Vascular Control Mechanisms in Normal and Lipopolysaccharide-treated Rats

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

Lisa Jolly, BSc (Hons)

2009

The University of Nottingham

Centre for Integrated Systems Biology and Medicine,
School of Biomedical Sciences
University of Nottingham Medical School
Queen's Medical Centre
Nottingham, NG7 2UH

Abstract

The development of sepsis is associated with complex cardiovascular changes, some of which can be micmked in animals by administration of lipopolysaccharide (LPS). Animal models have identified a number of mediators important in these changes. The work described within this thesis aimed to investigate the role of adrenomedullin (AM) and adenosine in regulating vascular function *in vivo* in normal and LPS-treated rats. Integrated haemodynamics were assessed in Sprague Dawley rats following implantation of pulsed Doppler flow probes, allowing changes in renal, mesenteric and hindquarters vascular conductance to be measured across time.

Adrenomedullin (AM), a hypotensive peptide involved in cardiovascular regulation, is upregulated in sepsis. Intermedin (IMD) is related to AM and shares some of its functions. However, the *in vivo* integrated responses to IMD have yet to be determined. In normal rats, both peptides caused marked vasodilatations in all regions, with hypotension and tachycardia. IMD was a more potent vasodilator than equimolar AM.

Next, mechanisms involved in IMD signalling were investigated and compared to AM. Both AM and IMD-mediated renal and mesenteric vasodilatation were attenuated by AM_{22-52} and some components of IMD were sensitive to L-NAME, suggesting IMD causes both endothelial-dependent and –independent vasodilatations. No role for K_{ATP} channels was found, but there was an enhanced response to AM in the presence of U37883A; this was due to

inhibition of the renin-angiotensin system as assessed by the angiotensin II receptor antagonist losartan.

To assess whether vascular sensitivity to AM and IMD was affected in an LPS model of endotoxaemia, rats were treated with LPS and responses to peptides were assessed at 1.5 h, 6 h and 25 h. Vascular hyporesponsiveness to both AM and IMD occurred at 1.5 h, but had returned by 25 h regardless of the LPS administration protocol. Thus, vascular hyporesponsiveness appears to be a common phenomenon during the early stages of LPS-induced endotoxaemia.

The role of adenosine was then examined in the haemodynamic sequelae of sepsis, since evidence suggests that adenosine-mediated vasodilatations help to maintain regional perfusion in animal models. In control rats, endogenous adenosine caused bradycardia and vasodilatation, whereas there was evidence of regional vasoconstriction in LPS-treated rats. In control animals, exogenous adenosine caused hypotension, tachycardia and vasodilatation, but in LPS-treated rats, the adenosine-induced renal (at 1.5 h) and hindquarters (at 6 h) vasodilatations were abolished. As enhanced A_1 receptor-mediated vasoconstriction could explain the results in LPS-treated rats, responsiveness to an A_1-receptor agonist (CCPA) or antagonist (DPCPX) was assessed. There was no evidence for enhanced vasoconstrictor responsiveness to CCPA in LPS-treated rats, but DPCPX caused renal vasodilatation, consistent with endogenous adenosine mediating renal vasoconstriction.

Finally, the effects of a subdepressor infusion of adenosine on the haemodynamic responses to AM and IMD were assessed, and vice versa, to determine whether any synergism exists between these agents. No synergism was found between adenosine and AM, but there was functional antagonism between adenosine and IMD in the mesenteric vasculature.

Collectively, these studies suggest that the development of novel cardiovascular therapies for treatment of sepsis should be designed to take into account the vascular region, and the time elapsed from onset.

Publications

Full papers

Jolly, L, March, JE, Kemp, PA, Bennett, T, Gardiner, SM (2009). Mechanisms involved in the regional haemodynamic effects of intermedin (adrenomedullin 2) compared with adrenomedullin in conscious rats. *Br. J. Pharmacol.* **157**: 1502-1513.

Jolly, L, March, JE, Kemp, PA, Bennett, T, Gardiner, SM (2008). Regional haemodynamic responses to adenosine receptor activation vary across time following lipopolysaccharide treatment in conscious rats. *Br. J. Pharmacol.* **154**: 1600-1610.

Abstracts

L. Jolly et al. (2008a). Regional haemodynamic effects of adenosine in normal and lipopolysaccharide-treated rats, http://www.pA2online.org/abstracts (Presented in Poster format to the British Pharmacological Society Winter Meeting 2007).

L. Jolly et al. (2008b). Adenosine A1 receptor-mediated haemodynamic responses in normal and lipopolysaccharide (LPS)-treated rats http://www.pA2online.org/abstracts (Presented in Poster format to the British Pharmacological Society Winter Meeting 2007).

L. Jolly et al. (2007). Vascular hypo-responsiveness to intermedin (IMD) following lipopolysaccharide (LPS) administration in conscious rats. http://www.pA2online.org/abstracts (Orally presented to the British Pharmacological Society Winter Meeting 2006).

Acknowledgments

First and foremost I wish to thank my supervisors Professors Sheila Gardiner and Terry Bennett for giving me the opportunity to complete this research, and for all their advice and guidance in writing this thesis. In particular I wish to thank Sheila for her support, and for giving me the confidence to be able to complete this PhD. I wish to thank Julie March for her help with the surgical aspect of this work; you truly have the patience of a saint! And finally, I wish to thank Philip Kemp for help with all technical aspects of this work, and for putting up with my moaning for the past 3 years!

No acknowledgment would be complete without thanking my parents. To my mum, thank you for always being there for me, you really are one in a million. Dad, thank you for passing to me a strong work ethic; you're the hardest working person I know and if I'm only a little like you then I'm happy. I know you will probably never read further than this page, but I love you both very much.

Finally, I wish to thank Christopher for putting up with me for the past few years, particularly during the writing of this thesis. You deserve a PhD yourself!

Abbreviations

8-PT	8-phenyltheophylline
ACh	Acetylcholine
ADA	Adenosine deaminase
ADM	Adrenomedullin
ADO	Adenosine
ADP	Adenosine diphosphate
AII	Angiotensin II
AMBP-1	Adrenomedullin binding protein-1
AMP	Adenosine monophosphate
APC	Activated protein C
ATP	Adenosine-5'- triphosphate
AV	Atrioventricular
AVP	Arginine vasopressin
CARS	Compensatory anti-inflammatory response syndrome
CCPA	2-choloro-N^6-cyclopentyladenosine
CL	Calcitonin receptor-like-receptor
CLP	Caecal ligation and puncture
cNOS	Constitutive nitric oxide synthase
CNS	Central nervous system
CO	Cardiac output
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
eNOS	Endothelial nitric oxide synthase
GPCR	G-protein-coupled-receptor
HIF-1	Hypoxia inducible factor-1
HR	Heart rate
i.a.	Intra-arterial
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravascular
ICU	Intensive care unit
IL-1/6/8/10	Interleukin-1/6/8/10
IMD	Intermedin
iNOS	Inducible nitric oxide synthase
K_{ATP}	ATP-sensitive potassium channel
LBP	LPS-binding protein
L-NAME	NG-nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
LV	Left ventricle
MAP	Mean arterial pressure
MODS	Multiple organ dysfunction syndrome
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern

PKA/B/C	Protein kinase A/B/C
PLC	Phospholipase C
PRR	Pattern recognition receptor
RAAS	Renin angiotensin system
RAMP	Receptor activity modifying protein
RAMP	Receptor activity modifying protein
s.c.	Subcutaneous
siRNA	Small interfering RNA
SIRS	Systemic inflammatory response syndrome
SVR	Systemic vascular resistance
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor alpha
TPR	Total peripheral resistance
VIP	Vasoactive intestinal peptide

Table of contents

CHAPTER 2: Methods

CHAPTER 3:

Results (I): ***Regional haemodynamic effects of adrenomedullin and intermedin***

CHAPTER 4:

Results (II): *Mechanisms of adrenomedullin and intermedin-mediated vasodilatation*

CHAPTER 5:

Results (III): ***Regional haemodynamic effects of adrenomedullin and intermedin in lipopolysaccharide-treated rats***

CHAPTER 6:

Results (IV): ***Cardiovascular responses to adenosine receptor activation and antagonism in normal and LPS-treated rats***

CHAPTER 7:

Results (V): ***Interactions between adenosine, adrenomedullin and intermedin in the cardiovascular system of conscious rats***

CHAPTER 8: General discussion

List of tables

CHAPTER 1: General Introduction

CHAPTER 2: General Methods

CHAPTER 3: Results (i)

CHAPTER 4: Results (ii)

CHAPTER 5: Results (iii)

CHAPTER 6: Results (iv)

CHAPTER 7: Results (v)

List of figures

CHAPTER 1: General Introduction

CHAPTER 2: General Methods

CHAPTER 3: Results (i)

Chapter 4: Results (ii)

Chapter 5: Results (iii)

Chapter 6: Results (iv)

Chapter 7: Results (v)

Chapter 1

General Introduction

1 General Introduction

The word 'sepsis' originates from the Greek for 'putrefaction', and was first used by Hippocrates (460-377 $_{BC}$) in reference to illness caused by an unknown poison which led to rapid and unpreventable death. Despite substantial medical and theoretical advancement over the intervening years, prognosis for patients suffering from sepsis is still poor, and it remains one of the leading causes of death worldwide. Sepsis refers to a syndrome characterised by an exaggerated and unpredictable systemic inflammatory response, and involves complex cardiovascular, immunological, respiratory, metabolic and neurological changes. The heterogeneous nature of the syndrome leads to difficulties in its correct diagnosis and management, with current treatments centred on supportive therapies that have limited efficacy if the patient presents symptoms of septic shock. Therefore, continued research into sepsis is crucial to identify novel therapeutic targets.

The development of sepsis is associated with biphasic changes in haemodynamics. A hyperdynamic circulation ensues during the onset of sepsis, when patients have an elevated cardiac output and a decrease in total peripheral resistance resulting in the maintenance of normal blood pressure. These features can be mimicked by administering low-dose lipopolysaccharide (LPS); a component of Gram-negative bacteria which is used routinely to model the disorder in human volunteers and animals. During late sepsis, as the status of the patient deteriorates, a hypodynamic circulation develops which is characterised by a low cardiac output and widespread regional vasodilatations that lead to resistant or 'unrelenting' hypotension. This can be mimicked by

higher doses of LPS. This involves impaired regulation of, and vascular hyporesponsiveness to, a wide variety of mediators, including adrenomedullin (AM), intermedin (IMD) and adenosine.

The calcitonin family of peptides includes AM and IMD. Both peptides elicit a potent hypotensive response when given to conscious and anaesthetised animals, and cause marked vasodilatations, mediated by endothelial-dependent and -independent mechanisms. The purine nucleoside, adenosine, also causes widespread vasodilatation and hypotension *in vivo,* and as a component of ATP, acts to couple metabolic demand to blood flow.

Impaired regulation of, and vascular hyporesponsiveness to, vasoactive mediators is involved in the development of resistant hypotension during sepsis. For example, protein levels of AM are increased in the plasma of septic patients and vascular responsiveness to the peptide is reduced in late sepsis. Endogenous adenosine has also been implicated in the development of regional vasodilatations during sepsis. Such findings have led researchers to suggest that both AM and adenosine are involved in the development of the haemodynamic sequelae associated with the pathophysiology of sepsis.

The first part of this Introduction will discuss sepsis, with reference to its epidemiology, pathophysiology and study. The second part will focus on AM and the recently discovered AM-2, or intermedin (IMD), and discuss what is currently known about their function in normal and pathophysiological states; with specific reference to sepsis. Finally, the third part will discuss the role of adenosine in the cardiovascular system and its role in the pathogenesis of sepsis.

1.1 Sepsis

1.1.1 Epidemiology

Septic shock is a major cause of mortality in Intensive Care Units (ICU) worldwide. A study by Angus *et al.* (2001) estimated there are 751,000 cases of severe sepsis each year in US hospitals, with a mortality rate of 28.6%; although some estimates put this as high as 40-50% (Tsiotou *et al.*, 2005; Vincent *et al.*, 2006). Furthermore, it was highlighted that in the US alone this amounts to an annual national cost of $16 billion, or $22,100 per case. An Intensive Care National Audit & Research Centre analysis of a clinical database over a 9-year period in the UK (1996-2004), calculated the number of admissions to ICUs with severe sepsis as 92,672, totalling 27% of all admissions. Between 1996 and 2004, the proportion of admissions increased from 23.5% to 28.7%. So, whilst the overall percentage mortality is slowly decreasing (from 34.3% in 1996, to 30.8% in 2004), the overall number of deaths is increasing (Harrison *et al.*, 2006). These data provide an indication of both the human cost, and the financial burden of sepsis on healthcare services worldwide. In addition, even if the patient survives the initial septic episode, there remains an increased risk of mortality for up to 5 years (Quartin *et al.*, 1997).

Difficulty lies with the diagnosis and treatment of sepsis; it is a heterogeneous condition with multiple causes, and involves the interplay of many systems. A Consensus Conference of American College of Chest Physicians/Society of Critical Care Medicine held in 1991, aimed to standardise definitions relating

to the sepsis syndrome and define specific parameters that should be present for a diagnosis to be made (Bone *et al.*, 1992). Traditionally used definitions such as 'septicaemia' were replaced with 'systemic inflammatory response syndrome' in cases with no documented infection, and 'sepsis' where infection is present. Furthermore, the terms 'sepsis', 'severe sepsis' and 'septic shock' were used to describe a continuum of the same disorder. The exact definitions for these terms, along with the symptoms necessary for diagnosis, are given in Table 1.1. In addition, diagnosis is complicated by the underlying status of the patient; pre-existing morbidities are common in patients who develop sepsis, particularly in those with a compromised immune system (i.e., increased risk in HIV patients) and those receiving chronic treatment through intravascular catheters (i.e., increased susceptibility in patients with a central venous catheter) (O'Brien *et al.*, 2007). Other factors affecting the development, progression and outcome of sepsis are age (i.e., increased risk in over 65s), gender (i.e., slightly increased risk in males) and ethnicity (i.e., Caucasian populations less susceptible than African Americans) (O'Brien *et al.*, 2007).

Whilst these guidelines have helped improve and standardise the diagnosis of sepsis, they fail to identify the nature of the underlying infection. A recent study of sepsis in European ICUs (SOAP study; Vincent *et al.*, 2006) found that only 60% of septic patients presented positive microbial cultures. Of these, the most common culture was positive for Gram-positive bacteria (40%), with *Staphylococcus* strains accounting for the majority of cases (including MRSA accounting for 14% of this subgroup). This was closely followed by cultures positive for Gram-negative bacteria (38%), of which

Pseudomonas strains and *Escherichia coli* were the most common species (14% and 13%, respectively). Fungi were identified in 17% of patients with a positive blood culture, whilst 18% presented a mixed culture (Vincent *et al.*, 2006). A reliable, clinically available test for Gram-negative bacteria is important since some antibiotics release endotoxin from micro-organisms (Jarisch-Herxheimer reaction), leading to induction of the cytokines TNF-α, IL-6 and IL-8 which may be detrimental to patients with already high endotoxin levels (for review see Lepper *et al.*, 2002).

Once a diagnosis of sepsis is made, volume resuscitation, broad-spectrum anti-microbial therapy, vasopressor treatment (commonly noradrenalin), life-support, where required are given (O'Brien *et al.*, 2007). The value of supportive treatments is limited once the patient has symptoms of severe sepsis or septic shock. The only therapy currently available that specifically targets the inflammatory response is the anti-coagulant, recombinant activated protein C (rAPC or drotrecogin alfa (activated)), since the ability to produce endogenous APC is impaired in septic patients. As this treatment can cause excessive bleeding in patients with pre-existing coagulation problems, it is used with caution and only in patients with a high risk of death (for review see O'Brien, 2007; Toltl, 2008).

The most common site of infection in patients presenting sepsis is the lungs (44-64%) (Angus *et al.*, 2001; Vincent *et al.*, 2006), followed by the abdomen

Table 1.1 *The Sepsis Syndrome*

Term	Definition	Diagnosis
Infection	Inflammatory response to microorganisms, or invasion of normally sterile host tissue by microorganisms	
Bacteraemia	Presence of viable bacteria in the blood	
SIRS	Systemic inflammation arising from an unknown cause	>1 of the following: • body temperature >38°C or <36°C • heart rate >90 beats min^{-1} • respiratory rate >20 breaths min^{-1} • white blood cell count >12 000 cu mm^{-1} or <4000 cu mm^{-1}
Sepsis	Systemic inflammation in the presence of infection	As above, with infection from a confirmed source
Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion or sepsis-induced hypotension	• Symptoms may include lactic acidosis, oliguria, acute alteration of mental state • Sepsis-induced hypotension: systolic BP of <90 mmHg or its reduction by 40 mm Hg
Septic shock	Persistent sepsis-induced hypotension (despite fluid resuscitation) plus hypoperfusion abnormalities and organ dysfunction	As above, with evidence of MODS
MODS	Continuum of organ dysfunction	• Altered organ function; organ(s) unable to maintain normal homeostasis • Can be primary or secondary

Adapted from Bone *et al.* (1992, 1993). SIRS: systemic inflammatory responses syndrome; MODS: multiple organ dysfunction syndrome

(22%), blood (20%) urinary tract (14%) and infections arising from intravascular catheters (8%) (Vincent *et al.*, 2006). Furthermore, loss of the intestinal epithelial barrier function in an inflammatory environment may allow the translocation of harmful bacteria from the gut to the blood in instances where the gut is not the main source of infection, leading to secondary infections and the development of systemic inflammation (for review see Rittirsch, 2008).

1.1.2 Pathophysiology of sepsis

In normal physiology, the immune system is tightly regulated to maintain the correct level of protective versus damaging responses in tissues. Conversely, host detection of a pathogen can cause overstimulation of the innate immune response; subsequent loss of inflammation control leads to the development of sepsis. The presence of pathogen-associated molecular patterns (PAMPs) on harmful microorganisms is identified by pattern recognition receptors (PRR); these include the Toll-like receptors (TLR), of which TLR-2 and TLR-4 are activated upon recognition of LPS (Kirschning *et al.*, 1998; Yang *et al.*, 1998; Rittirsch 2008). Interestingly, polymorphisms in proteins of the Toll-family may account for variable responses to septic insults in individuals, with the *TLR-4* Asp299Gly allele causing enhanced sensitivity to LPS and increased susceptibility to Gram-negative infections (Lorenz *et al.*, 2002). The signalling pathway induced by LPS binding to TLR-4 is shown in Figure 1.1. Briefly, recognition of the PAMP-containing pathogen, LPS, by TLR-4 triggers a signalling cascade, which leads to the translocation of NF-κB to the nucleus where it causes transcription and translation of pro-inflammatory mediators.

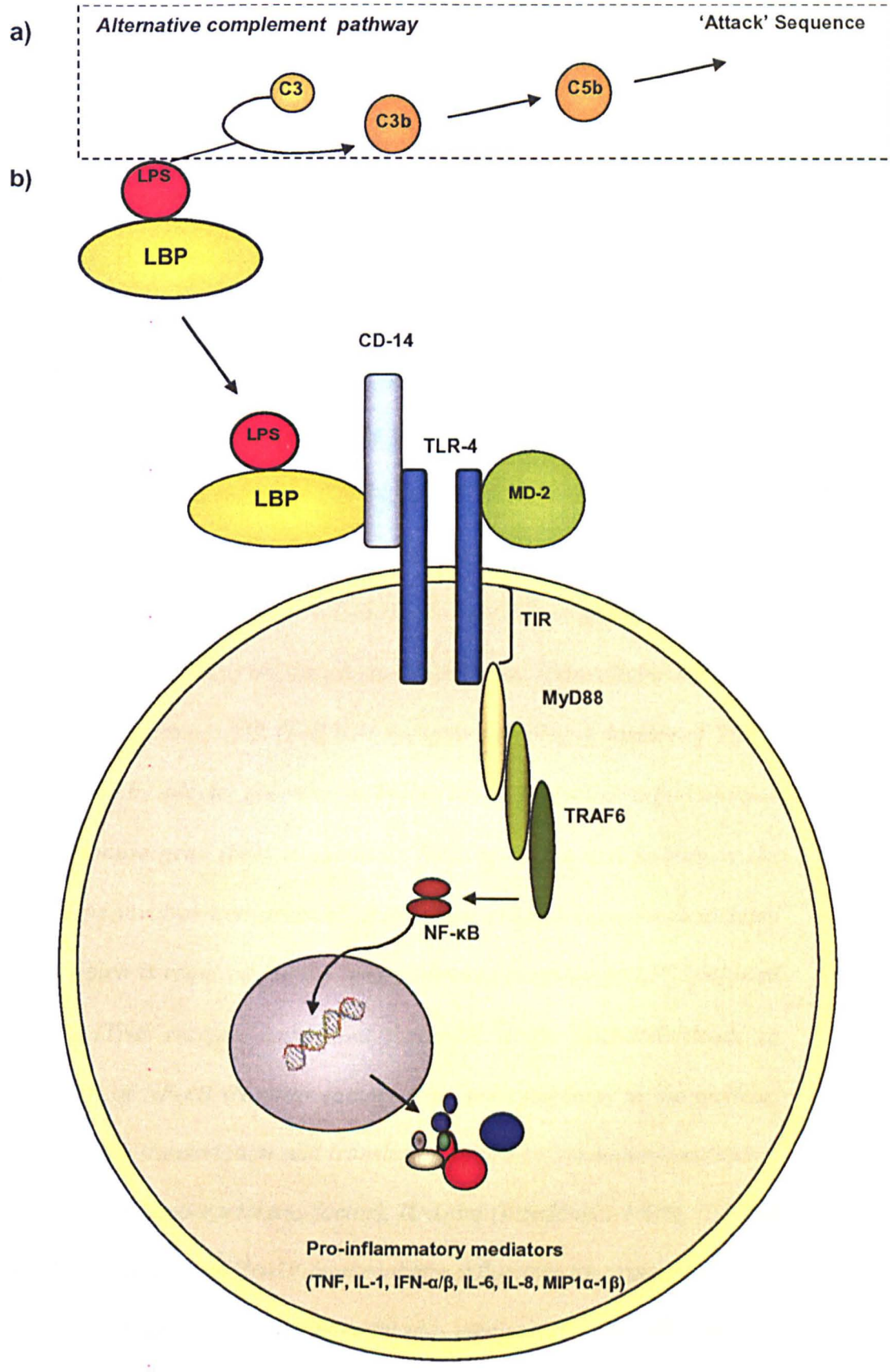

a)
Alternative complement pathway
'Attack' Sequence
C3
C3b
C5b
b)
LPS
LBP
CD-14
LPS
LBP
TLR-4
MD-2
TIR
MyD88
TRAF6
NF-κB
Pro-inflammatory mediators
(TNF, IL-1, IFN-α/β, IL-6, IL-8, MIP1α-1β)

Figure 1.1 *LPS signalling cascade*

a) *LPS activates the alternative pathway of the complement cascade, increasing levels of complement products, C3b and C5a, which function to enhance phagocytosis, and promote the development of a local inflammatory response, respectively.*

b) *LBP (LPS binding protein), produced by the liver, is constitutively active at low levels but upregulated after administration of LPS. LBP bound to LPS is detected by the opsonic receptor CD-14, which, along with LBP, is required for LPS sensing by the cell. The LPS/LBP/CD-14 complex activates TLR-4 which is bound to MD-2, a cell-surface molecule required for TLR-4 activation and positioning on the plasma membrane. Intracellular signalling requires the cytoplasmic TIR (Toll/IL-1 receptor homology) domain of TLR-4 which is bound by adapter proteins, including MyD88 (Myeloid differentiation primary response gene (88)) to facilitate binding of the next factors in the cascade. This involves activation of IRAK (Interleukin-1 receptor associated kinase 4) which is required for the innate immune response to LPS, followed by TRAF6 (TNF receptor-associated factor-6) which ultimately leads to translocation of NF-κB (Nuclear factor) from the cytoplasm to the nucleus. Here, it induces transcription and translation of pro-inflammatory mediators, including TNF (Tumour necrosis factor), IL-1/6/8 (Interleukin-1/6/8), IFN-α/β (Interferon- α/β) and MIP1α-1β (macrophage inflammatory protein-1) which leads to activation of pro-inflammatory mediators and cell-adhesion molecules.*

(For review see Cohen, 2002; Beutler & Rietschel, 2003).

Lipopolysaccharide is also an activator of the alternative pathway of the complement system leading to increased levels of the anaphylatoxin and pro-inflammatory product, C5b. These mechanisms act in concert to produce a pro-inflammatory environment, sometimes referred to as the systemic inflammatory response syndrome (SIRS) (Toltl *et al.*, 2007) (A potential source of confusion since this term is also used in the diagnosis of sepsis, section 1.1.1.) which is further exacerbated by down-regulation of anti-inflammatory mediators. In severe sepsis, there is an over- compensatory increase in anti-inflammatory mediators (such as IL-10 and IL-1ra), referred to as compensatory anti-inflammatory syndrome (CARS); this leads to immunosuppression in already ill patients and can result in further complications caused by secondary infections (for review see Toltl *et al.*, 2007).

Inflammation is also under control of the autonomic nervous system by the 'inflammation reflex'. Normally, inflammatory homeostasis is controlled by the two opposing arms of the autonomic nervous system. Activation of the cholinergic system suppresses the release of pro-inflammatory mediators, whilst activation of the adrenergic system leads to increased levels of circulating catecholamines and enhanced production of pro-inflammatory mediators. Dysregulation of these systems is involved in the development of sepsis; activation of the sympathetic nervous system in early sepsis enhances inflammation through an increase in circulating catecholamines (for review see Rittirsch *et al.*, 2008).

Enhanced inflammation leads to apoptosis of dendritic cells, lymphocytes, monocytes and neutrophils; this further exacerbates the pro-coagulatory environment in sepsis. Increased levels of apoptosis may be involved in the development of immunosuppression, loss of GI and respiratory tract cells and ultimately organ dysfunction in septic patients (O'Brien, 2007, review). In severe cases, sepsis-mediated dysregulation of coagulation results in disseminated intravascular coagulation, leading to microcirculatory impairment due to of increased fibrin deposition (Rittirsch, 2008).

Metabolic disturbances are a further complication of sepsis. In addition to tissue hypo-perfusion, impaired cellular oxygen extraction due to mitochondria failure ('cytopathic hypoxia') leads to the development of hypoxaemia and organ failure during sepsis (for review see Carré & Singer, 2008). The failure of tissues to extract oxygen leads to high venous O_2 measurements in septic patients (Sair *et al.*, 1996), and heterogeneity of tissue oxygenation in experimental models of shock (Dyson *et al.*, 2007). Many of the pathophysiological features of sepsis can be attributed to disturbances of the microcirculation; in fact, sepsis has been referred to as a 'disorder of the microcirculation' (Trzeciak *et al.*, 2008).

1.1.2.1 Cardiovascular changes during sepsis

The cardiovascular pathophysiology of sepsis and severe sepsis is marked by defined haemodynamic changes that rapidly deteriorate as the disorder progresses through the spectrum. These changes are biphasic and characterised by an early hyperdynamic phase (often called 'warm shock' as patients are

'flushed') which progresses into a hypodynamic stage ('cold shock' as patients are cold to the touch) as the status of the patient declines. A hyperdynamic circulation is characterised by an increase in cardiac output, regional perfusion, oxygen delivery and consumption. During this stage, patients remain normotensive due to the increase in cardiac output, in spite of the systemic vasodilatation. Surgical intervention, treatment with drugs and supportive therapy can be effective during the hyperdynamic phase. This distinct haemodynamic pattern is documented in adults with sepsis, and in paediatric sepsis arising through central venous catheter infection (Brierley & Peters, 2008). Severe sepsis is associated with a hypodynamic circulation which is characterised by a fall in cardiac output and blood flow leading to 'unrelenting' or 'resistant' hypotension. During this phase, the patient becomes increasingly unresponsive to treatment and the chance of recovery is significantly reduced (for review see Fowler *et al.*, 2003; Buras *et al.*, 2005; Tsiotou *et al.*, 2005).

Observed systemic cardiovascular changes during sepsis arise through unbalanced activation and inhibition of vasodilator and vasoconstrictor systems, impaired cardiac function and endothelial dysfunction. Endothelial cells release factors involved in coagulation homeostasis, and vascular smooth muscle relaxation and hyperpolarisation; therefore, endothelial dysfunction further exacerbates the hypotension and coagulopathy observed in sepsis (for review see Trzeciak *et al.*, 2008). For example, loss of vascular responsiveness to adrenergic agents is a common trait in both clinical and experimental sepsis, despite increased levels of circulating catecholamines; this contributes to the

resistant hypotension seen in the late stage of the disease. Diminished vascular responsiveness to α-adrenoceptor agonists, 5'-hydroxytryptomine, potassium and prostanoids, as well as the endothelial-dependent vasodilators acetylcholine (ACh), salbutamol and bradykinin have been demonstrated *in vivo* and *ex vivo* in animals or vessels treated with LPS (Beasley *et al.*, 1990; Parker *et al.*, 1994; Waller *et al.*, 1994; Piepot *et al.*, 2003). Impaired endothelial-dependent dilatation has also been demonstrated in healthy human volunteers treated with a mild-inflammatory stimulus (Hingorani *et al.*, 2000). Changes in vascular reactivity lead to alterations in blood flow distribution, regional hypoperfusion and contribute to tissue hypoxia and organ dysfunction. Evidence from *in vivo* and *in vitro* studies show enhanced nitric oxide (NO) production and excessive activation of ATP-sensitive potassium channels (K_{ATP} channels) are major factors in the development of vascular hyporesponsiveness in sepsis (Sorrentino *et al.*, 1999; O'Brien *et al.*, 2001; Wu *et al.*, 2004; for review see Buckley *et al.*, 2006).

A role for K_{ATP} channels in the development of regional vasodilatations, and vascular hyporesponsiveness during sepsis has been previously reported within 5 h of LPS administration, and at 24 h, in both conscious and anaesthetised rats (Gardiner *et al.*, 1999a; Sorrentino *et al.*, 1999). Enhanced K_{ATP} channel activity may be due to increased activation via the upregulation of mediators such as NO, adenosine and CGRP (Buckley *et al.*, 2006), and/or via increased K_{ATP} channel subunit expression, following LPS-treatment (Czaika *et al.*, 2000).

It is well documented that there are variable changes in NO synthase (NOS) activity during sepsis. An early increase in levels of the endothelial, constitutive isoform (eNOS), and a late increase in levels of the inducible isoform (iNOS), which peak between 4 and 12 h have been shown following LPS treatment in both *in vivo* and *in vitro* systems (Gardiner *et al.*, 1996; O'Brien *et al.*, 2001; Connelly *et al.*, 2005; Vo *et al.*, 2005; Yamaguchi *et al.*, 2005). Evidence suggests that eNOS-derived NO may act as a pro-inflammatory mediator in early sepsis, owing to its ability to stimulate iNOS expression (Connelly *et al.*, 2005; Vo *et al.*, 2005). Interestingly, eNOS knockout mice, as well as having decreased iNOS activity, also exhibited decreased mortality following a septic insult and more stable haemodynamics compared to wild type septic animals (Connelly *et al.*, 2005). Thus, endothelial-derived NO appears to be detrimental in sepsis due to its pro-inflammatory and depressor actions. The requirement of eNOS-derived NO for the induction of iNOS during sepsis is illustrated by studies which show that several hours exposure to LPS is required for iNOS upregulation (O'Brien *et al.*, 2001). Furthermore, endothelial derived NO is reduced by short-term LPS exposure due to impaired bioavailability of eNOS (Graier *et al.*, 1994). Therefore, early changes in eNOS expression may be responsible for impaired endothelial-dependent relaxation, whilst vascular smooth muscle contraction is affected following long-term LPS exposure and elevated iNOS expression.

Abnormal cardiac function has long been associated with infection and sepsis. Indeed, at the beginning of the eighteenth century, the 'heat of the blood' in septic patients was calculated by the 'pounding of the pulse' as the two were

believed to intrinsically linked (Mackenzie, 2001). It is now known that the development of sepsis results in cardiac abnormalities characterised by myocardial depression and impaired cardiomyocyte contractility. Although, cardiac contractility may be depressed, there is an early increase in cardiac output in sepsis owing to reduced afterload as a result of systemic vasodilatation. A reproducible pattern of cardiac dysfunction occurs 1-4 days from the onset of sepsis, and consists of reduced left ventricular (LV) ejection fraction, enhanced LV end-diastolic volume index, increased cardiac output and the maintenance of normal stroke volume index which was associated with ventricular dilatation (Parker *et al.*, 1995). Cardiac function of survivors was found to return to normal from day 4 onwards. Interestingly, non-survivors of sepsis maintained normal cardiac parameters throughout their time in the ICU (Parker *et al.*, 1985).

Parillo's group found that serum taken from septic patients with reduced LV ejection fraction contained a 'myocardial depressant substance' which depressed the contractility of rat cardiomyocytes *in vitro* (Parillo, 1989). The group later showed this 'myocardial depressant substance' to include synergism between the pro-inflammatory cytokines TNF-α and IL-1β, which caused increased production of NO by iNOS (Kumar *et al.*, 1996; 1999). A recent study by this group found the inflammation-induced depression of *in vitro* cardiomyocyte contractility could be prevented by the pleiotropic cytokine, transforming growth factor-β, suggesting a novel therapy for resolution of cardiac depression in sepsis (Kumar *et al.*, 2007).

1.1.3 Animal Models of Sepsis

To study the pathophysiological changes associated with sepsis *in vivo,* several animal models of the disease have been developed; these can be divided into two categories:

(i) Administration of a toxin/pathogen (e.g., LPS/*E.coli*)

(ii) Disruption of a natural protective barrier causing exposure to endogenous toxin/bacteria (e.g., by caecal ligation and puncture (CLP)

1.1.3.1 Administration of a toxin/pathogen

Gram-negative bacteria differ from Gram-positive bacteria by having a cell wall comprising inner and outer lipid membranes separated by a periplasmic space containing peptidoglycan. Lipopolysaccharide (LPS) is a component of the outer cell envelope found in all Gram-negative bacteria, but is absent from Gram-positive bacteria. Lipopolysaccharide is made up of an outer O-antigen component that is structurally diverse across strains, and a lipid-A component that serves to anchor LPS to the bacterial cell wall. Evidence suggests this lipid-A component is responsible for the toxicity of LPS and is recognised by the host immune system as 'non-self' (for review see Cohen, 2002).

Endotoxin can be detected using the *limulus* amebocyte lysate (LAL) assay or the chemiluminescent endotoxin activity assay (EAA) (Hurley *et al.,* 1994; Marshall *et al.,* 2002), and is found in patients suffering from sepsis as well as in a variety of other inflammatory conditions. Furthermore, endotoxaemia is not always associated with systemic inflammation, and has been reported in paediatric cardiopulmonary bypass patients, in pancreatitis and cirrhosis and

even in athletes' following long-distance marathons and triathlons (for review see Hurley, 1995). In the case of endurance athletes, this is often accompanied by gastrointestinal complaints as a consequence of reduced gut perfusion, subsequent mucosal damage and infiltration of endotoxin (Jeukendrup *et al.*, 2000).

When given to experimental animals and to human volunteers, endotoxin causes symptoms similar to those seen during clinical sepsis, including fever, tachycardia, tachypneoa and leukocytosis (Taviera de Silva *et al.*, 1993; Hurley, 1995; Remick *et al.*, 2005). There is one interesting report of a laboratory worker who developed symptoms within 3 h from self-administration of LPS (1mg kg^{-1}); these included a decrease in systemic vascular resistance, reduced blood pressure, elevated cardiac output, disseminated intravascular coagulation, renal and hepatic dysfunction and non-cardiogenic pulmonary oedema (Taviera de Silva *et al.*, 1993). LPS-based models of sepsis have been criticised on the basis that they lead to a rapid and substantial cytokine induction, causing a massive elevation in plasma levels of TNF-α, a cytokine which is only rarely detectable in clinical patients with sepsis. However, high LPS and TNF levels are seen in infants with meningococcal meningitis (for review see Riedermann, 2003). It is acknowledged as a drawback to this approach since the detection of endotoxaemia is variable in septic patients (for review see Hurley, 1995).

Gardiner *et al.* (1995a) developed an experimental model of Gram-negative endotoxaemia by chronically infusing Long-Evans rats with LPS (150μg kg^{-1} h^{-1} i.v.) for 32 h. At this dose, LPS infusion led to the development of a haemodynamic profile similar to that seen in clinical patients during the

establishment of the hyperdynamic phase. This model has been used to assess the relative contribution of vasoactive agents to the haemodynamic sequelae of sepsis, as evidence suggests there are clear temporal and regional variations (Waller *et al.*, 1994; Tarpey *et al.*, 1998; Bennett *et al.*, 2004). For example, using this model, vascular hyporesponsiveness to the vasoconstrictor effects of norepinephrine, angiotensin, endothelin and methoxamine was demonstrated in a time-dependent manner during LPS infusion (Gardiner *et al.*, 1996; Tarpey *et al.*, 1998; Bennett *et al.*, 2004). These findings are not restricted to vasoconstrictors; diminished vascular responses to the vasodilators acetylcholine, salbutamol and bradykinin have also been shown (Waller *et al.*, 1994) (Table 1.2).

Using this model it was shown that NO and angiotensin II were involved in the early (0-2 h) haemodynamic response during LPS infusion; endothelin was found to be involved from 2-8 h, whilst at 24 h arginine vasopressin activity was enhanced (Gardiner *et al.*, 1996; Tarpey *et al.*, 1998). Investigations into the role of NO in the haemodynamic sequelae of sepsis revealed that the peak period of iNOS activity did not correlate with the period of maximum haemodynamic stability indicating the involvement of mediators other than NO/or via mediators induced by NO. Furthermore, inhibition of iNOS activity, although preventing/limiting some regional vasodilatations during LPS infusion, also led to a detrimental mesenteric vasoconstriction, due to enhanced release of endothelin (Gardiner *et al.*, 1995). The overall finding of this study was that NOS inhibition could be beneficial during the early stages of endotoxaemia (0-8 h), but was detrimental during the later stages (8-24 h). A study using vasopressin-deficient Brattleboro rats found an important role

for this vasoconstrictor in the maintenance of haemodynamic stability during LPS-infusion (Tarpey *et al.*, 1998).

These studies show that LPS treatment leads to a changing haemodynamic profile in animal models and suggests that the changing influence of vasoconstrictor and vasodilator systems may have implications for the development of novel sepsis therapies.

In recent years, the incidence of Gram-positive sepsis has risen dramatically leading to an increase in sepsis-related mortality. In 2003, it was estimated that approximately 50% of reported sepsis cases in the US were related to Gram-positive bacteria (Wang *et al.*, 2003). This is largely due to the spread of antibiotic-resistant strains of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA). Two major wall components of Gram-positive bacteria, peptidoglycan (PepG) and lipoteichoic acid (LTA) can stimulate an inflammatory response *in vivo*, in a similar but less potent manner than LPS, although their exact roles in the development of sepsis are poorly understood. At present, there are little *in vivo* data available, since Gram-positive wall components are difficult to purify, poorly soluble and expensive, although their use in sepsis models is likely to increase in the near future (for review see Wang *et al.*, 2003).

Since the development of sepsis in human patients is not always associated with endotoxin, other models have been developed which use pure or mixed bacterial flora as the infecting agent(s). One model administers rats with faecal slurry (obtained from several donor rats) directly into the peritoneum (for

review see Buras *et al.*, 2005; Parker *et al.*, 2001). A drawback with this method is that it causes a variable response as the exact composition of bacterial flora present is not controlled. In order to control for dosage and content of the bacterial infecting agent, Goldfarb *et al.* (1996) developed a porcine model of bacterial sepsis which involved the implantation of an *E. coli*-laden fibrin clot into the peritoneal cavity, with the resultant cardiovascular changes being comparable to those seen in human sepsis (Goldfarb *et al.*, 1996).

1.1.3.2 Exposure to endogenous toxin/bacteria

The exposure of usually sterile tissue to harmful pathogens can occur through the disruption of a natural protective barrier. A commonly used sepsis model, which falls into this category, is induction of polymicrobial sepsis by caecal ligation and puncture (CLP). Ligation of the caecum below the ileocaecal valve and its subsequent puncture permits the leakage of faecal content into the peritoneum. This mimics the process of septic peritonitis and produces a biphasic cardiovascular response similar to that seen in clinical patients, with a hyperdynamic phase seen 2 to 10 h after CLP, followed by a hypodynamic phase seen after 16 h. Mortality rates in rats subjected to CLP are approximately 10% at 24 h and 94% at 48 h (for review see Fowler *et al.*, 2003; Buras *et al*, 2005). This model, although used routinely in sepsis research, has been criticised because the exact composition of infecting flora is difficult to control.

The CLP model more precisely mimics peritonitis and polymicrobial sepsis, whereas LPS administration models endotoxaemia (see above). Difficulty lies in drawing comparisons between studies which use different models as they differ in the infecting agent used, the disorder they describe and the inflammatory response they produce. It is therefore important to have several different animal models available to enable the study of sepsis in as many situations as are present in reality. This will become increasingly apparent as better diagnostic tests and personalised treatments become available.

	Renal			Mesenteric			Hindquarters		
Vasodilators	**2 h**	**6 h**	**25 h**	**2 h**	**6 h**	**25 h**	**2 h**	**6 h**	**25 h**
Acetylcholine[1]	↓	↓	↔	↓	↓	↔	↑	↔	↑
Salbutamol[1]	↓	↓	↔	↔	↔	↔	↓	↓	↓
Bradykinin[1]	↔	↓	↓	↑	↔	↔	↓	↓	↑
Vasoconstrictors	**2 h**	**6 h**	**25 h**	**2 h**	**6 h**	**25 h**	**2 h**	**6 h**	**25 h**
Norepinephrine[2]	↓	↓	↔	↓	↓	↔	↓	↓	↔
Vasopressin[2]	↔	↔	↔	↔	↑	↔	↔	↔	↑
Angiotensin[3]	↓	↓	↓	↓	↔	↓	↓	↓	↔
Endothelin[3]	↔	↓	↓	↔	↓	↓	↓	↓	↔
Methoxamine[1]	↔	↓	↔	↓	↓	↓	↓	↓	↔

Table 1.2 *Altered vascular responsiveness to vasoactive mediators in the presence of LPS as shown by the haemodynamic group at the University of Nottingham.*

↓ Reduced vascular responsiveness; ↔ no change in vascular responsiveness; ↑ enhanced vascular responsiveness

References: [1]Waller *et al.* (1994); [2]Bennett *et al.* (2004); [3]Tarpey *et al.*, (1998)

1.2 The calcitonin family of peptides

1.2.1 Introduction

The calcitonin family of peptides comprises, calcitonin, two calcitonin-gene-related-peptides (α-CGRP and β-CGRP), amylin, adrenomedullin (AM) and the recently discovered intermedin (IMD), (also called AM-2; see later) (Poyner *et al.*, 2002; Roh *et al.*, 2004; Takei *et al.*, 2004). These peptides arose early in vertebrate evolution (Ogoshi *et al.*, 2006), have diverse functions in physiological homeostasis and, apart from calcitonin, have potent vasodilator effects in the mammalian cardiovascular system (for review see Chang *et al.*, 2004). Amylin is co-secreted with insulin from pancreatic β-cells, and is essential for optimal glucose metabolism, calcitonin is required for calcium homeostasis, and is used therapeutically in the treatment of bone disorders, whilst CGRP and AM are involved in neurotransmission, as well as cardiovascular and respiratory regulation. Evidence suggests that AM is essential for vascular and lymphatic morphogenesis during embryonic development, as shown by the embryonic lethality of AM knockout mice (Shindo *et al.*, 2001; Fritz-six *et al.*, 2008).

Peptides related to calcitonin are of low molecular weight and share a unique structure consisting of an intramolecular ring of six amino acids flanked by a disulphide bond and an amidation signal at the C-terminus. Evidence suggests this structure confers the biological activity of these peptides (Sakata *et al.*, 1993; Lippton *et al.*, 1994; Gibbons *et al.*, 2006). Indeed, truncated fragments of these peptides which lack the amidated C-terminus have no biological

activity, and are used as receptor-specific antagonists (for review see Poyner *et al.*, 2002).

1.2.2 Calcitonin family receptors

Calcitonin family peptides signal through a unique receptor system comprising several distinct proteins. The first, a polypeptide comprising 7-transmembrane domains, is referred to as calcitonin-receptor-like-receptor (CL), and the second, a single membrane spanning domain protein is referred to as receptor-activity-modifying-protein (RAMP) (McLatchie *et al.*, 1998; for review see Poyner *et al.*, 2002; Martinez *et al.*, 2004). Together, these two proteins function as a receptor. The CL component is coupled to a type II G-Protein-Coupled-Receptor (GPCR), which, when activated, increases adenylate cyclase activity, leading to an increase in intracellular cAMP and subsequent smooth muscle relaxation. In contrast to CL, the RAMP proteins are located in the endoplasmic reticulum and transport the receptor complex to the cell surface where it can be activated by ligand(s) (McLatchie *et al.*, 1998; Wang, 2001). The combination of CL with a RAMP isoform confers ligand specificity; CL complexed with RAMP1 gives rise to a CGRP-specific receptor, termed $CGRP_1$ (which is blocked by the fragment $CGRP_{8-37}$), whilst CL with either RAMP2 or RAMP3 is AM specific (termed AM_1 and AM_2 receptors, respectively) (for review see Wang, 2001; Poyner *et al.*, 2002). A further GPCR (CT) can function as a calcitonin receptor by itself, or as an amylin receptor in combination with the RAMPs. CT bound to RAMPs also has some affinity for CGRP (for review see Poyner *et al.*, 2002; Brain and Grant, 2004) and may function as a $CGRP_2$ receptor which refers to the

component of CGRP signalling not blocked by $CGRP_{8-37}$ (for review see Brain and Grant, 2004).

Immunohistochemical analysis suggest that CL/RAMP combinations are found throughout the vasculature (Roh *et al.*, 2004), with different populations of receptor subtypes accounting for variation in tissue specificity of the ligands. Analysis of human and mouse transcriptomes showed the majority of CL to be complexed with RAMP2, to form an AM receptor, in normal physiology (Gibbons *et al.*, 2006). In addition to CL and RAMP, it was suggested that an accessory protein (RCP) may accompany CGRP receptors at the cell membrane and help couple the receptor to the signal transduction pathway (Nandha *et al.*, 1996).

Within the vasculature, CL/RAMP complexes are located on the endothelium and vascular smooth muscle, leading to both endothelial-dependent and -independent vasodilatation. Briefly, activation of receptors on endothelial cells, leads to an increase in intracellular Ca^{2+}, which, in turn, causes activation of endothelial NOS, and subsequent release of NOS through the conversion of L-arginine to L-citrulline. The released NO then diffuses into the underlying vascular smooth muscle where it activates soluble guanylate cyclase and causes relaxation through an increase in cGMP. Alternatively, NO may activate K_{ATP} channels in the vascular smooth muscle, leading to membrane hyperpolarisation and subsequent relaxation. Receptors located on the vascular smooth muscle, cause vascular relaxation through activation of the cAMP/PKA signalling pathway (for review see Poyner *et al.*, 2002).

1.2.3 Adrenomedullin (AM)

Adrenomedullin was discovered by Kitamura *et al.* (1993) in an assay developed to identify novel vasoactive proteins from human phaeochromocytoma tissue extracts. Using this assay, VIP and CGRP had previously been identified by their ability to raise cAMP levels in platelets (Kitamura *et al.*, 1993).

Adrenomedullin was classified as a calcitonin family peptide due to its slight sequence homology with CGRP (27%) and amylin (20%), which was restricted to the conserved C-terminal region of the mature peptide (Kitamura *et al.*, 1993). Adrenomedullin is formed from post-translational processing of the 185 amino acid precursor peptide, preproAM, which contains a 20 amino acid sequence for proadrenomedullin N-terminal 20 peptide (PAMP) (Sakata *et al.*, 1993), which also has some vasodilator activity (Champion *et al.*, 1996). Adrenomedullin is found in very high concentrations in phaeochromocytoma tissue (1900 ± 450 fmol mg^{-1}) and in normal individuals, AM is present in the highest concentration in the adrenal medulla (150 ± fmol mg^{-1}) and at significantly lower levels in the kidney and lung (Kitamura *et al.*, 1993). Adrenomedullin is also present in the plasma, and is produced from a variety of cells, including endothelial cells, vascular smooth muscle cells, adipocytes, cardiac myocytes, macrophages and fibroblasts (Zaks-Zilberman *et al.*, 1998; for review see Brain & Grant, 2004). Elevated plasma AM levels have been reported in a number of human diseases including heart failure, myocardial infarction, sepsis, renal failure, hypertension, hyperthyroidism, pulmonary hypertension and insulinoma (Shoji *et al.*, 1995; Cheung *et al.*, 2004; Ishimitsu *et al.*, 2006). During sepsis, AM levels can increase in excess of 20-

fold, whilst in pregnancy AM levels can increase 4-fold, although decreased AM levels have been identified in women with complicated pregnancies, including pre-eclampsia (Gibbons *et al.*, 2006). Recently, a pilot study found that co-infusion of AM and human atrial naturietic peptide had beneficial effects in patients with acute decompensated heart failure, these included a reduction in mean and pulmonary arterial pressure, reduced systemic and pulmonary vascular resistance, and elevated cardiac output without any change in heart rate. Furthermore, aldosterone was reduced and urine volume and sodium excretion was elevated (Nishikimi *et al.*, 2009). Thus, AM may present a novel therapy for the treatment of cardiovascular disease in humans.

1.2.3.1 Physiological functions of AM

Adrenomedullin is a multifunctional peptide which is most commonly known for its effects on the cardiovascular system. Shindo *et al.* (2001) demonstrated that AM null mouse embryos die *in utero* due to extreme vascular abnormalities, indicating that AM is essential for vascular development during embryogenesis (Shindo *et al.*, 2001). It was later shown that RAMP2 is essential for AM-mediated angiogenesis and maintenance of vascular integrity, as RAMP2 null mice also died mid-gestation, whilst heterozygotes had significantly impaired vascular integrity (Shindo *et al.*, 2008). Adrenomedullin is also essential for lymphatic development, and lymphatic endothelial cells from AM knockout mice undergo reduced proliferation (Fritz-Six *et al.*, 2008). Whilst mice heterozygous for the AM gene have normal survival rates, they have significantly higher blood pressure compared to wild type animals and this is associated with a decrease in NO production

(Shindo *et al.*, 2001). Furthermore, heterozygotes showed a greater infarction size and increased mortality compared to wild type animals when subjected to 30 min regional myocardial ischaemia and 120 min reperfusion. Additionally, this phenotype could be rescued by pre-treatment with exogenous AM, suggesting that this peptide exerts some protection against the deleterious effects of myocardial infarction, a role involving eNOS and PKB phosphorylation (Hamid & Baxter *et al.*, 2006).

Mice with only one copy of the AM gene also exhibit a more extreme cardiovascular response to CLP-induced sepsis which progresses directly into a hypodynamic state, compared to wild-type controls. These mice also have extreme oedema formation suggesting that AM may limit permeability of the endothelium (for review see Li *et al.*, 2007; Kahn *et al.*, 2008). A limitation in the study of AM is the lack of available tools to specifically antagonise endogenous AM, since AM knockout mice are lethal (see above). However, a preliminary study by Albertin *et al.* (2005), showed that it may be possible to target the AM gene by siRNAs, thus presenting a method by which knockout of AM function could be assessed in adult animals *in vivo* (Albertin *et al.*, 2005).

Adrenomedullin is important in fluid and electrolyte homeostasis, and has a number of effects on renal function, including promotion of sodium and urinary excretion at doses which have no effect on blood pressure (Charles *et al.*, 1997). Furthermore, vascular responsiveness to AM is significantly increased during pregnancy and is believed to be involved in generating the low blood pressure common during gestation (Ross & Yallampalli, 2007).

Adrenomedullin gene expression and production have also been observed in some human tumour cells, where it has been shown to promote growth and indirectly inhibit apoptosis (reviewed by Ishimitsu *et al.*, 2006). There is also some evidence that myocardial AM expression is upregulated by HIF-1 under hypoxic conditions, indicative of a protective role during myocardial infarction (Hofbauer *et al.*, 2002). Whilst AM is best characterised for its role in controlling vascular tone through vasodilatation, the peptide also affects the blood vessels in other ways. For example, AM can inhibit apoptosis in cultured rat endothelial cells, promote vascular regeneration and re-endothelisation during wound healing, and limit vascular damage by reducing the production of reactive oxygen species (for review see Kato *et al.*, 2005).

1.2.3.2 Cardiovascular responses to AM

Since AM shares some (27%) sequence homology with CGRP, the most potent vasodilator known, it was hypothesised that AM may have similar hypotensive and vasodilatory effects when given to experimental animals. A role for AM in the cardiovascular system has been confirmed *in vivo* and *in vitro*. The main findings of these studies are summarised below, and the mechanisms through which AM exerts these effects are summarised in Table 1.3.

(i) In vivo

In their 1993 paper, Kitamura *et al.* showed that AM caused a long-lasting hypotension when given to anaesthetised rats, confirming it to have comparable effects to CGRP, albeit being less potent (Kitamura *et al.*, 1993).

This has since been repeated and confirmed in a variety of species including rats (He *et al.*, 1995; Gardiner *et al.*, 1995; Nandha *et al.*, 1995), sheep (Charles *et al.*, 1997; Parkes *et al.*, 1995) and humans (Troughton *et al.*, 2000). These studies have also consistently shown that AM administration in different species of animals, including humans, causes an increase in cardiac output (He *et al.*, 1995; Parkes & May *et al.*, 1997; Charles et *al.*, 1997; Troughton *et al.*, 2000) and occasionally heart rate (Gardiner *et al.*, 1995), although the mechanism through which AM-mediated tachycardia occurs is unclear. Evidence that AM has little effect on heart rate in anaesthetised animals suggests that AM-mediated tachycardia is largely mediated by sympathetic activation (Nandha *et al.*, 1996; Parkes & May *et al.*, 1997; Sabates *et al.*, 1997). However, some studies using ganglion, sympathetic and parasympathetic blocking drugs are suggestive of a direct, albeit small, positive inotropic effect of AM (Parkes & May, 1997).

In vivo studies have shown that the blood pressure lowering effect of AM is a consequence of its potent vasodilator action in the rat renal, mesenteric and hindquarters vascular beds (Gardiner *et al.*, 1995; Elhawary *et al.*, 1995); cat pulmonary, mesenteric and hindquarters vascular beds (Lippton *et al.*, 1994; Champion *et al.*, 1996, 1997), dog coronary and renal vascular beds (Miura *et al.*, 1995; Sabates *et al.*, 1997), and sheep coronary vascular bed (Parkes & May, 1997). Using microspheres, He *et al.* (1995) demonstrated that AM administration in conscious rats led to significant increases in blood flow to the lungs, heart, liver, spleen, kidneys, adrenal gland and gastrointestinal tract, indicating AM-mediated vasodilatations in those regions. Interestingly, in that

study, AM administration caused reduced blood flow to skeletal muscles and testis and had no effect on cerebral blood flow (He *et al.*, 1995).

The mechanisms of AM's-action in the cardiovascular system are not fully understood but are known to involve activation of AM_1 and AM_2 receptors (see section 1.2.2) although it has been shown that AM can activate $CGRP_1$ receptors in some regions (for review see Table 1.3; Poyner *et al.*, 2002).

(i) In vitro

Administration of AM to perfused rat mesenteric beds caused concentration-dependent vasorelaxation which was approximately ten-times less marked than CGRP-mediated vasorelaxation, and was sensitive to treatment with the CGRP receptor antagonist $CGRP_{8-37}$, suggesting a role for $CGRP_1$ receptors (Nuki *et al.*, 1993). A vasorelaxant response to AM was demonstrated in isolated blood-perfused rat lung, which was independent of NO, $CGRP_1$ receptors, prostaglandins, K_{ATP} channels or adenosine A_1 receptors. It is possible that AM receptors were involved in AM-mediated pulmonary vasodilatation in that study, since the effects of the AM receptor antagonist, AM_{22-52}, were not investigated (Heaton *et al.*, 1995). Adrenomedullin is well known for its role in fluid and electrolyte homeostasis, and has been shown to dilate afferent and efferent arterioles to the same extent, and to decrease renal vascular resistance in isolated rat kidneys, through activation of $CGRP_1$ receptors and through NO release (Hirata *et al.*, 1995).

Mechanism		*Agent used*	*Species*	*Effect*	*Reference*
$CGRP_1$	*in vivo*	$CGRP_{8-37}$	Cat	No effect on AM-mediated hind-limb	Champion *et al.* (1997)
			Rat	vasodilatation	Elhawary *et al.* (1995);
				No effect on AM-mediated hypotension	Nandha *et al.* (1996)
			Dog	No effect on AM-mediated coronary vasodilatation	Sabates *et al.* (1997)
	in vitro	BIBN09BS	Mouse	Vasorelaxation of perfused mesentery	Grant *et al.* (2004)
$AM_{1/2}$	*in vivo*	AM_{22-52}	Rat	Inhibition of regional haemodynamic responses to AM	Gardiner *et al.* (1999)
	in vitro			Attenuation of AM-mediated increase in cAMP in mesenteric vessels from pregnant rats	Ross & Yallampalli (2007)
NO	*in vivo*	L-NAME	Cat	No effect on AM-mediated mesenteric relaxation	Champion *et al.* (1996)
			Rat	Some attenuation of AM-mediated hindquarters vasodilatation	Gardiner *et al.* (1995)
		L-NIO	Dog	Attenuation of AM-mediated renal vasodilatation	Miura *et al.* (1995)
	in vitro	L-NA	Rat	Attenuation of AM-mediated mesenteric vasorelaxation	
K_{ATP}	*in vivo*	U37883A	Cat	No effect on AM-mediated hind limb	Champion *et al.* (1997)
			Dog	vasodilatation	Sabates *et al.* (1997)
		Glibenclamide	Dog	Attenuation of AM-mediated coronary vasodilatation	Miura *et al.* (1995)
				No effect on AM-mediated renal vasodilatation	
	in vitro	Glibenclamide	Rat	Abolished AM-mediated mesenteric membrane hyperpolarisation	Goto *et al.* (2000)
Other	*in vivo*	Phosphodiesterase inhibition	Cat	Augmentation of AM response in cat hind limb	Champion *et al.* (1997)
		Adenosine receptor inhibition	Dog	Attenuation of AM-mediated coronary vasodilatation	Sabates *et al.* (1997)
	in vitro	Endothelial denudation	Rat	Attenuation of AM-mediated mesenteric vasorelaxation	Champion *et al.* (2001)

Table 1.3 *Mechanisms of AM action in the cardiovascular system*

It is well documented that AM has a direct stimulatory effect on juxtaglomerular cells, causing them to increase renin secretion independent of any effects on blood pressure or renal blood flow (Parkes *et al.*, 1995; Charles *et al.*, 1997; Troughton *et al.*, 2002). Adrenomedullin infusion in both healthy volunteers and sheep increased plasma renin activity 2-3 fold with no subsequent effect on the levels of aldosterone (Charles *et al.*, 2003). This failure of AM to cause a secondary increase in aldosterone or vasopressin, has led to some speculation that AM may be a functional antagonist of angiotensin II, causing uncoupling of the renin-angiotensin ratio (Yamaguchi *et al.*, 1995; 1996; Charles *et al.*, 1997; 2002; Troughton *et al.*, 2000). However, the effects of AM on aldosterone secretion are contradictory in the literature. Whilst some studies suggest AM has no effect on aldosterone levels (see above), others have shown a direct inhibition of aldosterone secretion (Yamaguchi *et al.*, 1995; 1996), or stimulation of aldosterone secretion from adrenal zona glomerulosa cells (Kapas *et al.*, 1998).

1.2.3.3 Adrenomedullin and sepsis

Adrenomedullin mRNA and protein are elevated in rats during CLP sepsis (Yang *et al.*, 2001) and LPS-induced endotoxaemia (Shoji *et al.*, 1995; Sugo *et al.*, 1995; Zaks-Zilberman *et al.*, 1998; Cheung *et al.*, 2004; Li *et al.*, 2005). Since mature AM is not stored in cells, its regulation occurs at the level of transcription. Shoji *et al.* (1995) found that AM gene transcription was elevated in the aorta, heart, lungs, adrenal glands, kidney, skeletal muscle, brain, thymus, submaxillary gland, liver and the gut, following i.p. injection of LPS in rats (Shoji *et al.*, 1995). Furthermore,

it was suggested that vascular endothelial and smooth muscle cells are a major source of circulating AM, and contribute to the elevated plasma levels of the peptide during sepsis (Shoji *et al.*, 1995).

Expression of AM mRNA was induced by the pro-inflammatory cytokines, IL-1β and TNF-α, and by iNOS in rat aortic smooth muscle cells, endothelial cells, mesengial cells and hepatocytes, possibly via binding of NFκB to the promoter region of the AM gene (Sugo *et al.*, 1995; Hofbauer *et al.*, 2002). It is not clear, however, whether AM was upregulated directly by pro-inflammatory cytokines or indirectly following upregulation of cytokine-induced iNOS expression. Nevertheless, since IL-1β, TNF-α and iNOS are all upregulated during sepsis (Yang *et al.*, 2001), this provides a mechanism to account for the sepsis-mediated increase in AM (see above). Moreover, evidence suggests that AM expression is directly upregulated by LPS in vascular smooth muscle cells (Sugo *et al.*, 1995). Therefore, any LPS-mediated increase in AM production may be amplified by the action of pro-inflammatory cytokines, which are themselves upregulated by LPS. Interestingly, AM has also been shown to limit the increase in pro-inflammatory cytokines following a septic insult, as mice heterozygous for the AM gene show an exaggerated inflammatory response to LPS, with elevated TNF-α and IL-1 levels, and increased liver damage relative to wild type mice (Dackor & Caron, 2007). Thus, AM appears to function as an anti-inflammatory mediator, providing negative feedback to limit the induction of TNF-α and IL-1 gene expression during sepsis.

Since the development of a hyperdynamic circulation in early sepsis is preceded by a rise in plasma AM, it has been suggested that this peptide may be an essential mediator of the haemodynamic changes in early sepsis (for review see Koo *et al.*,

2001; Wang, 2001). Administration of AM-specific antibodies to rats made septic by CLP prevented the development of the hyperdynamic circulation (that is, increased cardiac output and stroke volume, and decreased total peripheral resistance) (Wang *et al.*, 1998), supporting this hypothesis. When given prophylactically, AM was able to prevent the development of a hypodynamic circulation and limit the increase in arterial lactate accumulation in LPS-treated sheep. Furthermore, in the same study, when given therapeutically, it was shown that AM-treatment reversed a hypodynamic circulation back to a hyperdynamic state (Ertmer *et al.*, 2007). It has been suggested that myocardial dysfunction during hyperdynamic sepsis is prevented by a compensatory increase in AM which acts directly on cardiomyocytes to augment contractility and maintain cardiac output (Li *et al.*, 2005). There is some evidence to suggest that decreased vascular responsiveness to AM is responsible for the transition from a hyperdynamic to a hypodynamic circulation in late sepsis (i.e. after 16 h in CLP rats). This is supported by evidence showing reduced AM-mediated relaxation of isolated aortic rings and isolated perfused small intestine 20 h and 10 h from the onset of CLP sepsis, respectively (Wang *et al.*, 1999). Evidence suggests that an AM specific binding protein may be responsible for this.

Elsasser *et al.* (1999) identified a binding protein for AM in the plasma of several species including human and rat (Elasser *et al.*, 1999), which was subsequently found to be identical to human complement factor H (Pio *et al.*, 2001). Association of adrenomedullin binding protein (AMBP-1) with AM enhanced the AM-induced increase in cAMP levels in rat-2 fibroblasts and augmented AM-induced growth and proliferation of a human T-47D breast cancer cell line. Moreover, when in

association with AM, the co-factor activity of AMBP-1 (i.e. factor H), and hence cleavage of C3b, was significantly enhanced (Pio *et al.,* 2001). This mechanism may account for the anti-inflammatory effects of AM. AMBP-1 contains glycosaminoglycan binding sites which are involved in cell-surface attachment (Pio *et al.,* 2001). Therefore, as well as protecting AM from degradation, AMBP-1 may increase the concentration of AM at its receptors and prolong its effects. *In vitro* studies using aortae isolated from CLP-treated rats showed reduced vascular sensitivity to the vasorelaxant effects of AM associated with decreased levels of AMBP-1 rather than AM receptor components. Furthermore, treatment with AMBP-1 prevented the vascular hyporesponsiveness to AM in sepsis (Zhou *et al.,* 2002) suggesting that AMBP-1 was responsible for the loss of responsiveness to AM in later sepsis, and that therapeutic administration of this peptide may be helpful in preventing the transition from the hyperdynamic to the hypodynamic phase of sepsis (Zhou *et al.,* 2002). AMBP-1 gene expression was reduced in the liver (the main production site of the protein) at 20 h from CLP sepsis induction in rats, and AMBP-1 protein level is reduced in both the liver and the plasma at this juncture (Cui *et al.,* 2005). Furthermore, using a LPS-neutralizing agent, it was shown that LPS is the agent responsible for the downregulation of AMBP-1 during sepsis, a fact confirmed by significant reductions of the protein seen in LPS-treated animals (Cui *et al.,* 2005). This suggests that reduced vascular responsiveness to AM during late sepsis may be due to LPS-induced downregulation of AMBP-1.

In a series of studies by Ping Wangs group (Yang *et al.,* 2002; Zhou *et al.,*2004; 2007), it was found that co-administration of AM/AMBP-1 could limit the adverse effects caused by CLP-induced sepsis *in vivo.* For example, co-administration of AM/AMBP-1 in rats prevented the CLP-induced increase in total peripheral

resistance, decrease in oxygen delivery, and decrease in cardiac output and could limit liver damage and the increase in haematocrit at 20 h from the onset of sepsis, resulting in an overall reduction in mortality. Furthermore, the decrease in portal, intestinal and renal blood flow was prevented at this juncture, whereas coronary blood flow was elevated. A further study found there was increased apoptosis of aortic endothelial cells 20 h from the onset of CLP sepsis which could be prevented by co-treatment with AM and AMBP-1, suggesting a mechanism to account for the protective effects of these agents in sepsis. Co-administration of AM/AMBP-1 could also maintain vascular responsiveness to endothelial-dependent relaxation in isolated thoracic aorta and pulmonary arteries taken from rats 20 h after induction of CLP sepsis, by preventing the downregulation of eNOS. In terms of clinical relevance, this work identifies a novel approach to the treatment of bacterial, and Gram-negative sepsis, and suggests that AM and AMBP-1 may play an important role in the progression of sepsis into septic shock.

1.2.4 Intermedin (IMD)

In an attempt to identify peptides involved in osmoregulation, Ogoshi *et al.* (2003) identified 5 AM-like sequences from the pufferfish genome, and subsequently isolated cDNAs encoding AM like peptides which were named AM1-5 (Ogoshi *et al.*, 2003). The same group identified AM-2-like sequences in mouse, rat, and human genome databases, which they named AM-2 (Takei *et al.*, 2004). Simultaneously, a phylogenetic profiling approach identified AM-like sequences in the genomes of mammals and teleost fish which were similar to, but distinct from, AM (Roh *et al.*, 2004). Based on the detection of this peptide in the intermediate lobe of the pituitary, the product of this gene

was named intermedin (IMD) (Roh *et al.*, 2004). Sequence analysis has since found AM-2 and IMD to be the same peptide, which will be referred to in this thesis as IMD.

Sequence and structural analysis of IMD found it may be a novel member of the calcitonin family, homologous to AM, based on exon-intron structure and synteny of neighbouring genes (Roh *et al.*, 2004; Takei *et al.*, 2004b). Phylogenetic analysis clustered these peptides into a group, separate from CGRP and amylin, leading Takei *et al.* (2004) to suggest that, within the calcitonin family, there exists an AM subfamily, consisting of two peptides in vertebrates (named AM1 and 2) and 5 in fish (named AM1 to 5). Cloned IMD cDNA in mouse, rat and human, had 71% sequence homology between the mouse and pufferfish mature peptide sequence. This compared to 50% homology for AM between the same species. In addition, 90% similarity was reported between mouse and rat precursor sequences with only one amino acid differing between the mature sequences, suggesting IMD to be more highly conserved between species than AM (Takei *et al.*, 2004a).

In the same way that AM is formed from preproAM (Sakata *et al.*, 1993; see above), IMD is formed by proteolytic cleavage of the 148 amino acid precursor peptide, preproIMD, encoded by the IMD gene. Sequence analysis has revealed several biologically active fragments that can be derived through its cleavage. The mature form of human IMD is a 47 amino acid, $IMD_{1\text{-}47}$, although a shorter fragment, $IMD_{8\text{-}47}$, has also been identified (Roh *et al.*, 2004). Sequence analysis further revealed a putative cleavage site between Arg93-Arg94, producing a 53 amino acid fragment $IMD_{1\text{-}53}$ (Yang *et al.*, 2005). Whilst, the physiological significance of these different fragments is

yet to be fully understood, there is some evidence to suggest that IMD_{1-53} is an endogenously produced fragment (Yang *et al.*, 2005).

1.2.4.1 Tissue distribution of IMD

Intermedin mRNA was identified in the pituitary and stomach of rats and in the gastrointestinal tract of humans, with the highest levels found in the stomach and jejunum (Roh *et al.*, 2004). Furthermore, Takei *et al.* (2004) found high IMD expression in the submaxillary gland, kidney, mesentery, stomach, ovary (but not testis), lymphoid tissues and pancreas of mice. Interestingly, no expression was found within the adrenal gland, which contrasts with the high AM expression reported in this area (Kitamura *et al.*, 1993). The expression of IMD in the ovary suggests that there may be a role for this peptide either within the female reproductive system or during embryonic development (see later).

One study showed plasma levels of IMD to be significantly higher than circulating AM and CGRP in normal rats (205±59 pg ml^{-1} vs. 10-100 pg ml^{-1} and 2-25 pg ml^{-1}, respectively), although radioimmunoassay for AM may not take into account peptide bound to AMBP-1 (Elsasser *et al.*, 1999; Taylor *et al.*, 2004). Tissue examination found IMD protein to be most abundant in kidney (31±1 ng μg^{-1}) and stomach (30±1 ng μg^{-1}), and at lower levels in the hypothalamus (5±0.8 ng μg^{-1}). In contrast, Roh *et al.* (2004), found the highest IMD content in the pituitary (intermediate lobe) and stomach of mice, suggesting there may be some variation between species. In agreement with previous studies (Takei *et al.*, 2004; Roh *et al.*, 2004), the level of IMD protein

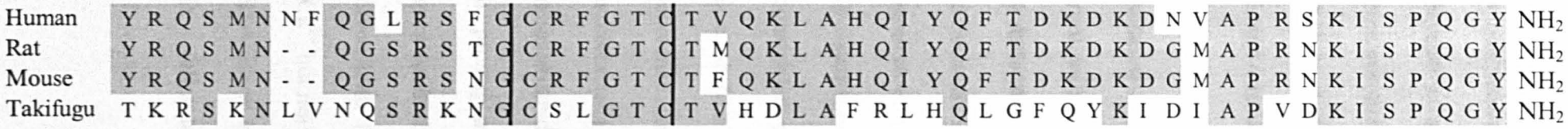
in the adrenal was found to be below the level of sensitivity (Taylor *et al.*, 2005). These results show a difference between the localisation of IMD protein in comparison to AM, which has highest expression levels in the lung, kidney, heart, spleen, duodenum and adrenal gland (Kitamura *et al.*, 1993). This raises the possibility that a number of the diverse functions originally attributed to AM, may actually be mediated by another member of the 'AM family', such as IMD, acting through the same receptors.

Human autopsy tissue showed IMD-like immunoreactivity in the hypothalamus, heart and kidney (Takahashi *et al.*, 2006: Morimoto *et al.*, 2007), and IMD expression was found in a number of cell types/tissues implicated in cardiovascular and central nervous system regulation (Morimoto *et al.*, 2007).

1.2.4.2 Cardiovascular responses to IMD

Since IMD shares some sequence and structural homology with AM, and can activate the same receptors (see above, and later), Roh *et al.* (2004) suggested that IMD may share some of the cardiovascular effects of AM and CGRP. A review of available *in vitro* and *in vivo* data from cardiovascular studies is summarised below.

Adrenomedullin



Intermedin$_{1\text{-}47}$

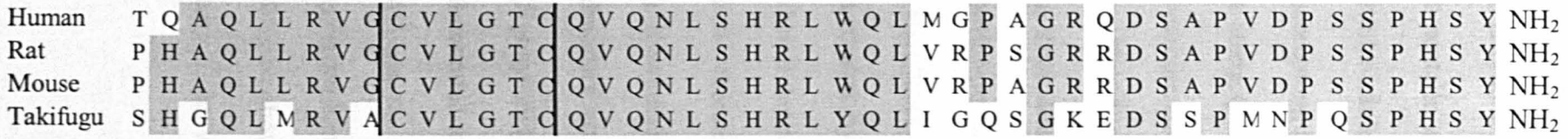


Figure 1.2 *Sequence homology between mammalian and Pufferfish (Takifugu) AM (top) and IMD (bottom).*

Lines denote a conserved disulphide bridge, flanking an intramolecular ring of 6 amino acids. Shaded areas denote amino acids conserved between 3 or more species. Letters denote amino acids. *Adapted from Takei et al. (2004)*

(i) In vitro studies

Kobayashi *et al.* (2004) observed IMD-mediated vasodilatation in isolated porcine coronary and carotid arteries which were equipotent to those induced by AM, and also in supramesenteric arteries. In the coronary artery, IMD-mediated vasorelaxation could be inhibited by $CGRP_{8-37}$, but not by AM_{22-52}, suggesting a role for CGRP receptors, but not AM receptors, in the coronary vasculature *in vitro*. In that study there was no effect of IMD on isolated femoral, pulmonary or renal porcine arteries. Coronary vasodilatation has also been demonstrated in pre-constricted rat aortic ring preparations (Pan *et al.*, 2005). Kandilci *et al.* (2006; 2008) studied the effects of IMD and selected antagonists on the isolated, buffer-perfused lung of Wistar rats, and found that IMD perfusion caused significant pulmonary vasodilatation (under preconstricted conditions) mediated by activation of $CGRP_1$ receptors and release of NO in an endothelial-dependent manner, which involved large conductance Ca^{2+} activated K^+ channels ($BKCa^{2+}$) but not K_{ATP} channels (Kandilci *et al.*, 2006; 2008). However, no IMD-induced vasodilatations were seen in isolated porcine pulmonary artery preparations when preconstricted with prostaglandin F2α (Kobayashi *et al.*, 2004). Intermedin-mediated vasorelaxation has been demonstrated in isolated rat mesenteric artery, which involves both the NO-cGMP and the AC-PKA pathways, with a role for $BKCa^{2+}$ but not K_{ATP} channels (Chauhan *et al.*, 2007), in line with the findings of Kandilci *et al.* (2006; 2008; see above). Therefore, evidence suggests that IMD stimulation causes activation of $BKCa^{2+}$ channels,

Mechanism		*Agent used*	*Species*	*Effect*	*Reference*
$CGRP_1$	*in vivo*	$CGRP_{8-37}$	Rat	Attenuation of IMD-mediated hypotension	Roh *et al.* (2004)
				Attenuation of IMD-mediated hypertension (CNS)	Ren *et al.* (2006)
	in vitro	$CGRP_{8-37}$	Rat	Inhibition of IMD-mediated cAMP increase in L6 skeletal myoblasts	Roh *et al.* (2004)
			Pig	Attenuation of IMD-mediated coronary vasorelaxation	Kobayashi *et al.* (2004)
			Rat	Attenuation of IMD-mediated pulmonary vasorelaxation	Kandilci *et al.* (2006)
			Rat	Inhibition of IMD-mediated increase in cAMP in spinal cord cells	Owji *et al.* (2008)
		BIBN4096	Rat	No effect (see above)	Owji *et al.* (2008)
$AM_{1/2}$	*in vivo*	AM_{22-52}	Rat	No effect on IMD-mediated hypotension	Roh *et al.* (2004)
				Attenuation of IMD-mediated hypertension (CNS)	Ren *et al.* (2006)
	in vitro		Pig	No effect on IMD-mediated aortic vasorelaxation	Kobayashi *et al.* (2004)
NO	*in vitro*	L-NAME	Rat	Attenuation of IMD-mediated pulmonary vasorelaxation	Kandilci *et al.* (2006)
			Rat	Attenuation of IMD-mediated mesenteric vasorelaxation	Chauhan *et al.* (2007)
K_{ATP}	*in vitro*	GLB	Rat	No effect on IMD-mediated pulmonary vasorelaxation	Kandilci *et al.* (2006)
Others	*in vitro*			IMD non-selectively activated $CGRP_1$, $AM_{1/2}$ receptors in transfected 293T cells	Roh *et al.* (2004)
		COX inhibition	Rat	No effect of meclonfenate on IMD-mediated pulmonary vasorelaxation	Kandilci *et al.* (2006)
		AC, GC inhibition	Rat	Attenuation of IMD-mediated mesenteric vasorelaxation by SQ22536, Rp-cAMPs and ODQ	Chauhan *et al.* (2007)

Table 1.4 *Mechanisms of IMD action in the cardiovascular system*

resulting in hyperpolarisation, subsequent inhibition of Ca^{2+} influx through voltage gated Ca^{2+} channels, resulting in relaxation of vascular smooth muscle.

In addition to the potent effects of IMD on the vasculature, there is increasing evidence of a direct role for IMD in modulating cardiac function. In isolated rat hearts, perfusion with IMD enhanced cardiac function, increased coronary blood flow and decreased intraventricular pressure, without any effect on heart rate (Yang *et al.*, 2005). However, a study in anaesthetised rats found that high doses of IMD inhibited cardiac function, whilst a low dose had no effect (Pan *et al.*, 2005). Studies on isolated murine ventricular myocytes indicate that IMD (as well as AM and CGRP) augmented cardiac contractility by enhancing intracellular Ca^{2+} release, in a PLC-dependent manner (Dong *et al.*, 2006). The authors suggested that whilst transient increases of IMD may have a cardioprotective effect, chronic IMD expression may be detrimental to cardiac function, increasing the risk of developing heart failure via chronic PKC activation (Dong *et al.*, 2006). Augmentation of cardiac contractility by IMD presents a direct mechanism through which the peptide may increase cardiac output *in vivo.*

Although at lower levels than AM, IMD and its receptor components were expressed in the hearts of normal rats, and were upregulated in left ventricular cardiomyocytes from spontaneously hypertensive rats, whilst AM levels were unaffected (Bell *et al.*, 2008; Zeng *et al.*, 2009). Furthermore, the role of IMD in enhancing cardiac contractility was blunted in left ventricular cardiomyocytes from hypertensive rats, despite increased levels of the peptide

(Bell *et al.*, 2008; Zeng *et al.*, 2006). Zhao *et al.* (2006) designed a study to assess whether myocardial IMD expression was affected in NO-deficient hypertension and found that preproIMD mRNA was elevated almost 9-fold compared to a modest 1.6-fold increase in preproAM expression. This is in contrast to normal hearts in which AM is expressed at higher levels than IMD in both ventricles. In accordance with the findings described above, left ventricles from hypertensive rats exhibited an increase in both mRNA expression and protein levels of all CL and RAMP receptor components (Zhao *et al.*, 2006). Thus, upregulation of both IMD and its receptor components may be a common cardioprotective phenomenon, at least in animal models of hypertension.

Yang *et al.* (2005) were the first to provide evidence that $IMD_{1\text{-}53}$ may be a physiologically significant endogenously-cleaved product of preproIMD, and found the fragment to have an almost identical cardioprotective effect to AM during cardiac ischaemia-reperfusion. Reperfusion with $IMD_{1\text{-}53}$ ameliorated ischaemia-reperfusion-induced bradycardia, myocardial injury and impairment of cardiac function, and increased myocardial cAMP content to the same extent as AM, suggesting that the cardioprotective effect of $IMD_{1\text{-}53}$ may be mediated via cAMP-dependent mechanisms. Furthermore, $IMD_{1\text{-}53}$ binding was also potentiated during ischaemia-reperfusion, with maximum binding capacity increased by 118%. In contrast, however, preproIMD protein content was reduced 4-fold in ischaemia-reperfused myocardia relative to control, suggesting the majority of IMD under such conditions is membrane bound. Like AM, $IMD_{1\text{-}53}$ was also found to limit oxidative stress induced by

ischaemia/reperfusion, as shown by reduced production of myocardial lipid peroxide in the presence of IMD_{1-53} (Yang *et al,* 2005).

(ii) In vivo studies

Whilst *in vitro* studies provide evidence of a cardiac role for IMD, studies investigating the systemic effects of the peptide are less clear, and it is currently unknown whether IMD has a direct effect on the heart to increase heart rate, or whether this is secondary to the IMD-mediated fall in blood pressure. Ganglion blockade with mecamylamine, inhibited IMD-induced tachycardia, suggesting this may be a reflex response to the fall in blood pressure (Abdelrahman *et al.*, 2006). In contrast, physical denervation of aortic baroreceptors in rats reduced IMD-induced tachycardia and renal sympathetic nerve activity, but did not abolish it (Fujisawa *et al.*, 2006), suggesting that whilst IMD causes autonomic nervous reflex activation, there may be some residual IMD action on the heart which is direct. However, since the cardiopulmonary baroreceptors were left intact, involvement of these low-pressure receptors cannot be ruled out (Fujisawa *et al.*, 2006).

When IMD_{1-47} and IMD_{8-47} (10 and 50nmol rat^{-1} each) were administered i.p. to normal and hypertensive rats, both forms dose-dependently decreased blood pressure and increased heart rate. $Intermedin_{1-47}$ was more potent than IMD_{8-47} at both doses, and high dose AM (50 nmol rat^{-1}) was more potent than both (Roh *et al.*, 2004). Comparison of the cardiovascular effects of IMD_{1-47} and IMD_{1-53} with AM showed that IMD_{1-47} caused the most potent fall in blood pressure, whilst IMD_{1-53} had an effect comparable to AM. Thus, evidence

suggests that different cleavage products of prepro-IMD may act on the cardiovascular system with different potencies (Ren *et al.*, 2006).

In anaesthetised mice, IMD caused a potent and long-lasting hypotensive response which was greater than that exerted by equimolar doses of AM (Takei *et al.*, 2004). Pan *et al.* (2005) showed that high doses of IMD (150 nmol i.v.) in Wistar rats caused a decrease in mean arterial pressure, decreased left ventricular end-systolic pressure and maximal rate of left-ventricular development, with no significant effect on heart rate. The dose used here, however, was considerably larger than that used by others. Intermedin infusion in conscious sheep led to significant reductions in blood pressure, increases in cardiac output and heart rate, with a concurrent fall in total peripheral resistance (Charles *et al.*, 2006). This was the first description of the haemodynamic effects of IMD in a large animal *in vivo,* and indicates its cardiovascular effects may be comparable between species.

Since IMD has been localised to the kidney (Takei *et al.*, 2004b; Taylor *et al.*, 2005; Takahashi *et al.*, 2006), it was suggested that it may have some role in renal function, particularly since intrarenal arterial AM infusion has been shown to dose-dependently increase renal blood flow, urinary output and urinary sodium excretion in anaesthetised dogs and rats (Hirata *et al.*, 1995; for review see Samson, 1999). Indeed, Fujisawa *et al.* (2004; 2006) showed that IMD significantly increased renal blood flow, urine volume and urinary sodium excretion to a level comparable to that seen following equimolar AM treatment, in both conscious and anaesthetised rats. The duration of the AM-

induced increase in renal blood flow was longer than that induced by IMD, in both studies (Fujisawa *et al.*, 2004; 2006).

In conscious sheep, IMD infusion was shown to have no significant effect on renal function (urine volume, urine sodium, potassium, or creatinine levels); consistent with an earlier study which found no significant effects of AM infusion on renal function in the same species (Charles *et al.*, 1997; 2006). This may indicate species differences in the renal actions of these peptides since both AM and IMD affected renal haemodynamics in rats and mice (Fujisawa *et al.*, 2004, 2006; Takei *et al.*, 2004). However, IMD infusion significantly increased plasma aldosterone levels in sheep, secondary to an increase in plasma renin activity, whereas AM caused no significant change in levels of aldosterone, despite a significant increase in plasma renin activity (Charles *et al.*, 1997; see above). Thus, angiotensin II inhibition may be a function unique to AM (see above), which is not shared by IMD (Charles *et al.*, 1997; see above).

The identification of IMD mRNA within the brain has led some researchers to propose a role for IMD in the central nervous system (CNS). Since AM and CGRP have hypertensive effects when administered intracerebroventricularly (i.c.v), Taylor *et al.* (2005) studied the effects of IMD in the CNS of rats. In contrast to its hypotensive effects when given both i.v and i.p, when administered i.c.v, IMD exerted dose-dependent hypertensive and tachycardic effects that were partly attenuated upon CNS administration of $CGRP_{8-37}$. In addition, IMD administered i.c.v. provoked an anorexic effect in fasted rats. Ren *et al.* (2006) compared the effects of i.c.v. IMD_{1-47}, IMD_{1-53} and AM in

rats and found both forms of IMD to increase blood pressure to a greater extent than AM, with IMD_{1-53} having more potent effects than IMD_{1-47}.

The potent hypertensive effect of IMD_{1-53} in the CNS was blocked by i.c.v. AM_{22-52} and $CGRP_{8-37}$, in line with the study of Taylor *et al.* (2005), which suggested a role for the CGRP receptor in the CNS. To examine the effects of endogenous IMD_{1-53} in central regulation of blood pressure, a prepro-IMD antibody that antagonised the effects of exogenous IMD_{1-53} in the CNS was used. However, in the absence of exogenous peptide, the antibody had no effect on either blood pressure or heart rate, suggesting endogenous IMD_{1-53} has little effect on CNS-mediated cardiovascular function in normal animals. The same methods are yet to be applied to the study of endogenous IMD in the periphery, or in pathophysiological conditions in which endogenous IMD may be upregulated.

1.2.4.3 Other functions of IMD

There is increasing evidence to support a role for IMD in the female reproductive system as an intermediate lobe-derived prolactin-releasing factor, since IMD expression is increased in lactating animals, and can be stimulated by estrogens. Furthermore, this function of IMD may be mediated by the AM_2 receptor (Chang *et al.*, 2004). Due to the homology between AM and IMD, it is tempting to speculate that IMD may also have some functional role during pregnancy, particularly since elevated mRNA levels have been seen in the ovaries of mice (Takei *et al.*, 2004); and a role for AM has been identified this, however, remains to be demonstrated. Like AM, vascular responsiveness

to IMD was enhanced in mesenteric arteries isolated from pregnant rats via enhanced NO production (Chauhan *et al.*, 2007).

1.3 Adenosine

1.3.1 Introduction

The physiological significance of adenosine was first realised in the 1920s, when it was shown to possess both coronary vasodilator and anti-arrhythmic properties in cat, dog and guinea pig hearts (Drury & Szent-Györgyi, 1929). A number of years later, Berne (1963) developed the 'Ado Hypothesis for the metabolic regulation of coronary blood flow', which described adenosine as an endogenous dilator of coronary vessels, released following a reduction in the myocardial O_2 supply-to-demand ratio (Berne, 1963). Further studies in the mid-1980s, on both humans and animals, demonstrated that an increase in adenosine plasma levels resulted in both bradycardia and hypotension (for review see Berne, 1986). Adenosine is now known to dilate multiple vascular beds, and is involved in many physiological processes including immune regulation, respiration, activation and coagulation of platelets, oxyradical production, neuronal signalling, inhibition of lipolysis and synaptic transmission (for review see Berne, 1986; Motew *et al,* 1998; Law *et al,* 2003; Adanin *et al,* 2002; Haskó & Cronstein, 2004). Adenosine is currently used clinically to treat patients with paroxysmal supraventricular tachycardia which involve re-entry through the atrioventricular node (DiMarco *et al.,* 1983; Riccardi *et al.,* 2008).

Adenosine is a nucleoside composed of the purine, adenine, linked via a glycosidic bond to a ribose sugar (Figure 1.3), and is found ubiquitously throughout the body. It is involved in both energy storage and transfer as a

component of ATP/ADP, and in signal transduction as a component of cAMP. As a degradation product of ATP, adenosine is released into both extra- and intracellular compartments following the dephosphorylation of AMP by 5-(ecto)-nucleosidase, and via S-adenosylhomocysteine (SAH) hydrolysis. The amount of adenosine produced via SAH hydrolysis is relatively small and accounts for the generation of physiological amounts of adenosine. Adenosine produced in this way is not sensitive to changes in cellular oxygen levels (for review see Conlon *et al.*, 2005). Typically, basal tissue levels of adenosine are < 50 nM, but can be elevated to levels of up to 1000 nM under conditions of increased metabolic demand (Rivkees *et al.*, 2001).

Adenosine has been described as a 'reactive molecule', 'reactive metabolite' or 'metabolic vasoregulator' since it exerts little effect on resting physiology. Periods of increased metabolic demand, such as exercise, or decreased oxygen supply, such ischemia or hypoxia, lead to transient accumulation of adenosine in those areas affected. Furthermore, it has been demonstrated that in conditions of reduced oxygen, activity of the enzyme 5'-nucleosidase (see above) is elevated, providing a direct link between metabolic demand and adenosine production (for review see Mubagwa *et al.*, 1996). High levels of adenosine activity are seen in skeletal muscle and hepatosplanchnic organs, which both require regular changes in perfusion to satisfy fluctuations in metabolic demand (Motew *et al.*, 1998).

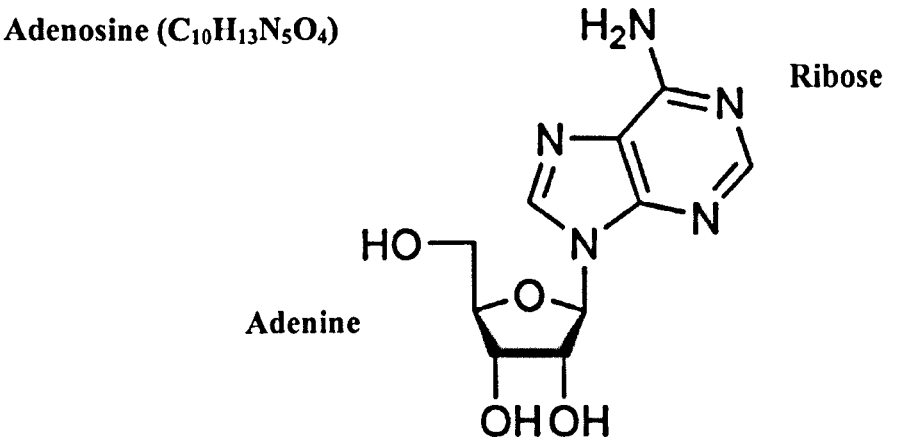


Figure 1.3: *Chemical structure of adenosine*

1.3.2 Adenosine receptors

Adenosine interacts with a family of four cell-surface, G-protein-coupled receptors belonging to the P_1 purinergic family, named A_1, A_{2a}, A_{2b} and A_3, and adenosine receptor-mediated effects have been confirmed experimentally in almost every tissue and system studied (for review see Conlon *et al.*, 2005). A_1, A_{2a} and A_{2b} receptors share approximately 80-95% sequence homology across species in which they have been identified, suggesting an important conserved role, whereas the A_3 subtype shows significant variation between species (for review see Conlon *et al.*, 2005). Expression of adenosine receptors in Chinese hamster ovary cells showed activation of A_1 and A_3 subtypes to cause inhibition of adenylate cyclase activity, whilst activation of both A_2 subtypes induced an increase in adenylate cyclase activity (Fredholm *et al.*, 2001). It was also shown that the order of potency of adenosine at these receptors was $A_1>A_3>A_{2a}>A_{2b}$, with A_1 receptor having the highest affinity for adenosine and A_{2b} the lowest. Therefore, A_1 activation occurs at physiological (low) levels of adenosine whilst A_{2b} activation requires pathophysiological

(high) levels of adenosine (Fredholm *et al.*, 2001); i.e., A_1 receptors are activated by adenosine in the nanomolar range whereas A_2 receptors require adenosine in the micromolar range (Murray & Churchill, 1984).

Adenosine receptors are non-selectively blocked by the alkylxanthines, caffeine and theophylline (Evoniuk *et al.*, 1987; Wormald *et al.*, 1989), in fact the physiological effects of caffeine are induced by its inhibitory effect on these receptors. In the 1980s, studies characterised the selectivity of adenosine receptor agonists and antagonists by their effect on 'A_2-mediated hypotension' and 'A_1-mediated bradycardia', with little or no consideration for regional effects - a view now known to be too simplistic (for example, Evoniuk *et al.*, 1987; Fredholm *et al.*, 1987).Whilst there are multiple pharmacological tools available for the study of adenosine receptors *in vivo* and *in vitro,* their use is often confounded due to poor selectivity, making interpretation of such studies difficult. For example, the non-xanthine adenosine receptor antagonist CGS-15943A, whilst non-selective, has higher affinity for A_2 than A_1-receptors (Ghai *et al.*, 1987). Using the A_2 agonist CV-1808, and the A_1-selective agonists CADO and CPA, Webb *et al.* (1990) showed A_2 activation leads to regional vasodilatation and increased renin release, whilst A_1-activation leads to bradycardia, mesenteric vasoconstriction and inhibition of renin secretion (Webb *et al.*, 1990). Each adenosine receptor subtype will now be discussed in more detail.

1.3.2.1 Adenosine A_1 receptors

Adenosine A_1 receptors are widely expressed in the brain, fat, and testis, and less prominently in the heart, kidneys, liver and bladder (Dixon *et al.*, 1996). A_1 receptors are linked to the inhibitory G-protein subunit G_i, leading to inhibition of adenylate cyclase and reduced intracellular cAMP formation. This has an inhibitory effect on the heart, leading to cardiac depression and bradycardia. Owing to the negative chronotropic effect of adenosine, A_1-selective antagonists have been developed for the treatment of cardiac disease and the resolution of supraventricular tachycardias (DiMarco *et al.*, 1983; Cotter *et al.*, 2008). The results of a Phase I trial piloting the A_1-selective antagonist, rolofylline, have recently been published (Cotter *et al.*, 2008) and suggest this drug may maintain renal function and enhance diuresis in patients with acute heart failure, due to inhibition of A_1-mediated afferent arteriolar constriction. The results of this study were promising and the drug is now in Phase II clinical trials (Cotter *et al.*, 2008). Although the effects of A_1 activation are most prominent in the heart, activation of vascular A_1 receptors also leads to renal and mesenteric vasoconstriction (Tagawa & Vandor, 1970; Webb *et al.*, 1990) In mice lacking the A_1 receptor the vasodilator effect of adenosine was augmented, suggesting activation of this receptor *in vivo* opposes A_2-mediated vasodilatation to some extent (Hansen *et al.*, 2005).

There is some evidence to suggest that A_1 activation can cause vasodilatation in some regions of skeletal muscle (Merkel *et al.*, 1992; Marshall *et al.*, 1993; see later; Danialou *et al.*, 1997). This adenosine receptor subtype can be activated by the selective agonists R-PIA and CCPA, and is antagonised by

the compounds DPCPX, WRC057 and NO861 (for review see Feoktistov & Biaggioni, 1997).

1.3.2.2 Adenosine A_2 receptors

Adenosine A_2 receptors were divided into A_{2a} and A_{2b} receptors due to their high and low affinity for adenosine in the rat brain (for review see Feoktistov & Biaggioni, 1997). A_{2a} receptors are located on vascular smooth muscle, endothelial cells, immune cells, in all brain regions and are widespread in the periphery (Dixon *et al.,* 1996). This subtype is linked to the stimulatory G protein, Gs, leading to activation of adenylate cyclase and cAMP formation when stimulated (for review see Mubagwa *et al,* 1996; Nemeth *et al,* 2006; Linden 2001). This subtype of receptors can be antagonised by ZM243185 and SCH58261, while APEC and CGS21680 act as selective A_{2a} agonists (for review see Linden, 2001).

Activation of the A_{2a} subtype induces vascular smooth muscle relaxation leading to vasodilatation of coronary arteries (Zhao *et al.,* 2003), renal afferent arterioles (Tagawa & Vandor, 1970; Murray & Churchill, 1985; Tang *et al.,* 1999), and mesenteric arteries (Zhao *et al.,* 2003). Systemic vasodilatation mediated by this subtype results in hypotension (Webb *et al.,* 1990). Activation of A_{2a} receptors on immune cells, also mediates the strong immunosuppressant effects of adenosine, and A_{2a} receptor activation on mast cells and macrophages leads to inhibition of histamine and TNF-α release via an increase in cAMP (Cronstein *et al.,* 1992; Ohta & Sitkovsky, 2001; Zhang *et al.,* 2005; Nemeth *et al,* 2006).

Although there are no selective A_{2b} agonists, these receptors can be antagonised by enprofylline (for review see Conlon, 2001). A_{2b} receptors are found ubiquitously in rats, and are expressed at various levels in all tissues studied, making it the most widespread adenosine receptor subtype in that species (Dixon *et al.*, 1996). A_{2b} receptors are expressed at the highest level in rat proximal colon and are widespread in the brain. As well as having ubiquitous tissue distribution, this receptor subtype is also found on immune cells including fibroblasts and mast cells. While both subtypes of A_2 receptors are linked to G_s, A_{2b} receptors may also be coupled to G_q, with activation leading to Ca^+ mobilisation and activation of mitogen-activated protein kinase (MAPK). Adenosine-mediated A_{2b} activation has been implicated in mast cell activation and asthma, vasodilatation and regulation of cell growth (for review see Feoktistov & Biaggioni, 1997; Linden, 2001).

1.3.2.3 Adenosine A_3 receptors

Adenosine A_3 receptors can be antagonised by MRS1067 and MRS1097, whilst IB-MECA acts as a selective agonist of this subtype (Feoktistov & Biaggioni, 1997). The A_3 receptor subtype is the least well characterised adenosine receptor. It is linked to G_i leading to inhibition of adenylate cyclase, and hence, vasoconstriction and mast cell degranulation *in vivo.* (Nieri *et al.*, 2001). The A_3 receptor is expressed only at low levels in rats in the testis, heart and retina (Dixon *et al.*, 1996; Fredholm *et al.*, 2001). Interestingly, apart from in sheep and human, A_3 receptors are not antagonised by methylxanthines, which may account for some of the discrepancies in

responses to adenosine antagonists in different species (for review see Feoktistov & Biaggioni, 1997).

A review of data concerning the cardiovascular responses to adenosine is given below, with specific reference given to the receptor(s) activated where known.

1.3.3 Adenosine and the cardiovascular system

The effects of adenosine on the cardiovascular system are complex, and vary depending upon the receptor(s) activated, the concentration of adenosine and the vascular region. When given to experimental animals, adenosine causes a fall in blood pressure which involves both cardiac and vascular effects, mediated by A_1- and A_2- receptors, respectively (Drury & Szent-Györgyi, 1929; Ohnishi *et al.*, 1986; Webb *et al.*, 1990; Tang *et al.*, 1999; Nieri *et al.*, 2001). Adenosine causes dilation of rat and rabbit mesenteric arteries (Mian & Marshall, 1995), dilatation, preceded by a transient constriction of rat and mouse renal arteries (Tagawa & Vander, 1970; Tang *et al.*, 1999; Hansen *et al.*, 2005) and dilatation of coronary arteries (Drury & Szent-Györgyi, 1929).

The study of Webb *et al.* showed that A_2-receptor activation leads to regional vasodilatation and systemic hypotension with reflex tachycardia and increased renin secretion, whilst activation of A_1-receptors causes profound bradycardia, depressed cardiac output and hypotension. Thus, while both A_1- and A_2-receptor activation leads to marked falls in blood pressure, in the case of the former this was mediated by cardiac events, whilst in the latter hypotension was mediated by vascular events (Webb *et al.*, 1990). The cardiac and vascular effects of adenosine will now be considered in turn.

1.3.3.1 Cardiac responses to adenosine

It has long been known that adenosine has a profound effect on the heart, and is used clinically to this effect to resolve paroxysmal supraventricular tachycardia (DiMarco *et al.*, 1983). Drury & Szent-Györgyi (1929) first described the cardiac slowing effect of adenine nucleotides in hearts taken from multiple species. A number of years later, it was shown that ATP, AMP and ADP rapidly reduced heart rate to a similar extent when perfused directly into the sinus node of isolated dog hearts. Moreover, this negative chronotropic effect was not affected by atropine, suggesting it was independent of parasympathetic activation. Since infusion of creatine phosphate had no effect on heart rate, it was concluded that adenosine was the agent responsible for the cardiac slowing effect of these nucleotides (James *et al.*, 1965). Studies in conscious animals have been valuable in elucidating the involvement of the autonomic nervous system in the cardiovascular response to adenosine. For example, in whole animals, adenosine has been observed to cause tachycardia, which is either reduced (Lappe *et al.*, 1991), abolished (Ohnishi *et al.*, 1985), or reverted to a bradycardia (Hintz *et al.*, 1985; Watt & Routledge, 1986; Belloni *et al.*, 1989) in the presence of cholinergic and adrenergic blockade. This suggests that adenosine-mediated tachycardia *in vivo* is largely baroreflex-mediated, and masks an underlying bradycardia (Hintz *et al.*, 1985; Ohnishi *et al.*, 1985). Interestingly, this response was shown in healthy volunteers, in which infusion of adenosine led to a transient bradycardia, which reverted to a tachycardia once respiration rate increased and blood pressure decreased (Watt & Routledge, 1986). This bradycardic

effect of adenosine is a consequence of a transient impairment of AV conduction (Hintz *et al.*, 1985; Watt & Routledge, 1986; Belloni *et al.*, 1989). Adenosine-mediated bradycardia is mediated by the A_1-receptor subtype, since isolated hearts from A_1-receptor knockout mice failed to respond to adenosine (Hansen *et al.*, 2005; Tawfik *et al.*, 2006). No difference was shown in the basal heart rate between A_1-receptor knockout and wild type mice suggesting that endogenous adenosine acting on this receptor is not involved in the maintenance of resting heart rate (Tawfik *et al.*, 2006). However, hearts from A_1–receptor knockout mice exhibited a higher basal coronary blood flow suggestive of A_1-receptor mediated vasoconstriction in this region in normal mice (Tawfik *et al.*, 2006).

1.3.3.2 Renal effects of adenosine

Systemically, adenosine acts as a potent vasodilator (Ohnishi *et al.*, 1985; Nieri *et al.*, 2001), indeed, it is this function of the nucleoside that couples metabolism to blood flow. However, in the kidney, adenosine has been reported to cause both an increase and a decrease in blood flow, owing to the opposing effects of A_2- and A_1-receptor stimulation, respectively (Tagawa & Vander, 1970; Hansen, *et al.*, 2005). Several studies have shown that adenosine causes a transient renal vasoconstriction which develops into a long-lasting renal vasodilatation (Tagawa & Vandor, 1970; Hansen *et al.*, 2005).

A study of adenosine receptor abundance in rat kidney showed that the A_1 and A_{2b} subtypes are abundant in the preglomerular circulation, with A_{2b} mRNA and protein being expressed throughout the whole kidney, whereas in that

study, A_{2a}- and A_3-receptor subtypes were barely detectable in the preglomerular circulation (Jackson *et al.*, 2002). Along with other studies, this suggests that the renal vasodilator response to adenosine is mediated by the A_{2b}-receptor (Tang *et al.*, 1999; Jackson *et al.*, 2002).

The overall renal effect of adenosine administration *in vivo* has been shown to be vasodilatation, suggesting that A_2-receptor activation predominates in this vascular bed, which may relate to high concentrations of exogenous adenosine and/or higher distribution of the A_2 receptor subtype in the kidney (Murray & Churchill, 1985; Jackson *et al.*, 2002). However, a study in conscious dogs showed that adenosine infusion led to renal vasoconstriction, and A_{2a}-receptor agonists had no effect, suggesting A_1-receptors predominate, at least in this model (Zhao *et al.*, 2003). As in other systems, the effect of adenosine on renal blood flow is concentration-dependent, since A_1-receptors have a higher affinity for adenosine than do A_{2a}-receptors (Tang *et al.*, 1999). At low concentrations, adenosine perfusion in isolated rat kidneys caused a transient DPCPX-sensitive vasoconstriction, whilst at higher concentrations adenosine caused vasodilatation which was sensitive to A_2-receptor blockade. Adenosine-mediated renal vasoconstriction is potent but transient, and is mediated by activation of afferent arteriolar A_1-receptors, an effect that can be mimicked and blocked by A_1-receptor selective agonists and antagonists, respectively (Tang *et al.*, 1999). The observation that adenosine-mediated vasoconstriction was absent in afferent arterioles isolated from A_1-receptor knockout mice, indicates that this response involves activation of A_1-receptor receptors in this region (Hansen *et al.*, 2005). Interestingly, when perfused into isolated kidneys at a high dose, the A_1-selective agonist, CHA, caused a

marked vasodilatation which was similar to that caused by high dose NECA (Murray & Churchill, 1985).

Acting via A_1- and A_2- receptors, adenosine leads to inhibition and stimulation of renin release, respectively (Tagawa & Vandor, 1970; Murray & Churchill, 1985; Weihprecht *et al.,* 1990), and has been described as a 'physiological brake on renin release' (Jackson *et al.,* 1991). Evidence suggests the inhibitory effect mediated via the A_1-receptor subtype is a direct effect of adenosine acting on the juxtaglomerular apparatus (Weihprecht *et al.,* 1990), whilst A_2-meditated renin stimulation is a result of increased sympathetic nerve activity, since this response is not seen in isolated preparations lacking neural input. The inhibitory effect on adenosine of renin secretion has been demonstrated in isolated rabbit juxtaglomerular cells (Weihprecht *et al.,* 1990), and in rat renal cortical slices (Churchill & Churchill, 1985). As with renal blood flow, a biphasic dose-dependent renin response is observed following adenosine administration *in vitro* and *in vivo*, with low concentrations of adenosine leading to inhibition of renin secretion and high concentrations stimulating renin release, directly via A_1- and indirectly via A_2-receptors, respectively (Tagawa & Vandor, 1970; Churchill & Churchill, 1985). The opposing roles of these receptor subtypes in regulating renal function have been revealed in studies using receptor specific agonists and antagonists. For example, A_1-receptor activation by CHA led to a marked decrease in renin release from perfused rabbit kidneys, an effect which was reversed by A_1-blockade by DPCPX (Weihprecht *et al.,* 1990).

1.3.3.3 Effects of adenosine in skeletal muscle

During extended periods of hypoxia, adenosine acts on endothelial, cardiac and central A_1-receptors to bring about femoral muscle vasodilatation, decrease in heart rate and decrease in tidal volume and respiratory rate, respectively (see Walsh & Marshall, 2006). In a series of studies, Janice Marshall's group showed that the hind limb vasodilatation induced during systemic hypoxia in anaesthetised rats, was mediated by adenosine receptor activation and subsequent opening of K_{ATP} channels (Marshall *et al.*, 1993). Using a selection of A_1- and A_2-receptor agonists and antagonists, they later showed that whilst both receptor subtypes were present in rat hindlimb and contribute equally to adenosine-induced muscle vasodilatation in normoxia, only A_1-receptors were activated by adenosine in hypoxia. Furthermore, DPCPX reversed the increase in femoral vascular conductance evoked by both hypoxia and exogenous adenosine suggesting the involvement of endogenous adenosine in this response (Bryan & Marshall, 1999). Adenosine-stimulated endothelial NO release has been shown from rat aorta in normoxia (Ray *et al.*, 2002) and from rat hindlimb in an A_1-dependent manner in both chronic and acute systemic hypoxia (Ray & Marshall, 2005; Walsh & Marshall, 2006). Interestingly, studying the effects of exercise on muscle vasodilatation, the same group showed there was no role for NO in adenosine-induced hyperaemia in exercise. Furthermore, during exercise, the hindlimb vasodilator response to adenosine was shown to involve direct activation of A_{2a}-receptors on vascular smooth muscle. It was therefore concluded that the mechanisms through which adenosine leads to muscle vasodilatation in exercise and during hypoxia are different (Ray & Marshall, 2009). In human

skeletal muscle, A_1-, A_{2a}- and A_{2b}-receptors are located on both vascular smooth muscle cells and endothelial cells, and both A_2-receptor subtypes were also found on the plasma membrane and in the cytosol of human skeletal muscle cells (Lynge & Hellsten, 2000). Evidence of A_1-mediated lower body vasodilatation has also been shown in conscious dogs (Zhao *et al.*, 2003).

Given that adenosine is critical in the regulation of local blood flows in response to fluctuations in metabolic demand, the observation that A_1-receptors mediate skeletal muscle vasodilatation is not surprising. Since A_1-receptors have a high affinity for adenosine, A_1-receptor mediated vasodilatation (as opposed to vasoconstriction for which this subtype is best characterised) would increase blood flow to skeletal muscle cells even when small amounts of adenosine have been liberated from ATP.

1.3.4 Adenosine in sepsis

Studies using the non-specific adenosine-receptor antagonist, 8-PT, have shown that endogenous adenosine is involved in the maintenance of perfusion in the hepatosplanchnic organs (stomach, pancreas, small intestine, colon, and caecum) and skeletal muscle 24 h after the induction of septic peritonitis in rats (Motew *et al.*, 1998; Adanin *et al.*, 2002). Thus, an increase in vascular resistance was observed following 8-PT treatment in those regions where endogenous adenosine was exerting a tonic vasodilator effect. Furthermore, this was shown to occur in an NO-dependent manner in both regions, and via an NO-independent manner in skeletal muscle (Sam *et al.*, 2000). Based on the work of Marshall *et al.* (see above) it is possible this NO-independent mechanism in skeletal muscle is adenosine-mediated opening of K_{ATP}

channels secondary to A_1-receptor activation (Marshall *et al.*, 1993; Bryan & Marshall, 1999).

Results show that plasma levels of adenosine were higher in patients admitted to an ICU with sepsis, severe sepsis and septic shock compared to healthy volunteers and those with hypovolaemic shock (Martin *et al.*, 2000). Furthermore, non-survivors always had significantly higher adenosine plasma levels than survivors. Interestingly, there were no significant differences in adenosine plasma levels between patients suffering from Gram-negative and Gram-positive sepsis, suggesting that increased adenosine release may be a common feature of clinical sepsis. Thus, the use of adenosine as a prognostic index for outcome in septic patients has been suggested, with higher plasma levels linked to a better prognosis (Martin *et al.*, 2000).

Transgenic studies have identified a protective role for adenosine in murine endotoxaemia and peritonitis. Mice lacking the A_{2a}-receptor had increased systemic inflammation, whilst A_1- and A_3-knockout mice had significantly higher mortality rates compared to wild type mice, associated with increased inflammation and enhanced hepatic and renal injury (Sullivan *et al.*, 2004; Gallos *et al.*, 2005; Lee *et al.*, 2006). Moreover, stimulation of A_{2a}-receptors was associated with decreased live bacteria in the blood, reduced inflammation and reduced neutrophil extravasation and adhesion and reduced coagulopathy in CLP and LPS-treated mice (Sullivan *et al.*, 2004).

Adenosine decreases TNF-α release from LPS-activated monocytes and human macrophages (Zhang *et al.*, 2005; Ryzhov *et al.*, 2008). Furthermore,

mice lacking the A_{2a}-receptor show increased susceptibility to even a mild inflammatory response which does not provoke a response in wild type animals (Ohta & Sitkovsky, 2001). This study showed that adenosine acting via the A_{2a}-receptor subtype plays a central role in limiting inflammation during sepsis by down-regulating pro-inflammatory cytokines and preventing tissue damage (Ohta & Sitkovsky, 2001). However, another study showed increased survival in CLP-induced sepsis following inactivation of A_{2a}-receptors (Németh *et al.*, 2006). Mice lacking the A_{2a}-receptor were shown to have improved antigen presentation, decreased apoptosis of lymphocytes in the spleen and decreased levels of IL-10 and IL-6, at 16 h and 48 h from the onset of sepsis (Németh *et al.*, 2006). It is possible that A_{2a}-receptor mediated immunosuppressant actions are beneficial during the exaggerated inflammatory stage of early sepsis, but detrimental during the immunosuppressive stage of late sepsis (see section 1.1). Furthermore, since the study of Sullivan *et al.* (2004), induced sepsis by administration of LPS or live *E.coli*, and that of Németh *et al.* (2006) by CLP, it is possible that the response to A_{2a}-reception activation differs according to the cytokine profile associated with each model.

1.3.4.1 Therapeutic potential of adenosine in sepsis

Since adenosine is able to limit the pro-inflammatory response in early sepsis, it has been suggested (Adanin *et al.*, 2002; Cohen *et al.*, 2002; Law *et al.*, 2003) that blocking the breakdown of adenosine may be a novel therapy in the ICU. Adenosine has a half life of seconds, due to its rapid catabolism by

adenosine deaminase (ADA) into ammonia and inosine (for review see Martin *et al.*, 2000). Adenosine deamination can be blocked by 2-deoxycoformycin (Pentostatin (PNT)) which binds ADA and prevents adenosine catabolism. Pentostatin has successfully been used in the treatment of hairy cell leukaemia and myocardial ischemia, and its therapeutic potential in sepsis has attracted some attention (Adanin *et al.*, 2002; Cohen *et al.*, 2002; Law *et al.*, 2003).

Evidence suggests that adenosine is as an important modulator of the early immune response to sepsis, since *in vitro* studies have shown that adenosine inhibits TNF-α production. Adanin *et al.* (2002) assayed TNF-α levels in serum and tissue in rats treated with LPS or faecal slurry, and with either PNT or the adenosine receptor antagonist 8-SPT. They showed that PNT significantly attenuated the LPS-induced increase in TNF-α in a dose-dependent manner, while 8-SPT significantly increased TNF-α levels 24 h after peritoneal sepsis induction (Adanin *et al.*, 2002). Furthermore, administration of PNT significantly attenuated microvascular dysfunction and increased survival in mice 48 h after induction of sepsis via CLP (Cohen *et al*, 2002), consistent with the ability of adenosine to limit microvascular dysfunction during sepsis by inhibiting neutrophil-mediated injury to endothelial cells.

Clinically, this suggests that prevention of adenosine breakdown via PNT could be used as a prophylactic treatment in high-risk scenarios, including in pre-surgical patients (Adanin *et al.*, 2002). A beneficial effect of PNT as a pre-treatment to LPS exposure was also shown by Law *et al* (2003) who found the drug to increase survival in a rat faecal slurry model of peritoneal sepsis (Law *et al.*, 2003). Due to the prolonged suppression of pro-inflammatory

cytokines by adenosine, a possible side effect of chronic ADA inhibition is immunosuppression. Plasma elimination of PNT in humans was reported to be 5 to 6 h, and white blood cell counts returned to pre-treatment levels after 24 h in septic rats. Thus, immunosuppression is only short-term following administration of PNT.

Overall, evidence suggests that adenosine plays an important role in limiting cellular injury during sepsis. However, whilst adenosine acting via A_{2a} receptors has a protective role during early sepsis, it also has detrimental effects in the later stages of severe sepsis and septic shock when systemic hypotension is marked and the immune system is suppressed.

1.4 General aim

The studies presented in this thesis were designed to delineate possible mechanisms involved in IMD and AM-mediated vascular responses, and to assess factors (AM, IMD, adenosine) contributing to regional changes in vascular tone across time, in normal and LPS-treated rats.

Against this background, the specific aims of the work contained in this thesis were:

1. To compare the regional haemodynamic responses to AM and IMD in normal animals.

2. To investigate possible mechanisms involved in mediating the cardiovascular effects of AM and IMD in normal animals.

3. To characterise regional haemodynamic changes across time in a rat model of endotoxaemia based on (a) a low-dose LPS bolus (1mg kg^{-1}) and (b) a continuous low-dose LPS infusion (15μg kg^{-1} h^{-1}), and to assess whether the vascular responses to AM and IMD are affected at selected time points in each model.

4. To investigate the regional haemodynamic responses to adenosine across time in normal and LPS-treated rats, and to establish the contribution of adenosine receptors to any changes in responsiveness during endotoxaemia.

5. To investigate any synergism between adenosine, AM and IMD in normal, conscious rats.

Chapter 2

General Methods

2.1 Introduction

The experiments described in this thesis were carried out on male Sprague-Dawley rats chronically implanted with miniature, pulsed Doppler flow probes to measure changes in regional vascular conductances. Specifically, changes in renal, mesenteric and hindquarters Doppler shift were measured by way of flow probes placed around the left renal artery, superior mesenteric artery and distal aorta, respectively. Between 10 and 14 days later, the same animals were implanted with intravascular catheters to allow administration of substances and the continuous recording of blood pressure and heart rate, and regional vascular conductances were derived from Doppler shift signals from renal, mesenteric and hindquarters probes. This system has been refined over several years, and allows integrated regional haemodynamics to be measured in conscious animals for up to 4 days.

2.2 Pulsed Doppler flowmetry

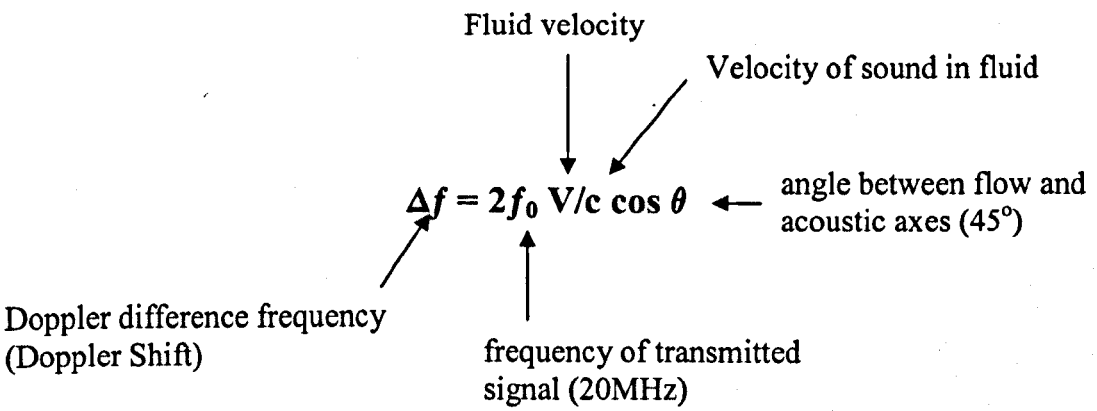
2.2.1 The Doppler method

The use of pulsed Doppler flowmetry to measure regional blood flow in small animals was developed by Hartley & Cole (1974), and later refined for use in rats by Haywood *et al.* (1981). Other methods for measuring blood flow, such as continuous wave Doppler flowmetry, or electromagnetic flowmetry, require bulky probes making them impractical for use in small animals. Pulsed Doppler flow probes in contrast, are much smaller, allowing several probes to

be implanted in a single animal. A complete probe comprises a single piezoelectric crystal with a diameter of 1mm (DBF-120A-XS; Crystal Biotech, Holliston, MA) which acts as both a transmitter and a receiver. The crystal is attached to wire leads and is mounted in a thin, lightweight cuff, which can be sutured around blood vessels measuring 1-5mm in diameter.

When the probe is connected to the flowmeter, the crystal emits a short burst of ultrasonic sound at a frequency of 20MHz; this is received back as an 'echo' signal by the crystal when the signal hits moving targets in its path (in this case, red blood cells). Since energy scattered at 20MHz is higher than that scattered at lower frequencies, smaller crystals can be used, again optimising this system for use in small animals (Hartley & Cole, 1974). The difference in frequency between the sound transmitted from the crystal and that received back is known as the 'Doppler shift' and can be expressed mathematically by the Doppler equation, which is shown below.

2.2.2 The Doppler equation



The Doppler equation indicates that blood velocity is directly proportional to blood flow providing that the diameter of the vessel beneath the probe remains constant. This is ensured by the growth of a fibrous capsule around the probe cuff during chronic implantation, keeping the crystal anchored to the vessel at a constant angle of 45°. Since f_0, c and θ are constant for any individual probe in any individual animal, then, Δf is proportional to V. Therefore, changes in Doppler shift are a measure of changes in blood flow velocity. Since the vessel diameter beneath the probe remains constant, changes in blood flow velocity can be used as an index of changes in blood flow distal to the probe. Changes in the Doppler shift signal from the probe are related to changes in flow in the total vascular bed, and hence flow, downstream of the probe. This system also has the benefit of being directionally sensitive allowing the direction of blood flow to be determined.

By manually adjusting the range gate, the time delay between the transmitted signal and the reflected signal is adjusted. Adjusting the signal to an optimum range allows the velocity of blood cells in the centre of the vessel, where it is at its highest, to be sampled.

The Nyquist or 'sampling' theorem states that correct reproduction of analogue-to-digital signals requires that the sampling frequency (pulse repetition frequency (PRF) or number of times a crystal emits and receives each second) must be greater than half the maximum frequency response. If the maximum frequency response is more than half the sampling frequency then the phenomenon of 'aliasing' occurs whereby the same sample values overlap and the high frequency components of the signal are lost (or appear as

an 'alias' signal). Hence, commercially available pulsed Doppler systems which use a PRF of 62.5 kHz per second are of limited value in systems measuring high velocities. Gardiner *et al.* (1990) showed that, in conscious rats implanted with aortic pulsed Doppler flow probes and treated with nitroprusside or methoxamine, a standard PRF of 62.5 kHz was insufficient to accurately measure changes in Doppler shift due to aliasing of the Doppler signal. In this system, only spectral components of 32.5 kHz or less (half the PRF) could be detected. By modifying this system and increasing the PRF to 125 KHz, the high velocity component of the signal was included (i.e., PRF/2 is 62.5 kHz; therefore frequencies up to this value could be measured). Therefore the experiments outlined here used a high-velocity module with a PRF of 125 kHz (Gardiner et al., 1990).

2.2.3 Construction of pulsed Doppler flow probes

Piezoelectric crystals (diameter of 1mm) with attached wires were purchased from Crystal Biotech Inc and complete flow probes were constructed in house by Julie March in a 3 stage process modified from that described by Haywood et al. (1981).

Stage 1: Mounting the crystal

Construction of flow probes begins by mounting the piezoelectric crystal into a piece of silicon tubing measuring 3-4mm in length. One end of the tubing is cut at an angle of 45° and the crystal is placed into the lumen using sharp forceps. This is the angle which the crystal will be at relative to the vessel, and

is the optimum angle for detecting changes in blood velocity. Melted dental wax is placed on the angled end of the tubing which holds the crystal in place until it is permanently fixed. The other end of the tubing is covered in silastic adhesive (Raumedic) onto which a small piece of expanded polystyrene is placed to act as an acoustic baffle. This prevents the crystal receiving interference from blood flow in adjacent regions. This subunit is then left overnight to allow the adhesive to set.

Stage 2: Silastic cuff formation

The subunit is mounted onto a needle such that the crystal is sitting at a 45° angle. The needle mimics the blood vessel which will eventually sit in that position and the use of different sized needles enable probes to be made for different sized vessels. The dental wax placed on the subunit in stage 1 is melted using a soldering iron and allowed to set on the needle, holding the unit in place. The silastic (Raumedic) cuff is then formed by building up silastic adhesive around the subunit and needle. This is again left overnight to set in place.

Stage 3: Probe preparation

The completed probe is removed from the needle by using a scalpel to cut the silastic cuff along the underside of the needle. The resulting flaps will eventually provide the opening through which the blood vessel is positioned with the crystal lying underneath. The dental wax is then removed and sutures

(6/0 ophthalmic silk, Ethicon, Edinburgh, UK) are sewn into each flap to provide ties which can be used to secure the probe onto the vessel.

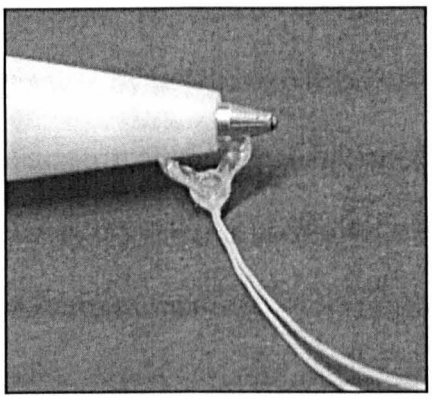


Figure 2.1: *A complete Doppler flow probe, shown against a pen nib for size comparison*

2.2.3 Measurement of blood pressure

In order for regional vascular conductances to be determined by the use of Doppler flow probes, Doppler shift must be recorded simultaneously with blood pressure. Continuous recording of blood pressure and heart rate in these experiments was achieved by the implantation of intra-arterial catheters. Conductance could then be calculated by dividing flow by pressure:

Conductance = flow/pressure

Blood pressure was measured by connecting a fluid-filled arterial catheter to a pressure transducer (Gould, type 4-442) and transducer amplifier (Gould, model 13-4615-50), which interfaced with a custom-designed Haemodynamic Data Acquisition System (HDAS; built by the Bioinstrumentation

Laboratories at the University of Maastricht, Netherlands). Pressure changes in the catheter system are detected as changes in resistance in strain gauges within the pressure transducer, and resistance changes are converted to voltage changes by a Wheatstone bridge circuit. The system can be calibrated using a mercury manometer such that changes in voltage are converted to changes in mmHg by HDAS, and there is a linear relationship between changes in pressure and voltage.

In order to measure pressure effectively, and to achieve optimal damping, the measuring system must be capable of following frequencies as high as the $\geq$ 5^{th} harmonic of the basic frequency of the signal, which in this case is the heart rate and is approximately 40Hz. This system is optimised to reduce damping by having the main part of the catheter (minus the 6 inches inserted into the caudal artery; see below) consist of rigid tubing (Portex Nylon tubing, with an internal diameter of 0.58mm), and a custom-designed low volume transducer dome containing de-gassed water to eliminate air bubbles and minimal air space.

2.4 Surgical procedures

2.4.1 Animals

All experiments were carried out on male Sprague-Dawley rats supplied by Charles River UK weighing 400-500g at the time of experimentation. Unrestrained animals were housed in groups of up to 4 for at least 1 week prior to surgery, and individually afterwards, with free access to food and

water throughout. Animals were kept in temperature-controlled holding areas, with a 12h light/dark cycle (6am to 6pm), in the Biomedical Services Unit at the University of Nottingham. Procedures were carried out with the approval of the University of Nottingham Ethical Review Committee and were performed in accordance with Home Office Licensing regulations.

All surgery was carried out under general anaesthesia, provided by fentanyl citrate (Sublimaze, supplied by Jansen-Cilac Ltd) and medetomidine (Domitor, supplied from Pfizer); 0.3 mg kg^{-1} of each i.p. Anaesthesia was reversed by atipamezole hydrochloride (1 mg kg^{-1} s.c.) (Antisedan, supplied by Pfizer) and buprenorphine (0.3 mg kg^{-1} s.c.) (Vetergesic, supplied by Alstoe Animal Health); buprenorphine also provided analgesia. Following surgery for probe implantation, a single i.m. injection of ampicillin trihydrate (150 mg s.c.) (Amoxycare LA) was given. Surgical plane of anaesthesia was assessed by the absence of an interdigit toe pinch, and top-up was provided as required.

2.4.2 Implantation of Doppler flow probes

At the first surgical stage, rats were chronically implanted with miniature, pulsed Doppler flow probes to measure changes in renal, mesenteric and hindquarters blood flows. Once rats reached an adequate level of anaesthesia, they were weighed and shaved to expose the midline abdomen, the left flank above the hindlimb, and at the level of the shoulder blades below the neck.

Rats were wrapped in cling film (which acted as a surgical drape), and placed in the dorsal recumbent position on a heated table to help prevent heat loss.

A ventral midline abdominal incision of 6-8cm was made along the line of the *linea alba* extending down the animal from the xiphoid cartilage. Clamps were inserted and secured using Transpore tape (Blenderm, 3M, USA) before the intestines were wrapped in a sterile saline-soaked paper towel and retracted to expose the abdominal cavity. Target vessels were identified under a microscope, isolated and freed of any connective tissue using two cotton wool buds and blunt dissection. For each vessel, an appropriately sized probe was chosen, coupling gel was placed on the face of the crystal and the target vessel lifted to allow the probe to be placed around. Signals were confirmed for each probe by adjusting the range gate on a Doppler flowmeter before the probe was sutured into place around the vessel. This procedure was followed for the left renal artery, superior mesenteric artery and the distal abdominal aorta, below the level of the ileocaecal artery. Wires were tunnelled through the body wall, exiting on the left side; excess wires were placed in an s.c. pouch, and the ends of the wires were tunnelled s.c. to exit at a single point at the nape of the neck where they were secured with tape and suture. The body wall was closed and the abdominal skin sutured and closed with Michel clips. The position of flow probes is shown on Figure 2.2.

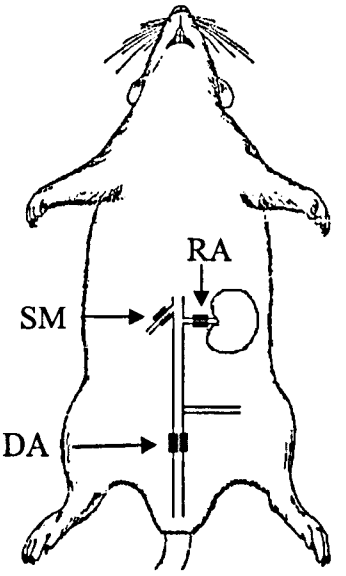


Figure 2.2: *Location of Doppler flow probes. Probes were sutured around the left renal artery, superior mesenteric artery and the distal aorta in rats under anaesthesia. RA=renal artery; DA=distal aorta; SM=superior mesenteric.*

2.4.3 Implantation of intravascular catheters

Venous, and i.p. catheters consisted of 150cm Portex polythene tubing, internal diameter of 0.28mm, dead space 0.1ml; venous catheters used for overnight infusions and arterial catheters comprised 2cm or 6cm Portex polythene tubing, respectively (as above) attached (heat sealed) to 85cm Portex Nylon tubing, with an internal diameter of 0.58mm. Arterial catheter patency was maintained by heparinised saline 30 units ml^{-1} saline; venous catheter patency was maintained by 15 units ml^{-1} saline (CP Pharmaceuticals Ltd).

Surgery for catheter implantation occurred after a recovery period of 10-14 days and after a satisfactory inspection by the Named Veterinary Surgeon. Rats were anaesthetised as above (see section 2.4.1), weighed, and an area of skin was shaved over the right jugular vein between the larynx and the

sternum. The probe wires were freed from the nape of the neck, stripped of insulation and soldered into a micro-connector (Microtech Inc., Boothwyn, PA, USA, part no GF-6), which was secured using a small piece of tape. Animals were then wrapped in cling film which acted as a surgical drape and also helped to maintain body temperature.

2.4.3.1 Intravenous catheters

An incision of approximately 1cm was made over the right jugular vein, which could be detected as a slight pulsation beneath the skin. The vein was isolated and cleared of connective tissue by blunt dissection. Catheters (2 or 3) were then tunnelled s.c. from the nape of the neck using the opening made during probe implantation, to the new incision over the vein. A small pair of forceps was used to lift the vessel clear of surrounding tissue and lignocaine was dripped onto the vein to prevent it going into spasm. Sutures were then used to tie off the rostral end of the jugular vein, a small cut was made in the upper surface of the vessel and the catheters were introduced with the aid of a wire introducer and advanced for a length of 2cm. Once all catheters were in, they were tied in place with 4/0 silk thread, and the excess lengths were pulled through the back of the neck and the incision was closed with sutures.

2.4.3.2 Intra-arterial catheters:

With the rat in the dorsal recumbent position, an incision of 1-2cm was made at the base of the tail on the ventral side. The caudal (tail) artery was isolated and cleared of connective tissue using blunt dissection. A small pair of forceps

was placed underneath the vessel, and lignocaine was dripped onto the artery to prevent it from going into spasm. The caudal end of the vessel was tied off using silk sutures and a small incision was made in the upper surface of the vessel. Using a wire introducer the catheter tip was inserted into the vessel and, with the aid of forceps, was advanced 6cm so the tip lay in the distal aorta (caudal to the flow probe). The catheter was then tied in place and tunnelled to exit the tail on the dorsal side and the ventral tail incision was closed using interrupted sutures. The catheter was then tunnelled s.c. with the aid of a blunt trochar to exit at the incision at the back of the neck (see above).

2.4.3.3 Intraperitoneal catheters

Some experiments required substances to be given via an i.p. catheter. In these cases an additional area of skin on the right hand flank above the hind limb was shaved whilst the animal was prepared for catheter implantation. The rat was placed on its left side and an incision of approximately 1cm was made using a scalpel blade. Connective tissue was then cleared using blunt dissection until the abdominal wall was accessible. The catheter was tunnelled from the incision at the back of the neck to the incision on the right flank. Whilst firmly holding the abdominal wall with forceps, a small incision was made into which the catheter could be advanced into the peritoneal cavity with the aid of a rod. Purse-string sutures were used to secure the catheter in place, and the skin on the flank was closed.

2.5 Experimental set-up

Following catheter implantation, animals were fitted with a custom-designed harness which was placed around their neck and secured around the body below the forelimbs. The micro-connector containing the probe wires was screwed into place on the top of the harness, and a custom-built probe-connector was attached. The catheters were tunnelled through a flexible spring, which was secured at one end to the top of the harness on the dorsal side of the rat, and at the other to a counter-balanced swivel system once the animal was placed into a cage; this allowed free movement within the cage (Figure 2.3).

The arterial catheter was attached to a single channel swivel which allowed a continuous infusion of heparinised saline (15 units ml^{-1}; 0.4 ml h^{-1}) to be given before and in between experimental periods to maintain catheter patency. In experiments where animals were to receive a continuous infusion of LPS, a double-channel swivel system was used which enabled 2 substances to be given simultaneously. These were made in house by Julie March based on the method described by Brown *et al.* (1976) for single channel swivels, and those described by Blair *et al.* (1980) for double channel swivels. These allow fluids to be infused without restraining the animals. Experiments began at least 24 h after catheter implantation, in unrestrained, conscious animals.

(a) (b)

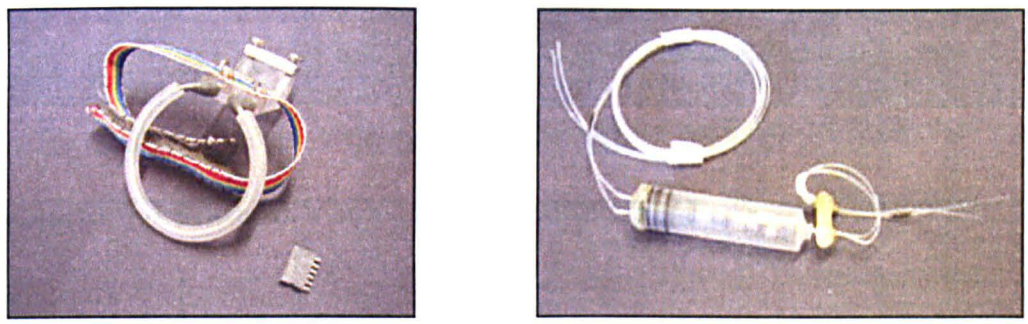


Figure 2.3: *Custom designed harness (a) and double channel swivel (b). Together these allow continuous cardiovascular measurements to be made in freely moving, conscious rats.*

On experimental days, the arterial catheter was disconnected from the swivel and attached to the transducer dome to allow blood pressure and heart rate to be measured. The probe connector was attached to leads on the Doppler flowmeter to allow regional haemodynamics to be measured. The signals from the probes interfaced with HDAS via the Doppler flowmeter (Crystal Biotech VF-1 mainframe fitted with high velocity (HVPD-20) modules). The signals were sampled by HDAS every 2ms, averaged every cardiac cycle and stored to disc at 5s intervals for later analysis, using custom-designed software (Datview; University of Limburg, Maastricht, Netherlands) allowing analysis of cardiovascular responses over selected intervals. The range gate was adjusted for each individual probe (see section 2.2.2) to ensure optimal signals were received by each crystal. Substances were then given via the i.v. and/or i.p. catheter(s) and cardiovascular responses recorded.

2.6 Statistical analysis

Since the data could not all be considered to be normally distributed, non-parametric statistical tests were used. Within-group analysis of data was performed using Friedman's test (non-parametric version of ANOVA), with $P<0.05$ taken as significant. Between-groups analysis was performed using the Wilcoxon test for comparison of paired sets of data, the Mann-Whitney U test for comparison of unpaired datasets, and the Kruskall-Wallis test for comparisons between multiple unpaired datasets.

2.7 Drugs

Details of drugs and peptides used in these experiments are given in Table 2.1.

Abbreviation brand name	Full name	Purchased from	Preparation/administration	Chapter(s)
AM	Adrenomedullin (rat)	Bachem (St Helens, UK)	• dissolved in distilled water to provide stock; diluted further with saline • given as 0.1ml i.v. bolus	3,4,5,7
Ado	Adenosine	Sigma (Dorset, UK)	• dissolved in distilled water • given as an i.v. infusion at a rate of 0.15ml min^{-1} for 3 min	6,7
AII	Angiotensin II	Bachem (St Helens, UK)	• dissolved in sterile isotonic saline • given as a continuous infusion 0.4ml h^{-1}	4
AM_{22-52}	Adrenomedullin $_{22-52}$	Peptide Institute Inc. (Scientific Marketing Associates, Barnet, UK)/ Bachem (St Helens, UK)	• dissolved in sterile isotonic saline, infused over 6 min at 0.4ml h^{-1}	4
Amoxycare LA	Amoxicillin trihydrate	Animalcare Ltd (York, UK)	• dissolved in sterile isotonic saline	3-7
Antisedan	Atipamezole hydrochloride	Pfizer (Sandwich, Kent, U.K.)	• dissolved in sterile isotonic saline	3-7
AVP	Arginine vasopressin	Bachem (St Helens, UK)	• dissolved in sterile isotonic saline • given as a continuous infusion at 0.4ml h^{-1}	4
CCPA	2-Chloro-N^6-cyclopentyladenosine	Sigma (Dorset, UK)	• dissolved in a vehicle containing 5% propylene glycol, 2% Tween-80 in saline • given as an i.v. infusion at a rate of 0.15ml min^{-1}	6

DPCPX	8-Cyclopentyl-1,3-dipropylxanthine	Tocris Cookson (Avonmouth)	• dissolved in a vehicle containing 5% propylene glycol, 2% Tween-80 in saline • given as 0.1ml i.v. bolus	6
$IMD_{(1-53)}$	Intermedin	Bachem (St Helens, UK)	• dissolved in distilled water to provide stock; diluted further with saline • given as 0.1ml i.v. bolus	3,4,5,7
L-NAME	NG-nitro-L-arginine methyl ester	Sigma (Dorset, UK)	• dissolved in sterile isotonic saline • given as a continuous infusion at 0.4ml h^{-1}	4
Losartan	Losartan potassium	Sequoia Research Products (Oxford, UK)	• dissolved in sterile isotonic saline • given as 0.1ml i.v. bolus	4,6
LPS	Lipopolysaccharide (*E coli* serotype 0127 B8)	Sigma (Dorset, UK)	• dissolved in sterile isotonic saline • administered as described in text	5,6
Sublimaze	Fentanyl citrate	Janssen-Cilag (High Wycombe, U.K)	• dissolved in sterile isotonic saline	3-7
U37883A		Biomol (Plymouth Meeting, PA, USA)	• dissolved in sterile isotonic saline • infused at a rate of 2ml h^{-1} for 30 min	4
Vetergesic	Buprenorphine	Alstoe Animal Health (York, UK)	• dissolved in sterile isotonic saline	3-7

Table 2.1 *Drugs used throughout the course of these experiments.*

Chapter 3

Results (I)

Regional haemodynamic effects of adrenomedullin and intermedin

Summary

Regional haemodynamic responses to AM in conscious rats have been described but, to date, no direct comparison has been made between AM and IMD. The work in this Chapter aimed to assess the regional haemodynamic effects of IMD, and compare them to those of AM, in conscious rats.

Responses to IMD (1nmol kg^{-1} i.v.) and AM (1 and 3nmol kg^{-1} i.v.) were assessed and compared in Sprague-Dawley rats, implanted with miniature pulsed Doppler flow probes to measure renal, mesenteric and hindquarters blood flows.

Intermedin, like AM, caused hypotension, tachycardia, and widespread vasodilatation. Intermedin-induced hypotension and mesenteric vasodilatation were significantly more marked than those of an equimolar dose of AM. The renal vasodilator response to IMD (1 nmol kg^{-1}) was intermediate between (and not different from) those of 1 and 3 nmol kg^{-1} AM and the hindquarters vasodilator response to IMD was not different from either dose of AM.

The work in this Chapter shows that, in normal animals, IMD is a strong peripheral vasodilator, and has actions in the cardiovascular system similar to those of the related peptide, AM.

3.1 Introduction

Adrenomedullin (AM) is a 52 amino acid peptide isolated from human phaeocytochroma and is involved in diverse physiological functions including cardiovascular and renal homeostasis (Kitamura *et al.*, 1993; Sakata *et al.*, 1993; Charles *et al.*, 1997; Ishimitsu *et al.*, 2006), blood and lymphatic vessel development (Fritz-Six *et al.*, 2008; Ichikawa-Shindo *et al.*, 2008), maintenance of vascular integrity (Shindo *et al.*, 2001; Kato *et al.*, 2005) and protection against oxidative stress (Ishimitsu *et al.*, 2006). *In vitro,* AM causes relaxation of isolated vascular beds (Nuki *et al.*, 1993) and *in vivo,* AM causes dose-dependent hypotension arising from widespread, regional vasodilatations (Kitamura *et al.*, 1993; Sakata *et al.*, 1993; Lippton *et al.*, 1994; Gardiner *et al.*, 1995; Hirata *et al.*, 1995; Charles *et al.*, 1997; see section 1.2.3).

Analysis of the teleost fish genome recently identified several AM-like genes which were named AM-1-5, (Ogoshi *et al.*, 2004), and AM-2-like sequences were found in mouse, rat, and human genome databases (Takei *et al.*, 2004). At the same time, Roh *et al.*, (2004) identified genes in mammals and pufferfish which shared some sequence homology with AM; expression of this gene in the intermedia of the pituitary led its product to be named intermedin (IMD) (Roh *et al.*, 2004). The amino acid sequences of AM-2 and IMD were found to be almost identical, leading researchers to accept they are the same peptide, referred to herein as IMD.

Proteolytic cleavage of preproIMD yields several biologically active fragments, including IMD long, consisting of 47 amino acids, IMD short, consisting of 40 amino acids, and IMD_{1-53}, which has unknown physiological

significance (Roh *et al.*, 2004; Yang *et al.*, 2005). Whilst most of the studies in the literature have used IMD_{1-47}, the studies to be described here have used the fragment IMD_{1-53}, since this was the form commercially available at the start of this work. Whilst the physiological significance of these preproIMD fragments are not yet understood, there is some evidence to suggest that IMD_{1-53} is a physiologically relevant cleavage product of the precursor peptide (Yang *et al.*, 2005; Ren *et al.*, 2006).

Since IMD was found to share some sequence and structural homology with AM and CGRP, which both have potent actions within the cardiovascular system, it was hypothesised that IMD may have similar effects. Indeed, when given *in vivo,* IMD has been shown to cause hypotension in conscious rats (Roh *et al.*, 2004; Abdelrahman *et al.*, 2006; Fujisawa *et al.*, 2006; Chauhan *et al.*, 2007) anaesthetised mice (Takei *et al.*, 2004) and conscious sheep (Charles *et al.*, 2006). This was associated with an increase in cardiac output (Charles *et al.*, 2006; Fujisawa *et al.*, 2006a), regional vasodilatations (Fujisawa *et al.*, 2007) and a decrease in total peripheral resistance (Charles *et al.*, 2006). Intermedin-induced tachycardia is reported in some studies (Roh *et al.*, 2004; Abdelrahman *et al.*, 2006; Charles *et al.*, 2006; Fujisawa *et al.*, 2006) but not all (Pan *et al.*, 2005; Chauhan *et al.*, 2007). *In vitro,* IMD dilates porcine and rat coronary arteries (Kobayashi *et al.*, 2004; Pan *et al.*, 2005), preconstricted rat pulmonary vessels (Kandilci *et al.*, 2006) and causes relaxation of isolated mesenteric arteries (Chauhan *et al.*, 2007).

Whilst IMD was shown to increase renal blood flow *in vivo* (Fujisawa *et al.*, 2004; Fujisawa *et al.*, 2006), only one study has reported effects of the peptide

on regional haemodynamics (Fujisawa *et al.*, 2007). Although that study showed IMD to cause regionally selective increases in blood flow, the radio labelled microsphere method was used, allowing IMD-mediated changes in blood flow to only be shown at one time point. Furthermore, the study by Fujisawa *et al.* (2007) did not compare the regional effects of IMD with those of AM. The first aim of this work therefore, was to assess the regional haemodynamic responses to $IMD_{1\text{-}53}$ in conscious, unrestrained rats, and to compare them to an equimolar dose of AM.

3.2 Methods

Five groups of Sprague-Dawley rats were chronically instrumented with miniature, pulsed, Doppler flow probes to measure renal, mesenteric and hindquarters vascular conductances, and intravascular catheters (i.a, i.v, & i.p.) to allow administration of substances and continuous derivation of blood pressure and heart rate. These procedures are outlined in Chapter 2. To investigate the regional haemodynamic responses to AM and IMD, the following protocols were followed.

Regional haemodynamic responses to AM and IMD (1nmol kg^{-1}) were assessed in rats that received saline (0.5ml i.p. bolus, or 0.4ml h^{-1} i.v. infusion) to control for the effects of LPS (i.p. bolus, or i.v. infusion) as will be described in Chapter 4. Peptides were given 1.5 h, 6 h and 25 h after a saline bolus, or from the onset of a saline infusion, but for the purpose of delineating the regional haemodynamic effects of the peptides in normal rats, responses described here are those from administration at the 1.5 h time point (i.e. in peptide-naïve animals). The dose of peptide (1nmol kg^{-1}) was chosen on the basis of previous experiments which showed AM (1nmol kg^{-1}) to cause marked and reproducible cardiovascular changes in Long Evans rats (Gardiner *et al.*, 1995) and pilot studies which showed IMD at this dose to cause robust and reproducible cardiovascular changes.

Since IMD (1nmol kg^{-1}) was given to 1 group of rats (n=8) administered saline (0.5ml i.p.) to control for the effects of a LPS bolus, and in a separate group of

rats (n=11) administered saline (0.4ml h^{-1}) to control for the effects of a LPS infusion, these 2 groups were combined (n=19) to describe the responses to IMD (1nmol kg^{-1}) in normal animals. Here, AM (1nmol kg^{-1}) was given following a saline bolus but not in later experiments in which animals received a saline infusion. Therefore, 1 group of rats (n=9) was used to assess the effects of AM at a dose of 1nmol kg^{-1} in normal animals.

Adrenomedullin (1nmol kg^{-1}) was given to 1 group of rats (n=9) to control for the effects of LPS bolus (see above and Chapter 5). However, since it was found that AM at a dose of 1nmol kg^{-1} had only modest and poorly reproducible effects, a higher dose of AM (3nmol kg^{-1}) was given to a group of rats that received a saline bolus to control for the effects of a LPS bolus (n=10), and to a separate group of rats that received a saline infusion to control for the effects of a LPS infusion (n=10), as described above. These 2 groups were combined (n=20) to describe responses to AM (3nmol kg^{-1}) in normal animals.

3.3 Results

Baseline cardiovascular variables for each group of animals are given in Table 3.1. Prior to administration of AM (1nmol kg^{-1} and 3nmol kg^{-1}), and IMD (1nmol kg^{-1}) in 3 separate groups of animals (see above), resting cardiovascular variables were similar ($P>0.05$ Kruskal Wallis test).

	HR (beats min^{-1})	MAP (mmHg)	RVC (units)	MVC (units)	HVC (units)
AM (1nmol kg^{-1})	320±7	107±3	84±5	77±9	37±3
AM (3nmol kg^{-1})	343±6	108±2	82±6	70±5	47±3
IMD (1nmol kg^{-1})	339±6	110±2	83±7	68±3	44±3

Table 3.1 *Resting cardiovascular variables prior to administration of AM (1 and 3nmol kg^{-1}) (n=9 and n=20, respectively) and IMD (1nmol kg^{-1}) (n=19) in conscious rats. Units for vascular conductance (VC) are kHz/mmHg x 10^3.*

3.3.1 Regional haemodynamic responses to IMD

The time-course of IMD responses in conscious rats is shown in Figure 3.3.1. Administration of IMD (1nmol kg^{-1}) caused a fall in blood pressure which was significant between 0-20 min and 40-50 min ($P<0.05$, Friedman's test); this was accompanied by an increase in heart rate, which remained elevated 50 min after IMD administration ($P<0.05$, Friedman's test), and marked increases in renal, mesenteric and hindquarters vascular conductances, indicating IMD-mediated vasodilatations in those regions. The most marked regional vasodilator response to IMD occurred in the mesenteric bed which remained

dilated 50 min from IMD administration ($P<0.05$, Friedman's test). The renal vascular bed also showed a marked increase in conductance which was sustained for 40 min ($P<0.05$, Friedman's test). Whilst there was IMD-mediated vasodilatation in the hindquarters, this was less marked and less long-lasting than in the other vascular beds, with conductance being significantly increased for 20 min from peptide administration. The rank order of potency for IMD therefore was mesentery = renal>hindquarters. The effects of IMD on Doppler shift (i.e. flow) were assessed since vascular conductance is determined by both blood flow and blood pressure (see Chapter 2). IMD at a dose of 1nmol kg^{-1} caused clear increases in Doppler shift (i.e. flow) in all 3 vascular beds (Figure 3.3.2).

3.3.2 Regional haemodynamic responses to AM

The time course of responses to AM (1nmol kg^{-1}) is shown in Figure 3.3.1a. Administration of AM at this dose caused tachycardia and increases in renal, mesenteric and hindquarters vascular conductances, which were most marked 1-2 min from administration and sustained for 20 min ($P<0.05$ Friedman's test) (Figure 3.3.1a). Despite causing regional vasodilatations at this dose, AM failed to cause any fall in blood pressure, in fact, from 30-50 min there was a small, but significant, increase in blood pressure ($P<0.05$ Friedman's test). At a dose of 1nmol kg^{-1}, AM caused increases in Doppler shift (i.e., flow) in all 3 vascular beds (Figure 3.3.2).

Since the 1nmol kg^{-1} dose of AM failed to cause hypotension and produced responses which were poorly reproducible, the protocol was repeated using

AM at a dose of 3nmol kg^{-1}. The time course of AM at this dose responses is shown in Figure 3.3.1b, and it is apparent that AM caused a marked hypotension (which was sustained across the whole 60 min recording period ($P<0.05$ Friedman's test)), tachycardia and increases in renal, mesenteric and hindquarters vascular conductances, all of which were sustained for 50-60 min from AM administration (Figure 3.3.1b). Regional responses to AM occurred with the rank order renal>mesenteric>hindquarters. Increases in Doppler shift in the renal, mesenteric and hindquarters vascular beds in response to AM (3nmol kg^{-1}), indicate AM-mediated increases in blood flow in those regions (Figure 3.3.2b).

3.3.3 Comparison of the regional haemodynamic responses to AM and IMD

A comparison of the integrated (0-10min) responses to AM (1 and 3nmol kg^{-1}) and IMD (1nmol kg^{-1}) 1.5 h after saline treatment is shown in Figure 3.3.3. Intermedin-induced hypotension, and increases in renal and mesenteric vascular conductance were significantly ($P<0.05$, Kruskall Wallis test) more marked than those of an equimolar dose of AM, whereas IMD-induced tachycardia, hypotension and renal vasodilatations were significantly less marked than those caused by the higher dose of AM (3nmol kg^{-1}). However, increases in mesenteric vascular conductance were not different between the IMD (1nmol kg^{-1}) and AM (3nmol kg^{-1}), groups and there was no difference in the hindquarters vasodilator response between any of the groups. However, when compared over 60 min, the integrated (0-60 min) change in hindquarters vascular conductance was significantly greater following treatment with AM

(3nmol kg^{-1}) when compared to IMD (1nmol kg^{-1}). The peak increase in vascular conductance occurred at 20 min in the AM-treated group and vasodilatation in this region was sustained over the whole 60 min recording period, whereas the IMD-mediated increase in hindquarters vascular conductance had returned to baseline levels by 30 min after administration. (Figure 3.3.1b)

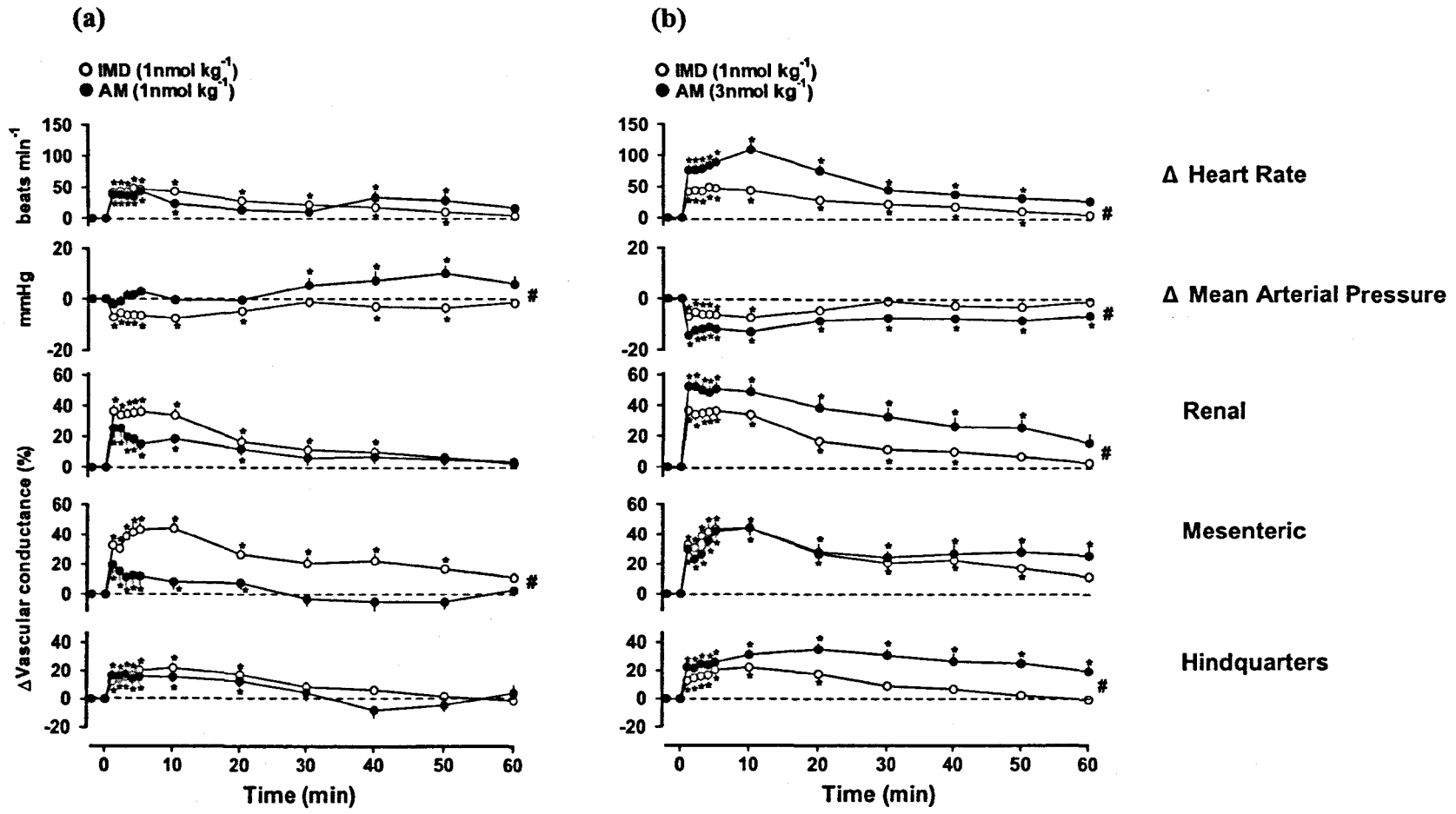


Figure 3.3.1 *Comparison of the regional haemodynamic responses to* **(a)** *AM (n=9) and IMD (n=19) (1nmol kg^{-1} i.v.) (n=9) and* **(b)** *AM (3nmol kg^{-1}) (n=20) and IMD (1nmol kg^{-1} i.v.) (n=19) in conscious rats.*

**P<0.05 versus original baseline (Friedman's test). #P<0.05 versus integrated (0-60 min) response to other peptide (Mann Whitney U test)*

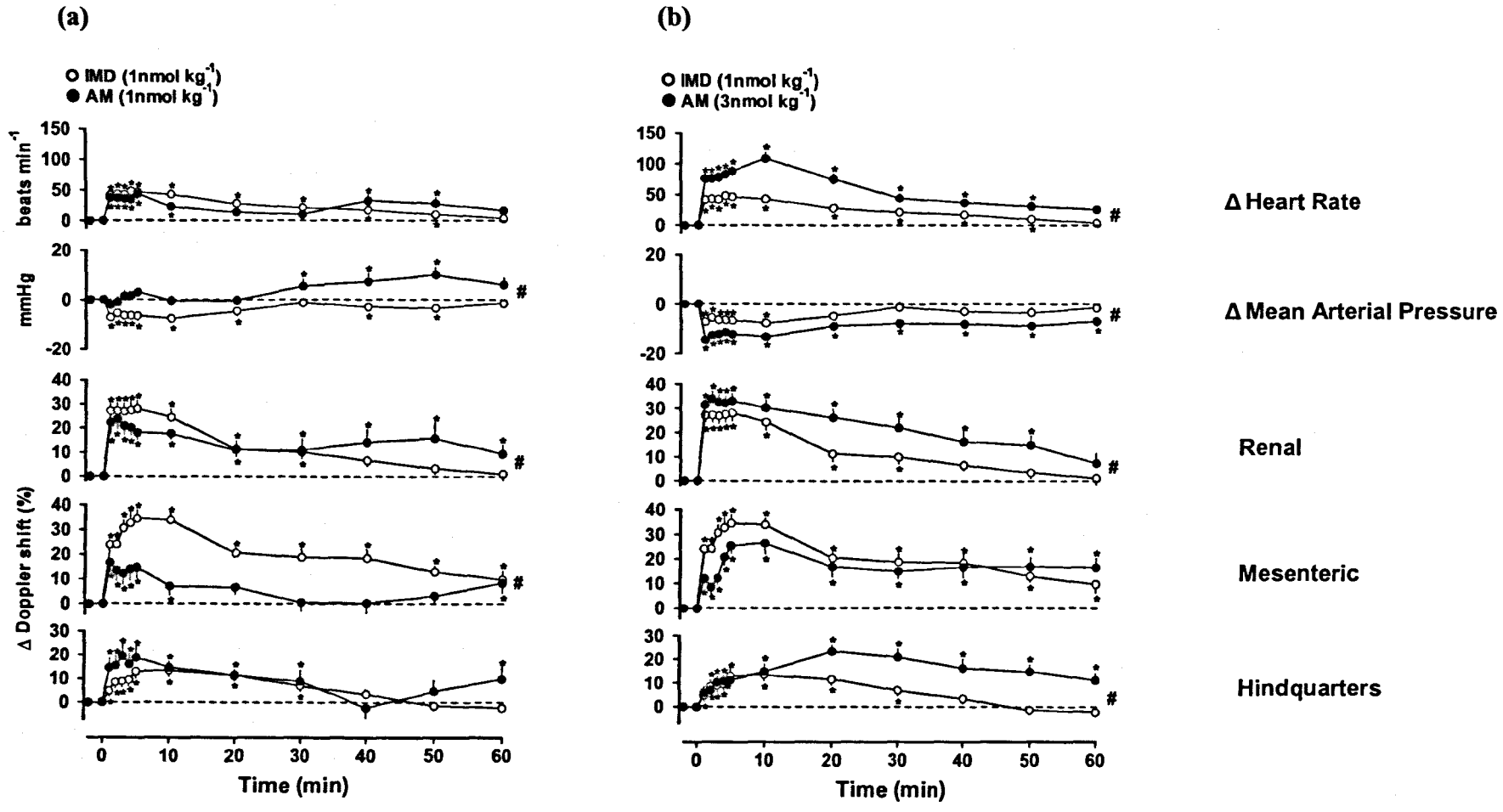


Figure 3.3.2 *Comparison of the regional haemodynamic responses to* **(a)** *AM (n=9) and IMD (n=19) (1nmol kg^{-1} i.v.) (n=9) and* **(b)** *AM (3nmol kg^{-1}) (n=20) and IMD (1nmol kg^{-1} i.v.) (n=19) in conscious rats, showing changes in Doppler shift as an index of flow.*

**P<0.05 versus original baseline (Friedman's test). #P<0.05 versus integrated (0-60 min) response to other peptide (Mann Whitney U test)*

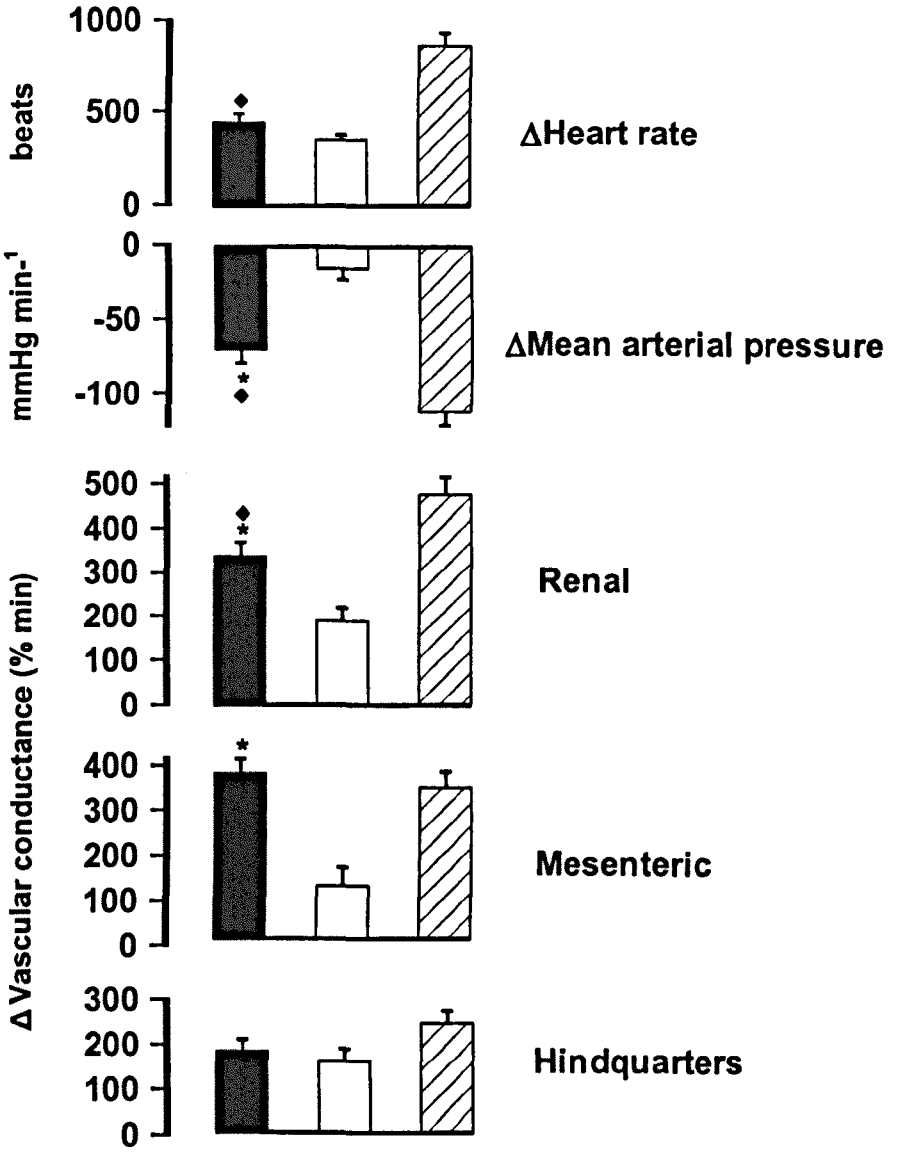


Figure 3.3.3 *Integrated (0-10 min) responses following IMD (1nmol kg^{-1}, solid bars) (n=19), AM (1nmol kg^{-1}, open bars) (n=9) and AM (3nmol kg^{-1}, hatched bars) (n=20) administration in saline-treated Sprague-Dawley rats.*

**P<0.05 vs. responses to AM (1nmol kg^{-1}); ♦P<0.05 vs. responses to AM (3nmol kg^{-1}) (Kruskal Wallis test)*

3.4 Discussion

Recent identification of novel AM-like genes in teleost fish and mammals has led researchers to postulate the existence of a distinct AM family of peptides (Takei *et al.*, 2004b; 2008). Adrenomedullin is a potent hypotensive peptide involved in cardiovascular regulation, and is upregulated in some pathophysiological conditions affecting the cardiovascular system, including sepsis and hypertension, and during pregnancy (Wang *et al.*, 2001; Ishimitsu *et al.*, 2006; Senna *et al.*, 2008); a similar role for the AM-related peptide, IMD, is not yet established. This study was designed to assess the regional vascular responses to IMD and compare them with those of AM in conscious rats.

Here, it is showed that, in normal animals, $IMD_{1\text{-}53}$, (1nmol kg^{-1}) caused vasodilatations in the renal, mesenteric and hindquarters vascular beds which were sufficient to cause a fall in blood pressure, and tachycardia. The most marked vasodilatations were seen in the mesenteric and renal vasculature, where the increases in vascular conductance were still pronounced at 50 and 40 min, respectively. The finding that IMD causes hypotension is consistent with previous reports, indeed, the first descriptions of the peptide showed it to have blood pressure lowering effects in conscious normotensive and hypertensive rats, and in anaesthetised mice (Roh *et al.*, 2004; Takei *et al.*, 2004). This finding has since been extended and confirmed in rats (Pan *et al.*, 2005; Abdelrahman *et al.*, 2006; Fujisawa *et al.*, 2006, 2007; Chauhan *et al.*, 2007), and sheep (Charles *et al.*, 2006). Evidence indicates that the

hypotensive effects of IMD are due to its action as a potent vasodilator. Studies have shown IMD-mediated renal vasodilatation *in vivo* (Fujisawa *et al.*, 2004), and IMD-mediated relaxation of isolated coronary (Kobayashi *et al.*, 2004; Pan *et al.*, 2005), carotid (Kobayashi *et al.*, 2004), mesenteric (Kobayashi *et al.*, 2004; Chauhan *et al.*, 2007) and pulmonary arteries (Kandilci *et al.*, 2006) *in vitro*. The present results are consistent with IMD being a hypotensive and vasodilator peptide, and extend findings of the above studies to the *in vivo* situation.

The findings reported here are consistent with those of Takei *et al.* (2004) who found IMD to have a greater hypotensive effect than an equimolar dose of AM in anaesthetised mice (Takei *et al.*, 2004). However, findings regarding the relative potencies of IMD and AM vary in the literature depending on the form of IMD used (IMD_{1-47}, IMD_{8-47} or IMD_{1-53}), the route and method of administration (i.v. or i.p., bolus or infusion), the method of blood pressure measurement (invasive or non-invasive) and the species used. For example, using the tail-cuff method, Roh *et al.* (2004) found that i.p. administration of AM caused a greater fall in systolic blood pressure than did an equimolar dose of IMD_{1-47}. In contrast, Ren *et al.* (2005) found that i.v. bolus administration of IMD_{1-47} caused a greater hypotensive response than equimolar IMD_{1-53} and AM in anaesthetised rats (Ren *et al.*, 2005), whilst in conscious rats, Taylor *et al* (2004) found i.v. administration of IMD reduced blood pressure by a comparable amount to CGRP, but to a lesser degree than did equimolar AM (Taylor *et al.*, 2004). Studies have found IMD reduced blood pressure and increased renal blood flow to a comparable extent to AM, with the effects of

the latter being considerably longer lasting (Fujisawa *et al.*, 2004). Whilst here the responses to IMD were significantly more marked than those to equimolar AM, some effects of the latter were longer lasting than those of the former, at the higher dose.

A previous study described the regional haemodynamic effects of IMD, but this was limited to changes at one time point, i.e., 10 min from onset of IMD infusion (Fujisawa *et al.*, 2007). Here, the full profile of regional haemodynamic changes following IMD administration is described and compared with those of AM in conscious rats. The finding that IMD caused renal vasodilatation is consistent with the findings of Fujisawa *et al.* (2007) who showed IMD increased blood flow to the kidney in both normotensive and hypertensive rats. They further showed that IMD significantly increased the % distribution of cardiac output to the kidney. However, they failed to show an increase in mesenteric blood flow following IMD-administration, instead showing a small but significant decrease in blood flow to this region in hypertensive rats. This is surprising, since there was a clear mesenteric hyperaemic vasodilator effect of IMD immediately after administration. As outlined above, it is possible that the method of peptide administration (infusion vs. bolus), or the strain of rat may have affected the outcome; furthermore, Fujisawa *et al.* (2007) did not state the form of the peptide used, and these have different potencies (see above).

In the present study, IMD caused a significant increase in heart rate in conscious rats. Although several studies have reported IMD-mediated

tachycardia (Abdelrahman & Pang, 2006; Charles *et al.*, 2006; Fujisawa *et al.*, 2006, 2007; Ren *et al.*, 2006) others have shown IMD had no effect on heart rate, irrespective of it causing a fall in blood pressure, or the presence/absence of anaesthesia (Pan *et al.*, 2005; Chauhan *et al.*, 2007). It is not yet clear whether IMD has a direct inotropic and/or chronotropic effect on the heart or whether the observed tachycardia is baroreflex-mediated, although *in vitro* data suggest that it has a direct effect on cardiac myocyte contractile function (Dong *et al.*, 2006). Studies have reported the presence of IMD and CL/RAMP mRNA and protein in the heart of mice, rat and humans (Takahashi *et al.*, 2006; Zhao *et al.*, 2006; Morimoto *et al.*, 2007; Bell *et al.*, 2008; Zeng *et al.*, 2009) supporting the view that IMD maybe directly involved in the control of cardiac function.

Cardiovascular responses to 1nmol kg^{-1} AM in conscious rats consisted of a tachycardia and increases in renal, mesenteric and hindquarters vascular conductances. Intermedin-induced vasodilatations were greater than those caused by equimolar AM in the renal and mesenteric vasculature, but not in the hindquarters, where the response to the 2 peptides was not different. Furthermore, IMD caused a significant fall in blood pressure which was not seen following treatment with an equimolar dose of AM. Since AM caused widespread vasodilatations, this failure to induce a fall in blood pressure indicates that cardiac output must have been increased. These results are consistent with studies which have shown AM had a modest effect on blood pressure but caused relatively large increases in heart rate and cardiac output (Parkes, 1995; Parkes *et al.*, 1997). Despite this lack of hypotension, AM at a

dose of 1nmol kg^{-1} led to a significant tachycardia suggesting it had a direct chronotropic effect on the heart.

The cardiovascular responses to a higher dose (3nmol kg^{-1}) of AM were assessed and given that, at this dose, there was a significant hypotension, and responses were more robust and more closely matched to those following administration of IMD (1nmol kg^{-1}); this higher dose was used in all subsequent experiments to be described in this thesis.

Since vascular conductance is determined by both blood flow and blood pressure, the effects of AM and IMD on flow alone (i.e. Doppler shift, refer to Chapter 2) were assessed to ascertain whether the observed increases in vascular conductance were really due to an increase in flow arising from peptide-mediated vasodilatation, or whether it was an autoregulatory response to the peptide-mediated hypotension (Figure 3.3.2). Since there were clear increases in flow (i.e. Doppler shift) in all vascular beds following treatment with AM (1 and 3nmol kg^{-1}) and IMD (1nmol kg^{-1}), it can be stated that both peptides caused active vasodilatations in those regions.

In conclusion, the present study shows that the newly discovered calcitonin family peptide, IMD, caused marked hypotension when given to conscious rats and regional vasodilatations which were equal to or more marked than those caused by an equimolar dose of AM. These findings are consistent with those of others who have reported IMD to be a potent hypotensive peptide, and suggest that, like AM and CGRP, IMD may be an important regulator of cardiovascular function.

Chapter 4

Results (II)

Mechanism(s) of adrenomedullin and intermedin-mediated vasodilatation

Summary

In Chapter 3, the regional haemodynamic responses to IMD were described and compared to those of AM. Both peptides were shown to elicit a comparable haemodynamic profile comprising widespread vasodilatation, decrease in blood pressure and a tachycardia. In this Chapter, possible underlying mechanisms involved in these actions of IMD and AM were investigated.

Measurements of blood pressure, heart rate and renal, mesenteric and hindquarters haemodynamics were made in conscious, chronically-instrumented rats.

The AM receptor antagonist, AM_{22-52}, was equally effective in attenuating the renal and mesenteric vasodilator effects of IMD (1 nmol kg^{-1}) and AM (3 nmol kg^{-1}), but inhibition of NO synthase was more effective at reducing the vasodilator effects of IMD than AM. Vascular K_{ATP} channel blockade with U-37883A did not inhibit the vasodilator effects of either peptide, although it did lead to augmentation of some of the vasodilator effects of AM. Using the AII receptor antagonist, losartan, this effect was found to involve inhibition of the renin-angiotensin system.

In vivo, the regional haemodynamic profile of IMD resembles that of AM, and some of the vasodilator effects of IMD are mediated by AM receptors and NO, but not by K_{ATP} channels.

4.1 Introduction

In the previous Chapter, it was shown that the *in vivo* integrated haemodynamic effects of IMD resemble those of AM in conscious rats. The aim of this Chapter was to determine whether the mechanisms involved in these vascular actions of IMD of AM were similar.

The calcitonin family of peptides (calcitonin, CGRP, amylin, AM and IMD) exert their effects on the vasculature via a unique receptor system comprising a G-protein coupled receptor, named calcitonin receptor-like receptor (CL), and one of three receptor activity-modifying proteins (RAMP1-3). The vascular effects of CGRP are mediated by activation of the $CGRP_1$ receptor, which consists of CL and RAMP1, and can be antagonised by the fragment $CGRP_{8-37}$. Adrenomedullin functions through two receptors, AM_1 and AM_2, which consist of CL complexed with RAMP2 and RAMP3, respectively. Adrenomedullin action through these receptors can be antagonised by the fragment AM_{22-52} (for review see Poyner *et al.*, 2002). Agonist binding to these receptors activates adenylate cyclase leading to accumulation of cAMP, relaxation of vascular smooth muscle and endothelial-independent vasodilatation. Studies have also shown that AM can initiate vasodilatation in an endothelial-dependent manner via the production of NO and by activation of K_{ATP} channels (Gardiner *et al.*, 1995; Sabates *et al.*, 1997; Goto *et al.*, 2000; Terata *et al.*, 2000). Whilst the mechanism(s) of AM and CGRP-mediated vasodilatation are well documented (for review see Poyner *et al.*, 2002), only a limited number of studies have examined those of IMD. Since IMD has some structural and sequence homology with both AM and CGRP, some

researchers' have suggested that IMD and AM may signal through a common pathway(s).

Currently, evidence suggests that IMD is a non-selective agonist of the CL/RAMP system, functioning through CL complexed to any of the three RAMPS (Roh *et al.*, 2004). Furthermore, some findings indicate the existence evidence of a unique and unidentified IMD receptor that is distinct from this system (Owji *et al.*, 2008; Kandilci *et al.*, 2008). The first evidence that IMD was able to interact non-discriminately with CL/RAMP complexes came from studies by Roh *et al.* (2004) utilising transfected 293T cells, which showed IMD could increase cAMP levels through each of the three receptor complexes. Furthermore, co-application of $CGRP_{8-37}$ and AM_{22-52} to isolated rat pulmonary arteries caused an additive attenuation of IMD-mediated vasorelaxation. The remaining IMD-mediated vasodilatation which was insensitive to both $CGRP_{8-37}$ and AM_{22-52} matched that seen upon addition of the IMD fragment, IMD_{17-47}, which is believed to act as an antagonist at specific 'IMD-receptors' (Roh *et al.*, 2004; Kandilci *et al.*, 2008). Along with evidence from *in vitro* and *in vivo* studies, this suggests a role for both $CGRP_{8-37}$-sensitive (Kobayashi *et al.*, 2004; Roh *et al.*, 2004; Taylor *et al.*, 2004; Ren *et al.*, 2006; Kandilci *et al.*, 2006, 2008) and/or AM_{22-52}-sensitive receptors (Ren *et al.*, 2006; Kandilci *et al.*, 2008) in regional IMD-mediated vasodilatations, as well as a distinct IMD_{17-47}-sensitive receptor yet to be identified. A study using isolated perfused rat lung also found evidence of a role for NO in IMD-mediated relaxation under pre-constricted conditions, but no role for K_{ATP} channels (Kandilci *et al.*, 2006), both of which are believed to be involved in AM-mediated vasodilatation (Sabates *et al.*, 1997).

The aim of the following experiments therefore, was to examine the mechanisms through which AM and IMD exert their regional vascular effects in conscious rats. To assess the contribution of AM receptors, the AM receptor antagonist, AM_{22-52}, and the $CGRP_1$ receptor antagonist $CGRP_{8-37}$, were used. The non-selective NOS inhibitor, L-NAME, and the K_{ATP} channel blocker, U37883A, were used to investigate the role of NO and K_{ATP} channels, respectively. Due to some unexpected responses to AM following treatment with U37883A, which could possibly be explained by the effects of these two substances on renin secretion, the AT_1 receptor antagonist, losartan, was used to assess the role of renin-angiotensin system in AM and IMD-mediated vasodilatation. Experiments were performed with IMD at a dose of 1nmol kg^{-1}, and AM at a dose of 3nmol kg^{-1} since AM (1nmol kg^{-1}) was ineffective at causing a fall in blood pressure (Chapter 3).

4.2 Methods

Eight groups of rats (n=5-10) were used in the following experiments which were completed according to the methods described in Chapter 2. Briefly, rats were anaesthetised, and miniature Doppler flow probes were placed around the left renal artery, the superior mesenteric artery and the distal aorta allowing changes in renal, mesenteric and hindquarters vascular conductances to be calculated in response to peptide challenges. Rats were later implanted with i.v. and i.a. catheters to allow substance administration and continuous derivation of blood pressure and heart rate in unrestrained animals. Experiments were performed on Days 1-4, starting 24 h from catheter implantation. To investigate the mechanism(s) responsible for mediating the vascular effects of AM and IMD the following protocols were followed; all substances were given i.v.

4.2.1 Experiment 1: Effects of repeated dosing of AM and IMD

Since some of the protocols required repeat dosing with AM and IMD (see below), the first experiment was designed to assess whether any tachyphylaxis or cross-tachyphylaxis occurred following repeated dosing of AM and IMD. A group of rats (n=8) were randomised to receive AM (3nmol kg^{-1}) on Days 1 and 3, and IMD (1nmol kg^{-1}) on Days 2 and 4, or IMD on Days 1 and 3, and AM on Days 2 and 4, with animals only receiving 1 treatment each day. This protocol was used as it is consistent with the way in which AM and IMD administration was randomised in the experiments described in this chapter. This protocol is shown in diagrammatic form below (Figure 4.2.1).

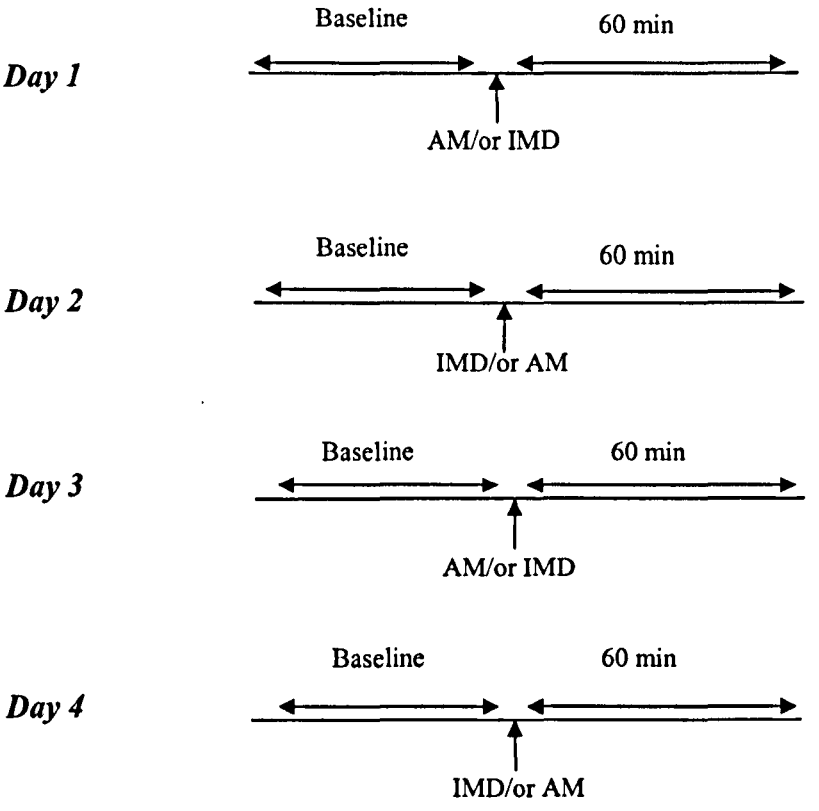


Figure 4.2.1 *Following a period of approximately 60 min for measurements of resting cardiovascular variables to be taken, animals received either AM on Day 1 and 3, and IMD on Day 2 and 4; or IMD on Day 1 and 3, and AM on Day 2 and, this was followed by a recording period of 60 min*

4.2.2 Experiment 2: Involvement of $CGRP_{8-37}$-sensitive receptors

The involvement of $CGRP_1$ receptors in the regional vascular responses to AM and IMD was assessed using the CGRP peptide fragment, $CGRP_{8-37}$.

Animals (n=6) received a 6 min infusion of $CGRP_{8-37}$ (500 nmol kg^{-1} h^{-1}) followed by IMD (1nmol kg^{-1}) given as a 0.1ml bolus between 5 and 10 min

later. A separate group of animals (n=5) was used in control experiments, and received a 6 min infusion of saline (0.4ml h^{-1}) followed by IMD (1nmol kg^{-1}).

Responses to AM in the presence of $CGRP_{8-37}$ were assessed in a group of animals (n=5) who received an infusion of $CGRP_{8-37}$ (500nmol kg^{-1} h^{-1} given over 6 mins), followed by bolus administration of AM (1nmol kg^{-1}) between 5 and 10 min later. A separate group of animals (n=5) were challenged with AM (1nmol kg^{-1}) 5-10 min after infusion of saline (0.4 ml h^{-1} for 6 mins)

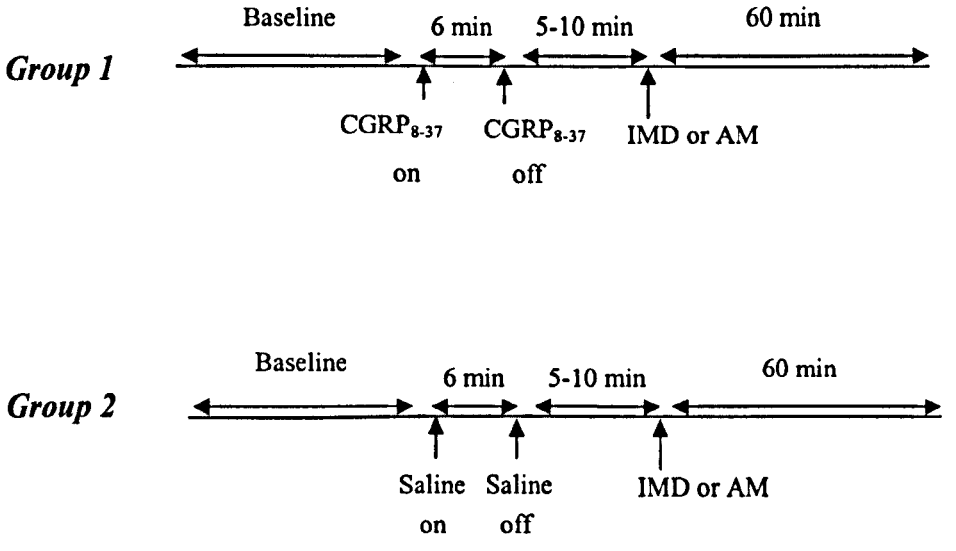


Figure 4.2.2 *Animals were pre-treated with $CGRP_{8-37}$ for 5-10 min before administration of IMD or AM. A separate group were pre-treated with saline before administration of IMD or AM.*

4.2.3 Experiment 3: Involvement of AM_{22-52}-sensitive receptors

The involvement of AM receptors in the regional vascular responses to AM and IMD was assessed using the AM peptide fragment, AM_{22-52}, which has greater selectivity for AM than CGRP receptors (for review see Poyner *et al.*, 2002).

A group of animals (n=6) received a 6 min infusion of AM_{22-52} (500 nmol kg^{-1} h^{-1}) followed by IMD (1nmol kg^{-1}) given as a 0.1ml bolus between 5 and 10 min later. A separate group of animals (n=5) was used in control experiments, and received a 6 min infusion of saline (0.4ml h^{-1}) followed by IMD (1nmol kg^{-1}). The dose of AM_{22-52} has previously been shown to attenuate the regional vasodilatations to AM (1nmol kg^{-1}) *in vivo* (Gardiner *et al.*, 1999); this protocol is illustrated below (Figure 4.2.3).

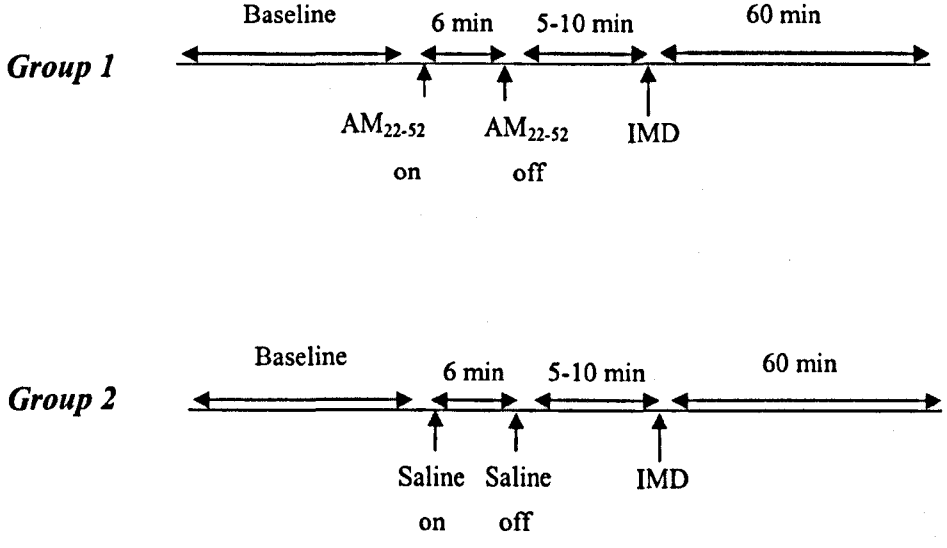


Figure 4.2.3 *A group of animals were pre-treated with AM_{22-52} 5-10 min before administration of IMD. A separate group were pre-treated with saline before administration of IMD.*

Effects of AM_{22-52} on responses to AM were assessed in a single group of animals (n=8) randomised to receive either a 6 min infusion of saline (0.4 ml h^{-1}) or AM_{22-52}, followed by bolus administration of AM between 5 and 10 min later. This was a paired experiment, conducted on a single day with peptide challenges occurring 3-4 h apart. This protocol is illustrated in Figure 4.2.3a.

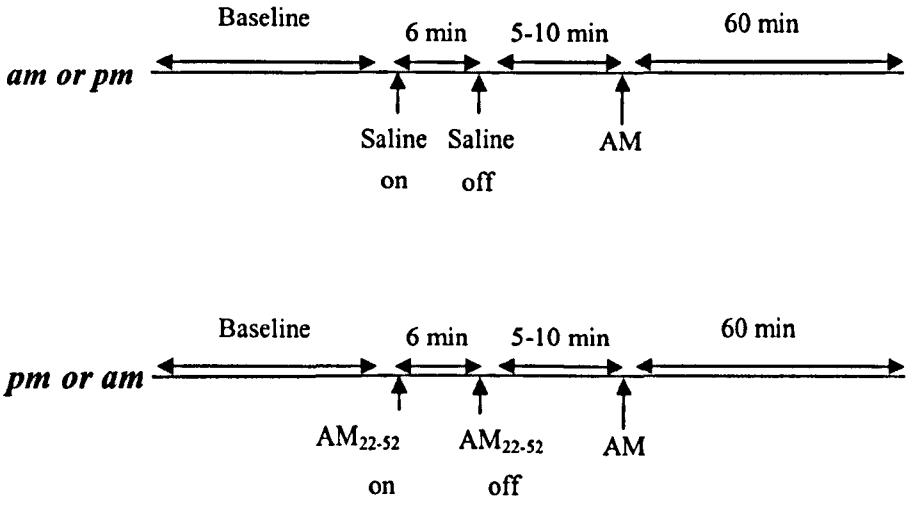


Figure 4.2.3a *Animals were pre-treated with either saline in the morning and AM_{22-52} in the afternoon, or AM_{22-52} in the morning and saline in the afternoon, followed 5-10 min later by administration of AM.*

4.2.4 Experiment 4: Involvement of Nitric Oxide

Involvement of NO in the vascular responses to AM and IMD was assessed using the non-selective NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 3mg kg^{-1} h^{-1}). This dose and method of administration has been used routinely in the Nottingham haemodynamics laboratory over several years, and has been shown to inhibit NO-mediated vasodilatation (Gardiner *et al.*, 1991a/b/c, 1995, 2006; Wakefield *et al.*, 2003). Since L-NAME itself leads to vasoconstriction and hypertension through inhibition of NOS, a suitable control was required which changes baseline haemodynamics in a similar manner to that of L-NAME. Control animals were therefore treated with a co-infusion of angiotensin II (AII) (200 ng kg^{-1} h^{-1}) and arginine vasopressin (AVP) (20 ng kg^{-1} h^{-1}). The doses were chosen based on previous work by the group in which AII/AVP co-infusion had vasopressor and regional haemodynamic effects consistent with those seen during L-NAME infusion (Gardiner *et al.*, 2006).

Animals received a co-infusion of AII/AVP and a challenge of AM (3 nmol kg^{-1}) or IMD (1 nmol kg^{-1}) on Day 1 and the other peptide on Day 2, 90 min after the onset of the AII/AVP infusion. This protocol was repeated on Days 3 and 4 with animals receiving an infusion of L-NAME instead of AII/AVP. In this way, each animal received each of the 4 treatments and acted as its own control. L-NAME was always given on Days 3 or 4 because of its very long duration of action. This protocol is illustrated in Figure 4.2.4.

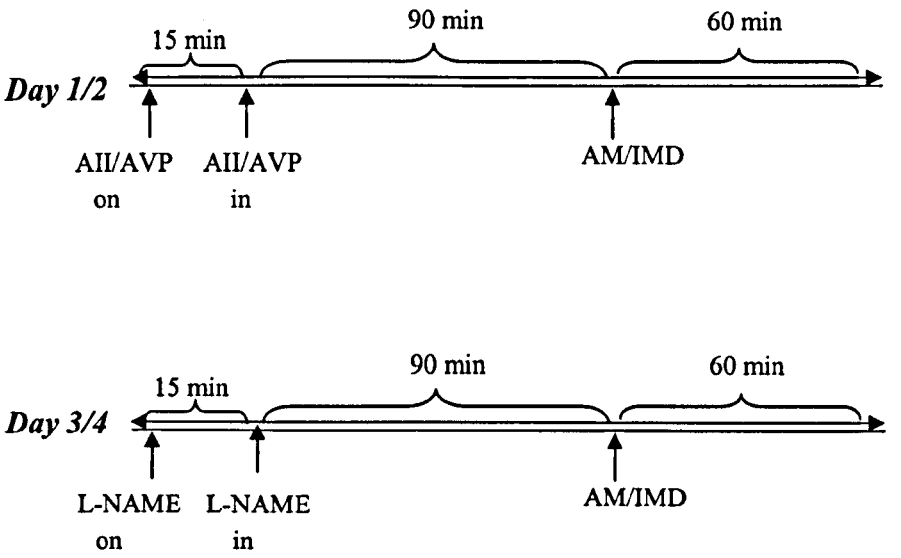


Figure 4.2.4 *Animals were pre-treated with AII/AVP for 90 min before receiving either AM or IMD on Day 1, and the other peptide on Day 2. On Days 3 and 4 this protocol was repeated in the presence of L-NAME. The 15 min period at the start of the AII/AVP and L-NAME infusion represents the time taken for the solution to infuse through the dead space of the catheter (i.e. 0.1 ml)*

4.2.5 Experiment 5: Involvement of K_{ATP} channels

The role of K_{ATP} channels in AM and IMD-mediated vasodilatations was assessed using the K_{ATP} channel blocker, U37883A (Teramoto, 1996). This antagonist was chosen as evidence suggests it is relatively selective for vascular K_{ATP} channels. The dose chosen was based on published studies showing attenuation of the effects of the K_{ATP} channel opener levcromakalim (LCK) at the given dose (Dabisch *et al.*, 2004).

A group of animals (n=9) was randomised so each animal received saline and U37883A (5 mg kg^{-1}) given as a 1ml infusion over 30 min, followed 20 min later by administration of AM (3 nmol kg^{-1}) or IMD (1 nmol kg^{-1}). Over the 4 experimental days, each animal received all treatments and was able to act as its own control. Effectiveness of U37883A at blocking K_{ATP} channels was assessed by the use of LCK (10 μg kg^{-1} min^{-1} for 3 min). Figure 4.3.5 illustrates the protocol followed.

Whilst U37883A was unable to completely antagonise the effects of LCK, it attenuated responses when given 2h after the onset of U37883A infusion. Thus, U37883A reduced the LCK-mediated fall in blood pressure by 35% when compared to responses to LCK alone (0-3min integrated absolute change). Also, LCK-mediated renal, mesenteric and hindquarters vasodilatations were reduced by 23%, 18% and 65%, respectively (0-3min integrated absolute changes) in the presence of U37883A. This is consistent with studies showing that U37883A attenuated, but did not abolish the responses to LCK (Dabisch *et al.*, 2004).

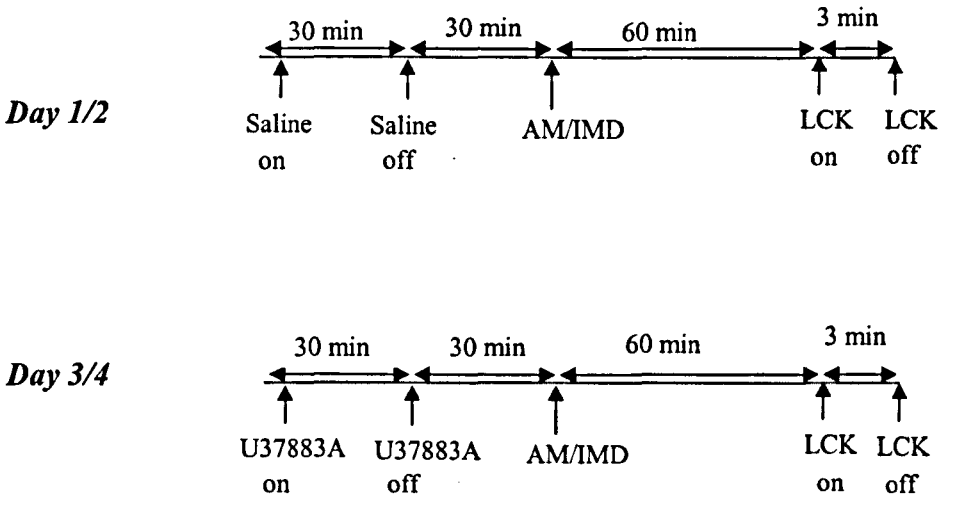


Figure 4.2.5 Animals were pre-treated with saline for 30 min followed 30 min later by AM or IMD on Day 1 or 2. The protocol was repeated on Day 3 and 4 in the presence of U37883A pre-treatment. At least 60 min after peptide administration animals received a 3 min infusion of LCK.

4.2.6 Experiment 6: Interactions between NO and K_{ATP} channels

To further examine the contribution of K_{ATP} channels to AM and IMD-mediated vasodilatations in the absence of NO, a separate group of animals (n=9) were co-administered L-NAME (3mg kg h^{-1} pre-treated for 90 min) and saline (Days 1 and 2), or U37883A (Days 3 and 4) (30 min infusion) beginning 30 min after the start of the L-NAME infusion. This was followed by AM or IMD challenges 30 min after the end of the U37883A or saline infusion; the protocol is shown in Figure 4.2.6.

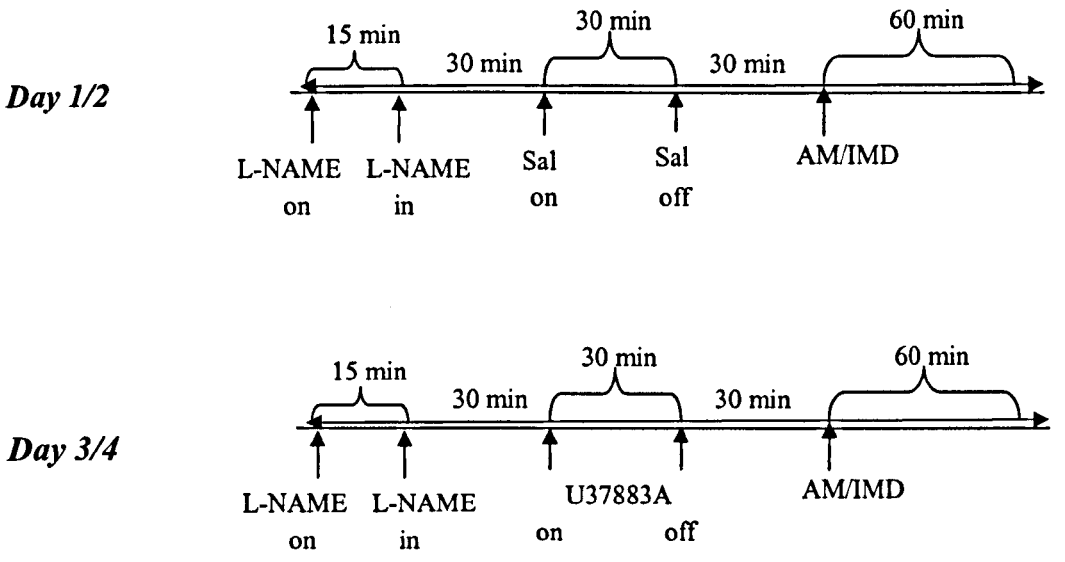


Figure 4.2.6 *Animals were pre-treated with L-NAME in combination with saline (Days 1 and 2) or U37883A (Days 3 and 4) prior to administration of AM on Day 1 and 3, and IMD on Day 2 and 4; or IMD on Day 1 and 3 and AM on Day 2 and 4.*

4.2.7 Experiment 7: Involvement of the renin-angiotensin system

Since some of the results obtained in earlier experiments (see results) could be explained by differential activation of the renin-angiotensin system (see Results), the effects of the angiotensin II (AT_1) receptor antagonist, losartan, were assessed on the regional haemodynamic responses to AM and IMD.

A group of animals (n=9) was randomised to receive a 0.1ml saline bolus followed 60 min later by AM (3 nmol kg^{-1}) or IMD (1 nmol kg^{-1}) on either Day 1 or Day 2. On Days 3 and 4, animals were given a 0.1 ml bolus of losartan (10 mg kg^{-1}) followed 60 min later by either AM or IMD (as above).

Losartan was always given on the final experimental days because of its long duration of action. This protocol is illustrated in Figure 4.2.7.

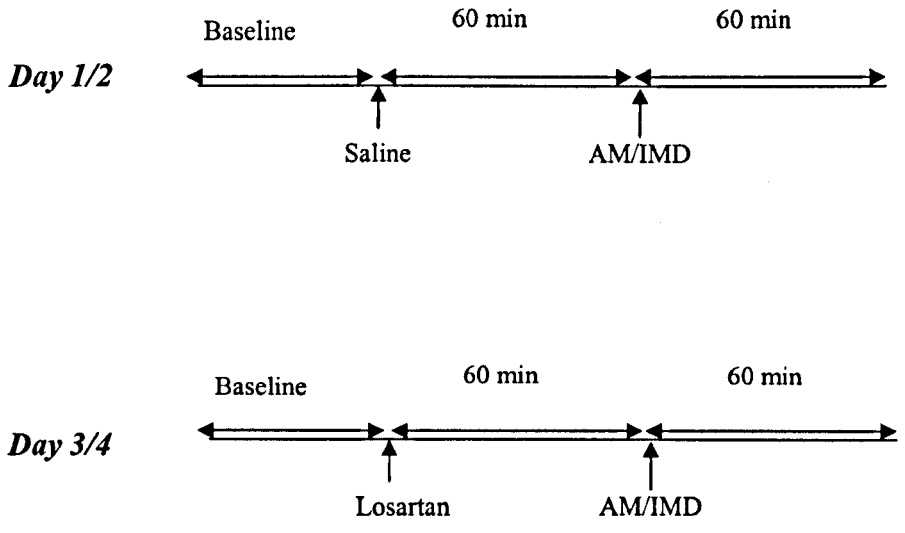


Figure 4.2.7 *Animals received a bolus of saline on Days 1 and 2 followed by administration of either AM or IMD. On Days 3 and 4, animals were pre-treated with losartan followed by administration of either AM or IMD.*

4.3 Results

Resting cardiovascular variables for all groups of animals used in these experiments are shown in Table 4.1.

4.3.1 Experiment 1: Effects of repeated dosing of AM and IMD

There were no differences in baseline cardiovascular variables in animals prior to administration of AM on Days 1 or 2, and Days 3 or 4; this was also true for animals that were to receive IMD (Table 4.1).

Following the first treatment with AM (3 nmol kg^{-1}), there was a significant tachycardia, hypotension and renal, mesenteric and hindquarters vasodilatations. These responses were unchanged following the second treatment with AM (3 nmol kg^{-1}) ($P>0.05$ for integrated 0-30 min changes for each variable) (Figure 4.3.1a).

The first treatment with IMD (1 nmol kg^{-1}) caused a significant tachycardia, hypotension and renal, mesenteric and hindquarters vasodilatations; these responses were not different from those seen following the second treatment with IMD (1 nmol kg^{-1}) ($P>0.05$ for integrated 0-30 min changes for each variable) (Figure 4.3.1b). Thus, for both AM and IMD, the haemodynamic changes were reproducible following repeated administration.

4.3.2 Experiment 2: Involvement of $CGRP_{8-37}$-sensitive receptors

Following pre-treatment with either $CGRP_{8-37}$ or saline, there were no differences in resting cardiovascular variables between groups. In the control group, AM (1nmol kg^{-1}) caused a tachycardia but no other significant changes

in haemodynamics (see Chapter 3). Following pre-treatment with the $CGRP_1$ antagonist $CGRP_{8-37}$, AM-mediated tachycardia was abolished (Figure 4.3.2a). In animals who had received a pre-treatment of saline, IMD (1nmol kg^{-1}) caused tachycardia, marked increases in renal, mesenteric and hindquarters vascular conductances, but no change in blood pressure. Administration of $CGRP_{8-37}$ had no effect on the haemodynamic responses to IMD (Figure 4.3.2b). These data suggest that IMD does not activate $CGRP_1$ receptors in conscious rats.

4.3.3 Experiment 3: Involvement of AM_{22-52}-sensitive receptors

There were no differences in resting cardiovascular variables in animals prior to administration of AM (3n mol kg^{-1}) following administration of saline or AM_{22-52}. In animals that were to receive IMD (1 nmol kg^{-1}) following AM_{22-52}, there was a significantly higher renal vascular conductance at baseline compared to the separate group of animals that were to receive IMD (1nmol kg^{-1}) following saline (Table 4.1).

Under control conditions, AM (3nmol kg^{-1}) caused a marked tachycardia, hypotension and increases in renal, mesenteric and hindquarters vascular conductances (Figure 4.3.3a). Following treatment with AM_{22-52}, AM-mediated tachycardia, hypotension and hindquarters vasodilatation were not different from control, but there was a significant attenuation of the AM-mediated increase in renal vascular conductance ($P<0.05$ for integrated 0-30min change). Furthermore, the marked AM-mediated increase in mesenteric vascular conductance seen in control conditions was abolished (Figure 4.3.3a),

indicating a prominent role for AM_{22-52}-sensitive receptors in AM-mediated vasodilatation in this region.

In control animals, IMD (1 nmol kg^{-1}) caused a tachycardia, small but significant hypotension and increases in renal, mesenteric and hindquarters vascular conductances. Following treatment with AM_{22-52}, IMD-mediated tachycardia, hypotension and hindquarters vasodilatation were unaffected ($P>0.05$ for 0-30min responses), but IMD-mediated renal and mesenteric vasodilatations were significantly attenuated ($P<0.05$ for integrated 0-30 min responses) (Figure 4.3.3b).

4.3.4 Experiment 4: Involvement of Nitric Oxide

Prior to administration of AM or IMD, resting cardiovascular variables were matched in the control (AII/AVP infused) condition and in the presence of L-NAME (Table 4.1).

Administration of AM during AII/AVP co-infusion caused a marked tachycardia and fall in blood pressure accompanied by large increases in renal, mesenteric and hindquarters vascular conductances (Figure 4.3.4a). These responses to AM were unaffected by L-NAME ($P>0.05$ for the integrated 0-30 min changes for each variable).

In animals infused with AII/AVP, IMD caused renal, mesenteric and hindquarters vasodilatations, along with a fall in blood pressure and tachycardia (Figure 4.3.4b). In the presence of L-NAME, there was no difference in the IMD-induced tachycardia or hypotension and, although there was some attenuation of the IMD-mediated mesenteric vasodilatation, this failed to reach statistical significance. However, the renal vasodilator response

to IMD was significantly attenuated in animals receiving L-NAME ($P<0.05$ for integrated 0-30min changes) suggesting a role for NO in IMD-mediated vasodilatation in this region. Whilst IMD-mediated hindquarters vasodilatation was abolished (Friedman's test), the integrated (0-30 min) changes were not different between the L-NAME and AII/AVP infused conditions (Figure 4.3.4b).

4.3.5 Experiment 5: Involvement of K_{ATP} channels

Resting cardiovascular variables are shown in Table 4.1. Prior to administration of AM, there was a significant bradycardia, hypertension and hindquarters vasoconstriction in U37883A-treated animals compared to the saline-treated condition. This pattern was also seen at baseline in animals that were to be given IMD except on this occasion mesenteric, but not hindquarters vascular conductance was significantly less in the presence of U37883A (Table 4.1).

The control response to AM (3nmol kg^{-1}) was tachycardia, hypotension and marked increases in renal, mesenteric and hindquarters vascular conductances, as described above. Following treatment with U37883A, there was no change in AM-mediated tachycardia, hypotension and hindquarters vasodilatation. However, contrary to expectation, pre-treatment with U37883A led to significant augmentation of AM-mediated renal and mesenteric vasodilatations when compared to controls ($P<0.05$ for integrated 0-30 min changes) (Figure 4.3.5a).

The control response to IMD was tachycardia, hypotension and renal, mesenteric and hindquarters vasodilatations, as described above, and these

responses were unaffected by pre-treatment with U37883A (P>0.05 for the integrated 0-30min changes) (Figure 4.3.5b).

4.3.6 Experiment 6: Interactions between NO and K_{ATP} channels

A further group of animals (n=9) was pre-treated with L-NAME plus either saline or U37783A prior to administration of either AM or IMD. Resting cardiovascular variables in those animals before peptide administration are shown in Table 4.1. Additional treatment with U37883A in the presence of L-NAME caused a further increase in blood pressure with hindquarters vasoconstriction ($P<0.05$), bradycardia and mesenteric vasoconstriction ($P<0.05$ in rats to be given IMD only).

In rats treated with L-NAME together with either saline or U37883A, administration of AM caused tachycardia, hypotension, and marked increases in renal, mesenteric and hindquarters vascular conductances. The integrated (0-30 min) responses were not different in the absence or presence of U37883A, with the exception of the tachycardia which was significantly ($P<0.05$) less in the presence of U37883A (Figure 4.3.6a).

In rats treated with L-NAME and either saline or U37883A, IMD administration caused tachycardia, hypotension and increases in renal, mesenteric and hindquarters vascular conductances. In the presence of U37883A, IMD-mediated hypotension along with renal and mesenteric vasodilatations were significantly ($P<0.05$) augmented when compared to responses in the L-NAME and saline condition (Figure 4.3.6b).

4.3.7 Experiment 7: Involvement of the renin-angiotensin system

Resting cardiovascular variables for each group are given in Table 4.1. Prior to administration of AM there were no differences at baseline in the control group and the losartan-treated group. However, prior to administration of IMD, blood pressure was significantly lower, and hindquarters vascular conductance was significantly higher, in the losartan-treated condition (Table 4.1).

When given to the saline-treated group, AM caused tachycardia, hypotension and marked increases in renal, mesenteric and hindquarters vascular conductances, as described above. Following pre-treatment with losartan, AM-mediated hypotension was significantly augmented as were AM-mediated renal and mesenteric vasodilatations ($P<0.05$ for integrated 0-30 min changes). The augmentation of AM-mediated mesenteric vasodilatation was particularly marked in the presence of losartan with a maximum increase of ~70% from baseline between 5 and 10 minutes from administration (Figure 4.3.7a).

In the absence of losartan, IMD caused tachycardia and marked increases in renal, mesenteric and hindquarters vascular conductances, as described above, although, in this experiment, the IMD-induced fall in blood pressure was not significant (Friedman's test). Following pre-treatment with losartan, there was no change in the regional haemodynamic responses to IMD (Figure 4.3.7b).

	Expt. 1		Expt. 2		Expt. 3		Expt. 4		Expt. 5		Expt. 6		Expt. 7	
	Dose 1	*Dose 2*	*Saline*	*CGRP* $_{8\text{-}37}$	*Saline*	*AM* $_{22\text{-}52}$	*AII/AVP*	*L-NAME*	*Saline*	*U37883A*	*L-NAME*	*L-NAME + U37883A*	*Saline*	*Losartan*
AM														
HR	374±18	377±21	321± 5	316± 17	382±14	391±8	307±13	282±9	359±14	294±8*	285±10	267±11	355±13	369±17
MAP	110±4	117±4	111±4	110±3	111±4	107±5	135±5	146±8	115±3	127±2*	141±4	159±5*	111±2	104±4
RVC	93±7	93±7	66±3	78±9	94±8	107±5	56±7	53±12	81±9	73±7	40±4	40±4	93±12	81±14
MVC	71±8	65±7	69±11	64±11	69±11	95±14	31±3	28±5	68±4	60±6	34±4	26±3	77±7	83±7
HVC	49±3	46±3	43±8	37±4	47±4	46±4	30±3	30±2	50±4	35±3*	23±2	17±2*	42±5	47±5
IMD														
HR	349±13	354±15	329±8	334±13	347±10	338±9	296±12	287±9	353±12	315±10*	298±10	254±9*	336±9	355±7
MAP	111±3	108±4	107±4	113±4	113±7	114±3	133±5	138±6	113±3	129±3*	138±4	159±2*	111±2	98±4*
RVC	99±9	89±8	69±4	80±8	62±8	92±9†	57±9	57±9	86±9	76±8	41±4	39±4	90±10	105±12
MVC	61±5	64±6	75±12	69±9	75±11	82±9	36±5	31±5	70±4	57±4*	40±3	28±4*	74±4	89±9
HVC	49±5	48±4	42±8	37±5	43±8	36±3	35±5	34±2	45±4	37±3	24±2	16±2*	37±4	50±5*

Table 4.1. *Resting cardiovascular variables prior to administration of AM or IMD in Experiments 1-6.*

Units for vascular conductance (VC) are kHz mmHg^{-1} x 10^{3}. n=5-10 group^{-1}.

**P<0.05 vs. control (Wilcoxon Test; paired experiment). †P<0.05 vs. control (Mann Whitney U Test; unpaired experiment).*

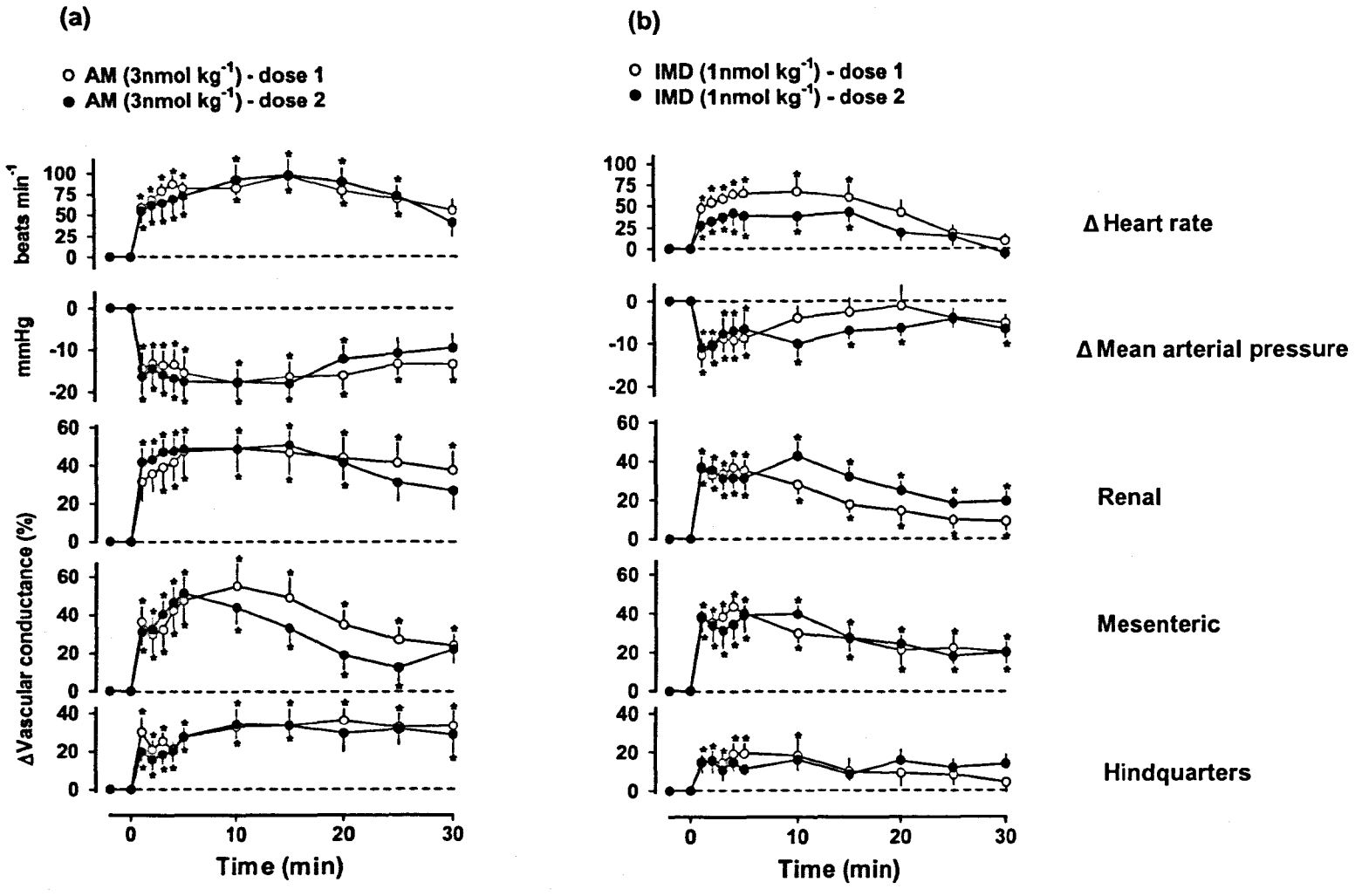


Figure 4.3.1 *Effects of repeated dosing of* **(a)** *AM (3 nmol kg^{-1} i.v.) (n=8) and* **(b)** *IMD (1 nmol kg^{-1} i.v.) (n=8) on regional haemodynamics in conscious rats.*

**P<0.05 versus original baseline (Friedman's test).*

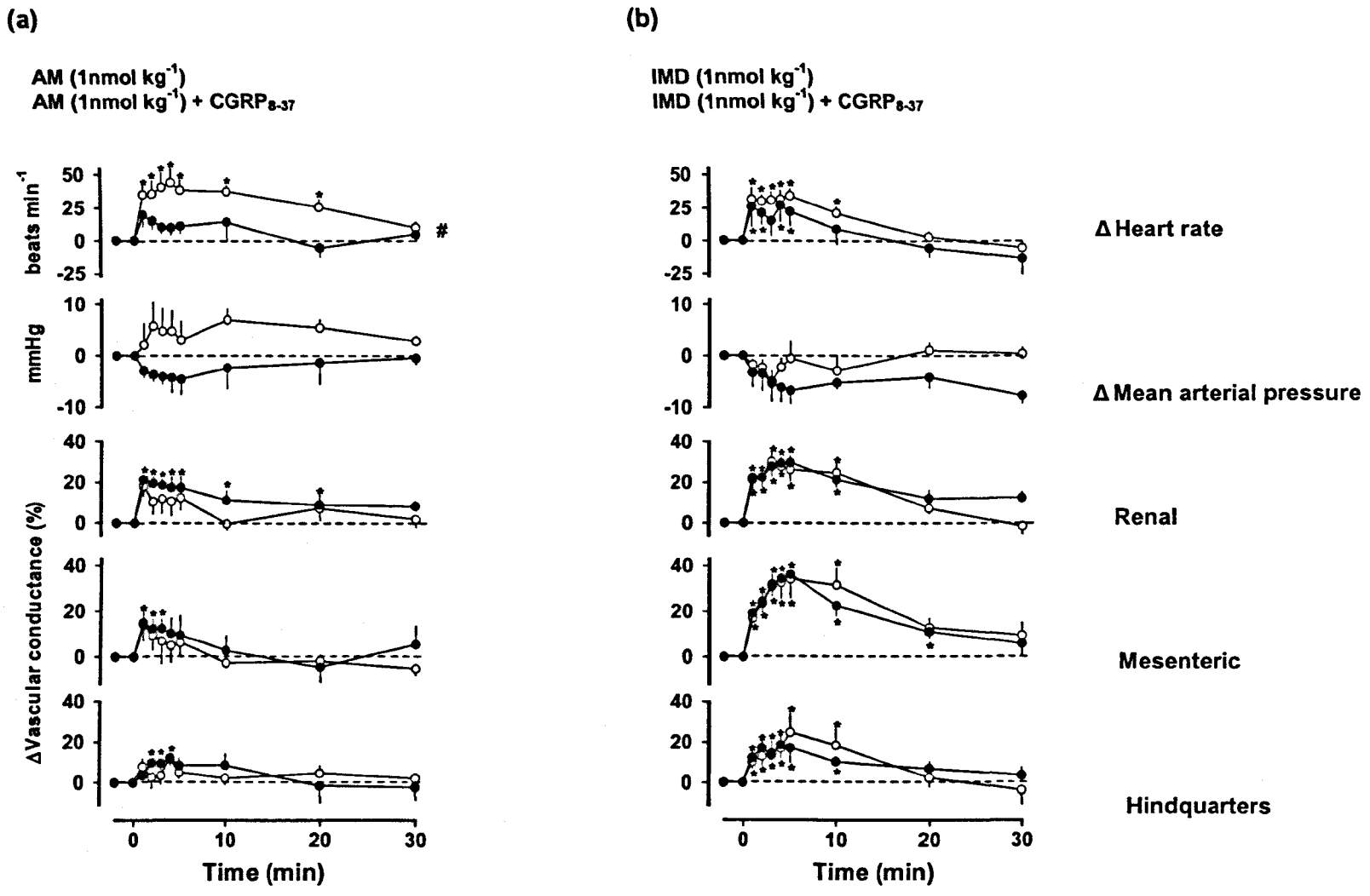


Figure 4.3.2 *Effects of $CGRP_{8-37}$ on cardiovascular responses to AM (1nmol kg^{-1} i.v.) (n=6)* ***(a)*** *or IMD (1nmol kg-1 i.v.) (n=6)* ***(b)*** *in conscious rats.* *$P<0.05$ *versus original baseline (Friedman's Test).* #$P<0.05$ *versus control ADM/IMD responses (n=5) (Mann Whitney U Test).*

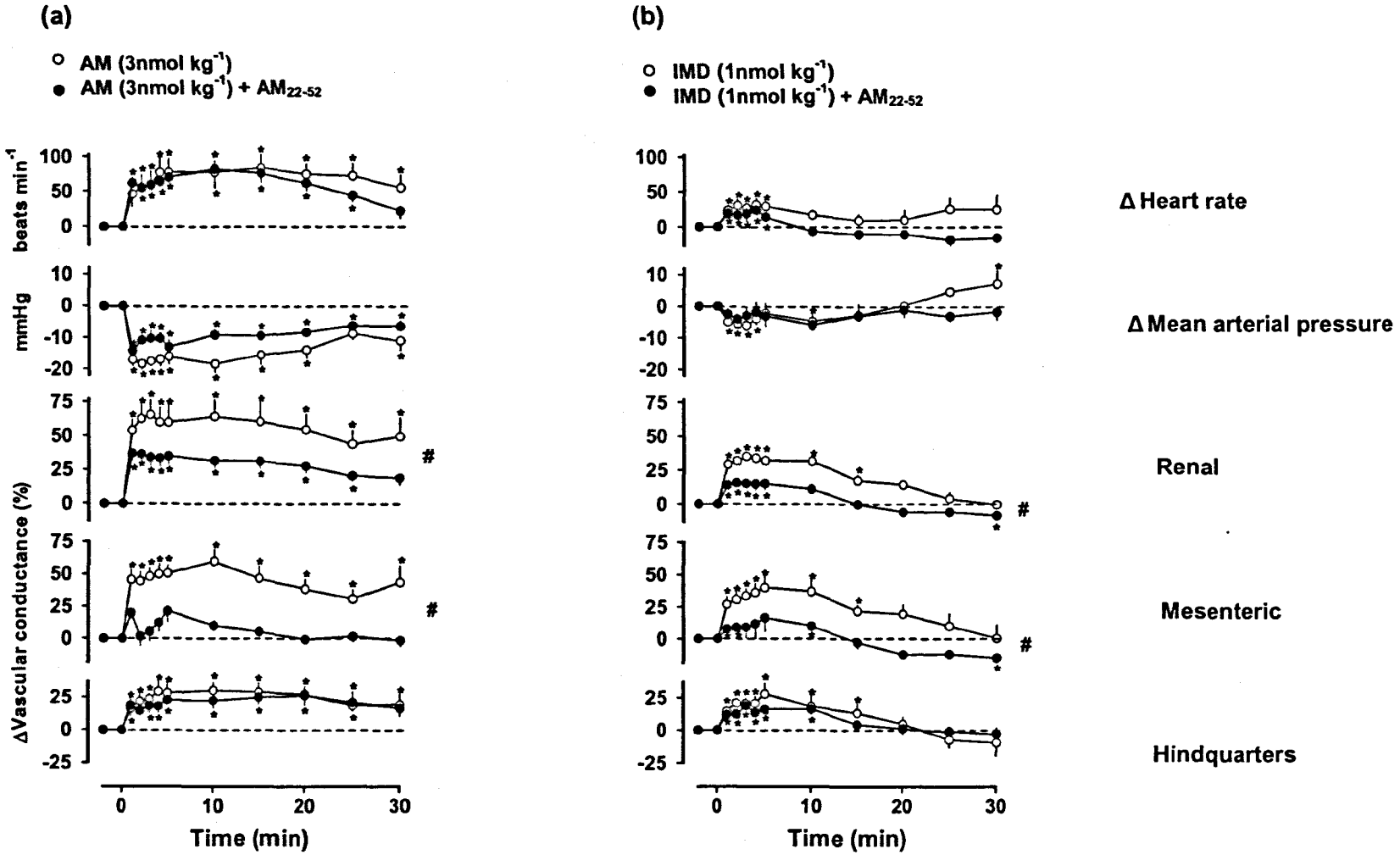


Figure 4.3.3 *Effects of AM_{22-52} on responses to* **(a)** *AM (3 nmol kg^{-1} i.v.) (n=8) or* **(b)** *IMD (1 nmol kg^{-1} i.v.) (n=6) in conscious rats.*

**P<0.05 versus original baseline (Friedman's test). #P<0.05 versus control AM (Wilcoxon test) and IMD (Mann Whitney U test) responses.*

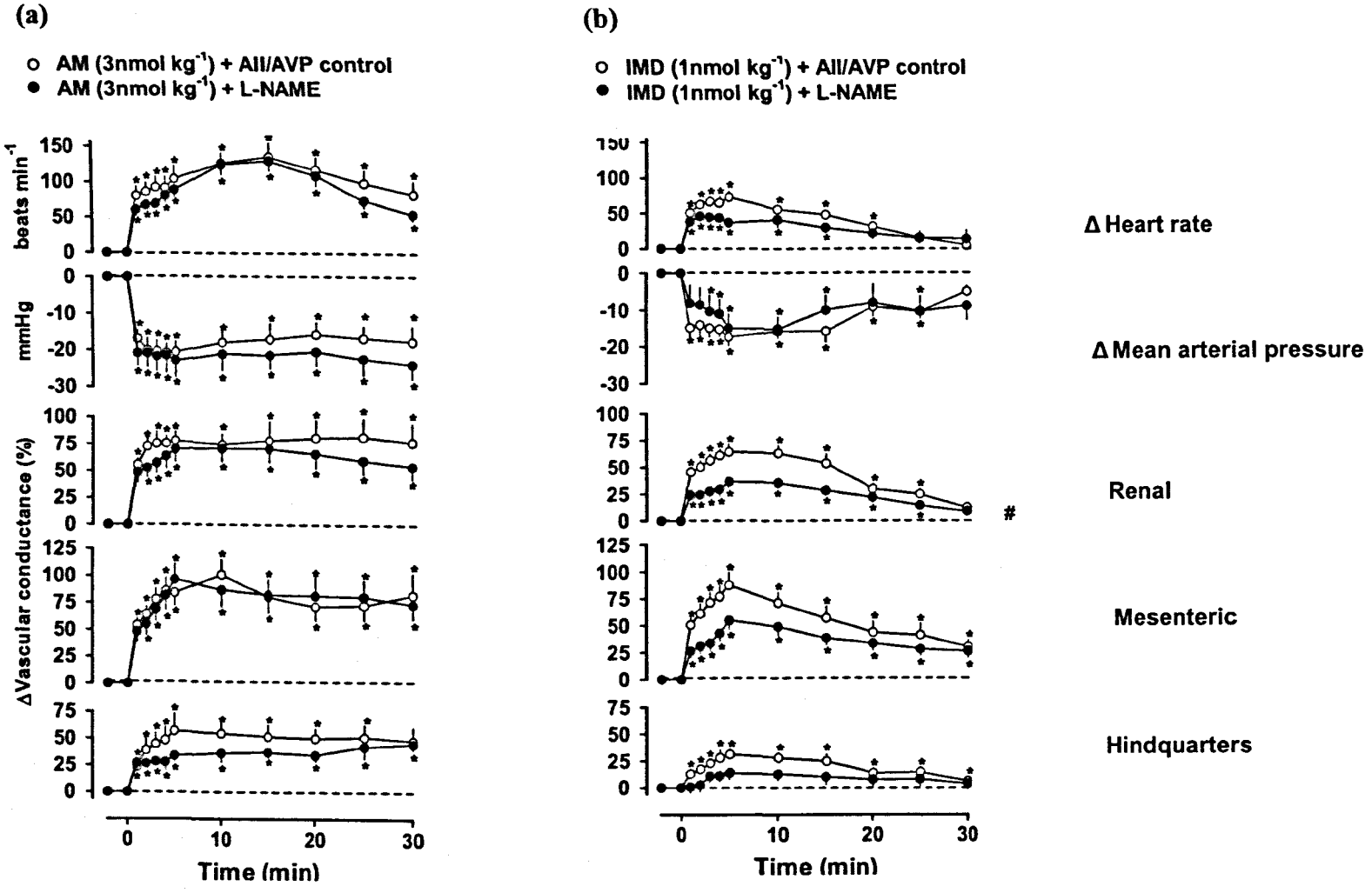


Figure 4.3.4 *Effects of L-NAME (3mg kg^{-1}h^{-1}) on cardiovascular responses to* **(a)** *AM (3nmol kg^{-1} i.v.) (n=9) or* **(b)** *IMD (1nmol kg^{-1} i.v.) (n=8) (b) in conscious rats.*

**P<0.05 versus original baseline (Friedman's test). #P<0.05 versus control (AII 0.2μg ml^{-1}/AVP 0.02μg ml^{-1}) AM (n=9)/IMD (n=8) (Wilcoxon test (n=8)*

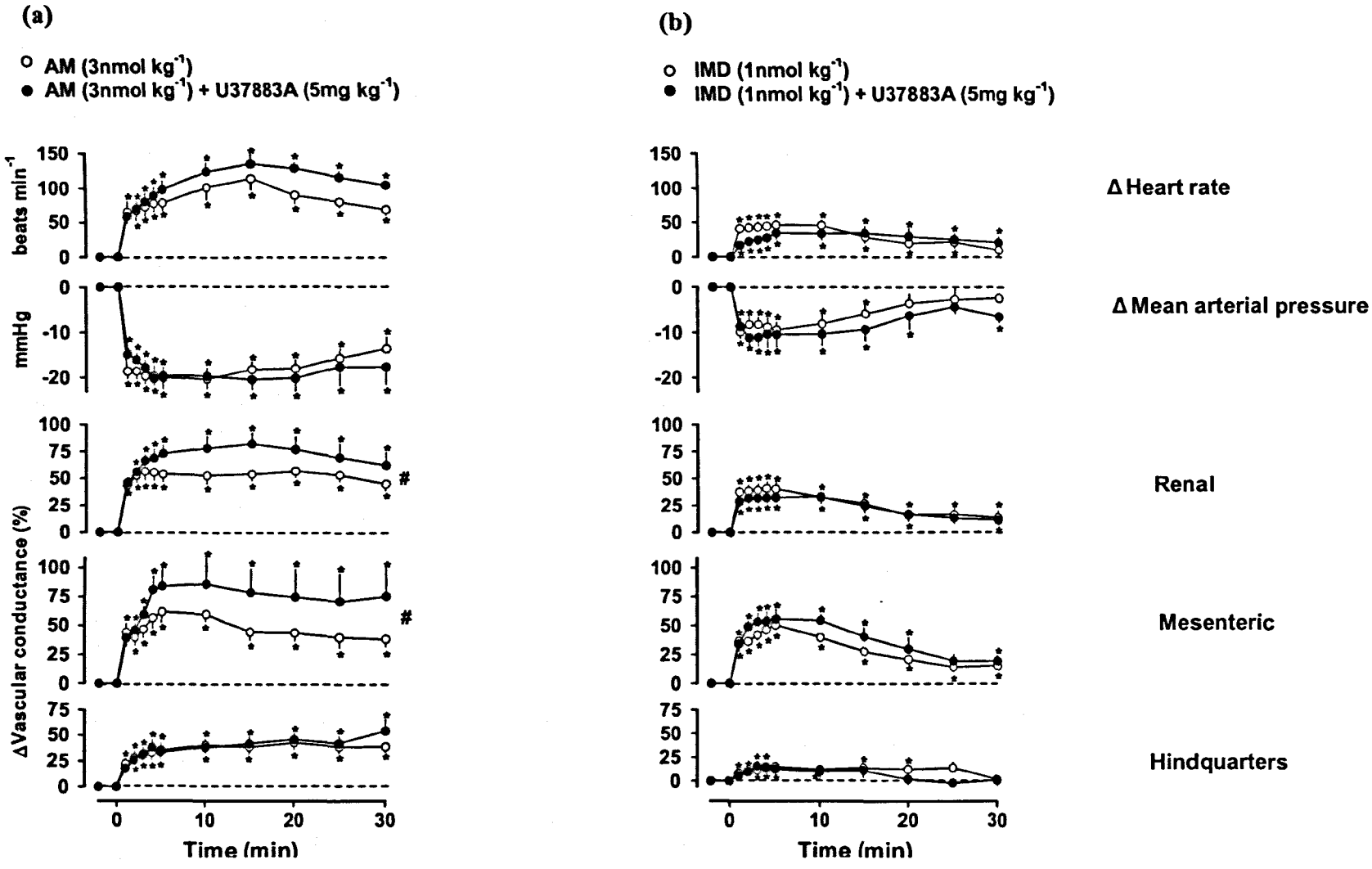


Figure 4.3.5 *Effects of U37883A (5mg kg^{-1}) on cardiovascular responses to* **(a)** *AM (3nmol kg^{-1} i.v.) (n=9) or* **(b)** *IMD (1nmol kg^{-1} i.v.) (n=9) in conscious rats.*

**P<0.05 versus original baseline (Friedman's test). #P<0.05 versus control AM/IMD responses (n=9) (Wilcoxon test)*

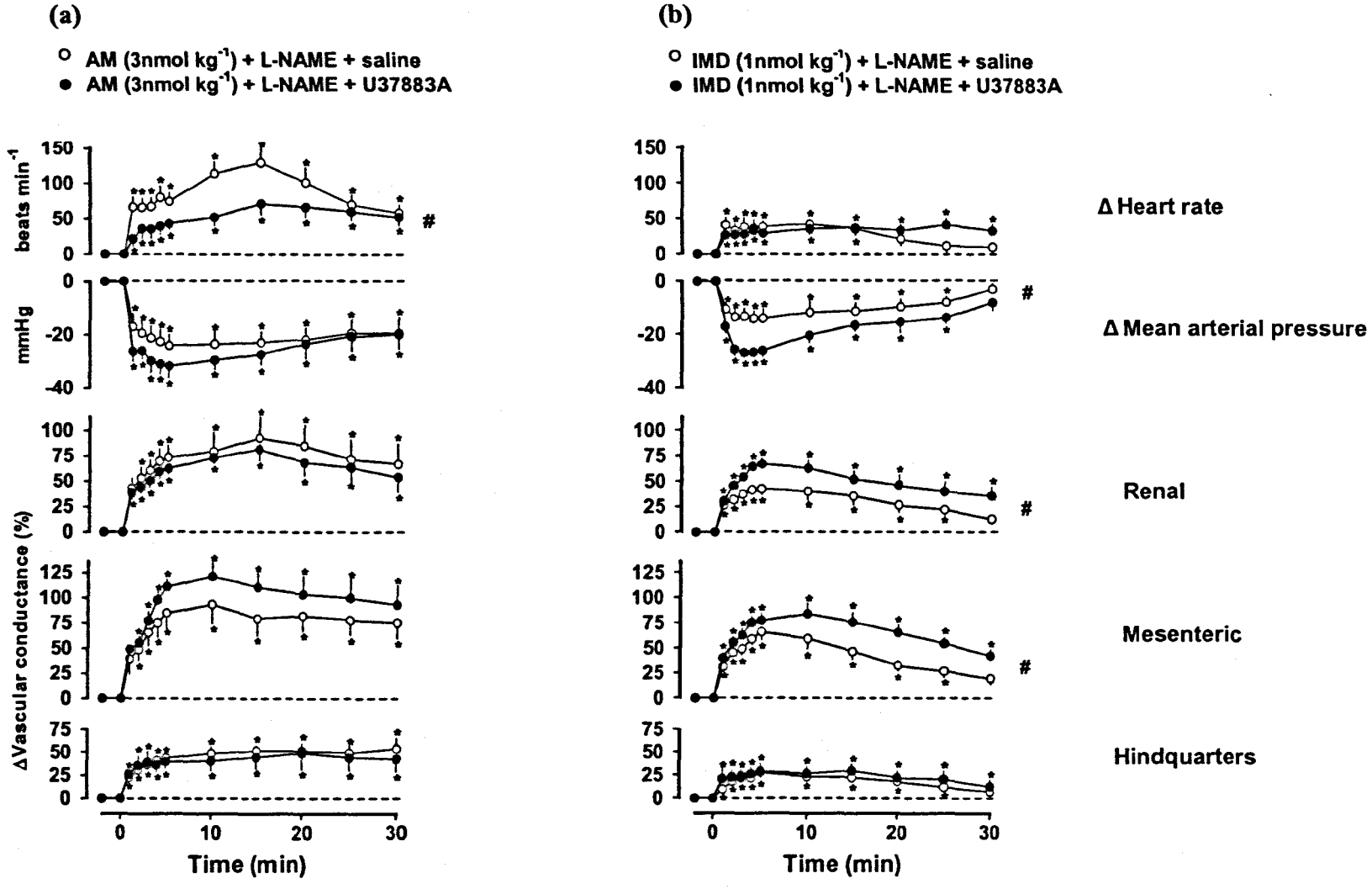


Figure 4.3.6 *Effects of L-NAME (3 mg kg^{-1} h^{-1}) alone, and in combination with U37883A (5 mg kg^{-1}) on cardiovascular responses to* **(a)** *AM (3 nmol kg^{-1} i.v.) (n=9) or* **(b)** *IMD (1 nmol kg^{-1} i.v.) (n=9) in conscious rats.*

**P<0.05 versus original baseline (Friedman's test). #P<0.05 versus control (Wilcoxon test)*

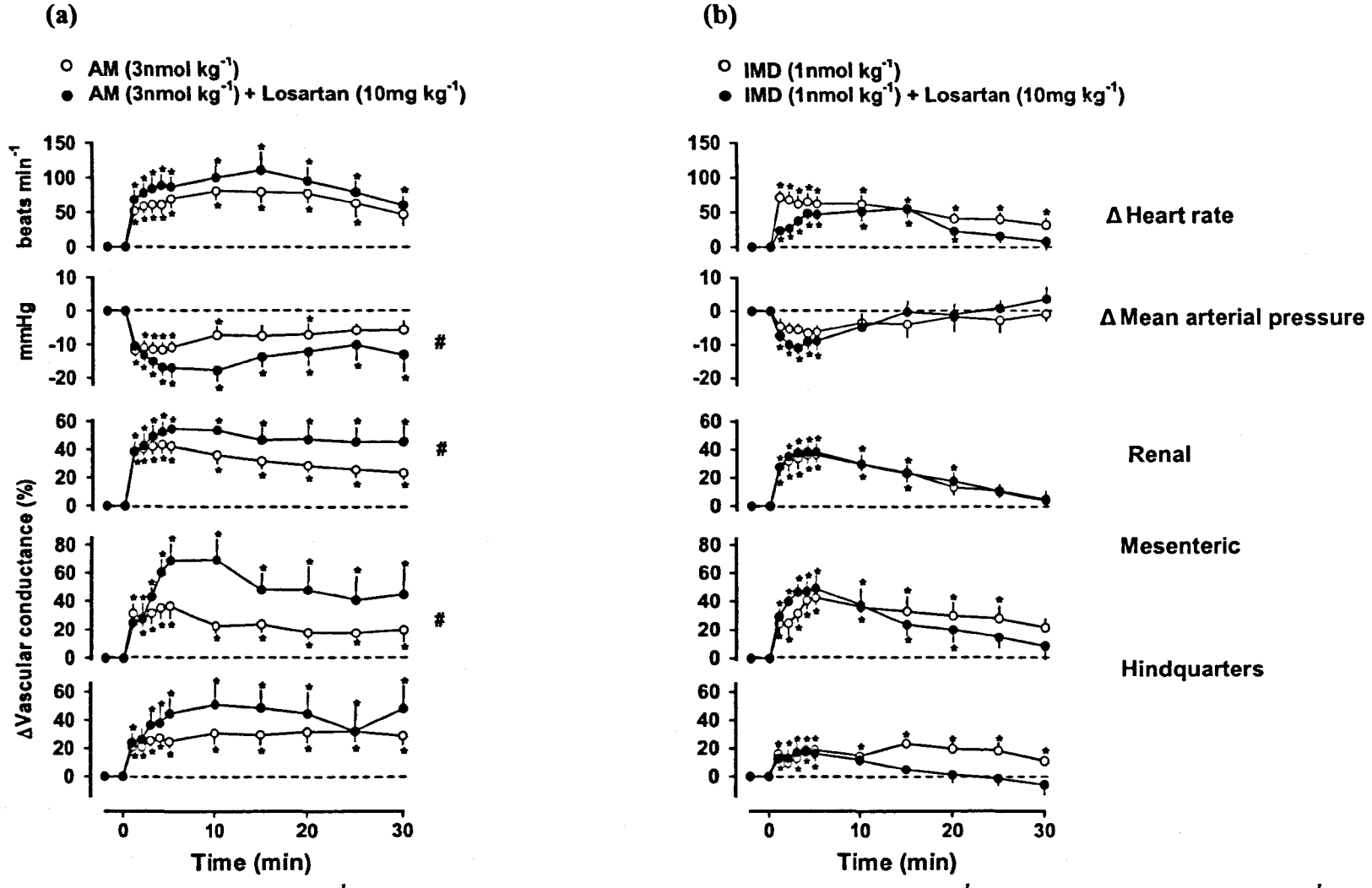


Figure 4.3.7 *Effects of losartan (10 mg kg^{-1}) on the cardiovascular responses to* **(a)** *AM (3 nmol kg^{-1} i.v.) (n=9)* **(b)** *IMD (1nmol kg^{-1} i.v.) (n=9) in conscious rats. *P<0.05 versus original baseline (Friedman's test). #P<0.05 versus control (Wilcoxon test)*

4.4 Discussion

Adrenomedullin is a potent vasoactive peptide which causes widespread vasodilatation and hypotension *in vivo* (Lippton *et al.*, 1994; Gardiner *et al.*, 1995; Nandha *et al.*, 1996; Parkes and May *et al.*, 1997), and relaxation of vascular smooth muscle *in vitro* (Nuki *et al.*, 1993; Santiago *et al.*, 1995). The vasorelaxant effects of AM occur through increases in receptor-mediated cAMP production, release of NO and activation of K_{ATP} channels (for review see Brain and Grant, 2004). Intermedin is a recently identified peptide, which shares some structural, sequence and functional homology with AM and is also called AM-2 (Roh *et al.*, 2004; Takei *et al.*, 2004). Mechanisms of IMD-mediated vasodilatation have yet to be fully elucidated but are thought to involve the non-selective activation of CL/RAMP complexes and activation of an unidentified IMD receptor (Roh *et al.*, 2004; Takei *et al.*, 2004).; there is also limited evidence suggestive of a role for NO in IMD-mediated vasorelaxation in isolated rat mesenteric and pulmonary vessels (Kandilci *et al.*, 2006, 2008; Chauhan *et al.*, 2007). The aim of these experiments therefore, was to compare the mechanisms through which AM and IMD lead to regional vasodilatations in conscious rats.

Earlier studies in this thesis compared the responses of equimolar AM and IMD (1 nmol kg^{-1}) and found that whilst IMD caused robust responses at this dose, AM did not (Chapter 3). The studies described in this chapter therefore used AM at a dose of 3 nmol kg^{-1}. Although it was intended that responses to each peptide would be matched, it was noted that throughout the course of the present experiments, the responses to AM (3 nmol kg^{-1}) appeared to be

significantly more marked than those to IMD (1 nmol kg^{-1}); the reasons for this are unclear.

The first set of experiments described in this Chapter show there was no tachyphylaxis following repeated dosing of AM or IMD (Figure 4.3.1), and hence, the results were not affected by the AM/IMD dosing regimen used. Using the AM-receptor antagonist, AM_{22-52}, evidence was found of AM_{22-52}-sensitive receptor activation in IMD-mediated vasodilatation in the renal and mesenteric beds but not in the hindquarters. This pattern is consistent with the observed attenuation of AM-mediated vasodilatations in those regions following pre-treatment with AM_{22-52}. This suggests that in normal rats, IMD predominantly signals through $AM_{1/2}$ receptors in the renal and mesenteric vascular beds. Interestingly, role was found for $CGRP_{8-37}$-sensitive receptors in the regional vasodilator response to IMD (Figure 4.3.2b) suggesting that IMD was predominantly activating AM_{22-52}-sensitive or IMD-specific receptors, at least in this system. Whilst there is evidence in the literature that IMD action can be inhibited by AM_{22-52}, this is somewhat limited (Ren *et al.*, 2006; Cui *et al.*, 2008; Kandilci *et al.*, 2008). Furthermore, the lack of effect of a $CGRP_1$ antagonist is inconsistent with studies which have shown regional and systemic responses to IMD were attenuated by $CGRP_{8-37}$ (Kobayashi *et al.*, 2004; Roh *et al.*, 2004; Taylor *et al.*, 2004; Kandilci *et al.*, 2006, 2008; Ren *et al.*, 2006).

Study of the CL/RAMP receptor system is complicated by a lack of selective antagonists, and interpretation of results using these agents should be

approached with caution. For example, although there is evidence to suggest that AM_{22-52} is more selective for AM_1 (CL/RAMP2) than AM_2 (CL/RAMP3) receptors (Hay *et al.*, 2003), there are no tools available to discern the true contribution of each. AM_{22-52} can also inhibit CGRP-, but not AM-mediated vasodilatation in the cat hindlimb (Champion *et al.*, 1997). Furthermore, AM_{22-52} by itself has weak agonist activity at AM receptors in the mesenteric vascular bed, leading to small vasodilatation in this region during infusion of the antagonist (Gardiner *et al.*, 1999). This was also seen here and was apparent in baseline data (Table 4.1, Expt. 3), although the change was not sufficient to be significant. Adrenomedullin also has some affinity for the $CGRP_1$ receptor, and CGRP has some affinity for both subtypes of AM receptors. Thus, it has been shown that application of the selective $CGRP_1$ receptor antagonist, BIBN4096BS, blocked the vasodilator action of AM in isolated perfused murine mesentery, but not in the aorta, implicating $CGRP_1$ receptors in AM-mediated vasodilatation in selected regions (Grant *et al.*, 2004). Interpretation of results from molecular studies should also be cautious, as species differences in combinations of CL and RAMP have been shown to cause variable results (Hay *et al.*, 2003). Nonetheless, our findings clearly show that both AM and IMD-mediated renal and mesenteric vasodilatations were attenuated by AM_{22-52}, suggesting AM_1 and/or AM_2 receptors were activated by both peptides in a regionally-selective manner.

In addition to a role for AM_{22-52}-sensitive receptors, an involvement for NO in IMD-mediated vasodilatation was demonstrated. Although it only reached significance in the renal vascular bed, there was a tendency for all vasodilator

responses to IMD to be reduced by L-NAME (Figure 4.3.4). Hence, the mesenteric vasodilatation was blunted, and the hindquarters vasodilatation was abolished in the presence of L-NAME (Friedman's test), but the integrated (0-30 min) area was not significantly different from control. This is in line with a study showing sensitivity of IMD-mediated relaxation in isolated pulmonary and mesenteric arteries to L-NAME (Kandilci *et al.*, 2006, 2008; Chauhan *et al.*, 2007). Whilst it might be expected that the reduced vasodilatations would be manifest as an overall reduction in the blood pressure response, it is likely that along with the overall smaller peripheral vasodilatation, there was a smaller increase in cardiac output. This is consistent with studies which found no contribution of NO to IMD-mediated hypotension (Taylor *et al.*, 2004; Abdelrahman *et al.*, 2007). Clearly findings here demonstrate a role for NO in the regional haemodynamic responses to IMD, and illustrate that results can be misleading if only blood pressure is measured.

No role for NO in AM-mediated vascular responses was found in the present study, which is at odds with previous work showing a role for NO in AM's action (Miura *et al.*, 1995; Gardiner *et al.*, 1995; Terata *et al.*, 2000). Gardiner *et al.* (1995) found AM-mediated hindquarters vasodilatation was attenuated by L-NAME in conscious rats, whereas here AM-mediated hindquarters vasodilatation was reduced in the presence of L-NAME, but this was not significantly different from control conditions. However, the study by Gardiner *et al.* (1995) used a lower dose of AM (1 nmol kg^{-1}) and compared integrated responses over 15 min, rather than the 30 min areas compared here. It is possible here that the hindquarters response to AM in the presence of L-

NAME may have been significant if responses were compared over 15 min (Figure 4.3.4).

To summarise, the present findings are consistent with activation of AM_{22-52}-sensitive receptors in responses to both AM and IMD in the renal and mesenteric vascular beds, and a role for NO in IMD-mediated renal vasodilatation, and to a lesser extent in the mesenteric and hindquarters vascular beds. No role for NO in the vascular responses to AM was found, although there was a tendency for hindquarters vasodilatation to be reduced in the presence of L-NAME (Figure 4.3.4). Kandilci *et al.* (2008) showed that endothelial removal abolished IMD-mediated vasorelaxation in isolated rat pulmonary artery, consistent with our findings which suggest IMD caused vasodilatation via endothelial-dependent mechanisms, at least in some vascular beds.

There is some evidence that the cardiovascular responses to AM are sensitive to glibenclamide and U37883A, indicating a role for K_{ATP} channels (Sabates *et al.*, 1997; Goto *et al.*, 2000). However, other studies found glibenclamide had no effect on the vascular responses to AM (Miura *et al.*, 1995; Terata *et al.*, 2000). A role for K_{ATP} channel activation in the response to IMD has not yet been demonstrated, although some studies have shown no effect of glibenclamide on the vasorelaxant response to IMD in isolated vessels (Kandilci *et al.*, 2006; Chauhan *et al.*, 2007). Blocking K_{ATP} channels with U37883A should attenuate peptide-mediated vasodilatation in areas where these channels are involved. In the present study, U37883A caused no attenuation of either AM or IMD-mediated responses, consistent with the

suggestion that K_{ATP} channels are not involved in the vasodilator responses. However, contrary to expectation, when AM was administered following treatment with U37883A, there was a significant *augmentation* of AM-mediated increases in renal and mesenteric vascular conductances. Since administration of U37883A caused an increase in blood pressure and vasoconstriction, it could be that the augmented vasodilatation was due to this preconstricted state. However, this is unlikely, firstly, because the augmentation was evident when the data were analysed as absolute rather than % changes (data not shown), and secondly, because the augmentation was regionally-selective and not seen in the hindquarters vascular bed significantly constricted by U37883A. Furthermore, IMD-mediated vasodilatation was not augmented by U37883A, suggesting that the phenomenon was unique to AM and not due to a change in baseline status.

One difference between AM and IMD may be in relation to their influence on the renin-angiotensin system. Hypotension usually causes reflex activation of this system, but there is also good evidence that AM has a direct renin-stimulatory effect on renal juxtaglomerular cells (Parkes *et al.*, 1995; Charles *et al.*, 1997, 2002; Troughton *et al.*, 2002). Although there is no evidence for a similar effect of IMD, this peptide has been shown to increase aldosterone levels secondary to an increase in plasma renin activity (Charles *et al.*, 2006); it is not known if this is a direct effect of IMD, and/or is secondary to its hypotensive action. Since U37883A has been shown to inhibit renin release, by a direct effect on renal juxtaglomerular cells (Humphrey and Ludens, 1998; Vallon *et al.*, 1998), it was hypothesised that U37883A might inhibit the direct

AM-induced renin release, thereby augmenting the AM-mediated renal and mesenteric vasodilatations. To test this hypothesis, a separate group of animals were pre-treated with the AT_1 receptor antagonist, losartan, and subsequently given AM and IMD. Losartan pretreatment had no effect on the vascular responses to IMD but AM-mediated hypotension and renal and mesenteric vasodilatation were significantly augmented (Figure 4.3.7) in its presence, consistent with the above hypothesis. Although it has been suggested that AM might be a functional antagonist of angiotensin II (AII) (Yamaguchi *et al.*, 1995; 1996; Charles *et al.*, 1997; 2002 Troughton *et al.*, 2000), the present results do not support such an effect. While the present findings indicate that the renin-angiotensin system did not modulate IMD action, it is possible that the hypotensive response to IMD in this study was insufficient to trigger a reflexive renin release.

Although an involvement of K_{ATP} channels in AM or IMD-mediated vasodilatation was not apparent, the possibility existed that there may be cross-talk between vasodilator mechanisms such that in the absence of one (e.g., NO) another (e.g., K_{ATP} channels) became more active. Thus, to assess the overall contribution of NO and K_{ATP} channels to the vascular effects of AM and IMD, a group of animals were co-administered L-NAME and U37883A prior to peptide challenges. In this series of experiments, the cardiovascular responses to IMD in the presence of L-NAME and U37883A were surprising. Thus, rather than any expected inhibition of vasodilator responses, there was augmentation of IMD-mediated renal and mesenteric vasodilatation following combined inhibition of NOS and K_{ATP} channel

blockade when compared to inhibition of NOS alone. Augmentation of these vasodilator responses was reflected in a greater hypotension in the experimental group relative to control (Figure 4.3.6b). Since such augmentation was not seen with AM, it is plausible that increased vasodilator responses to IMD under these conditions were due to baseline vasoconstrictions and increased blood pressure which was more apparent in the group given IMD in the presence of L-NAME together with U37883A, than in the group given AM under those conditions. Consistent with this suggestion, the increase in mesenteric vascular conductance was not augmented when the data were analysed as absolute rather than % changes. However, this does not explain the enhanced response observed to IMD in the renal vasculature following NOS inhibition and K_{ATP} channel blockade, since there was not an enhanced baseline renal vasoconstriction under these conditions. There is some evidence that IMD can directly activate large-conductance Ca^{2+}-activated K^+-channels (BK_{Ca} channels) (Kandilci *et al.*, 2006, 2007; Chauhan *et al.*, 2007); these channels can also be activated by NO leading to hyperpolarisation and relaxation of vascular smooth muscle. During treatment with L-NAME therefore, the proportion of closed BK_{Ca} channels may increase due to the removal of an activating stimulus, as a result of which there may be an increased number of 'available' channels which IMD can activate leading to augmentation of IMD-mediated vasodilatation in those vascular beds, which have a high population of BK_{Ca} channels. Whilst L-NAME by itself attenuated IMD-mediated vasodilatation, the conflicting results upon addition of U37883A may have been due to an enhanced response

to IMD in the presence of a 'depolarised' environment; this clearly warrants further investigation.

Another surprising observation was that blockade of K_{ATP} channels during NOS inhibition attenuated AM-mediated tachycardia when compared to animals treated with L-NAME alone. This suggests that cardiac K_{ATP} channels were activated in a cAMP-PKA-dependent manner by AM, which is surprising as U37883A is believed to be selective for vascular K_{ATP} channels (Surah-Narwal *et al.*, 1999; Wellman *et al.*, 1999) and U37883A does not have such an effect when given alone. This response is unrelated to differences in baseline heart rate; when absolute changes are compared, the differences remain significant regardless of some differences at rest (Table 4.1). Furthermore, as the addition of U37883A had no effect on the blood pressure response to AM, this attenuated tachycardia was unlikely due to reduced sympathetic activation. This is consistent with the findings of Vallon *et al.* (1998).

In conclusion, the present studies identified a role for AM_{22-52}-sensitive receptors in mediating IMD action in the renal and mesenteric vascular beds, and a role for NO in the renal vascular bed, and to a lesser extent in the mesenteric and hindquarters vascular beds. To further assess the contribution of AM and CGRP receptors, the two fragments (AM_{22-52} and $CGRP_{8-37}$) could be given together. Since AM responses sensitive to AM_{22-52} were apparent in the renal and mesenteric vascular beds, like those of IMD, it is plausible that effects in these regions that have previously been attributed to AM may have in part been mediated by IMD activating the same receptors. Again, the lack

of suitable antagonists of this system will hinder further research into this area until agents that are more selective are produced.

Chapter 5

Results (III)

Regional haemodynamic effects of adrenomedullin and intermedin in lipopolysaccharide-treated rats

Summary

The experiments described in this chapter aimed to investigate the regional and temporal haemodynamic responses to AM and IMD in LPS-treated rats, as others (Wang *et al.*, 1999) have shown a change in vascular responsiveness to AM in animal models of sepsis.

Male, Sprague-Dawley rats were implanted with Doppler flow probes to measure renal, mesenteric and hindquarters blood flows; catheters were implanted in the jugular vein, caudal artery and i.p. to allow administration of substances. Responses to bolus doses of LPS (1mg kg^{-1} and 3mg kg^{-1} i.p.) or LPS infusion (15μg kg^{-1} h^{-1}) were assessed in conscious rats, and vascular responses to AM and IMD were determined.

Following LPS administration (1mg kg^{-1}, i.p), there was a marked loss of responsiveness to AM and IMD in the renal, mesenteric and hindquarters vascular beds at 1.5h, with some recovery at 6h. At 25h, AM and IMD-induced increases in renal and mesenteric vascular conductances were normal, but there remained some hyporesponsiveness in the hindquarters. To assess the contribution of the LPS administration protocol, the experiment was repeated in animals treated with a continuous low dose infusion of LPS. In this model, vascular responsiveness to AM and IMD was still evident at 1.5 h but had returned by 6 h. At 25 h there was some enhancement of the vascular responses to both peptides which may have been related to an increase in resting vascular tone.

These results suggest that loss of vascular responsiveness to AM and IMD is an early event following LPS-administration in conscious rats. The mechanisms responsible for this vascular hyporesponsiveness remain to be determined.

5.1 Introduction

The calcitonin family of peptides, including AM and the recently discovered IMD, are involved in cardiovascular and renal homeostasis, and studies indicate that AM is significantly upregulated in disorders affecting the cardiovascular system, including sepsis (for review see Ishimitsu *et al.*, 2006; Bell & McDermott, 2008, and Introduction).

Lipopolysaccharide (LPS) is found in the cell wall of Gram-negative bacteria and is believed to be involved in the pathogenesis of human Gram-negative endotoxaemia. When LPS is administered to experimental animals at an appropriate dose, it causes pathophysiological changes that mimic those seen in clinical patients, including those with a hyperdynamic circulation (for review see Parker & Watkins, 2001). Thus, LPS is used to study the cardiovascular sequelae of early sepsis. Gardiner *et al.* (1995) developed an experimental model of hyperdynamic endotoxaemia by continuously infusing Long Evans rats with LPS (150μg kg^{-1} h^{-1}) over 32 h (Waller *et al.*, 1994; Gardiner *et al.*, 1995). This model was characterised by a biphasic tachycardia and hypotension, with renal, mesenteric and hindquarters vasodilatations, the pattern of which changed across time. There was a progressive increase in cardiac and stroke index, and total peripheral conductance, 3 h from the onset of LPS infusion (Gardiner *et al.*, 1995). This model has since been used to assess the involvement of a variety of endogenous mediators during rat endotoxaemia (see Chapter 1, Table 1.2) (Waller *et al.*, 1994; Gardiner *et al.*, 1996; Bennett *et al.*, 2004).

A role for the calcitonin family peptide adrenomedullin (AM), in influencing cardiovascular function in sepsis has been suggested, since high AM plasma levels have been seen in clinical patients (Wang, 2001), and AM mRNA and protein levels are upregulated following LPS-treatment *in vivo* (Shoji *et al.*, 1995; Hattori *et al.*, 1998; Yang *et al.*, 2001; Cheung *et al.*, 2004; Li *et al.*, 2005) and *in vitro* (Sugo *et al.*, 1995; Zaks-Zilberman *et al.*, 1998). Evidence suggests that AM has some involvement in the development of a hyperdynamic circulation in early sepsis (Wang *et al.*, 1999; Koo *et al.*, 2001) and that the transition to a hypodynamic circulation in late sepsis may be due to a decrease in vascular sensitivity to the peptide (Wang *et al.*, 1999; Ertmer *et al.*, 2007). Furthermore, mice heterozygous for the AM gene have increased sensitivity to the inflammatory effects of LPS (Dackor & Caron, 2007), whilst mice over expressing AM in their vasculature, are resistant to some of the deleterious effects of LPS (Shindo *et al.*, 2000). Since the newly discovered member of the calcitonin family IMD shares some sequence and structural homology with AM, can activate AM receptors, and has an effect on the cardiovascular system comparable to that of AM (Roh *et al.*, 2004; Takei *et al.*, 2004; see Chapters 3 & 4), it is possible that IMD may share some of the functions of AM during sepsis, although IMD has yet to be studied in this context.

The aims of this study were, therefore, to establish whether or not regional vascular sensitivity to the vasodilator effects of AM and IMD were affected across time, in LPS-treated rats. But it was first necessary to establish the

optimal LPS dosing regimen in Sprague Dawley rats. In their study, Gardiner *et al.* (1995) used Long Evans rats bred in house in Nottingham, but, as these were not available at the onset of the present work, outbred Sprague Dawley rats were obtained from Charles River UK. For some reason, the latter were very sensitive to the effects of LPS leading to an unacceptable number of deaths when it was given at a dose of 150μg kg^{-1} h^{-1}. Thus, studies were first completed to determine an appropriate LPS-administration protocol in this strain, prior to an evaluation of the effects of repeated administration of AM and IMD across time following LPS administration.

Because the results of the studies with AM and IMD showed reduced vascular sensitivity to the peptides during early sepsis (i.e., 1.5 h, see later), a time when endogenous AM levels have been shown to be elevated (for review see Wang *et al.*, 1999), a further experiment was conducted using the AM receptor antagonist, AM_{22-52}, to assess the role of endogenous AM in the haemodynamic changes 1.5h after LPS administration.

5.2 Methods

The following experiments were performed in 9 groups of Sprague-Dawley rats, prepared for haemodynamic recording with the 2-stage surgical procedure outlined in Chapter 2. Briefly, animals were anaesthetised and chronically implanted with miniature pulsed Doppler flow probes which were sutured around the left renal artery, the superior mesenteric artery and the distal aorta, to measure changes in renal, mesenteric, and hindquarters Doppler shift; this was used as an index of blood flow. After a recovery period of 10-14 days, animals were again anaesthetised and implanted with catheters (i.a, i.v, & i.p.) to allow continuous derivation of blood pressure and heart rate, and administration of substances.

5.2.1 Experiment 1: Cardiovascular effects of LPS administration by i.p. bolus or i.v. infusion

To determine the optimum LPS dosing regime for producing an hyperdynamic circulation in Sprague Dawley rats, 3 separate groups of animals received either LPS given as an i.p. bolus at a dose of 1mg kg^{-1} (n=9) or 3mg kg^{-1} (n=8), or a saline bolus (0.5ml i.p.) (n=7). A further group of animals received a saline infusion (0.4 ml h^{-1}) for 25 h beginning on Day 1, and a continuous LPS infusion for 25 h beginning on Day 3, at a dose of 15μg kg^{-1} h^{-1} i.v. which is 10-fold lower than that used by Gardiner *et al.* (1995). Cardiovascular variables were measured for up to 26 h.

5.2.2 Experiment 2: Cardiovascular responses to AM/IMD in endotoxaemia

5.2.2.1 Cardiovascular responses to AM/IMD following bolus administration of LPS

In the light of results from the experiment above (see Results), the following protocols administered LPS as an i.p. bolus at a dose of 1mg kg^{-1}.

To assess whether the regional haemodynamic responses to AM or IMD were affected in a time-dependent manner during endotoxaemia, rats were treated with saline (0.5ml i.p.) at time 0 on Day 1 (as outlined in Chapter 3.2), or LPS (1mg kg^{-1} i.p.) at time 0 on Day 3, and challenges of AM (3nmol kg^{-1}) (n=9) or IMD (1nmol kg^{-1}) (n=9) were given at 1.5 h, 6 h and 25 h after saline or LPS administration. These time points were chosen as they represent a time when a hyperdynamic circulation was beginning to develop (1.5 h), was fully developed (6 h) or when cardiovascular variables were starting to return to normal (25 h) (see later). This protocol is illustrated in Figure 5.2.2.

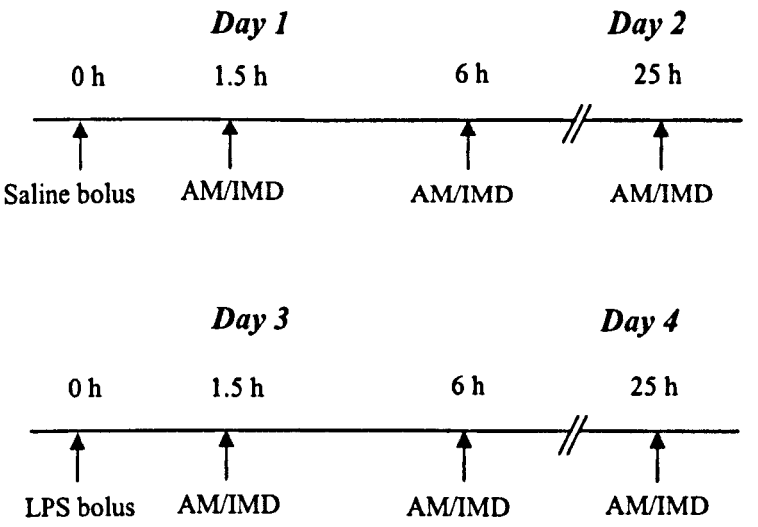


Figure 5.2.2 *Animals received AM or IMD 1.5 h, 6 h and 25 h from administration of saline at time 0 on Day 1, and LPS at time 0 h on Day 3,*

5.2.2.2 Cardiovascular responses to AM/IMD during a continuous infusion of LPS

The above experimental protocol was repeated in 2 separate groups of animals (n=10 each group) in the presence of a continuous i.v. infusion of saline (0.4ml h^{-1}) or LPS (15μg kg^{-1} h^{-1} i.v.). Each animal received 1 peptide throughout the course of the experiment. The LPS infusion protocol followed is shown in Figure 5.2.2a.

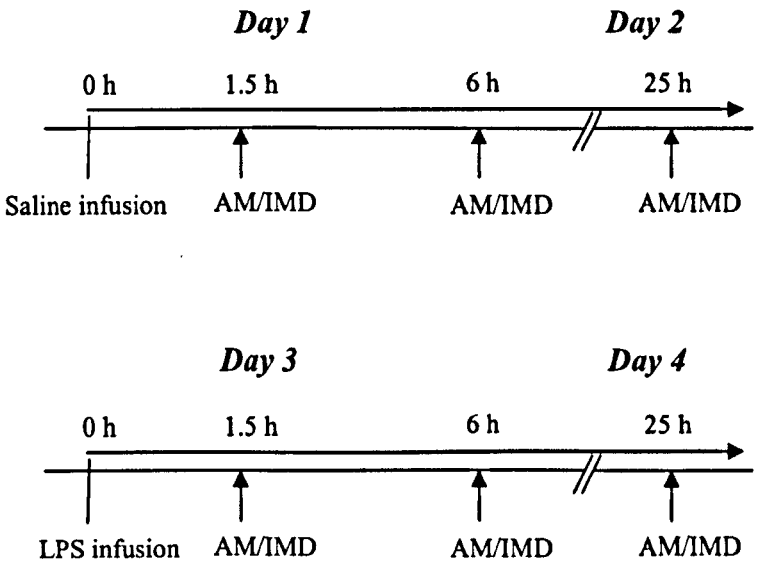


Figure 5.2.2a *A continuous 26 h infusion of saline began at time 0 on Day 1, and LPS at time 0 h on Day 3. Animals were administered AM or IMD 1.5 h, 6 h and 25 h after the onset of the infusion.*

5.2.4 Experiment 3: Cardiovascular responses to AM_{22-52} in LPS-treated rats

To assess whether endogenous AM contributed to the haemodynamic profile in LPS-treated rats, a separate group of animals (n=8) were infused with the AM fragment, AM_{22-52} (500nmol kg^{-1} h^{-1}), for 6 min, beginning 1.5 h, 6 h and 25 h after LPS treatment (1mg kg^{-1} i.p. bolus). This fragment has some selectivity for AM-sensitive receptors and is currently the only available AM receptor antagonist (for review see Poyner *et al.*, 2002). The dose chosen has been shown to antagonise some of the cardiovascular effects of exogenous AM (Chapter 4). This protocol is illustrated in Figure 5.2.4.

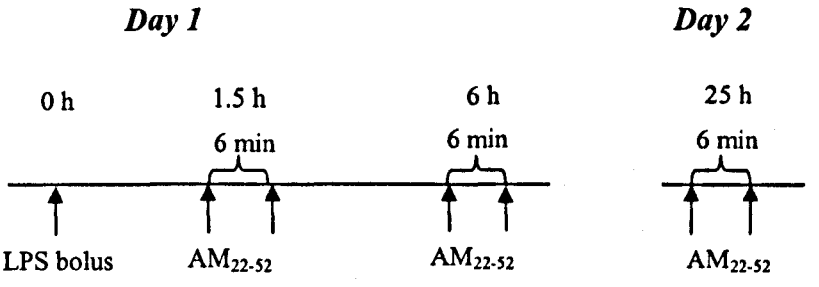


Figure 5.2.4 *Animals were treated with a LPS bolus at time 0 h, and were administered AM_{22-52} 1.5 h, 6 h and 25 h later.*

5.3 Results

5.3.1 Experiment 1: Cardiovascular effects of LPS administration

Baseline cardiovascular variables in groups of animals that were to receive LPS at a dose of 1mg kg^{-1} or 3mg kg^{-1}, or saline are shown in Table 5.1. Prior to bolus administration of LPS (1mg kg^{-1} i.p.) there was a slightly higher resting heart rate, and lower resting renal vascular conductance, in comparison to the group who were to receive saline ($P<0.05$, Kruskall Wallis test).

5.3.1.1 Cardiovascular changes following treatment with saline (0.5ml i.p.)

In animals given a saline bolus, there was a small rise in blood pressure ($P<0.05$, 24 h to 25 h) and a progressive hindquarters vasoconstriction from 6 h onwards, but no other changes in haemodynamics during the 26 h recording period (Figure 5.3.1).

5.3.1.2 Cardiovascular changes following treatment with LPS (1 mg kg^{-1} i.p.)

Cardiovascular changes 0-25 h following administration of LPS (1mg kg^{-1} i.p. bolus) are shown in Figure 5.3.1. There was a tachycardia relative to the original baseline ($P<0.05$, Friedman's test), which was sustained over the first 8 h, peaking 6h after LPS administration, but by 25 h the resting heart rate was no longer different from baseline ($P>0.05$, 24 h and 25 h). The tachycardia was accompanied by a triphasic blood pressure response, comprising an early rise ($P<0.05$, 0.25 h and 0.5 h) followed by a fall ($P<0.05$, 1 h to 1.75 h), with blood pressure returning to baseline by 2h. There was then a second period of hypotension ($P<0.05$, 5 h to 7 h), after which blood pressure returned to

baseline. In the renal vascular bed, there was a sustained increase in vascular conductance which remained elevated relative to original baseline ($P<0.05$, Friedman's test) from 0 to 24 h after LPS-administration. Changes in the mesenteric and hindquarters vascular beds mirrored the changes in blood pressure. That is, an early (0.25 to 0.5 h) transient vasoconstriction ($P<0.05$ in the hindquarters), followed by a marked transient vasodilatation ($P<0.05$, 1 h to 1.25 h in the mesentery, and 1 h to 2 h in the hindquarters), before returning to baseline (hindquarters), or a second phase of vasoconstriction (mesenteric). Both vascular beds underwent a second phase of vasodilatation ($P<0.05$, 5 h to 8 h), with vascular conductance returning to baseline levels by 24 h.

5.3.1.3 Cardiovascular changes following treatment with LPS (3mg kg^{-1} i.p.)

Cardiovascular changes 0-25 h following administration of LPS (3mg kg^{-1} i.p. bolus), are shown in Figure 5.3.1. Responses to LPS at this dose were similar to those seen with 1mg kg^{-1}, comprising a sustained tachycardia ($P<0.05$, 0 h to 8 h) which was accompanied by a triphasic blood pressure response. There was a sustained increase in renal vascular conductance, which remained elevated relative to original baseline 1h from LPS onwards ($P<0.05$, 1 h to 25 h). Changes in the mesenteric and hindquarters vascular beds followed the triphasic pattern observed using the lower dose of LPS, except the early mesenteric vasoconstriction was more marked ($P<0.05$, 1.25 h to 1.75 h) and the first phase of mesenteric vasodilatation was less marked than that seen at the lower dose ($P>0.05$, 1 h to 1.5 h) In the hindquarters, the second phase of vasodilatation was sustained for the remainder of the recording period ($P<0.05$, 5 h to 25 h).

Since LPS at a dose of 1mg kg^{-1} caused widespread vasodilatations which were similar to those caused by LPS at a dose of 3mg kg^{-1}, with relatively small changes in blood pressure this was the bolus LPS dose used in subsequent experiments.

5.3.1.4 Cardiovascular changes during a continuous saline or LPS infusion (15μg kg^{-1} h^{-1})

Baseline cardiovascular variables were not different between animals prior to the onset of a LPS infusion or a saline infusion; these baseline data are shown in Table 5.1.

The only significant deviation from baseline in saline infused rats was a fall in hindquarters vascular conductance from 24 h to 25 h (Figure 5.3.1a).

A continuous infusion of LPS (15μg kg^{-1} h^{-1}) caused a tachycardia, which was sustained for the entire recording period ($P<0.05$, Friedman's test) and a transient hypotension ($P<0.05$, 1.25 to 1.75 h). An increase in renal vascular conductance was sustained from 0.75 h onwards, whilst mesenteric vascular conductance was reduced from 1.5 h to 5.5 h, and hindquarters vascular conductance was transiently elevated between 1 h and 2h , and again between 24 h and 25 h ($P<0.05$, Friedman's test).

At the 1.5 h time-point, regional haemodynamics were similar between those animals given LPS as a bolus (1mg kg^{-1} and 3mg kg^{-1}), and those given LPS as

an infusion, except that in the latter there was no change in mesenteric vascular conductance. At 6 h the haemodynamic profile in groups treated with bolus LPS were similar, but differed from that observed in the LPS-infused group inasmuch as the latter displayed no deviation from baseline in blood pressure, mesenteric or hindquarters vascular conductance. At 25 h, animals given LPS as an infusion were tachycardic and vasodilated in all 3 vascular beds, whilst those administered LPS as a bolus were no longer tachycardic, or vasodilated in the mesenteric (both LPS doses) or the hindquarters (1mg kg^{-1} only) vascular beds.

5.3.2 Experiment 2: Regional haemodynamic responses to AM and IMD in endotoxaemia

Baseline cardiovascular measurements were taken in all groups of animals before the onset of either saline bolus or infusion or LPS bolus or infusion to determine whether there were any differences between the groups at rest (Table 5.2, 0 h). The only significant differences were a lower resting heart rate prior to LPS bolus administration in the AM group, and a lower renal vascular conductance prior to LPS bolus administration in the IMD group ($P<0.05$).

5.3.2.1 Cardiovascular responses to AM/IMD following an LPS bolus

Resting values for cardiovascular variables prior to administration of AM (3nmol kg^{-1}) 1.5 h, 6 h and 25 h after LPS are given in Table 5.2, and all baseline data for experiments in this chapter are summarised in Table 5.3.

Control responses to AM in the presence of saline are detailed in Chapter 3; briefly, AM (3nmol kg^{-1}) caused tachycardia, a significant fall in blood pressure and marked increases in renal, mesenteric and hindquarters vascular conductances (Figure 3.3.1). At 1.5 h after administration of LPS (1mg kg^{-1} i.p.), all cardiovascular responses to AM were blunted ($P<0.05$ for 0-10min integrated changes for all variables), the effect being particularly marked in the mesenteric and hindquarters vascular beds. At 6 h after LPS, the AM-induced tachycardia and mesenteric and hindquarters vasodilatations remained significantly attenuated compared to control but the renal vasodilatation and blood pressure response were no longer different between the groups ($P<0.05$ for 0-10 min integrated changes). At 25 h after LPS, the AM-induced increase in hindquarters vascular conductance remained reduced in the LPS group but there were no other differences between the groups (Figure 5.3.2).

Resting cardiovascular variables prior to administration of IMD 1.5 h (n=9), 6 h (n=9) and 25 h (n=9) following saline or LPS bolus administration are shown in Table 5.2, and summarised in Table 5.3.

Control responses to IMD in saline-treated animals are detailed in Chapter 3, and comprised a tachycardia, fall in blood pressure and increases in renal, mesenteric and hindquarters vascular conductances (Figure 3.3.1). At 1.5 h after LPS administration, all cardiovascular responses to IMD were significantly attenuated ($P<0.05$, for 0-10min integrated changes), this effect being most marked in the mesenteric and hindquarters vascular beds where IMD-mediated vasodilatations were abolished. Six h after LPS, all

cardiovascular responses to IMD remained attenuated but these effects only reached significance in the mesenteric and hindquarters vascular beds ($P<0.05$, for 0-10min integrated changes). At 25 h, the hindquarters vasodilator response to IMD remained attenuated ($P<0.05$), but all other responses were not different from those in the saline condition. At this juncture, the IMD-mediated fall in blood pressure appeared to be augmented, but this effect was not significant (Figure 5.3.3).

To assess the effects of the mode of LPS administration on vascular responses to AM and IMD the above experimental protocol was repeated in animals given a continuous LPS infusion ($15\mu g\ kg^{-1}\ h^{-1}$ i.v.) .

5.3.2.2 Cardiovascular responses to AM/IMD during an LPS infusion

Resting cardiovascular variables prior to the onset of either a saline or LPS infusion were not different in those animals that were to receive AM or those that were to receive IMD (Table 5.2). Baseline cardiovascular variables prior to administration of AM at 1.5 h, 6 h and 25 h are given in Table 5.2, and summarised in Table 5.3.

At 1.5 h from the onset of LPS infusion, all cardiovascular responses to AM were significantly attenuated ($P<0.05$, for 0-10min integrated changes for all variables), with AM-mediated vasodilatations in the mesenteric and hindquarters vascular beds being most affected. At 6 h, the renal and hindquarters vasodilator responses remained attenuated in the LPS-group, whilst the mesenteric vasodilatation and tachycardia were no longer

significantly altered from control ($P>0.05$ for 0-10 min integrated changes). Interestingly, at 25 h, AM-mediated vasodilatations in the renal and mesenteric vascular beds were augmented, but this only reached statistical significance in the latter ($P<0.05$ for 0-10 min integrated changes) (Figure 5.3.4).

Baseline cardiovascular measurements 1.5 h, 6 h and 25 h from the onset of a saline or LPS infusion in those animals that were to receive IMD are shown in Table 5.2, and summarised in Table 5.3.

When IMD was administered 1.5 h after the onset of LPS infusion, there was attenuation of cardiovascular responses to IMD (Figure 5.3.5), with IMD-mediated mesenteric and hindquarters vasodilatations being abolished. IMD caused renal, mesenteric and hindquarters vasodilatations at 6 h which were not different from controls, although the tachycardia was significantly attenuated whilst the hypotensive response was significantly augmented ($P<0.05$, for 0-10 min changes). In the absence of any enhancement of the vasodilator effects of IMD this last result is surprising, but an enhanced IMD-mediated hypotension was also seen in the LPS groups at 25 h ($P<0.05$ for 0-10 min integrated changes), although IMD-mediated renal and mesenteric vasodilatations were not significantly enhanced ($P>0.05$ for 0-10 min integrated changes). There was no difference in the IMD-induced tachycardia and there was an attenuated vasodilator response in the hindquarters ($P<0.05$, for 0-10 min changes).

5.3.3 Experiment 3: Cardiovascular responses to AM_{22-52} in LPS-treated rats

The AM fragment, AM_{22-52}, was used to assess the possible contribution of endogenous AM acting at AM_{22-52}-sensitive receptors in LPS-treated rats. Prior to administration of AM_{22-52}, there were vasodilatations in the renal and hindquarters, but not in the mesenteric, vascular beds (data not shown). At 1.5 h after LPS, infusion of AM_{22-52}, at a dose shown to antagonise the effects of exogenous AM (500nmol kg^{-1} h^{-1}) (Chapter 4), had no effect on the developing, LPS-induced changes in regional haemodynamics (Table 5.4). The only significant difference between those animals receiving AM_{22-52} 1.5 h after LPS and the time- matched LPS controls was a small tachycardia in the AM_{22-52} group 10 min from the end of the infusion (Table 5.4).

When AM_{22-52} was given 6 h and 25 h after LPS (1mg kg^{-1} i.p.) administration, there were no changes in any haemodynamic variable throughout the course of AM_{22-52} infusion at either time-point (Table 5.5). However, lack of a suitable LPS time control for these experiments did not permit a full analysis of these data; this is acknowledged as a draw-back to this study.

	Bolus protocol (i.p.)			*Infusion protocol (i.v.)*	
	Saline (0.5ml)	**LPS (1mg kg^{-1})**	**LPS (3mg kg^{-1})**	**Saline (0.4ml h^{-1})**	**LPS (15µg kg^{-1} h^{-1})**
HR(beats/min)	337±10	359±7*	349±7	356±11	356±12
MAP(mmHg)	103±3	107±2	111±4	113±2	114±3
RVC (units)	97±11	72±7*	91±5	69±5	69±6
MVC units)	76±10	66±6	62±1	58±7	50±8
HVC (units)	48±6	41±2	40±5	49±5	45±5

Table 5.1 *Resting cardiovascular variables in groups of rats prior to bolus administration of saline (0.5ml i.p.) (n=7), LPS at a dose of 1mg kg^{-1} i.p. (n=9) or 3mg kg^{-1} (n=8), and prior to the onset of a saline (0.4ml h^{-1} i.v.) or LPS (15µg kg^{-1} h^{-1} i.v.) infusion in a separate group of rats (n=10).*

*VC=vascular conductance. Units for VC are kHz $mmHg^{-1}$ x 10^3. *P<0.05 vs. saline bolus (Kruskall-Wallis test)*

		HR (beats/min)		MAP (mmHg)		RVC (units)		MVC (units)		HVC (units)	
		Sal	LPS	Sal	LPS	Sal	LPS	Sal	LPS	Sal	LPS
AM *(LPS bolus)*	0h	350±5	312±5*	107±2	104±2	66±4	66±5	65±5	66±5	45±3	42±3
	1.5h	342±9	434±10*	109±3	101±3	66±5	81±8	64±5	55±5	46±4	59±4
	6h	351±11	405±9*	107±2	98±3*	66±5	97±9*	57±5	85±8*	43±3	60±5*
	25h	351±11	358±13	114±3	107±4*	64±5	81±6*	57±3	71±7*	40±3	57±3*
AM *(LPS infusion)*	0h	349±11	354±15	111±2	106±3	92±9	90±9	69±6	67±6	48±4	46±1
	1.5h	344±9	400±13*	108±3	95±4*	98±9	122±15*	76±9	63±6	48±6	77±7*
	6h	350±9	387±11*	112±2	116±5	94±7	122±12*	69±8	47±5*	43±5	41±7
	25h	342±15	377±17	107±3	126±6*	93±8	100±13	66±7	47±7	46±6	52±8
IMD *(LPS bolus)*	0h	340±5	334±7	112±3	111±3	94±10	78±8*	67±5	67±6	38±3	34±3
	1.5h	340±8	414±16*	112±4	104±4	90±10	102±7	71±5	59±9	37±4	46±6
	6h	340±10	413±16*	111±3	103±3	93±9	126±9*	64±6	86±8*	34±5	49±5*
	25h	323±10	347±8	117±3	111±4	77±7	100±7*	57±6	66±6*	29±3	50±6*
IMD *(LPS infusion)*	0h	339±7	333±15	106±1	104±2	84±12	81±12	74±5	77±5	52±6	43±5
	1.5h	339±9	445±10*	108±2	96±3*	76±9	103±17	66±4	54±6	49±4	75±7*
	6h	348±13	422±12*	107±3	114±5	80±8	100±7*	65±4	58±9	43±3	39±5
	25h	337±9	403±22	108±1	122±3*	78±9	81±12	71±5	55±8*	44±4	47±6

Table 5.2. *Resting cardiovascular variables, at selected time points, prior to administration of AM or IMD in rats treated with a saline/LPS bolus (1mg kg^{-1}) and a saline/LPS infusion, n=8-10/group*

*Units for vascular conductance (VC) are kHz $mmHg^{-1}$ x 10^3. *P<0.05 vs. Saline (Wilcoxon test).*

(a) LPS bolus

	Control			AM group			IMD group		
	1.5h	6h	25h	1.5h	6h	25h	1.5h	6h	25h
HR (beats min^{-1})	↑	↑	↔	↑	↑	↔	↑	↑	↔
MAP (mmHg)	↓	↔	↔	↔	↓	↑	↔	↔	↔
RVC (units)	↑	↑	(↑)	↔	↑	↑	↔	↑	↑
MVC (units)	↑	↑	↔	↔	↑	↑	↔	↑	↑
HVC (units)	↑	↑	↔	↔	↑	↑	↔	↑	↑

(b) LPS infusion

	Control			AM group			IMD group		
	1.5h	6h	25h	1.5h	6h	25h	1.5h	6h	25h
HR (beats min^{-1})	↑	↑	↑	↑	↑	↑	↑	↑	↑
MAP (mmHg)	↓	↔	↔	↓	↔	↑	↓	↔	↑
RVC (units)	↑	↑	↑	↑	↑	↑	↑	↑	↔
MVC (units)	↓	↓	↔	↔	↓	↔	↔	↔	↓
HVC (units)	↑	↔	↑	↑	↔	↔	↑	↔	↔

Table 5.3 *Summary of baseline cardiovascular variables at selected time points after administration of* **(a)** *bolus LPS (1mg kg^{-1})* **(b)** *from the onset of LPS infusion (15μg kg^{-1} h^{-1}), in control groups and in animals that were to receive peptide challenges.*

↔ denotes that there was no change in that variable vs. saline time control; ↑ denotes an increase and ↓ a decrease.

VC=vascular conductance. Units for VC are kHz $mmHg^{-1}$ x 10^3.

		+/-AM_{22-52} infusion		Time from end of infusion					
		start	end	5min	10min	15min	20min	25min	30min
HR (beats/min)	+AM_{22-52}	425±10	424±10	415±8	414±8*	436±5	441±8	443±6	446±8
	-AM_{22-52}	429±9	435±6	434±9	436±10	433±11	452±11	455±11	453±11
MAP (mmHg)	+AM_{22-52}	103±3	107±4	108±3	108±3	106±2	108±4	107±3	105±3
	-AM_{22-52}	102±3	108±2	110±2	111±3	111±2	113±3	111±4	110±4
RVC (units)	+AM_{22-52}	96±14	95±15	96±15	95±15	100±16	100±15	103±16	104±16
	-AM_{22-52}	88±8	87±9	86±8	85±9	87±8	88±9	84±7	88±7
MVC (units)	+AM_{22-52}	62±9	47±7	46±7	47±8	47±8	48±7	53±9	55±8
	-AM_{22-52}	64±9	50±7	45±5	42±4	45±4	46±4	46±3	49±3
HVC (units)	+AM_{22-52}	62±5	53±6	52±6	50±5	51±5	50±5	52±6	54±6
	-AM_{22-52}	54±4	48±4	47±4	44±4	45±4	41±3	42±4	41±4

Table 5.4 *Cardiovascular variables 1.5 h after treatment with LPS, in the presence (n=8) and absence (n=8) of AM_{22-52} (500nmol kg^{-1} h^{-1}, for 6 min). Units for vascular conductance (VC) are kHz $mmHg^{-1}$ x 10^3.*

**P<0.05 vs. LPS time control (Mann Whitney U test).*

		AM_{22-52} infusion	
		Start	*End*
HR (beats min^{-1})	6h	426±7	444±10
	25h	386±9	407±12
MAP (mmHg)	6h	97±5	101±5
	25h	105±3	105±5
RVC (units)	6h	113±20	113±5
	25h	99±15	104±16
MVC (units)	6h	113±14	111±14
	25h	86±9	94±1
HVC (units)	6h	65±10	65±10
	25h	56±7	55±6

Table 5.5 *Cardiovascular changes at the beginning and end of AM_{22-52} (500nmol kg^{-1} h^{-1}, for 6 min) administration, 6 h and 25 h (n=8, each time point) from LPS-treatment in conscious rats.*

VC=vascular conductance. Units for VC are kHz $mmHg^{-1}$ x 10^3.

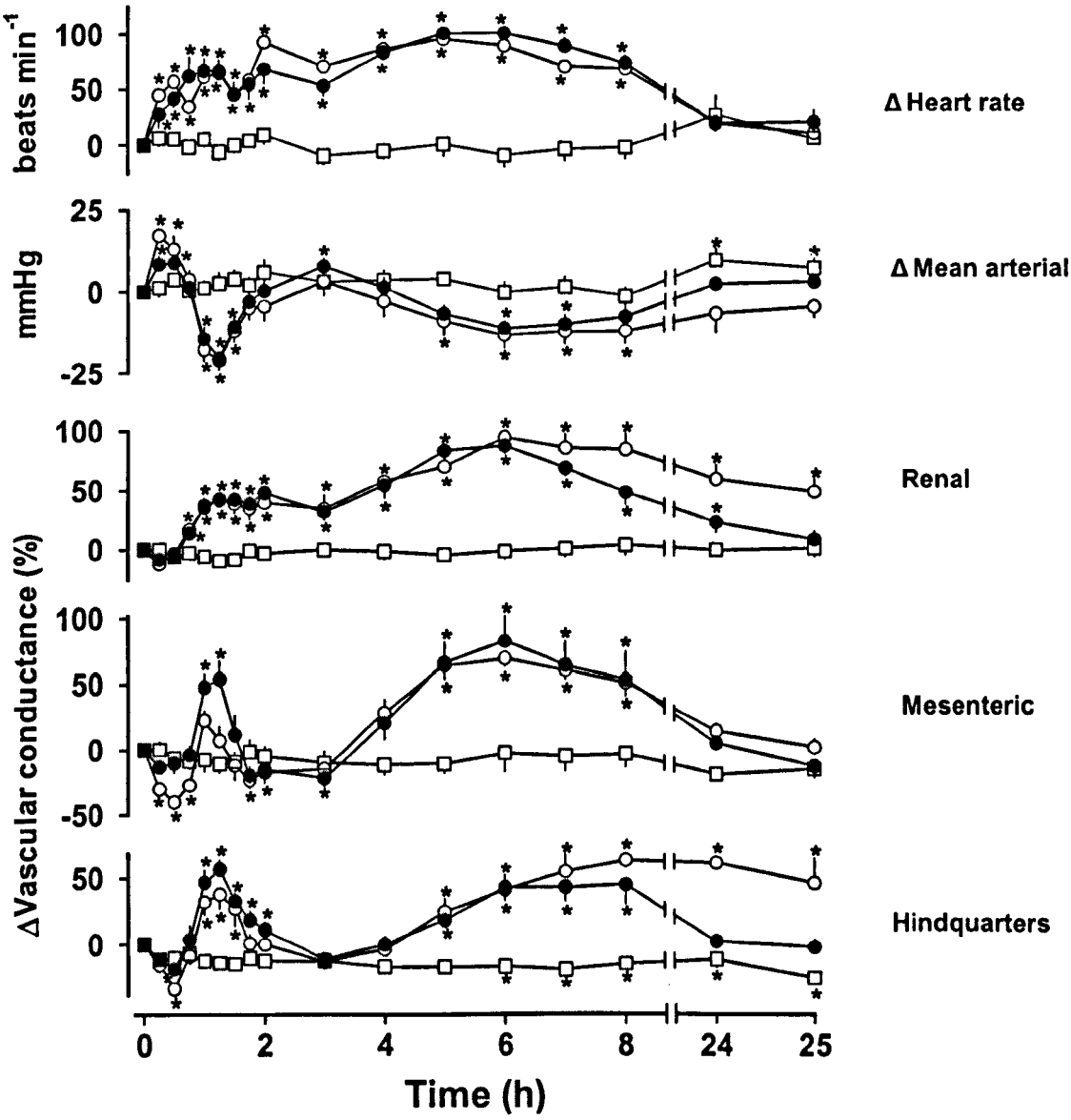


Figure 5.3.1 *Cardiovascular changes in Sprague-Dawley rats, over 25 h following administration of LPS 1mg kg^{-1} i.p. bolus (n=9) (closed circles), LPS 3mg kg^{-1} i.p. bolus(n=8) (open circles) or saline 0.5ml i.p. bolus (n=7) (open squares)*

**P<0.05 versus original baseline (Friedman's test)*

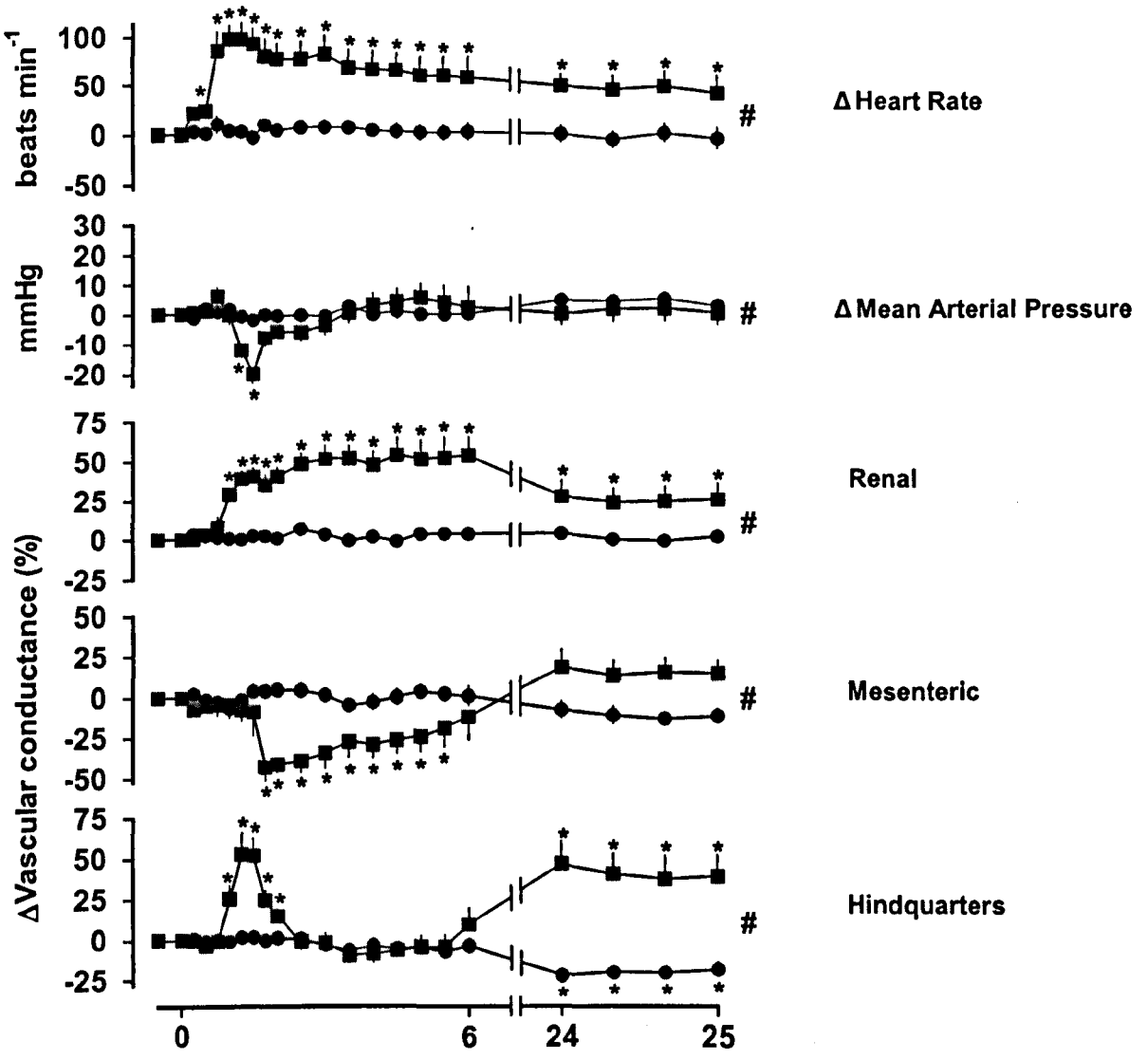


Figure 5.3.1a *Cardiovascular changes during a 25 h continuous infusion of LPS (15μg kg^{-1} h^{-1} i.v.) (n=8) (closed squares), or saline (0.4ml h^{-1}) (n=8) (closed circles).*

**P<0.05 versus original baseline (Friedman's test..#P<0.05 for integrated between-group differences (Mann Whitney test).*

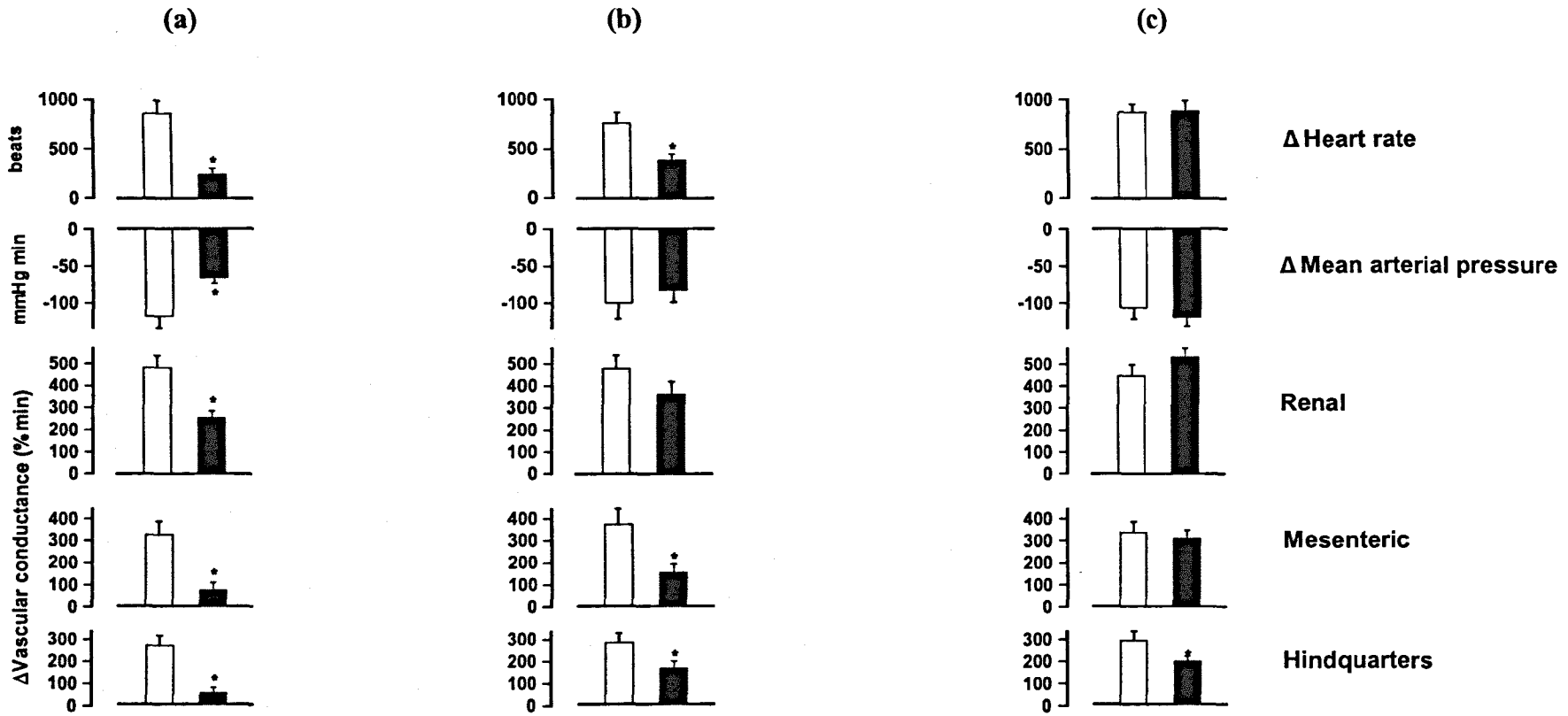


Figure 5.3.2 *Integrated (0-10min) cardiovascular changes following AM (3nmol kg^{-1} i.v.) administration* **(a)** *1.5 h,* **(a)** *6h and* **(c)** *25 h after saline (0.5 ml i.p.) (open bars) or LPS (1mg kg^{-1} i.p.) (closed bars) treatment in conscious Sprague Dawley rats (n=10). Values are mean and vertical bars represent s.e.m.*

**P<0.05 vs. original baseline (Friedman's test). #P<0.05 vs. integrated (0-10 min) changes between groups (Wilcoxon's test).*

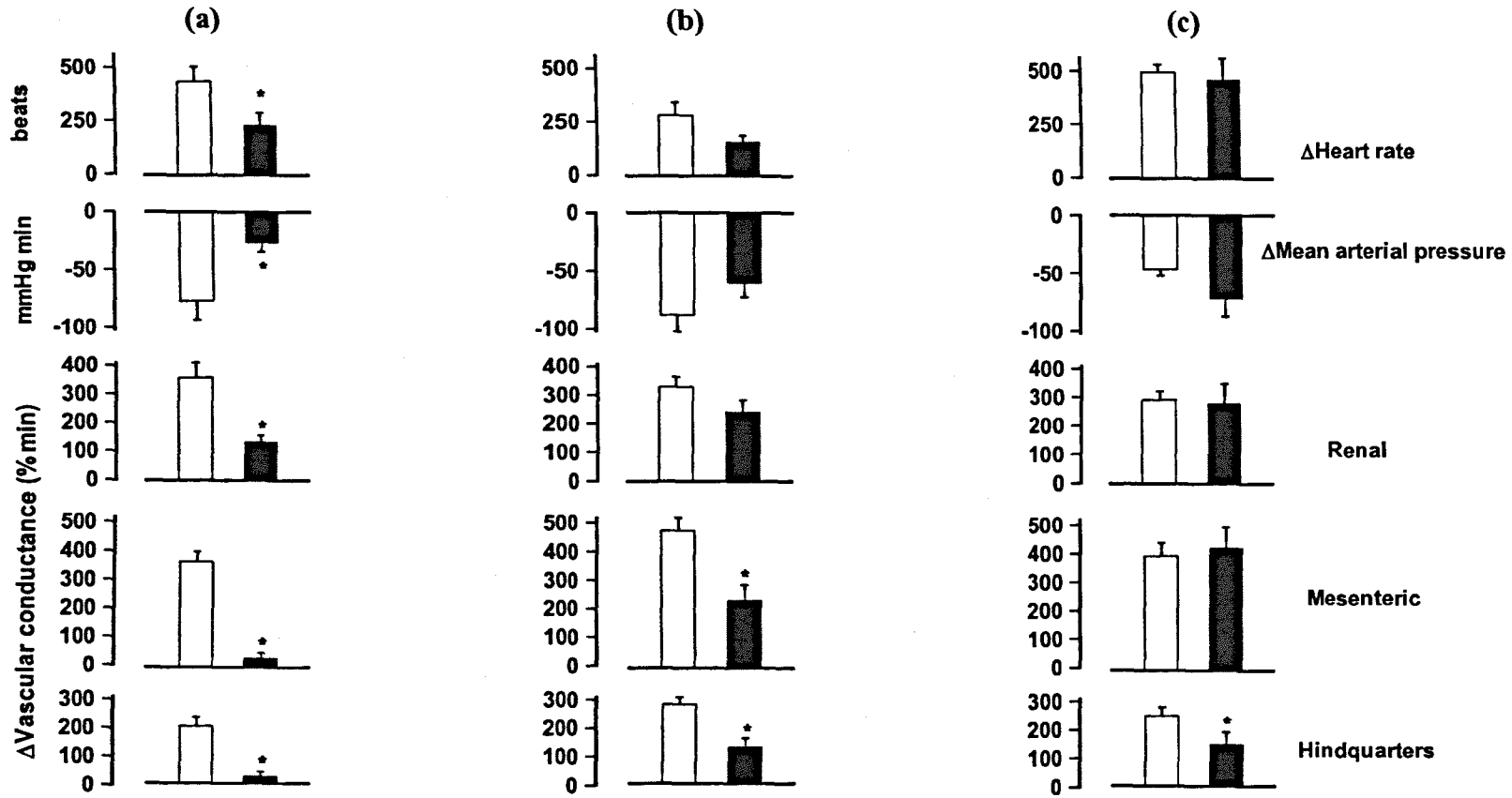


Figure 5.3.3 *Integrated (0-10min) cardiovascular changes following IMD (1nmol kg^{-1} i.v.) administration* **(a)***1.5 h (n=8),* **(b)** *6h (n=9) and* **(c)** *25 h (n=9) after saline (0.5 ml i.p.) (open bars) or LPS (1mg kg^{-1} i.p.) (closed bars) treatment in conscious Sprague Dawley rats. Values are mean and vertical bars represent the s.e.m.*

$^{*}P<0.05$ vs integrated (0-10 min) changes between groups (Wilcoxon's test).

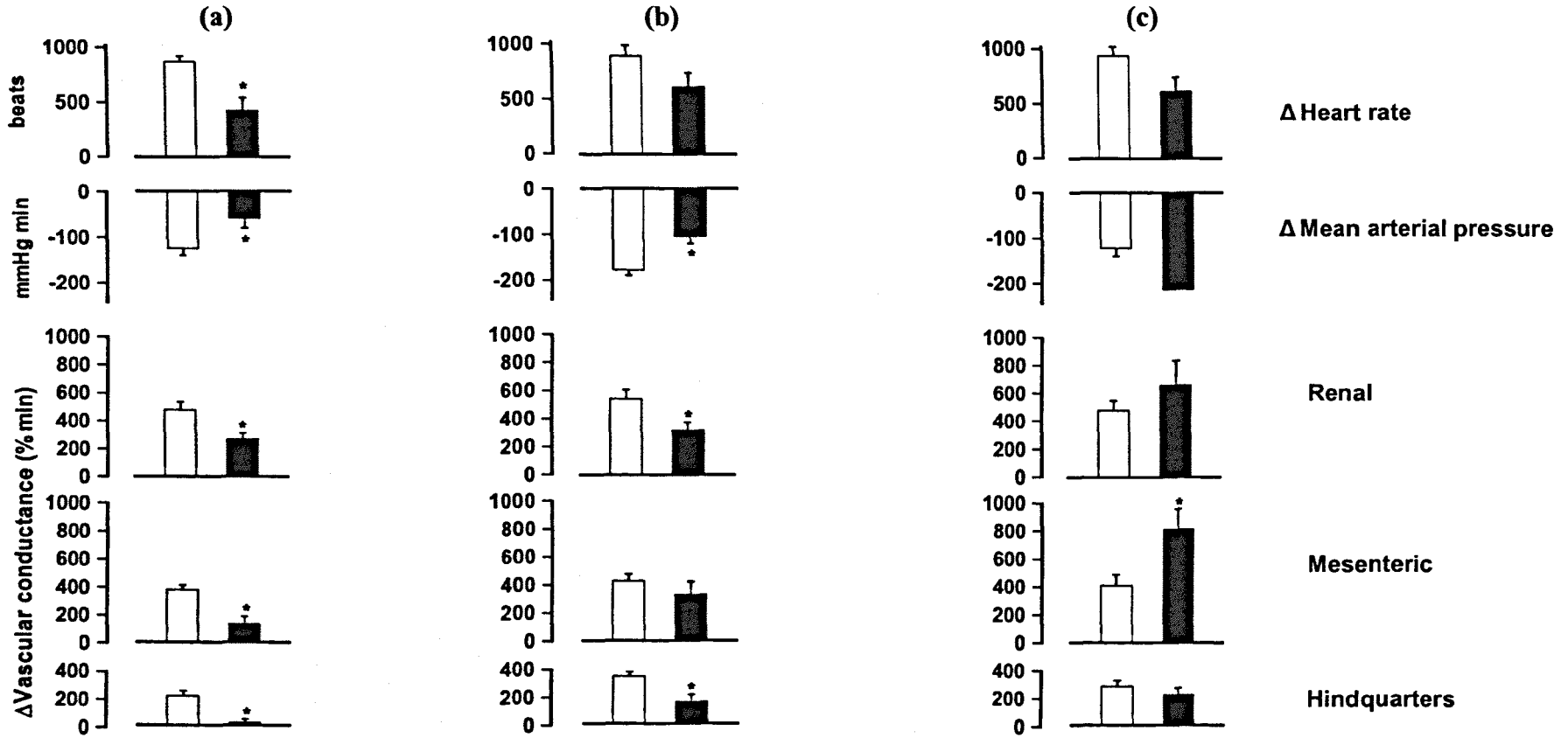


Figure 5.3.4 *Integrated (0-10min) cardiovascular changes following AM (3nmol kg^{-1} i.v.) administration* **(a)** *1.5 h (n=9),* **(b)** *6h (n=9) and* **(c)** *25 h (c) (n=8) from the onset of saline (0.4 ml h^{-1}.) (open bars) or LPS (15 μg kg^{-1} h^{-1} i.v.) (closed bars) treatment in conscious Sprague Dawley rats. Values are mean and vertical bars represent the s.e.m.*

**P<0.05 vs. integrated (0-10 min) changes between groups (Wilcoxon's test).*

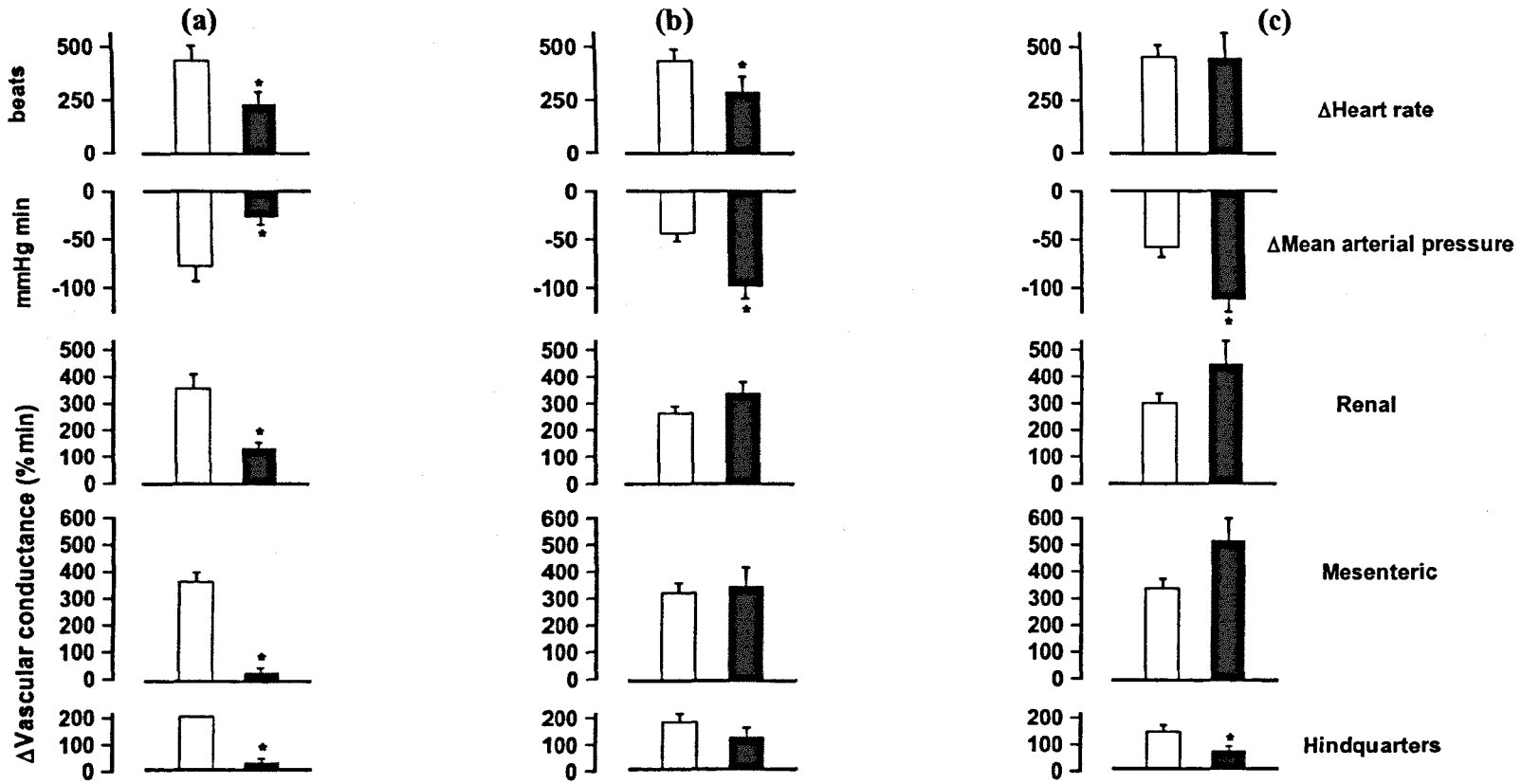


Figure 5.3.5 *Integrated (0-10min) cardiovascular changes following IMD (1nmol kg^{-1} i.v.) administration* **(a)** *1.5 h (n=8),* **(b)** *6h (n=9) and* **(c)** *25 h (n=9) from the onset of saline (0.4 ml h^{-1}.) (open columns) or LPS (15 μg kg^{-1} h^{-1} i.v.) (closed columns) treatment in conscious Sprague Dawley rats. Values are mean and vertical bars represent the s.e.m.*

**P<0.05 vs integrated (0-30 min) changes between groups (Wilcoxon's test).*

5.4 Discussion

Clinical sepsis is associated with a complex haemodynamic profile, the nature of which changes as the severity of the disorder increases. Animal models have attempted to mimic some of the haemodynamic manifestations of clinical sepsis, commonly using administration of LPS. Whilst high doses of LPS reportedly lead to the development of an hypodynamic circulation and rapid cardiovascular collapse (for review see Buras *et al.*, 2005), lower doses lead to the development of an hyperdynamic circulation which is often observed during early clinical sepsis. The present chapter describes the haemodynamic profiles associated with three LPS administration protocols in conscious, Sprague-Dawley rats.

Each 'model' described here was associated with tachycardia (0-8 h for LPS bolus; 0-25 h for LPS infusion), an early, transient fall in blood pressure, sustained renal vasodilatation and a biphasic hindquarters vasodilatation. The response in the mesenteric vascular bed differed such that that there was a biphasic vasodilatation in response to bolus administration of LPS, but a marked vasoconstriction (from 1-5 h) when LPS was given as an infusion. The mesenteric vascular response in Long-Evans rats infused with LPS at a dose 10-fold higher than that used here (i.e. 150 $\mu g\ kg^{-1}\ h^{-1}$), was somewhat intermediate between the different responses reported here, comprising a small vasoconstriction from 2-3 h, followed by a small vasodilatation (Gardiner *et al.*, 1995). Thus, the response to LPS in this vascular bed appears to be the most variable. At the 24 h time-point, the LPS-infused ($15\mu g\ kg^{-1}\ h^{-1}$) group was dilated in all 3 vascular beds, and was tachycardic, although normotensive, thus displaying the hallmarks of a hyperdynamic circulation.

Animals given LPS as a bolus were normotensive and somewhat vasodilated at 24h, as were the Long-Evans rats studied by Gardiner *et al.* (1995). Regional and temporal differences in vascular conductance suggest that opposing vasodilator and vasoconstrictor systems are differentially regulated depending upon the LPS administration protocol.

Since LPS caused changes in haemodynamics which were both regionally and temporally variable (see above), peptide challenges were assessed at multiple time points from the onset of sepsis to determine whether responses were affected in a time-dependent manner. The time points chosen were 1.5 h from LPS, representing the first phase of hypotension and vasodilatation, at 6 h, representing the second phase of hypotension and vasodilatation, and at 25 h, when haemodynamics were stable (infusion model) or are beginning to return to normal (bolus model).

The LPS-administration protocols used in the present study were assessed because Sprague-Dawley rats turned out to be more sensitive to the effects of LPS than were the Long-Evans rats studied previously in the Nottingham Haemodynamics Laboratory (Gardiner *et al.*, 1995). Furthermore, it became clear there were differences in the cardiovascular responses to LPS between groups in the present study (summarised in Table 5.3). Thus, the control LPS bolus (1mg kg^{-1}) group was widely vasodilatated and hypotensive at the 1.5 h time point, whilst the LPS-treated groups which were to be administered peptide, were not. Since the latter received LPS on Day 3 after catheter implantation, and the control LPS group received LPS on Day 1 (see section 5.2), it is feasible that the differences were due to the different times elapsed

after anaesthesia and surgery. Furthermore, whilst the LPS control group were naïve to peptides, the AM and IMD groups were not, since these were paired experiments, this may also help to explain the baseline differences between the groups. Although the haemodynamic response to LPS was qualitatively similar between groups, it was not always quantitatively the same at any given time point, and this is acknowledged as a drawback to these studies.

Vascular hyporesponsiveness to AM has been reported in a rat model of haemorrhagic shock, 1.5 h after fluid resuscitation (Wu *et al.*, 2005), and 20 h from induction of CLP-sepsis, despite increased expression of AM gene and protein (Wang *et al.*, 1999; Yang *et al.*, 2002; Zhou *et al.*, 2002). Furthermore, evidence suggests that decreased responsiveness to AM is involved in the transition from a hyper- to a hypo-dynamic circulation in late CLP-sepsis (i.e. from 20 h onwards) (Wang *et al.*, 1999). The newly identified AM-like peptide, IMD, has also been shown to have marked renal and cardiovascular effects (see Chapter 3; Bell & McDermott, 2008), but nothing is presently known about the peptide in endotoxaemia. Since IMD has yet to be studied in this context, one of the aims of these experiments was to investigate whether regional vascular responsiveness to IMD was affected across time from LPS treatment, and, if so, how any changes compared to those seen with AM, in conscious rats.

The present work showed a fall in vascular responsiveness to both AM and IMD 1.5 h from LPS treatment, prior to the development of regional LPS-induced vasodilatations (Table 5.1). At 6h there was some return of

responsiveness, despite the animals showing renal, mesenteric and hindquarters vasodilatations at that stage. Hence, although it might be expected that pre-existing vasodilatations would diminish vasodilator responses, this was not the case with AM and IMD. By 25 h, responsiveness to both peptides had almost returned to control levels, and, although there was still a significantly lower resting blood pressure with renal, mesenteric and hindquarters vasodilatations in the LPS group, these effects were less marked than they were at 6 h (Figure 5.3.2 and 5.3.3; Table 5.1). It is possible that this progressive return of vascular responsiveness to AM and IMD was, due to the passage of time from bolus administration of LPS, in line with the finding that the prolonged presence of LPS *in vitro* sustains the period of vascular hyporeactivity to vasoconstrictors, and is more important than the dose of LPS (O'Brien *et al.*, 2001), but it is not known if vasodilators are affected in similar way.

To determine if the maintained presence of LPS affected vascular sensitivity to AM and IMD *in vivo,* experiments were repeated in rats treated with a low dose LPS-infusion over 24 h vascular hyporesponsiveness to AM and IMD was apparent 1.5 h from the onset of a low-dose LPS infusion (15μg kg^{-1} h^{-1} i.v.), as was observed at this juncture in the bolus model. At the 6 h time-point, although some loss of responsiveness to AM and IMD was seen in the LPS bolus model, regional vasodilator responses were not different from controls in animals receiving a low dose LPS infusion (Figure 5.3.4 and 5.3.5), thus vascular responsiveness returned more rapidly in this model. Interestingly, at this time point, there was an augmented hypotensive response to IMD in the LPS-infused group, without any difference in the changes in regional vascular

conductance. Since the IMD-mediated tachycardia was also reduced, this suggests some depression of cardiac function at this time point in this model. At 25 h from the onset of LPS infusion, vasodilator responses to AM and IMD were augmented in some vascular beds. Since, mesenteric vascular conductance was reduced at this time it is possible that augmentation of vasodilator responses was due to this pre-existing vasoconstriction (Table 5.1).

Results from Chapter 4 indicated that NO was involved in IMD-mediated vasodilatation in the mesenteric, and to some extent in the renal, vascular bed (Figure 4.3.4). Hence, since IMD-mediated mesenteric vasodilatation was impaired to a greatest extent than that to AM 1.5 h from LPS administration, and no support of a role for NO was found in AM-mediated vasodilatation, it is possible that reduced NO release, possibly via impaired eNOS bioactivity (see Introduction), had some involvement in the vascular hyporesponsiveness to IMD observed here in LPS-treated rats. Since endothelial-dependent vasodilatation is impaired in sepsis, but responses directly stimulating the vascular smooth muscle are unaffected (Cameron *et al.*, 1998; Hingorani *et al.*, 2000; Piepot *et al.*, 2003), this finding may suggest that IMD and AM-mediated mesenteric and hindquarters vasodilatations are largely endothelium-dependent, whilst IMD and AM-mediated renal vasodilatation has a component which is endothelium-independent. To further study the mechanisms responsible, experiments could be conducted using known endothelium-dependent (ACh and/or BK) and -independent (SNP) agonists to assess whether regional vascular responses were impaired at different time

points from LPS exposure. Antagonists of the cGC/cGMP pathway could also be employed to determine which (if any) step in this pathway was impaired.

Excessive activation of K_{ATP} channels, and increased channel subunit expression are believed to be involved in the development of vascular hyporeactivity during endotoxaemia (Sorrentino *et al.*, 1999; Buckley *et al.*, 2006) and this is linked to enhanced NO production in the early stages. Indeed, a role for K_{ATP} channels in the development of regional vasodilatations has been previously reported within 5 h of LPS treatment, and at 24 h, in both conscious and anaesthetised rats, (Gardiner *et al.*, 1999; Sorrentino *et al.*, 1999) and may be reflected in the baseline vasodilatations reported here in LPS-treated animals. It has been suggested that LPS modifies K_{ATP} channel pharmacology, preventing effective functioning of mediators involved in their control (Buckley *et al.*, 2006). While it is possible that LPS-induced changes in K_{ATP} channel pharmacology or configuration were responsible for the changes in responses to AM and IMD observed here, the K_{ATP} channel blocker, U37883A, had no effect on responses to AM or IMD in normal animals (Chapter 4). Thus, it appears unlikely that changes in K_{ATP} channels underlie changes in responses to AM or IMD following LPs administration

Alternatively, a receptor-mediated mechanism could account for diminished AM and IMD-mediated vasodilatations in LPS-treated rats. Calcitonin family peptides signal through a unique receptor system comprising the calcitonin-receptor-like-receptor (CL) and one of three receptor-activity-modifying-proteins (RAMP1-3) (as discussed in Chapter 4). Nagoshi *et al.* (2004)

showed that RAMP1, RAMP2 and CL mRNA levels were significantly reduced in human coronary artery smooth muscle cells following incubation with TNF-α, which was associated with reduced AM and CGRP receptor activity, as determined by an assay for peptide-induced increases in cAMP. Hence, TNF-α-mediated inhibition of CL/RAMP expression could be responsible for reducing responses to AM and IMD in the presence to LPS, since TNF-α expression is induced by LPS (Nagoshi *et al.*, 2004), and peaks by 1.5 h (Waller *et al.,* 1995). In addition, because CL and RAMP2 levels in the lungs of LPS-treated mice declined by 95%, after 12 h, and since the majority of CL has been shown to be complexed with RAMP2 in normal mice (Gibbons *et al.*, 2006), this could partly account for the loss of vascular responsiveness to these peptides 1.5 h after LPS-treatment. A further study found reduced RAMP2 expression in the lungs of mice, with progressive falls from 0.5 h to 3 h after LPS injection. In terms of AM signalling, this was compensated for by a later (from 6 h onwards) increase in RAMP3 mRNA. The authors suggest that reduced receptor-bound AM, due to reduced expression of receptor components, accounted for the increased plasma concentration of AM in early sepsis (Ono *et al.* 2000). Since AM and IMD have been shown to activate the same receptors (Roh *et al.*, 2004; see Chapter 4), LPS-mediated down-regulation of their receptor components may, in part, account for the loss of vascular responsiveness to these peptides, as reported here.

Increased levels of endogenous AM have been reported during the early stages of sepsis (for review see Wang *et al.*,1999), so it is possible that the observed

vascular hyporesponsiveness to exogenous AM 1.5 h from LPS-treatment was due to increased levels of the endogenous peptide already bound to AM receptors. To address this point, the effects of the AM receptor antagonist, AM_{22-52}, was assessed in animals treated with LPS (1mg kg^{-1} i.p.). This antagonist would be expected to evoke vasoconstriction in regions where endogenous AM was causing vasodilatation through AM_{22-52}-sensitive receptors, but we found no effect of AM_{22-52} on regional haemodynamic responses following LPS-treatment in conscious rats (Table 5.2). This may suggest that, in this model, endogenous AM was not contributing to the regional haemodynamic responses to LPS. However, evidence suggests that AM_{22-52} has greater selectivity for AM_1 (CL/RAMP2) than for AM2 (CL/RAMP3) receptors (Hay *et al.*, 2003). As discussed above, the RAMP2 component of the AM_1 receptor is down regulated following exposure to LPS (Nagoshi *et al.*, 2004; Gibbons *et al.*, 2006). Therefore, if RAMP2 levels are reduced during early endotoxaemia, there is an unavailability of endogenous, AM_{22-52}-sensitive receptors due to them being down regulated rather than to their being occupied by endogenous AM. If reduced receptor levels are indeed responsible for the reduction of vascular sensitivity to AM during early endotoxaemia (i.e. at 1.5 h from onset), then antagonists selective for such receptors will tell us little about activity of endogenous peptides at these targets. Furthermore, it is possible that high levels of AM were responsible for vasodilatation through AM2 receptors and were not affected by AM_{22-52}. Mazzocchi *et al.* (2000) co-administered AM_{22-52} (3nmol kg^{-1}) to rats with LPS (500μg kg^{-1} i.p.) and showed that the LPS-induced fall in blood pressure was reduced to 50% of that observed with LPS alone, by 2-3 h from treatment.

Between 3 h and 6 h, LPS-induced hypotension was reversed when it was co-administered with AM_{22-52} (Mazzocchi *et al.*, 2000). This clearly suggests a role for endogenous AM in LPS-induced hypotension. The discrepancy between the above study and the one presented here is the method of AM_{22-52} administration, with the former giving the antagonist as a pre-treatment prior to the rise in plasma AM, whilst in the latter it was given at a time when AM levels may already have been high. Thus, it is possible that, at the point AM_{22-52} was given in the present study, AM was already acting on its receptors and could not be displaced by the antagonist.

In conclusion, the present studies showed that vascular responsiveness to AM and IMD were significantly attenuated 1.5 h from LPS treatment in conscious rats, and that responsiveness returned as a function of time after LPS administration. Whilst several mechanisms may have been involved (see above), until the pharmacology of IMD is more fully characterised, the exact role for each in IMD action during endotoxaemia, and any therapeutic value this may have, remain to be seen. Since AM has been implicated in the development of a hyperdynamic circulation during early sepsis, it is possible that there may be a similar role for IMD in the clinical condition.

Chapter 6

Results (IV)

Cardiovascular responses to adenosine receptor activation and antagonism in normal and LPS-treated rats

Summary

In this chapter, the regional haemodynamic responses to adenosine agonists and antagonists were examined in normal and LPS-treated rats, since studies have shown that adenosine-mediated vasodilatations play an important role in the maintenance of regional perfusion during sepsis.

Male Sprague–Dawley rats were chronically instrumented with pulsed Doppler flow probes to measure regional haemodynamic responses to adenosine-receptor agonists (adenosine, 2-choloro-N^6-cyclopentyladenosine (CCPA)) and antagonists (8-phenyltheophylline (8-PT), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX)), at selected time points in control and LPS-treated rats.

The responses to 8-PT were consistent with endogenous adenosine causing bradycardia, and renal and hindquarters vasodilatation in control rats, whereas in LPS-treated rats, there was evidence for endogenous adenosine causing renal (at 1.5 h) and hindquarters (at 6 h) vasoconstriction. In control animals, exogenous adenosine caused hypotension, tachycardia and widespread vasodilatation, whereas in LPS-treated rats, the adenosine-induced renal (at 1.5 h) and hindquarters (at 6 h) vasodilatations were abolished. As enhanced A_1 receptor-mediated vasoconstriction could explain the results in LPS-treated rats, vascular responsiveness to a selective A_1-receptor agonist (CCPA) or antagonist (DPCPX) was assessed. There was no evidence for enhanced vasoconstrictor responsiveness to CCPA in LPS-treated rats, but DPCPX

caused renal vasodilatation, consistent with endogenous adenosine mediating renal vasoconstriction under these conditions.

The results show changes in adenosine receptor-mediated cardiovascular effects in endotoxaemia that may have implications for the use of adenosine-based therapies in sepsis

6.1 Introduction

The development of clinical sepsis is associated with complex haemodynamic changes which include a hyperdynamic phase and a hypodynamic stage as the severity of the condition increases. During the hyperdynamic phase, patients may remain normotensive, despite systemic vasodilatation, due to an increase in cardiac output. A role for adenosine in the haemodynamic changes associated with sepsis during this early stage has been suggested by a number of researchers (Martin *et al.*, 2000; Law *et al.*, 2003; Conlon *et al.*, 2005).

Adenosine interacts with 4 receptors (A_1, A_{2a}, A_{2b} and A_3; see Introduction) (Fredholm *et al.*, 2001b), and evidence suggests that A_1 and A_2-receptors are primarily responsible for the cardiovascular actions of adenosine (Fredholm *et al.*, 2001a). A_1-receptors are linked to G_i causing inhibition of cAMP activity, which leads to cardiac depression, a reduction in heart rate, renal vasoconstriction (Evans *et al.*, 1982; Webb *et al.*, 1990; Fredholm *et al.*, 2001a; Hansen *et al.*, 2005) and skeletal muscle vasodilatation (Marshall *et al.*, 1993; Ray *et al.*, 2002; Ray *et al.*, 2006). A_{2a}-receptors are located on vascular smooth muscle, endothelial cells and on immune cells, and are linked to G_s, leading to cAMP activation (Mubagwa *et al.*, 1996; Fredholm *et al.*, 2001a; Nemeth *et al.*, 2006), vasodilatation and immunosuppression (Webb *et al.*, 1990; Nemeth *et al.*, 2006). A_{2b} receptor activation may also lead to regionally-selective vasodilatation, but the lack of pharmacological probes selective for A_{2b} receptors means this subtype is poorly characterised (Feoktistov *et al.*, 1997).

Endogenous adenosine has been shown to contribute to changes in regional blood flow distribution during sepsis, thereby contributing to the development of a hyperdynamic circulation. For example, Motew *et al.* (1998) showed that, 24 h after a septic challenge, the non-selective adenosine receptor antagonist, 8-phenyltheophylline (8-PT), caused vasoconstriction (selective for skeletal muscle, hepatic portal and cerebral circulations), indicating endogenous adenosine-mediated vasodilator tone (Motew *et al.*, 1998). However, in that study, the animals were not vasodilated at the time of antagonist administration, and the antagonist responses were only considered at one time point (i.e. 24 h) following the septic challenge.

To extend those observations, the aim of the present study was to determine the effects of adenosine receptor agonism and antagonism at different times, in a model of endotoxaemia (lipopolysaccharide (LPS) administration) in which regionally and temporally-selective vasodilatation occurs. It was hypothesised that adenosine receptor antagonism would cause vasoconstriction in regions where endogenous adenosine was acting as a vasodilator. However, the results of the first experiments, contrary to expectation, were consistent with up-regulation of A_1 receptor-mediated vasoconstrictor effects following LPS administration. Therefore, further experiments were performed to assess the hemodynamic responses to A_1 receptor activation and antagonism. It is well reported that A_1 receptor activation causes inhibition of renin release (Tagawa *et al.*, 1970; Churchill *et al.*, 1985; Weihprecht *et al.*, 1990), and since there was a vasoconstrictor effect of A_1 receptor antagonism in the control rats (see Results), a final set of experiments was performed in the presence of the angiotensin (AT_1) receptor

antagonist, losartan, to assess if the vasoconstrictor effects of A_1 receptor antagonism were secondary to renin-angiotensin system activation.

6.2 Methods

All experiments were performed in male Sprague-Dawley rats, as outlined in Chapter 2. At the first surgical stage, Doppler flow probes (to measure renal, mesenteric and hindquarters blood flows) were sutured around the left renal artery, the superior mesenteric artery and the distal abdominal aorta below the level of the ileocaecal artery (for hindquarters). At least 10 days after probe implantation, catheters were inserted in the right jugular vein (up to 3 catheters) and the peritoneal cavity for substance administration, and in the distal aorta (via the caudal artery), for continuous recording of arterial blood pressure and heart rate. Experiments began at least 24 h after catheter implantation, in unrestrained, conscious animals.

6.2.1 Experiment 1: Cardiovascular effects of 8-phenyltheophylline (8-PT) in normal and LPS-treated rats

Responses to the non-selective adenosine receptor antagonist, 8-PT (4mg kg^{-1} in saline given as a 0.1ml bolus), were assessed 1.5 h, 6 h and 25 h after bolus administration of saline (0.5 ml i.p.) (n=9), or LPS (1 mg kg^{-1} i.p.) (n = 9) to determine whether endogenous adenosine affected resting vascular tone in normal or endotoxaemic animals, and whether any effects of 8-PT changed across time. This antagonist shows equal selectivity for A_1- and A_2-receptors (Fredholm *et al.*, 1987), and the dose was that used by Motew et al. (1998). In pilot experiments it was shown to fully antagonise the cardiovascular effects of adenosine (300 μg kg^{-1} min^{-1} i.v.) for up to 60 min. Those pilot experiments also indicated that adenosine-mediated responses had returned to normal

within 6 h following 8-PT administration. This experimental protocol is illustrated below.

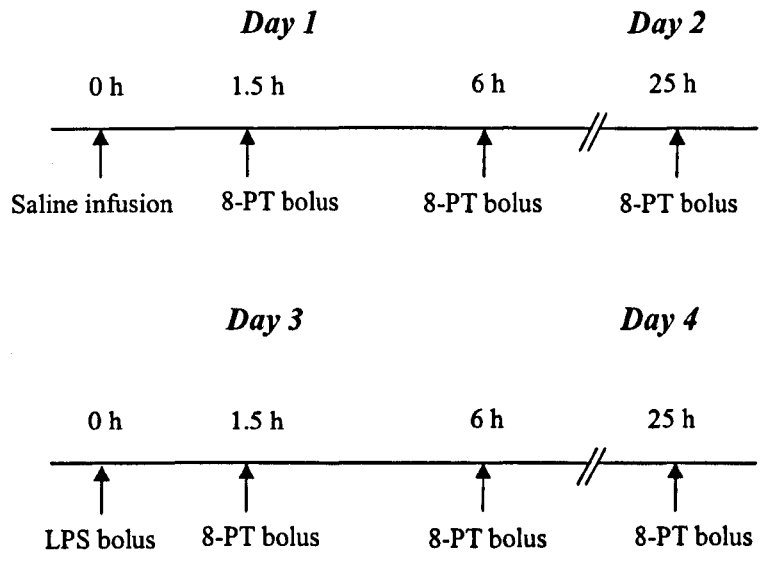


Figure 6.2.1 *Saline was given as a bolus at time 0 on Day 1, and LPS at time 0 h on Day 3. Animals were administered 8-PT 1.5 h, 6 h and 25 h later.*

6.2.2 Experiment 2: Cardiovascular responses to adenosine in normal and LPS-treated rats

To determine whether the vascular sensitivity to adenosine was altered following LPS treatment, cardiovascular changes during 3 min infusions of adenosine (300μg kg^{-1} min^{-1} i.v. in saline 0.15 ml min^{-1}) were assessed 1.5 h, 6 h and 25 h following bolus administration of saline (0.5 ml i.p.) on day 1 and following LPS (1 mg kg^{-1} i.p.) on day 3 (n=9). The dose of adenosine was chosen on the basis of pilot studies which showed it to cause robust and reproducible vasodilatations in normal animals.

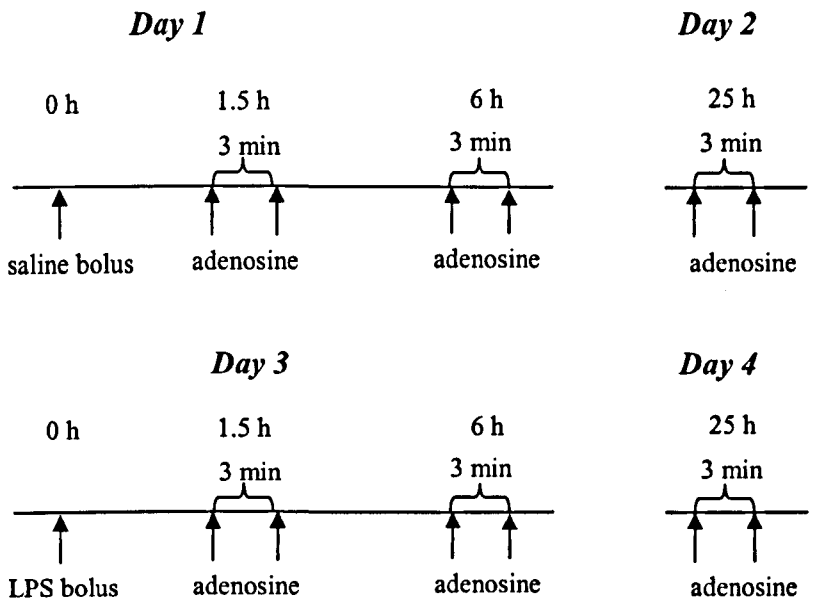


Figure 6.2.2 *Animals were treated with a saline and LPS bolus at time 0 h on Day 1 and 3 respectively, and were administered adenosine 1.5 h, 6 h and 25 h later.*

Since the responses to 8-PT and adenosine had returned to baseline 25 h after administration of LPS (see Results), the following experiments were only performed at 1.5 h and at 6 h. This protocol is shown in Figure 6.2.2.

6.2.3 Experiment 3: Cardiovascular responses to 2-choloro-N^6-cyclopentyladenosine (CCPA)

Haemodynamic responses during 3 min infusions of the A_1-receptor agonist, CCPA (1.4 μg kg^{-1} min^{-1} i.v. (0.15 ml min^{-1} in vehicle containing 5% propylene glycol, 2% Tween-80 in saline) were assessed in one group of animals (n=8) at 1.5 h after saline (0.5 ml i.p.) on day 1 and 1.5 h after LPS (1 mg kg^{-1} i.p.) on day 3, and in another group of animals (n=8) at 6 h after saline

(as above) on day 1 and 6 h after LPS (as above) on day 3. Results from pilot studies showed that, at the dose used, CCPA caused reproducible changes in regional vascular conductances which were consistent with A_1-receptor activation as reported in the literature. Different animals were used at the 2 time points because of desensitisation to this agonist (Casati *et al.*, 1994). This protocol is illustrated below.

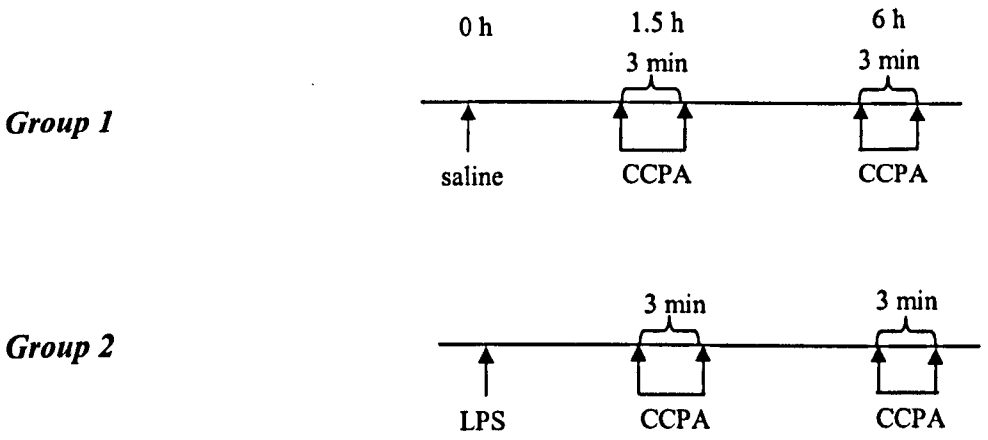


Figure 6.2.3 *Animals were given either saline or LPS at time 0, followed by a 3 min infusion of CCPA 1.5 h and 6 h later.*

6.2.4 Experiment 4: Cardiovascular effects of 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) in normal and LPS-treated rats

Responses to the A_1-receptor antagonist, DPCPX (0.1 mg kg^{-1} in 0.1ml of vehicle containing 5% propylene glycol, 2% Tween-80 in saline), were assessed at 1.5 h and 6 h following saline (0.5 ml i.p.) on Day 1 and at 1.5 h and 6 h after LPS (1 mg kg^{-1} i.p.) on Day 3 (n=8). The dose and method of delivery of DPCPX was chosen on the basis of the literature showing

successful administration of the antagonist *in vivo* (Kellett *et al.*, 1989; Bryan *et al.*, 1999), and on pilot studies conducted prior to beginning these experiments. Difficulty in dissolving the antagonist limited its usability when given as an infusion, but at the dose given, we were able to completely antagonise the effects of CCPA, and the antagonism had reversed by 6 h; this time course of action is consistent with the literature (Kellett *et al.*, 1989).

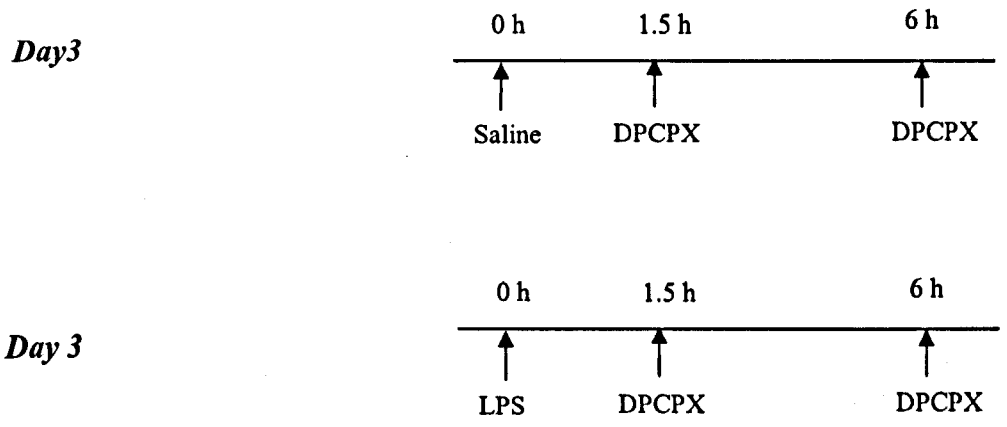


Figure 6.2.4 *Animals were given saline or LPS at time 0 on Day 1 and Day 3 respectively, followed by bolus administration of DPCPX 1.5 h and 6 h later.*

6.2.5 Experiment 5: Cardiovascular effects of DPCPX following pre-treatment with losartan

Antagonism of A_1 receptors can cause renin release, and hence any cardiovascular effect could be secondary to activation of the renin-angiotensin system (Tagawa *et al.*, 1970; Jackson *et al.*, 1991). To assess whether the regional haemodynamic responses to DPCPX were due to direct A_1-receptor antagonism or due to renin-angiotensin system activation, the effects of

DPCPX were measured in the presence of the angiotensin (AT_1) receptor antagonist losartan (10 mg kg^{-1} in distilled water given as a 0.1 ml i.v. bolus). Losartan was given 30 min prior to i.p. administration of saline or LPS (as above) and DPCPX (0.1 mg kg^{-1} as above) was given 1.5 h and 6 h later. In pilot experiments it became clear that the marked effects of losartan on baseline haemodynamic status in LPS-treated rats confounded interpretation of results using DPCPX and therefore, only results from control animals are presented.

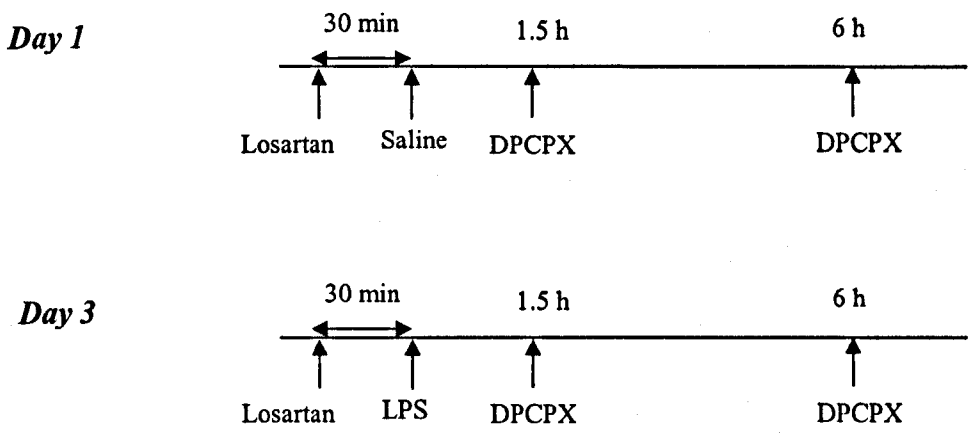


Figure 6.2.5 *Animals were pre-treated with losartan before administration of saline on Day 1 and LPS on Day 3. DPCPX was then given 1.5 h and 6 h later.*

6.3 Results

6.3.1 Experiment 1: Cardiovascular effects of 8-PT in normal and LPS-treated rats

Cardiovascular responses to the non-selective adenosine receptor antagonist, 8-PT (4mg kg^{-1} i.v. bolus) after LPS or saline treatment are shown in Figure 2. In saline-treated animals, administration of 8-PT at all 3 time points caused significant tachycardia, which was sometimes accompanied by a rise in blood pressure ($P<0.05$ at 6 h and 25 h), a fall in renal vascular conductance ($P<0.05$ at 6 h and 25 h) and a rise in hindquarters vascular conductance ($P<0.05$ at 1.5 h and 25 h).

At 1.5 h after LPS treatment, 8-PT caused tachycardia, hypotension, renal and hindquarters vasodilatation (Figure 6.3.1a); the integrated (0-10 min) changes in blood pressure and renal vascular conductance were significantly different from those seen in the saline-treated rats (Figure 6.3.1a). At 6 h after LPS treatment, 8-PT caused no tachycardia but there was hypotension and hindquarters vasodilatation (Figure 6.3.1b); the integrated (0-10 min) changes in heart rate, blood pressure and hindquarters vascular conductance were significantly different from those seen in the saline-treated rats. The integrated (0-10 min) change in renal vascular conductance in response to 8-PT was also different in LPS-treated rats at this juncture; inasmuch as the renal vasoconstrictor responses seen in the controls was absent. Twenty-five h following LPS treatment, the integrated (0-10 min) responses to 8-PT were not different between the saline and LPS-treated animals (Figure 6.3.1c).

6.3.2 Experiment 2: Cardiovascular responses to adenosine in normal and LPS-treated rats

Cardiovascular responses to adenosine (300μg kg^{-1} min^{-1}) are shown in Figure 6.3.2. In saline-treated rats, adenosine caused tachycardia, a fall in blood pressure, and increases in vascular conductance in the renal, mesenteric and hindquarters vascular beds.

At 1.5 h following LPS-treatment (Figure 6.3.2a), the adenosine-induced tachycardia was abolished and this was accompanied by a hypotension which took longer to develop than in the saline group. There was a tendency for an initial renal vasoconstriction but there was a small vasodilatation by the end of the infusion, and robust increases in mesenteric and hindquarters vascular conductances. The integrated (0-3min) changes in heart rate and renal vascular conductance in response to adenosine at 1.5 h were significantly ($P<0.05$) less in the LPS group than in the control group, but the integrated changes in mesenteric and hindquarters vascular conductance were not significantly different in the 2 groups. At 6 h after LPS (Figure 6.3.2b), adenosine caused a slight tachycardia although the integrated (0-3 min) change was still less than the control. The accompanying hypotension was again slower in onset but the integrated change was not different from the control. At this juncture, the renal vasodilatation was still significantly different from control, the mesenteric vasodilatation was not different from control, but the hindquarters vasodilator response to adenosine was lost (Figure 6.3.2b). By 25 h after the administration of LPS the cardiovascular responses to adenosine were not different between the saline and LPS-treated groups (Figure 6.3.2c).

6.3.3 Experiment 3. Cardiovascular responses to CCPA

Separate groups of animals were given CCPA at 1.5 h or 6 h since others (Casati *et al.*, 1994; Saura *et al.*, 1998) found evidence of desensitisation following repeated administration of this agonist.

In saline-treated animals, CCPA caused bradycardia, hypotension, mesenteric and renal vasoconstriction and hindquarters vasodilatation (Figure 6.3.3). For no obvious reason, although the changes were qualitatively similar at 1.5 h and at 6 h, the magnitude of change in blood pressure, heart rate and renal and mesenteric vascular conductances in response to CCPA were smaller at 6 h than at 1.5 h ($P<0.05$ for integrated 0-3min responses in both saline and LPS-groups), despite the use of naive animals at the 2 time points (Figure 6.3.3).

At 1.5 h after LPS administration, the integrated (0-3 min) bradycardic response to CCPA was greater, but the hypotension and mesenteric vasoconstriction were less than in the saline-treated animals at that time (Figure 6.3.3a). At 6 h after LPS treatment, the integrated bradycardia was still greater, but the renal and mesenteric vasoconstrictions were also greater than those in the corresponding saline control (Figure 6.3.3b). The hindquarters vasodilator response to CCPA did not differ between saline- and LPS-treated rats at any stage.

6.3.4 Experiment 4: Cardiovascular effects of DPCPX in normal and LPS-treated rats

Haemodynamic responses to DPCPX treatment in saline and LPS-treated animals are shown in Figure 6.3.4. In the saline-treated group, responses were quite variable but DPCPX caused a small (significant at 1.5h only) rise in blood pressure with mesenteric and hindquarters vasoconstriction (significant at both time points).

In the LPS-treated group, at 1.5 h, the integrated (0-10 min) mesenteric and hindquarters vasoconstrictor responses to DPCPX were not different from the saline-treated group but there was renal vasodilatation (not seen in the control group) and the pressor response was lost (Figure 6.3.4a). Six h after LPS treatment, there were no significant changes in any haemodynamic variable following administration of DPCPX (Figure 6.3.4b), and the integrated (0-10 min) changes in blood pressure and renal and hindquarters vascular conductances were significantly less than in the saline-treated rats.

6.3.5 Experiment 5: Cardiovascular effects of DPCPX following pre-treatment with losartan

In losartan-treated rats, 1.5 h and 6 h after saline, resting cardiovascular variables were: heart rate 358±6 and 368±15 beats min^{-1}, blood pressure 100±3 and 95±2 mmHg, renal vascular conductance (109±10 and 110±10 (kHz $mmHg^{-1}$)10^3), mesenteric vascular conductance (75±5 and 89±10 (kHz $mmHg^{-1}$)10^3) hindquarters vascular conductance (49±5 and 53±4 (kHz $mmHg^{-1}$)10^3).

In the presence of losartan, DPCPX had no significant effect on blood pressure or heart rate, but caused a fall in mesenteric vascular conductance ($P<0.05$ at 1.5 h and 6 h), and a fall in hindquarters vascular conductance ($P<0.05$ at 1.5 h only). At 1.5 h, the integrated (0-10 min) fall in mesenteric vascular conductance in response to DPCPX was significantly smaller following losartan (-76 ± 20%) than in the control condition (-136 ± 24%), and at both times, the DPCPX-induced fall in hindquarters vascular conductance was smaller after losartan (at 1.5 h -67±16%, at 6 h -31 ± 18%) than in the control condition (-97 ± 20%, -100 ± 15% respectively) although the difference was only significant at 6h.

	Expt 1		Expt 2		Expt 3a		Expt 3b		Expt 4	
1.5h	**Sal**	**LPS**	**Sal**	**LPS**	**Sal**	**LPS**	**Sal**	**LPS**	**Sal**	**LPS**
Heart rate (beats/min)	347±7	402±16*	340±10	429±14*	336±6	385±8*	352±7	427±9*	351±7	415±10*
Mean BP (mmHg)	113±3	104±5	102±2	104±2	110±2	108±5	109±9	103±3*	110±2	116±6
Renal VC (units)	77±10	99±9	75±8	87±10	94±5	109±7	74±8	90±9*	91±9	95±5
Mesenteric VC (units)	69±8	59±5	77±9	49±7*	66±5	50±9	72±7	62±8*	64±4	35±4*
Hindquarters VC (units)	44±4	62±7*	54±3	56±5	45±5	52±6	45±6	47±9	47±2	53±4
6h	**Sal**	**LPS**	**Sal**	**LPS**	**Sal**	**LPS**	**Sal**	**LPS**	**Sal**	**LPS**
Heart rate (beats/min)	353±12	445±12*	343±10	429±14*	337±8	399±14*	360±11	416±6*	340±7	399±8*
Mean BP (mmHg)	112±3	92±6*	102±2	92±3	107±1	102±3	111±3	99±3	111±2	114±7
Renal VC (units)	78±10	134±15*	74±7	109±16*	89±3	112±6*	75±8	98±11*	96±9	107±14
Mesenteric VC (units)	57±6	128±10*	83±9	108±14*	62±2	84±9*	70±8	106±1*	59±5	57±9
Hindquarters VC (units)	43±4	67±6*	52±3	67±3*	42±4	48±6	42±5	53±5*	42±2	45±3
25h	**Sal**	**LPS**	**Sal**	**LPS**						
Heart rate (beats/min)	351±7	383±21	356±10	352±11						
Mean BP (mmHg)	114±3	101±4*	107±1	100±2						
Renal VC (units)	79±7	87±5	70±9	81±14						
Mesenteric VC (units)	68±7	83±7	68±11	76±9						
Hindquarters VC (units)	43±4	61±6*	45±3	63±4*						

Table 6.1 Cardiovascular variables prior to administration of adenosine agonists and antagonists. Values are mean ± s.e.m. Units for vascular conductance (VC) are kHz/mmHg x 10^3. n = 8-9/group

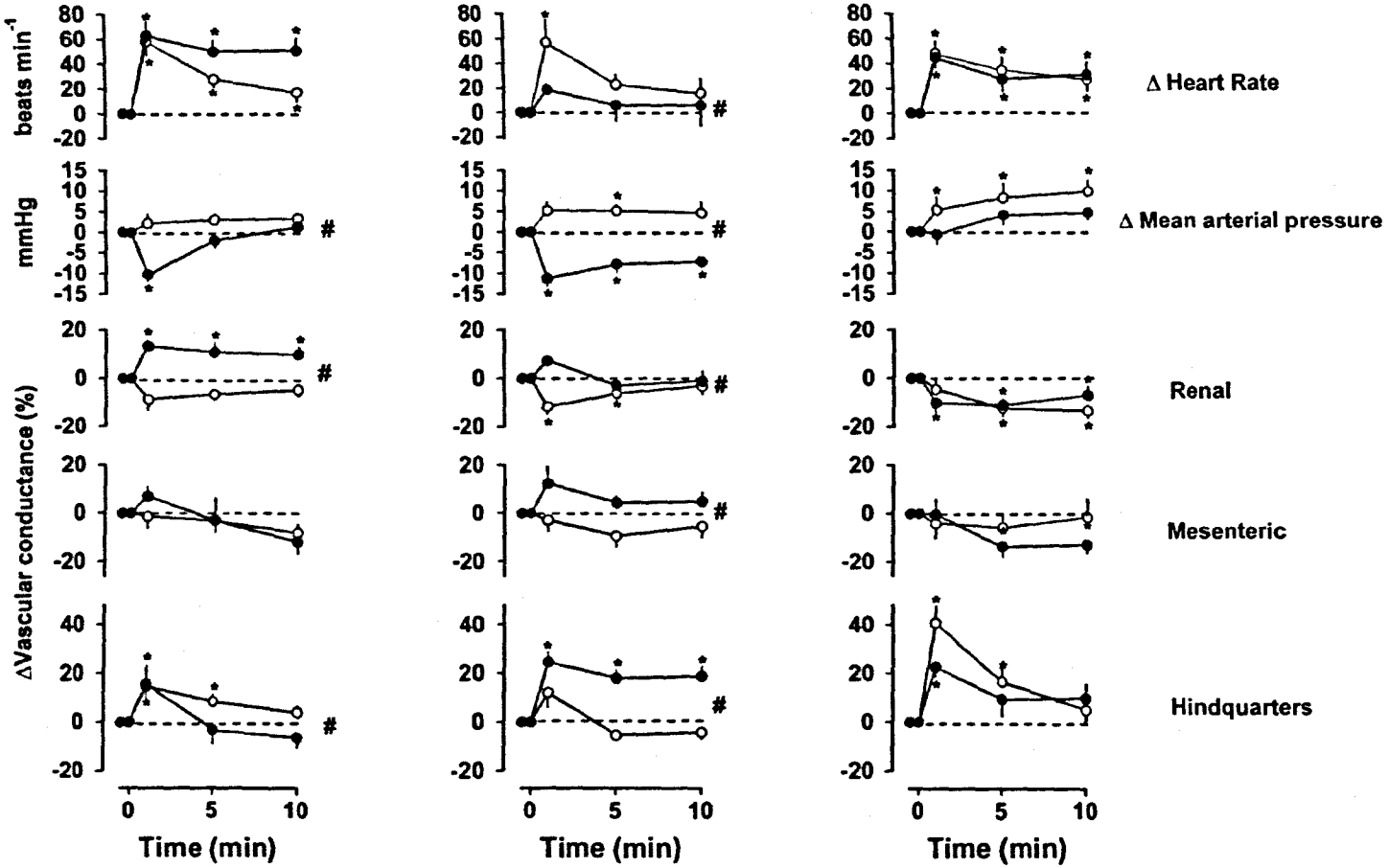


Figure 6.3.1 *Cardiovascular changes following 8-PT administration (4mg kg^{1} i.v.)1.5h* ***(a)****, 6h* ***(b)*** *and 25h* ***(c)*** *after saline (open circles) (n=9)or LPS (1mg kg i.p.) (closed circles) (n=9) treatment in conscious Sprague Dawley rats. *P<0.05 versus original baseline (Friedman's Test). #P<0.05 vs saline-treated animals (Mann-Whitney Test).*

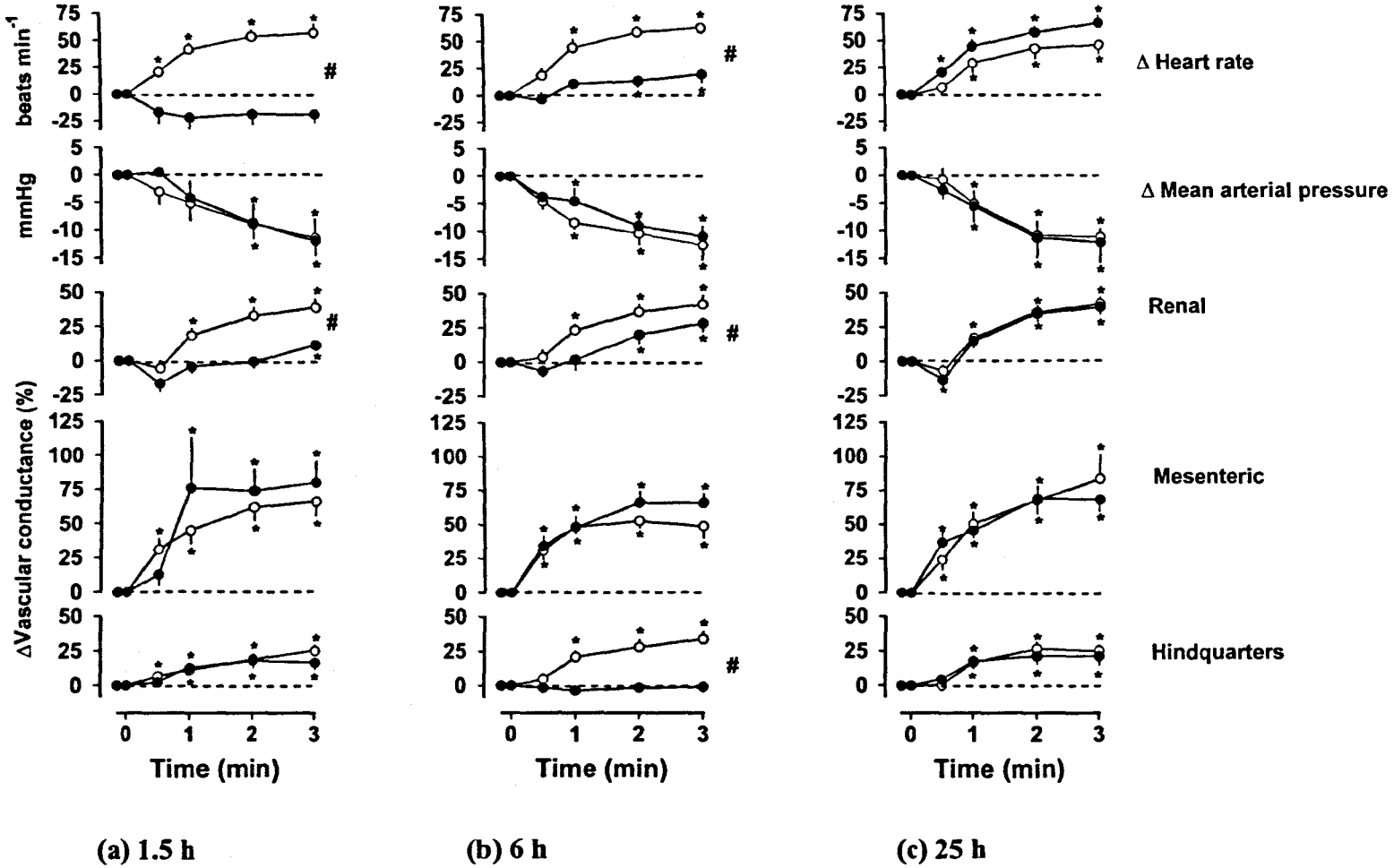


Figure 6.3.2 *Cardiovascular changes during 3 min infusion of adenosine (300μg kg^{-1} min^{-1} i.v.)* **(a)** *1.5 h,* **(b)** *6h, and (c) 25 h after saline (open circles) or LPS (1mg kg^{-1} i.p.) (closed circles) (n=9) treatment in conscious Sprague-Dawley rats.*

**P<0.05 versus original baseline (Friedman's test). #P<0.05 vs saline treated animals (Wilcoxon test).*

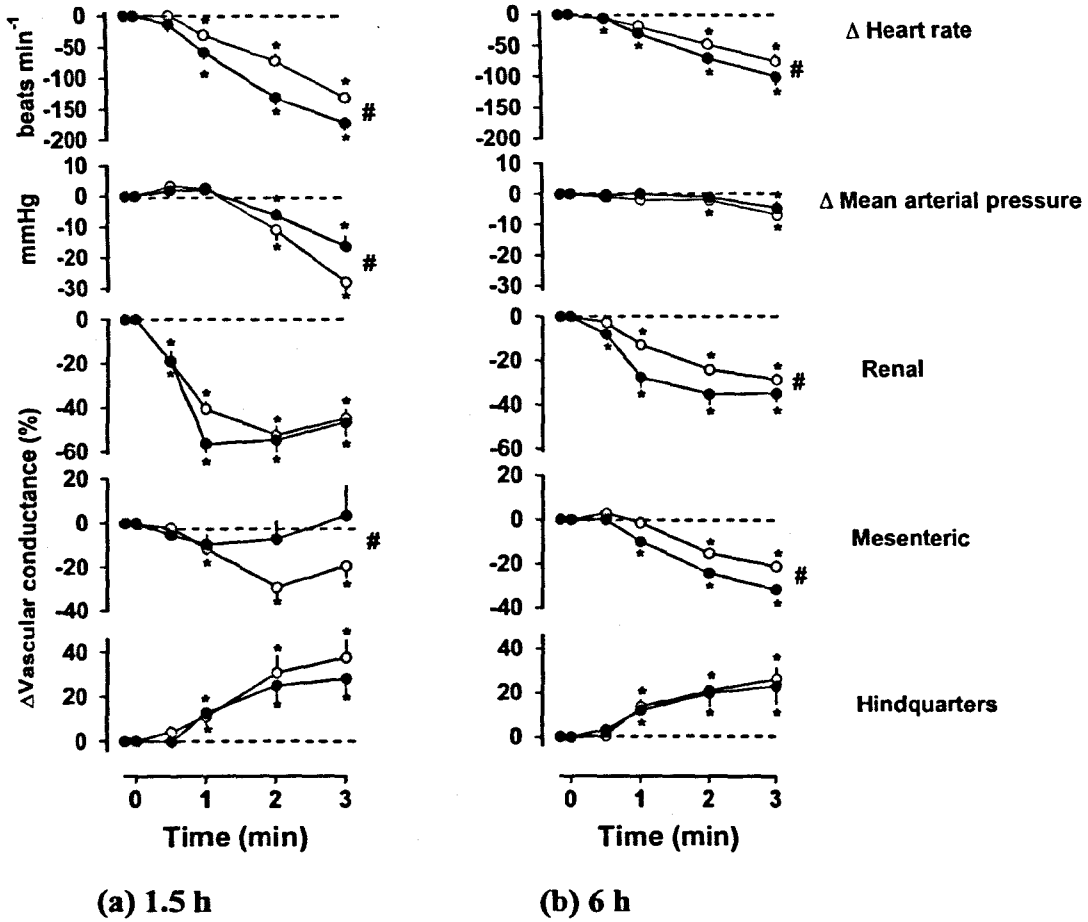


Figure 6.3.3 *Cardiovascular changes during 3 minute infusions of CCPA (1.4μg kg^{-1} min^{-1} i.v.)*(**a**) *1.5h, and* (**b**) *6h after saline (open circles) (n=8) or LPS (1mg kg^{-1} i.p.) (closed circles) (n=8) treatment in conscious Sprague Dawley rats.*

**P<0.05 versus original baseline (Friedman's Test). #P<0.05 vs saline-treated animals (Wilcoxon Test)*

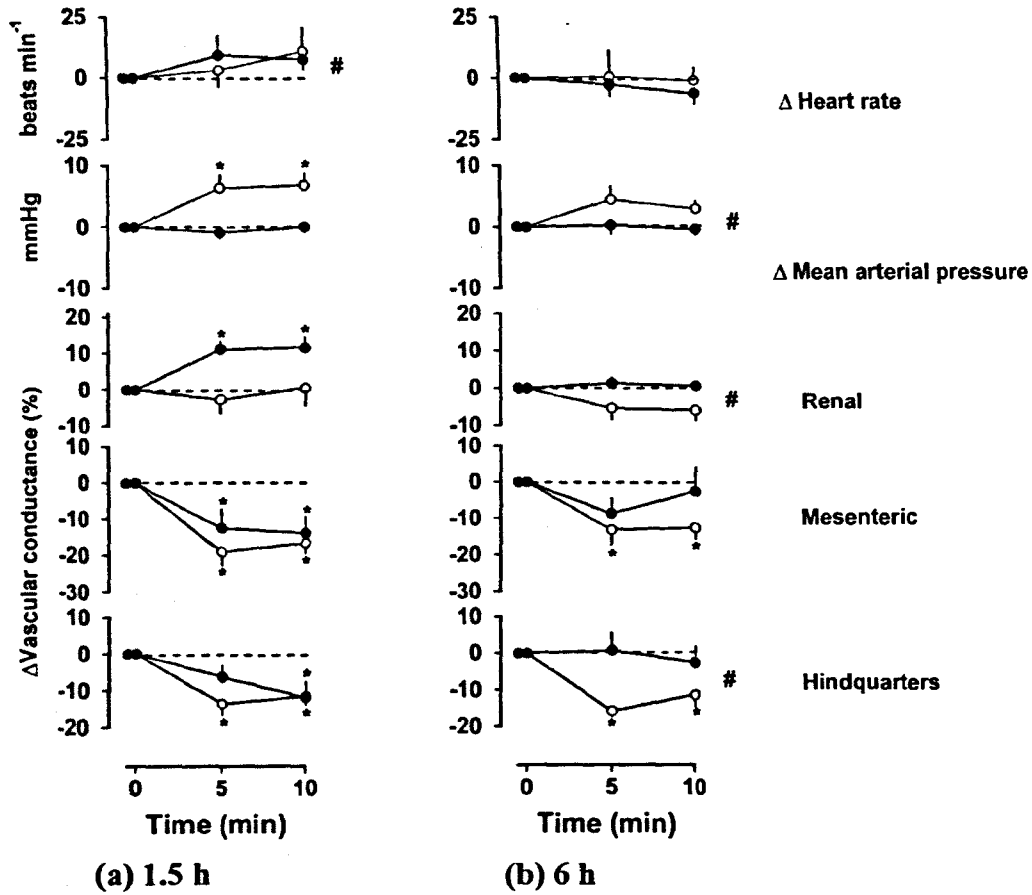


Figure 6.3.4 *Cardiovascular changes following DPCPX administration (0.1mg kg^{-1} min^{-1} i.v.) 1.5h* ***(a)*** *and 6h* ***(b)*** *after saline (open circles) or LPS (closed circles) (n=8) treatment in conscious Sprague Dawley rats.*

**P<0.05 versus original baseline (Friedman's Test). # = P<0.05 between vs. saline-treated animals or LPS (Wilcoxon Test)*

	HR	MAP	RVC	MVC	HVC
8-PT	↑	↔	↔	↔	↑
Adenosine	↑	↓	↑	↑	↑
CCPA	↓	↓	↓	↓	↑
DPCPX	↔	↑	↔	↓	↓

Table 6.2 *Summary of the cardiovascular responses to adenosine receptor agonists and antagonists.* ↑ *denotes an increase from baseline;* ↓ *denotes a decrease from baseline;* ↔ *denotes no change from baseline. Responses taken from the 1.5 h time point, 1 min from administration.*

6.4 Discussion

The haemodynamic responses to adenosine are complex and involve interaction of the nucleoside with four cell-surface G-protein coupled adenosine receptors (A_1, A_{2a}, A_{2b}, A_3). Cardiovascular responses to adenosine vary depending upon which receptor is activated, the local concentration of adenosine, and the relative density of each receptor subtype. Studies suggest that physiological levels of endogenous adenosine are low and contribute little to resting vascular tone. To assess this in normal animals, the non-selective adenosine receptor antagonist 8-PT was used. In saline-treated animals, the effects of 8-PT were consistent with endogenous adenosine causing bradycardia (Evoniuk *et al.*, 1987) and vasodilatation in the hindquarters. The magnitude of the responses to 8-PT varied across time (Figure 6.3.1), possibly due to variations in levels of endogenous adenosine which may reflect changes in the energy status of animals following surgery. These responses differed from those caused by exogenous adenosine which led to marked vasodilatations in the renal, mesenteric and hindquarters vascular beds, a robust hypotension and tachycardia, which were reproducible across time. Whilst the responses mediated by endo- and exogenous adenosine appear to be contradictory, both are consistent with the opposing effects of A_1 and A_2 receptors mediated by activation at low and high concentrations of adenosine, respectively (see Chapter 1.3).

To delineate the role of the adenosine A_1 receptor in the cardiovascular responses to both endogenous and exogenous adenosine, the selective A_1 agonist, 2-choloro-N^6-cyclopentyladenosine (CCPA), and antagonist, 8-

cyclopentyl-1, 3-dipropylxanthine (DPCPX), were used. Administration of the A_1-selective agonist to normal animals caused bradycardia, hypotension, renal and mesenteric vasoconstriction and hindquarters vasodilatation. These findings are consistent with those reported by Webb *et al.* in spontaneously hypertensive rats (Webb *et al.*, 1990), showing that A_1 receptor-mediated vasoconstriction (Tagawa *et al.*, 1970; Barrett *et al.*, 1993; Hansen *et al.*, 2005) is not restricted to the renal vasculature. The hindquarters vasodilator response to CCPA is also consistent with reports by others showing A_1 receptor-mediated vasodilatation in skeletal muscle (Marshall *et al.*, 1993; Ray *et al.*, 2002; Ray *et al.*, 2006).

In the final set of experiments, DPCPX was used to study endogenous A_1-receptor activation, and in normal animals, caused a modest increase in blood pressure, together with mesenteric and hindquarters vasoconstriction, with no significant effect on renal vascular conductance. The decrease in hindquarters vascular conductance following administration of DPCPX is internally consistent with the hindquarters vasodilatation elicited by CCPA. Collectively, these data indicate a role for A_1-receptor activation in mediating hindquarters blood flow. Furthermore, the effects of DPCPX and CCPA on blood pressure support a hypotensive role for adenosine acting via A_1-receptors. Since the mesenteric and hindquarters vasoconstrictor effects of DPCPX were reduced by losartan, it is likely that these responses were, in part, secondary to the removal of A_1-mediated inhibition of renin release (Tagawa *et al.*, 1970; Jackson *et al.*, 1991). The lack of response to DPCPX in the renal vasculature indicates that levels of endogenous adenosine were insufficient to activate renal A_1-receptors in normal animals. This is in line with a study by Kellet *et*

al. (Kellett *et al.*, 1989) who found that DPCPX had no effect on renal blood flow under normal conditions, and suggests that the observed responses to 8-PT in control animals may have been mediated by inhibition of A_2 receptors. These responses are summarised in Table 6.2.

Previous reports have shown a vasodilator role for adenosine 24 h after the induction of sepsis by caecal slurry injection (Motew et al., 1998, Sam et al., 2000a and b). To extend those observations, the present studies were designed to investigate the role of adenosine in the early cardiovascular changes seen in a rat model of endotoxaemia which is characterised by regionally-selective vasodilatation, the pattern of which varies across time (Chapter 5).

In LPS-treated animals, there were some notable changes in responses to 8-PT which, in this condition, caused hypotension associated with renal vasodilatation at 1.5 h, and hindquarters vasodilatation at 6 h. By 25 h after LPS administration, responses to 8-PT had returned to normal. Since Motew *et al.* reported vasoconstriction in response to 8-PT in sepsis (Motew *et al.*, 1998), our results showing 8-PT-induced vasodilatation were unexpected. These findings would be consistent with enhanced vasoconstriction and/or impaired vasodilatation in response to endogenous adenosine in LPS-treated animals, mediated by A_1 or A_2 receptors, respectively. In addition, we found that the vasodilator response to exogenous adenosine in normal animals was lost in the renal vascular bed 1.5 h after LPS administration, and was absent in the hindquarters vascular bed 6 h after LPS administration. Others have described A_1-receptor-mediated constriction of renal afferent arterioles in normal animals (Tagawa *et al.*, 1970; Webb *et al.*, 1990; Tang *et al.*, 1999),

and here we also found that, in the control animals, adenosine caused some fall in renal vascular conductance prior to development of the vasodilatation. The findings of attenuated vasodilator responses to adenosine in the renal vascular bed at 1.5 h, and in the hindquarters at 6 h following LPS administration are internally consistent with the effects of 8-PT (see above), and suggest either an LPS–induced enhancement of A_1-receptor-mediated vasoconstriction or impairment of A_2-receptor-mediated vasodilatation. Interestingly, adenosine-induced tachycardia as observed in normal animals was also substantially reduced in the LPS group. Since there was a resting tachycardia following LPS treatment (Table 1), one explanation for this difference is that the resting heart rate was already at its upper limit. However, this is unlikely as the LPS group showed tachycardic responses to 8-PT (Experiment 1), and from previous experiences, the level of heart rate in the LPS-treated rats in this experiment (~420 beats min^{-1}) was not maximal for a conscious rat.

Because adenosine caused a similar fall in blood pressure in control and LPS-treated rats, the lack of tachycardia in the latter could indicate some impairment of the baroreflex, reduction of the direct effects of A_2-receptors on the heart (Lappe *et al.*, 1992) and/or enhanced direct action of A_1-receptors on the heart.

Since one interpretation of the findings from the experiments using 8-PT and adenosine was that there were enhanced A_1-receptor-mediated responses in LPS-treated rats, further experiments were performed using CCPA and DPCPX in endotoxaemic rats. In the light of experiments with 8-PT and adenosine (see above), it was hypothesised that there would be enhanced

CCPA-induced renal vasoconstriction 1.5h after LPS treatment, enhanced hindquarters vasoconstriction (or diminished vasodilatation) 6h after LPS, and enhanced bradycardia at both time-points. The data did indeed show enhanced CCPA-induced bradycardia in the LPS-treated group which is consistent with enhancement of A_1-receptor-mediated negative chronotropic actions. However, there was no enhancement of the CCPA-induced renal vasoconstriction at 1.5h, and no change in the hindquarters vascular response at 6 h after LPS. Similarly, 6 h after LPS administration, there was no evidence for enhanced A_1-mediated vasoconstriction in the hindquarters. Interestingly, 1.5h after LPS, the mesenteric vasoconstrictor response to CCPA was diminished at a time when the bradycardia was enhanced and yet the hypotension was unaffected. This must indicate differential changes in cardiac output in response to CCPA in control vs. LPS-treated rats at this time, with a larger reduction in cardiac output in the former, possibly due to the larger increase in afterload secondary to the greater mesenteric vasoconstriction.

In contrast to the results from the previous experiment, DPCPX caused renal vasodilatation at 1.5 h in the LPS group, consistent with the results from the experiments with 8-PT. This suggests that endogenous adenosine was acting at renal A_1-receptors to cause vasoconstriction. However, unlike 8-PT, DPCPX did not cause vasodilatation in the hindquarters vascular bed at 6 h after LPS which suggests that the results with 8-PT and adenosine may be explained by reduced A_2-mediated vasodilatation in this instance.

It was noted that rats given DPCPX at 1.5h did not develop mesenteric vasodilatation across the 2-6 h period following LPS treatment (Table 6.1),

suggesting that the earlier A_1-receptor antagonism blunted the development of the hyperdynamic circulation. One explanation for this attenuated baseline vasodilatation in the DPCPX-treated group is that there was increased activation of the renin-angiotensin system, consequent upon removal of the normal A_1 receptor-mediated inhibition of this system (Weihprecht *et al.*, 1990; Lappe *et al.*, 1992).

Adenosine receptors are widely distributed throughout the central nervous system and A_{2a} receptors are implicated in control of baroreceptor-mediated reflexes (Thomas *et al.*, 2000). Furthermore, Schindler *et al.* (2005) showed that in conscious rats, whilst the hypotensive and bradycardic effects of i.p. A_1 receptor agonist administration were due to peripheral A_1 receptor activation, receptor-mediated hypotension was due to activation of peripheral receptors, but the tachycardia was, at least in part, due to activation of centrally located receptor (Schindler *et al.*, 2005). Therefore, it is possible that some of the results presented here reflect activation of central as well as peripheral adenosine receptors. Indeed, since, the integrity of the blood brain barrier is compromised during sepsis; an increased involvement of centrally located adenosine receptors following LPS treatment in our studies is feasible.

Whilst adenosine-based compounds are not currently used in the treatment of sepsis; their potential usefulness is under discussion (for reviews see Skrabanja *et al.*, 2005; Sands & Palmer, 2005). The present studies show that the regional haemodynamic effects of adenosine receptor activation and antagonism change in a regionally distinct manner, across time, following

LPS-treatment in conscious rats. These results show that enhancement of A_1-receptor-mediated effects could only partly explain the changed cardiovascular response to adenosine at 1.5 h and 6 h after LPS treatment, and we therefore suggest that impaired A_2 receptor mediated action may also have been involved. Thus, the cardiovascular effects of adenosine during LPS-induced endotoxaemia appear to vary according to the receptor activated, the vascular bed, and the time elapsed since LPS exposure.

Chapter 7

Results (V)

Interactions between adenosine, adrenomedullin and intermedin in the cardiovascular system of conscious rats

Summary

Evidence suggests that the vasodepressor effect of adenosine is potentiated in the presence of several other vasoactive mediators, including AM. In this Chapter, possible synergism between the regional haemodynamic responses to adenosine, AM and IMD was investigated *in vivo*.

Sprague Dawley rats were chronically implanted with miniature pulsed Doppler flow probes and intravascular catheters, to measure changes in regional blood flows along with blood pressure and heart rate.

In the presence of a low dose infusion of AM, responses to adenosine were not different from those in the presence of saline. Similarly, the regional haemodynamic responses to AM were not changed when administered in the presence of a low-dose infusion of adenosine. However, when adenosine challenges were given in the presence of a low-dose infusion of IMD, there was attenuation of adenosine-mediated mesenteric vasodilatation. The inhibitory interaction between these two agents was also observed in the cardiovascular response to adenosine in the presence of IMD.

In conclusion, these results do not support a synergistic relationship between adenosine and AM, but they do indicate a possible inhibitory interaction between adenosine and IMD. The reason(s) for this remain to be determined but highlight an interesting difference between AM and IMD.

7.1 Introduction

Endogenous mediators such as peptides, neuropeptides and amines interact to maintain cardiovascular homeostasis by controlling regional blood flow. The mediators include those acting in an endocrine manner to control peripheral blood flow, and those acting in an autocrine and/or paracrine fashion in response to local fluctuations in energy requirements. Adenosine is an example of a vasoactive mediator which acts locally; it is a nucleoside which possesses potent vasodilatory and hypotensive actions, and is released by the hydrolysis of ATP in metabolically active tissues, coupling the energy requirements of the cell to blood flow (for review see Fredholm *et al.*, 2001).

There is some evidence to suggest that adenosine-mediated hypotension is potentiated in the presence of other mediators, including vasoactive intestinal peptide (VIP), CGRP and AM; this interaction is believed to involve K_{ATP} channels. Thus, in a series of experiments, Sakai and Saito (1998a, b, c, d) showed that adenosine-mediated hypotension could be enhanced in anesthetised rats during infusion of CGRP, at a dose which, by itself, had no effect on blood pressure. They further demonstrated that VIP, CGRP and adenosine could interact synergistically to lower blood pressure, an effect which could be blocked by pretreatment with the K_{ATP} channel blocker, glibenclamide. Furthermore, infusion of the K_{ATP} channel opener, levcromakalim, significantly enhanced the depressor effects of VIP, CGRP and adenosine, indicating that activation of K_{ATP} channels was central to this effect. Further evidence came from the finding that co-infusion of the anti-

anginal drug nicorandil, which activates of K_{ATP} channels, with sub-depressor doses of VIP or CGRP further potentiated the adenosine-mediated fall in blood pressure. These studies also demonstrated that the hypotensive action of adenosine could be potentiated by infusing rats with AM, at a dose which by itself had no effect on blood pressure. This effect appeared to be mediated by K_{ATP} channel activation since the interaction was not seen following treatment with glibenclamide (Sakai *et al.*, 1998d). Furthermore, a study in open-chest dogs, demonstrated that AM-mediated increases in coronary blood flow could be inhibited be addition of the K_{ATP} channel blocker, U37883A, indicating that K_{ATP} channels were involved in AM-mediated vasodilatation. Interestingly, the vasodilator action of AM in the coronary artery could also be inhibited by addition of the non-selective adenosine-receptor antagonist, xanthine amine congener (XAC), leading the authors to speculate that AM-mediated coronary vasodilatation was mediated by AM-induced opening of K_{ATP} channels via the prior activation of adenosine receptors (Sabates *et al.*, 1997). These results are at odds with those found here, which showed no role for K_{ATP} channel activation in mediating the vasorelaxant effects of AM (Chapter 5). However, the different vascular bed and different species studied may, in part, account for these discrepancies.

Since the above studies suggest there may be some synergism between AM and adenosine in anaesthetised animals, with a possible role for adenosine receptors and K_{ATP} channels, the present study was designed to assess whether any such interaction exists in conscious animals. Furthermore, whilst synergism was demonstrated for the hypotensive actions of these agents, nothing is known about these interactions with regard to regional

haemodynamics, i.e., it is not known if the synergistic effects on blood pressure were due to synergistic vasodilatation in the same, or in different, vascular beds. Thus, the first set of experiments aimed to measure the regional haemodynamic response to adenosine (see Chapter 6) in the absence and presence of a sub-depressor dose of AM. Since the newly identified peptide, IMD, has been shown to share some of the cardiovascular actions of AM (Chapter 3), and has been shown to activate the same receptor system (Chapter 4), we extended the above experiment to assess the effect of a sub-depressor dose of IMD on the cardiovascular responses to adenosine. For completeness, the second set of experiments was designed to determine if a sub-depressor dose of adenosine had any effect on the cardiovascular responses to AM and IMD in the conscious, freely moving rat.

7.2 Methods

Male Sprague-Dawley rats were chronically instrumented with pulsed Doppler flow probes to allow continuous measurements of regional (renal, mesenteric and hindquarters) haemodynamics, as described in Chapter 2. Briefly, under anaesthesia, 3 miniaturised probes were sutured around the left renal artery, superior mesenteric artery and distal aorta to allow changes in renal, mesenteric and hindquarters vascular conductances to be assessed. Animals then underwent a recovery period of 10-14 days before being anaesthetised again to allow implantation of intravascular catheters. Up to 3 catheters were placed in the jugular vein for administration of drugs and 1 was placed in the caudal artery and advanced into the distal aorta to allow continuous recording of blood pressure and heart rate. Animals were fitted with a harness and attached to a swivel system, allowing them to move freely throughout the experimental period, which began 24 h after catheter implantation.

7.2.1 Cardiovascular responses to adenosine during infusion of AM or IMD

A group (n=9) of animals was infused with saline (0.4ml h^{-1} i.v.) on Day 1, with AM (1ng kg^{-1} min^{-1} i.v.) on Day 2 and with IMD (1ng kg^{-1} min^{-1}) on Day 3, for 20 min prior to 3 min challenges of adenosine (300µg kg^{-1} min^{-1} i.v.). The saline or peptide infusions continued during the adenosine challenge. The dose of adenosine was chosen because it was used in previous studies (see Chapter 6), and is known to cause robust cardiovascular responses. The dose of peptides was the same as the dose of AM used by Sakai & Saito (1998),

which they showed to have no effect on blood pressure but was able to enhance adenosine-mediated hypotension. This experimental protocol is outlined in Figure 7.2.1.

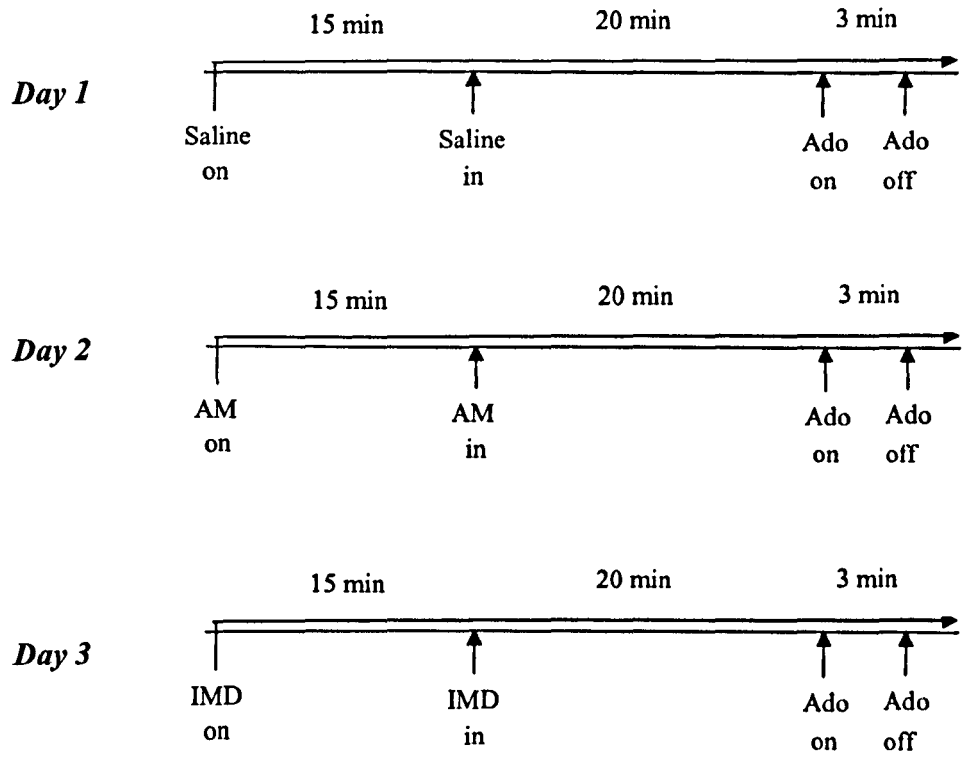


Figure 7.2.1 *Animals were pre-treated for 20 min with saline on Day 1, with AM on Day 2, and with IMD on Day 3, prior to a 3 min challenge of adenosine (Ado). The 15 min period at the start of the saline/AM/IMD infusion represents the time taken for the solution to advance through the dead space of the catheter (i.e. 0.1ml)*

7.2.2 Cardiovascular responses to AM & IMD during infusion of adenosine

To assess whether adenosine had any effect on the cardiovascular response to AM and IMD, a group (n=11) of animals received a pre-treatment of saline (0.4ml h^{-1} i.v.) or adenosine (3μg kg^{-1} min^{-1} i.v.), infused for 20 min prior to administration of peptide. AM (3nmol kg^{-1} i.v.) or IMD (1nmol kg^{-1} i.v.) were then given as a 0.1ml bolus and cardiovascular responses were recorded for 30 min during which animals continued to receive saline or adenosine. The dose of adenosine was that used by Sakai et al. (1998) and the doses of peptides were those used in previous studies (see Chapters 3-5), in which they caused robust cardiovascular responses. Since AM and IMD at the doses given cause marked vasodilatations, the above protocol was repeated using lower doses of peptides (AM 1nmol kg^{-1}; IMD 0.3nmol kg^{-1} i.v.), as any synergism could have been be difficult to observe if peptide administration had caused maximal vasodilatation. The protocol was randomised, such that each animal received all treatments over the 4 day experimental period, with 1 treatment each morning, and 1 treatment each afternoon. In cases where animals received 2 doses of a peptide in 1 day, they were always given the lower dose in the morning and the higher dose in the afternoon. An example protocol is illustrated in Figure 7.2.2.

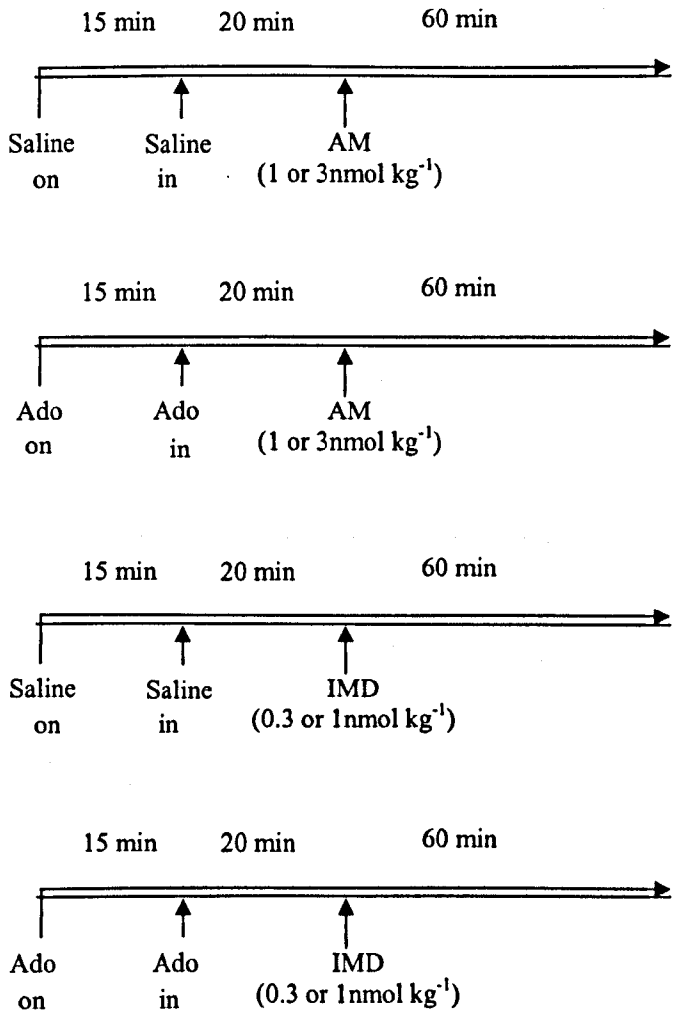


Figure 7.2.2 *Animals were pre-treated for 20 min with saline, or adenosine (Ado), prior to administration of either AM or IMD, cardiovascular changes were measured for 60 min during which the saline/adenosine infusion was present. The 15 min period at the start of the saline/Ado infusion represents the time taken for the solution to advance through the dead space of the catheter (i.e. 0.1ml).*

7.3 Results

7.3.1 Cardiovascular responses to adenosine during infusion of AM or IMD

Baseline cardiovascular variables, prior to the onset of saline/AM/IMD infusion, and prior to administration of adenosine are given in Table 7.1. There were no differences in any groups indicating that low dose AM or IMD infusion had no effect on baseline blood pressure or regional haemodynamics. The cardiovascular changes during a 3 min adenosine infusion, in the presence of saline were the same as those reported in Chapter 6, namely, tachycardia and hypotension, and renal, mesenteric and hindquarters vasodilatations (Figure 7.1a). The cardiovascular response to adenosine was not changed in the presence of AM infusion ($P>0.05$, for 0-3 min integrated changes for all variables), although there appeared to be some attenuation of adenosine-mediated mesenteric vasodilatation, this was not statistically significant (Figure 7.1a).

Cardiovascular changes during a 3 min infusion of adenosine in the presence of an infusion of IMD are shown in Figure 7.1b, which illustrates that the adenosine-mediated fall in blood pressure and renal vasodilatation were significantly attenuated ($P<0.05$, for 0-3 min integrated changes). There was also some attenuation of IMD-mediated vasodilatation in the mesenteric and hindquarters vascular beds, but this failed to reach significance ($P>0.05$, for integrated changes).

7.3.2 Cardiovascular responses to AM & IMD during infusion of adenosine

Responses to AM in the presence of adenosine

Baseline haemodynamics preceding saline or adenosine infusion and administration of peptides are given in Table 7.2. There were no significant differences between groups indicating that pre-treatment with low dose adenosine had no effect on cardiovascular variables.

Cardiovascular responses to AM (1 and 3nmol kg^{-1}) during a saline infusion are shown in Figure 7.2. Cardiovascular responses to AM, at each dose, were similar to those reported previously (Chapter 3-5), i.e., a fall in blood pressure and tachycardia with increases in renal, mesenteric and hindquarters vascular conductances; these effects were sustained for a longer period time following administration of AM at the higher dose. Cardiovascular responses to AM in the presence of a low-dose infusion of adenosine, are shown in Figure 7.2; they were not different from those observed during infusion of saline ($P>0.05$, for 0-30 min integrated changes for all variables).

Responses to IMD in the presence of low-dose adenosine

Resting cardiovascular variables prior to administration of IMD are shown in Table 7.2. Intermedin at a dose of 0.3nmol kg^{-1} caused a modest fall in blood pressure and a tachycardia, and increases in renal, mesenteric and hindquarters vascular conductances; all variables returned to baseline values by 10-20 min (Figure 7.3a). At a dose of 1nmol kg^{-1}, IMD caused a fall in blood pressure and tachycardia and increases in renal, mesenteric and hindquarters vascular

conductances which were significantly more marked than those evoked by the lower dose ($P<0.05$, for 0-10 min integrated changes for each variable) (Figure 7.3b), as noted in Chapter 3- 5. In the presence of adenosine there was no change in the cardiovascular response to IMD at a dose of 0.3nmol kg^{-1} ($P>0.05$, for 0-10 min integrated changes for each variable). However, at the higher dose of IMD (1nmol kg^{-1}), low dose adenosine caused significant attenuation of IMD-mediated mesenteric, but not renal or hindquarters vasodilatation. Furthermore, although there was no significant IMD-mediated fall in blood pressure in the presence of adenosine (Friedman's test), the integrated (0-10 min) change was not significantly different from the blood pressure response in the saline-infused condition. The effect of IMD on heart rate was not changed in the presence of adenosine (Figure 7.3).

	+ saline infusion		*+ AM infusion*		*+ IMD infusion*	
	(a)	**(b)**	**(a)**	**(b)**	**(a)**	**(b)**
HR (beats min^{-1})	372±10	364±12	355±11	348±11	343±9	360±8
MAP (mmHg)	114±4	116±5	115±5	114±4	105±2	107±2
RVC (units)	79±8	77±7	76±9	74±7	76±9	76±8
MVC (units)	71±8	64±6	65±8	63±8	73±9	74±8
HVC (units)	42±4	38±3	43±2	40±1	49±4	48±4

Table 7.1 *Baseline cardiovascular variables prior to the onset of saline or AM or IMD infusion* ***(a)*** *and prior to administration of adenosine* ***(b)*** *in conscious rats (n=9). Values are mean ± SE.*

Units for vascular conductance (VC) are kHz $mmHg^{-1}$ x 10^3.

	Saline + AM				*Adenosine + AM*			
	1nmol kg^{-1}		**3nmol kg^{-1}**		**1nmol kg^{-1}**		**3nmol kg^{-1}**	
	(a)	**(b)**	**(a)**	**(b)**	**(a)**	**(b)**	**(a)**	**(b)**
HR (beats min^{-1})	328±8	325±6	320±7	341±10*	320±8	325±7	337±12	330±8
MAP (mmHg)	102±1	102±2	100±2	104±3	102±2	106±2	102±2	103±3
RVC (units)	73±9	71±9	71±9	71±9	73±7	70±7	78±7	75±7
MVC (units)	73±7	73±7	70±4	71±8	71±6	71±7	74±7	72±8
HVC (units)	45±2	43±3	43±2	43±3	42±3	41±2	44±3	44±3
	Saline + IMD				*Adenosine + IMD*			
	0.3nmol kg^{-1}		**1nmol kg^{-1}**		**0.3nmol kg^{-1}**		**1nmol kg^{-1}**	
	(a)	**(b)**	**(a)**	**(b)**	**(a)**	**(b)**	**(a)**	**(b)**
HR (beats min^{-1})	331±7	334±10	323±6	323±4	333±17	333±16	323±5	327±9
MAP (mmHg)	103±4	105±4	99±2	101±2	103±3	105±5	101±2	100±2
RVC (units)	75±11	81±10	76±8	75±8	77±11	79±9	72±7	71±8
MVC (units)	67±11	73±10	72±9	71±9	75±8	74±9	72±6	73±7
HVC (units)	46±4	46±3	47±3	42±3	45±3	43±2	41±2	42±2

Table 7.2 *Resting cardiovascular variables prior to the onset of saline or adenosine infusion* ***(a)*** *and prior to administration of AM or IMD* ***(b)*** *in conscious rats (n=11). Values are mean ± SE. Units for vascular conductance (VC) are kHz mmHg^{-1} x 10^{3}.*

**P<0.05 vs. value at onset of saline/adenosine infusion.*

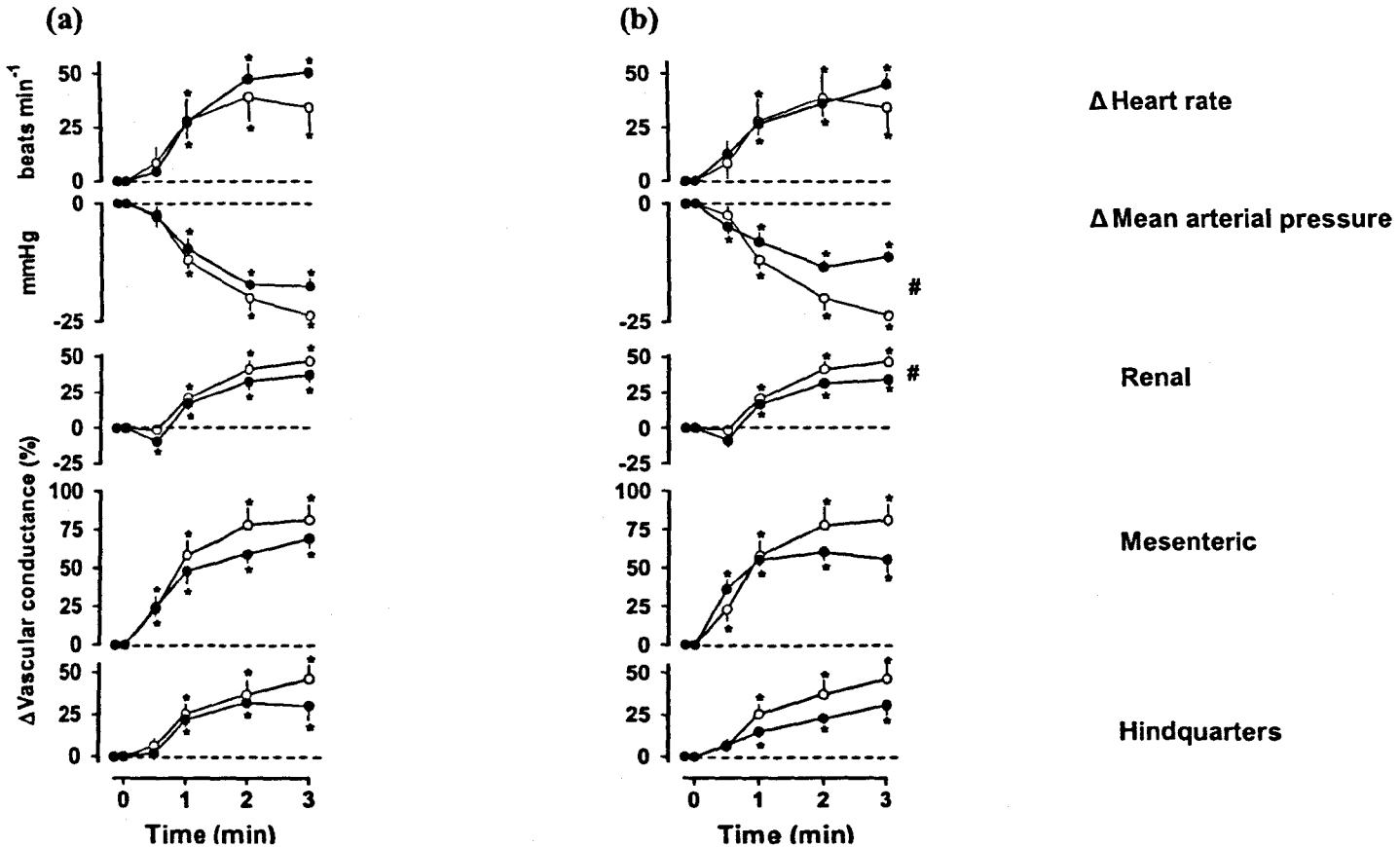


Figure 7.1 *Cardiovascular responses to adenosine (300μg kg^{-1} min^{-1} i.v.) during continuous infusion of saline (open circles),* **(a)** *AM (1ng kg^{-1} min^{-1}) (closed circles) or* **(b)** *IMD (1ng kg^{-1} min^{-1}) (closed circles) in conscious rats (n=11).*

**P<0.05 versus original baseline (Friedman's test). #P<0.05 vs. saline (Wilcoxon test).*

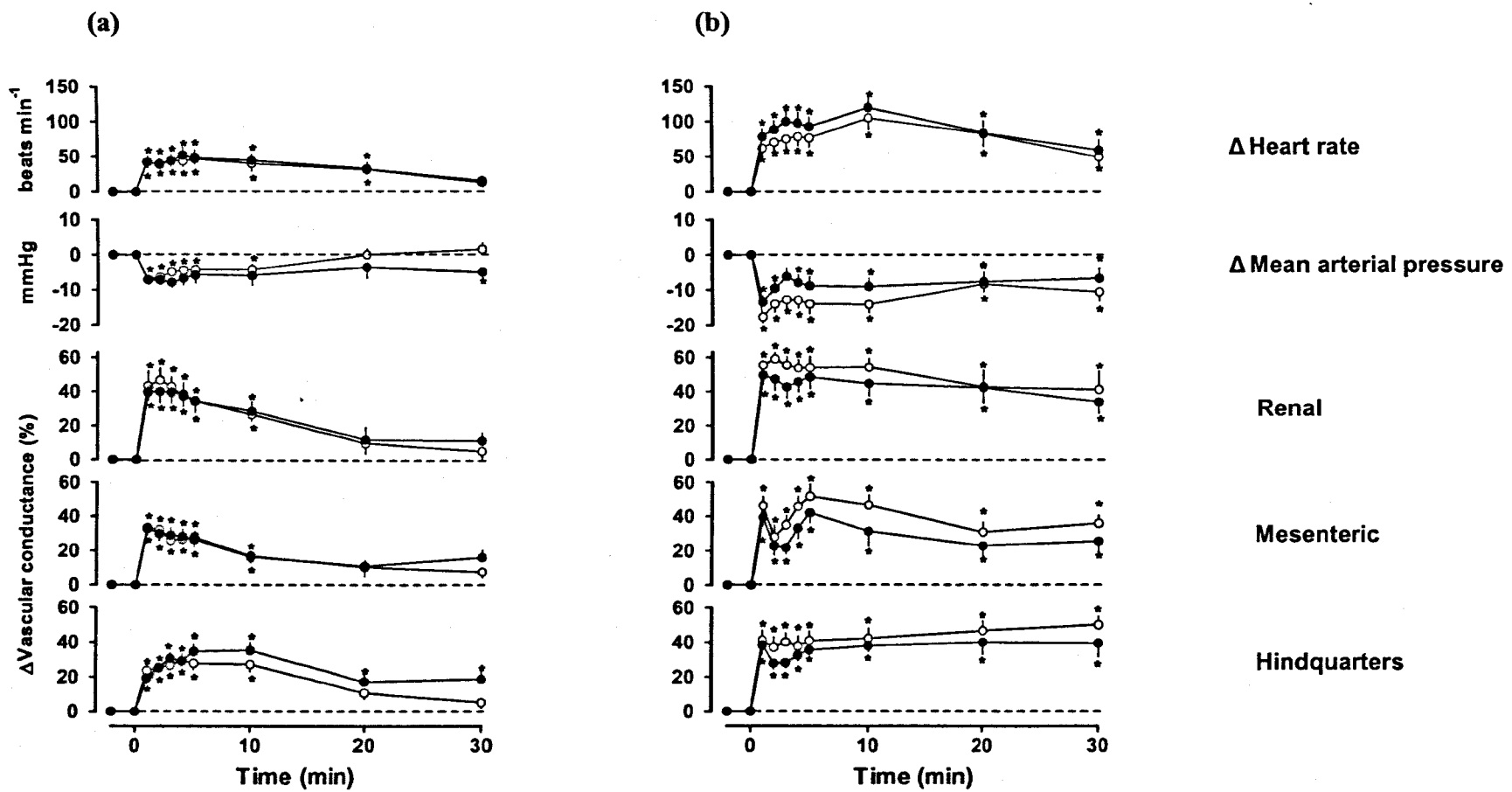


Figure 7.2 *Cardiovascular responses to* **(a)** *AM 1nmol kg^{-1} and* **(b)** *3nmol kg^{-1} during a continuous infusion of saline (open circles) or adenosine (3μg kg^{-1} min^{-1} i.v.) (closed circles) in conscious rats (n=9).*

**P<0.05 versus original baseline (Friedman's test)*

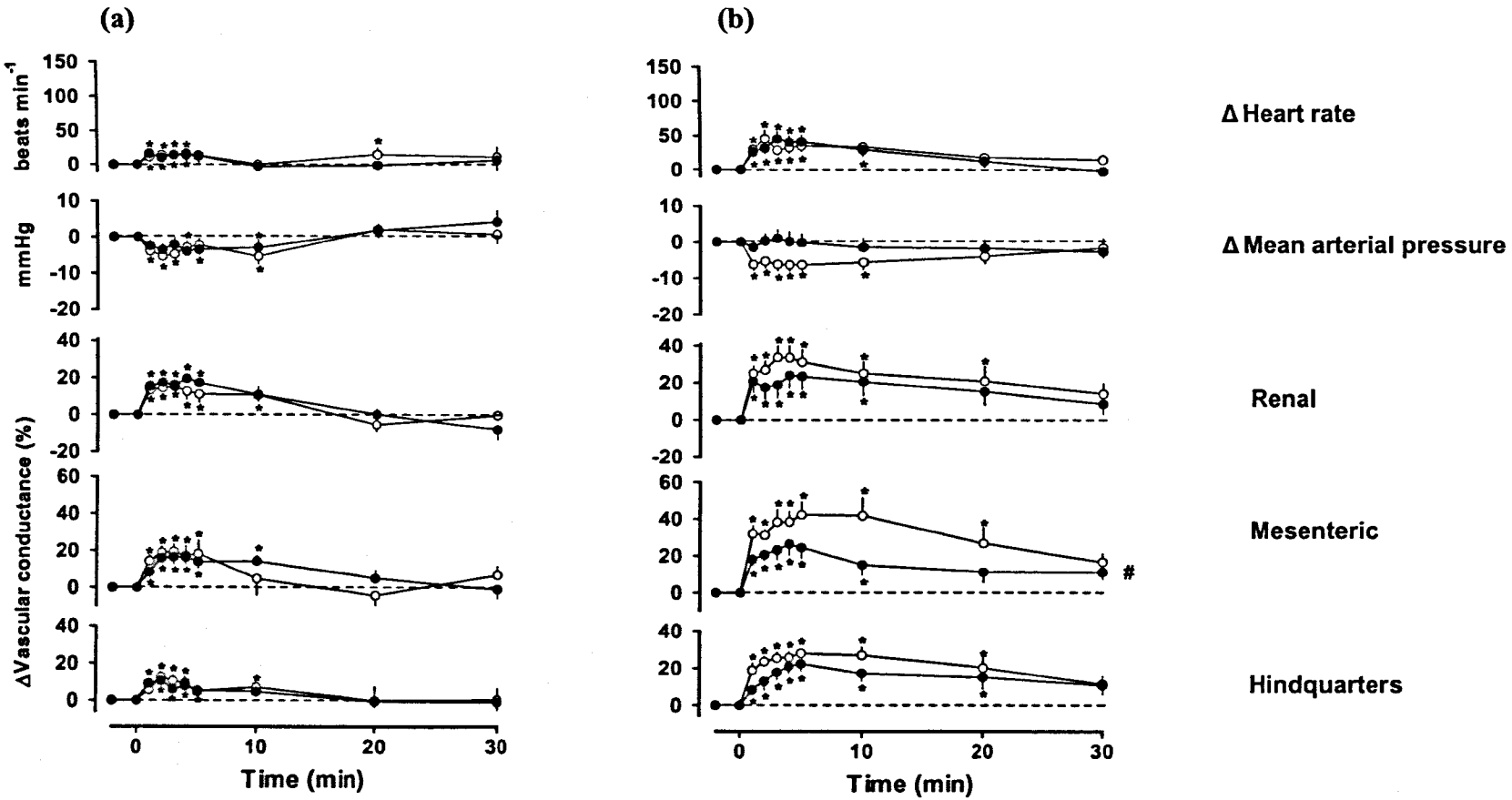


Figure 7.3 *Cardiovascular responses to* **(a)** *IMD 0.3nmol kg^{-1} and* **(b)** *1nmol kg^{-1} during a continuous infusion of saline (open circles) or adenosine (3μg kg^{-1} min^{-1} i.v.) (closed circles) in conscious rats (n=9).*

**P<0.05 versus original baseline (Friedman's test). #P<0.05 versus saline (Wilcoxon test)*

7.4 Discussion

In a series of experiments, Sakai and Saito. (1998) showed that adenosine, VIP, CGRP and AM could reciprocally interact, such that they enhanced each other's vasodepressor actions: this effect was mediated via the activation of K_{ATP} channels (Sakai *et al.*, 1998a,b,c,d). The present study aimed to replicate these findings in conscious, freely moving rats, and to extend this by determining if there was any synergism between adenosine, AM and IMD in the cardiovascular system. Thus, responses to adenosine were assessed in the presence of a sub-depressor dose of AM or IMD, and the cardiovascular responses to AM and IMD were assessed in the presence of a sub-depressor dose of adenosine. Contrary to the findings of Sakai *et al.* (1998), no evidence was found for a synergistic interaction between adenosine and AM. Thus, the regional haemodynamic responses to both low (1nmol kg^{-1}) and high (3nmol kg^{-1}) dose AM were not different between animals receiving an infusion of adenosine and those receiving saline. Furthermore, the cardiovascular response to adenosine was not changed when co-infused with AM, further indicating that there was no synergistic interaction between these two agents.

In their studies, Sakai & Saito (1998) showed that the reciprocal interaction between AM and adenosine was mediated via the opening of K_{ATP} channels in a manner which caused amplification of membrane hyperpolarisation (Sakai *et al.*, 1998d). This was shown following i.v. administration of adenosine and AM, and in isolated smooth muscle cells isolated from rat mesenteric vascular bed. The role of K_{ATP} channels in AM-mediated vasodilatation and hypotension has been confirmed in some studies (Sabates *et al.*, 1997; Goto *et*

al., 2000) but not in others (Miura *et al.*, 1995; Terata *et al.*, 2000). Using the K_{ATP} channel blocker, U37783A, no role for K_{ATP} channels was found in AM-mediated renal, mesenteric or hindquarters vasodilatation (Chapter 4). Thus, the present results are internally consistent, i.e., there was no evidence for K_{ATP}-channel involvement in AM-signalling (Chapter 4), and no synergism was found between AM and adenosine (this Chapter). Since Sakai *et al.* (1998) used anaesthetised animals in their studies, whereas conscious rats were used in the present experiments, it is possible that this may account for the disparate findings.

The experiments reported here also assessed possible synergism between adenosine and IMD, which shares some sequence homology with AM and has effects on the cardiovascular system similar to those of AM (Chapters 3-5; Takei et al., 2005; Roh et al., 2004). The cardiovascular responses to the low dose of IMD (0.3nmol kg^{-1}) were unaffected in the presence of adenosine, but, contrary to expectation, adenosine infusion caused a significant attenuation of the mesenteric vasodilatation induced by the high dose of IMD (1nmol kg^{-1}). The inhibitory interaction between these two agents was also observed in the cardiovascular response to adenosine in the presence of IMD. In these experiments there was significant attenuation of adenosine-mediated vasodilatation in the renal vascular bed, and to some extent in the mesentery and hindquarters, although in the case of the latter two this failed to reach statistical significance. Furthermore, the reduced adenosine-mediated vasodilatations were reflected in the blood-pressure response, such that in the presence of IMD, adenosine-mediated hypotension was abolished. Since, at the doses used, neither adenosine nor IMD pretreatment had any effect on

resting cardiovascular variables (Table 7.1 and 7.2), the observed negative synergism between IMD and adenosine can not be explained by any differences in baseline haemodynamics between the groups. Interestingly, despite the lack of hypotensive response to IMD in the presence of adenosine, there was still a significant tachycardia, which suggests IMD may have some positive chronotropic effect on the heart (Chapter 3). The exact physiological role of IMD in the heart remains speculative, but our findings are suggestive of a direct action of IMD on the heart, and support those of Dong et al. (2006) who showed that IMD had a direct effect on myocyte contractile function, and those of Fujisawa et al. (2006) who showed that there was a residual IMD-induced increase in heart rate following surgical denervation of aortic baroreceptors in rats (Dong et al., 2006; Fujisawa et al., 2006).

There are relatively few studies examining the effects of IMD on regional haemodynamics, and the mechanisms through which IMD leads to vasorelaxation remain unclear. Others have identified IMD as a non-selective agonist of the CL/RAMP receptor system, which also binds CGRP and AM in a selective manner (Roh *et al.*, 2004; Pan *et al.*, 2005; Kandilci *et al.*, 2006; Dong *et al.*, 2006; Chapter 4). We have identified that IMD-mediated vasodilatation is sensitive to AM_{22-52} in the renal and mesenteric vascular beds (Chapter 4), and others have found a role for $CGRP_{8-37}$-senstive receptors (Kobayashi *et al.*, 2004; Roh *et al.*, 2004; Taylor *et al.*, 2004; Kandilci *et al.*, 2006, 2008; Ren *et al.*, 2006). We have also demonstrated that a component of IMD-mediated vasodilatation in the renal and mesenteric vascular beds is sensitive to L-NAME, (Chapter 4), although no such role was found for NO in

AM-mediated vasodilatation in this study. As adenosine can influence vascular tone in an endothelial manner via production of NO (Barrett & Droppleman, 1983; Ray *et al.*, 2002; Hansen *et al.*, 2005), it is possible that limitation of the available NO is responsible for the observed functional antagonism between IMD and adenosine.

Chapter 8

General Discussion

8.1 Summary of main findings

In Chapter 3, the regional haemodynamic responses to AM and IMD were assessed and compared. Intermedin evoked widespread vasodilatations which were sufficient to cause a fall in blood pressure and an increase in heart rate, consistent with the findings of others (Roh *et al.*, 2004; Takei *et al.*, 2004; Charles *et al.*, 2006; Ren *et al.*, 2006; Fujisawa *et al.*, 2007; Chauhan *et al.*, 2007). These responses were similar, but more marked, than those caused by equimolar (1nmol kg^{-1}) AM, which at this dose failed to cause a reproducible fall in blood pressure. In an attempt to match the responses, AM at a dose of 3nmol kg^{-1} was used. At this dose, AM caused marked hypotension accompanied by widespread vasodilatation, which was more marked than that caused by IMD in the renal vasculature.

In Chapter 4, IMD-mediated vasodilatation was shown to be attenuated by AM_{22-52} and L-NAME in the renal and mesenteric, and renal vascular beds, respectively. This suggests that activation of AM_{22-52}-sensitive receptors and release of NO were involved in mediating some of the vascular effects of IMD. L-NAME attenuated but did not abolish the vasodilator action of IMD, suggesting that the peptide stimulates both endothelial-dependent and –independent pathways. While some aspects of AM signalling were affected by AM_{22-52}, (renal and mesenteric vasodilatation), they were unaffected by L-NAME. Thus, evidence was found of IMD-mediated endothelial dependent vasodilatation, but not AM. No role for K_{ATP} channels was found for either peptide, although there was an enhanced vasodilatation response to AM in the

renal and mesenteric vasculature following treatment with U37883A. With the use of losartan, it was shown that this response was mediated by inhibition of the renin-angiotensin system. Several studies have shown that IMD is a non-selective agonist of the CL/RAMP system, and its cardiovascular effects are sensitive to the $CGRP_1$ receptor antagonist $CGRP_{8-37}$ (Kobayashi *et al.*, 2004; Roh *et al.*, 2004; Taylor *et al.*, 2004; Ren *et al.*, 2006; Kandilci *et al.*, 2006; 2008). However, we found no role for this receptor in mediating the vascular effects of either peptide.

Although interest in this group of peptides is high, research is difficult due to a lack of selective agonists and antagonists. Knockout mice have been of some value in the study of AM function, and have shown it to be essential for vascular development. Furthermore, mice heterozygous for the AM gene have increased blood pressure and reduced levels of NO, suggesting that AM is involved in cardiovascular homeostasis in normal animals (Shindo *et al.*, 2001). Since RAMP2 was found to be essential for this function of AM (Shindo *et al.*, 2008), and we (chapter 4), and others (see above) have shown IMD can activate RAMP2-containing AM receptors, it is possible that this function is shared by IMD.

In Chapter 5, vascular responsiveness to AM and IMD was assessed across time in LPS-treated rats. By itself, LPS (1mg kg^{-1} i.p.) led to the development of widespread vasodilatations, the pattern of which was both temporally and spatially distinct, without a corresponding fall in blood pressure. When given 1.5 h after LPS-administration, there was a significant loss of vascular responsiveness to both AM and IMD; but responses had almost recovered by

25 h. The next set of experiments aimed to replicate these results in rats treated with a continuous low dose LPS infusion, to determine whether the gradual return in responsiveness to the peptides was a consequence of the waning effects of LPS. At 1.5 h there was vascular hyporesponsiveness to both AM and IMD, but responsiveness returned sooner than was found following LPS bolus administration, and peptide responses were augmented in some cases by the 25 h time point. Enhanced vascular tone at baseline could have explained these results, perhaps due to enhanced involvement of vasoconstrictor systems, such as endothelin.

Increased production of AM following stimulation with LPS could have accounted for the vascular hyporesponsiveness to exogenous AM at 1.5 h after LPS and to investigate this, the AM receptor antagonist, $AM_{22\text{-}52}$, was administered 1.5 h from the onset of endotoxaemia. There was no response to the antagonist, but this could have been either because endogenous AM was not exerting a tonic effect at this time point, or because the receptor components to which this antagonist binds were down regulated. Thus, downregulation of RAMP2 could explain the loss of vascular responsiveness to AM and IMD (since we show in chapter four that AM receptors comprised of this accessory protein are involved in both AM and IMD-mediated vasodilatation), and the lack of results with $AM_{22\text{-}52}$.

In Chapter 6, the role of adenosine in the cardiovascular sequelae of endotoxaemia was examined. Using the non-selective adenosine-receptor antagonist, 8-PT, a clear role was demonstrated for endogenous adenosine in the maintenance of vascular tone in normal, conscious animals, and in the

changing cardiovascular response to LPS. The response to exogenous adenosine was altered in the presence of LPS in such a way that it could have been due to either an up-regulation of adenosine A_1 receptor responses, and/or to a down-regulation of adenosine A_2 mediated responses. To investigate this further, the selective A_1 receptor agonist, CCPA, and the antagonist DPCPX were used. The results indicated that whilst altered A_1 receptor function could account for some of the altered responses to adenosine in LPS-treated rats, it could not account for them all. Hence, a changing influence of both A_1 and A_2 adenosine receptors is likely in this model of endotoxaemia.

Finally, in Chapter 7, experiments were performed which were designed to replicate the findings of Sakai *et al.* (1998a,b,c), who showed that the vasodepressor response to adenosine was potentiated in the presence of sub-depressor doses of a number of vasoactive peptides, including AM. However, no evidence was found for synergism between adenosine and AM. Contrary to the results with adenosine and AM, a negative interaction between adenosine and IMD was observed. The reasons for these remain to be explored, but the findings highlight an interesting difference between the two peptides.

In summary, the work contained within this thesis is consistent with other studies which have shown IMD to have comparable effects on the cardiovascular system to AM, due, in part to IMD's ability to activate the same receptors. However, the lack of pharmacological tools to study this receptor system limits the extent to which it can be studied, and the true

contribution of each receptor in mediating the haemodynamic effects of IMD remain to be determined. This work has also extended previous findings by the haemodynamic group at the University of Nottingham, which have shown changing vascular responses to vasoactive mediators during LPS-induced endotoxaemia in conscious rats. As both adenosine receptor ligands and AM are being studied as potential novel treatments in clinical sepsis, these findings are important, because they indicate that the point in the progression of sepsis should be considered together with putative regional haemodynamic effects, as well as the region targeted.

8.2 Suggestions for further research

In the future, the following studies would be of interest to extend the work contained within this thesis:

1. To measure levels of AM, IMD and adenosine in plasma and tissues taken from LPS-treated rats.

2. Determination of the mechanisms responsible for the loss of vascular responsiveness to AM and IMD 1.5 h from the onset of endoxaemia. This could be achieved in the following ways:

    a) Assessment of responses to agents which cause vasodilatation by a known mechanism. For example, the extent of endothelial dysfunction could be determined using known endothelial-dependent vasodilators, such as salbutamol and acetylcholine. The role of the NO pathway could be assessed using the NO donor, SNP, and LCK could be used to assess the function of K_{ATP} channels.

    b) Molecular and/or cellular biology techniques could be used to measure the levels of CL/RAMP receptor components in harvested tissues taken from rats 1.5 h from the onset of endotoxaemia (and to quantify levels of adenosine receptors at each time point, see Chapter six).

c) As co-administration of AM and AMBP-1 has been shown to reverse endothelial dysfunction in models of sepsis, AMBP-1 could be administered with AM or IMD, to determine if this combination restored vascular responsiveness to these peptides at the 1.5 h time point. It would also be of interest to assess the impact of AMBP-1 on IMD function; indeed, it is not known whether IMD binds with this, or a similar, binding protein. Furthermore, if vascular responsiveness is restored at the 1.5 h time point by AM/AMBP-1 co-administration, it would be of interest to administer AMBP-1 as a pre-treatment and then to assess whether this combination was able to restore vascular responsiveness in a global fashion.

3. To determine if regional loss of vascular responsiveness to peptides (and/or other agents) occurred in the conscious rat in other models of sepsis. This would identify common features of such models which may have clinical relevance.

4. To develop a Gram-positive model of sepsis. This has increasing clinical relevance due to the spread of hospital-acquired infections.

References

Abdelrahman, AM, Pang, CC (2006). Effect of intermedin/adrenomedullin-2 on venous tone in conscious rats. *Naunyn-Schmiedeberg's Arch.s Pharmacol* **373:** 376-380.

Abdelrahman, AM, Pang, CC (2007). Vasodilator mechanism of intermedin/adrenomedullin-2 in anesthetized rats. *Proc. West. Pharmacol. Soc* **50:** 43-46.

Ackland, G, Grocott, MP, Mythen, MG (2000). Understanding gastrointestinal perfusion in critical care: so near, and yet so far. *Crit. Care* **4:** 269-281.

Adanin, S, Yalovetskiy, IV, Nardulli, BA, Sam, AD, 2nd, Jonjev, ZS, Law, WR (2002). Inhibiting adenosine deaminase modulates the systemic inflammatory response syndrome in endotoxemia and sepsis. *Am. J. Physiol.* **282:** R1324-R1332.

Albertin, G, Carraro, G, Nussdorfer, GG (2005). Human adrenomedullin gene silencing by short interfering RNAs: a preliminary study. *Int. J. Mol. Med* .**15:** 579-583.

Angus, DC, Linde-Zwirble, WT, Lidicker, J, Clermont, G, Carcillo, J, Pinsky, MR (2001). Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit. Care Med.* **29:** 1303-1310.

Balk, RA (2002). Endotoxemia in critically ill patients: why a reliable test could be beneficial. *Crit Care* **6:** 289-290.

Barrett, RJ, Droppleman, DA (1993). Interactions of adenosine A1 receptor-mediated renal vasoconstriction with endogenous nitric oxide and ANG II. *Am. J. Physiol.* **265:** F651-659.

Beasley, D, Cohen, RA, Levinsky, NG (1990). Endotoxin inhibits contraction of vascular smooth muscle in vitro. *Am. J. Physiol.* **258:** H1187-1192.

Belardinelli, L, Shryock, J, West, GA, Clemo, HF, DiMarco, JP, Berne, RM (1984). Effects of adenosine and adenine nucleotides on the atrioventricular node of isolated guinea pig hearts. *Circulation* **70:** 1083-1091.

Belardinelli, L, Giles, WR, West, A (1988). Ionic mechanisms of adenosine actions in pacemaker cells from rabbit heart. *J. Physiol.* **405:** 615-633.

Bell, D, Zhao, Y, McCoy, FP, Devine, A, McDermott, BJ (2008). Expression of the counter-regulatory peptide intermedin is augmented in the presence of oxidative stress in hypertrophied cardiomyocytes. *Cell. Physiol. Biochem.* **21:** 409-420.

Belloni, FL, Belardinelli, L, Halperin, C, Hintze, TH (1989). An unusual receptor mediates adenosine-induced SA nodal bradycardia in dogs. *Am. J. Physiol.* **256:** H1553-1564.

Bennett, T, Mahajan, RP, March, JE, Kemp, PA, Gardiner, SM (2004). Regional and temporal changes in cardiovascular responses to norepinephrine and vasopressin during continuous infusion of lipopolysaccharide in conscious rats. *Br. J. Anaesth.* **93:** 400-407.

Berne, RM (1963). Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. *Am. J. Physiol.* **204:** 317-322.

Beutler, B, Rietschel, ET (2003). Innate immune sensing and its roots: the story of endotoxin. *Nat. Rev. Immunol.* **3:** 169-176.

Blair, R, Fishman, B, Amit, Z, Weeks, JR (1980). A simple double channel swivel for infusions of fluids into unrestrained animals. *Pharmacol. Biochem. Behav.* **12:** 463-466.

Bone, RC, Balk, RA, Cerra, FB, Dellinger, RP, Fein, AM, Knaus, WA, Schein, RM, Sibbald, WJ (1992). Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* **101:** 1644-1655.

Bone, RC, Sibbald, WJ, Sprung, CL (1992). The ACCP-SCCM consensus conference on sepsis and organ failure. *Chest* **101:** 1481-1483.

Borovikova, LV, Ivanova, S, Zhang, M, Yang, H, Botchkina, GI, Watkins, LR, Wang, H, Abumrad, N, Eaton, JW, Tracey, KJ (2000). Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* **405:** 458-462.

Brain, SD, Grant, AD (2004). Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol. Rev.* **84:** 903-934.

Brierley, J, Peters, MJ (2008). Distinct hemodynamic patterns of septic shock at presentation to pediatric intensive care. *Pediatrics* **122:** 752-759.

Brown, ZW, Amit, Z, Weeks, JR (1976). Simple flow-thru swivel for infusions into unrestrained animals. *Pharmacol. Biochem. Behav.* **5:** 363-365.

Bryan, PT, Marshall, JM (1999). Adenosine receptor subtypes and vasodilatation in rat skeletal muscle during systemic hypoxia: a role for A1 receptors. *J. Physiol.* **514:** 151-162.

Buckley, JF, Singer, M, Clapp, LH (2006). Role of K_{ATP} channels in sepsis. *Cardiovasc. Res.* **72:** 220-230.

Buras, JA, Holzmann, B, Sitkovsky, M (2005). Animal models of sepsis: setting the stage. *Nat. Rev. Drug. Discov.* **4:** 854-865.

Cameron, EM, Wang, SY, Fink, MP, Sellke, FW (1998). Mesenteric and skeletal muscle microvascular responsiveness in subacute sepsis. *Shock* **9:** 184-192.

Carre, JE, Singer, M (2008). Cellular energetic metabolism in sepsis: the need for a systems approach. *Biochim. Biophys. Acta.* **1777:** 763-771.

Carrizo, GJ, Wu, R, Cui, X, Dwivedi, AJ, Simms, HH, Wang, P (2007). Adrenomedullin and adrenomedullin-binding protein-1 downregulate inflammatory cytokines and attenuate tissue injury after gut ischemia-reperfusion. *Surgery* **141:** 245-253.

Casati, C, Monopoli, A, Dionisotti, S, Zocchi, C, Bonizzoni, E, Ongini, E (1994). Repeated administration of selective adenosine A1 and A2 receptor agonists in the spontaneously hypertensive rat: tolerance develops to A1-mediated hemodynamic effects. *J. Pharmacol. Exp. Ther.* **268:** 1506-1511.

Champion, HC, Erickson, CC, 3rd, Simoneaux, ML, Bivalacqua, TJ, Murphy, WA, Coy, DH, Kadowitz, PJ (1996). Proadrenomedullin NH2-terminal 20 peptide has cAMP-mediated vasodilator activity in the mesenteric vascular bed of the cat. *Peptides* **17:** 1379-1387.

Champion, HC, Santiago, JA, Murphy, WA, Coy, DH, Kadowitz, PJ (1997). Adrenomedullin $_{(22-52)}$ antagonizes vasodilator responses to CGRP but not adrenomedullin in the cat. *Am. J. Physiol.* **272:** R234-R242.

Champion, HC, Pierce, RL, Bivalacqua, TJ, Murphy, WA, Coy, DH, Kadowitz, PJ (2001). Analysis of responses to hAmylin, hCGRP, and hADM in isolated resistance arteries from the mesenteric vascular bed of the rat. *Peptides* **22:** 1427-1434.

Chang, CL, Roh, J, Hsu, SY (2004). Intermedin, a novel calcitonin family peptide that exists in teleosts as well as in mammals: a comparison with other calcitonin/intermedin family peptides in vertebrates. *Peptides* **25:** 1633-1642.

Charles, CJ, Rademaker, MT, Richards, AM, Cooper, GJ, Coy, DH, Jing, NY, Nicholls, MG (1997). Hemodynamic, hormonal, and renal effects of adrenomedullin in conscious sheep. *Am. J. Physiol.* **272:** R2040-2047.

Charles, CJ, Lainchbury, JG, Nicholls, MG, Rademaker, MT, Richards, AM, Troughton, RW (2003). Adrenomedullin and the renin-angiotensin-aldosterone system. *Regul. Pept.* **112:** 41-49.

Charles, CJ, Rademaker, MT, Richards, AM (2006). Hemodynamic, hormonal, and renal actions of adrenomedullin-2 in normal conscious sheep. *Endocrinology* **147:** 1871-1877.

Chauhan, M, Ross, GR, Yallampalli, U, Yallampalli, C (2007). Adrenomedullin-2, a novel calcitonin/calcitonin-gene-related peptide family peptide, relaxes rat mesenteric artery: influence of pregnancy. *Endocrinology* **148:** 1727-1735.

Cheung, BM, Hwang, IS, Li, CY, O, WS, Tsang, KW, Leung, RY, Kumana, CR, Tang, F (2004). Increased adrenomedullin expression in lungs in endotoxaemia. *J. Endocrinol.* **181:** 339-345.

Churchill, PC, Churchill, MC (1985). A1 and A2 adenosine receptor activation inhibits and stimulates renin secretion of rat renal cortical slices. *J. Pharmacol. Exp. Ther.* **232:** 589-594.

Churchill, PC, Bidani, AK, Schwartz, MM (1987). Renal effects of endotoxin in the male rat. *Am. J. Physiol.* **253:** F244-250.

Cohen, ES, Law, WR, Easington, CR, Cruz, KQ, Nardulli, BA, Balk, RA, Parrillo, JE, Hollenberg, SM (2002). Adenosine deaminase inhibition attenuates microvascular dysfunction and improves survival in sepsis. *Am. J. Respir. Crit. Care Med.* **166:** 16-20.

Cohen, J (2002). The immunopathogenesis of sepsis. *Nature* **420:** 885-891.

Cohen, J (2000). The detection and interpretation of endotoxaemia. *Intensive Care Med.* **26:** S51-56.

Collin, M, Murch, O, Thiemermann, C (2006). Peroxisome proliferator-activated receptor-gamma antagonists GW9662 and T0070907 reduce the protective effects of lipopolysaccharide preconditioning against organ failure caused by endotoxemia. *Crit. Care Med.* **34:** 1131-1138.

Conlay, LA, Evoniuk, G, Wurtman, RJ (1988). Endogenous adenosine and hemorrhagic shock: effects of caffeine administration or caffeine withdrawal. *Proc. Natl. Acad. Sci. USA* **85:** 4483-4485.

Conlon, BA, Ross, JD, Law, WR (2005). Advances in understanding adenosine as a plurisystem modulator in sepsis and the systemic inflammatory response syndrome (SIRS). *Front. Biosci.* **10:** 2548-2565.

Connelly, L, Madhani, M, Hobbs, AJ (2005). Resistance to endotoxic shock in endothelial nitric-oxide synthase (eNOS) knock-out mice: a pro-inflammatory role for eNOS-derived no in vivo. *J. Biol. Chem.* **280:** 10040-10046.

Cotter, G, Dittrich, HC, Weatherley, BD, Bloomfield, DM, O'Connor, CM, Metra, M, Massie, BM (2008). The PROTECT pilot study: a randomized, placebo-controlled, dose-finding study of the adenosine A1 receptor antagonist rolofylline in patients with acute heart failure and renal impairment. *J. Card. Fail.* **14:** 631-640.

Cronstein, BN, Levin, RI, Philips, M, Hirschhorn, R, Abramson, SB, Weissmann, G (1992). Neutrophil adherence to endothelium is enhanced via adenosine A1 receptors and inhibited via adenosine A2 receptors. *J. Immunol.* **148:** 2201-2206.

Cui, H, Kohsaka, A, Waki, H, Gouraud, S, Nakamura, T, Yukawa, K, Hatada, A, Bhuiyan, ME, Maeda, M (2008). Adrenomedullin 2 microinjection into the nucleus tractus solitarius elevates arterial pressure and heart rate in rats. *Auton. Neurosci.* **142:** 45-50.

Cui, X, Wu, R, Zhou, M, Dong, W, Ulloa, L, Yang, H, Wang, H, Tracey, KJ, Simms, HH, Wang, P (2005). Adrenomedullin and its binding protein attenuate the proinflammatory response after hemorrhage. *Crit. Care Med.* **33:** 391-398.

Cui, Y, Ji, Y, Wu, R, Zhou, M, Wang, P (2006). Adrenomedullin binding protein-1 is downregulated during polymicrobial sepsis in the rat. *Int J Mol Med* **17:** 925-929.

Cunha, RA (2001). Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem. Int.* **38:** 107-125.

Czaika, G, Gingras, Y, Zhu, E, Comtois, AS (2000). Induction of the ATP-sensitive potassium (uK(ATP)-1) channel by endotoxemia. *Muscle Nerve.* **23:** 967-969.

Dabisch, PA, Liles, JT, Taylor, JT, Sears, BW, Saenz, R, Kadowitz, PJ (2004). Role of potassium channels in the nitric oxide-independent vasodilator response to acetylcholine. *Pharmacol. Res.* **49:** 207-215.

Dackor, R, Caron, K (2007). Mice heterozygous for adrenomedullin exhibit a more extreme inflammatory response to endotoxin-induced septic shock. *Peptides* **28**(11): 2164-2170.

Danialou, G, Vicaut, E, Sambe, A, Aubier, M, Boczkowski, J (1997). Predominant role of A1 adenosine receptors in mediating adenosine induced vasodilatation of rat diaphragmatic arterioles: involvement of nitric oxide and the ATP-dependent K+ channels. *Br. J. Pharmacol.* **121:** 1355-1363.

Danner, RL, Elin, RJ, Hosseini, JM, Wesley, RA, Reilly, JM, Parillo, JE (1991). Endotoxemia in human septic shock. *Chest* **99:** 169-175.

DiMarco, JP, Sellers, TD, Berne, RM, West, GA, Belardinelli, L (1983). Adenosine: electrophysiologic effects and therapeutic use for terminating paroxysmal supraventricular tachycardia. *Circulation* **68:** 1254-1263.

Dixon, AK, Gubitz, AK, Sirinathsinghji, DJ, Richardson, PJ, Freeman, TC (1996). Tissue distribution of adenosine receptor mRNAs in the rat. *Br. J. Pharmacol.* **118:** 1461-1468.

Dong, F, Taylor, MM, Samson, WK, Ren, J (2006). Intermedin (adrenomedullin-2) enhances cardiac contractile function via a protein kinase C- and protein kinase A-dependent pathway in murine ventricular myocytes. *J. Appl. Physiol.* **101:** 778-784.

Drury, AN, Szent-Gyorgyi, A (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J. Physiol.* **68:** 213-237.

Dwivedi, AJ, Wu, R, Nguyen, E, Higuchi, S, Wang, H, Krishnasastry, K, Marini, CP, Ravikumar, TS, Wang, P (2007). Adrenomedullin and adrenomedullin binding protein-1 prevent acute lung injury after gut ischemia-reperfusion. *J. Am. Coll. Surg.* **205:** 284-293.

Dyson, A, Stidwill, R, Taylor, V, Singer, M (2007). Tissue oxygen monitoring in rodent models of shock. *Am J Physiol.* **293:** H526-533.

Eastridge, BJ, Darlington, DN, Evans, JA, Gann, DS (1994). A circulating shock protein depolarizes cells in hemorrhage and sepsis. *Ann. Surg.* **219:** 298-305.

Elhawary, AM, Poon, J, Pang, CC (1995). Effects of calcitonin gene-related peptide receptor antagonists on renal actions of adrenomedullin. *Br. J. Pharmacol.* **115:** 1133-1140.

Elsasser, TH, Kahl, S, Martinez, A, Montuenga, LM, Pio, R, Cuttitta, F (1999). Adrenomedullin binding protein in the plasma of multiple species: characterization by radioligand blotting. *Endocrinology* **140:** 4908-4911.

Ertmer, C, Morelli, A, Rehberg, S, Lange, M, Hucklenbruch, C, Van Aken, H, Booke, M, Westphal, M (2007). Exogenous adrenomedullin prevents and reverses hypodynamic circulation and pulmonary hypertension in ovine endotoxaemia. *Br. J. Anaesth.* **99:** 830-836.

Evans, DB, Schenden, JA, Bristol, JA (1982). Adenosine receptors mediating cardiac depression. *Life Sci.* **31:** 2425-2432.

Evoniuk, G, Jacobson, KA, Shamim, MT, Daly, JW, Wurtman, RJ (1987). A1- and A2-selective adenosine antagonists: in vivo characterization of cardiovascular effects. *J. Pharmacol. Exp. Ther.* **242:** 882-887.

Farmer, MR, Roberts, RE, Gardiner, SM, Ralevic, V (2003). Effects of in vivo lipopolysaccharide infusion on vasoconstrictor function of rat isolated mesentery, kidney, and aorta. *J. Pharmacol. Exp. Ther.* **306:** 538-545.

Feoktistov, I, Biaggioni, I (1997). Adenosine A2B receptors. *Pharmacol. Rev.* **49:** 381-402.

Fernandes, D, da Silva-Santos, JE, Duma, D, Villela, CG, Barja-Fidalgo, C, Assreuy, J (2006). Nitric oxide-dependent reduction in soluble guanylate cyclase functionality accounts for early lipopolysaccharide-induced changes in vascular reactivity. *Mol Pharmacol* **69:** 983-990.

Fowler, DE, Wang, P (2002). The cardiovascular response in sepsis: proposed mechanisms of the beneficial effect of adrenomedullin and its binding protein (review). *Int. J. Mol. Med.* **9:** 443-449.

Fowler, DE, Yang, S, Zhou, M, Chaudry, IH, Simms, HH, Wang, P (2003). Adrenomedullin and adrenomedullin binding protein-1: their role in the septic response. *J. Surg. Res.* **109:** 175-181.

Fozard, JR, Hannon, JP (1999). Adenosine receptor ligands: potential as therapeutic agents in asthma and COPD. *Pulm. Pharmacol. Ther.* **12:** 111-114.

Fredholm, BB, Ap, IJ, Jacobson, KA, Klotz, KN, Linden, J (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* **53:** 527-552.

Fredholm, BB, Jacobson, KA, Jonzon, B, Kirk, KL, Li, YO, Daly, JW (1987). Evidence that a novel 8-phenyl-substituted xanthine derivative is a cardioselective adenosine receptor antagonist in vivo. *J. Cardiovasc. Pharmacol.* **9:** 396-400.

Fredholm, BB, Irenius, E, Kull, B, Schulte, G (2001). Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochem. Pharmacol.* **61:** 443-448.

Fritz-Six, KL, Dunworth, WP, Li, M, Caron, KM (2008). Adrenomedullin signaling is necessary for murine lymphatic vascular development. *J. Clin. Invest.* **118:** 40-50.

Fuentes, JM, Talamini, MA, Fulton, WB, Hanly, EJ, Aurora, AR, De Maio, A (2006). General anesthesia delays the inflammatory response and increases survival for mice with endotoxic shock. *Clin. Vaccine. Immunol.* **13:** 281-288.

Fujisawa, Y, Nagai, Y, Miyatake, A, Takei, Y, Miura, K, Shoukouji, T, Nishiyama, A, Kimura, S, Abe, Y (2004). Renal effects of a new member of adrenomedullin family, adrenomedullin2, in rats. *Eur. J. Pharmacol.* **497:** 75-80.

Fujisawa, Y, Nagai, Y, Miyatake, A, Miura, K, Shokoji, T, Nishiyama, A, Kimura, S, Abe, Y (2006). Roles of adrenomedullin 2 in regulating the cardiovascular and sympathetic nervous systems in conscious rats. *Am. J. Physiol.* **290:** H1120-H1127.

Fujisawa, Y, Nagai, Y, Miyatake, A, Miura, K, Nishiyama, A, Kimura, S, Abe, Y (2007). Effects of adrenomedullin 2 on regional hemodynamics in conscious rats. *European journal of pharmacology* **558:** 128-132.

Gallos, G, Ruyle, TD, Emala, CW, Lee, HT (2005). A1 adenosine receptor knockout mice exhibit increased mortality, renal dysfunction, and hepatic injury in murine septic peritonitis. *Am. J.Physiol.* **289:** F369-F376.

Gardiner, SM, Compton, AM, Bennett, T (1989). Regional hemodynamic changes following hypovolemia in conscious rats. *Am. J. Physiol.* **256:** R1076-R1083.

Gardiner, SM, Compton, AM, Kemp, PA, Bennett, T (1991). Effects of NG-nitro-L-arginine methyl ester or indomethacin on differential regional and cardiac haemodynamic actions of arginine vasopressin and lysine vasopressin in conscious rats. *Br. J. Pharmacol.* **102:** 65-72.

Gardiner, SM, Kemp, PA, Bennett, T (1991). Effects of NG-nitro-L-arginine methyl ester on vasodilator responses to acetylcholine, 5'-N-ethylcarboxamidoadenosine or salbutamol in conscious rats. *Br. J. Pharmacol.* **103:** 1725-1732.

Gardiner, SM, Kemp, PA, Bennett, T (1991). Effects of NG-nitro-L-arginine methyl ester on vasodilator responses to adrenaline or BRL 38227 in conscious rats. *Br. J. Pharmacol.* **104:** 731-737.

Gardiner, SM, Kemp, PA, March, JE, Bennett, T (1995). Cardiac and regional haemodynamics, inducible nitric oxide synthase (NOS) activity, and the effects of NOS inhibitors in conscious, endotoxaemic rats. *Br. J. Pharmacol.* **116:** 2005-2016.

Gardiner, SM, Kemp, PA, March, JE, Bennett, T (1995). Regional haemodynamic effects of human and rat adrenomedullin in conscious rats. *Br. J. Pharmacol.* **114:** 584-591.

Gardiner, SM, Kemp, PA, March, JE, Bennett, T (1996). Temporal differences between the involvement of angiotensin II and endothelin in the cardiovascular responses to endotoxaemia in conscious rats. *Br. J. Pharmacol.* **119:** 1619-1627.

Gardiner, SM, Kemp, PA, March, JE, Bennett, T (1999). Regional haemodynamic responses to infusion of lipopolysaccharide in conscious rats: effects of pre- or post-treatment with glibenclamide. *Br. J. Pharmacol.* **128:** 1772-1778.

Gardiner, SM, March, JE, Kemp, PA, Bennett, T (1999). Influence of CGRP (8-37), but not adrenomedullin (22-52), on the haemodynamic responses to lipopolysaccharide in conscious rats. *Br. J. Pharmacol.* **127:** 1611-1618.

Gardiner, SM, March, JE, Kemp, PA, Bennett, T (2006). Effects of nitric oxide synthase inhibition with or without cyclooxygenase-2 inhibition on resting haemodynamics and responses to exendin-4. *Br. J. Pharmacol.* **149:** 802-809.

Ghai, G, Francis, JE, Williams, M, Dotson, RA, Hopkins, MF, Cote, DT, Goodman, FR, Zimmerman, MB (1987). Pharmacological characterization of CGS 15943A: a novel nonxanthine adenosine antagonist. *J. Pharmacol. Exp. Ther.* **242:** 784-790.

Gibbons, C, Dackor, R, Dunworth, W, Fritz-Six, K, Caron, KM (2006). "RAMP"ing up Adrenomedullin Signaling. *Mol. Endocrinol.* **21:** 783-796.

Goldfarb, RD, Glock, D, Kumar, A, McCarthy, RJ, Mei, J, Guynn, T, Matushek, M, Trenholme, G, Parrillo, JE (1996). A porcine model of peritonitis and bacteremia simulates human septic shock. *Shock* **6:** 442-451.

Goto, K, Fujii, K, Onaka, U, Abe, I, Fujishima, M (2000). Effects of adrenomedullin and PAMP on membrane potential and neurotransmission. *Peptides* **21:** 257-263.

Graier, WF, Myers, PR, Rubin, LJ, Adams, HR, Parker, JL (1994). Escherichia coli endotoxin inhibits agonist-mediated cytosolic Ca^{2+} mobilization and nitric oxide biosynthesis in cultured endothelial cells. *Circ. Res.* **75:** 659-668.

Granger, HJ, Norris, CP (1980). Role of adenosine in local control of intestinal circulation in the dog. *Circ. Res.* **46:** 764-770.

Grant, AD, Tam, CW, Lazar, Z, Shih, MK, Brain, SD (2004). The calcitonin gene-related peptide (CGRP) receptor antagonist BIBN4096BS blocks CGRP and adrenomedullin vasoactive responses in the microvasculature. *Br. J. Pharmacol.* **142:** 1091-1098.

Hamid, SA, Baxter, GF (2006). A critical cytoprotective role of endogenous adrenomedullin in acute myocardial infarction. *J. Mol. Cell Cardiol.* **41:** 360-363.

Hansen, PB, Hashimoto, S, Oppermann, M, Huang, Y, Briggs, JP, Schnermann, J (2005). Vasoconstrictor and vasodilator effects of adenosine in the mouse kidney due to preferential activation of A1 or A2 adenosine receptors. *J. Pharmacol. Exp. Ther.* **315:** 1150-1157.

Harrison, DA, Welch, CA, Eddleston, JM (2006). The epidemiology of severe sepsis in England, Wales and Northern Ireland, 1996 to 2004: secondary analysis of a high quality clinical database, the ICNARC Case Mix Programme Database. *Crit. Care* **10:** R42.

Hartley, CJ, Cole, JS (1974). An ultrasonic pulsed Doppler system for measuring blood flow in small vessels. *J. Appl. Physiol.* **37:** 626-629.

Hasko, G, Cronstein, BN (2004). Adenosine: an endogenous regulator of innate immunity. *Trends. Immunol.* **25:** 33-39.

Hattori, Y, Murakami, Y, Atsuta, H, Minamino, N, Kangawa, K, Kasai, K (1998). Glucocorticoid regulation of adrenomedullin in a rat model of endotoxic shock. *Life Sci.* **62:** PL181-PL189.

Hay, DL, Howitt, SG, Conner, AC, Schindler, M, Smith, DM, Poyner, DR (2003). CL/RAMP2 and CL/RAMP3 produce pharmacologically distinct adrenomedullin receptors: a comparison of effects of adrenomedullin$_{22-52}$, CGRP$_{8-37}$ and BIBN4096BS. *Br. J. Pharmacol.* **140:** 477-486.

Haywood, JR, Shaffer, RA, Fastenow, C, Fink, GD, Brody, MJ (1981). Regional blood flow measurement with pulsed Doppler flowmeter in conscious rat. *Am. J. Physiol.* **241:** H273-H278.

He, H, Bessho, H, Fujisawa, Y, Horiuchi, K, Tomohiro, A, Kita, T, Aki, Y, Kimura, S, Tamaki, T, Abe, Y (1995). Effects of a synthetic rat adrenomedullin on regional hemodynamics in rats. *Eur. J. Pharmacol.* **273:** 209-214.

Heaton, J, Lin, B, Chang, JK, Steinberg, S, Hyman, A, Lippton, H (1995). Pulmonary vasodilation to adrenomedullin: a novel peptide in humans. *Am. J. Physiol.* **268:** H2211-H2215.

Hiley, CR, Bottrill, FE, Warnock, J, Richardson, PJ (1995). Effects of pH on responses to adenosine, CGS 21680, carbachol and nitroprusside in the isolated perfused superior mesenteric arterial bed of the rat. *Br. J. Pharmacol.* **116:** 2641-2646.

Hingorani, AD, Cross, J, Kharbanda, RK, Mullen, MJ, Bhagat, K, Taylor, M, Donald, AE, Palacios, M, Griffin, GE, Deanfield, JE, MacAllister, RJ, Vallance, P (2000). Acute systemic inflammation impairs endothelium-dependent dilatation in humans. *Circulation* **102:** 994-999.

Hintze, TH, Belloni, FL, Harrison, JE, Shapiro, GC (1985). Apparent reduction in baroreflex sensitivity to adenosine in conscious dogs. *Am. J. Physiol.* **249:** H554-H559.

Hirata, Y, Hayakawa, H, Suzuki, Y, Suzuki, E, Ikenouchi, H, Kohmoto, O, Kimura, K, Kitamura, K, Eto, T, Kangawa, K, et al. (1995). Mechanisms of adrenomedullin-induced vasodilation in the rat kidney. *Hypertension* **25:** 790-795.

Hofbauer, KH, Schoof, E, Kurtz, A, Sandner, P (2002). Inflammatory cytokines stimulate adrenomedullin expression through nitric oxide-dependent and -independent pathways. *Hypertension* **39:** 161-167.

Humphrey, SJ, Ludens, JH (1998). K-ATP-blocking diuretic PNU-37883A reduces plasma renin activity in dogs. *J. Cardiovasc. Pharmacol.* **31:** 894-903.

Hurley, JC (1994). Concordance of endotoxemia with gram-negative bacteremia in patients with gram-negative sepsis: a meta-analysis. *J. Clin. Microbiol.* **32:** 2120-2127.

Hurley, JC (1995). Endotoxemia: methods of detection and clinical correlates. *Clin. Microbiol. Rev.* **8:** 268-292.

Ichikawa-Shindo, Y, Sakurai, T, Kamiyoshi, A, Kawate, H, Iinuma, N, Yoshizawa, T, Koyama, T, Fukuchi, J, Iimuro, S, Moriyama, N, Kawakami, H, Murata, T, Kangawa, K, Nagai, R, Shindo, T (2008). The GPCR modulator protein RAMP2 is essential for angiogenesis and vascular integrity. *J. Clin. Invest.* **118:** 29-39.

Ishimitsu, T, Ono, H, Minami, J, Matsuoka, H (2006). Pathophysiologic and therapeutic implications of adrenomedullin in cardiovascular disorders. *Pharmacol.Ther.* **111:** 909-927.

Jackson, EK (1991). Adenosine: a physiological brake on renin release. *Annu. Rev. Pharmacol. Toxicol.* **31:** 1-35.

Jackson, EK, Zhu, C, Tofovic, SP (2002). Expression of adenosine receptors in the preglomerular microcirculation. *Am. J. Physiol.* **283:** F41-F51.

Jackson, EK, Mi, Z, Zacharia, LC, Tofovic, SP, Dubey, RK (2007). The pancreatohepatorenal cAMP-adenosine mechanism. *J. Pharmacol. Exp. Ther.* **321:** 799-809.

James, TN (1965). The chronotropic action of ATP and related compounds studied by direct perfusion of the sinus node. *J. Pharmacol. Exp. Ther.* **149:** 233-247.

Jeukendrup, AE, Vet-Joop, K, Sturk, A, Stegen, JH, Senden, J, Saris, WH, Wagenmakers, AJ (2000). Relationship between gastro-intestinal complaints and endotoxaemia, cytokine release and the acute-phase reaction during and after a long-distance triathlon in highly trained men. *Clin Sci (Lond)* **98:** 47-55.

Johansson, B, Halldner, L, Dunwiddie, TV, Masino, SA, Poelchen, W, Gimenez-Llort, L, Escorihuela, RM, Fernandez-Teruel, A, Wiesenfeld-Hallin, Z, Xu, XJ, Hardemark, A, Betsholtz, C, Herlenius, E, Fredholm, BB (2001). Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A1 receptor. *Proc. Natl. Acad. Sci. USA* **98:** 9407-9412.

Jolly, L, March, JE, Kemp, PA, Bennett, T, Gardiner, SM (2008). Regional haemodynamic responses to adenosine receptor activation vary across time following lipopolysaccharide treatment in conscious rats. *Br. J. Pharmacol.* **154**: 1600-1610.

Jolly, L, March, JE, Kemp, PA, Bennett, T, Gardiner, SM (2009). Mechanisms involved in the regional haemodynamic effects of intermedin (adrenomedullin 2) compared with adrenomedullin in conscious rats. *Br J Pharmacol* **157:** 1502-1513.

Kaiser, SM, Quinn, RJ (1999). Adenosine receptors as potential therapeutic targets. *Drug Discov. Today* **4:** 542-551.

Kandilci, HB, Gumusel, B, Wasserman, A, Witriol, N, Lippton, H (2006). Intermedin/adrenomedullin-2 dilates the rat pulmonary vascular bed: Dependence on CGRP receptors and nitric oxide release. *Peptides* **27:** 1390-1396.

Kandilci, HB, Gumusel, B, Lippton, H (2008). Intermedin/adrenomedullin-2 (IMD/AM2) relaxes rat main pulmonary arterial rings via cGMP-dependent pathway: role of nitric oxide and large conductance calcium-activated potassium channels (BK(Ca)). *Peptides* **29:** 1321-1328.

Kapas, S, Martinez, A, Cuttitta, F, Hinson, JP (1998). Local production and action of adrenomedullin in the rat adrenal zona glomerulosa. *J. Endocrinol.* **156:** 477-484.

Kato, J, Tsuruda, T, Kita, T, Kitamura, K, Eto, T (2005). Adrenomedullin: a protective factor for blood vessels. *Arterioscle. Thromb. Vasc Biol.* **25:** 2480-2487.

Kellett, R, Bowmer, CJ, Collis, MG, Yates, MS (1989). Amelioration of glycerol-induced acute renal failure in the rat with 8-cyclopentyl-1,3-dipropylxanthine. *Br. J. Pharmacol.* **98:** 1066-1074.

Kirschning, CJ, Wesche, H, Merrill Ayres, T, Rothe, M (1998). Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J. Exp. Med.* **188:** 2091-2097.

Kitamura, K, Kangawa, K, Kawamoto, M, Ichiki, Y, Nakamura, S, Matsuo, H, Eto, T (1993). Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem. Biophys. Res. Commun.* **192:** 553-560.

Kitamura, K, Sakata, J, Kangawa, K, Kojima, M, Matsuo, H, Eto, T (1993). Cloning and characterization of cDNA encoding a precursor for human adrenomedullin. *Biochem. Biophys. Res. Commun.* **194:** 720-725.

Kobayashi, Y, Liu, YJ, Gonda, T, Takei, Y (2004). Coronary vasodilatory response to a novel Peptide, adrenomedullin 2. *Clin. Exp. Pharmacol. Physiol.* **31:** S49-S50.

Koo, DJ, Zhou, M, Chaudry, IH, Wang, P (2001). The role of adrenomedullin in producing differential hemodynamic responses during sepsis. *J. Surg. Res.* **95:** 207-218.

Kristof, AS, Noorhosseini, H, Hussain, SN (1997). Attenuation of endothelium-dependent hyperpolarizing factor by bacterial lipopolysaccharides. *Eur. J. Pharmacol.* **328:** 69-73.

Kuan, CJ, Herzer, WA, Jackson, EK (1992). An experimental paradigm for investigating the role of endogenous adenosine/A1 receptor interactions in vivo. *J. Pharmacol. Exp. Ther.* **263:** 657-662.

Kumar, A, Brar, R, Wang, P, Dee, L, Skorupa, G, Khadour, F, Schulz, R, Parrillo, JE (1999). Role of nitric oxide and cGMP in human septic serum-induced depression of cardiac myocyte contractility. *Am. J. Physiol.* **276:** R265-R276.

Kumar, A, Paladugu, B, Mensing, J, Parrillo, JE (2007). Transforming growth factor-beta1 blocks in vitro cardiac myocyte depression induced by tumor necrosis factor-alpha, interleukin-1beta, and human septic shock serum. *Crit. Care Med.* **35:** 358-364.

Kumar, A, Thota, V, Dee, L, Olson, J, Uretz, E, Parrillo, JE (1996). Tumor necrosis factor alpha and interleukin 1beta are responsible for in vitro myocardial cell depression induced by human septic shock serum. *J. Exp. Med.* **183:** 949-958.

Lappe, RW, Sheldon, JH, Cox, BF (1992). Selective adenosine-2 agonist produces both direct and reflex tachycardia in normotensive rats. *J. Cardiovasc. Pharmacol.* **19:** 460-463.

Law, WR (2006). Adenosine receptors in the response to sepsis: what do receptor-specific knockouts tell us? *Am. J. Physiol.* **291:** R957-R958.

Law, WR, Valli, VE, Conlon, BA (2003). Therapeutic potential for transient inhibition of adenosine deaminase in systemic inflammatory response syndrome. *Crit. Care Med.* **31:** 1475-1481.

Lee, HT, Kim, M, Joo, JD, Gallos, G, Chen, JF, Emala, CW (2006). A3 adenosine receptor activation decreases mortality and renal and hepatic injury in murine septic peritonitis. *Am. J. Physiol.* **291:** R959-R969.

Lepper, PM, Held, TK, Schneider, EM, Bölke, E, Gerlach, H, Trautmann, M (2002). Clinical implications of antibiotic-induced endotoxin release in septic shock. *Intensive Care Med.* **28:** 824-833.

Li, YY, Wong, LY, Cheung, BM, Hwang, IS, Tang, F (2005). Differential induction of adrenomedullin, interleukins and tumour necrosis factor-alpha by lipopolysaccharide in rat tissues in vivo. *Clin. Exp. Pharmacol. Physiol.* **32:** 1110-1118.

Li, YY, Cheung, BM, Wong, LY, Hwang, IS, Kumana, CR, Tang, F (2005). Adrenomedullin gene expression and levels in the cardiovascular system after treatment with lipopolysaccharide. *Neuropeptides* **39:** 73-80.

Linden, J (2001). Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. *Annu. Rev. Pharmacol. Toxicol.* **41:** 775-787.

Lippton, H, Chang, JK, Hao, Q, Summer, W, Hyman, AL (1994). Adrenomedullin dilates the pulmonary vascular bed in vivo. *J. Appl. Physiol.* **76:** 2154-2156.

Llewelyn, MJ, Cohen, J (2007). Tracking the microbes in sepsis: advancements in treatment bring challenges for microbial epidemiology. *Clin. Infect. Dis.* **44:** 1343-1348.

Londos, C, Cooper, DM, Wolff, J (1980). Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. USA* **77:** 2551-2554.

Lorenz, E, Mira, JP, Frees, KL, Schwartz, DA (2002). Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. *Arch. Intern. Med.* **162:** 1028-1032.

Luodonpaa, M, Leskinen, H, Ilves, M, Vuolteenaho, O, Ruskoaho, H (2004). Adrenomedullin modulates hemodynamic and cardiac effects of angiotensin II in conscious rats. *Am. J. Physiol.* **286:** R1085-R1092.

Lynge, J, Hellsten, Y (2000). Distribution of adenosine A1, A2A and A2B receptors in human skeletal muscle. *Acta. Physiol. Scand.* **169:** 283-290.

MacKenzie, IM (2001). The haemodynamics of human septic shock. *Anaesthesia* **56:** 130-144.

Marshall, JC, Walker, PM, Foster, DM, Harris, D, Ribeiro, M, Paice, J, Romaschin, AD, Derzko, AN (2002). Measurement of endotoxin activity in critically ill patients using whole blood neutrophil dependent chemiluminescence. *Crit. Care* **6:** 342-348.

Marshall, JM, Thomas, T, Turner, L (1993). A link between adenosine, ATP-sensitive K+ channels, potassium and muscle vasodilatation in the rat in systemic hypoxia. *J. Physiol.* **472:** 1-9.

Martin, C, Leone, M, Viviand, X, Ayem, ML, Guieu, R (2000). High adenosine plasma concentration as a prognostic index for outcome in patients with septic shock. *Crit. Care Med.* **28:** 3198-3202.

Martinez, A, Oh, HR, Unsworth, EJ, Bregonzio, C, Saavedra, JM, Stetler-Stevenson, WG, Cuttitta, F (2004). Matrix metalloproteinase-2 cleavage of adrenomedullin produces a vasoconstrictor out of a vasodilator. *Biochem. J.* **383:** 413-418.

Mathot, RA, van Schaick, EA, Langemeijer, MW, Soudijn, W, Breimer, DD, Ijzerman, AP, Danhof, M (1994). Pharmacokinetic-pharmacodynamic relationship of the cardiovascular effects of adenosine A1 receptor agonist N6-cyclopentyladenosine in the rat. *J. Pharmacol. Exp. Ther.* **268:** 616-624.

Matsuda, N, Hattori, Y (2007). Vascular biology in sepsis: pathophysiological and therapeutic significance of vascular dysfunction. *J. Smooth Muscle Res.* **43:** 117-137.

Mazzocchi, G, Albertin, G, Nussdorfer, GG (2000). Adrenomedullin (ADM), acting through ADM (22-52)-sensitive receptors, is involved in the endotoxin-induced hypotension in rats. *Life Sci* **66:** 1445-1450.

McLatchie, LM, Fraser, NJ, Main, MJ, Wise, A, Brown, J, Thompson, N, Solari, R, Lee, MG, Foord, SM (1998). RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* **393:** 333-339.

Merkel, LA, Lappe, RW, Rivera, LM, Cox, BF, Perrone, MH (1992). Demonstration of vasorelaxant activity with an A1-selective adenosine agonist in porcine coronary artery: involvement of potassium channels. *J. Pharmacol. Exp. Ther.* **260:** 437-443.

Merx, MW, Weber, C (2007). Sepsis and the heart. *Circulation* **116:** 793-802.

Mian, R, Marshall, JM (1995). The role of adenosine in mediating vasodilatation in mesenteric circulation of the rat in acute and chronic hypoxia. *J. Physiol.* **489:** 225-234.

Miksa, M, Wu, R, Cui, X, Dong, W, Das, P, Simms, HH, Ravikumar, TS, Wang, P (2007). Vasoactive hormone adrenomedullin and its binding protein: anti-inflammatory effects by up-regulating peroxisome proliferator-activated receptor-gamma. *J. Immunol.* **179:** 6263-6272.

Miura, K, Ebara, T, Okumura, M, Matsuura, T, Kim, S, Yukimura, T, Iwao, H (1995). Attenuation of adrenomedullin-induced renal vasodilatation by NG-nitro L-arginine but not glibenclamide. *Br. J. Pharmacol.* **115:** 917-924.

Morimoto, R, Satoh, F, Murakami, O, Totsune, K, Suzuki, T, Sasano, H, Ito, S, Takahashi, K (2007). Expression of adrenomedullin2/intermedin in human brain, heart, and kidney. *Peptides* **28:** 1095-1103.

Motew, SJ, Sam, AD, Mourelatos, MG, Sharma, AC, Alden, KJ, Ferguson, JL, Law, WR (1998). Adenosine receptor antagonism affects regional resting vascular resistance during rat peritoneal sepsis. *J. Surg. Res.* **80:** 326-332.

Mubagwa, K, Mullane, K, Flameng, W (1996). Role of adenosine in the heart and circulation. *Cardiovasc. Res.* **32:** 797-813.

Murray, RD, Churchill, PC (1984). Effects of adenosine receptor agonists in the isolated, perfused rat kidney. *Am. J. Physiol.* **247:** H343-H348.

Murray, RD, Churchill, PC (1985). Concentration dependency of the renal vascular and renin secretory responses to adenosine receptor agonists. *J Pharmacol Exp Ther* **232:** 189-193.

Nagoshi, Y, Kuwasako, K, Cao, YN, Imamura, T, Kitamura, K, Eto, T (2004). Tumor necrosis factor-alpha downregulates adrenomedullin receptors in human coronary artery smooth muscle cells. *Peptides* **25:** 1115-1121.

Nandha, KA, Taylor, GM, Smith, DM, Owji, AA, Byfield, PG, Ghatei, MA, Bloom, SR (1996). Specific adrenomedullin binding sites and hypotension in the rat systemic vascular bed. *Regul. Pept.* **62:** 145-151.

Nemeth, ZH, Csoka, B, Wilmanski, J, Xu, D, Lu, Q, Ledent, C, Deitch, EA, Pacher, P, Spolarics, Z, Hasko, G (2006). Adenosine A2A receptor inactivation increases survival in polymicrobial sepsis. *J. Immunol.* **176:** 5616-5626.

Newby, AC (1984). Adenosine and the concept of retaliatory metabolites. *Trends Biochem. Sci.* **9.**

Nieri, P, Martinotti, E, Calderone, V, Breschi, MC (2001). Adenosine-mediated hypotension in in vivo guinea-pig: receptors involved and role of NO. *Br. J. Pharmacol.* **134:** 745-752.

Nishikimi, T, Karasawa, T, Inaba, C, Ishimura, K, Tadokoro, K, Koshikawa, S, Yoshihara, F, Nagaya, N, Sakio, H, Kangawa, K, Matsuoka, H (2009). Effects of long-term intravenous administration of adrenomedullin (AM) plus hANP therapy in acute decompensated heart failure. *Circ. J.* **73:** 892-898.

Nishiyama, A, Miura, K, Miyatake, A, Fujisawa, Y, Yue, W, Fukui, T, Kimura, S, Abe, Y (1999). Renal interstitial concentration of adenosine during endotoxin shock. *Eur. J. Pharmacol.* **385:** 209-216.

Nuki, C, Kawasaki, H, Kitamura, K, Takenaga, M, Kangawa, K, Eto, T, Wada, A (1993). Vasodilator effect of adrenomedullin and calcitonin gene-related peptide receptors in rat mesenteric vascular beds. *Biochem. Biophys. Res. Commun.* **196:** 245-251.

O'Brien, AJ, Wilson, AJ, Sibbald, R, Singer, M, Clapp, LH (2001). Temporal variation in endotoxin-induced vascular hyporeactivity in a rat mesenteric artery organ culture model. *Br. J. Pharmacol.* **133:** *351-360.*

O'Brien, JM, Jr., Ali, NA, Aberegg, SK, Abraham, E (2007). Sepsis. *Am. J. Med.* **120:** 1012-1022.

Ogoshi, M, Inoue, K, Naruse, K, Takei, Y (2006). Evolutionary history of the calcitonin gene-related peptide family in vertebrates revealed by comparative genomic analyses. *Peptides* **27:** 3154-3164.

Ogoshi, M, Inoue, K, Takei, Y (2003). Identification of a novel adrenomedullin gene family in teleost fish. *Biochem. Biophys. Res. Commun.* **311:** 1072-1077.

Ohnishi, A, Biaggioni, I, Deray, G, Branch, RA, Jackson, EK (1986). Hemodynamic effects of adenosine in conscious hypertensive and normotensive rats. *Hypertension* **8:** 391-398.

Ohta, A, Sitkovsky, M (2001). Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* **414:** 916-920.

Ono, Y, Okano, I, Kojima, M, Okada, K, Kangawa, K (2000). Decreased gene expression of adrenomedullin receptor in mouse lungs during sepsis. *Biochem. Biophys.Res.Commun.***271:** 197-202.

Owji, AA, Chabot, JG, Dumont, Y, Quirion, R (2008). Adrenomedullin-2/intermedin induces cAMP accumulation in dissociated rat spinal cord cells: evidence for the existence of a distinct class of sites of action. *J. Mol. Neurosci.* **35:** 355-361.

Pan, CS, Yang, JH, Cai, DY, Zhao, J, Gerns, H, Yang, J, Chang, JK, Tang, CS, Qi, YF (2005). Cardiovascular effects of newly discovered peptide intermedin/adrenomedullin 2. *Peptides* **26:** 1640-1646.

Parker, JL, Myers, PR, Zhong, Q, Kim, K, Adams, HR (1994). Inhibition of endothelium-dependent vasodilation by Escherichia coli endotoxemia. *Shock* **2:** 451-458.

Parker, MM, Cunnion, RE, Parrillo, JE (1985). Echocardiography and nuclear cardiac imaging in the critical care unit. *JAMA* **254:** 2935-2939.

Parker, SJ, Watkins, PE (2001). Experimental models of gram-negative sepsis. *Br. J. Surg.* **88:** 22-30.

Parkes, DG (1995). Cardiovascular actions of adrenomedullin in conscious sheep. *Am. J. Physiol.* **268:** H2574-H2578.

Parkes, DG, May, CN (1997). Direct cardiac and vascular actions of adrenomedullin in conscious sheep. *Br. J. Pharmacol.* **120:** 1179-1185.

Parratt, JR (1998). Nitric oxide in sepsis and endotoxaemia. *J. Antimicrob. Chemother.* **41:** 31-39.

Parrillo, JE (1989). The cardiovascular pathophysiology of sepsis. *Annu. Rev. Med.* **40:** 469-485.

Pennanen, MF, Bass, BL, Dziki, AJ, Harmon, JW (1994). Adenosine: differential effect on blood flow to subregions of the upper gastrointestinal tract. *J. Surg. Res.* **56:** 461-465.

Piepot, HA, Groeneveld, AB, van Lambalgen, AA, Sipkema, P (2003). Endotoxin impairs endothelium-dependent vasodilation more in the coronary and renal arteries than in other arteries of the rat. *J. Surg. Res.* **110:** 413-418.

Pio, R, Martinez, A, Unsworth, EJ, Kowalak, JA, Bengoechea, JA, Zipfel, PF, Elsasser, TH, Cuttitta, F (2001). Complement factor H is a serum-binding protein for adrenomedullin, and the resulting complex modulates the bioactivities of both partners. *J. Biol. Chem.* **276:** 12292-12300.

Porciatti, F, Pelzmann, B, Cerbai, E, Schaffer, P, Pino, R, Bernhart, E, Koidl, B, Mugelli, A (1997). The pacemaker current I(f) in single human atrial myocytes and the effect of beta-adrenoceptor and A1-adenosine receptor stimulation. *Br. J. Pharmacol.* **122:** 963-969.

Poulsen, SA, Quinn, RJ (1998). Adenosine receptors: new opportunities for future drugs. *Bioorg. Med. Chem.* **6:** 619-641.

Poyner, DR, Sexton, PM, Marshall, I, Smith, DM, Quirion, R, Born, W, Muff, R, Fischer, JA, Foord, SM (2002). International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol. Rev.* **54:** 233-246.

Prentice, DJ, Payne, SL, Hourani, SM (1997). Activation of two sites by adenosine receptor agonists to cause relaxation in rat isolated mesenteric artery. *Br. J. Pharmacol.* **122:** 1509-1515.

Quartin, AA, Schein, RM, Kett, DH, Peduzzi, PN (1997). Magnitude and duration of the effect of sepsis on survival. Department of Veterans Affairs Systemic Sepsis Cooperative Studies Group. *JAMA* **277:** 1058-1063.

Ray, CJ, Abbas, MR, Coney, AM, Marshall, JM (2002). Interactions of adenosine, prostaglandins and nitric oxide in hypoxia-induced vasodilatation: in vivo and in vitro studies. *J. Physiol.* **544:** 195-209.

Ray, CJ, Marshall, JM (2006). The cellular mechanisms by which adenosine evokes release of nitric oxide from rat aortic endothelium. *J. Physiol.* **570:** 85-96.

Ray, CJ, Marshall, JM (2005). Measurement of nitric oxide release evoked by systemic hypoxia and adenosine from rat skeletal muscle in vivo. *J. Physiol.* **568:** 967-978.

Ray, CJ, Marshall, JM (2009). Nitric oxide (NO) does not contribute to the generation or action of adenosine during exercise hyperaemia in rat hindlimb. *J. Physiol.* **587:** 1579-1591.

Remick, DG, Newcomb, DE, Bolgos, GL, Call, DR (2000). Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide vs. cecal ligation and puncture. *Shock* **13:** 110-116.

Remick, DG, Ward, PA (2005). Evaluation of endotoxin models for the study of sepsis. *Shock* **24:** 7-11.

Ren, YS, Yang, JH, Zhang, J, Pan, CS, Yang, J, Zhao, J, Pang, YZ, Tang, CS, Qi, YF (2006). Intermedin 1-53 in central nervous system elevates arterial blood pressure in rats. *Peptides* **27:** 74-79.

Riccardi, A, Arboscello, E, Ghinatti, M, Minuto, P, Lerza, R (2008). Adenosine in the treatment of supraventricular tachycardia: 5 years of experience (2002-2006). *Am. J. Emerg. Med.* **26:** 879-882.

Riedemann, NC, Guo, RF, Ward, PA (2003). Novel strategies for the treatment of sepsis. *Nat. Med.* **9:** 517-524.

Rittirsch, D, Flierl, MA, Ward, PA (2008). Harmful molecular mechanisms in sepsis. *Nat. Rev. Immunol.* **8:** 776-787.

Rittirsch, D, Hoesel, LM, Ward, PA (2007). The disconnect between animal models of sepsis and human sepsis. *J. Leukoc. Biol.* **81:** 137-143.

Rivkees, SA, Zhao, Z, Porter, G, Turner, C (2001). Influences of adenosine on the fetus and newborn. *Mol. Genet. Metab.* **74:** 160-171.

Roh, J, Chang, CL, Bhalla, A, Klein, C, Hsu, SY (2004). Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. *J. Biol. Chem.* **279:** 7264-7274.

Ross, GR, Yallampalli, C (2007). Vascular hyperresponsiveness to adrenomedullin during pregnancy is associated with increased generation of cyclic nucleotides in rat mesenteric artery. *Biol. Reprod.* **76:** 118-123.

Ryzhov, S, Zaynagetdinov, R, Goldstein, AE, Novitskiy, SV, Blackburn, MR, Biaggioni, I, Feoktistov, I (2008). Effect of A2B adenosine receptor gene ablation on adenosine-dependent regulation of proinflammatory cytokines. *J. Pharmacol. Exp. Ther.* **324:** 694-700.

Sabates, BL, Pigott, JD, Choe, EU, Cruz, MP, Lippton, HL, Hyman, AL, Flint, LM, Ferrara, JJ (1997). Adrenomedullin mediates coronary vasodilation through adenosine receptors and KATP channels. *J. Surg. Res.* **67:** 163-168.

Sair, M, Etherington, PJ, Curzen, NP, Winlove, CP, Evans, TW (1996). Tissue oxygenation and perfusion in endotoxemia. *Am. J. Physiol.* **271:** H1620-H1625.

Sakai, K, Saito, K, Akima, M (1998). Synergistic effect of calcitonin gene-related peptide on adenosine-induced vasodepression in rats. *Eur. J. Pharmacol.* **344:** 153-159.

Sakai, K, Saito, K (1998). Reciprocal interactions among neuropeptides and adenosine in the cardiovascular system of rats: a role of K(ATP) channels. *Eur. J. Pharmacol.* **345:** 279-284.

Sakai, K, Saito, K (1998). Synergism between low-dose nicorandil and neuropeptides on adenosine-induced vasodepression in rats. *Eur. J. Pharmacol.* **352:** 229-236.

Sakai, K, Saito, K, Ishizuka, N (1998). Adrenomedullin synergistically interacts with endogenous vasodilators in rats: a possible role of K(ATP) channels. *European journal of pharmacology* **359:** 151-159.

Sakata, J, Shimokubo, T, Kitamura, K, Nakamura, S, Kangawa, K, Matsuo, H, Eto, T (1993). Molecular cloning and biological activities of rat adrenomedullin, a hypotensive peptide. *Biochem. Biophys. Res. Commun.* **195:** 921-927.

Sam, AD, 2nd, Sharma, AC, Rice, AN, Ferguson, JL, Law, WR (2000). Adenosine and nitric oxide regulate regional vascular resistance via interdependent and independent mechanisms during sepsis. *Crit. Care Med.* **28:** 1931-1939.

Samson, WK (1999). Adrenomedullin and the control of fluid and electrolyte homeostasis. *Annu. Rev. Physiol.* **61:** 363-389.

Sands, WA, Palmer, TM (2005). Adenosine receptors and the control of endothelial cell function in inflammatory disease. *Immunol. Lett.* **101:** 1-11.

Santiago, JA, Garrison, E, Purnell, WL, Smith, RE, Champion, HC, Coy, DH, Murphy, WA, Kadowitz, PJ (1995). Comparison of responses to adrenomedullin and adrenomedullin analogs in the mesenteric vascular bed of the cat. *Eur.J. Pharmacol.* **272:** 115-118.

Saura, CA, Mallol, J, Canela, EI, Lluis, C, Franco, R (1998). Adenosine deaminase and A1 adenosine receptors internalize together following agonist-induced receptor desensitization. *J. Biol. Chem.* **273:** 17610-17617.

Schindler, CW, Karcz-Kubicha, M, Thorndike, EB, Muller, CE, Tella, SR, Ferre, S, Goldberg, SR (2005). Role of central and peripheral adenosine receptors in the cardiovascular responses to intraperitoneal injections of adenosine A1 and A2A subtype receptor agonists. *Br. J. Pharmacol.* **144:** 642-650.

Senna, AA, Zedan, M, el-Salam, GE, el-Mashad, AI (2008). Study of plasma adrenomedullin level in normal pregnancy and preclampsia. *Medscape. J. Med.* **10:** 29.

Shindo, T, Kurihara, H, Maemura, K, Kurihara, Y, Kuwaki, T, Izumida, T, Minamino, N, Ju, KH, Morita, H, Oh-hashi, Y, Kumada, M, Kangawa, K, Nagai, R, Yazaki, Y (2000). Hypotension and resistance to lipopolysaccharide-induced shock in transgenic mice overexpressing adrenomedullin in their vasculature. *Circulation* **101:** 2309-2316.

Shindo, T, Kurihara, Y, Nishimatsu, H, Moriyama, N, Kakoki, M, Wang, Y, Imai, Y, Ebihara, A, Kuwaki, T, Ju, KH, Minamino, N, Kangawa, K, Ishikawa, T, Fukuda, M, Akimoto, Y, Kawakami, H, Imai, T, Morita, H, Yazaki, Y, Nagai, R, Hirata, Y, Kurihara, H (2001). Vascular abnormalities and elevated blood pressure in mice lacking adrenomedullin gene. *Circulation* **104:** 1964-1971.

Shoji, H, Minamino, N, Kangawa, K, Matsuo, H (1995). Endotoxin markedly elevates plasma concentration and gene transcription of adrenomedullin in rat. *Biochem. Biophys. Res. Commun.* **215:** 531-537.

Sibbald, WJ, Fox, G, Martin, C (1991). Abnormalities of vascular reactivity in the sepsis syndrome. *Chest* **100:** 155S-159S.

Simpson, AJ (2001). Melioidosis: a clinical model for gram-negative sepsis. *J. Med. Microbiol.* **50:** 657-658.

Skrabanja, AT, Bouman, EA, Dagnelie, PC (2005). Potential value of adenosine 5'-triphosphate (ATP) and adenosine in anaesthesia and intensive care medicine. *Br. J. Anaesth.* **94:** 556-562.

Somani, SM, Gupta, P (1988). Caffeine: a new look at an age-old drug. *Int. J. Clin. Pharmacol. Ther. Toxicol.* **26:** 521-533.

Sorrentino, R, d'Emmanuele di Villa Bianca, R, Lippolis, L, Sorrentino, L, Autore, G, Pinto, A (1999). Involvement of ATP-sensitive potassium channels in a model of a delayed vascular hyporeactivity induced by lipopolysaccharide in rats. *Br. J. Pharmacol.* **127:** 1447-1453.

Sugo, S, Minamino, N, Shoji, H, Kangawa, K, Kitamura, K, Eto, T, Matsuo, H (1995). Interleukin-1, tumor necrosis factor and lipopolysaccharide additively stimulate production of adrenomedullin in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **207:** 25-32.

Sullivan, GW, Fang, G, Linden, J, Scheld, WM (2004). A2A adenosine receptor activation improves survival in mouse models of endotoxemia and sepsis. *J. Infect. Dis.* **189:** 1897-1904.

Surah-Narwal, S, Xu, SZ, McHugh, D, McDonald, RL, Hough, E, Cheong, A, Partridge, C, Sivaprasadarao, A, Beech, DJ (1999). Block of human aorta Kir6.1 by the vascular KATP channel inhibitor U37883A. *Br. J. Pharmacol.* **128:** 667-672.

Tagawa, H, Vander, AJ (1970). Effects of adenosine compounds on renal function and renin secretion in dogs. *Circ. Res.* **26:** 327-338.

Takahashi, K, Kikuchi, K, Maruyama, Y, Urabe, T, Nakajima, K, Sasano, H, Imai, Y, Murakami, O, Totsune, K (2006). Immunocytochemical localization of adrenomedullin 2/intermedin-like immunoreactivity in human hypothalamus, heart and kidney. *Peptides* **27:** 1383-1389.

Takei, Y, Hashimoto, H, Inoue, K, Osaki, T, Yoshizawa-Kumagaye, K, Tsunemi, M, Watanabe, TX, Ogoshi, M, Minamino, N, Ueta, Y (2008). Central and peripheral cardiovascular actions of adrenomedullin 5, a novel member of the calcitonin gene-related peptide family, in mammals. *J. Endocrinol.* **197:** 391-400.

Takei, Y, Hyodo, S, Katafuchi, T, Minamino, N (2004). Novel fish-derived adrenomedullin in mammals: structure and possible function. *Peptides* **25:** 1643-1656.

Takei, Y, Inoue, K, Ogoshi, M, Kawahara, T, Bannai, H, Miyano, S (2004). Identification of novel adrenomedullin in mammals: a potent cardiovascular and renal regulator. *FEBS letters* **556:** 53-58.

Tang, L, Parker, M, Fei, Q, Loutzenhiser, R (1999). Afferent arteriolar adenosine A2a receptors are coupled to KATP in in vitro perfused hydronephrotic rat kidney. *Am J Physiol* **277:** F926-F933.

Tarpey, SB, Bennett, T, Randall, MD, Gardiner, SM (1998) Differential effects of endotoxaemia on pressor and vasoconstrictor actions of angiotensin II and arginine vasopressin in conscious rats. *Br. J. Pharmacol.* **123:** 1367-1374.

Taveira da Silva, AM, Kaulbach, HC, Chuidian, FS, Lambert, DR, Suffredini, AF, Danner, RL (1993). Brief report: shock and multiple-organ dysfunction after self-administration of Salmonella endotoxin. *N. Engl. J. Med.* **328:** 1457-1460.

Tawfik, HE, Teng, B, Morrison, RR, Schnermann, J, Mustafa, SJ (2006). Role of A1 adenosine receptor in the regulation of coronary flow. *Am. J. Physiol.* **291:** H467-H472.

Taylor, MM, Bagley, SL, Samson, WK (2005). Intermedin/adrenomedullin-2 acts within central nervous system to elevate blood pressure and inhibit food and water intake. *Am. J. Physiol.* **288:** R919-R927.

Terata, K, Miura, H, Liu, Y, Loberiza, F, Gutterman, DD (2000). Human coronary arteriolar dilation to adrenomedullin: role of nitric oxide and K(+) channels. *Am. J. Physiol.* **279:** H2620-H2626.

Thomas, T, St Lambert, JH, Dashwood, MR, Spyer, KM (2000). Localization and action of adenosine A2a receptors in regions of the brainstem important in cardiovascular control. *Neuroscience* **95:** 513-518.

Toltl, LJ, Shin, LY, Liaw, PC (2007). Activated protein C in sepsis and beyond: update 2006. *Front. Biosci.* **12:** 1963-1972.

Troughton, RW, Lewis, LK, Yandle, TG, Richards, AM, Nicholls, MG (2000). Hemodynamic, hormone, and urinary effects of adrenomedullin infusion in essential hypertension. *Hypertension* **36:** 588-593.

Trzeciak, S, Cinel, I, Phillip Dellinger, R, Shapiro, NI, Arnold, RC, Parrillo, JE, Hollenberg, SM (2008). Resuscitating the microcirculation in sepsis: the

central role of nitric oxide, emerging concepts for novel therapies, and challenges for clinical trials. *Acad. Emerg. Med.* **15:** 399-413.

Tsiotou, AG, Sakorafas, GH, Anagnostopoulos, G, Bramis, J (2005). Septic shock; current pathogenetic concepts from a clinical perspective. *Med. Sci. Monit.* **11:** RA76-RA85.

Vallon, V, Albinus, M, Blach, D (1998). Effect of KATP channel blocker U37883A on renal function in experimental diabetes mellitus in rats. *J. Pharmacol. Exp. Ther.* **286:** 1215-1221.

Vallon, V, Muhlbauer, B, Osswald, H (2006). Adenosine and kidney function. *Physiol. Rev.* **86:** 901-940.

Vincent, JL, Sakr, Y, Sprung, CL, Ranieri, VM, Reinhart, K, Gerlach, H, Moreno, R, Carlet, J, Le Gall, JR, Payen, D (2006). Sepsis in European intensive care units: results of the SOAP study. *Crit. Care Med.* **34:** 344-353.

Vo, PA, Lad, B, Tomlinson, JA, Francis, S, Ahluwalia, A (2005). autoregulatory role of endothelium-derived nitric oxide (NO) on Lipopolysaccharide-induced vascular inducible NO synthase expression and function. *J. Biol. Chem.* **280:** 7236-7243.

Wakefield, ID, March, JE, Kemp, PA, Valentin, JP, Bennett, T, Gardiner, SM (2003). Comparative regional haemodynamic effects of the nitric oxide synthase inhibitors, S-methyl-L-thiocitrulline and L-NAME, in conscious rats. *Br. J. Pharmacol.* **139:** 1235-1243.

Waller, J, Gardiner, SM, Bennett, T (1994). Regional haemodynamic responses to acetylcholine, methoxamine, salbutamol and bradykinin during lipopolysaccharide infusion in conscious rats. *Br. J. Pharmacol.* **112:** 1057-1064.

Waller, J, Gardiner, SM, Jose, J, Bennett, T (1995). Lack of effect of TNF antibodies on the cardiovascular sequelae of lipopolysaccharide infusion in conscious rats. *Br J Pharmacol* **116:** 2487-2495.

Walsh, MP, Marshall, JM (2006). The role of adenosine in the early respiratory and cardiovascular changes evoked by chronic hypoxia in the rat. *J. Physiol.* **575:** 277-289.

Walsh, MP, Marshall, JM (2006). The early effects of chronic hypoxia on the cardiovascular system in the rat: role of nitric oxide. *J Physiol* **575:** 263-275.

Wang, JE, Dahle, MK, McDonald, M, Foster, SJ, Aasen, AO, Thiemermann, C (2003). Peptidoglycan and lipoteichoic acid in gram-positive bacterial sepsis: receptors, signal transduction, biological effects, and synergism. *Shock* **20:** 402-414.

Wang, P (2001). Andrenomedullin and cardiovascular responses in sepsis. *Peptides* **22:** 1835-1840.

Wang, P, Chaudry, I (1998). A Single Hit Model of Polymicrobial Sepsis: Cecal Ligation and Puncture. *Sepsis* 227-233.

Wang, P, Yoo, P, Zhou, M, Cioffi, WG, Ba, ZF, Chaudry, IH (1999). Reduction in vascular responsiveness to adrenomedullin during sepsis. *J. Surg. Res.* **85:** 59-65.

Watt, AH (1986). Adenosine in atrial arrhythmias. *J. Am. Coll. Cardiol.* **8:** 463-464.

Webb, RL, McNeal, RB, Jr., Barclay, BW, Yasay, GD (1990). Hemodynamic effects of adenosine agonists in the conscious spontaneously hypertensive rat. *J. Pharmacol. Exp. Ther.* **254:** 1090-1099.

Weihprecht, H, Lorenz, JN, Schnermann, J, Skott, O, Briggs, JP (1990). Effect of adenosine1-receptor blockade on renin release from rabbit isolated perfused juxtaglomerular apparatus. *J. Clin. Invest.* **85:** 1622-1628.

Wellman, GC, Barrett-Jolley, R, Koppel, H, Everitt, D, Quayle, JM (1999). Inhibition of vascular K(ATP) channels by U-37883A: a comparison with cardiac and skeletal muscle. *Br. J. Pharmacol.* **128:** 909-916.

White, MM, Samson, WK (2007). Intermedin 17-47 does not function as a full intermedin antagonist within the central nervous system or pituitary. *Peptides* **28:** 2171-2178.

Wormald, A, Bowmer, CJ, Yates, MS, Collis, MG (1989). Pharmacokinetics of 8-phenyltheophylline in the rat. *J. Pharm. Pharmacol.* **41:** 418-420.

Wu, CC, Chen, SJ, Garland, CJ (2004). NO and KATP channels underlie endotoxin-induced smooth muscle hyperpolarization in rat mesenteric resistance arteries. *Br. J. Pharmacol.* **142:** 479-484.

Wu, R, Zhou, M, Wang, P (2003). Adrenomedullin and adrenomedullin binding protein-1 downregulate TNF-alpha in macrophage cell line and rat Kupffer cells. *Regul. Pept.* **112:** 19-26.

Wu, R, Cui, X, Dong, W, Zhou, M, Simms, HH, Wang, P (2005). Mechanisms responsible for vascular hyporesponsiveness to adrenomedullin after hemorrhage: the central role of adrenomedullin binding protein-1. *Ann. Surg.* **242:** 115-123.

Wu, R, Dong, W, Zhou, M, Cui, X, Simms, HH, Wang, P (2005). A novel approach to maintaining cardiovascular stability after hemorrhagic shock: beneficial effects of adrenomedullin and its binding protein. *Surgery* **137:** 200-208.

Wu, R, Dong, W, Qiang, X, Ji, Y, Cui, T, Yang, J, Zhou, M, Blau, S, Marini, CP, Ravikumar, TS, Wang, P (2008). Human vasoactive hormone adrenomedullin and its binding protein rescue experimental animals from shock. *Peptides* **29:** 1223-1230.

Wu, R, Higuchi, S, Dong, W, Ji, Y, Zhou, M, Marini, CP, Ravikumar, TS, Wang, P (2009). Reversing established sepsis in rats with human vasoactive hormone adrenomedullin and its binding protein. *Mol Med* **15:** 28-33.

Yaar, R, Jones, MR, Chen, JF, Ravid, K (2005). Animal models for the study of adenosine receptor function. *J. Cell Physiol.* **202:** 9-20.

Yamaguchi, N, Jesmin, S, Zaedi, S, Shimojo, N, Maeda, S, Gando, S, Koyama, A, Miyauchi, T (2006). Time-dependent expression of renal vaso-regulatory molecules in LPS-induced endotoxemia in rat. *Peptides* **27:** 2258-2270.

Yamaguchi, T, Baba, K, Doi, Y, Yano, K (1995). Effect of adrenomedullin on aldosterone secretion by dispersed rat adrenal zona glomerulosa cells. *Life Sci.* **56:** 379-387.

Yamaguchi, T, Baba, K, Doi, Y, Yano, K, Kitamura, K, Eto, T (1996). Inhibition of aldosterone production by adrenomedullin, a hypotensive peptide, in the rat. *Hypertension* **28:** 308-314.

Yang, JH, Jia, YX, Pan, CS, Zhao, J, Ouyang, M, Yang, J, Chang, JK, Tang, CS, Qi, YF (2005). Effects of intermedin(1-53) on cardiac function and ischemia/reperfusion injury in isolated rat hearts. *Biochem. Biophys. Res. Commun.* **327:** 713-719.

Yang, JH, Qi, YF, Jia, YX, Pan, CS, Zhao, J, Yang, J, Chang, JK, Tang, CS (2005). Protective effects of intermedin/adrenomedullin2 on ischemia/reperfusion injury in isolated rat hearts. *Peptides* **26:** 501-507.

Yang, RB, Mark, MR, Gray, A, Huang, A, Xie, MH, Zhang, M, Goddard, A, Wood, WI, Gurney, AL, Godowski, PJ (1998). Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* **395:** 284-288.

Yang, S, Zhou, M, Chaudry, IH, Wang, P (2002). Novel approach to prevent the transition from the hyperdynamic phase to the hypodynamic phase of sepsis: role of adrenomedullin and adrenomedullin binding protein-1. *Ann. Surg.* **236:** 625-633.

Yang, S, Zhou, M, Chaudry, IH, Wang, P (2001). The role of lipopolysaccharide in stimulating adrenomedullin production during polymicrobial sepsis. *Biochim. Biophys. Acta.* **1537:** 167-174.

Yang, S, Zhou, M, Fowler, DE, Wang, P (2002). Mechanisms of the beneficial effect of adrenomedullin and adrenomedullin-binding protein-1 in sepsis: down-regulation of proinflammatory cytokines. *Crit. Care Med.* **30:** 2729-2735.

Yang, JH, Pan, CS, Jia, YX, Zhang, J, Zhao, J, Pang, YZ, Yang, J, Tang, CS, Qi, YF (2006). Intermedin1-53 activates L-arginine/nitric oxide synthase/nitric oxide pathway in rat aortas. *Biochemical and biophysical research communications* **341:** 567-572.

Zaks-Zilberman, M, Salkowski, CA, Elsasser, T, Cuttitta, F, Vogel, SN (1998). Induction of adrenomedullin mRNA and protein by lipopolysaccharide and paclitaxel (Taxol) in murine macrophages. *Infect. Immun.* **66:** 4669-4675.

Zeng, Q, Yuan, Y, Wang, X, Wu, HM, Fan, L, Qi, YF, Tang, CS, Cai, Y, Pan, CS (2009). Upregulated expression of intermedin and its receptor in the myocardium and aorta in spontaneously hypertensive rats. *Peptides* **30:** 391-399.

Zhang, JG, Hepburn, L, Cruz, G, Borman, RA, Clark, KL (2005). The role of adenosine A2A and A2B receptors in the regulation of TNF-alpha production by human monocytes. *Biochem. Pharmacol.* **69:** 883-889.

Zhao, G, Linke, A, Xu, X, Ochoa, M, Belloni, F, Belardinelli, L, Hintze, TH (2003). Comparative profile of vasodilation by CVT-3146, a novel A2A receptor agonist, and adenosine in conscious dogs. *J. Pharmacol. Exp. Ther.* **307:** 182-189.

Zhao, Y, Bell, D, Smith, LR, Zhao, L, Devine, AB, McHenry, EM, Nicholls, DP, McDermott, BJ (2006). Differential expression of components of the cardiomyocyte adrenomedullin/intermedin receptor system following blood pressure reduction in nitric oxide-deficient hypertension. *J. Pharmacol. Exp. Ther.* **316:** 1269-1281.

Zhou, M, Wang, P, Chaudry, IH (1997). Endothelial nitric oxide synthase is downregulated during hyperdynamic sepsis. *Biochim Biophys Acta* **1335:** 182-190.

Zhou, M, Ba, ZF, Chaudry, IH, Wang, P (2002). Adrenomedullin binding protein-1 modulates vascular responsiveness to adrenomedullin in late sepsis. *Am. J. Physiol.* **283:** R553-R560.

Zhou, M, Simms, HH, Wang, P (2004). Adrenomedullin and adrenomedullin binding protein-1 attenuate vascular endothelial cell apoptosis in sepsis. *Ann. Surg.* **240:** 321-330.

Zhou, M, Maitra, SR, Wang, P (2007). Adrenomedullin and adrenomedullin binding protein-1 protect endothelium-dependent vascular relaxation in sepsis. *Mol. Med.* **13:** 488-494.