# RESEARCH PAPER

# Mechanisms involved in the regional haemodynamic effects of intermedin (adrenomedullin 2) compared with adrenomedullin in conscious rats

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**Background and purpose:** Intermedin (IMD) is a newly identified member of the calcitonin family of peptides that shares structural and functional homology with adrenomedullin (AM). *In vivo* cardiovascular effects of AM have been described, but relatively little is known of the *in vivo* actions of IMD. The purpose of this study was to compare the regional haemodynamic effects of IMD with those of AM in conscious rats, and investigate possible underlying mechanisms.
**Experimental approach:** Measurements of blood pressure, heart rate and renal, mesenteric and hindquarters haemodynamics were made in conscious, chronically-instrumented rats.
**Key results:** IMD caused tachycardia and vasodilatation in all three vascular beds, associated with modest hypotension. At an equimolar dose (1 nmol·kg$^{-1}$), most of the cardiovascular effects of IMD were greater than those of AM. The AM receptor antagonist, AM$_{22–52}$, was equally effective in attenuating the renal and mesenteric vasodilator effects of IMD (1 nmol·kg$^{-1}$) and AM (3 nmol·kg$^{-1}$), but inhibition of NO synthase was more effective at reducing the vasodilator effects of IMD than AM. Vascular K$_{ATP}$ channel blockade with U-37883A did not inhibit the vasodilator effects of either peptide.
**Conclusions and implications:** *In vivo*, the regional haemodynamic profile of IMD resembles that of AM, and some of the vasodilator effects of IMD are mediated by AM receptors and NO, but not by K$_{ATP}$ channels. The cardiovascular effects of AM have been implicated in various pathological conditions, but whether or not endogenous IMD fulfils a similar role remains to be determined.
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## Introduction

A new member of the calcitonin peptide superfamily [which includes calcitonin, calcitonin gene-related peptide (CGRP), adrenomedullin (AM) and amylin], was described by Roh *et al.* (2004) and named intermedin (IMD), since high levels of expression were seen in the intermediate lobe of the pituitary. An independent group had previously isolated cDNAs encoding five AM-like peptides in pufferfish (Ogoshi *et al.*, 2003) and in 2004, described cDNA encoding a new member of the

AM family in mammals that they named AM2 (Takei *et al.*, 2004). It is now generally understood that IMD and AM2 are the same peptide, herein referred to as IMD.

The receptor system involved in mediating the actions of CGRP and AM comprises a calcitonin receptor (CL) together with one of three receptor activity modifying proteins (RAMP1, 2 or 3) (McLatchie *et al.*, 1998; receptor nomenclature follows Alexander *et al.*, 2008). The CL/RAMP1 complex forms a CGRP receptor which is blocked by the CGRP fragment, CGRP$_{8–37}$, whereas CL/RAMP2 and 3 complexes form AM$_1$ and AM$_2$ receptors, respectively, the former being blocked more effectively than the latter by the AM fragment, AM$_{22–52}$ (Hay *et al.*, 2003). Although the sequence homology between IMD and AM or CGRP is not strong (~30% and 20%, respectively), there is evidence to suggest that many of the

actions of IMD are also mediated by CL/RAMP complexes. Thus, IMD can act non-selectively as an agonist at all three CL/RAMP complexes (Roh *et al.*, 2004), but with a pharmacological profile different from that of either CGRP or AM (Bell and McDermott, 2008). Recently, there has been some evidence published which suggests that IMD might also act at a distinct class of receptor (Owji *et al.*, 2008).

It is clear that CGRP and AM can have important cardiovascular actions *in vitro* and *in vivo* (reviewed by Brain and Grant, 2004), and there is now accumulating evidence showing some similar cardiovascular actions of IMD (reviewed by Bell and McDermott, 2008). Thus, *in vitro*, vasorelaxant effects of IMD have been reported (Kobayashi *et al.*, 2004; Pan *et al.*, 2005; Chauhan *et al.*, 2007; Kandilci *et al.*, 2008), and, *in vivo*, hypotensive effects have been shown in anaesthetized (Fujisawa *et al.*, 2004; Takei *et al.*, 2004; Pan *et al.*, 2005; Ren *et al.*, 2006) and conscious (Roh *et al.*, 2004; Abdelrahman and Pang, 2006; Charles *et al.*, 2006; Fujisawa *et al.*, 2006; 2007) animals. It is generally assumed that IMD-induced hypotension is secondary to vasodilatation, but, although total peripheral resistance has been shown to fall in sheep following IMD administration (Charles *et al.*, 2006), to our knowledge only one study has reported regional haemodynamic effects of IMD (Fujisawa *et al.*, 2007), and those measurements were restricted to one point in time during IMD infusion. Thus, the first aim of the present study was to describe the regional haemodynamic profile for IMD in conscious rats and compare it with that of AM. Furthermore, to determine the extent to which AM receptors were involved in the vasodilator actions of the peptides, the effects of the AM receptor antagonist, $AM_{22-52}$, on responses to AM and IMD were assessed.

There is some evidence that AM can cause vasodilatation in some vascular beds via the production of NO and/or by activation of $K_{ATP}$ channels (Gardiner *et al.*, 1995; Lang *et al.*, 1997; Sabates *et al.*, 1997; Goto *et al.*, 2000; Terata *et al.*, 2000). *In vitro* studies have shown a role for NO in the vasorelaxant effects of IMD (Kandilci *et al.*, 2006; Chauhan *et al.*, 2007), possibly due to up-regulation of NO synthesis (Yang *et al.*, 2006), but, *in vivo*, the depressor response to IMD is reportedly unaffected by NO synthase inhibition (Taylor *et al.*, 2005; Abdelrahman and Pang, 2007), although the accompanying regional vascular effects have not yet been evaluated. Therefore, our second aim was to compare the effects of NO synthase inhibition [with $N^G$ nitro L arginine methyl ester (L-NAME)] on the regional haemodynamic effects of AM and IMD. In addition, although $K_{ATP}$ channels are reportedly not involved in the vasorelaxant effects of IMD *in vitro* (Kandilci *et al.*, 2006; Chauhan *et al.*, 2007; Kandilci *et al.*, 2008), the effects of vascular $K_{ATP}$ channel inhibition on cardiovascular responses to IMD *in vivo* have not been studied to date. Hence, our third aim was to compare the effects of vascular $K_{ATP}$ channel inhibition with U-37883A (Meisheri *et al.*, 1993) on the regional haemodynamic effects of AM and IMD. Finally, in the light of some unexpected effects of U-37883A on responses to AM but not to IMD, which could possibly be explained by an influence of the renin-angiotensin system, we compared the effects of angiotensin receptor antagonism with losartan on responses to AM and IMD.

## Methods

### Animals and surgery

All animal care and procedures were approved by the University of Nottingham Ethical Review Committee and were performed under Home Office Project and Personal Licence authority. Male Sprague-Dawley rats (350–400 g) were purchased from Charles River (Margate, Kent, UK) and housed in the Biomedical Services Unit, University of Nottingham for at least 10 days after delivery, with free access to standard rat chow (Teklad Global 18% protein rodent diet, Bicester, Oxon, UK) and water. Room temperatures were maintained at $21 \pm 2°C$, and lights were on from 06.00 h to 18.00 h.

All surgery was performed under general anaesthesia (fentanyl and medetomidine, $300 \, \mu g \cdot kg^{-1}$ of each i.p.). Anaesthetic reversal and the provision of analgesia was achieved using atipamezole and buprenorphine ($1 \, mg \cdot kg^{-1}$ and $0.03 \, mg \cdot kg^{-1}$, respectively, s.c.). At the first surgical stage, miniaturized pulsed Doppler flow probes were sutured around the left renal and superior mesenteric arteries, and around the distal abdominal aorta (to monitor hindquarters flow). The wires from the probes were secured at the nape of the neck and animals were returned to their home cages with free access to food and water. At least 10 days later, and following satisfactory inspection by the Named Veterinary Surgeon, the animals were anaesthetized (as above) and catheters were implanted in the distal abdominal aorta (via the ventral caudal artery) for monitoring arterial blood pressure and heart rate, and in the right jugular vein for the administration of substances. At this stage, the animals were fitted with custom-designed harnesses with a counter-balanced spring attached, to protect the catheters and allow the animals freedom of movement in their home cage with access to food and water *ad libitum*. The arterial catheters were connected to fluid-filled swivels for overnight infusion of heparinized saline ($15 \, U \cdot mL^{-1}$, $0.4 \, mL \cdot h^{-1}$) to maintain catheter patency.

### Data acquisition and analysis

Experiments began 24 h after catheterization. Cardiovascular variables [heart rate, arterial blood pressure, renal, mesenteric and hindquarters Doppler shifts (flow) ] were recorded continuously throughout each experimental day, using a customized, computer-based system [Haemodynamics Data Acquisition System (HDAS), University of Limburg, Maastricht, The Netherlands] connected to the transducer amplifier (Gould model 13-4615-50) and the Doppler flowmeter [Crystal Biotech VF-1 mainframe (pulse repetition frequency 125 kHz) fitted with high velocity (HVPD-20) modules]. Data were sampled by HDAS every 2 ms, averaged each cardiac cycle and stored to disc every 5 s, and analysed off-line (Datview, University of Maastricht, The Netherlands).

Data are expressed as mean $\pm$ SEM. As not all data were normally distributed, within-group analyses were carried out by a non-parametric equivalent of ANOVA allowing for multiple comparisons (Friedman's test) (Theodorsson-Norheim, 1987), and between-group analyses were performed on the baseline values and the integrated responses to the peptides (areas under or over curves) using Wilcoxon's test or Mann–Whitney $U$-test as appropriate. $P \leq 0.05$ was taken as significant.

*Experimental protocols*
*Experiment 1: Comparison of the regional haemodynamic effects of IMD and AM.* After a period of baseline recording, rats were given an equimolar dose (1 nmol·kg⁻¹ i.v. in 0.1 mL sterile saline) of either IMD ($n = 8$) or AM ($n = 9$) (Experiment 1a). As that dose of AM was found to have relatively modest and poorly reproducible effects (see Results), an additional experiment (Experiment 1b) was run in which the effects of IMD (1 nmol·kg⁻¹; $n = 11$) were compared with those of a higher dose of AM (3 nmol·kg⁻¹, $n = 10$). All further experiments used IMD at a dose of 1 nmol·kg⁻¹ and AM at a dose of 3 nmol·kg⁻¹.

*Experiment 2: Effects of repeated doses of IMD and AM.* As some of the protocols required repeat dosing with AM and IMD (see below), it was necessary to assess the reproducibility of responses under those conditions. A group of rats ($n = 8$) was randomized to receive AM (3 nmol·kg⁻¹) on day 1 and 3 and IMD (1 nmol·kg⁻¹) on day 2 and 4, or IMD on day 1 and 3, and AM on day 2 and 4, with animals only receiving one treatment each day.

*Experiment 3: Involvement of AM₂₂₋₅₂-sensitive receptors.* Rats were given a 6 min i.v. infusion of saline (0.4 mL·h⁻¹) or AM₂₂₋₅₂ (500 nmol·kg⁻¹·h⁻¹) followed by IMD (1 nmol·kg⁻¹) or AM (3 nmol·kg⁻¹) between 5 and 10 min later. The dose of AM₂₂₋₅₂ has previously been shown to attenuate the regional vasodilatations to AM (1 nmol·kg⁻¹) *in vivo* (Gardiner *et al.*, 1999).

*Experiment 4: Involvement of NO.* Because NO synthase inhibition with L-NAME causes vasoconstriction and hypertension, the control condition in this experiment involved co-infusion of angiotensin II (AII) (200 ng·kg⁻¹·h⁻¹) and arginine vasopressin (AVP) (20 ng·kg⁻¹·h⁻¹), which we have shown previously to match the regional haemodynamic effects of L-NAME (3 mg·kg⁻¹·h⁻¹) infusion (Gardiner *et al.*, 2005). Animals ($n = 8$) were given 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 start of the AII + AVP infusion. This protocol was repeated on days 3 and 4 with L-NAME infused instead of AII + AVP. L-NAME was always given on days 3 or 4 because of its extremely long duration of action.

*Experiment 5: Involvement of K_ATP channels.* Rats ($n = 9$) were given AM (3 nmol·kg⁻¹) or IMD (1 nmol·kg⁻¹) 30 min after administration of U-37883A (5 mg·kg⁻¹; Meisheri *et al.*, 1993; Champion *et al.*, 2002) or vehicle (1 mL sterile saline infused over 30 min). The experiments ran over 4 days with each animal receiving only one treatment on each experimental day in random order. Effectiveness of U-37883A at blocking K_ATP channels was assessed using the K_ATP channel opener, levcromakalim (10 µg·kg⁻¹·min⁻¹ for 3 min). Whilst U-37883A was unable to completely antagonize the effects of levcromakalim, it attenuated responses to levcromakalim given 30 min after the U-37883A infusion. Thus, U-37883A reduced the levcromakalim-mediated fall in blood pressure by 75% and attenuated the renal, mesenteric and hindquarters vasodilatations by 52, 33 and 88% respectively.

*Experiment 6: Effects of angiotensin (AT₁) receptor antagonism.* As some of the results obtained in earlier experiments could be explained by differential activation of the renin-angiotensin system (see Experiment 5 Results), the effects of the angiotensin II (AT₁) receptor antagonist, losartan, on the regional haemodynamic responses to AM and IMD were measured.

A group of animals ($n = 9$) was given either AM (3 nmol·kg⁻¹) or IMD (1 nmol·kg⁻¹) on day 1 and the other peptide on day 2, 60 min after administration of losartan vehicle (0.1 mL sterile saline). On days 3 and 4, peptide administration was repeated 60 min after administration of losartan (10 mg·kg⁻¹). This dose of losartan causes complete inhibition of the cardiovascular effects of AII (50 ng) for at least 9 h (Batin *et al.*, 1991). Losartan was always given on the final experimental days because of its long duration of action.

*Materials*
Adrenomedullin (rat), intermedin (1–53), angiotensin II and arginine vasopressin were from Bachem (St Helens, UK). Adrenomedullin ₂₂₋₅₂ was either from Peptide Institute Inc. (Scientific Marketing Associates, Barnet, UK) or Bachem (St Helens, UK). L-NAME was from Sigma (Poole, Dorset, UK), losartan potassium was from Sequoia Research Products (Oxford, UK), U-37883A was from Biomol (Plymouth Meeting, PA, USA). All drugs were dissolved in sterile saline; stock solutions of peptides were made up in sterile water for injection, and diluted in sterile saline. Injection volumes were 0.1 mL and infusion rates were 0.4 mL·h⁻¹, except in the case of U37883A that was infused at a rate of 2 mL·h⁻¹ for 30 min.

Fentanyl citrate was from Janssen-Cilag (High-Wycombe, UK); medetomidine hydrochloride (Domitor) and atipamezole hydrochloride (Antisedan) were from Pfizer (Sandwich, Kent, UK); buprenorphine (Vetergesic) was from Alstoe Animal Health (York, UK)

## Results

*Experiment 1: Comparison of the regional haemodynamic effects of IMD and AM*
Resting cardiovascular variables and integrated (0–30 min) responses to IMD and AM are shown in Table 1. In the first experiment (Experiment 1a), administration of IMD (1 nmol·kg⁻¹) caused a fall in blood pressure associated with tachycardia and hyperaemic vasodilatation in all three vascular beds, whereas the equimolar dose of AM had no significant effect on blood pressure and caused smaller increases in renal and mesenteric vascular conductances and a more short-lived tachycardia [$P < 0.05$ for integrated (0–30 min) changes] (Figure 1A; Table 1). In the second experiment (Experiment 1b), the higher dose of AM (3 nmol·kg⁻¹) caused significantly [$P < 0.05$ for integrated (0–30 min) changes] greater hypotension, tachycardia and renal vasodilatation compared to IMD at a dose of 1 nmol·kg⁻¹, but the integrated mesenteric and hindquarters vasodilator effects were not different (Figure 1B; Table 1). In the light of these results and because the effects of AM (1 nmol·kg⁻¹) were not highly reproducible (data not shown), all subsequent experiments used 1 nmol·kg⁻¹ IMD and 3 nmol·kg⁻¹ AM.

**Table 1** Resting cardiovascular variables and integrated responses to IMD and AM (Experiment 1)

| | Experiment 1a | | Experiment 1b | |
| --- | --- | --- | --- | --- |
| | IMD 1 nmol·kg⁻¹ | AM 1 nmol·kg⁻¹ | IMD 1 nmol·kg⁻¹ | AM 3 nmol·kg⁻¹ |
| *Resting cardiovascular variables* | | | | |
| Heart rate (beats·min⁻¹) | 340 ± 8 | 320 ± 7[a] | 339 ± 9 | 344 ± 9 |
| Blood pressure (mmHg) | 112 ± 4 | 107 ± 3 | 108 ± 2 | 108 ± 3 |
| Renal VC (units) | 90 ± 10 | 84 ± 5 | 76 ± 9 | 98 ± 9 |
| Mesenteric VC (units) | 71 ± 5 | 77 ± 9 | 66 ± 4 | 76 ± 9 |
| Hindquarters VC (units) | 37 ± 4 | 37 ± 3 | 49 ± 4 | 48 ± 6 |
| *Integrated (0–30 min) responses* | | | | |
| Heart rate (beats) | +1030 ± 132 | +706 ± 91[a] | +1136 ± 159 | +2697 ± 168[a] |
| Blood pressure (mmHg min) | −195 ± 52 | −45 ± 13[a] | −180 ± 44 | −357 ± 48[a] |
| Renal DS (% min) | +638 ± 142 | +460 ± 58 | +514 ± 81 | +932 ± 126[a] |
| Renal VC (% min) | +828 ± 140 | +467 ± 69[a] | +706 ± 114 | +1443 ± 190[a] |
| Mesenteric DS (% min) | +705 ± 97 | +278 ± 75[a] | +828 ± 90 | +682 ± 121 |
| Mesenteric VC (% min) | +943 ± 101 | +307 ± 86[a] | +1049 ± 124 | +1142 ± 134 |
| Hindquarters DS (% min) | +361 ± 87 | +417 ± 64 | +352 ± 78 | +532 ± 88 |
| Hindquarters VC (% min) | +567 ± 91 | +450 ± 63 | +528 ± 121 | +954 ± 135 |

In the first experiment (a) IMD (1 nmol·kg⁻¹, *n* = 8) or AM (1 nmol·kg⁻¹, *n* = 9) were given. In the second experiment (b) IMD (1 nmol·kg⁻¹, *n* = 11) or AM (3 nmol·kg⁻¹, *n* = 10) were given. Values are mean ± SEM, *n* = 8–11 per group. Units for VC are (kHz·mmHg⁻¹) 10³.
[a]*P* < 0.05 versus corresponding IMD group (Mann–Whitney *U*-test).
AM, adrenomedullin; DS, Doppler shift; IMD, intermedin; VC, vascular conductance.
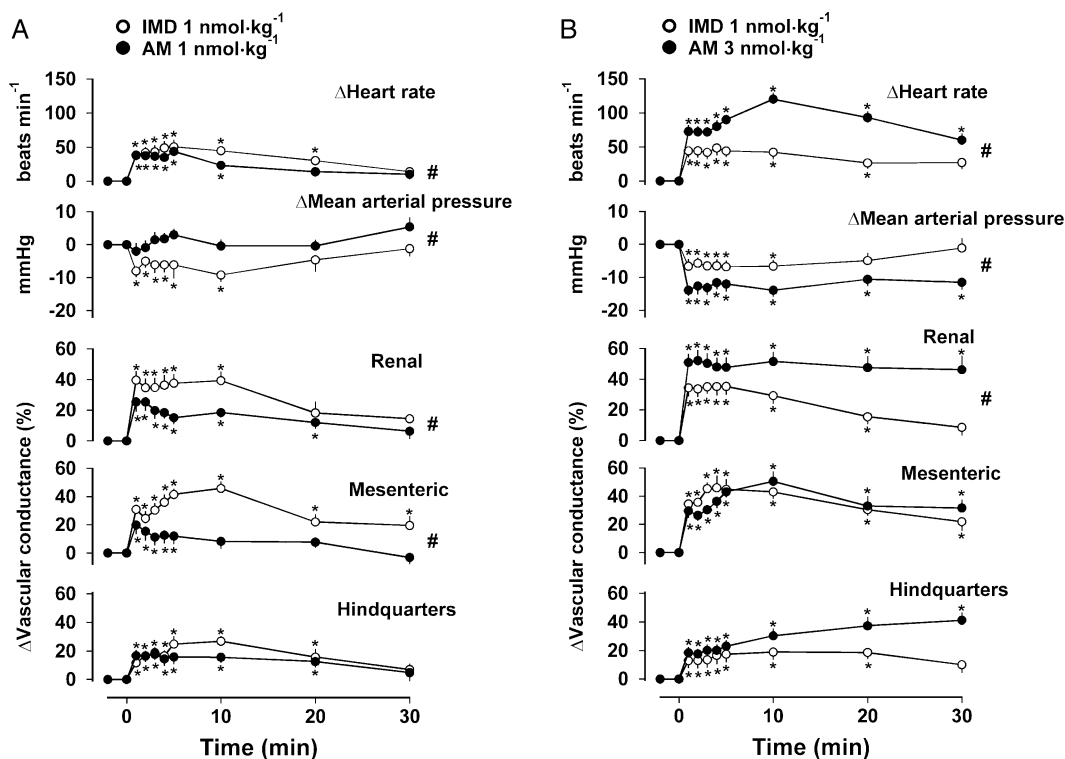


**Figure 1** Cardiovascular responses to IMD [1 nmol·kg⁻¹, *n* = 8 (A) or 11 (B)] and AM [1 nmol·kg⁻¹, *n* = 9 (A) or 3 nmol·kg⁻¹, *n* = 10 (B)] in conscious rats. Values are mean and vertical bars represent SEM. **P* < 0.05 versus baseline (Friedman's test), #*P* < 0.05 for integrated (0–30 min) changes (Mann–Whitney *U*-test). AM, adrenomedullin; IMD, intermedin.

*Experiment 2: Effects of repeated doses of IMD and AM*
Resting cardiovascular variables and the integrated responses to IMD or AM on the two occasions were not different (Table 2).

*Experiment 3: Involvement of AM₂₂–₅₂-sensitive receptors*
Administration of AM₂₂–₅₂ caused a small increase in mesenteric vascular conductance as reported previously (Gardiner *et al.*, 1999), but the effect was not significant, hence resting

**Table 2** Resting cardiovascular variables and integrated responses to repeated doses of IMD and AM (Experiment 2)

| | IMD (1)<br>1 nmol·kg⁻¹ | IMD (2)<br>1 nmol·kg⁻¹ | AM (1)<br>3 nmol·kg⁻¹ | AM(2)<br>3 nmol·kg⁻¹ |
|---|---|---|---|---|
| *Resting cardiovascular variables* | | | | |
| Heart rate (beats·min⁻¹) | $349 \pm 13$ | $354 \pm 15$ | $374 \pm 18$ | $377 \pm 21$ |
| Blood pressure (mmHg) | $111 \pm 3$ | $108 \pm 4$ | $110 \pm 4$ | $117 \pm 4$ |
| Renal VC (units) | $99 \pm 9$ | $89 \pm 8$ | $93 \pm 7$ | $93 \pm 7$ |
| Mesenteric VC (units) | $61 \pm 5$ | $64 \pm 6$ | $71 \pm 8$ | $65 \pm 7$ |
| Hindquarters VC (units) | $49 \pm 5$ | $48 \pm 4$ | $49 \pm 4$ | $46 \pm 3$ |
| *Integrated (0–30 min) responses* | | | | |
| Heart rate (beats) | $+1425 \pm 284$ | $+951 \pm 252$ | $+2315 \pm 242$ | $+2368 \pm 431$ |
| Blood pressure (mmHg min) | $-184 \pm 52$ | $-218 \pm 58$ | $-453 \pm 92$ | $-436 \pm 82$ |
| Renal VC (% min) | $+643 \pm 73$ | $+868 \pm 130$ | $+1328 \pm 290$ | $+1257 \pm 221$ |
| Mesenteric VC (% min) | $+854 \pm 106$ | $+854 \pm 117$ | $+1185 \pm 240$ | $+939 \pm 175$ |
| Hindquarters VC (% min) | $+454 \pm 123$ | $+415 \pm 92$ | $+947 \pm 164$ | $+908 \pm 191$ |

Values are mean $\pm$ SEM, $n = 8$. Units for VC are (kHz·mmHg⁻¹) $10^3$.
AM, adrenomedullin; IMD, intermedin; VC, vascular conductance.

**Table 3** Resting cardiovascular variables prior to administration of IMD or AM in animals pre-treated with saline or AM$_{22-52}$ (Experiment 3)

| | Saline | AM$_{22-52}$ | Saline | AM$_{22-52}$ |
|---|---|---|---|---|
| | IMD (1 nmol·kg⁻¹) | | AM (3 nmol·kg⁻¹) | |
| Heart rate (beats·min⁻¹) | $347 \pm 10$ | $338 \pm 9$ | $382 \pm 14$ | $391 \pm 8$ |
| Blood pressure (mmHg) | $113 \pm 7$ | $114 \pm 3$ | $111 \pm 4$ | $107 \pm 5$ |
| Renal VC (units) | $62 \pm 8$ | $92 \pm 9^a$ | $94 \pm 8$ | $107 \pm 5$ |
| Mesenteric VC (units) | $75 \pm 11$ | $82 \pm 9$ | $69 \pm 11$ | $95 \pm 14$ |
| Hindquarters VC (units) | $43 \pm 8$ | $36 \pm 3$ | $47 \pm 4$ | $46 \pm 4$ |

Values are mean $\pm$ SEM, $n = 6$–8 per group. Units for VC are (kHz·mmHg⁻¹) $10^3$.
[a]$P < 0.05$ versus corresponding saline group (Mann–Whitney *U*-test).
AM, adrenomedullin; IMD, intermedin; VC, vascular conductance.

cardiovascular variables immediately before administration of AM or IMD following treatment with saline or AM$_{22-52}$ were not different, with the exception of renal vascular conductance that was higher prior to IMD in the animals treated with AM$_{22-52}$ (Table 3); this was not an effect of AM$_{22-52}$ inasmuch as the renal vascular conductance was higher in this group prior to AM$_{22-52}$ administration.

In saline-treated animals IMD (1 nmol·kg⁻¹) caused tachycardia, hypotension and increases in renal, mesenteric and hindquarters vascular conductances, as described above. Following treatment with AM$_{22-52}$, IMD-mediated tachycardia, hypotension and hindquarters vasodilatation were unaffected [$P > 0.05$ for integrated (0–30 min) responses] but the renal and mesenteric vasodilatations were significantly attenuated [$P < 0.05$ for integrated (0–30 min) responses] (Figure 2A). In saline-treated animals, AM (3 nmol·kg⁻¹) caused a tachycardia, hypotension and increases in renal, mesenteric and hindquarters vascular conductances as described above. 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 increase in renal vascular conductance [$P < 0.05$ for integrated (0–30 min) change], and the marked AM-mediated increase in mesenteric vascular conductance seen in control conditions was abolished (Figure 2B).

*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).

In animals infused with AII + AVP, IMD caused tachycardia, hypotension and marked renal, mesenteric and hindquarters vasodilatations (Figure 3A). In the presence of L-NAME, the integrated (0–30 min) IMD-induced tachycardia and hypotension were not different from the changes seen in the presence of AII + AVP, whereas the renal vasodilator response was significantly reduced, and there was some attenuation of the IMD-mediated mesenteric vasodilatation, although this failed to reach statistical significance [$P > 0.05$ for integrated (0–30 min) change]. In addition, IMD-mediated hindquarters vasodilatation was abolished (Friedman's test) although the integrated (0–30 min) changes were not different between the L-NAME and AII + AVP infused conditions (Figure 3A).

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 3B). These responses to AM were unaffected by L-NAME [$P > 0.05$ for the integrated (0–30 min) changes for each variable].

*Experiment 5: Involvement of K$_{ATP}$ channels*
Resting cardiovascular variables are shown in Table 5. Administration of U-37883A on both occasions caused a significant ($P < 0.05$) rise in blood pressure and fall in heart rate; these changes were accompanied by falls in vascular conductance ($P < 0.05$ for mesenteric vascular conductance prior to IMD and hindquarters conductance prior to AM).
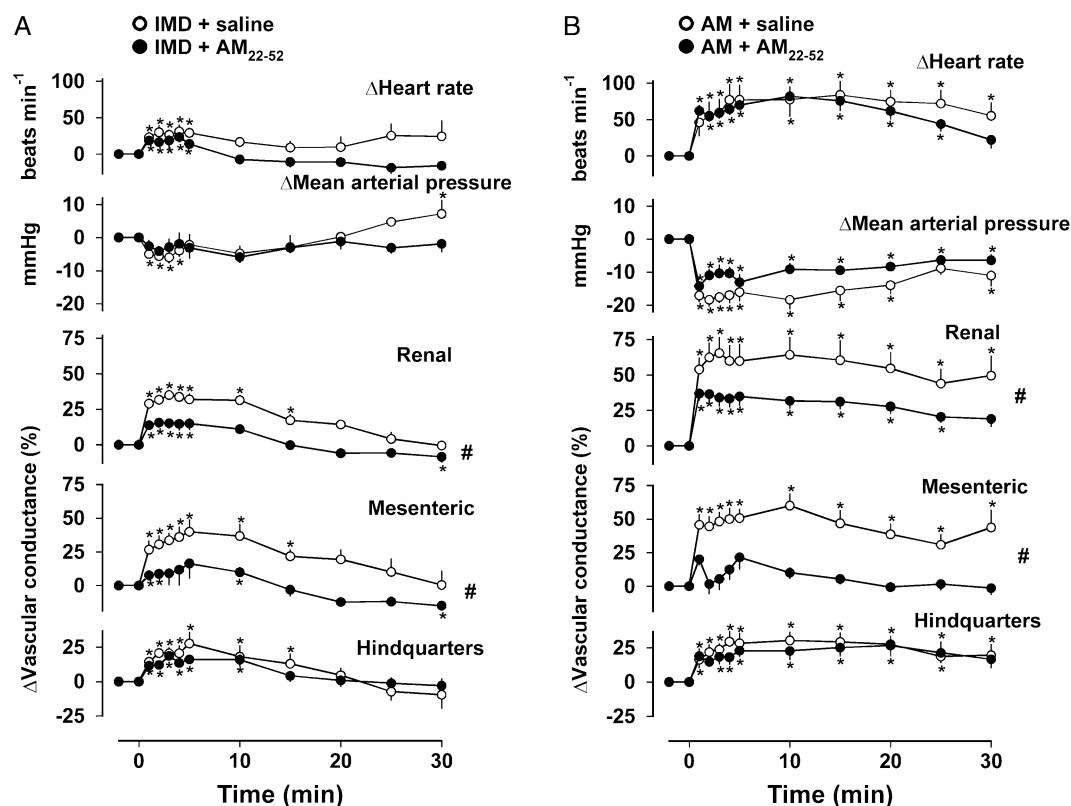
Intermedin and adrenomedullin *in vivo*
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**Figure 2** Cardiovascular responses to (A) IMD (1 nmol·kg$^{-1}$) or (B) AM (3 nmol·kg$^{-1}$) following administration of saline (0.4 mL·h$^{-1}$) or AM$_{22-52}$ (500 nmol·kg$^{-1}$·h$^{-1}$). Values are mean and vertical bars represent SEM, $n = 6–8$ per group. *$P < 0.05$ versus baseline (Friedman's test), #$P < 0.05$ for integrated (0–30 min) changes (Mann–Whitney $U$-test). AM, adrenomedullin; IMD, intermedin.

**Table 4** Resting cardiovascular variables prior to administration of IMD or AM in animals pre-treated with co-infusion of AII+AVP or L-NAME (Experiment 4)

| | AII + AVP | L-NAME | AII + AVP | L-NAME |
|---|---|---|---|---|
| | IMD (1 nmol·kg$^{-1}$) | | AM (3 nmol·kg$^{-1}$) | |
| Heart rate (beats·min$^{-1}$) | 296 ± 12 | 287 ± 9 | 307 ± 13 | 282 ± 9 |
| Blood pressure (mmHg) | 133 ± 5 | 138 ± 6 | 135 ± 5 | 146 ± 8 |
| Renal VC (units) | 57 ± 9 | 57 ± 9 | 56 ± 7 | 53 ± 12 |
| Mesenteric VC (units) | 36 ± 5 | 31 ± 5 | 31 ± 3 | 28 ± 5 |
| Hindquarters VC (units) | 35 ± 5 | 34 ± 2 | 30 ± 3 | 30 ± 2 |

Values are mean ± SEM, $n = 9$. Units for VC are (kHz·mmHg$^{-1}$) 10$^3$.
AII, angiotensin II; AM, adrenomedullin; IMD, intermedin; VC, vascular conductance.

The control response to IMD comprised tachycardia, hypotension and renal, mesenteric and hindquarters vasodilatations, as described above, and these responses were unaffected by pre-treatment with U-37883A [$P > 0.05$ for the integrated (0–30 min) changes] (Figure 4A). Administration of AM under control conditions caused tachycardia, hypotension and increases in renal, mesenteric and hindquarters vascular conductances, as described above. Following treatment with U-37883A, there was no change in AM-mediated tachycardia, hypotension and hindquarters vasodilatation. However, contrary to expectation, pre-treatment with U-37883A 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 4B).

*Experiment 6: Effects of angiotensin (AT$_1$) receptor antagonism*
Pre-treatment with losartan had no substantial effects on resting haemodynamics although there was a slight reduction in blood pressure and increase in hindquarters vascular conductance ($P < 0.05$ prior to administration of IMD only) (Table 6).

In the absence of losartan, cardiovascular responses to IMD and AM were as described above although on this occasion, 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 5A), whereas AM-mediated hypotension and renal and mesenteric vasodilatations were significantly augmented [$P < 0.05$ for integrated (0–30 min) changes] (Figure 5B).
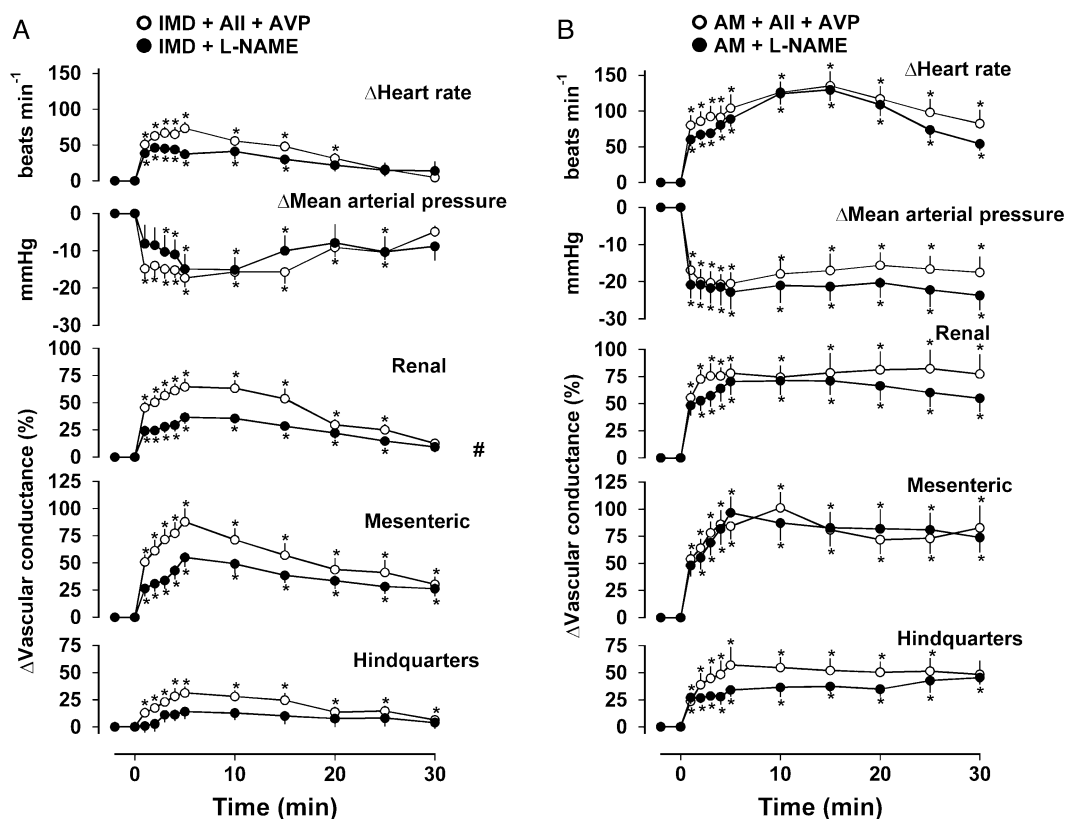
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**Intermedin and adrenomedullin *in vivo***
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**Figure 3** Cardiovascular responses to (A) IMD (1 nmol·kg⁻¹) or (B) AM (3 nmol·kg⁻¹) in the presence of angiotensin II (AII, 200 ng·kg⁻¹·h⁻¹) + AVP (20 ng·kg⁻¹·h⁻¹) or L-NAME (3 mg·kg⁻¹·h⁻¹). Values are mean and vertical bars represent SEM, $n = 8$ *$P < 0.05$ versus baseline (Friedman's test), #$P < 0.05$ for integrated (0–30 min) changes (Wilcoxon's test). AM, adrenomedullin; AVP, arginine vasopressin; IMD, intermedin.

**Table 5** Resting cardiovascular variables prior to administration of IMD or AM in animals pre-treated with saline or U-37883A (Experiment 5)

| | *Saline* | *U-37883A* | *Saline* | *U-37883A* |
|---|---|---|---|---|
| | *IMD (1 nmol·kg⁻¹)* | | *AM (3 nmol·kg⁻¹)* | |
| Heart rate (beats·min⁻¹) | 353 ± 12 | 315 ± 10[a] | 359 ± 14 | 294 ± 8[a] |
| Blood pressure (mmHg) | 113 ± 3 | 129 ± 3[a] | 115 ± 3 | 127 ± 2[a] |
| Renal VC (units) | 86 ± 9 | 76 ± 8 | 81 ± 9 | 73 ± 7 |
| Mesenteric VC (units) | 70 ± 4 | 57 ± 5[a] | 68 ± 4 | 60 ± 6 |
| Hindquarters VC (units) | 45 ± 4 | 37 ± 3 | 50 ± 4 | 35 ± 2[a] |

Values are mean ± SEM, $n = 9$. Units for VC are (kHz·mmHg⁻¹) 10³.
[a]$P < 0.05$ versus corresponding saline group (Wilcoxon's test).
AM, adrenomedullin; IMD, intermedin; VC, vascular conductance.

## Discussion

IMD (also known as AM2) is a newly identified member of the calcitonin family of peptides which shares some structural, sequence, and functional homology with AM (Roh *et al.*, 2004; Takei *et al.*, 2004). AM is a potent vasoactive peptide which causes relaxation of vascular smooth muscle *in vitro*, and vasodilatation and hypotension *in vivo*. The vasorelaxant effects of AM occur through increases in CL/RAMP receptor complex-mediated cAMP production, and may, to some extent, involve release of NO and activation of K$_{ATP}$ channels (for review see Brain and Grant, 2004).

The aims of the present studies were to measure the regional haemodynamic effects of IMD and AM in conscious animals, and to compare possible mechanisms involved in the effects observed. The main findings were that, like AM, IMD causes widespread vasodilatation with a clear role for AM$_{22-52}$-sensitive receptors in the renal and mesenteric, but not the hindquarters vasodilator responses, and no evidence for an involvement of K$_{ATP}$ channels in the vasodilator responses. However, there was a significant role for NO in the renal vasodilator effects of IMD but not those of AM. Furthermore, there was an unexpected enhancement of the renal and mesenteric vasodilator actions of AM in the presence of a K$_{ATP}$ channel blocker which could be explained by an inhibitory effect of the latter on AM-mediated renin release (see below).

To our knowledge, only one study prior to this one has described the regional haemodynamic effects of IMD
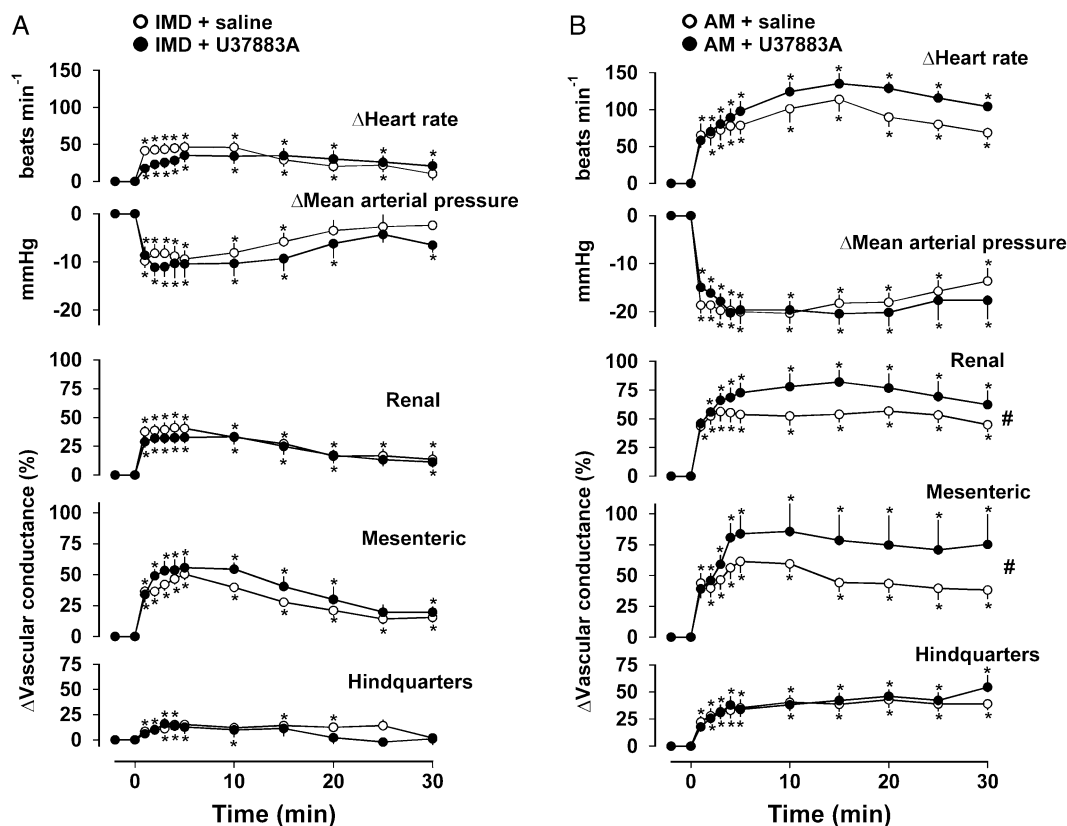
**Figure 4** Cardiovascular responses to (A) IMD (1 nmol·kg⁻¹) or (B) AM (3 nmol·kg⁻¹) following administration of saline (1 mL infused over 30 min) or U-37883A (5 mg·kg⁻¹). Values are mean and vertical bars represent SEM, $n = 9$ *$P < 0.05$ versus baseline (Friedman's test), #$P < 0.05$ for integrated (0–30 min) changes (Wilcoxon's test). AM, adrenomedullin; IMD, intermedin.

**Table 6** Resting cardiovascular variables prior to administration of IMD or AM in animals pre-treated with saline or losartan (Experiment 6)

| | Saline | losartan | Saline | losartan |
|---|---|---|---|---|
| | IMD (1 nmol·kg⁻¹) | | AM (3 nmol·kg⁻¹) | |
| Heart rate (beats·min⁻¹) | 336 ± 9 | 355 ± 7 | 355 ± 13 | 369 ± 17 |
| Blood pressure (mmHg) | 111 ± 2 | 98 ± 4[a] | 111 ± 2 | 104 ± 4 |
| Renal VC (units) | 90 ± 10 | 105 ± 12 | 93 ± 12 | 81 ± 14 |
| Mesenteric VC (units) | 74 ± 4 | 89 ± 9 | 77 ± 7 | 83 ± 7 |
| Hindquarters VC (units) | 37 ± 4 | 50 ± 5[a] | 42 ± 5 | 47 ± 5 |

Values are mean ± SEM, $n = 9$. Units for VC are (kHz·mmHg⁻¹) 10³.
[a]$P < 0.05$ versus corresponding saline group (Wilcoxon's test).
AM, adrenomedullin; IMD, intermedin; VC, vascular conductance.

(Fujisawa *et al.*, 2007). Although, because of the nature of the measurements (i.e. radiolabelled microspheres), that study covered more vascular beds than studied here, and was restricted to one point in time during an IMD infusion. Our experiments delineated the temporal profile of response to bolus injections and showed that IMD, like AM, caused widespread vasodilatation with a rank order of potency of mesenteric ≥ renal > hindquarters. In contrast, the previous study found renal vasodilatation to be more prominent than mesenteric vasodilatation, the latter not being accompanied by an increase in flow (Fujisawa *et al.*, 2007). Differences between the experiments which could explain the disparate findings include the strain of rat, the form of peptide (see

below), and perhaps more importantly, the mode of peptide administration (i.e. bolus administration vs. continuous infusion).

In the first experiments we compared equimolar doses of IMD and AM and found the former to be more potent than the latter. Other studies comparing the potency of these peptides have produced variable results. Thus, Roh *et al.* (2004) described the blood pressure lowering effects of IMD and AM as similar, although the effect of IMD appeared less marked, consistent with the results of Taylor *et al.* (2005). In contrast, Takei *et al.* (2004) and Ren *et al.* (2006) showed more potent hypotensive effects of IMD than AM. Fujisawa *et al.* (2006) found similar initial hypotensive effects of AM and IMD but
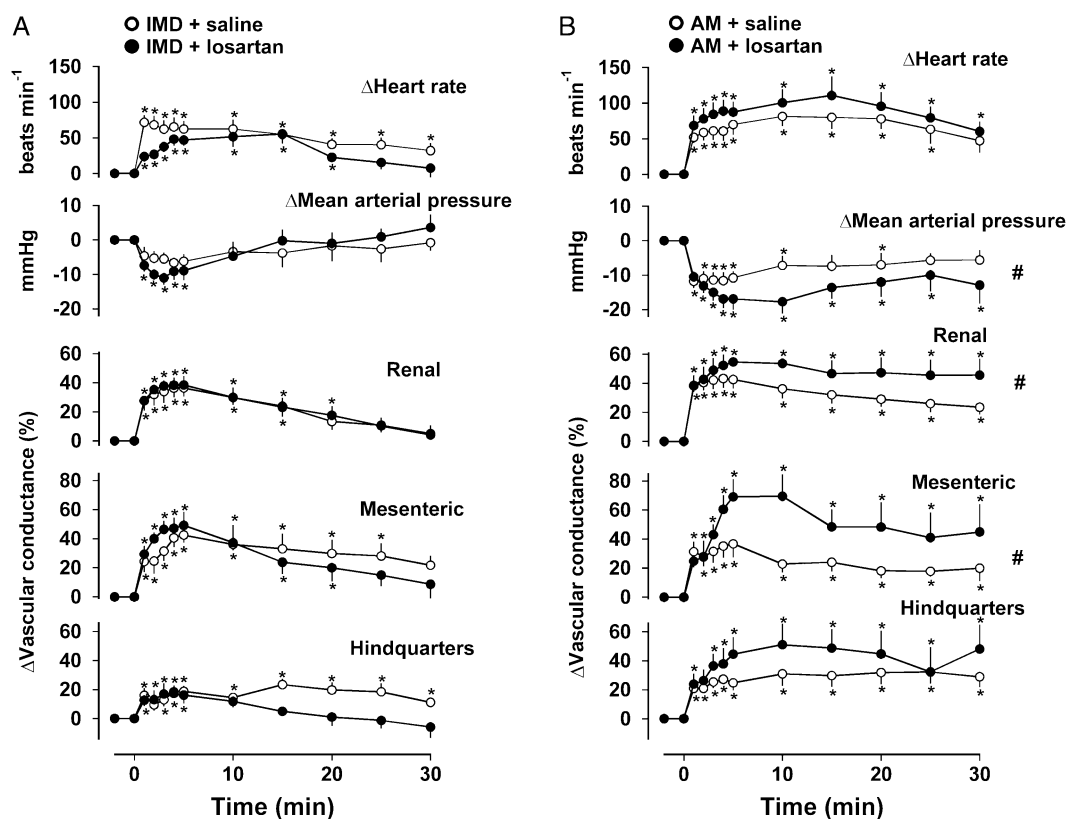
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**Intermedin and adrenomedullin *in vivo***
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**Figure 5** Cardiovascular responses to (A) IMD (1 nmol·kg$^{-1}$) or (B) AM (3 nmol·kg$^{-1}$) following administration of saline (0.1 mL) or losartan (10 mg·kg$^{-1}$). Values are mean and vertical bars represent SEM, $n = 9$ *$P < 0.05$ versus baseline (Friedman's test), #$P < 0.05$ for integrated (0–30 min) changes (Wilcoxon's test). AM, adrenomedullin; IMD, intermedin.

the effects of AM were more long lasting. It is possible that some of these apparent differences could be due to the form of the peptides used. In the original description (Roh *et al.*, 2004), the mature peptides identified were the 47 amino acid prepro-IMD$_{101-147}$ (IMD$_{1-47}$, also known as IMD long) and the 40 amino acid prepro-IMD$_{108-147}$ (IMD$_{8-47}$, also known as IMD short). However, subsequently (Yang *et al.*, 2005), another fragment was identified, that is prepro-IMD$_{95-147}$ (IMD$_{1-53}$). Most of the studies described above used IMD$_{1-47}$ (where specified), whereas our studies used IMD$_{1-53}$ since that was the only commercially available form at the time our work was initiated. However, Ren *et al.* (2006) compared the hypotensive effects of IMD$_{1-47}$ with those of IMD$_{1-53}$ and AM, and found the rank order to be IMD$_{1-47}$ > IMD$_{1-53}$ = AM. In contrast, we did not find the hypotensive action of IMD$_{1-53}$ to be equipotent to that of AM although we compared 1 nmol·kg$^{-1}$ doses of the peptides, whereas the previous study compared 3 nmol·kg$^{-1}$ doses (Ren *et al.*, 2006). In the light of the results of these first experiments, for the mechanistic studies, we chose to compare a 1 nmol·kg$^{-1}$ dose of IMD with 3 nmol·kg$^{-1}$ AM. These doses delivered into a plasma volume of ~0.4 mL·kg$^{-1}$ would give a theoretical peak plasma concentration of about 25 or 75 pmol·mL$^{-1}$ (not allowing for binding or clearance), which are clearly higher than the normal circulating levels of IMD or AM (~35 and 20 fmol·mL$^{-1}$, respectively) (Bell and McDermott, 2008). Although the initial mesenteric and hindquarters vasodilator effects of the chosen doses of IMD and AM were matched, the renal vasodilator and

hypotensive actions were greater for AM. This unavoidable mismatching is clearly a limitation to the study.

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 possibly also activation of an unidentified IMD receptor (Roh *et al.*, 2004; Takei *et al.*, 2004; Owji *et al.*, 2008). In isolated cell preparations, IMD appeared to show greater activity at CL/RAMP1 and CL/RAMP$_3$ complexes than at CL/RAMP2, perhaps consistent with a more effective antagonism of the hypotensive effects with CGRP$_{8-37}$ (which acts at CL/RAMP1) than AM$_{22-52}$ (which acts at CL/RAMP2) (Roh *et al.*, 2004). Furthermore, *in vitro*, coronary vasodilator responses to IMD have been shown to be inhibited by CGRP$_{8-37}$ but not by AM$_{22-52}$ (Kobayashi *et al.*, 2004). However, here we found evidence of AM$_{22-52}$-sensitive vasodilator effects of IMD in the renal and mesenteric vascular beds, but not in the hindquarters. This pattern is consistent with that observed for AM following pre-treatment with AM$_{22-52}$, and suggests that IMD signals, at least in part, through AM receptors in those regions. Although AM$_{22-52}$ attenuated renal and mesenteric vasodilatations, the effect was not sufficient to inhibit the accompanying fall in blood pressure, possibly because the expected increase in cardiac output was less, due to the smaller fall in afterload and/or a change in the effects on preload (Abdelrahman and Pang, 2006). Thus, the previous studies showing minimal effects of AM$_{22-52}$ on the hypotensive effects of IMD (Roh *et al.*, 2004) are not inconsistent with ours, although our interpretation is

different. Interestingly, in pilot studies, we found no effect of $CGRP_{8-37}$ on the regional vasodilator response to IMD (data not shown), in line with our earlier findings with AM (Gardiner *et al.*, 1995). The lack of effect of $AM_{22-52}$ on the hindquarters vasodilator response to AM is consistent with results of others in the cat hindlimb where AM-induced vasodilatation was resistant to blockade with either $AM_{22-52}$ or $CGRP_{8-37}$ (Champion *et al.*, 1997).

The study of the CL/RAMP receptor system is complicated by a lack of selective antagonists, and although $AM_{22-52}$ is acknowledged to be weak and lacking in specificity, it is the only available tool. Nonetheless, our findings clearly show that both AM- and IMD-mediated renal and mesenteric vasodilatations were attenuated by $AM_{22-52}$, suggesting $AM_1$ and/or $AM_2$ receptors are activated by both peptides in a regionally selective manner.

*In vitro* studies have shown up-regulation of NO synthesis by IMD (Yang *et al.*, 2006) and some degree of attenuation of IMD-induced relaxation by NO synthase inhibition (Kandilci *et al.*, 2006; Chauhan *et al.*, 2007), but *in vivo*, the depressor response to IMD is reportedly unaffected by NO synthase inhibition (Taylor *et al.*, 2005; Abdelrahman and Pang, 2007), although the accompanying regional vascular effects have not previously been evaluated. We therefore assessed the effects of NO synthase inhibition with L-NAME on regional haemodynamic responses to IMD and AM, under conditions where controls were made for the baseline cardiovascular effects of L-NAME. Those experiments showed a tendency for all vasodilator responses to IMD to be reduced by L-NAME, although this was only significant in the renal vascular bed. While it might be expected that the reduced vasodilatations would have been manifested 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. Our findings, therefore, agree with those previously published showing no effect of NO synthase inhibition on IMD-mediated hypotension (Taylor *et al.*, 2005; Abdelrahman and Pang, 2007), but because of the additional measurements we made, our interpretation is different, and we can conclude that there is an involvement of NO in IMD-mediated vasodilatation, particularly in the renal vascular bed.

No significant role for NO in AM-mediated vascular responses was found in the present study which is at odds with our previous studies (Gardiner *et al.*, 1995), where we found AM-mediated hindquarters vasodilatation to be attenuated by L-NAME in conscious rats. In the present study, AM-mediated hindquarters vasodilatation was reduced in the presence of L-NAME, but this was not significantly different from control conditions. However, our earlier study used a lower dose of AM (1 nmol·kg⁻¹) and compared integrated responses over 15 min rather then the 30 min compared here, so it is possible that the hindquarters response to AM in the presence of L-NAME would have been significantly less in the present study if responses had been compared over 15 min (Figure 3B). Nonetheless, it appears from our results that the involvement of NO may be greater for IMD than AM, although it is possible that this apparent difference was due to the differential hypotensive effects of the peptides.

There is some evidence that the cardiovascular responses to AM are sensitive to glibenclamide and U-37883A, suggesting a role for $K_{ATP}$ channels (Sabates *et al.*, 1997; Goto *et al.*, 2000), although other studies have found $K_{ATP}$ channel inhibition to have no effect on the vascular responses to AM (Miura *et al.*, 1995; Champion *et al.*, 1997; Terata *et al.*, 2000). *In vitro* studies have failed to show an effect of glibenclamide on the vasorelaxant response to IMD (Kandilci *et al.*, 2006; Chauhan *et al.*, 2007), but to our knowledge there have been no *in vivo* studies on the possible role for vascular $K_{ATP}$ channels in the vasodilator effects of IMD. Blocking $K_{ATP}$ channels with U-37883A would be expected to attenuate peptide-mediated vasodilatation in areas where those channels were involved. In the present study there was no attenuation of either AM or IMD-mediated responses by U-37883A, consistent with the suggestion that $K_{ATP}$ channels are not involved in the vasodilator responses to either peptide. However, contrary to expectation, when AM was administered following treatment with U37883A, there was a significant *augmentation* of AM-mediated renal and mesenteric vasodilator responses. As administration of U-37883A caused an increase in blood pressure and vasoconstriction, it could be that the augmented vasodilatation was due to animals being in a pre-constricted state. However, this is an unlikely explanation, firstly because the augmentation was still 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 vascular bed that was significantly constricted by U-37883A (i.e. the hindquarters). Thirdly, IMD-mediated vasodilatation was not augmented by U-37883A 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 causes reflex activation of this system, but there is evidence that AM also has a direct stimulatory effect on renin release from renal juxtaglomerular cells (Jensen *et al.*, 1997). No such evidence exists for a similar effect of IMD although IMD has been shown to increase aldosterone levels secondary to an increase in plasma renin activity (Charles *et al.*, 2006), but it is not yet known whether this is a direct action of IMD, or secondary to its hypotensive effect. As U-37883A has been shown to inhibit renin release (Humphrey and Ludens, 1998; Vallon *et al.*, 1998), we hypothesized that U-37883A might be inhibiting AM-induced renin release. Thus, while it has been suggested that AM might be a functional antagonist of AII (Charles *et al.*, 2000), it was feasible that the observed augmentation of AM-mediated renal and mesenteric vasodilatations seen following treatment with U-37883A, was due to a decrease in renin activity and the consequent diminution of the vasoconstrictor effects of AII. To test this hypothesis, a separate group of animals was pre-treated with the $AT_1$ receptor antagonist, losartan, and subsequently given AM and IMD. Interestingly, losartan pre-treatment had no effect on the vascular responses to IMD, but significantly augmented AM-mediated hypotension and renal and mesenteric vasodilatation. These data suggest that the hypotensive and vasodilator effects of AM are attenuated by its renin-stimulatory action, and when $AT_1$ receptors are blocked, AM-mediated vasodilatations are significantly augmented in regions where there is high sensitivity to the vasoconstrictor effects of AII. Thus, the enhanced vasodilator effects of AM in the presence

of U-37883A could be due to the latter's ability to decrease renin secretion (Humphrey and Ludens, 1998; Vallon *et al.*, 1998), offsetting AM-mediated increase in renin secretion. The results of the present study do not support a similar role of the renin-angiotensin system in IMD action, although it is possible that the modest hypotensive response to IMD in this study was insufficient to trigger reflex renin release.

In conclusion, it appears that IMD and AM are similar in causing renal and mesenteric vasodilatations which are sensitive to AM$_{22-52}$, suggesting that AM$_1$ and/or AM$_2$ receptors are activated by both peptides in a regionally selective manner, although a fuller analysis of the receptor types must await the availability of more selective antagonists. While it appears that K$_{ATP}$ channels do not contribute to the vasodilator action of either IMD or AM, an involvement of NO is more apparent in the vasodilator actions of IMD than AM, at least at the doses used here. Moreover, the peptides also differ in that U-37883A enhances the renal and mesenteric vasodilator responses to AM, but not those to IMD, possibly due to an involvement of the renin-angiotensin system in the haemodynamic effects of the former but not the latter. The mechanisms involved in the vasodilator effects of AM and IMD which were not sensitive to AM$_{22-52}$, and not mediated by NO or K$_{ATP}$ channels, are unknown. In cats, Champion *et al.* (1997) showed hindlimb vasodilator responses to AM which involved activation of cAMP, but were resistant to antagonism by AM$_{22-52}$, CGRP$_{8-37}$, NO synthase inhibition and K$_{ATP}$ channel blockade. Whether or not these vasodilatations involve CL/RAMP receptor complexes that are not effectively antagonized by the available tools, or in the case of IMD, the recently described distinct class of receptor (Owji *et al.*, 2008), remains to be determined.

## Acknowledgement

## Conflict of interest

None.

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