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**A comparison between the cardiovascular actions of
urocortin 1 and urocortin 2 (stresscopin related peptide) in
conscious rats.**

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Running title: Cardiovascular effects of urocortins

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Abstract

The aims of the study were, in conscious Sprague-Dawley rats, i) to compare the effects of stresscopin-related peptide (SRP) and urocortin 1 (UCN1) on blood pressure, heart rate and regional hemodynamics, ii) to determine whether or not there were residual tachycardic effects of SRP or UCN1 after cardiac autonomic blockade and iii) to investigate a possible involvement of corticotropin releasing factor type 1 (CRF₁) receptor-mediated histamine release in the vasodilator actions of UCN1. SRP and UCN1 (both at 3nmol kg⁻¹ i.v.) caused hypotension, tachycardia and mesenteric and hindquarters vasodilatation, but the magnitude and/or duration of the effects of UCN1 were generally greater than those of SRP. Pre-treatment with atropine plus propranolol abolished the tachycardic effects of SRP and UCN1, and, under those conditions, the hypotensive effect of SRP, but not that of UCN1, was enhanced, probably because the hindquarters vasodilator effect of the latter was also reduced. Pre-treatment with mepyramine plus cimetidine had no effect on the hemodynamic actions of either SRP or UCN1. It is concluded that, in conscious rats, the tachycardic effects of SRP and UCN1 are autonomically-mediated and likely to be largely reflex in origin. There is no evidence for an involvement of CRF₁ receptor-mediated histamine release in the vasodilator actions of UCN1, but a propranolol-sensitive hindquarters vasodilator action of UCN, but not of SRP, was identified.

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Four members of the corticotropin-releasing factor (CRF) family of peptides have been identified, namely CRF, urocortin (UCN) 1, UCN2 (analogous to stresscopin-related peptide (SRP)) and UCN3 (analogous to stresscopin). In addition, two receptor types have been cloned, namely, CRF₁ and CRF₂. The CRF₁ receptor is expressed largely, but not exclusively, in the central nervous system, whereas CRF₂ receptor expression is mainly localised to peripheral tissues (for review see Dautzenberg and Hauger, 2002). The binding affinities of the CRF peptides for the different receptors vary, with CRF showing more affinity for CRF₁ than CRF₂ receptors, UCN1 showing equal affinity at CRF₁ and CRF₂ receptors, and UCN2 and UCN3 showing almost exclusive binding to CRF₂ receptors (for review see Dautzenberg and Hauger, 2002).

In the original description of the identification of UCN1 (Vaughan et al., 1995), hypotensive and tachycardic effects were described in conscious rats. The hypotension has since been attributed to vasodilatation, and the tachycardia has been suggested to be a reflex response to the hypotension, because it was absent in thiobutabarbital-anesthetised rats (Abdelrahman and Pang, 2003). However, this suggestion is not consistent with the finding that, in sheep, the tachycardic effects of UCN1 are unaffected by ganglion blockade (reviewed in Parkes et al., 2001). Hypotensive (Chen et al., 2003; Mackay et al., 2003) and vasodilator (Gardiner et al., 2005) effects of UCN2/SRP have also been described in conscious rats, and shown to be mediated by CRF₂ receptors. In the latter study it was noted that there was dissociation between the extent of the hypotension and the degree of tachycardia. Specifically, CRF had modest hypotensive effects relative to an equimolar dose of UCN2, but caused the same degree of tachycardia, suggesting that the tachycardia

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may not be entirely reflex in origin. Others have reported cardiac effects of UCN1 (Terui et al., 2001) and UCN2 (Bale et al., 2004) which were insensitive to β -adrenoceptor antagonism. Thus, the first aim of the present study was to compare the integrated cardiovascular effects of UCN1 and SRP in the absence and presence of cardiac autonomic blockade, with atropine and propranolol, to determine whether or not there were any tachycardic effects independent of the autonomic nervous system.

The similarity between the cardiovascular actions of UCN1 and UCN2, together with the peripheral localisation of CRF₂ receptors, and the effectiveness of selective CRF₂ receptor antagonism, has led to the conclusion that the cardiovascular actions of all peripherally-administered UCN peptides are likely to be CRF₂ receptor-mediated (reviewed by Hashimoto et al., 2004). However, there is evidence to suggest that activation of CRF₁ receptors causes degranulation of mast cells (reviewed by Theoharides et al., 2004), and that the resulting release of histamine contributes to the vasodilator effects of CRF in human skin (Crompton et al., 2003). Thus, it is feasible that CRF₁ receptor-mediated histamine release may contribute to the cardiovascular actions of UCN1, but not UCN2, since the latter has no affinity for CRF₁ receptors (see above). This was the second hypothesis tested in the current study, in which we assessed the effects of histamine (H₁ and H₂) receptor antagonism on the cardiovascular actions of UCN1 and SRP.

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Methods

Animals and surgery

Prior to any surgical intervention, male, Sprague-Dawley rats (350-400g, Charles River, Margate, Kent, U.K) were 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 $21 \pm 2^\circ\text{C}$, and there was a 12h light/dark cycle (06.00h to 18.00h).

Surgery was performed in two stages under general anesthesia (fentanyl and medetomidine, $300\mu\text{g kg}^{-1}$ of each i.p.). Anesthetic reversal and the provision of analgesia was achieved using atipamezole and buprenorphine (0.5 mg kg^{-1} and 0.05 mg kg^{-1} , respectively, s.c.). Firstly, 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). Secondly, 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. The surgical stages were separated by at least 10 days, and between stages, the fitness of the animals was certified by the named Veterinary Surgeon. The procedures were approved by the University of Nottingham Ethical Review Committee and were performed under Home Office Project and Personal Licence authority.

After catheterisation, the animals were fitted with custom-designed harnesses with a counter-balanced spring attached, to protect the catheters and allow the animals

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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 i.a. infusion of heparinised (15 U ml^{-1} , 0.4 ml h^{-1}) saline to maintain catheter patency.

Data acquisition and analysis

Experiments began 24 h after catheterization. On each experimental day, continuous recordings of cardiovascular variables (heart rate, arterial blood pressure, renal, mesenteric and hindquarters Doppler shifts (flow)), were made using a customized, computer-based system (Hemodynamics 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 2ms, averaged each cardiac cycle and stored to disc every 5s, throughout the experimental period.

Data were analysed off-line (Datview, University of Maastricht, The Netherlands). Measurements were made under resting conditions and at intervals up to 240 min after peptide administration. Data are expressed as mean \pm S.E.M. Within-group analyses were carried out by a non-parametric equivalent of ANOVA allowing for multiple comparisons (Friedman's test), (Theodorsson-Norheim, 1987). Between-group analyses were performed on the maximal changes and the times at which these occurred (calculated for each animal), and on the integrated responses (areas under or over curves) using Wilcoxon's test (paired), or Mann-Whitney U test (unpaired), as appropriate. $P \leq 0.05$ was taken as significant.

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Experiment 1. Regional hemodynamic effects of UCN1 and SRP in the absence and presence of atropine and propranolol

Two groups of rats were used (n=8 in each), one group receiving UCN1 and SRP in the presence of saline, and the other group receiving UCN1 and SRP in the presence of atropine and propranolol. On Day 1, either UCN1 or SRP was administered at a dose of 3 nmol kg⁻¹ i.v., 90 min after the onset of administration of either saline (0.1ml bolus, 0.4 ml h⁻¹ infusion) or a combination of propranolol (1 mg kg⁻¹ bolus, 0.5 mg kg⁻¹ h⁻¹ infusion) plus atropine (1 mg kg⁻¹ bolus, 1 mg kg⁻¹ h⁻¹ infusion). As a time control, on Day 2, saline (0.1ml) was administered 90 min after the start of saline (as above) or atropine plus propranolol (as above). On Day 3, the animals that had received UCN1 on day 1 were given SRP and vice versa, in the presence of either saline or atropine plus propranolol.

In an additional group of animals (n=7) the effects of atropine alone on responses to UCN1 and SRP were examined, in a protocol which involved administration of UCN1 or SRP in the presence of saline (as above) on Days 1 (n=3 UCN, n=4 SRP) and 2 (n=4 UCN, n=3 SRP), and in the presence of atropine (as above) on Days 3 and 4.

Experiment 2. Regional hemodynamic effects of UCN1 and SRP in the absence and presence of mepyramine and cimetidine

Rats were given 3 nmol kg⁻¹ UCN1 (n=6) or SRP (n=5) after administration of saline on Day 1, and the same peptide was re-administered on Day 3, 15 min after the end of administration (1ml infused i.v. over 30 min) of a combination of i.v. mepyramine (3 mg kg⁻¹) and cimetidine (30mg kg⁻¹) (Vleeming et al., 2000). No substances were administered on Day 2.

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Experiment 3. Effects of antisauvagine-30 on the hemodynamic responses to UCN1

In the light of the findings in the initial experiments (see Results), an additional experiment was performed (n=6) in which the effects of UCN 1 (3nmol kg⁻¹) were tested 30min after the onset of a primed infusion of the CRF₂ receptor antagonist, antisauvagine-30 (50µg kg⁻¹ bolus, 50 µg kg⁻¹ h⁻¹ infusion; Gardiner et al., 2005).

Peptides and Drugs

Urocortin 1 (human) was from Bachem (St Helens, UK) and SRP (human) was from the Peptide Institute Inc (Scientific Marketing Associates, Barnet, UK). Stock solutions of peptides were made up in sterile water for injection, and diluted in sterile saline. Injection volumes were 0.1ml and infusion rates were 0.4ml h⁻¹ except in the case of mepyramine/cimetidine which was given as a mixture at a rate of 2ml h⁻¹ for 30 min. Atropine methyl nitrate, propranolol hydrochloride, mepyramine maleate and cimetidine were from Sigma Chemical (Poole, Dorset, UK)

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. Regional hemodynamic effects of UCN1 and SRP in the absence and presence of atropine and propranolol

In rats receiving saline infusion, resting cardiovascular variables immediately before administration of UCN1 and SRP were not different (Table 1). Bolus injection of SRP (3 nmol kg⁻¹) caused tachycardia, hypotension, and marked vasodilatation in the mesenteric and hindquarters vascular beds, with little or no change in the renal vascular bed (Figure 1a), as described previously (Gardiner et al., 2005). The effects of UCN1 (3 nmol kg⁻¹) were qualitatively similar to those of SRP, but there were some differences in the time to peak, maximum change and duration of action (Figure 1b). Thus, for heart rate and blood pressure, the maximum effects of SRP (+166 ± 9 beats min⁻¹, -30 ± 2 mmHg,) occurred at 6.2 ± 1.2 min and 9.6 ± 0.6 min respectively, whereas for UCN1, the maximum changes (+170 ± 6 beats min⁻¹, -32 ± 3 mmHg) were similar, but occurred significantly (P<0.001, Wilcoxon's test) later (at 14 ± 1.1 min and 29 ± 2.3 min, respectively), and lasted longer (compare Figures 1a & b). The maximum increase in hindquarters vascular conductance in response to SRP also occurred significantly (P<0.001) sooner (at 17 ± 2.7 min) than the corresponding response to UCN1 (at 102 ± 15.3 min), but, in addition, the magnitude of change was greater for UCN1 (+160 ± 9%) than for SRP (+95 ± 9%; P<0.001), and the latter was more long-lasting (compare Figures 1a & b) In contrast, the maximum increase in mesenteric vascular conductance following SRP (+116 ± 9% at 4.1 ± 0.6 min) occurred at the same time and was of similar magnitude to the response to UCN1 (+89 ± 7% at 4.9 ± 0.9 min), although the duration of action of UCN1 on mesenteric vascular conductance was greater than SRP (compare Figures 1a & b). As a result of these differences, the integrated (0-240 min) increase in heart rate (+17454

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± 1871 beats), fall in blood pressure (-6106 ± 398 mmHg min) and increase in mesenteric vascular conductance ($+10258 \pm 1421$ % min) and hindquarters vascular conductance ($+27240 \pm 1576$ % min) in response to UCN1 were significantly (Wilcoxon's test) greater than the corresponding changes following SRP ($+7134 \pm 618$ beats; -3196 ± 276 mmHg min; $+6058 \pm 1615$ % min; $+10953 \pm 1093$ % min). In the renal vascular bed, UCN1 caused an initial small vasodilatation followed by vasoconstriction (Figure 1b).

In rats receiving atropine plus propranolol, cardiovascular variables immediately prior to administration of UCN1 or SRP were not different, and did not differ from baseline variables in the saline-infused rats (Table 1). In the presence of atropine plus propranolol, the tachycardic response to SRP was virtually abolished, the mesenteric and hindquarters vasodilatations were unaffected, but the integrated (0-240 min) fall in blood pressure (-4167 ± 453 mmHg min) was significantly greater than in the presence of saline (Figure 1a). In contrast, although the tachycardic response to UCN1 was virtually abolished by atropine and propranolol, the integrated fall in blood pressure was unaffected, possibly because the integrated increase in hindquarters vascular conductance ($+15991 \pm 2521$ % min) was significantly reduced (Figure 1b).

In rats receiving atropine, prior to administration of SRP or UCN1, resting heart rate was significantly elevated (369 ± 8 beats min^{-1} and 363 ± 11 and beats min^{-1} , respectively) compared to the corresponding values in the presence of saline (339 ± 7 beats min^{-1} and 325 ± 8 beats min^{-1}), but all other cardiovascular variables were not different (data not shown). In the presence of atropine, the tachycardic responses to SRP ($+46 \pm 9$ beats min^{-1} at 30 min) and UCN1 ($+97 \pm 10$ beats min^{-1} at 30 min) were

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reduced by about 50% ($P < 0.05$) compared to the corresponding changes in the presence of saline ($+71 \pm 10$ beats min^{-1} and $+135 \pm 23$ beats min^{-1} , respectively), but the hypotensive and regional vascular changes were not different (data not shown).

Experiment 2. Regional hemodynamic effects of UCN1 and SRP in the absence and presence of mepyramine and cimetidine

Resting cardiovascular variables in the 2 groups of animals prior to administration of UCN1 and SRP are shown in Table 1. The cardiovascular responses to SRP and UCN1 in these groups of animals were as described above, and the combination of cimetidine and mepyramine had no effect on the hemodynamic responses to either peptide (Figure 2).

Experiment 3. Effects of antisauvagine-30 on the hemodynamic responses to UCN1

In the presence of antisauvagine-30, the hemodynamic effects of UCN1 were markedly reduced or abolished. Thus, 30 min after administration of UCN1, there was no significant change in blood pressure ($+2 \pm 3$ mmHg), renal vascular conductance ($-5 \pm 7\%$) or mesenteric vascular conductance ($-1 \pm 2\%$), and the changes in heart rate ($+13 \pm 6$ beats min^{-1}) and hindquarters vascular conductance ($+15 \pm 6\%$) were substantially less than in animals not given antisauvagine-30 (see Figures 1 & 2).

Discussion

The design of these experiments enabled, firstly, a direct comparison of the regional hemodynamic effects of equimolar doses of UCN1 and SRP in the same animals, secondly, an evaluation of the extent to which the tachycardic effects of these two peptides are dependent on the autonomic nervous system and, thirdly, assessment of the possible involvement of CRF₁ receptor-mediated histamine release in the vasodilator effects of UCN1. The following discussion will deal with each of these aspects of the study in turn.

Comparison of the hemodynamic effects of UCN1 and SRP

In conscious sheep, integrated cardiovascular effects of UCN1 (Rademaker et al., 2002), UCN2 (Rademaker et al., 2005) and UCN3 (Rademaker et al., 2006) have been described in separate studies. Qualitatively, the effects of the three urocortin peptides are similar in this large animal model, and whilst those studies did not provide a direct comparison between them, Rademaker et al (2005) noted that the hemodynamic effects of UCN2 took less time to reach a maximum and were of shorter duration of action than UCN1. They suggested that the difference could be explained by differences in volume of distribution and clearance of the peptides (Rademaker et al., 2005), although, given their similarities in size (UCN1 40 amino acids, UCN2 38 amino acids), it is not clear why this should be.

Consistent with the observations of Rademaker and colleagues, the present study shows, for the first time in the same animals, that the regional hemodynamic effects of UCN1 and SRP in conscious rats were qualitatively similar, but the effects of UCN1 on heart rate, blood pressure and hindquarters vascular conductance took longer to reach a maximum and were more long-lasting than those of an equimolar dose of UCN2. Interestingly, the same did not apply to the mesenteric vasodilator response

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which, for both peptides, reached a maximum within the first 5 min of administration, although the duration of effect of UCN1 was greater. Elsewhere, we have commented on the difference in time-course of onset and duration of effect of UCN2 in the mesenteric and hindquarters vascular beds, with the response in the latter being slower in onset and of longer duration than that in the mesenteric vascular bed (Gardiner et al., 2005). In that study we suggested that the mesenteric vasodilator effect might represent a direct action at vascular CRF_2 receptors, whereas the hindquarters vasodilatation could be secondary to a metabolic action (Gardiner et al., 2005). The present findings are consistent with UCN1 and SRP having similar affinity for CRF_2 receptors in the mesenteric vascular bed, but with the two peptides having differential effects on the putative indirect mechanisms responsible for the hindquarters vasodilatation (see below). The longer-lasting hypotensive effect of UCN1 corresponded with the more persistent hindquarters vasodilatation.

Effects of atropine and propranolol on responses to UCN1 and SRP

The tachycardic effects of both peptides were abolished in the presence of atropine and propranolol, consistent with the increase in heart rate being due to sympathoexcitation and vagal withdrawal, and experiments involving administration of atropine alone, indicated a significant and similar contribution from both components of the autonomic nervous system. This contrasts with findings in sheep where the tachycardic effects of UCN1 were reported to be unaffected by ganglion blockade (Parkes et al., 2001), but, in that species, UCN1-induced tachycardia was accompanied by a rise rather than a fall in blood pressure, with no peripheral vasodilatation (Rademaker et al., 2002). Given the closeness of the time-course of blood pressure and heart rate changes in the present study, it seems likely that the

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tachycardia was a reflex response to the fall in blood pressure, although we cannot exclude a contribution from centrally-mediated sympathoexcitation to the response to UCN1, as shown for CRF (Overton and Fisher, 1991).

In the presence of atropine and propranolol, the hypotensive effects of SRP were enhanced, whereas the mesenteric and hindquarters vasodilator effects were unchanged, indicating that reflex cardiac responses to SRP normally acted to oppose the hypotension. In contrast, the hypotensive response to UCN1 was not enhanced when the tachycardia was abolished in the presence of atropine and propranolol, most likely because the hindquarters vasodilator response to UCN1 was reduced under those conditions. Theoretically, the inhibition of the hindquarters vasodilator effect could have been due to propranolol antagonising adrenomedullary, β_2 -adrenoceptor-mediated, vasodilatation, or to atropine inhibiting cholinergic vasodilatation. However, since an additional experiment showed that administration of atropine alone had no effect on the hindquarters vasodilator effect of UCN1, it is most likely that the effects we observed with atropine plus propranolol were due to an effect of propranolol. Therefore, the results indicate that UCN1 may cause sympathoadrenal activation which contributes to its hindquarters vasodilator and hypotensive effects, but that this effect is not seen with SRP. The results of the experiment we performed using antisauvagine-30 indicate that UCN1-induced cardiovascular effects are primarily, if not exclusively, mediated via CRF₂ receptors, consistent with our previous work using CRF (Gardiner et al., 2005). Therefore, the additional, propranolol-sensitive vasodilator effect of UCN1 observed here is likely to be CRF₂ receptor-mediated, and may be explained by a more effective activation of

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sympathoadrenal activity by that ligand, possibly due to different pharmacokinetics (see above and Rademaker et al., 2005).

Effects of mepyramine and cimetidine on responses to UCN1 and SRP

As explained in the Introduction, findings in the literature are consistent with activation of CRF₁ receptors causing mast cell degranulation and histamine release, and, given the affinity of UCN1 for CRF₁ receptors, and the lack of such affinity of SRP, then it was feasible the cardiovascular effects of UCN1 involved histamine release, whereas those of SRP did not. However, in the presence of mepyramine and cimetidine, to block H₁ and H₂ receptors, respectively, there was remarkably little change in the responses to UCN1 or SRP, indicating no involvement of endogenous histamine via H₁ or H₂ receptors in the vasodilator responses. Histamine can also affect cardiac function importantly through H₃ receptors (see Levi & Smith, 2000), and since we did not use an H₃ receptor antagonist, we cannot exclude an effect of histamine via this receptor sub-type. Furthermore, vasoactive mediators other than histamine may be released from mast cells, and we cannot exclude a contribution from those to the effects observed, although histamine has been shown to be the principal mediator of CRH-induced skin vasodilatation in man (Crompton et al., 2003).

In conclusion, the present results are consistent with SRP and UCN1 causing similar, CRF₂ receptor-mediated mesenteric vasodilator responses, and indirect, autonomically-mediated, tachycardic effects. While both peptides cause hindquarters vasodilatation which is resistant to propranolol and atropine, an additional propranolol-sensitive vasodilator effect of UCN1 has been identified.

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Footnotes

- a) This work was supported by the British Heart Foundation
- b) Reprint requests to Professor SM Gardiner, School of Biomedical Sciences, Floor E, Medical School, University of Nottingham NG7 2UH. UK

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Legends for figures

Figure 1. Hemodynamic effects of SRP (a) and UCN1 (b) in the presence of saline (closed circles) and propranolol plus atropine (open circles) in conscious, male Sprague-Dawley rats. Peptides were given as an i.v. bolus dose of 3 nmol kg⁻¹. Values are mean and vertical bars show S.E.M.; n=8 in each group. * P<0.05 vs baseline (Friedman's test). Between-group statistical comparisons of integrated responses are given in the text.

Figure 2. Hemodynamic effects of SRP (a; n=5) and UCN1 (b; n=6) in the presence of saline (closed circles) and mepyramine plus cimetidine (open circles) in conscious, male Sprague-Dawley rats. Peptides were given as an i.v. bolus dose of 3 nmol kg⁻¹. Values are mean and vertical bars show S.E.M. * P<0.05 vs baseline (Friedman's test).

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Table 1. Resting cardiovascular variables

Experiment 1	Saline	ATR+PROP	Saline	ATR+PROP
	UCN1	UCN1	SRP	SRP
	n=8	n=8	n=8	n=8
Heart rate (beats min ⁻¹)	316±6	341±12	326±6	347±12
Mean BP (mmHg)	107±2	111±2	111±3	112±3
Renal VC ([kHz mmHg ⁻¹] 10^3)	72±4	77±6	80±4	79±8
Mesenteric VC ([kHz mmHg ⁻¹] 10^3)	57±4	56±7	54±4	56±9
Hindquarters VC ([kHz mmHg ⁻¹] 10^3)	30±4	41±3	36±3	43±3
Experiment 2	Saline	MEP+CIM	Saline	MEP+CIM
	UCN1	UCN1	SRP	SRP
	n=6	n=6	n=5	n=5
Heart rate (beats min ⁻¹)	343±9	303±12	316±10	307±13
Mean BP (mmHg)	111±2	111±3	115±9	107±6
Renal VC ([kHz mmHg ⁻¹] 10^3)	80±7	86±11	89±8	94±11
Mesenteric VC ([kHz mmHg ⁻¹] 10^3)	63±4	68±6	58±4	54±4
Hindquarters VC ([kHz mmHg ⁻¹] 10^3)	37±3	35±6	37±1	39±2

Values (mean ± S.E.M.) are those obtained prior to administration of the peptide, 90 min after the onset of saline or atropine plus propranolol (ATR+PROP; Experiment 1), or 15 min after administration of mepyramine plus cimetidine (MEP+CIM; Experiment 2). VC=vascular conductance.

Figure 1

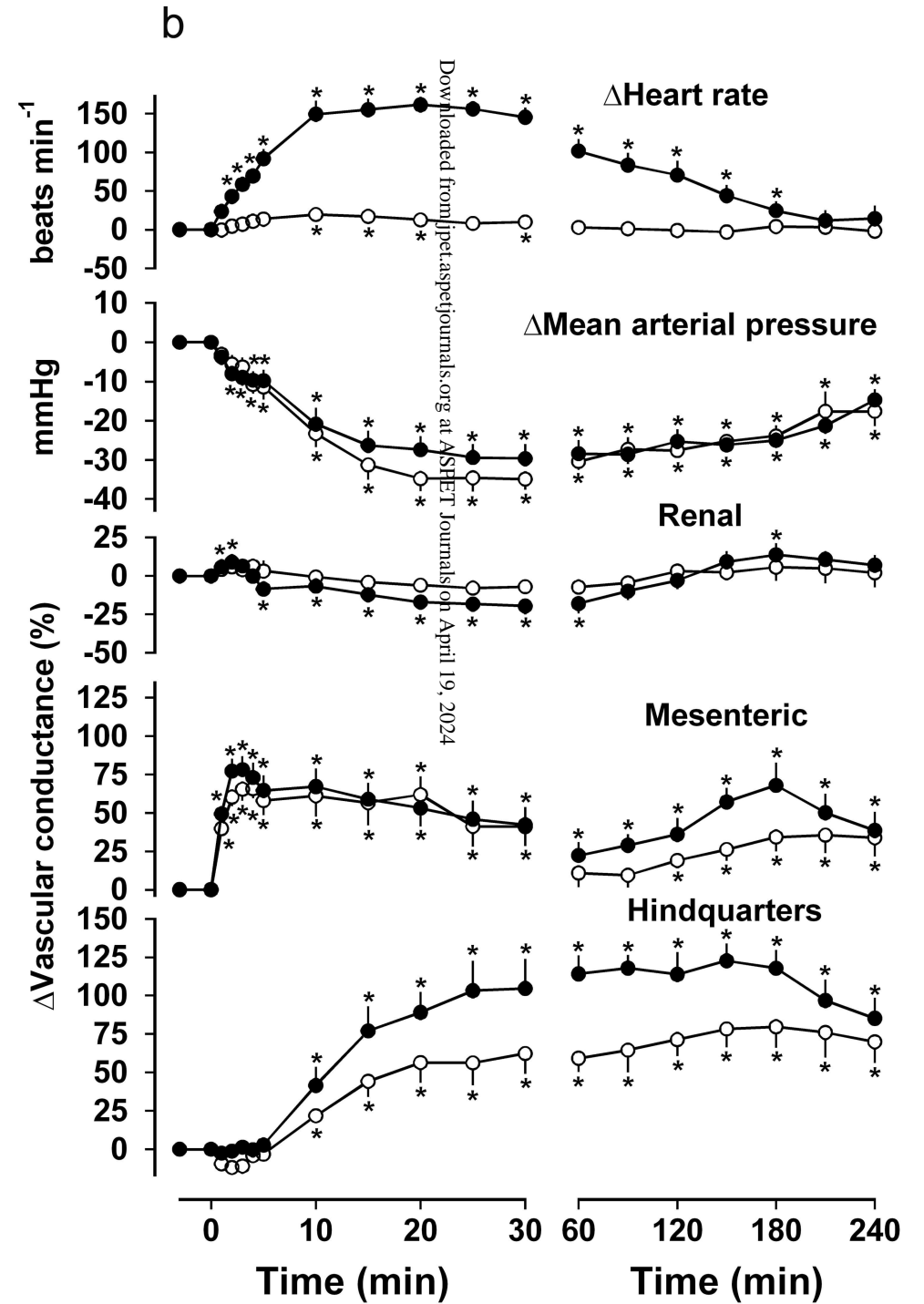
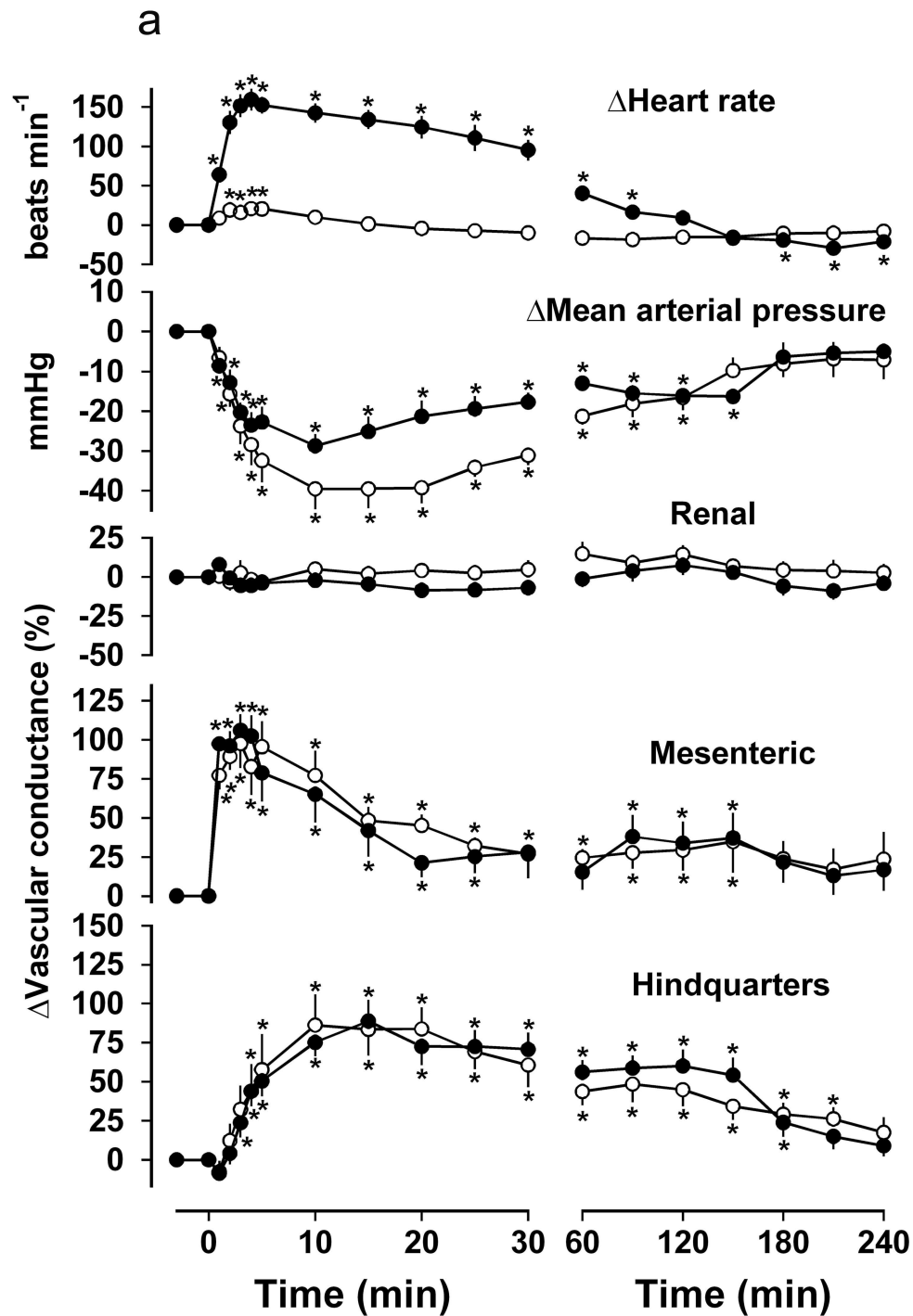


Figure 2

