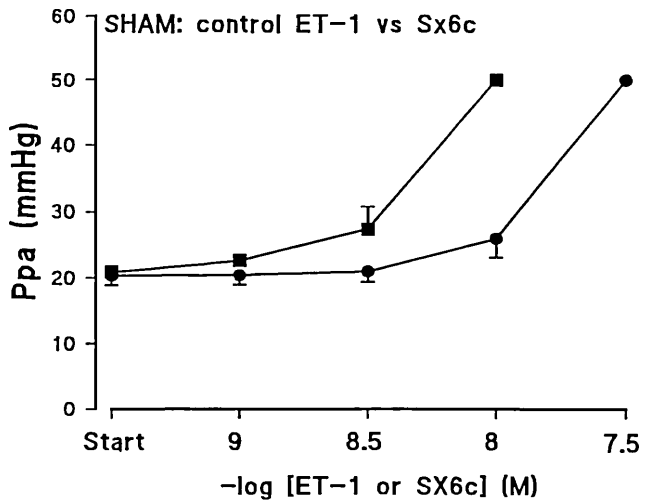
Comparison between the effects of ET-1 (10^{-9} to $3x10^{-8}$M) (circles) and sarafotoxin S6c (10^{-9} to 10^{-8}M) (squares) on Ppa in sham (UPPER panel; solid symbols) or LPS-treated (MIDDLE panel; open symbols) rats. In all groups, n=6.

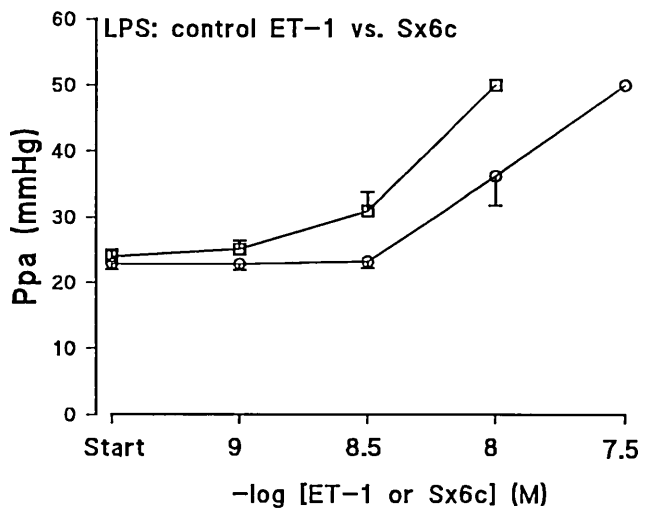
Note that at the final concentration of either ET-1 or S6c, only the peak pressure is shown in these graphs.

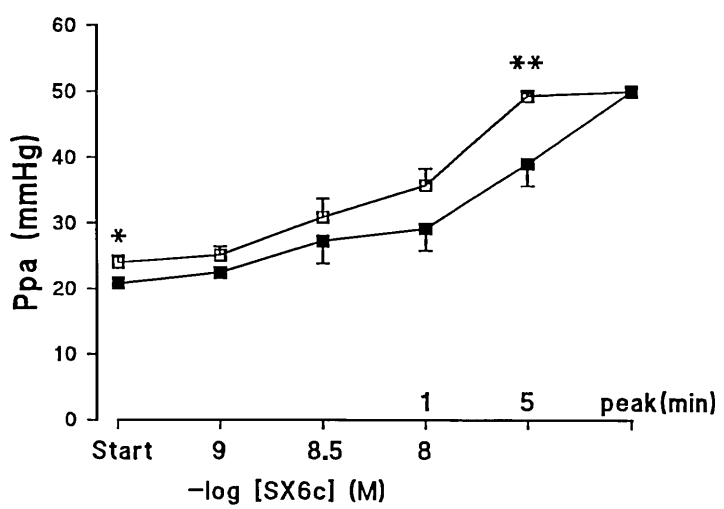
LOWER PANEL

Effects of S6c in sham (solid squares) and LPS-treated (open squares) rats. At the final concentration, Ppa is given at 3 time points to allow for differences in the rate of rise in Ppa: 1 and 5 minutes and at the peak pressure (maximum limited as 50mmHg - see text).

Comparison was made by unpaired *t* tests: ** $p<0.01$.


SHAM: control ET−1 vs Sx6c
Ppa (mmHg)
60
50
40
30
20
10
0
Start
9
8.5
8
7.5
−log [ET−1 or SX6c] (M)


LPS: control ET−1 vs. Sx6c
Ppa (mmHg)
60
50
40
30
20
10
0
Start
9
8.5
8
7.5
−log [ET−1 or Sx6c] (M)


Ppa (mmHg)
60
50
40
30
20
10
0
*
**
1
5
peak(min)
Start
9
8.5
8
−log [SX6c] (M)

Figures show the actual traces obtained (reduced by 70% of original size) by transducing the pulmonary artery pressure (Ppa, labelled **p**) and the movement of the upper lobe of the right lung (**i**) from which the amplitude index was derived: thus,

Amplitude index at a particular pressure = (Ap/Ab x 100) %

where: Ap is the amplitude at that pressure

Ab is the amplitude at baseline

[A] Trace obtained from a sham rat by perfusing the IBPL with its own blood for 45 minutes without any intervention.

[B] Trace obtained from a sham rat treated with 4 increasing concentrations of ET-1. Note that the Ppa increases, but there is little change in the amplitude of lung movement.

[C] Trace obtained from a sham rat treated with 3 increasing concentrations of sarafotoxin S6c. The Ppa rises rapidly after the final dose of S6c, and the amplitude of lung movement decreases simultaneously. This reduction is matched by the macroscopic development of pulmonary oedema and the wet:dry lung weight ratio. At the point marked by the arrows, the recording speed is increased to make the change in pressure and amplitude clearer.

The reason for drift of the amplitude trace is unknown.

Figure 6.8 **Typical traces for amplitude index** (For legend see opposite)

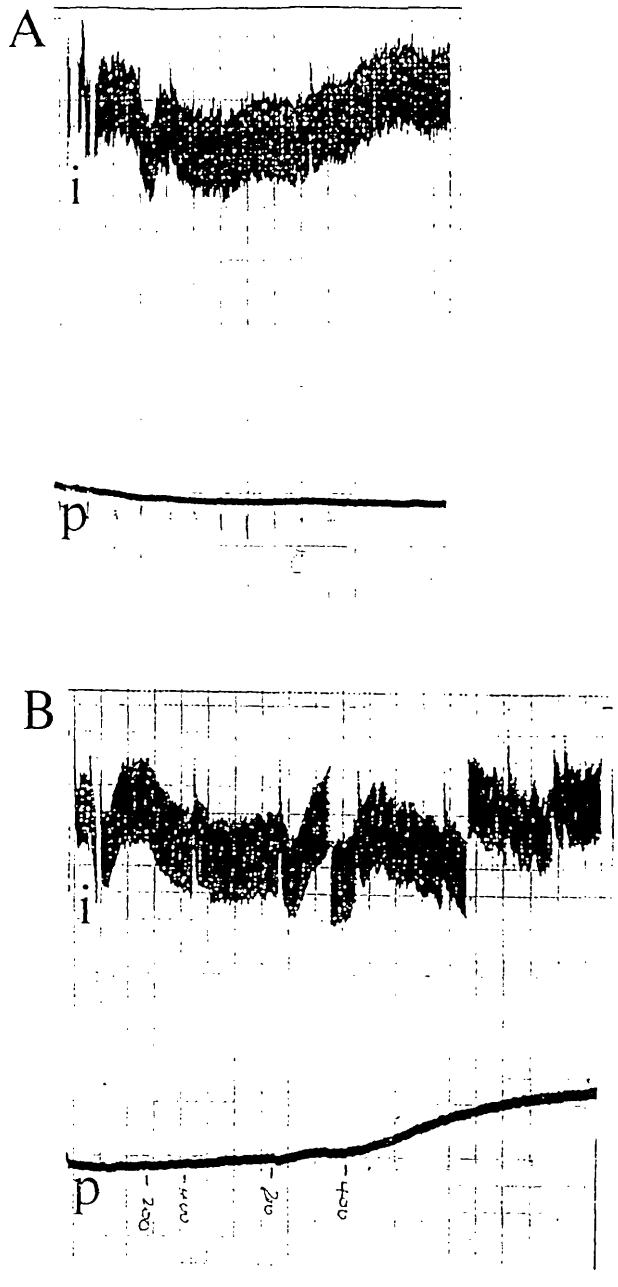
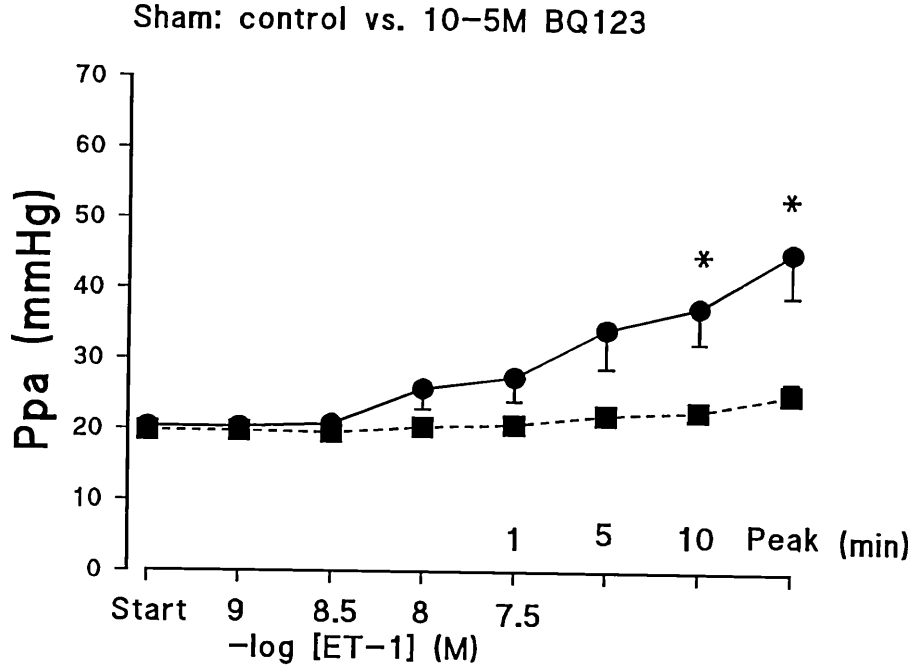


Figure 6.9 **Pulmonary artery responses to ET-1: effects of BQ123**

Starting pulmonary artery pressure (Ppa) and effect of increasing concentrations of ET-1 in sham (UPPER PANEL) or LPS-treated (LOWER PANEL) IBPL following pretreatment with 10^{-5}M BQ123 (squares; interrupted connect) or vehicle (circles). All groups were n=6.
p values vs. vehicle: * <0.05; ** <0.01; *** <0.001.



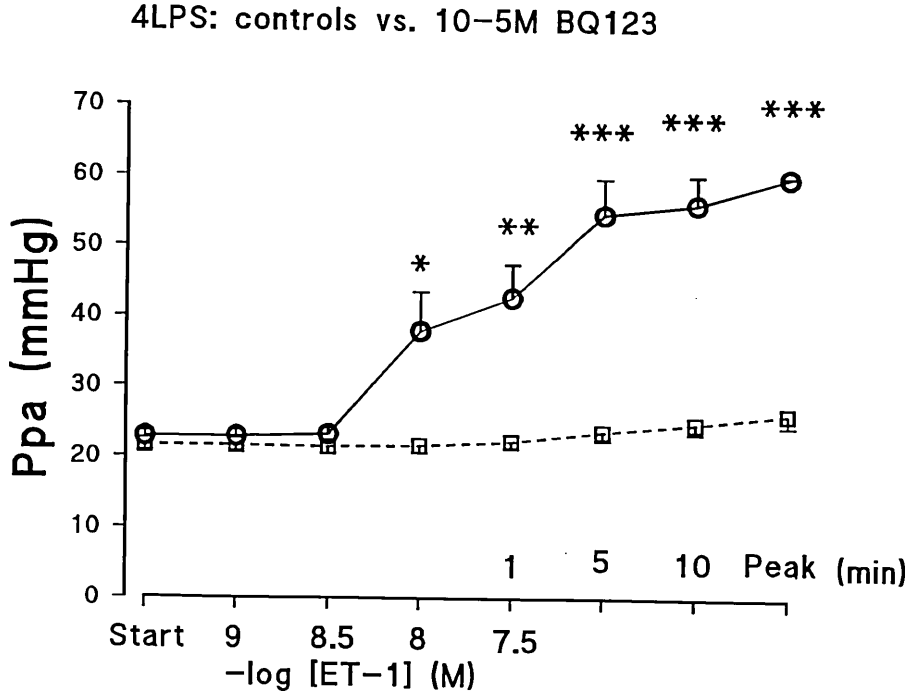


Figure 6.10 **Pulmonary artery responses to sarafotoxin S6c: effects of bosentan**

Starting pulmonary artery pressure (Ppa) and effect of increasing concentrations of sarafotoxin S6c in sham (UPPER PANEL) or LPS-treated (LOWER PANEL) IBPL following pretreatment with either 10^{-5}M bosentan (triangles; interrupted connect) or vehicle (solid connect). All groups n≥5.
p values vs. vehicle: * <0.05; ** <0.01; *** <0.001.

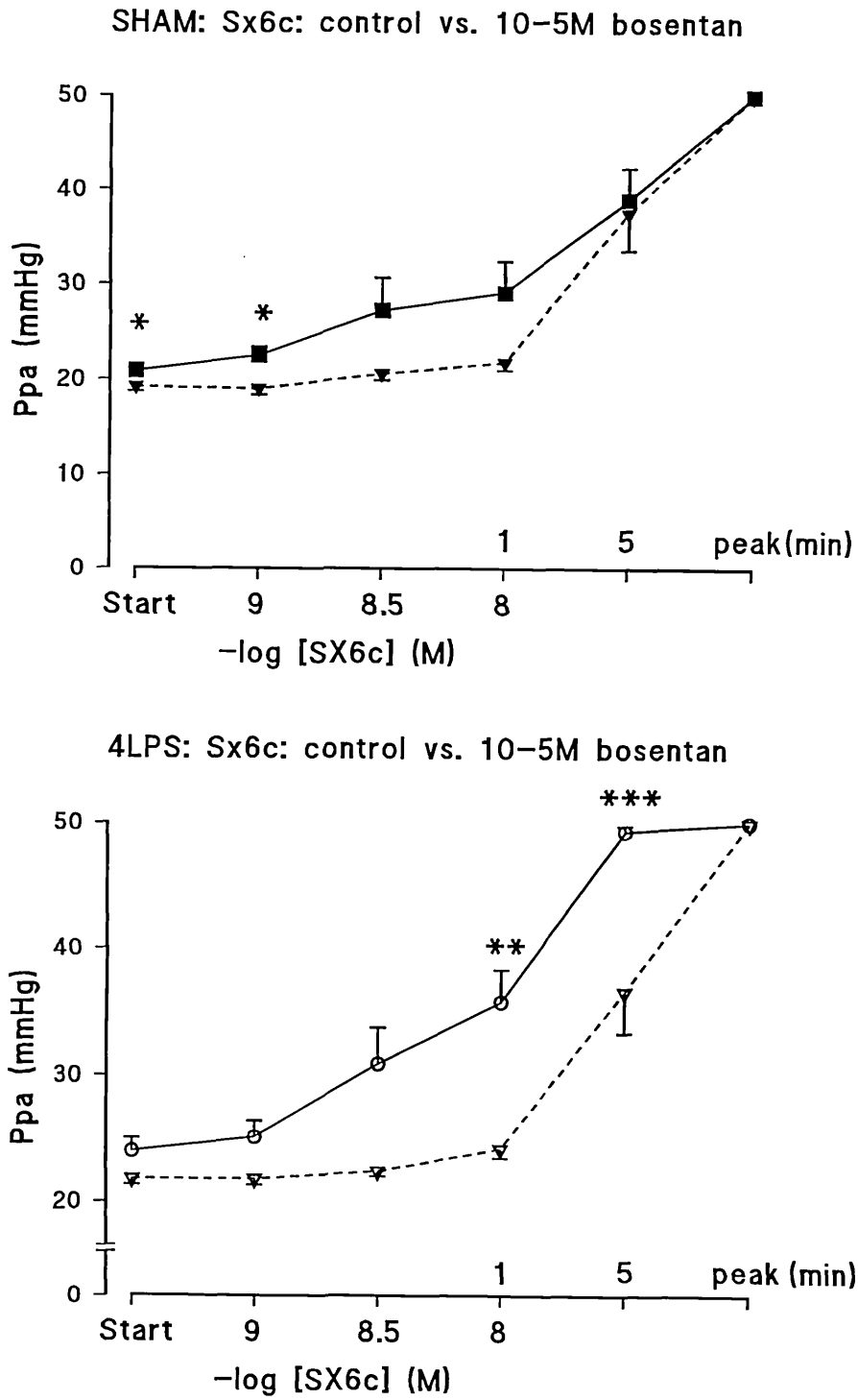
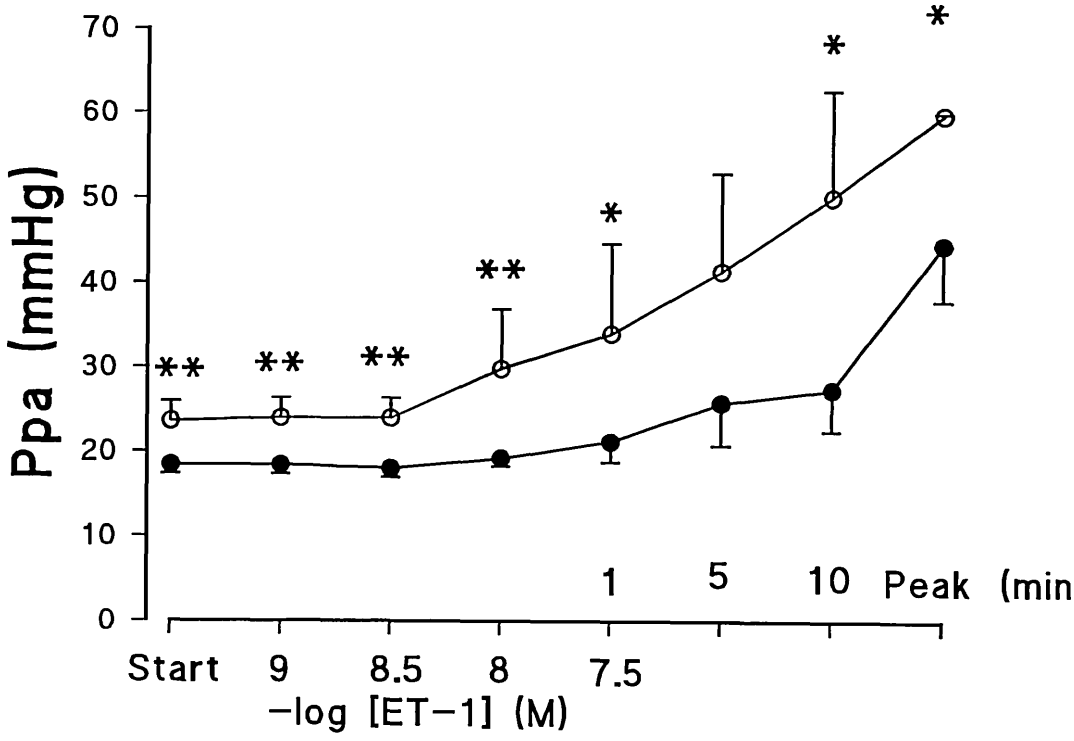


Figure 6.11 **ET-1-induced increases in pulmonary artery pressure in sham and LPS-treated rats perfused with cross-transfused blood**

Starting Ppa and response to 4 concentrations of ET-1 in sham rats perfused with blood from LPS-treated rats (solid circles; n=5) and LPS rats perfused with blood from sham animals (open circles; n=5). Results are expressed as mean±SEM.
* $p<0.05$; ** $p<0.01$.



CHAPTER 7

CHARACTERISATION OF THE EFFECTS OF ET RECEPTOR ACTIVATION *IN VIVO* IN SHAM AND LPS-TREATED RATS

7.1 INTRODUCTION

The systemic haemodynamic consequences of intravenous (*iv*) administration of ET-1 are established in conscious (Bazil et al. 1992; Filep et al. 1994a), anaesthetised (Spokes et al. 1989; Cristol et al. 1993) and pithed (Guc et al. 1990) rats. Following *iv* ET-1, there is a biphasic pattern of response in mean arterial blood pressure (MAP) consisting of: (a) transient hypotension accompanied by tachycardia, then (b) a more sustained pressor response of slower onset accompanied by bradycardia. These responses are qualitatively, but not quantitatively consistent. Slow infusion of ET-1, by contrast, usually results in sustained hypertension, and is not accompanied by the transient hypotensive phase. The mechanisms underlying these systemic haemodynamic phases are also understood. Thus, ET_B receptor-mediated NO release causes the transient hypotension, attenuated by pre-administration of NOS inhibitors (Filep et al. 1993), and has also been reproduced using ET_B receptor agonists (Filep et al. 1994a; Cristol et al. 1993). The pressor phase is largely due to ET_A receptor activation, although there appears to be a component of this rise in MAP that cannot be removed by ET_A receptor antagonists (McMurdo et al. 1993a) and there is also evidence of a contribution from ET_B receptors (Filep et al. 1994a). Fewer data are available regarding the effects of exogenous ET-1 on Ppa in rats. A modest, slow-onset increase in PVR has been demonstrated in response to

high dose *iv* ET-1 (Raffestin et al. 1991), but apart from this most evidence about the behaviour of the pulmonary vascular bed is derived from experiments using IPRL, or vascular tissue *in vitro* (Rodman et al. 1992; Uhlig et al. 1995). There is clear evidence that ET-1 is a pulmonary vasoconstrictor in the rat, acting with greater potency on veins than arteries (Toga et al. 1992), even allowing for experiments in isolated rat lung preparations which demonstrate that ETs-1 and -3 can act as a vasodilator under conditions of increased basal tone, for example in hypoxia (Eddahibi et al. 1993) or following preconstriction with U46619 (Crawley et al. 1992; Hasunuma et al. 1990). One reason for the paucity of data on Ppa responses to ET-1 in the rat *in vivo* is the technical difficulty of siting a cannula in the PA and maintaining it. Larger mammals are easier from this viewpoint, and ET-1 has been shown to increase Ppa in anaesthetised cats, rising to a peak within 3 minutes (Minkes et al. 1990), and in pigs and man (Weitzberg, 1993) in a slow onset sustained rise. There are more data available on chronic PHT in the rat, and several studies have demonstrated the ability of ET_A or combined ET_A/ET_B receptor antagonists to attenuate the development of PHT in rats exposed to hypoxia (Chen et al. 1995; Bonvallet et al. 1993) or other insults such as monocrotaline (Miyauchi et al. 1993). In man, low dose ET-1 (4pmol/kg/min infused over 20 min) caused increases in Ppa and PVR by 20% and 67% respectively (Weitzberg, 1993), and accompanying rises in MAP (7%) and SVR (25%). By contrast, the same dose of ET-1 caused a small decrease in Ppa (12%) in pigs, despite similar changes in MAP (Weitzberg, 1993). At a much higher dose (20pmol/kg/min over 20 min), however, MAP, SVR, Ppa and PVR increased by 10%, 90%, 15% and 71% respectively. These results illustrate how difficult it is to extrapolate conclusions from animal models to man.

Several studies have assessed the effect of LPS on responses to either endogenous or exogenous ET-1 in the rat. The administration of an infusion of *E.coli* endotoxin (250μg/kg/hr) to rats results in a fall in MAP, and impaired vasodepressor responses to ET-1, 5-HT, ACh, bradykinin, sodium nitroprusside (SNP) and salbutamol (Guc et al. 1990; Guc et al. 1991). Interestingly, whilst pressor responses were attenuated (ie. shifted to the right) to phenyelphrine, arginine vasopressin, clonidine and 5-HT, there was no blunting of the pressor response to ET-1 in the same model. In conscious, chronically-instrumented rats, a co-infusion of a non-selective ET receptor antagonist, SB 209670, together with a 24 hour, low-dose LPS infusion (150μg/kg/h), resulted in a significant enhancement of the fall in MAP seen with LPS alone, and a conversion of mesenteric vasoconstriction to vasodilatation (Gardiner et al. 1995). These data imply that endogenous ET-1 antagonises the tendency for systemic hypotension in response to slow LPS infusion in rats. In pigs, infusion of LPS (15μg/kg/h) causes a reduction in MAP after 30 minutes accompanied by a biphasic Ppa response; peaking within 30 minutes, followed by a return towards baseline value and then a slow-onset, sustained rise. The first of these rises in Ppa can be attenuated by COX inhibitors, such as diclofenac (Weitzberg, 1993), and the second by bosentan, the combined ET_A/ET_B receptor antagonist, at a dose of 5mg/kg *iv* (Lundberg et al. 1995), despite an increase in arterial plasma [ET-1] induced by bosentan.

Therefore, there is good evidence that not only is endogenous ET-1 present in the circulation in increased quantity in animal models of sepsis (Chapters 1 & 3), but also that systemic haemodynamic responses to both endogenous and exogenous ET-1 are modulated by the inflammatory vascular

response to sepsis in the rat, and that both pulmonary and systemic haemodynamic responses are altered under similar circumstances in the pig.

In these experiments, the systemic and pulmonary haemodynamic (MAP and Ppa) effects of exogenous ET-1 and sarafotoxin S6c were assessed, and compared with responses to angiotensin II (AII) and SNP. Finally, the contribution of the elevated endogenous ET-1 to basal MAP and Ppa parameters, and the modulation of the responses to ET-1 injection, were assessed by the administration of bosentan.

7.2 EXPERIMENTAL PROTOCOLS

7.2.1 Effects of ET-1 on pulmonary artery and systemic arterial pressures in sham and LPS-treated rats

ET-1 was dissolved in 0.1% acetic acid to make a stock solution, and diluted to the required dose using distilled water. Dose-response curves were studied to increasing, cumulative *iv* bolus injections (0.1, 0.5, 1.0 nmol/kg). Following the initial dose, subsequent injections were administered when the systemic pressor response had reached its peak for the previous dose. After the final dose, the experiment was terminated either at peak pressor response or 10 mins following the 1.0 nmol/kg dose of ET-1, depending on which occurred first. In two separate groups, three consecutive doses of distilled water (vehicle) of equivalent volumes to the ET-1 doses were given at least 5 mins apart as controls.

7.2.2 Effects of ET_A/ET_B antagonism

7.2.2.1 Effects of a combined ET_A/ET_B antagonist on baseline arterial pressures, and on ET-1 dose-responses

In a further set of experiments, the combined ET_A/ET_B receptor antagonist, bosentan, was administered at a dose of 10 mg/kg 10 mins before subsequent ET-1 dose response studies (as described above). This dose of bosentan had been shown to produce effective ET receptor antagonism *in vivo* (Filep et al. 1994a; Clozel et al. 1994).

7.2.2.2 Effects of a combined ET_A/ET_B antagonist on ET-1-induced contraction of isolated aortic and PA rings from sham and LPS-treated rats

Experiments were performed on isolated vessels *in vitro* in order to establish that bosentan remained an effective antagonist of ET-1-induced contractile responses in systemic and pulmonary arteries under endotoxaemic conditions. In separate studies, aortic and PA rings had contractile responses to increasing concentrations of ET-1 (10^{-11} to 10^{-6}M) evaluated in cumulative fashion. In each experiment, half the rings were pretreated with bosentan (10^{-5}M), and half with distilled water, 10 minutes before the ET-1 concentration-contraction response was started. Results are expressed as tension generated above baseline in grams.

7.2.3 Effects of ET_B receptor activation on pulmonary and systemic arterial pressures in sham and LPS-treated rats

Sarafotoxin S6c was dissolved in distilled water. MAP and Ppa responses were studied to increasing, cumulative *iv* injections (0.1, 0.5, 1.0nmol/kg). Following the initial concentration, subsequent doses were given when the systemic pressor response had reached its peak for the previous dose. Results are expressed for changes in hemodynamic parameters seen with the two lower doses, since there was a high mortality associated with the 1.0nmol/kg dose.

7.2.4 Effects of angiotensin II on pulmonary and systemic arterial pressures in sham and LPS-treated rats

Angiotensin II was administered in two consecutive doses (0.25, 0.5mg), which preliminary experiments had shown to provide an appropriate pressor response. Continuous pressure monitoring was continued until maximal responses had been observed.

7.2.5 Effects of SNP on pulmonary and systemic arterial pressures in sham and LPS-treated rats

A single dose of SNP (100µl of a 10^{-3}M solution made up immediately before each experiment), chosen after preliminary experiments (data not shown), was administered and continuous monitoring maintained until after the MAP response had resolved.

7.3 RESULTS

Values for MAP and Ppa in all groups before any intervention (following the equilibration period) are shown in **Table 7.1**. There was no significant difference between any groups.

Pooling all the available arterial blood gas (ABG) data for rats studied following either saline (n=21) or LPS 4h previously (n=20) revealed a significantly lower $PaCO_2$ (sham, 5.72±0.17 *vs.* LPS, 4.92±0.12 kPa respectively; p <0.01) and a significantly higher PaO_2 (sham, 11.60±0.43 *vs.* LPS, 13.86±0.41 kPa; p <0.01) in the endotoxaemic rats when compared with their controls. The arterial hydrogen ion concentration ($[H^+]$) (sham, 46.39±1.22; LPS, 48.69±0.60 mmol/L; p=0.10) was not significantly different.

7.3.1 Effects of ET-1 on pulmonary artery and systemic pressures in sham and LPS-treated rats

Intravenous ET-1 produced characteristic haemodynamic changes in all groups that were not seen in their controls **[Figures 7.1 & 7.2]**. Within 30s of injection there was a rapid, transient fall in MAP, which returned to baseline within 1 minute. This was followed by a slower rise in MAP to a peak 5-10 mins post dose. By contrast, Ppa rose transiently and rapidly as MAP fell **[Figure 7.1]**. This rise in Ppa was much greater than that seen in the DH_2O group, in which it was assumed to be an injection arterfact. Following a return towards baseline, Ppa then rose more slowly, often not reaching a detectable peak before the next dose of ET-1 was administered at the peak of the MAP response for that dose. Six haemodynamic parameters were therefore compared in the ET-1 experiments, namely: (a)

transient depressor response in MAP, (b) secondary pressor response in MAP, (c) transient pressor response in Ppa, (d) secondary pressor response in Ppa; with (a)-(d) being parameters for each ET-1 dose; and finally overall (e) MAP and (f) Ppa responses to all three doses **[Figure 7.3]**.

Treatment with *ip* LPS 4h prior to anaesthesia significantly reduced the transient fall in MAP as well as the corresponding transient rise in Ppa **[Figure 7.4]**. LPS failed to significantly alter the end-of-dose systemic pressor responses, or the overall change in MAP, but it did cause a significant attenuation of the Ppa response to 0.5nmol/kg ET-1 in LPS rats, and a significantly lower overall change in Ppa in this group.

7.3.2 Effects of bosentan

7.3.2.1 Effects of bosentan on baseline arterial pressures and responses to exogenous ET-1

Bosentan produced a small initial fall in MAP in both groups, but 10 minutes later the MAP had fallen 5.30 ± 1.81mmHg and 3.25 ± 1.26mmHg in the sham and 4h LPS groups respectively (p=0.39). At the same time point, Ppa had fallen 0.90 ± 0.82 mmHg in the sham rats and 0.88 ± 0.69 mmHg in the LPS group (p=0.98).

In sham rats, pretreatment with bosentan (10mg/kg) significantly attenuated all subsequent ET-1-induced responses, such that all 6 of the pressure parameters studied were no different to those obtained using DH_2O **[Figure 7.3]**. In LPS rats, however, there were fewer significant differences in the parameters studied between ET-1 alone comparing them to their LPS DH_2O controls **[Figure 7.5]**, although the trend was for the all of the DH_2O control group values to be lower.

Furthermore, bosentan pretreatment in LPS rats did not significantly attenuate any of the ET-1-induced hemodynamic changes **[Figure 7.5]**.

7.3.2.2 **Effects of bosentan on ET-1-induced contractile responses of isolated aortic and PA from sham and LPS-treated rats**

Both aortic and PA rings from LPS-treated rats displayed significant hyporesponsiveness to ET-1, demonstrable by shifts to the right in the concentration-contraction response curves **[Table 7.2 & Figure 7.6]**. In all groups except aortic rings from LPS-treated rats, however, the contraction achieved at 10^{-6}M ET-1 was not significantly different to that of their control rings. In these LPS aortic rings the contraction at this ET-1 concentration was still significantly less than in its control group.

In all vessel groups, bosentan (10^{-5}M) produced a marked shift in the concentration-contraction response curves to the right **[Figure 7.6 a,b,c,d]**. EC_{50} calculations were not possible in these bosentan groups, because no plateau was achieved at the maximum concentration.

7.3.3 <u>Effects of Sarafotoxin S6c on pulmonary and systemic arterial pressures in sham and LPS-treated rats</u>

In both sham and LPS rats, the mortality associated with the administration of the final dose of S6c (1.0nmol/kg) was over 70%. Results are therefore shown only for 0.1 and 0.5nmol/kg doses. In sham rats, there was an early, transient systemic hypotensive response of the same degree as that seen in the ET-1 sham group **[Figure 7.7a]**, both of these being significantly different from the DH_2O controls. Following this, there was a small pressor response that did not achieve statistical

significance compared with controls **[Figure 7.7b]**, and which at 0.5nmol/kg was significantly less than for ET-1. Intravenous S6c elicited very small early increases in Ppa which were not different from those of the water controls, and which were significantly smaller than those associated with ET-1 **[Figure 7.7d]**. Similarly, the end-of-dose Ppa responses were not different from controls, and were again smaller than those of the equivalent ET-1 group **[Figure 7.7c]**. A typical response in a single rat is shown in **Figure 7.8a**.

In the LPS animals, the initial depressor response in MAP for both doses was attenuated compared with the sham S6c responses, but were larger than those of the equivalent ET-1 group **[Figure 7.9a & 7.10]**. Subsequently, there was again a small systemic pressor response, but even at the higher dose this was not significantly different from the controls, although it was significantly higher than the response to S6c in the sham group **[Figure 7.9b & 7.10]**. The maximum early Ppa response was greater than controls, without reaching statistical significance between either control or LPS ET-1 groups **[Figure 7.9d & 7.10]**. The end-of-dose Ppa responses showed small reductions for both doses that did not differ from the control or LPS ET-1 groups **[Figure 7.9c]**. These end-of-dose Ppa responses in LPS rats treated with S6c were, however, significantly different from the equivalent S6c sham responses **[Figure 7.10]**.

7.3.4 Effects of Angiotensin II on pulmonary and systemic arterial pressures in sham and LPS-treated rats

Angiotensin II caused rapid elevations in MAP and Ppa in all animals studied. Baseline MAP and Ppa were not significantly different between groups. The systemic pressor response to AII was attenuated in the LPS rats (n=7) compared

to the sham (n=8), achieving significance at the lower dose of 0.25µg (LPS, 23.4±4.6 mmHg *vs.* sham, 48.6±6.2mmHg; $p<0.01$), but not the higher dose (LPS, 41.0±6.0 mmHg *vs.* sham, 62.5±8.1mmHg; $p=0.2$) **[Figure 7.11 & 7.12]**. The Ppa increase to AII was, however, greater in the LPS-treated animals than in their controls, again this difference being significant only at the lower dose (LPS, 22.7±1.8 mmHg *vs.* sham, 14.9±2.1mmHg; $p=0.02$), but not at 0.5µg (LPS, 23.4±2.0 mmHg *vs.* sham, 18.0±2.2mmHg; $p=0.09$).

7.3.5 Effects of SNP on pulmonary and systemic arterial pressures in sham and LPS-treated rats

SNP produced rapid reductions in MAP and Ppa in all rats studied. The response of both MAP (sham, 79.0±5.7 *vs.* LPS, 56.6±7.0: $p=0.03$) and Ppa (sham, 5.6±1.4 *vs.* LPS, 2.7±0.9: $p=0.10$) to the single dose of SNP (100µl 10^{-3}M) was attenuated in the LPS group, although this only reached significance in the case of MAP.

7.4 DISCUSSION

Previous studies in rats *in vivo* have demonstrated the effects of exogenous ET-1 on systemic haemodynamics, both in untreated (Bazil et al. 1992; Filep et al. 1994a; Spokes et al. 1989; Cristol et al. 1993) and in endotoxaemic (Guc et al. 1990; Guc et al. 1991) animals. In addition, the effects of bolus intravenous injections of ET-1 on pulmonary and systemic haemodynamics have been studied *in vivo* in otherwise untreated rats (Raffestin et al. 1991). This is the first time that measurements of pulmonary and systemic arterial pressure have been made to assess the effects of ET receptor blockade and exogenous ET-1 in a rat model of endotoxaemia. The rat exhibits considerable resistance to the development of systemic hypotension in response to LPS administered by this route. In fact, depression in MAP has been previously demonstrated but only following intravenous infusions of LPS at relatively high doses (Guc et al. 1990). In addition, this model does not exhibit elevation of Ppa, although it has been demonstrated that continuous infusion of *E.coli* endotoxin produces significant elevations in Ppa in rats but only from 24h onwards (Kirton and Jones, 1987). The starting pressures were not significantly different in LPS animals compared with sham rats. However, the arterial blood gas parameters were significantly different, endotoxaemic animals having a higher PaO_2, but lower $PaCO_2$. These differences are likely to reflect the extent of the pathophysiological changes induced by the extensive inflammatory response induced by LPS, and specifically, the macroscopic and microscopic changes consistent with this inflammatory reaction in the lung (Read and Meyrick, 1994) (Section 3.3.1). The same changes have been reported before in rats treated with endotoxin intravenously (Law et al. 1985). The decision to accept these inter-group

discrepancies was based upon the desire to reproduce genuine, *in vivo* differences in the response to LPS, which may contribute to alterations in vasoactive responses; rather than to use mechanical ventilation and bicarbonate treatment to artificially "standardize" arterial blood gas tensions, as was done in the IBPL preparation (Chapter 6). As a result, however, a 4h LPS distilled water group was included as an additional control. Preliminary attempts to study rats 6h after LPS challenge were terminated because the animals were intolerant of the extensive surgical and catheterisation manoeuvres required.

The systemic pressure changes seen in response to increasing doses of ET-1 in these experiments were broadly similar to those found by others. Thus, transient hypotension followed by a slower and longer-lasting pressor response produces the characteristic biphasic pattern previously reported. However, the magnitude of these responses was different, the current study demonstrating larger hypotensive reactions and, possibly because of this, smaller subsequent elevations in MAP. Such differences almost certainly relate to variations in anaesthesia, ventilation, surgical preparation and the origin of the ET-1 used. However, the Ppa response to ET-1 is a novel finding. The early transient rise in Ppa was unexpected, although the subsequent slower pressor response was not. Previously, ET-1 has been shown to increase Ppa in anaesthetized, non-endotoxaemic cats (Minkes et al. 1990), although this response reached its peak about 3 minutes after the injection, and there was no sign of the early rise in Ppa that was observed in these experiments. In a previous study of the response of Ppa in conscious rats no obvious changes were detectable up to 45 minutes after intravenous bolus injections of ET-1 (Raffestin et al. 1991), despite this dose range overlapping those used in the current study. The reason for this discrepancy is unclear. These experiments are limited by not having

measurements of cardiac output (CO), although the early and short-lived changes observed in MAP and Ppa would render assessment of CO by dye- or thermo-dilution impossible. The additional surgical preparation and equipment required for the implantation of direct flow probes was also deemed not to be feasible. Nevertheless, it seems unlikely that changes in CO from the left ventricle during the systemic hypotensive phase could be invoked as an explanation for the simultaneous sharp rise in Ppa. The possibility that the intravenous injection resulted in direct increases in Ppa with consequent reductions in "left-sided" CO was controlled for by using similar volumes of distilled water. To further investigate the interrelationship between the responses of the two sides of the circulation, AII and SNP experiments were performed. From the AII data, it was seen that a potent, direct vasoconstrictor could produce profound rises in both Ppa and MAP simultaneously. Administration of the NO donor, SNP, confirmed that it is also possible to produce systemic hypotension and pulmonary hypotension simultaneously. Currently, the systemic hypotension induced by ET-1 is thought to be the result of ET_B-mediated release of vasodilators such as NO and PGI_2 from endothelial cells. Indeed, it is possible to attenuate this response using inhibitors of NO synthesis such as L-NMMA (Filep et al. 1993). If this is the correct explanation for this transient hypotensive response, either the vasodilators involved are short-acting, or the initial hypotensive effect that they produce is subsequently overtaken by the direct vasoconstrictor effects of ET-1 itself, principally as a result of ET_A receptor stimulation. Some workers have, however, been unable to completely attenuate the ET-1 MAP pressor response using ET_A receptor antagonists (McMurdo et al. 1993a; Filep et al. 1994a; Gardiner et al. 1994). Taken together with data using ET_B agonists, which also produce a secondary systemic pressor response, it

seems that a proportion of this systemic hypertension is produced either by ET_B receptors or via stimulation of one or more non-ET_A, non-ET_B receptors. In fact, these experiments with S6c clearly confirm that the early hypotensive response to ET-1 in sham rats is produced as a result of ET_B receptor stimulation. The subsequent pressor response of the systemic circulation to S6c, however, was not significantly different from the DH_2O controls. Presumably, this sequence of ET-1-induced systemic vasodilatation (secondary to ET_B activation) followed by direct ET_A-mediated vasoconstriction does not occur in the pulmonary circulation, in which the observed early elevations in Ppa in the sham rats were followed by smaller overall pressor responses by the end of any particular dose of ET-1. Further investigation is required to find out whether this is because no (or less) vasodilators are released, because the direct effects of ET-1 are relatively more potent, or because vasoconstrictors are released by ET-1 in these vessels. The S6c data in the sham group imply that this early ET-1-induced transient rise in Ppa is a non ET_B-mediated phenomenon, since it was absent in the S6c group.

The difference between the MAP responses of the sham and LPS groups are also broadly similar to the findings of previous studies (Guc et al. 1990; Guc et al. 1991), in that there was significant attenuation only in the hypotensive response to the ET-1 in the LPS-treated rats. This pattern was mimicked in the S6c groups, although the actual depressor response to S6c in the 4h LPS group was greater than for the equivalent ET-1 doses in LPS-treated rats. The attenuation may either reflect a dampening of ET_B-mediated NO release, or of the effect of any NO that is released, possibly because of the hugely increased amounts of NO that are produced in these circumstances; or may reflect a change in the population of ET_B receptors, either numerically or via functional down-regulation. Down-regulation of ET_B receptor mRNA has in fact already

been demonstrated in pulmonary hypertension (Yorikane et al. 1993). In the LPS group, the maximum early rise in Ppa to ET-1 was also attenuated, to the point where these responses were not significantly different to those obtained in the equivalent S6c group. Again this could be explained by either a down-regulation of the specific receptor sub-type responsible for this response (i.e. either ET_A or non-ET_A, non-ET_B), or more simply by a "swamping" effect of the initial constrictor reaction by the large quantities of locally-released NO. Again, however, there was no significant reduction in the subsequent Ppa pressor response to each dose of ET-1, although there was a significant fall in the overall three-dose rise in Ppa. In the S6c groups, LPS abolished the secondary Ppa pressor response, so that there was a reduction of Ppa at the end of each dose as well as overall. This is further evidence for a shift in effector status of the responsible receptor populations. These findings contrast sharply with the AII responses in LPS rats, when, in comparison to sham controls, there was a differential increase in pulmonary vascular reactivity accompanying the hyporesponsiveness of the systemic circulation. This model thus failed to demonstrate a selective increase in pulmonary vasoreactivity to ET-1 *in vivo* in endotoxaemia, but, importantly, such differential reactivity has been demonstrated using AII. These results are thus at odds with the effects of ET-1 in the IBPL preparation (Chapter 6).

The *in vitro* experiments confirmed two main points. Firstly, they demonstrated that aortic, as well as PA rings from LPS-treated rats exhibit hyporesponsiveness to exogenous ET-1, when compared to those of sham animals. This confirms the previous findings in rat PA (Chapters 4 & 5), and is unsurprising in view of a similarly reduced responsiveness to agents such as phenylephrine that has previously been demonstrated in rat aortic and PA rings

(Griffiths et al. 1993; Griffiths et al. 1995a). Secondly, bosentan pretreatment produced large shifts to the right in the ET-1 concentration-contraction curves of all ring types tested, thus confirming it to be an effective antagonist at ET receptors, regardless of the endotoxaemic status of the animal. This is the first time that bosentan has been used in these circumstances, and it was important to confirm that it retains its effectiveness in vessels from LPS-treated rats.

Previous studies on anaesthetized rats and dogs have shown no effect on resting MAP following administration of bosentan (Richard et al. 1994; Teerlink et al. 1995). Only a small effect was also observed in these experiments, and, surprisingly, the effect was no greater in the LPS-treated rats. This small haemodynamic effect of bosentan, even in the context of elevated ET-1 production, underlines the suspicion that any explanation of the role of ET-1 in endotoxaemia is unlikely to be straightforward. The release of ET-1 from endothelial cells, the main site of production, is polar, being directed basally (towards the underlying smooth muscle), rather than luminally (Wagner et al. 1992), and most evidence suggests that it acts mainly as an autocrine or paracrine agent rather than as a circulating hormone. This result adds weight to the hypothesis that the observed increases in plasma [ET-1] concentration in sepsis merely reflected increased production, and not necessarily changes in functional status. It is also possible that more ET-1 is available and bound to its receptors, and that bosentan fails to affect this process, because in order to do so it would have to interfere with an established ET-1-receptor complex. Recently published data suggest that ET-1 has a tonic pressor influence in the anaesthetised rat which is not demonstrated by bosentan treatment alone, but only unmasked when NO synthesis is inhibited (Richard et al. 1995). This finding may also be relevant in endotoxaemia when NO production is massively

increased (Griffiths et al. 1994). In sham rats *in vivo* bosentan effectively removed differences between the arterial pressure responses to ET-1 and to distilled water, so that responses in the sham ET-1/bosentan group became indistinguishable from those of the controls. In the LPS group, however, this was not observed in a clear cut or significant fashion, despite data from the *in vitro* experiments. For most of the parameters studied, non-significant reductions in response were observed in the bosentan group compared with the ET-1 (alone) group, but in no instance did these differences reach significance. Although this may be because the standard errors are large amongst the LPS groups, thus reducing the chances of achieving significance, it may also be that responses are produced via different mechanisms in these rats which are not so efficiently antagonized by bosentan *in vivo*. Specifically, it is possible that differences in receptor populations and degrees of activation occur in endotoxaemia, an hypothesis that demands further investigation.

In conclusion, these experiments demonstrated that despite the confirmed increase in vascular ET-1 mRNA and in plasma [ET-1] in rats treated with LPS, treatment of such rats with the combined ET receptor antagonist, bosentan, did not affect their baseline arterial pressures compared to controls, suggesting that the role of ET-1 in modulating vascular tone in these circumstances is not directly related to increased production or circulating levels in the plasma. These experiments also demonstrated novel differential effects of ET-1 on the pulmonary circulation *in vivo*, an early sharp rise in Ppa mirroring the expected transient fall in MAP, although the latter (as expected) was ET_B receptor mediated, whereas the former was not. Furthermore, in LPS-treated rats, the hypotensive phase of the systemic response to ET-1, and the

hypertensive phase of the Ppa response were attenuated, but the systemic pressor phase was unaffected; despite documented *in vitro* hyporesponsiveness to ET-1 of both aortic and PA rings. Importantly, whilst angiotensin II elicits an increased sensitivity of Ppa responses in LPS treated animals, this was not demonstrable in the case of ET-1. In addition, the efficiency with which bosentan could attenuate ET-1 effects in sham rats *in vivo* was considerably diluted in the endotoxaemic animals.

These findings may have important implications for the application of ET receptor antagonism *in vivo*, since they raise the possibility that the status of the relevant effector receptor populations changes under endotoxaemic conditions, and suggest that further investigation regarding the role of ET-1 in the vascular response to sepsis should probably be aimed primarily at the receptor and secondary messenger levels.

Table 7.1 **Starting MAP and Ppa in all groups**

Group	n	MAP (mmHg)	Ppa (mmHg)
Sham: ET-1	6	114.67±5.57	22.17±1.91
4h LPS: ET-1	6	125.50±6.57	19.00±1.57
Sham: SX6c	6	133.67±3.38	20.00±1.00
4h LPS:SX6c	5	125.80±3.33	20.20±2.08
Sham: DH_2O	8	134.67±3.41	21.67±1.61
4h LPS: DH_2O	6	120.00±4.47	19.17±0.98
Sham: BOS + ET-1	6	124.33±5.55	21.00±0.86
4h LPS: BOS + ET-1	5	129.00±4.68	18.20±0.80

Abbreviations:

n	number in group
ET-1	endothelin-1
LPS	lipopolysaccharide/endotoxin
DH_2O	distilled water
h	hour
SX6c	sarafotoxin S6c
BOS	bosentan

For statistical comparisons see text under "Results".

Table 2 **EC_{50} values for ET-1 concentration-contraction response curves in aortic & pulmonary artery rings.**

	AORTA	PULMONARY.A
E+ LPS-	7.29±0.09	8.37±0.13
	(n=4)	(n=5)
E+ LPS+	6.96±0.10 *	7.70±0.22^{+}
	(n=4)	(n=4)

Abbreviations:

EC_{50} concentration at which 50% of maximum tension generated was achieved.

E+ endothelium intact

LPS- sham treated

LPS+ endotoxin treated

Statistical analysis between the LPS- and LPS+ group for each vessel type was undertaken by Student's unpaired *t* test: * $p = 0.04$; $^{+}p = 0.03$.

Figure 7.1 **Typical MAP and Ppa responses to ET-1**

Typical changes in mean systemic (MAP; squares) and pulmonary (Ppa; triangles) arterial pressures in a sham rat (UPPER PANEL) and a LPS-treated rat (LOWER PANEL) to 3 doses of ET-1 (0.1, 0.5, 1.0 nmol/kg).

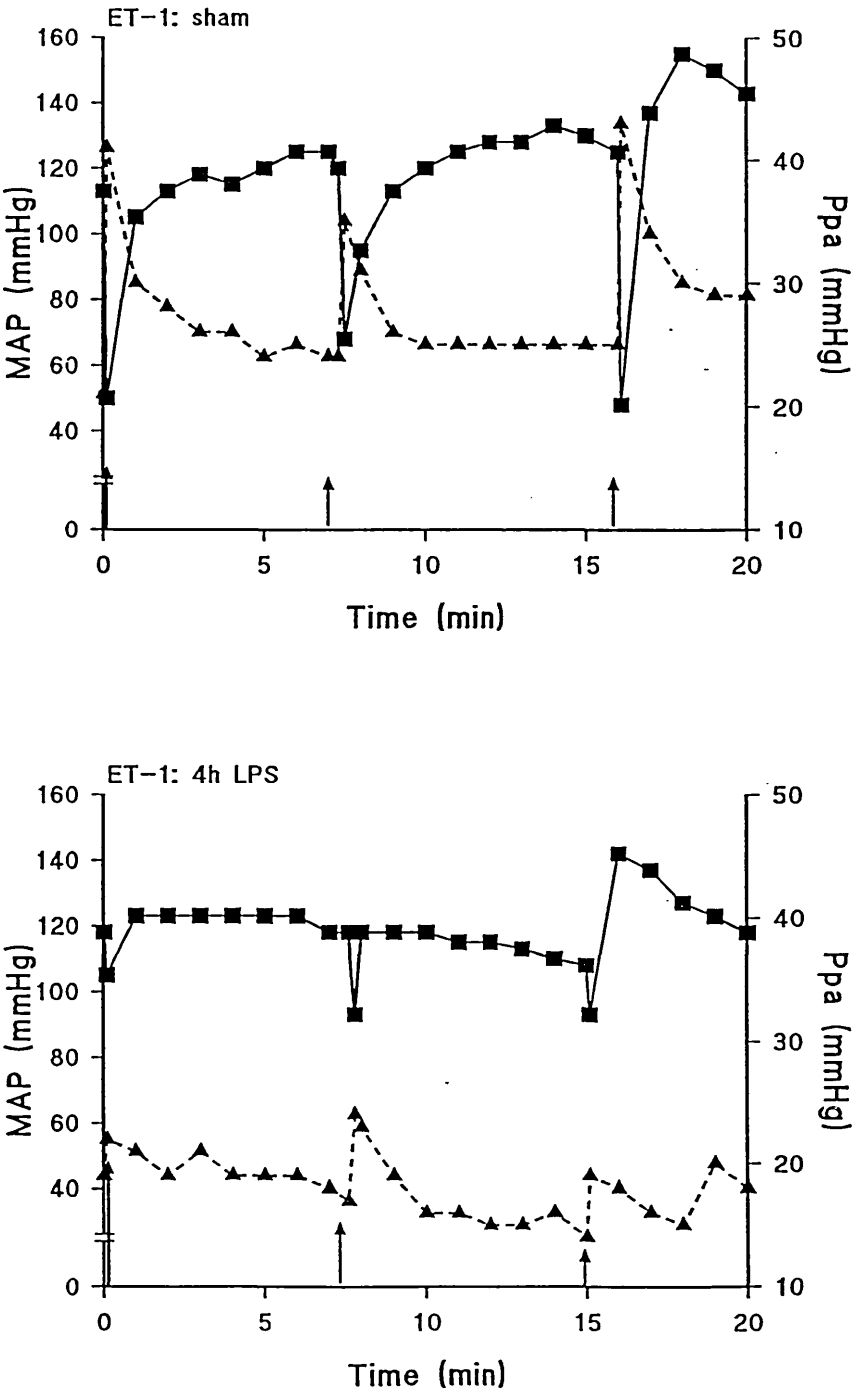


Figure 7.2 **Typical MAP and Ppa responses to distilled water**

Typical changes in mean systemic (MAP; sqaures) and pulmonary (Ppa; triangles) arterial pressures in a sham rat (UPPER PANEL) and a LPS-treated rat (LOWER PANEL) to three 300μL boluses of distilled water (DH_2O).

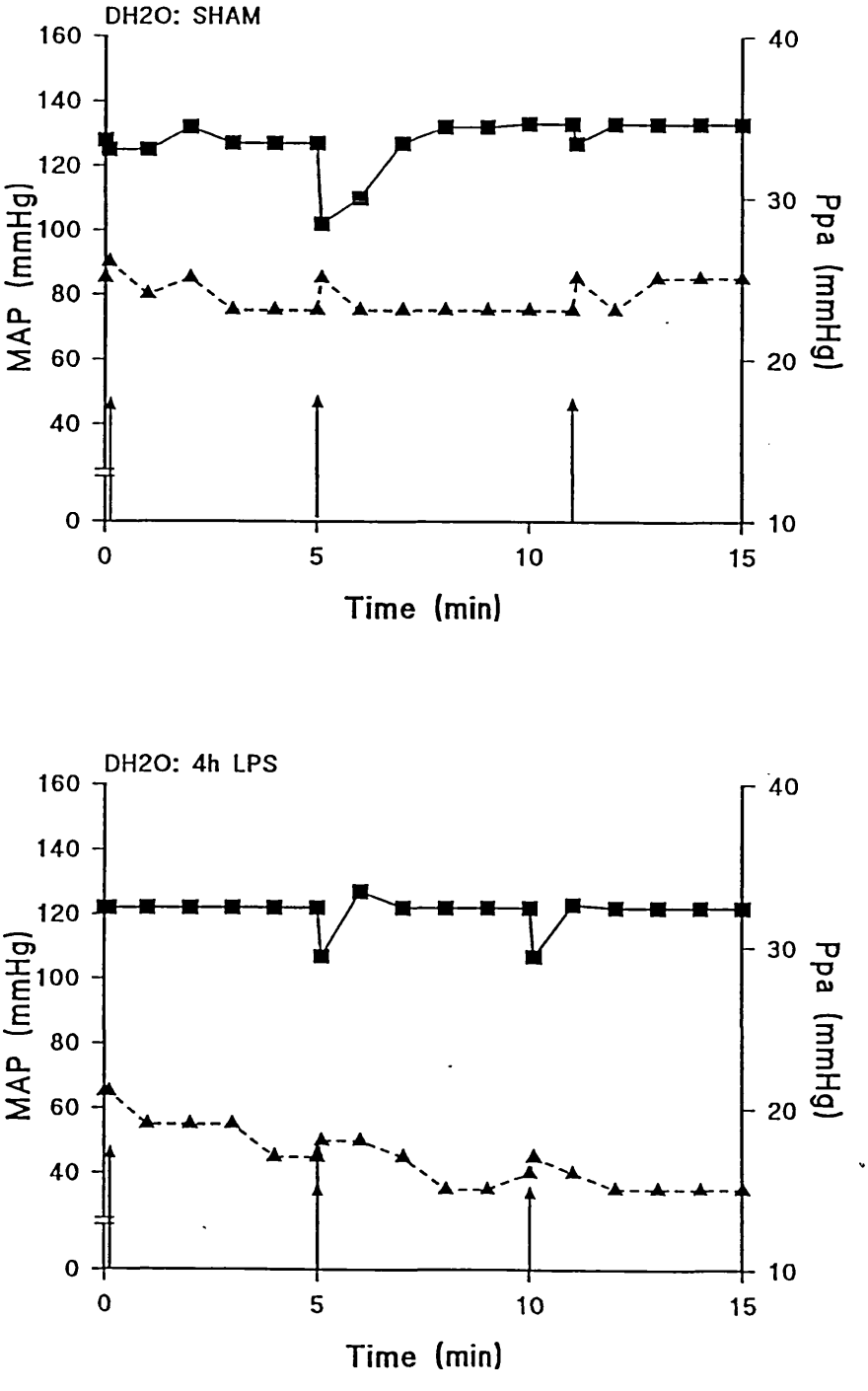


Figure 7.3 **Haemodynamic responses to ET-1 in sham rats, with and without bosentan**

MAP and Ppa responses in sham rats to ET-1 (open bars; n=6), DH2O in 3 identical volumes (striped bars; n=6) and ET-1 10 mins following pretreatment with 10mg/kg bosentan (solid bars; n=6).

(A) represents initial systemic hypotensive response, and (D) represents the initial Ppa pressor response to ET-1. The subsequent end-of-dose systemic (B) and Ppa (C) responses are also shown. In (E), the overall responses to the three doses are given.

Results are expressed as means±SEM. Statistical analysis was undertaken using ANOVA with Tukey-Kramer tests for multiple comparisons. *p* values are for responses of groups compared to those of ET-1 (alone): * < 0.05; ** < 0.01; ϕ < 0.001.

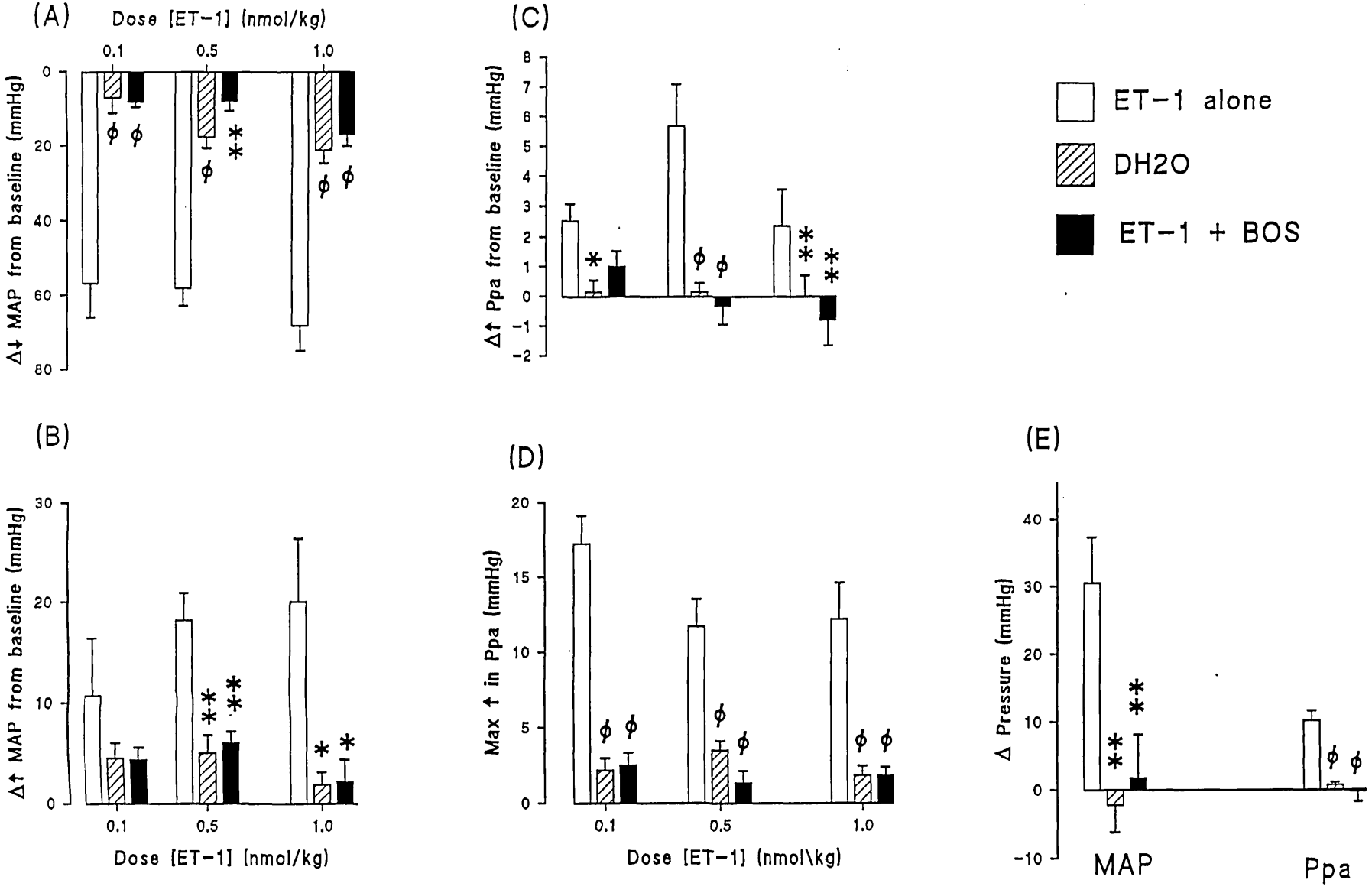

(A)
Dose [ET-1] (nmol/kg)
0.1
0.5
1.0
Δ↓ MAP from baseline (mmHg)
0
20
40
60
80
(B)
Δ↑ MAP from baseline (mmHg)
0
10
20
30
0.1
0.5
1.0
Dose [ET-1] (nmol/kg)
(C)
Δ↑ Ppa from baseline (mmHg)
8
7
6
5
4
3
2
1
0
−1
−2
(D)
Max ↑ in Ppa (mmHg)
0
5
10
15
20
0.1
0.5
1.0
Dose [ET-1] (nmol\kg)
ET−1 alone
DH2O
ET−1 + BOS
(E)
Δ Pressure (mmHg)
40
30
20
10
0
−10
MAP
Ppa

Figure 7.4 **Haemodynamic responses to ET-1 in sham and LPS-treated rats**

MAP and Ppa responses to ET-1 in sham (open bars; n=6), and 4h post LPS (cross-hatched bars; n=6).

(A) represents initial systemic hypotensive response, and (D) represents the initial Ppa pressor response to ET-1. The subsequent end-of-dose systemic (B) and Ppa (C) responses are also shown. In (E), the overall responses to the three doses are given.

Results are expressed as means±SEM. Statistical analysis was undertaken using ANOVA with Tukey-Kramer tests for multiple comparisons. *p* values for responses compared to those of sham: * < 0.05; ** < 0.01.

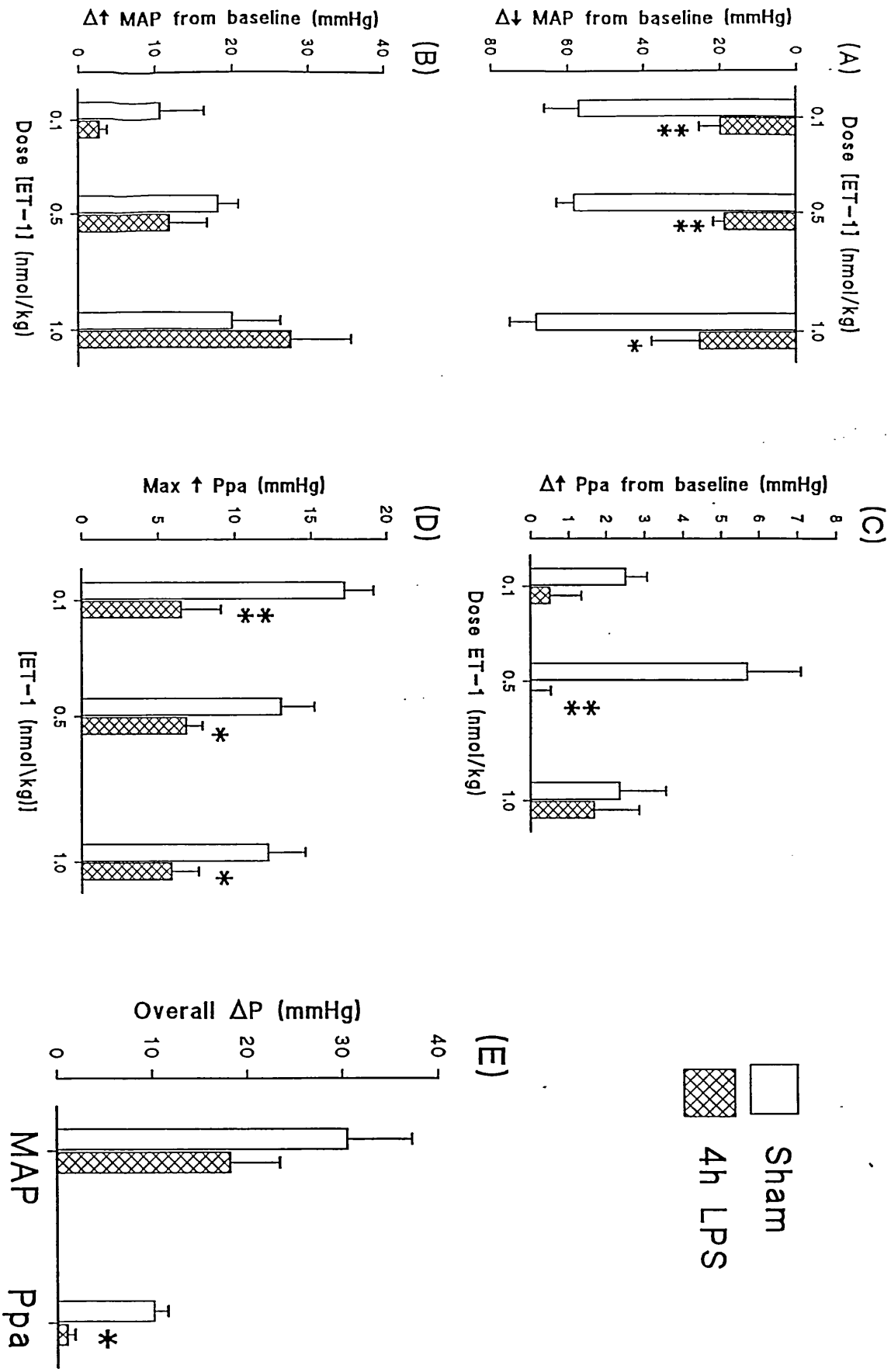

(A)
Δ↓ MAP from baseline (mmHg)
Dose [ET-1] (nmol/kg)
(B)
Δ↑ MAP from baseline (mmHg)
Dose [ET-1] (nmol/kg)
(C)
Δ↑ Ppa from baseline (mmHg)
Dose ET-1 (nmol/kg)
(D)
Max ↑ Ppa (mmHg)
[ET-1 (nmol\kg)]
(E)
Overall ΔP (mmHg)
MAP
Ppa
Sham
4h LPS

Figure 7.5 **Haemodynamic responses to ET-1 in LPS-treated rats with and without bosentan**

MAP and Ppa responses in rats treated with *ip* LPS 4h previously to ET-1 (open bars; n=6), DH2O in 3 identical volumes (striped bars; n=6) and ET-1 10 mins following pretreatment with 10mg/kg bosentan (solid bars; n=5).

(A) represents initial systemic hypotensive response, and (D) represents the initial Ppa pressor response to ET-1. The subsequent end-of-dose systemic (B) and Ppa (C) responses are also shown. In (E), the overall responses to the three doses are given.

Results are expressed as means±SEM. Statistical analysis was undertaken using ANOVA with Tukey-Kramer tests for multiple comparisons. *p* values are for responses when compared to those of ET-1 (alone): * < 0.05; ** < 0.01.

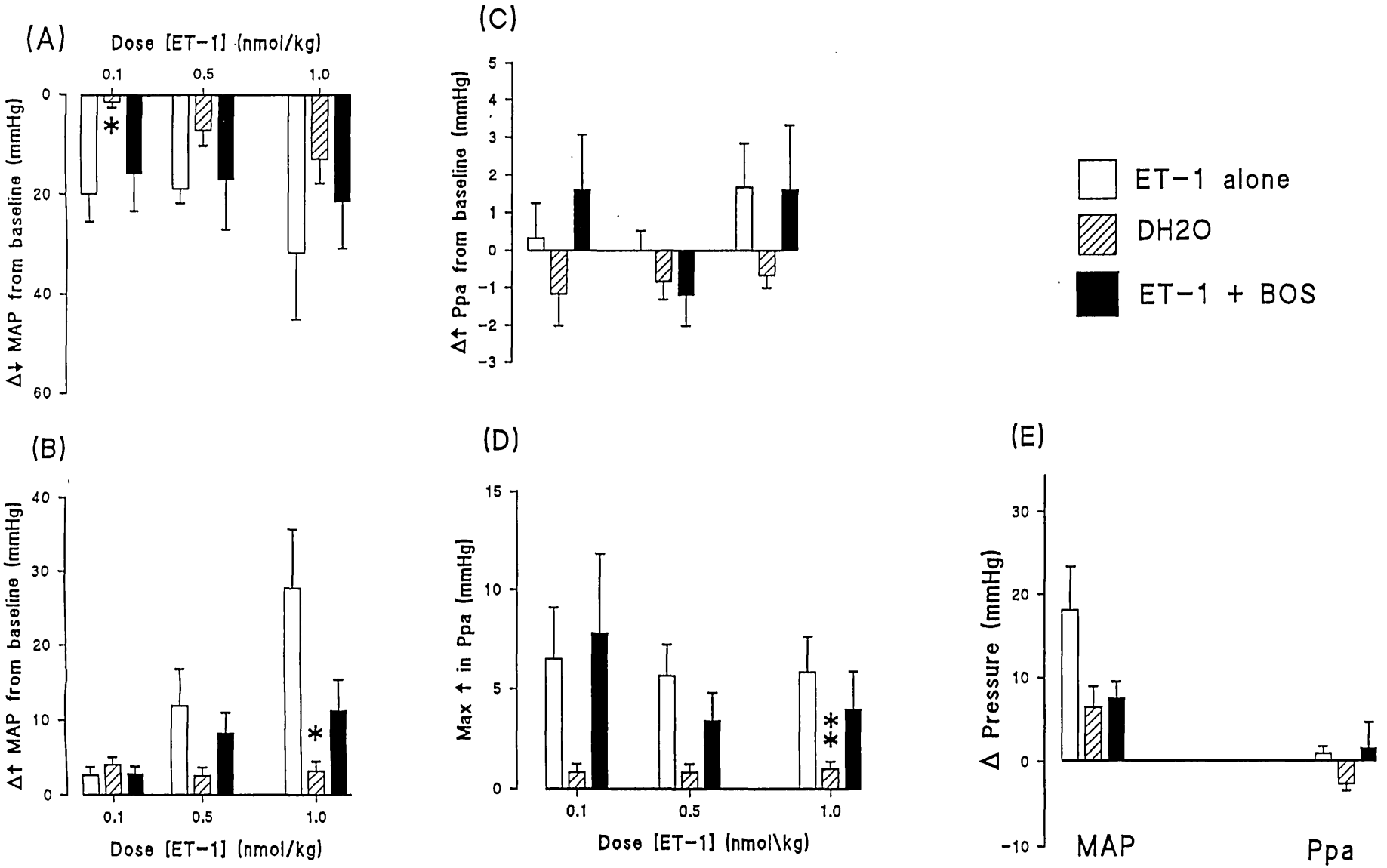
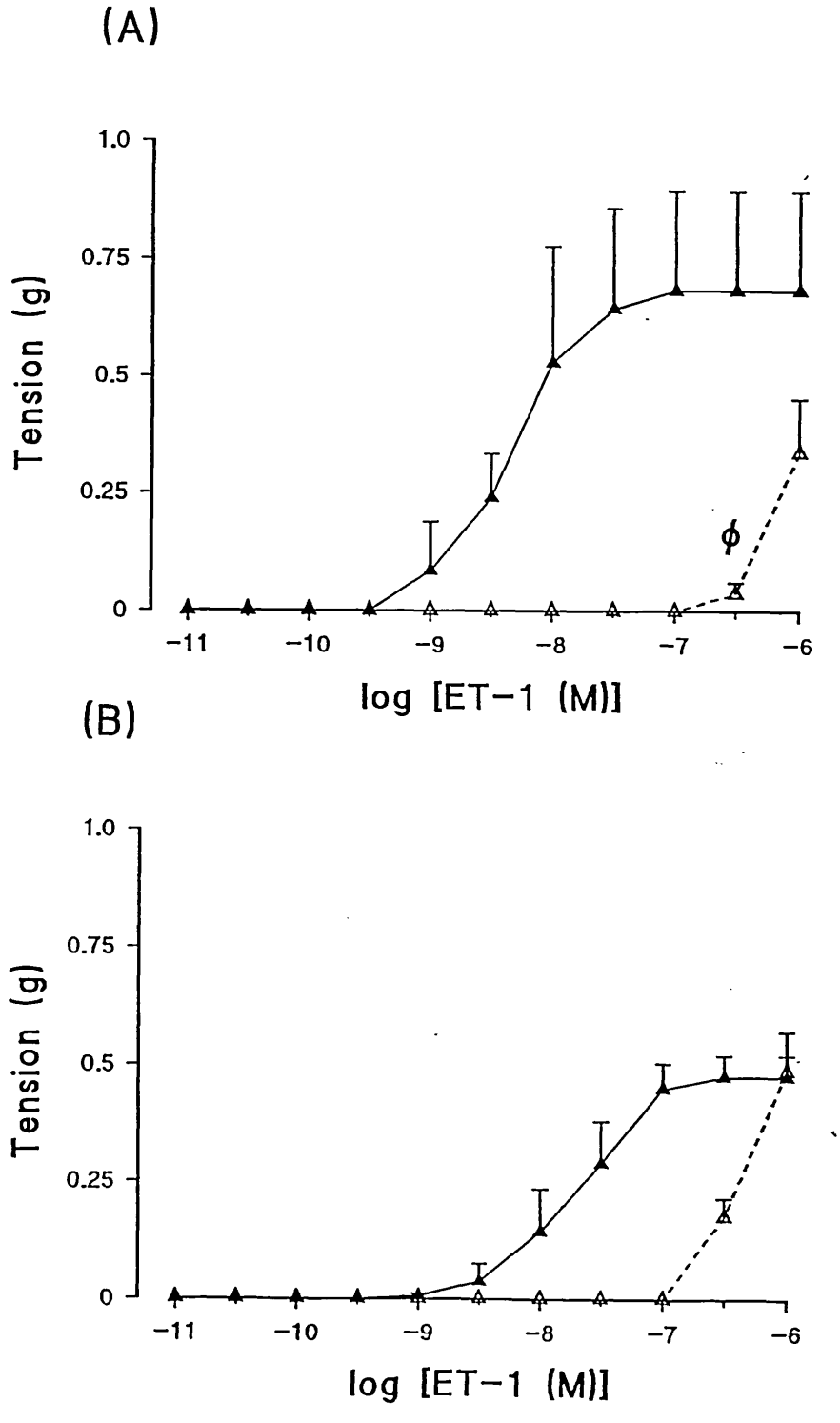
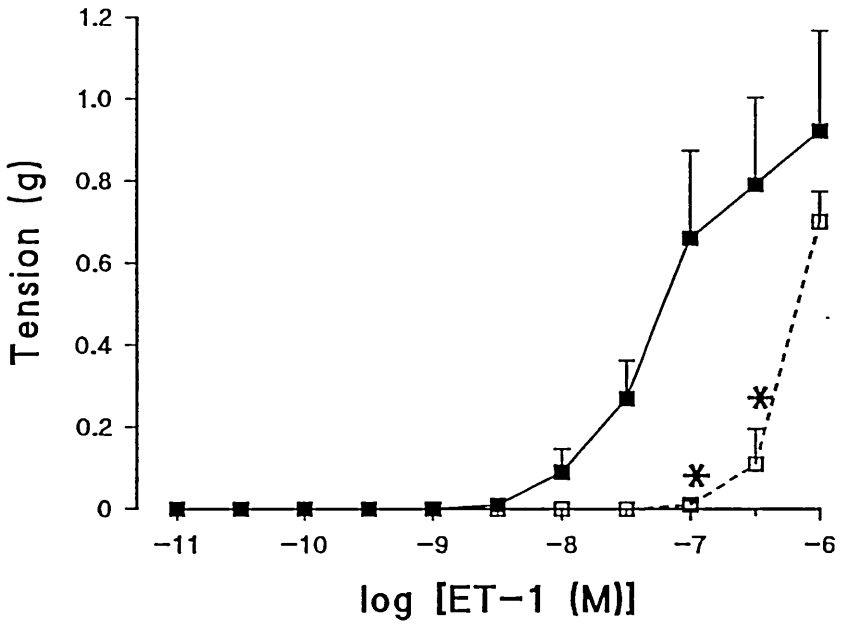

(A)
Dose [ET-1] (nmol/kg)
0.1
0.5
1.0
Δ↓ MAP from baseline (mmHg)
0
20
40
60
*
(B)
Δ↑ MAP from baseline (mmHg)
0
10
20
30
40
*
0.1
0.5
1.0
Dose [ET-1] (nmol/kg)
(C)
Δ↑ Ppa from baseline (mmHg)
5
4
3
2
1
0
-1
-2
-3
(D)
Max ↑ in Ppa (mmHg)
15
10
5
0
**
0.1
0.5
1.0
Dose [ET-1] (nmol\kg)
ET-1 alone
DH2O
ET-1 + BOS
(E)
Δ Pressure (mmHg)
30
20
10
0
-10
MAP
Ppa

Figure 7.6 **ET-1-induced contractile responses of PA and aortic rings from sham and LPS-treated rats: effects of bosentan**

Cumulative concentration-contraction responses to ET-1 in isolated PA (A&B) or aortic (C&D) rings pretreated with either vehicle (solid symbol, solid connect) or 10^{-5}M bosentan (open symbol, broken connect). Vessels in A & C are from sham rats, and those from B and D are from rats treated with LPS. Results are mean±SEM for n=5 in all groups. *p* values: * <0.05; ** <0.01; ϕ <0.001.



(C)


1.2
1.0
0.8
0.6
0.4
0.2
0
Tension (g)
−11
−10
−9
−8
−7
−6
log [ET−1 (M)]
*
*

(D)

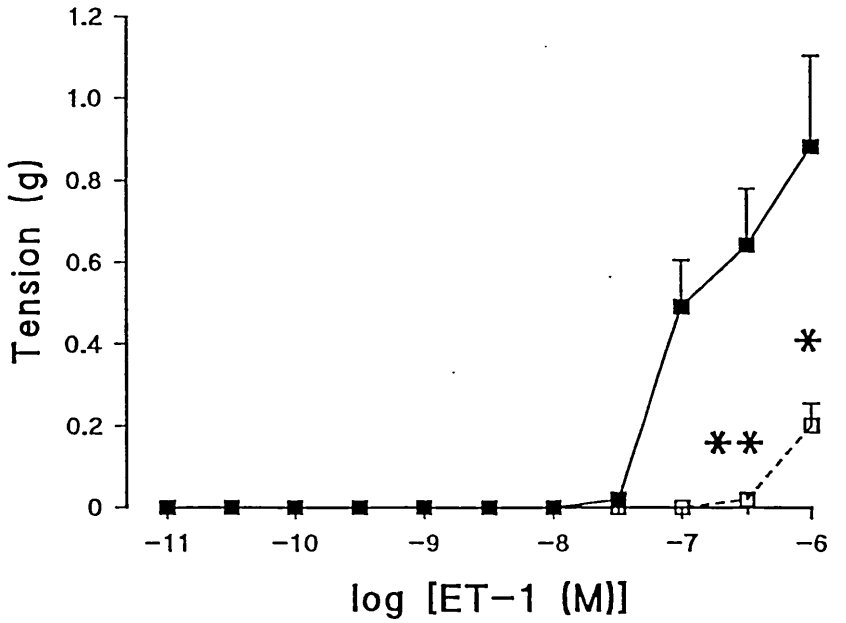

1.2
1.0
0.8
0.6
0.4
0.2
0
Tension (g)
−11
−10
−9
−8
−7
−6
log [ET−1 (M)]
*
**

Figure 7.7 **Haemodynamic responses to sarafotoxin δc in sham rats**

MAP and Ppa responses in sham rats to ET-1 (open bars; n=6), DH2O in 3 identical volumes (striped bars; n=6) and the 2 smaller doses of sarafotoxin 6c (Sx6c) (lined bars; n=6).

(A) represents initial systemic hypotensive response, and (D) represents the initial Ppa pressor response to ET-1. The subsequent end-of-dose systemic (B) and Ppa (C) responses are also shown.

Results are expressed as means±SEM. Statistical analysis was undertaken using ANOVA with Tukey-Kramer tests for multiple comparisons. *p* values are for *treatment groups vs. DH2O controls* unless otherwise shown: * < 0.05; ** < 0.01; ϕ < 0.001.

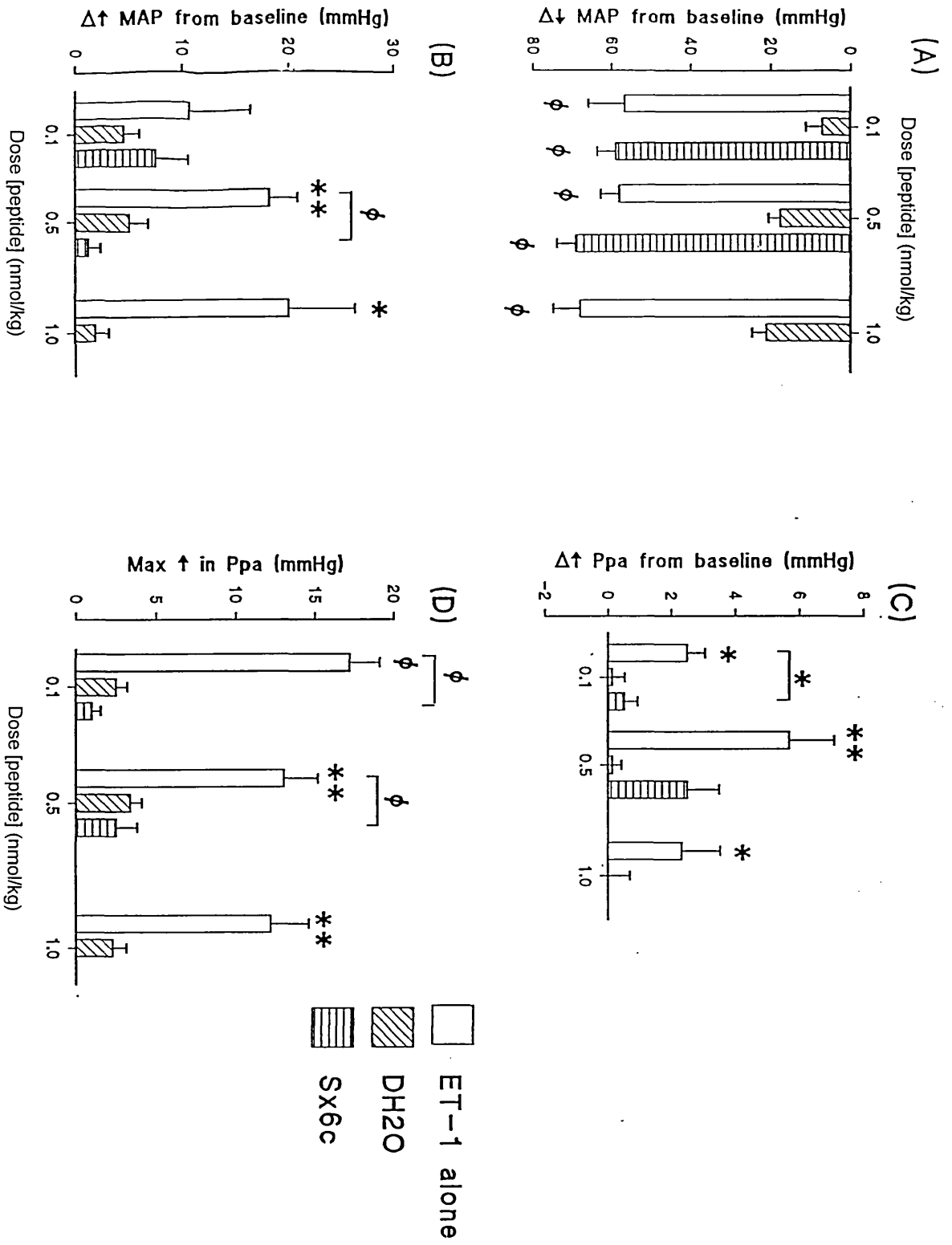

(A)
Δ↓ MAP from baseline (mmHg)
Dose [peptide] (nmol/kg)
(B)
Δ↑ MAP from baseline (mmHg)
Dose [peptide] (nmol/kg)
(C)
Δ↑ Ppa from baseline (mmHg)
(D)
Max ↑ in Ppa (mmHg)
Dose [peptide] (nmol/kg)
ET-1 alone
DH2O
Sx6c

Figure 7.8 **Typical MAP and Ppa responses to Sarafotoxin 6c**

Typical changes in mean systemic (MAP; squares) and pulmonary (Ppa; triangles) arterial pressures in a sham rat (UPPER PANEL) and a LPS-treated rat (LOWER PANEL) to 3 doses of sarafotoxin S6c.

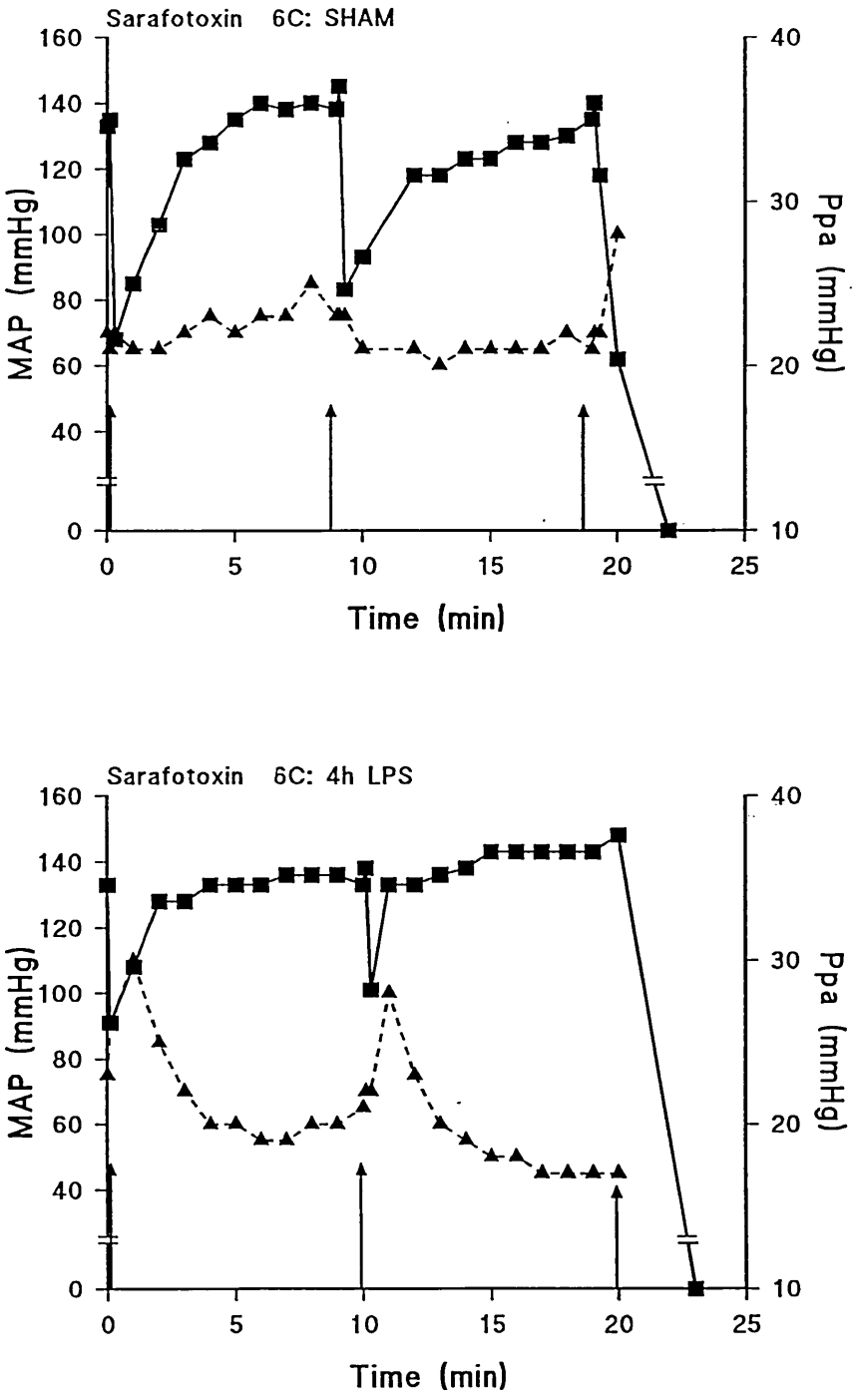


Figure 7.9 **Haemodynamic responses to sarafotoxin 6c in LPS-treated rats**

MAP and Ppa responses in rats treated 4h previously with *ip* LPS to ET-1 (open bars; n=6), DH2O in 3 identical volumes (striped bars; n=6) and the 2 smaller doses of sarafotoxin 6c (Sx6c) (solid bars; n=5).

(A) represents initial systemic hypotensive response, and (D) represents the initial Ppa pressor response to ET-1. The subsequent end-of-dose systemic (B) and Ppa (C) responses are also shown.

Results are expressed as means±SEM. Statistical analysis was undertaken using ANOVA with Tukey-Kramer tests for multiple comparisons. *p* values are for *treatment groups vs. DH2O controls* unless otherwise shown: * < 0.05; ** < 0.01.

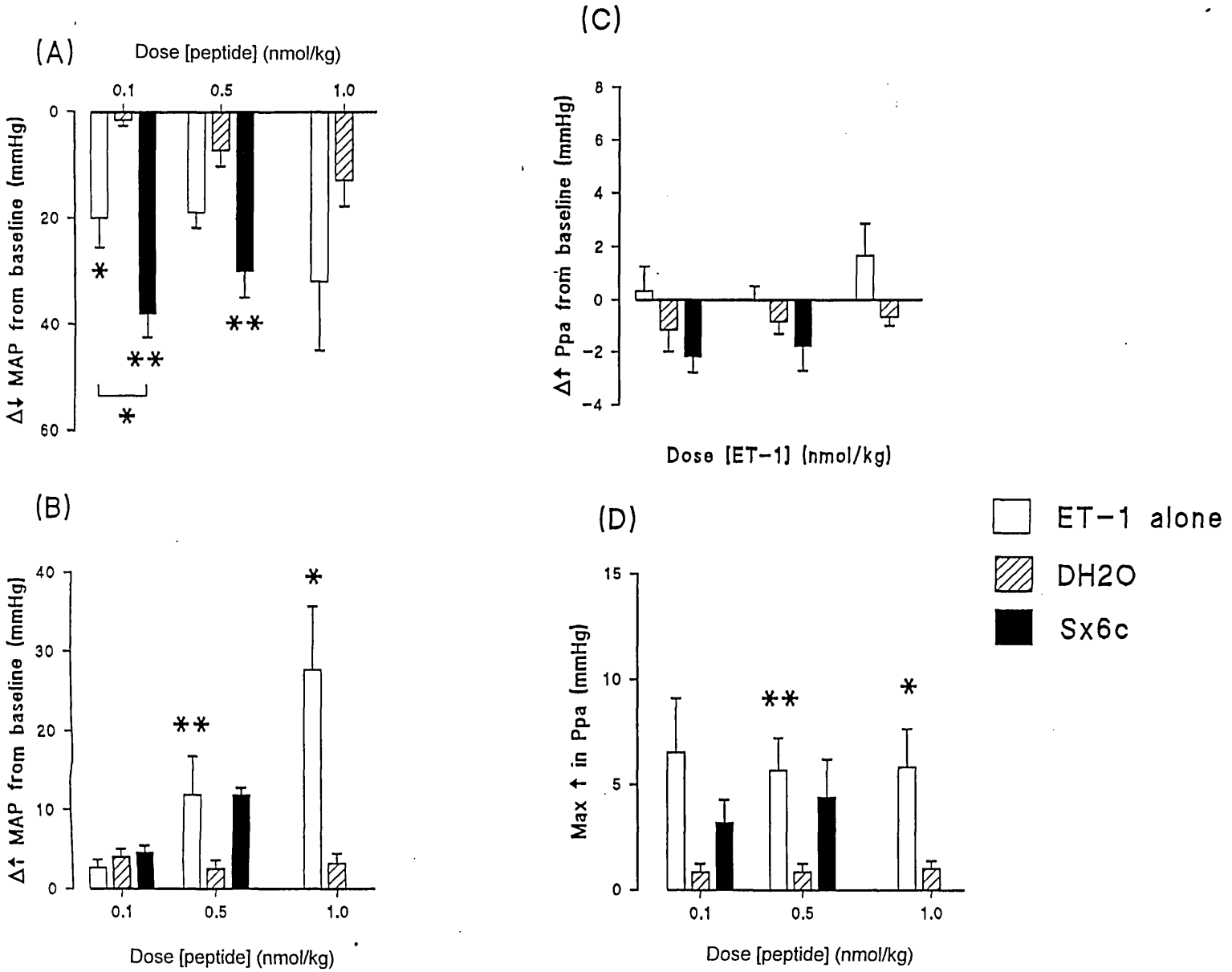

(A)
Dose [peptide] (nmol/kg)
0.1
0.5
1.0
Δ↓ MAP from baseline (mmHg)
0
20
40
60
*
**
*
**
(C)
Δ↑ Ppa from baseline (mmHg)
8
6
4
2
0
−2
−4
Dose [ET-1] (nmol/kg)
(B)
Δ↑ MAP from baseline (mmHg)
40
30
20
10
0
**
*
0.1
0.5
1.0
Dose [peptide] (nmol/kg)
(D)
Max ↑ in Ppa (mmHg)
15
10
5
0
**
*
0.1
0.5
1.0
Dose [peptide] (nmol/kg)
ET−1 alone
DH2O
Sx6c

Figure 7.10 **Comparison of haemodynamic responses to sarafotoxin 6c in sham and LPS-treated rats**

MAP and Ppa responses to 2 doses of sarafotoxin 6c (Sx6c) in sham (lined bars; n=6) or 4h post LPS rats (solid bars; n=5). (A) represents initial systemic hypotensive response, and (D) represents the initial Ppa pressor response to ET-1. The subsequent end-of-dose systemic (B) and Ppa (C) responses are also shown. (E) represents overall response after both doses. Results are expressed as means±SEM. Statistical analysis was undertaken using Students *t* test. *p* values : * < 0.05; ** < 0.01; ϕ < 0.001.

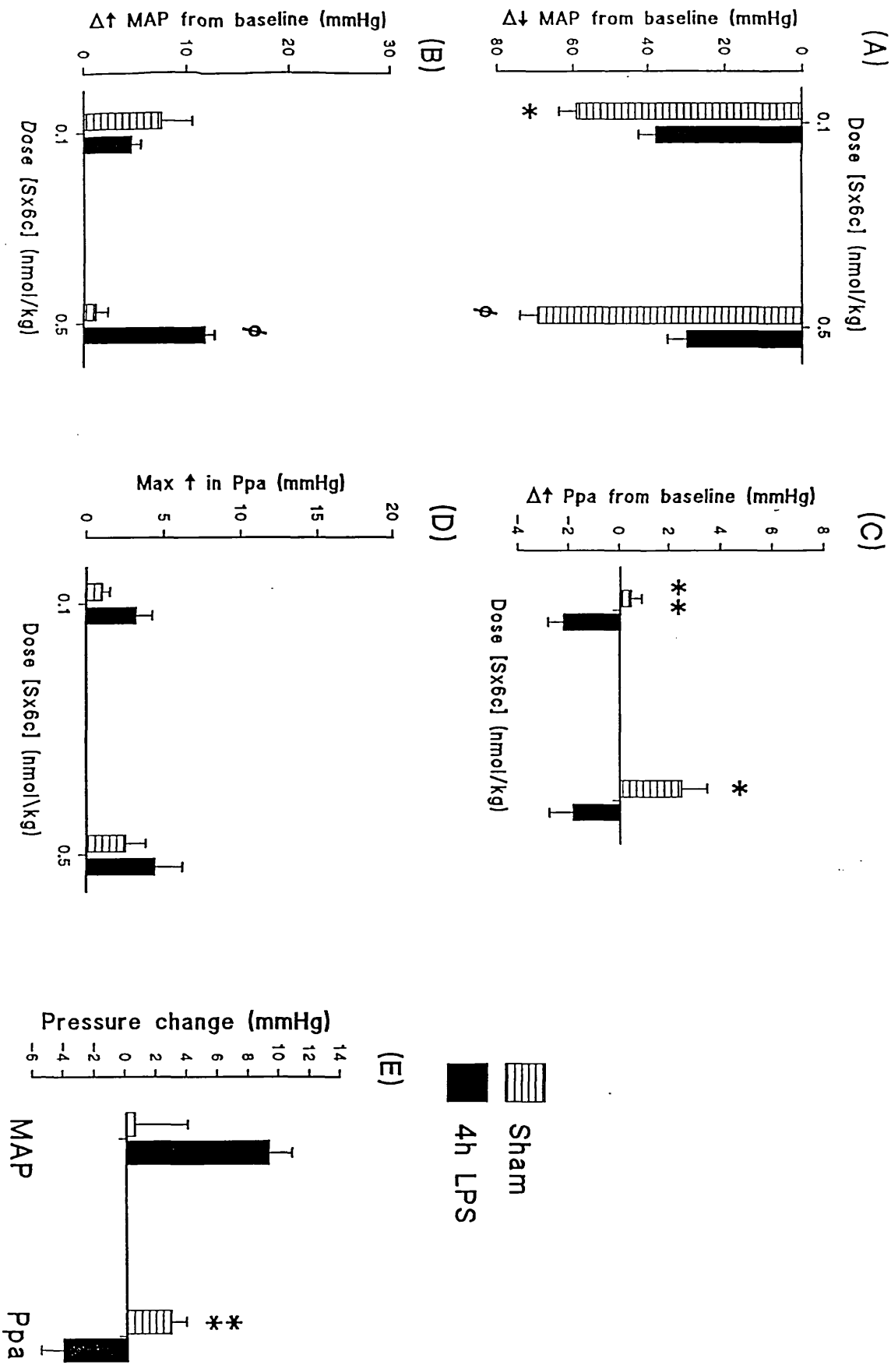

(A)
Δ↓ MAP from baseline (mmHg)
80
60
40
20
0
Dose [Sx6c] (nmol/kg)
0.1
0.5
*
ϕ
(B)
Δ↑ MAP from baseline (mmHg)
0
10
20
30
Dose [Sx6c] (nmol/kg)
0.1
0.5
ϕ
(C)
Δ↑ Ppa from baseline (mmHg)
−4
−2
0
2
4
6
8
Dose [Sx6c] (nmol/kg)
**
*
(D)
Max ↑ in Ppa (mmHg)
0
5
10
15
20
Dose [Sx6c] (nmol\kg)
0.1
0.5
(E)
Pressure change (mmHg)
−6
−4
−2
0
2
4
6
8
10
12
14
MAP
Ppa
**
Sham
4h LPS

Figure 7.11 **Typical MAP and Ppa responses to Angiotensin II**

Typical changes in mean systemic (MAP; squares) and pulmonary (Ppa; triangles) arterial pressures in a sham rat (LOWER PANEL) and a LPS-treated rat (UPPER PANEL) to 2 doses of angiotensin II.

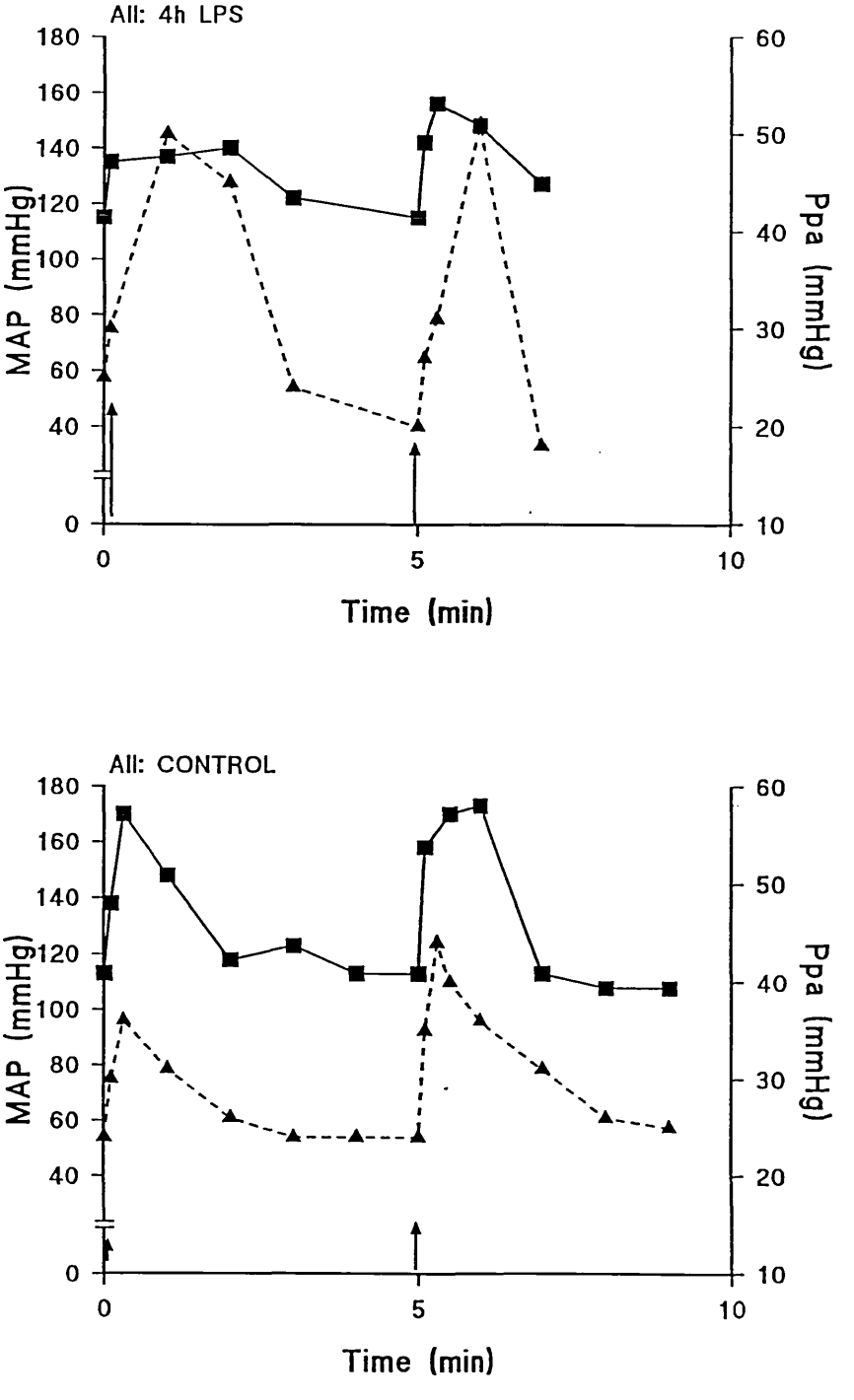
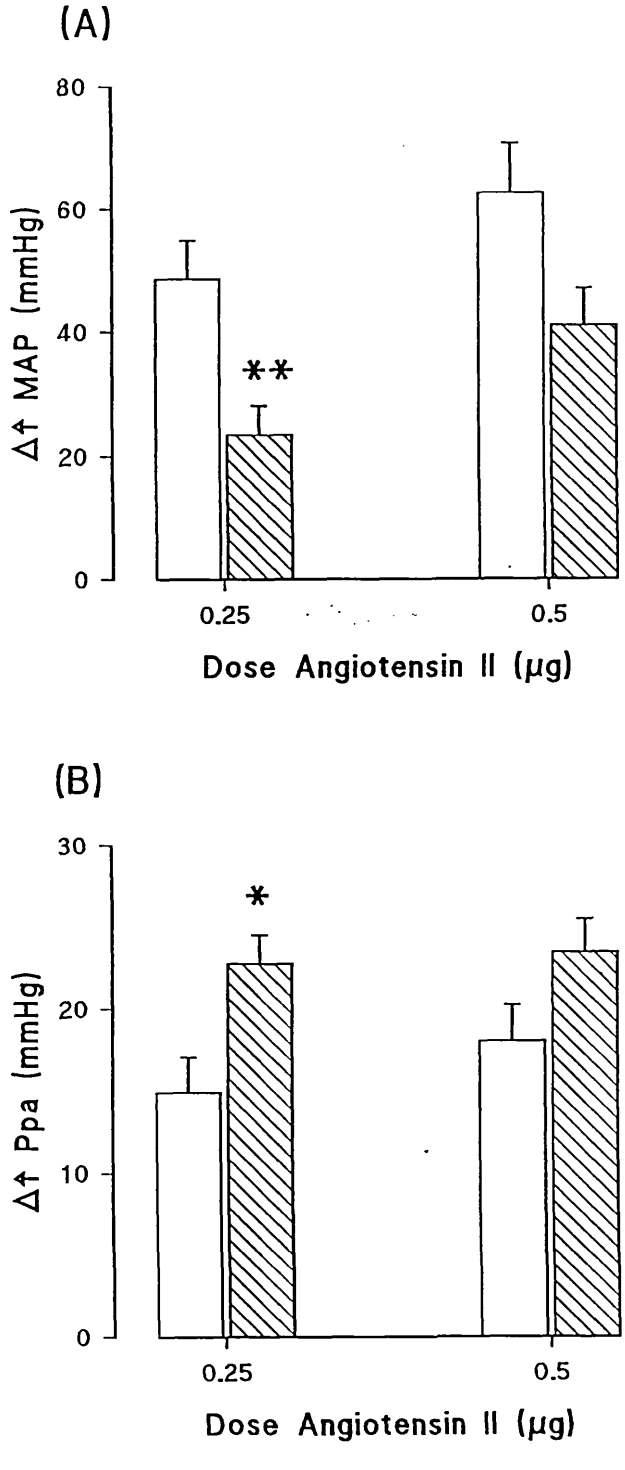


Figure 7.12 **Haemodynamic responses to angiotensin II in sham and LPS-treated rats**

Changes in mean systemic (UPPER PANEL) and pulmonary arterial (LOWER PANEL) pressures to 2 doses of angiotensin II in sham (open bars) and LPS-treated (striped bars) rats. Statistical analysis was made by Student's *t* test. *p* values: * <0.05; ** <0.01.



CHAPTER 8
GENERAL DISCUSSION

8.1 SUMMARY OF RESULTS

The administration of LPS as a 20mg/kg *ip* bolus induced an inflammatory response that includes neutrophil recruitment and infiltration into the interstitium of the lung, as well as copious NO production. Production of ET-1 was increased in this model following LPS challenge as assessed by both molecular and protein expression. Specifically, the expression of pre-pro-ET-1 mRNA was increased in heart, lung and aorta at 6h, in pulmonary artery and aorta at 1h, and not at all in skeletal muscle and kidney. There was no evidence of a specific steroid-suppressible step in the production of ET-1, in contrast to that of iNOS. Arterial plasma levels of ET-1 rose from 0.5h after LPS challenge, and had not plateaued by 6h.

Studies employing isolated PA yielded the following data. Firstly, ET-1-induced contraction of these vessels was modified by the endothelium. Secondly, *in vivo* LPS treatment induced hyporesponsiveness of these vessels to ET-1 as well as to PE, a hyporesponsiveness that could be almost completely reversed by the NOS inhibitor, L-NAME. In addition, a component of the ability of ET-1 to constrict intact PA rings from LPS-treated rats was dependent upon a cyclooxygenase product, even though contraction was unequivocally mediated by ET_A receptors. Thirdly, in these large conduit vessels, the hyporesponsiveness to ET-1 did not differ from those of thoracic aortae.

By contrast, the isolated, perfused lung preparation demonstrated increased sensitivity to exogenous ET-1 in LPS-treated rats, which was

potentiated by prior treatment with L-NMMA. The advantage of this system is that it studies the pulmonary circulation as a unit, rather than looking at just the large conduit arteries contained within it. There was a small, but significant increase in baseline Ppa in LPS-treated rats, which was not due to a circulating factor, and was not reversed by BQ123 or bosentan (although the pretreatment period for each was only 15 minutes). It was surprising that whilst BQ123 virtually completely attenuated the Ppa responses to ET-1; the ET_B antagonist, sarafotoxin S6c, produced an even greater elevation in Ppa, on a molar basis, than ET-1 itself. This ET_B-mediated response was also hypersensitive in LPS-treated rats, although, paradoxically, there was less pulmonary oedema formation. It is unclear why ET-1 itself did not have more ET_B agonist effects, unless the latter were produced by the ET_B agonist via non ET_A/nonET_B receptors.

In vivo the effects of ET receptor activation on MAP were similar to those that have already been described. LPS treatment significantly attenuated the rise in Ppa that was induced by ET-1, which is a novel finding. It appears that there is a greater attenuation of ET_B-mediated effects in endotoxaemia than of ET_A effects. Importantly, whilst no hypersensitivity was demonstrated in the Ppa response to ET-1, such differential hypersensitivity was seen with angiotensin II.

Despite evidence confirming increased production of ET-1, the reductions in MAP and Ppa *in vivo* seen after administration of bosentan (at a dose that unequivocally achieved adequate receptor antagonism) were small, and not significantly greater than those in sham animals. In addition, administration of bosentan and BQ123 in the isolated, blood-perfused lung preparation also failed to affect baseline Ppa, even in LPS-treated rats. This is an important combination of results, because it implies that ET-1, despite increased production, has little

effect on basal vascular tone in endotoxaemic conditions. Several factors need to be considered before this conclusion is accepted. The dynamics of the interaction of the peptide with its receptor may mean that an acutely administered receptor antagonist may take longer to unmask the effects of endogenous ET-1 that is already complexed to its receptor (and intracellularly sited) than it does to competitively inhibit the activity of exogenously administered ET-1. The first consideration, therefore, is that the time allowed following each of these receptor antagonists may have been inadequate to effect disruption of the already complexed endogenous ET-1. This suspicion is strengthened by data suggesting that reversal of established responses to ET-1, both *in vivo* and *in vitro* takes longer than 10-15 minutes (Warner et al. 1994). Further, in this study the attenuation of ET-1-induced pressor effects by the combined ET_A/ET_B antagonist, PD 145065, was much less after 60 minutes than by the ET_A antagonist BQ123. Secondly, if an antagonist such as bosentan were given before the LPS-induced increase in ET-1 production, this may have unmasked unsuspected effects of ET-1 on vascular tone. For example, the baseline Ppa and MAP values were no different in LPS-treated than in sham rats, but it is possible that ET-1 was contributing to these pressures in the LPS group, and that if this effect were removed then MAP and Ppa would have been lower. Indeed, since these experiments were performed, it has been reported that in conscious rats, the non-selective ET antagonist, SB 209670, caused significant potentiation of the fall in MAP during an infusion of LPS (Gardiner et al. 1995). A third important consideration is the increasingly reported interaction between NO and ET-1 release. NO can reduce ET-1 release, and a recent report suggests that some of the L-NMMA-induced pressor effects *in vivo* are mediated by ET-1 (Gardiner et al. 1996). This may be of particular relevance in

endotoxaemia, in which NO production is high, because NO may affect the potency of ET-1 as well.

8.2 Limitations of these experiments

8.2.1 Animal model

It is always important to be circumspect about applying laboratory-derived results to clinical conditions. Clearly, there are similarities between this model and the patient with clinical sepsis:

- In healthy lungs of rats and humans, iNOS activity is low, but increases in response to endotoxin and cytokines (Douglas et al. 1995; Liu et al. 1993; Griffiths et al. 1995a).
- In septic patients (Pittet et al. 1991; Voerman et al. 1992; Sanai et al. 1996), and in this model, arterial plasma [ET-1] are elevated.
- The starting Ppa was elevated in the LPS rats in the IBPL experiments, mimicking the elevated Ppa seen in many septic patients.
- Rats were generally studied at a time (4h post LPS) when a septic inflammatory response was already *established.* Pharmacological interventions at that stage were therefore a closer reflection of the clinical situation than the more artificial scenario in which interventions are made in animal models in order to *prevent* the sequelae of LPS-induced inflammation.

Just as clearly, however, there are fundamental differences between rats and patients. At 4h, the time at which functional data was obtained, there was no systemic hypotension, and no pulmonary hypertension, *in vivo* in the LPS-treated rats. This therefore reflects poorly the clinical state. Although the IBPL

preparation, with its fixed flow rate, did exhibit elevated Ppa, and hypersensitivity to ET-1, it was not possible to identify the regions of the pulmonary vascular bed that were responsible for these effects.

8.2.2 **Specific experiments**

It is not possible to determine which cell type was responsible for the increased expression of pre-pro-ET-1 mRNA using the RNase protection assay is that. In particular, this would be interesting to know in the vasculature.

The experiments *in vivo* would have been enhanced by measurements of cardiac output. Measurement of MAP and Ppa provides valuable information, but data about the vascular resistances of the systemic and pulmonary circulations would be extremely useful. Since the *in vivo* experiments were performed, this group has invested in equipment with which thermodilution cardiac output can be measured, and together with the pulmonary artery catheter, further detailed investigation of the properties of ET-1 in rat endotoxaemia can now be undertaken.

In the IBPL preparation, different volumes of blood were removed from control and LPS-treated rats. The total circulating volume was, however, standardised so that the final concentration achieved by each dose of drug/chemical was the same. The cross-transfusion experiments were performed in order to examine the potential effects of these differing blood volumes, and provided considerable reassurance that the vasoactive properties of the pulmonary circulation remained unchanged.

8.3 Therapeutic implications

Two of the key target areas for therapeutic advance in intensive care medicine are the reversal of hyporesponsiveness of systemic vessels in sepsis, with the aim of restoring adequate mean blood pressure, and reduction of PVR, with the expectation that this would both reduce mortality and improve pulmonary gas exchange. NOS inhibitors have already been given in patients with septic shock in order to restore systemic blood pressure (Griffiths et al. 1994). However, despite evidence that blood pressure is indeed improved in patients who appear virtually unresponsive to conventional pressor agents (particularly catecholamines) (Griffiths et al. 1994; Petros et al. 1991; Petros et al. 1994), trials are yet awaited that promise to provide data on the outcome of such patients. One of the theoretical concerns about this increasingly utilised therapy is that it may cause detrimental increases in PVR (Lorente et al. 1993; Griffiths et al. 1994). The results obtained in Chapters 5 and 6 using non-specific NOS inhibitors in both isolated PA, and in the IBPL, lend weight to this theoretical concern, since in both cases the constrictor responses to ET-1 were potentiated following pretreatment with such agents. Whether some of the increase in starting Ppa that was observed in the LPS-treated rats following 10^{-3}M L-NMMA is due to endogenous ET-1 release (Gardiner et al. 1996) is so far unclear, but these data encourage scepticism about the success of global non-specific NOS inhibition, particularly because similar considerations must be made with regard to the renal and splanchnic beds (Hutcheson et al. 1990; Walder et al. 1991).

A second therapeutic consideration concerns the potential benefit of ET receptor antagonists in attenuating the rise in PVR seen in critically ill patients. Both ET_A (Okada et al. 1995; Bonvallet et al. 1993) and combined ET_A/ET_B antagonists (Chen et al. 1995) have been shown to attenuate PHT in

experimental conditions, but in these cases the aetiology of the PHT was different, and the time course more prolonged. Whilst the results using the ET_A antagonist, BQ123, in Chapter 6, show that it is via these receptors that ET-1 achieves its pressor effects in the rat pulmonary circulation, it is important to note that it had no effect on the baseline Ppa after 15 minutes. Whilst this may be an inadequate time course in which to interfere with the interaction between endogenous ET-1 and its receptors, the results as they stand do not lend support to the therapeutic benefit of such a strategy. More experimental data is now required, particularly because global ET_A receptor blockade may have adverse effects upon blood flow in other organs, such as the kidney.

Finally, these data sound a warning about the projected use of either ET-1 or angiotensin II as pressor agents in critically ill patients with refractory hypotension (Ryding et al. 1995; Thomas and Nielsen, 1991). The results from administration of either of these agents in the pulmonary circulation of LPS-treated rats (Chapters 6 & 7) demonstrate the potential for large, rapid rises in Ppa in such circumstances. Careful assessment of the effects on PVR is required in patients in whom angiotensin II, in particular, is used as a "rescue" pressor agent.

8.5 Future experiments

Using modern haemodynamic monitoring equipment, the priority must be to examine in more detail the functional role of the *endogenous* ET-1 that is produced in the pulmonary vasculature in sepsis. Administration of selective antagonists over at least 60 minutes would be expected to unmask its vasoactive effects. In addition, a closer examination of the interplay between NO and ET-1 release and effector systems may prove valuable, particularly in the light of

current therapeutic strategies that employ both NOS inhibitors and inhaled NO! It is already important to study the effects of such treatments on the balance between endogenously produced vasoactive mediators, including NO, ET-1, prostanoids and possibly isoprostanes.

Much of the data presented in Chapters 4,5,6 and 7 implies that there is a change in numerical or functional ET receptor status as a result of LPS-induced inflammation. Receptor binding studies would yield specific information about the patterns of such changes.

If it proves possible to produce viable ET-1-gene-deleted rodents, then this would provide an exciting opportunity to examine the effect of LPS-challenge in an *in vivo* model that is unable to produce endogenous ET-1, as well as ET receptors. Coupled to this, more specific inhibitors of the various ECE enzymes may become available as the system is better understood, and these may provide a more selective way to interefere with the unwanted effects of ET-1 whilst preserving the beneficial effects.

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