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Vascular Control Mechanisms in Normal and Lipopolysaccharide-treated Rats

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

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Abstract

The development of sepsis is associated with complex cardiovascular changes, some of which can be mimicked 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 AM22-52 and some components of IMD were sensitive to L-NAME, suggesting IMD causes both endothelial-dependent and -independent vasodilatations. No role for KATP 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


Abstracts


Acknowledgments

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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!
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>8-PT</td>
<td>8-phenyltheophylline</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
</tr>
<tr>
<td>ADM</td>
<td>Adrenomedullin</td>
</tr>
<tr>
<td>ADO</td>
<td>Adenosine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>AMBP-l</td>
<td>Adrenomedullin binding protein-1</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Activated protein C</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'- triphosphate</td>
</tr>
<tr>
<td>AV</td>
<td>Atrioventricular</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
</tr>
<tr>
<td>CARS</td>
<td>Compensatory anti-inflammatory response syndrome</td>
</tr>
<tr>
<td>CCPA</td>
<td>2-chloro-N⁶-cyclopentyladenosine</td>
</tr>
<tr>
<td>CL</td>
<td>Calcitonin receptor-like-receptor</td>
</tr>
<tr>
<td>CLP</td>
<td>Caecal ligation and puncture</td>
</tr>
<tr>
<td>cNOS</td>
<td>Constitutive nitric oxide synthase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>DPCPX</td>
<td>8-cyclopentyl-1,3-dipropylxanthine</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled-receptor</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia inducible factor-1</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>i.a.</td>
<td>Intra-arterial</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravascular</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IL-1/6/8/10</td>
<td>Interleukin-1/6/8/10</td>
</tr>
<tr>
<td>IMD</td>
<td>Intermedin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt;</td>
<td>ATP-sensitive potassium channel</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS-binding protein</td>
</tr>
<tr>
<td>L-NAME</td>
<td>NG-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MODS</td>
<td>Multiple organ dysfunction syndrome</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PKA/B/C</td>
<td>Protein kinase A/B/C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin angiotensin system</td>
</tr>
<tr>
<td>RAMP</td>
<td>Receptor activity modifying protein</td>
</tr>
<tr>
<td>RAMP</td>
<td>Receptor activity modifying protein</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SVR</td>
<td>Systemic vascular resistance</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TPR</td>
<td>Total peripheral resistance</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
</tbody>
</table>
CHAPTER 1: General introduction

General Introduction ........................................................................................................... 2
1.1 Sepsis ............................................................................................................................ 4
  1.1.1 Epidemiology ................................................................................................... 4
  1.1.2 Pathophysiology ................................................................................................. 8
    1.1.2.1 Cardiovascular changes during sepsis ............................................ 12
  1.1.3 Animal models ................................................................................................. 17
    1.1.3.1 Administration of a toxin/pathogen ................................................... 17
    1.1.3.2 Exposure to endogenous toxin/bacteria ............................................. 21
  1.2 The calcitonin family of peptides ............................................................................ 24
    1.2.1 Introduction .................................................................................................... 24
    1.2.2 Calcitonin family receptors ............................................................................. 25
    1.2.3 Adrenomedullin (AM) .................................................................................... 27
      1.2.3.1 Physiological functions of AM ......................................................... 28
      1.2.3.2 Cardiovascular responses to AM ...................................................... 30
      1.2.3.3 Adrenomedullin in sepsis .................................................................. 34
    1.2.4 Intermedin (IMD) ........................................................................................... 38
      1.2.4.1 Tissue distribution of IMD ................................................................ 40
      1.2.4.2 Cardiovascular responses to IMD ..................................................... 41
      1.2.4.3 Other functions of IMD .................................................................... 50
  1.3 Adenosine ................................................................................................................. 52
    1.3.1 Introduction .................................................................................................... 52
    1.3.2 Adenosine receptors ......................................................................................... 54
3.2 Methods ..................................................................................................................... 95
3.3 Results ........................................................................................................................ 97
   3.3.1 Regional haemodynamic responses to IMD .................................................... 97
   3.3.2 Regional haemodynamic responses to AM ...................................................... 98
   3.3.3 Comparison of the regional haemodynamic responses to AM and IMD ......... 99
3.4 Discussion ...................................................................................................... 104

CHAPTER 4:

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

Summary .......................................................................................................................... 110

4.1 Introduction .............................................................................................................. 111
4.2 Methods ................................................................................................................... 114
   4.2.1 Experiment 1: Effects of repeated dosing of AM and IMD .................... 114
   4.2.2 Experiment 2: Involvement of CGRP$_{8-37}$ sensitive receptors .......... 115
   4.2.3 Experiment 3: Involvement of AM$_{22-52}$ sensitive receptors .............. 117
   4.2.4 Experiment 4: Involvement of nitric oxide ............................................. 119
   4.2.5 Experiment 5: Involvement of K$_{ATP}$ channels ..................................... 121
   4.2.6 Experiment 6: Interactions between NO and K$_{ATP}$ channels .......... 122
   4.2.7 Experiment 7: Involvement of the renin-angiotensin system ............... 123
4.3 Results ...................................................................................................................... 125
   4.3.1 Experiment 1 .......................................................... 125
   4.3.2 Experiment 2 .......................................................... 125
   4.3.3 Experiment 3 .......................................................... 126
   4.3.4 Experiment 4 .......................................................... 127
   4.3.5 Experiment 5 .......................................................... 128
   4.3.6 Experiment 6 .......................................................... 129
   4.3.7 Experiment 7 .......................................................... 130
4.4 Discussion ................................................................................................................ 139
CHAPTER 5:

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

Summary .......................................................................................................................... 150

5.1 Introduction.............................................................................................................. 152
5.2 Methods ................................................................................................................... 155
   5.2.1 Experiment 1: Cardiovascular effects of LPS administration ....................... 155
   5.2.2 Experiment 2: Cardiovascular responses to AM/IMD in endotoxaemia .... 156
      5.2.2.1 Bolus LPS ........................................................................................ 156
      5.2.2.2 Continuous infusion of LPS............................................................. 157
   5.2.3 Experiment 3: Cardiovascular responses to AM_{22,52} in LPS-treated rats ..... 158
5.3 Results ...................................................................................................................... 159
   5.3.1 Experiment 1: Cardiovascular effects of LPS administration ....................... 159
      5.3.1.1 Saline (0.5 ml i.p.) ............................................................................ 159
      5.3.1.2 LPS (1 mg kg^{-1} i.p.) ................................................................... 159
      5.3.1.3 LPS (3 mg kg^{-1} i.p.) ................................................................... 160
      5.3.1.4 LPS infusion (15 \mu g kg^{-1} h^{-1}) ................................................. 161
   5.3.2 Experiment 2: Responses to AM/IMD in endotoxaemia .............................. 162
      5.3.2.1 LPS bolus (1 mg kg^{-1}) .................................................................. 162
      5.3.2.2 LPS infusion (15 \mu g kg^{-1} h^{-1}) .................................................. 164
   5.3.3. Experiment 3: Experiment 3: AM_{22,52} in LPS-treated rats ................. 166
5.4 Discussion ............................................................................................................... 178

CHAPTER 6:

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

Summary .......................................................................................................................... 188

6.1 Introduction.............................................................................................................. 190
6.2 Methods ................................................................................................................... 193
   6.2.1 Experiment 1: CV effects of 8-PT in normal/LPS rats ............................... 193
   6.2.2 Experiment 2: CV responses adenosine in normal/LPS rats ..................... 194
   6.2.3 Experiment 3: CV responses to CCPA in normal/LPS rats ....................... 195
   6.2.4 Experiment 4: CV effects of DPCPX in normal/LPS rats ......................... 196
6.2.5 Experiment 5: CV effects of DPCPX + losartan in normal/LPS rats .......... 197
6.3 Results................................................................. 199
  6.3.1 Experiment 1: ................................................. 199
  6.3.2 Experiment 2: .................................................. 200
  6.3.3 Experiment 3: .................................................. 201
  6.3.4 Experiment 4: .................................................. 202
  6.3.5 Experiment 5: .................................................. 202
6.4 Discussion ............................................................. 210

CHAPTER 7:

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

Summary................................................................. 219
7.1 Introduction.......................................................... 220
7.2 Methods .............................................................. 223
  7.2.1 Cardiovascular responses to adenosine during infusion of AM or IMD ..... 223
  7.2.2 Cardiovascular responses to AM or IMD during infusion of adenosine .... 225
7.3 Results................................................................. 227
  7.3.1 Cardiovascular responses to adenosine during infusion of AM or IMD ..... 227
  7.3.2 Cardiovascular responses to AM and IMD during infusion of adenosine... 228
7.4 Discussion............................................................. 235

CHAPTER 8: General discussion

8.1 Summary of main findings ....................................... 240
8.2 Suggestions for further research ................................ 245

References............................................................... 247
List of tables

CHAPTER 1: General Introduction
Table 1.1: The sepsis syndrome ................................................................. 7
Table 1.2: Altered responses to vasoactive mediators in the presence of LPS .... 23
Table 1.3: Mechanisms of AM action in the cardiovascular system .............. 33
Table 1.4: Mechanisms of IMD action in the cardiovascular system ............... 44

CHAPTER 2: General Methods
Table 2.1: Drugs used throughout the course of these experiments .............. 88

CHAPTER 3: Results (i)
Table 3.1: Resting cardiovascular variables prior to administration of AM and IMD .... 97

CHAPTER 4: Results (ii)
Table 4.1: Resting cardiovascular variables prior to administration of AM and IMD (Experiments 1-7) ................................................................. 131

CHAPTER 5: Results (iii)
Table 5.1: Resting cardiovascular variables in groups of rats prior to bolus administration of saline or LPS ................................................................. 167
Table 5.2: Resting cardiovascular variables at selected time points prior to administration of AM or IMD in normal and LPS-treated rats ......................... 168
Table 5.3: Summary of baseline cardiovascular variables at selected time points after administration of LPS ........................................................................ 169
Table 5.4: Mechanisms Cardiovascular variables 1.5 h after treatment with LPS in the presence and absence of AM<sub>22-32</sub> .............................................. 170
Table 5.5: Cardiovascular changes at the beginning and end of AM<sub>22-32</sub> administration 6h and 25 h from LPS-treatment .................................................. 171
CHAPTER 6: Results (iv)

Table 6.1: Cardiovascular variables prior to administration of adenosine agonists and antagonists ................................................................. 204

Table 6.2: Summary of responses to adenosine receptor agonists and antagonists........ 226

CHAPTER 7: Results (v)

Table 7.1: Baseline cardiovascular variables prior to the onset of saline or AM or IMD Infusion, and prior to administration of adenosine ............................................. 230

Table 7.2: Resting cardiovascular variables prior to the onset of saline or adenosine Infusion, and prior to administration of AM or IMD................................. 231
List of figures

CHAPTER 1: General Introduction

Figure 1.1: LPS signaling cascade ................................................................. 9
Figure 1.2: Sequence homology between mammalian and pufferfish AM and IMD .... 42
Figure 1.3: Chemical structure of adenosine .................................................. 54

CHAPTER 2: General Methods

Figure 2.1: A complete Doppler flow probe .................................................. 78
Figure 2.2: Location of implanted Doppler flow probe .................................... 82
Figure 2.3: Custom designed harness and double-channel swivel .................... 86

CHAPTER 3: Results (i)

Figure 3.3.1: Comparison of the regional haemodynamic responses to AM and IMD . 101
Figure 3.3.2: Comparison of the regional haemodynamic responses to AM and IMD (Doppler Shift) ............................................................ 102
Figure 3.3.3: Integrated (0-10 min) responses following AM or IMD ................. 103

Chapter 4: Results (ii)

Figure 4.2.1: Protocol diagram for experiment 1 .............................................. 115
Figure 4.2.2: Protocol diagram for experiment 2 .............................................. 116
Figure 4.2.3: Protocol diagram for experiment 3 (IMD) .................................. 117
Figure 4.2.3a: Protocol diagram for experiment 3 (AM) .................................. 118
Figure 4.2.4: Protocol diagram for experiment 4 .............................................. 120
Figure 4.2.5: Protocol diagram for experiment 5 .............................................. 122
Figure 4.2.6: Protocol diagram for experiment 6 .............................................. 123
Figure 4.2.7: Protocol diagram for experiment 7 .............................................. 124
Figure 4.3.1: Effects of repeated dosing of AM or IMD .................................. 132
Figure 4.3.2: Effects of CGRP4-37 on responses to AM or IMD ..................... 133
Figure 4.3.3: Effects of AM22-52 on responses to AM or IMD ....................... 134
Figure 4.3.4: Effects of L-NAME on cardiovascular responses to AM or IMD ..... 135
Chapter 5: Results (iii)

Figure 5.2.2: Protocol diagram for 5.2.2.1 ................................................................. 156
Figure 5.2.2a: Protocol diagram for 5.2.2.2 ................................................................. 157
Figure 5.2.4: Protocol diagram for 5.2.3 ................................................................. 158
Figure 5.3.1: Cardiovascular changes over 25 h following LPS administration ........ 172
Figure 5.3.1a: Cardiovascular changes during a 25 h infusion of LPS ....................... 173
Figure 5.3.2: Integrated (0-10 min) cardiovascular changes following AM administration 1.5h, 6h and 25h after saline or LPS ................................................................. 174
Figure 5.3.3: Integrated (0-10 min) cardiovascular changes following IMD administration 1.5h, 6h and 25h after saline or LPS ................................................................. 175
Figure 5.3.4: Integrated (0-10 min) cardiovascular changes following AM administration 1.5h, 6h and 25h from onset saline or LPS ................................................................. 176
Figure 5.3.5: Integrated (0-10 min) cardiovascular changes following IMD administration 1.5h, 6h and 25h from onset of saline or LPS ................................................................. 177

Chapter 6: Results (iv)

Figure 6.2.1: Protocol diagram for experiment 1 ........................................................... 194
Figure 6.2.2: Protocol diagram for experiment 2 ........................................................... 195
Figure 6.2.3: Protocol diagram for experiment 3 ........................................................... 196
Figure 6.2.4: Protocol diagram for experiment 4 ........................................................... 197
Figure 6.2.5: Protocol diagram for experiment 5 ........................................................... 198
Figure 6.3.1: Cardiovascular changes following 8-PT administration 1.5h, 6h and 25h after saline or LPS treatment ................................................................. 205
Figure 6.3.2: Cardiovascular changes during 3 min infusion of adenosine 1.5h, 6h and 25 h after saline or LPS treatment ................................................................. 206
Figure 6.3.3: Cardiovascular changes during 3 min infusion of CCPA 1.5h, and 6h after saline or LPS treatment ................................................................. 207
Figure 6.3.4: Cardiovascular responses to DPCPX, 6h and 25 h after saline or LPS treatment ................................................................. 208
Chapter 7: Results (v)

Figure 7.2.1: Protocol diagram for 7.2.1 ................................................................. 224
Figure 7.2.2: Protocol diagram for 7.2.2 ................................................................. 226
Figure 7.1: Cardiovascular response to adenosine 8-PT administration during infusion of saline, AM or IMD ................................................................. 232
Figure 7.2: Cardiovascular response to AM during infusion of saline or adenosine ... 233
Figure 7.3: Cardiovascular response to IMD during infusion of saline or adenosine .. 234
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

Sepsic 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 antimicrobial 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 drotrecog in 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

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
<th>Diagnosis</th>
</tr>
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<tbody>
<tr>
<td><strong>Infection</strong></td>
<td>Inflammatory response to microorganisms, or invasion of normally sterile</td>
<td>&gt;1 of the following:</td>
</tr>
<tr>
<td></td>
<td>host tissue by microorganisms</td>
<td>• body temperature $&gt;38^\circ$C or $&lt;36^\circ$C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• heart rate $&gt;90$ beats min$^{-1}$</td>
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<td></td>
<td></td>
<td>• respiratory rate $&gt;20$ breaths min$^{-1}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• white blood cell count $&gt;12000$ cu mm$^{-1}$ or $&lt;4000$ cu mm$^{-1}$</td>
</tr>
<tr>
<td><strong>Bacteraemia</strong></td>
<td>Presence of viable bacteria in the blood</td>
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<td><strong>SIRS</strong></td>
<td>Systemic inflammation arising from an unknown cause</td>
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<td></td>
<td></td>
<td>• Symptoms may include lactic acidosis, oliguria, acute alteration of</td>
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<td></td>
<td></td>
<td>mental state</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Sepsis-induced hypotension: systolic BP of $&lt;90$ mmHg or its reduction</td>
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<tr>
<td></td>
<td></td>
<td>by $40$ mm Hg</td>
</tr>
<tr>
<td><strong>Sepsis</strong></td>
<td>Systemic inflammation in the presence of infection</td>
<td>As above, with infection from a confirmed source</td>
</tr>
<tr>
<td><strong>Severe sepsis</strong></td>
<td>Sepsis associated with organ dysfunction, hypoperfusion or sepsis-induced</td>
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<tr>
<td></td>
<td>hypotension</td>
<td></td>
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<tr>
<td><strong>Septic shock</strong></td>
<td>Persistent sepsis-induced hypotension (despite fluid resuscitation) plus</td>
<td>As above, with evidence of MODS</td>
</tr>
<tr>
<td></td>
<td>hypoperfusion abnormalities and organ dysfunction</td>
<td></td>
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<tr>
<td><strong>MODS</strong></td>
<td>Continuum of organ dysfunction</td>
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<tr>
<td></td>
<td></td>
<td>• Altered organ function; organ(s) unable to maintain normal homeostasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Can be primary or secondary</td>
</tr>
</tbody>
</table>

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.
**Alternative complement pathway**

- C3
- C5b

**‘Attack’ Sequence**

**Pro-inflammatory mediators**

- TNF
- IL-1
- IFN-α/β
- IL-6
- IL-8
- MIP1α-1β

**TLR-4**

**MD-2**

**CD-14**

**LBP**

**LPS**

**NF-κB**

**MyD88**

**TRAF6**

**TIR**

**Pro-inflammatory mediators**

(TNF, IL-1, IFN-α/β, IL-6, IL-8, MIP1α-1β)
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 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 O₂ 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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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, tachypnoea 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.
# Table 1.2

<table>
<thead>
<tr>
<th>Vasodilators</th>
<th>Renal 2h</th>
<th>6h</th>
<th>25h</th>
<th>Mesenteric 2h</th>
<th>6h</th>
<th>25h</th>
<th>Hindquarters 2h</th>
<th>6h</th>
<th>25h</th>
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<tbody>
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<td>Acetylcholine¹</td>
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<td>Salbutamol¹</td>
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</table>

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: ¹Waller et al. (1994); ²Bennett et al. (2004); ³Tarpey 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\textsubscript{1} (which is blocked by the fragment CGRP\textsubscript{8-37}), whilst CL with either RAMP2 or RAMP3 is AM specific (termed AM\textsubscript{1} and AM\textsubscript{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\textsubscript{2} receptor which refers to the
component of CGRP signalling not blocked by CGRP\textsubscript{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\textsuperscript{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\textsubscript{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 Physiology 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 AM₁ and AM₂ receptors (see section 1.2.2) although it has been shown that AM can activate CGRP₁ 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 CGRP₈-₃₇, suggesting a role for CGRP₁ receptors (Nuki et al., 1993). A vasorelaxant response to AM was demonstrated in isolated blood-perfused rat lung, which was independent of NO, CGRP₁ receptors, prostaglandins, K_{ATP} channels or adenosine A₁ 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, AM₂₂.₅₂, 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 CGRP₁ receptors and through NO release (Hirata et al., 1995).
<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Agent used</th>
<th>Species</th>
<th>Effect</th>
<th>Reference</th>
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<tbody>
<tr>
<td>CGRP&lt;sub&gt;1&lt;/sub&gt; in vivo</td>
<td>CGRP&lt;sub&gt;8-37&lt;/sub&gt;</td>
<td>Cat</td>
<td>No effect on AM-mediated hind-limb vasodilatation</td>
<td>Champion et al. (1997)</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td>No effect on AM-mediated hypotension</td>
<td>Elhawary et al. (1995); Nandha et al. (1996)</td>
</tr>
<tr>
<td>Dog</td>
<td></td>
<td></td>
<td>No effect on AM-mediated coronary vasodilatation</td>
<td>Sabates et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>BIBN09BS</td>
<td>Mouse</td>
<td>Vasorelaxation of perfused mesentery</td>
<td>Grant et al. (2004)</td>
</tr>
<tr>
<td>AM&lt;sub&gt;1/2&lt;/sub&gt; in vivo</td>
<td>AM&lt;sub&gt;2-32&lt;/sub&gt;</td>
<td>Rat</td>
<td>Inhibition of regional haemodynamic responses to AM</td>
<td>Gardiner et al. (1999)</td>
</tr>
<tr>
<td>in vitro</td>
<td></td>
<td></td>
<td>Attenuation of AM-mediated increase in cAMP in mesenteric vessels from pregnant rats</td>
<td>Ross &amp; Yallampalli (2007)</td>
</tr>
<tr>
<td>NO in vivo</td>
<td>L-NAME</td>
<td>Cat</td>
<td>No effect on AM-mediated mesenteric relaxation</td>
<td>Champion et al. (1996)</td>
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<tr>
<td>Rat</td>
<td></td>
<td></td>
<td>Some attenuation of AM-mediated hindquarters vasodilatation</td>
<td>Gardiner et al. (1995)</td>
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<td></td>
<td>L-NIO</td>
<td>Dog</td>
<td>Attenuation of AM-mediated mesenteric vasorelaxation</td>
<td>Miura et al. (1995)</td>
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<td></td>
<td>L-NA</td>
<td>Rat</td>
<td>Attenuation of AM-mediated mesenteric vasorelaxation</td>
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<td>K&lt;sub&gt;ATP&lt;/sub&gt; in vivo</td>
<td>U37883A</td>
<td>Cat</td>
<td>No effect on AM-mediated hind limb vasodilatation</td>
<td>Champion et al. (1997)</td>
</tr>
<tr>
<td>Dog</td>
<td></td>
<td></td>
<td>Attenuation of AM-mediated coronary vasodilatation</td>
<td>Sabates et al. (1997)</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>Dog</td>
<td></td>
<td>No effect on AM-mediated renal vasodilatation</td>
<td>Miura et al. (1995)</td>
</tr>
<tr>
<td>in vitro</td>
<td>Glibenclamide</td>
<td>Rat</td>
<td>Abolished AM-mediated mesenteric membrane hyperpolarisation</td>
<td>Goto et al. (2000)</td>
</tr>
<tr>
<td>Other in vivo</td>
<td>Phosphodiesterase inhibition</td>
<td>Cat</td>
<td>Augmentation of AM response in cat hind limb</td>
<td>Champion et al. (1997)</td>
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<td>Adenosine receptor inhibition</td>
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<td>Sabates et al. (1997)</td>
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<tr>
<td>in vitro</td>
<td>Endothelial denudation</td>
<td>Rat</td>
<td>Attenuation of AM-mediated mesenteric vasorelaxation</td>
<td>Champion et al. (2001)</td>
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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.,
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$_{1-47}$, although a shorter fragment, IMD$_{8-47}$, 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$_{1-53}$ (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.
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)
**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 F2$\alpha$ (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,
<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Agent used</th>
<th>Species</th>
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<tr>
<td>CGRP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>CGRP&lt;sub&gt;8-37&lt;/sub&gt;</td>
<td>Rat</td>
<td>Attenuation of IMD-mediated hypotension</td>
<td>Roh et al. (2004)</td>
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<td>Attenuation of IMD-mediated hypertension (CNS)</td>
<td>Ren et al. (2006)</td>
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<td>CGRP&lt;sub&gt;8-37&lt;/sub&gt;</td>
<td>Rat</td>
<td>Inhibition of IMD-mediated cAMP increase in L6 skeletal myoblasts</td>
<td>Roh et al. (2004)</td>
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<td>Pig</td>
<td>Attenuation of IMD-mediated coronary vasorelaxation</td>
<td>Kandilci et al. (2006)</td>
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<td>Rat</td>
<td>Attenuation of IMD-mediated pulmonary vasorelaxation</td>
<td>Kandilci et al. (2006)</td>
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<td></td>
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<td>Rat</td>
<td>Inhibition of IMD-mediated increase in cAMP in spinal cord cells</td>
<td>Owji et al. (2008)</td>
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<td>BIBN4096</td>
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<td>Rat</td>
<td>No effect (see above)</td>
<td>Owji et al. (2008)</td>
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<td>AM&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>AM&lt;sub&gt;22-52&lt;/sub&gt;</td>
<td>Rat</td>
<td>No effect on IMD-mediated hypotension</td>
<td>Roh et al. (2004)</td>
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<td>Attenuation of IMD-mediated hypertension (CNS)</td>
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<td>Pig</td>
<td>No effect on IMD-mediated aortic vasorelaxation</td>
<td>Kandilci et al. (2006)</td>
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<td>NO</td>
<td>L-NAME</td>
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<td>Kandilci et al. (2006)</td>
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<td></td>
<td></td>
<td>Rat</td>
<td>Attenuation of IMD-mediated mesenteric vasorelaxation</td>
<td>Chauhan et al. (2007)</td>
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<td>K&lt;sub&gt;K&lt;sub&gt;ATP&lt;/sub&gt;&lt;/sub&gt;</td>
<td>GLB</td>
<td>Rat</td>
<td>No effect on IMD-mediated pulmonary vasorelaxation</td>
<td>Kandilci et al. (2006)</td>
</tr>
<tr>
<td>Others</td>
<td></td>
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<td>IMD non-selectively activated CGRP&lt;sub&gt;1&lt;/sub&gt;, AM&lt;sub&gt;1/2&lt;/sub&gt; receptors in transfected 293T cells</td>
<td>Roh et al. (2004)</td>
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<td></td>
<td>COX</td>
<td>Rat</td>
<td>No effect of meclofenate on IMD-mediated pulmonary vasorelaxation</td>
<td>Kandilci et al. (2006)</td>
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<td></td>
<td>AC, GC</td>
<td>Rat</td>
<td>Attenuation of IMD-mediated mesenteric vasorelaxation by SQ22536, Rp-cAMPs and ODQ</td>
<td>Chauhan et al. (2007)</td>
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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
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_{1-53} 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_{1-53} 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_{1-53} may be mediated via cAMP-dependent mechanisms. Furthermore, IMD_{1-53} 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_{1-53} 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 50 nmol 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 intracerebroventriculally (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$_{8\text{-}37}$. 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$_{1\text{-}47}$, IMD$_{1\text{-}53}$ and AM in
rats and found both forms of IMD to increase blood pressure to a greater extent than AM, with IMD\textsubscript{1-53} having more potent effects than IMD\textsubscript{1-47}.

The potent hypertensive effect of IMD\textsubscript{1-53} in the CNS was blocked by i.c.v. AM\textsubscript{22-52} and CGRP\textsubscript{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\textsubscript{1-53} in central regulation of blood pressure, a prepro-IMD antibody that antagonised the effects of exogenous IMD\textsubscript{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\textsubscript{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\textsubscript{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).
1.3.2 Adenosine receptors

Adenosine interacts with a family of four cell-surface, G-protein-coupled receptors belonging to the P₁ purinergic family, named A₁, A₂a, A₂b and A₃, and adenosine receptor-mediated effects have been confirmed experimentally in almost every tissue and system studied (for review see Conlon et al., 2005). A₁, A₂a and A₂b receptors share approximately 80-95% sequence homology across species in which they have been identified, suggesting an important conserved role, whereas the A₃ 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 A₁ and A₃ subtypes to cause inhibition of adenylate cyclase activity, whilst activation of both A₂ 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 A₁>A₃>A₂a>A₂b, with A₁ receptor having the highest affinity for adenosine and A₂b the lowest. Therefore, A₁ activation occurs at physiological (low) levels of adenosine whilst A₂b 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 'A2-mediated hypotension' and 'A1-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 A2 receptors

Adenosine A2 receptors were divided into A2a and A2b receptors due to their high and low affinity for adenosine in the rat brain (for review see Feoktistov & Biaggioni, 1997). A2a 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 A2a agonists (for review see Linden, 2001).

Activation of the A2a 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 A2a receptors on immune cells, also mediates the strong immunosuppressant effects of adenosine, and A2a 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 A₁-receptor subtype, since isolated hearts from A₁-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 A₁-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 A₁-receptor knockout mice exhibited a higher basal coronary blood flow suggestive of A₁-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 A₂- and A₁-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 & Vander, 1970; Hansen et al., 2005).

A study of adenosine receptor abundance in rat kidney showed that the A₁ and A₂b subtypes are abundant in the preglomerular circulation, with A₂b 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<sub>1</sub>- and A<sub>2</sub>- receptors, adenosine leads to inhibition and stimulation of renin release, respectively (Tagawa & Vandor, 1970; Murray & Churchill, 1985; Weihprech 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<sub>1</sub>-receptor subtype is a direct effect of adenosine acting on the juxtaglomerular apparatus (Weihprech et al., 1990), whilst A<sub>2</sub>-mediated 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 (Weihprech 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<sub>1</sub>- and indirectly via A<sub>2</sub>-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<sub>1</sub>-receptor activation by CHA led to a marked decrease in renin release from perfused rabbit kidneys, an effect which was reversed by A<sub>1</sub>-blockade by DPCPX (Weihprech 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 A1-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 $K_{\text{ATP}}$ 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, A₁-, A₂a- and A₂b-receptors are located on both vascular smooth muscle cells and endothelial cells, and both A₂-receptor subtypes were also found on the plasma membrane and in the cytosol of human skeletal muscle cells (Lynge & Hellsten, 2000). Evidence of A₁-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 A₁-receptors mediate skeletal muscle vasodilatation is not surprising. Since A₁-receptors have a high affinity for adenosine, A₁-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-$\alpha$ release from LPS-activated monocytes and human macrophages (Zhang et al., 2005; Ryzhov et al., 2008). Furthermore,
mice lacking the A2a-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 A2a-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 A2a-receptors (Németh et al., 2006). Mice lacking the A2a-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 A2a-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 A2a-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

\[
\Delta f = 2f_0 \frac{V}{c} \cos \theta
\]

- \( \Delta f \) = Doppler difference frequency (Doppler Shift)
- \( f_0 \) = frequency of transmitted signal (20MHz)
- \( V \) = Fluid velocity
- \( c \) = Velocity of sound in fluid
- \( \theta \) = angle between flow and acoustic axes (45°)
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 $45^\circ$. Since $f_0, c$ and $\theta$ are constant for any individual probe in any individual animal, then, $\Delta f$ 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:

\[
\text{Conductance} = \frac{\text{flow}}{\text{pressure}}
\]

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 \textit{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.
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 1 cm 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 2 cm. 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 1-2 cm 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 tunneled 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.
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.
<table>
<thead>
<tr>
<th>Abbreviation brand name</th>
<th>Full name</th>
<th>Purchased from</th>
<th>Preparation/administration</th>
<th>Chapter(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM</td>
<td>Adrenomedullin (rat)</td>
<td>Bachem (St Helens, UK)</td>
<td>• dissolved in distilled water to provide stock; diluted further with saline given as 0.1ml i.v. bolus</td>
<td>3, 4, 5, 7</td>
</tr>
<tr>
<td>Ado</td>
<td>Adenosine</td>
<td>Sigma (Dorset, UK)</td>
<td>• dissolved in distilled water given as an i.v. infusion at a rate of 0.15ml min⁻¹ for 3 min</td>
<td>6, 7</td>
</tr>
<tr>
<td>AII</td>
<td>Angiotensin II</td>
<td>Bachem (St Helens, UK)</td>
<td>• dissolved in sterile isotonic saline given as a continuous infusion 0.4ml h⁻¹</td>
<td>4</td>
</tr>
<tr>
<td>AM₂₂-₅₂</td>
<td>Adrenomedullin ₂₂-₅₂</td>
<td>Peptide Institute Inc. (Scientific Marketing Associates, Barnet, UK)/Bachem (St Helens, UK)</td>
<td>• dissolved in sterile isotonic saline, infused over 6 min at 0.4ml h⁻¹</td>
<td>4</td>
</tr>
<tr>
<td>Amoxycare LA</td>
<td>Amoxicillin trihydrate</td>
<td>Animalcare Ltd (York, UK)</td>
<td>• dissolved in sterile isotonic saline</td>
<td>3-7</td>
</tr>
<tr>
<td>Antisedan</td>
<td>Atipamezole hydrochloride</td>
<td>Pfizer (Sandwich, Kent, U.K.)</td>
<td>• dissolved in sterile isotonic saline</td>
<td>3-7</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
<td>Bachem (St Helens, UK)</td>
<td>• dissolved in sterile isotonic saline given as a continuous infusion at 0.4ml h⁻¹</td>
<td>4</td>
</tr>
<tr>
<td>CCPA</td>
<td>2-Chloro-N⁶-cyclopentyladenosine</td>
<td>Sigma (Dorset, UK)</td>
<td>• 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</td>
<td>6</td>
</tr>
<tr>
<td>Drug</td>
<td>Description</td>
<td>Supplier</td>
<td>Preparation</td>
<td>Methodology</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| 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                                       | 4                                                                         |
| Losartan     | Losartan potassium                                                           | Sequoia Research Products (Oxford, UK)       | • dissolved in sterile isotonic saline                                       | 4,6                                                                       |
| LPS          | Lipopolysaccharide (E. coli serotype 0127 B8)                                | Sigma (Dorset, UK)                            | • dissolved in sterile isotonic saline                                       | 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                                       | 4                                                                         |
| Vetersgesic  | 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 phaeocytoma 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\textsubscript{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 IMD₁₋₄₇, the studies to be described here have used the
fragment IMD₁₋₅₃, 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 IMD₁₋₅₃
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<sub>1.53</sub> 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).

<table>
<thead>
<tr>
<th></th>
<th>HR (\text{beats min}^{-1})</th>
<th>MAP (\text{mmHg})</th>
<th>RVC (\text{units})</th>
<th>MVC (\text{units})</th>
<th>HVC (\text{units})</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM (1nmol kg(^{-1}))</td>
<td>320±7</td>
<td>107±3</td>
<td>84±5</td>
<td>77±9</td>
<td>37±3</td>
</tr>
<tr>
<td>AM (3nmol kg(^{-1}))</td>
<td>343±6</td>
<td>108±2</td>
<td>82±6</td>
<td>70±5</td>
<td>47±3</td>
</tr>
<tr>
<td>IMD (1nmol kg(^{-1}))</td>
<td>339±6</td>
<td>110±2</td>
<td>83±7</td>
<td>68±3</td>
<td>44±3</td>
</tr>
</tbody>
</table>

*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 1 nmol 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 \( (1 \text{nmol kg}^{-1}) \) 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 1 nmol kg\(^{-1}\), AM caused increases in Doppler shift (i.e., flow) in all 3 vascular beds (Figure 3.3.2).

Since the 1 nmol 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_{1-53}, (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\textsuperscript{-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\textsuperscript{-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\textsuperscript{-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\textsuperscript{-1}) and IMD (1nmol kg\textsuperscript{-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\textsubscript{22-52}, was equally effective in attenuating the renal and mesenteric vasodilator effects of IMD (1 nmol kg\textsuperscript{-1}) and AM (3 nmol kg\textsuperscript{-1}), but inhibition of NO synthase was more effective at reducing the vasodilator effects of IMD than AM. Vascular $K\text{\textsubscript{ATP}}$ 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.

\textit{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 $K\text{\textsubscript{ATP}}$ 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 CGRP₁ receptor, which consists of CL and RAMP1, and can be antagonised by the fragment CGRP₈-₃₇. Adrenomedullin functions through two receptors, AM₁ and AM₂, which consist of CL complexed with RAMP2 and RAMP3, respectively. Adrenomedullin action through these receptors can be antagonised by the fragment AM₂₂-₅₂ (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<sub>ATP</sub> 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 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 CGRP8-37 and AM22-52 to isolated rat pulmonary arteries caused an additive attenuation of IMD-mediated vasorelaxation. The remaining IMD-mediated vasodilatation which was insensitive to both CGRP8-37 and AM22-52 matched that seen upon addition of the IMD fragment, IMD17-47, 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 CGRP8-37-sensitive (Kobayashi et al., 2004; Roh et al., 2004; Taylor et al., 2004; Ren et al., 2006; Kandilci et al., 2006, 2008) and/or AM22-52-sensitive receptors (Ren et al., 2006; Kandilci et al., 2008) in regional IMD-mediated vasodilatations, as well as a distinct IMD17-47-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 KATP 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).
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 sub 8-37-sensitive receptors

The involvement of CGRP1 receptors in the regional vascular responses to AM and IMD was assessed using the CGRP peptide fragment, CGRP sub 8-37.

Animals (n=6) received a 6 min infusion of CGRP sub 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)

![Graph showing the experiment flows for Groups 1 and 2](image)

**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 \(^{-1}\) 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 (AT₁) 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 pretreated 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 CGRP1 antagonist CGRP8-37, 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 CGRP8-37 had no effect on the haemodynamic responses to IMD (Figure 4.3.2b). These data suggest that IMD does not activate CGRP1 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$_{\text{ATP}}$ 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).
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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 10³. n=5-10 group¹.

*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) $AM \ (3 \text{nmol kg}^{-1} \text{i.v.}) \ (n=8)$ and (b) $IMD \ (1 \text{nmol kg}^{-1} \text{i.v.}) \ (n=8)$ on regional haemodynamics in conscious rats.

*P<0.05 versus original baseline (Friedman's test).
Figure 4.3.2 Effects of CGRP8-37 on cardiovascular responses to AM (1 nmol kg⁻¹ i.v.) \( (n=6) \) (a) or IMD (1 nmol 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\textsubscript{22-52} on responses to (a) AM (3 nmol kg\textsuperscript{-1} i.v.) (n=8) or (b) IMD (1 nmol kg\textsuperscript{-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 (3 mg kg\(^{-1}\) h\(^{-1}\)) on cardiovascular responses to (a) AM (3 nmol kg\(^{-1}\) i.v.) (n=9) or (b) IMD (1 nmol kg\(^{-1}\) i.v.) (n=8) (b) in conscious rats.

*P<0.05 versus original baseline (Friedman's test). #P<0.05 versus control (AI 0.2 μg ml\(^{-1}\)/AVP 0.02 μg 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 (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)
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 KATP 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 AM22-52 is more selective for AM1 (CL/RAMP2) than AM2 (CL/RAMP3) receptors (Hay et al., 2003), there are no tools available to discern the true contribution of each. AM22-52 can also inhibit CGRP-, but not AM-mediated vasodilatation in the cat hindlimb (Champion et al., 1997). Furthermore, AM22-52 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 CGRP1 receptor, and CGRP has some affinity for both subtypes of AM receptors. Thus, it has been shown that application of the selective CGRP1 receptor antagonist, BIBN4096BS, blocked the vasodilator action of AM in isolated perfused murine mesentery, but not in the aorta, implicating CGRP1 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 vasodilations were attenuated by AM22-52, suggesting AM1 and/or AM2 receptors were activated by both peptides in a regionally-selective manner.

In addition to a role for AM22-52-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 AT1 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 (AngII) (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\textsubscript{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\textsuperscript{2+}-activated K\textsuperscript{+}-channels (BK\textsubscript{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\textsubscript{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\textsubscript{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, AM22-52, 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 15\(\mu\)g 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.

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>1.5 h</td>
</tr>
<tr>
<td>Saline bolus</td>
<td>AM/IMD</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Day 3</th>
<th>Day 4</th>
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<tbody>
<tr>
<td>0 h</td>
<td>1.5 h</td>
</tr>
<tr>
<td>LPS bolus</td>
<td>AM/IMD</td>
</tr>
</tbody>
</table>
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 (0.4 ml h⁻¹) or LPS (15 µg 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](image)

**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 \((P<0.05, \text{Kruskall Wallis test})\).

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 \((P<0.05, \text{24 h to 25 h})\) 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 \((P<0.05, \text{Friedman's test})\), which was sustained over the first 8 h, peaking 6 h after LPS administration, but by 25 h the resting heart rate was no longer different from baseline \((P>0.05, \text{24 h and 25 h})\). The tachycardia was accompanied by a triphasic blood pressure response, comprising an early rise \((P<0.05, \text{0.25 h and 0.5 h})\) followed by a fall \((P<0.05, \text{1 h to 1.75 h})\), with blood pressure returning to baseline by 2 h. There was then a second period of hypotension \((P<0.05, \text{5 h to 7 h})\), 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 (15\(\mu\)g 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 (15\(\mu\)g 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.
<table>
<thead>
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<td>359±7*</td>
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<td>MVC (units)</td>
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</tr>
<tr>
<td>HVC (units)</td>
<td>48±6</td>
<td>41±2</td>
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</table>

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 (15\(\mu\)g 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)
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<th>MAP (mmHg)</th>
<th>RVC (units)</th>
<th>MVC (units)</th>
<th>HVC (units)</th>
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<td>Sal LPS</td>
<td>Sal LPS</td>
<td>Sal LPS</td>
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</tr>
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<td>358±13</td>
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<td>107±4*</td>
<td>64±5</td>
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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 10⁷. *P<0.05 vs. Saline (Wilcoxon test).
(a) **LPS bolus**

<table>
<thead>
<tr>
<th>Control</th>
<th>AM group</th>
<th>IMD group</th>
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<tbody>
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<td>25h</td>
</tr>
<tr>
<td>HR (beats min⁻¹)</td>
<td>↑ ↑ ← ←</td>
<td>↑ ↑ ← ←</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>↓ ← ← ←</td>
<td>↓ ← ↑ ↑</td>
</tr>
<tr>
<td>RVC (units)</td>
<td>↑ ↑ (↑)</td>
<td>↑ ↑ ↑</td>
</tr>
<tr>
<td>MVC (units)</td>
<td>↑ ↑ ← ←</td>
<td>↑ ↑ ↑</td>
</tr>
<tr>
<td>HVC (units)</td>
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<td>↑ ↑ ↑</td>
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</table>

(b) **LPS infusion**

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<th>IMD group</th>
</tr>
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<td>25h</td>
</tr>
<tr>
<td>HR (beats min⁻¹)</td>
<td>↑ ↑ ↑ ↑</td>
<td>↑ ↑ ↑ ↑</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>↓ ← ← ←</td>
<td>↓ ← ← ↑</td>
</tr>
<tr>
<td>RVC (units)</td>
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<td>↑ ↑ ↑</td>
</tr>
<tr>
<td>MVC (units)</td>
<td>↓ ↓ ← ←</td>
<td>↓ ↓ ← ←</td>
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<tr>
<td>HVC (units)</td>
<td>↑ ← ← ↑</td>
<td>↑ ← ← ←</td>
</tr>
</tbody>
</table>

**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 10⁴.
<table>
<thead>
<tr>
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<th>+/-AM\textsubscript{22-52} infusion</th>
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<td></td>
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<tr>
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<tr>
<td>+AM\textsubscript{22-52}</td>
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<td>424±10</td>
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<tr>
<td>-AM\textsubscript{22-52}</td>
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<td>435±6</td>
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<tr>
<td>MAP (mmHg)</td>
<td></td>
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<tr>
<td>+AM\textsubscript{22-52}</td>
<td>103±3</td>
<td>107±4</td>
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<td>48±4</td>
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Table 5.4 Cardiovascular variables 1.5 h after treatment with LPS, in the presence (n=8) and absence (n=8) of AM\textsubscript{22-52} (500nmol kg\textsuperscript{-1} h\textsuperscript{-1}, for 6 min). Units for vascular conductance (VC) are kHz mmHg\textsuperscript{-1} x 10\textsuperscript{3}.

*P<0.05 vs. LPS time control (Mann Whitney U test).
### AM$_{22-52}$ infusion

<table>
<thead>
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<th></th>
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<th>End</th>
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<tbody>
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<td></td>
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<td>97±5</td>
</tr>
<tr>
<td></td>
<td>25h</td>
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<td>RVC (units)</td>
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<td>25h</td>
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<td>HVC (units)</td>
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<tr>
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<td>25h</td>
<td>56±7</td>
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</table>

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$^{1}$. 

171
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 \(\mu\)g 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 (0-10 min) cardiovascular changes following IMD (1nmol kg\(^{-1}\) i.v.) administration (a) 1.5 h (n=8), (b) 6 h (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 µg 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 (15µg 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 $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ channel pharmacology, preventing effective functioning of mediators involved in their control (Buckley et al., 2006). While it is possible that LPS-induced changes in $K_{\text{ATP}}$ channel pharmacology or configuration were responsible for the changes in responses to AM and IMD observed here, the $K_{\text{ATP}}$ channel blocker, U37883A, had no effect on responses to AM or IMD in normal animals (Chapter 4). Thus, it appears unlikely that changes in $K_{\text{ATP}}$ 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, AM22-52, 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 AM22-52-sensitive receptors, but we found no effect of AM22-52 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 AM22-52 has greater selectivity for AM₁ (CL/RAMP2) than for AM2 (CL/RAMP3) receptors (Hay et al., 2003). As discussed above, the RAMP2 component of the AM₁ 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, AM22-52-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 AM22-52. Mazzocchi et al. (2000) co-administered AM22-52 (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 AM22-52 (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 AM22-52 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 AM22-52 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-chloro-\(N^6\)-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 (A₁, A₂a, A₂b and A₃; see Introduction) (Fredholm et al., 2001b), and evidence suggests that A₁ and A₂-receptors are primarily responsible for the cardiovascular actions of adenosine (Fredholm et al., 2001a). A₁-receptors are linked to Gₛ 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). A₂a-receptors are located on vascular smooth muscle, endothelial cells and on immune cells, and are linked to Gₛ, 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). A₂b receptor activation may also lead to regionally-selective vasodilatation, but the lack of pharmacological probes selective for A₂b 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 A₁ receptor-mediated vasoconstrictor effects following LPS administration. Therefore, further experiments were performed to assess the hemodynamic responses to A₁ receptor activation and antagonism. It is well reported that A₁ 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 A₁ receptor antagonism in the control rats (see Results), a final set of experiments was performed in the presence of the angiotensin (AT₁) receptor
antagonist, losartan, to assess if the vasoconstrictor effects of $\text{A}_1$ 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 \(\mu\)g 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 \(\mu\)g 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 A₁-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 A₁-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 A₁ 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 A₁-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 pretreatment 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)10³), mesenteric vascular conductance (75±5 and 89±10 (kHz mmHg⁻¹)10³) hindquarters vascular conductance (49±5 and 53±4 (kHz mmHg⁻¹)10³).
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.
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<td>45±6   47±9</td>
<td>47±2   53±4</td>
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<td>343±10 429±14*</td>
<td>337±8  399±14*</td>
<td>360±11 416±6*</td>
<td>340±7  399±8*</td>
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<td>102±2  92±3</td>
<td>107±1  102±3</td>
<td>111±3  99±3</td>
<td>111±2  114±7</td>
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<td>74±7   109±16*</td>
<td>89±3   112±6*</td>
<td>75±8   98±11*</td>
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<td>Mesenteric VC (units)</td>
<td>57±6   128±10*</td>
<td>83±9   108±14*</td>
<td>62±2   84±9*</td>
<td>70±8   106±1*</td>
<td>59±5   57±9</td>
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<tr>
<td>Hindquarters VC (units)</td>
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<td>52±3   67±3*</td>
<td>42±4   48±6</td>
<td>42±5   53±5*</td>
<td>42±2   45±3</td>
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<tr>
<td>Heart rate (beats/min)</td>
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<td>356±10 352±11</td>
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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 10³. 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 (0.1 mg kg\(^{-1}\) min\(^{-1}\) i.v.) 1.5 h (a) and 6 h (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)
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<td>↔</td>
<td>↑</td>
<td>↔</td>
<td>↓</td>
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</tr>
</tbody>
</table>

**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<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, A<sub>3</sub>). 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<sub>1</sub> and A<sub>2</sub> receptors mediated by activation at low and high concentrations of adenosine, respectively (see Chapter 1.3).

To delineate the role of the adenosine A<sub>1</sub> receptor in the cardiovascular responses to both endogenous and exogenous adenosine, the selective A<sub>1</sub> agonist, 2-choloro-N<sup>6</sup>-cyclopentyladenosine (CCPA), and antagonist, 8-
cyclopentyl-1, 3-dipropylxanthine (DPCPX), were used. Administration of the
A₁-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 A₁ 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 A₁
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 A₁-
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 A₁-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 A₁-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 A₁-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 A₁-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<sub>2</sub> 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<sub>1</sub> or A<sub>2</sub> 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<sub>1</sub>-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 A1-receptor-mediated vasoconstriction or impairment of A2-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 A2-receptors on the heart (Lappe et al., 1992) and/or enhanced direct action of A₁-receptors on the heart.

Since one interpretation of the findings from the experiments using 8-PT and adenosine was that there were enhanced A₁-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 A₁-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 A₁-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 A₁-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 A₂-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 A₁-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 A₁ 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 A₂a 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. A₁ receptor agonist administration were due to peripheral A₁ 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 $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ channel blocker, glibenclamide. Furthermore, infusion of the $K_{\text{ATP}}$ channel opener, levemakalim, significantly enhanced the depressor effects of VIP, CGRP and adenosine, indicating that activation of $K_{\text{ATP}}$ channels was central to this effect. Further evidence came from the finding that co-infusion of the anti-
anginal drug nicorandil, which activates of $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ 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 by addition of the $K_{\text{ATP}}$ channel blocker, U37883A, indicating that $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ 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 (300\(\mu\)g 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 \( (P<0.05, \text{ for 0-10 min integrated changes for each variable}) \) (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\(^{-1}\) \( (P>0.05, \text{ for 0-10 min integrated changes for each variable}) \). 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).
<table>
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<th>+ AM infusion</th>
<th>+ IMD infusion</th>
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<td>HVC (units)</td>
<td>42±4</td>
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**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\(^{3}\).
<table>
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<th></th>
<th>Saline + AM</th>
<th>Adenosine + AM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1nmol kg⁻¹</td>
<td>3nmol kg⁻¹</td>
</tr>
<tr>
<td>(a) (b)</td>
<td>328±8</td>
<td>325±6</td>
</tr>
<tr>
<td>HR (beats min⁻¹)</td>
<td>320±8</td>
<td>325±7</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>102±1</td>
<td>102±2</td>
</tr>
<tr>
<td>RVC (units)</td>
<td>73±9</td>
<td>71±9</td>
</tr>
<tr>
<td>MVC (units)</td>
<td>73±7</td>
<td>73±7</td>
</tr>
<tr>
<td>HVC (units)</td>
<td>45±2</td>
<td>43±3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saline + IMD</td>
<td>Adenosine + IMD</td>
</tr>
<tr>
<td></td>
<td>0.3nmol kg⁻¹</td>
<td>1nmol kg⁻¹</td>
</tr>
<tr>
<td>(a) (b)</td>
<td>331±7</td>
<td>334±10</td>
</tr>
<tr>
<td>HR (beats min⁻¹)</td>
<td>333±17</td>
<td>333±16</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>103±4</td>
<td>105±4</td>
</tr>
<tr>
<td>RVC (units)</td>
<td>75±11</td>
<td>81±10</td>
</tr>
<tr>
<td>MVC (units)</td>
<td>67±11</td>
<td>73±10</td>
</tr>
<tr>
<td>HVC (units)</td>
<td>46±4</td>
<td>46±3</td>
</tr>
</tbody>
</table>

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 10⁴.

*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 (3\(\mu\)g kg\(^{-1}\) min\(^{-1}\) i.v.) (closed circles) in conscious rats (n=9).

\(*P<0.05\) versus original baseline (Friedman's test). \(\dagger P<0.05\) 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\textsubscript{ATP} channel blocker, U37783A, no role for K\textsubscript{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\textsubscript{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\textsuperscript{-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\textsuperscript{-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 CLRAMP 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\textsubscript{22,52} in the renal and mesenteric vascular beds (Chapter 4), and others have found a role for CGRP\textsubscript{8,37}-sensitive 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 CGRP₁ receptor antagonist CGRP₈₋₃₇ (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, AM22-52, 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 AM22-52.

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<sub>1</sub> receptor responses, and/or 
to a down-regulation of adenosine A<sub>2</sub> mediated responses. To investigate this 
further, the selective A<sub>1</sub> receptor agonist, CCPA, and the antagonist DPCPX 
were used. The results indicated that whilst altered A<sub>1</sub> 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<sub>1</sub> and A<sub>2</sub> 
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 endotoxaemia. 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


279


