RESEARCH PAPER

Hypotensive effects of ghrelin receptor agonists mediated through a novel receptor

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BACKGROUND AND PURPOSE

Some agonists of ghrelin receptors cause rapid decreases in BP. The mechanisms by which they cause hypotension and the pharmacology of the receptors are unknown.

EXPERIMENTAL APPROACH

The effects of ligands of ghrelin receptors were investigated in rats *in vivo*, on isolated blood vessels and on cells transfected with the only molecularly defined ghrelin receptor, growth hormone secretagogue receptor 1a (GHSR1a).

KEY RESULTS

Three agonists of GHSR1a receptors, ulimorelin, capromorelin and CP464709, caused a rapid decrease in BP in the anaesthetized rat. The effect was not reduced by either of two GHSR1a antagonists, JMV2959 or YIL781, at doses that blocked effects on colorectal motility, *in vivo*. The rapid hypotension was not mimicked by ghrelin, unacylated ghrelin or the unacylated ghrelin receptor agonist, AZP531. The early hypotension preceded a decrease in sympathetic nerve activity. Early hypotension was not reduced by hexamethonium or by baroreceptor (sino-aortic) denervation. Ulimorelin also relaxed isolated segments of rat mesenteric artery, and, less potently, relaxed aorta segments. The vascular relaxation was not reduced by JMV2959 or YIL781. Ulimorelin, capromorelin and CP464709 activated GHSR1a in transfected HEK293 cells at nanomolar concentrations. JMV2959 and YIL781 both antagonized effects in these cells, with their pA₂ values at the GHSR1a receptor being 6.55 and 7.84.

CONCLUSIONS AND IMPLICATIONS

Our results indicate a novel vascular receptor or receptors whose activation by ulimorelin, capromorelin and CP464709 lowered BP. This receptor is activated by low MW GHSR1a agonists, but is not activated by ghrelin.

Abbreviations

RSNA, renal sympathetic nerve activity; UAG, unacylated ghrelin.

Introduction

The major source of the acylated peptide hormone, ghrelin, is gastric endocrine cells. Ghrelin is formed from its precursor,

unacylated ghrelin (UAG), by the enzymic addition of an octanoyl group. After its discovery, ghrelin, but not UAG, was found to be an agonist at the growth hormone secretagogue receptor 1a (GHSR1a), the only molecularly identified ghrelin

receptor (Kojima *et al.*, 1999; Kojima and Kangawa, 2010; receptor nomenclature follows Alexander *et al.*, 2013). Surprisingly, the concentration of UAG in the circulation is about 20 times that of ghrelin (Van Der Lely *et al.*, 2004). Further investigation of effects of ghrelin and UAG have revealed actions that can best be explained by actions at receptors other than GHSR1a, including the UAG receptor that is activated by UAG and AZP531, but not by ghrelin (Tsubota *et al.*, 2005; Li *et al.*, 2006; Delhanty *et al.*, 2007; Granata *et al.*, 2012). Because of their range of effects, ghrelin and ghrelin receptor ligands have been either tested or proposed for the treatment of a very wide range of conditions, including congestive heart failure, chronic obstructive pulmonary disease, functional dyspepsia; gastroparesis, constipation, end-stage renal disease, osteoarthritis, other inflammatory conditions, memory deficits and some cancers (Andrews, 2011; Chopin *et al.*, 2011; DeBoer, 2011; Isgaard and Granata, 2011; Nass *et al.*, 2011; Akamizu and Kangawa, 2012; Warzecha and Dembinski, 2012).

The receptor GHSR1a has proven to be a relatively easy target for drug development and a large number of compounds of differing structure have been found to be effective at this receptor (Chollet *et al.*, 2009; 2012). Although these do not act equally at all sites where GHSR1a is expressed (Ferens *et al.*, 2010; Hassouna *et al.*, 2013), there has been little investigation of other targets of synthetic GHSR1a ligands. Because of their potential therapeutic uses, it is important to determine whether agonists that are targeted to GHSR1a also act at other sites.

We had previously reported a brief, unexplained, decrease in BP in the rat following i.v. injection of a low MW, non-peptide GHSR1a agonist, CP464709 (Ferens *et al.*, 2010). This fall in BP was not affected by autonomic blockade with hexamethonium (Ferens *et al.*, 2010), indicating that it is not due to an alteration in activity of autonomic pathways that affect BP. Peripheral injection of ghrelin itself gives a slower BP decrease that is accompanied by a decrease in sympathetic nerve discharge (Callaghan *et al.*, 2012). Ghrelin also causes a parallel decrease in BP and sympathetic nerve activity when it is injected i.c.v. (Matsumura *et al.*, 2002).

In the current work, we describe effects on BP of four chemically distinct GHSR1a agonists, capromorelin and CP464709, which are structurally related pyrazolinone-piperidine dipeptides (Carpino *et al.*, 2003), ulimorelin, which is a dissimilar tripeptide cyclised using a non-peptide tethering compound (Hoveyda *et al.*, 2011) and GSK894490, an unrelated non-peptide, low MW, agonist (Witherington *et al.*, 2008). We have investigated the mechanism and pharmacology of the early hypotensive effects of these chemically unrelated GHSR1a agonists.

Methods

BP recording

All animal care and experimental procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee and the Austin Health Animal Ethics Committee and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All

studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 155 animals were used in the experiments described here.

Adult male Sprague-Dawley rats (300–400 g) from Animal Resources Centre, Perth, Australia, were sedated with ketamine hydrochloride (50–60 mg·kg⁻¹, i.m.) and anaesthesia was induced with α -chloralose (60 mg·kg⁻¹, i.v.). The femoral artery was then cannulated for the infusion of anaesthetic and BP recording, and the femoral and jugular veins were cannulated for delivery of drugs. BP and heart rate (HR) were recorded with a PowerLab recording system using Chart 5 software (both from ADInstruments, Sydney, Australia). Anaesthesia was maintained by intra-arterial infusion of α -chloralose (12–20 mg·kg⁻¹·h⁻¹) plus ketamine (3–5 mg·kg⁻¹·h⁻¹) in PBS (0.15 M NaCl containing 0.01 M sodium phosphate buffer, pH 7.2). Colonic motility was recorded as previously described (Shimizu *et al.*, 2006). The distal colon was cannulated at the colonic flexure, which in the rat is at the junction of the proximal and distal colon, where formed faecal pellets are first observed. A second cannula was placed at the anus. The colon was left *in situ*, and the muscle and skin were closed around the proximal cannula. The proximal cannula was connected to a Marriotte bottle filled with warm PBS, and the distal cannula to a pressure transducer via a one-way valve. The baseline intraluminal pressure was maintained at 3–5 mmHg by adjusting the heights of the Marriotte bottle and outlet. Expelled fluid was collected in a cylinder distal to the one-way valve, and measured by weighing with a force transducer.

Bilateral sino-aortic denervation was performed while rats were under anaesthesia. On each side, the carotid sinus nerve was located and severed close to its branch point from the glossopharyngeal nerve (McDonald, 1983). The aortic depressor nerve was located close to the junction of the vagus and superior laryngeal nerve and it was also cut bilaterally.

Combined arterial pressure (AP) and renal nerve recording

Experiments were carried out on male Sprague-Dawley rats (550–700 g). These were anaesthetized in an isoflurane chamber and subsequently artificially ventilated through a tracheostomy with 100% O₂ containing 1.5–1.7% isoflurane (How *et al.*, 2013). The jugular vein was cannulated for i.v. administration of drugs and the right brachial artery for AP and HR measurement. For sympathetic nerve recordings, the left renal nerve was isolated via a retroperitoneal approach, placed onto a silver wire electrode and embedded in silicone sealant. The incision was closed and the wires externalized. Nerve signals were amplified and filtered (30–3000 Hz band-pass; gain $\times 10$ k), full-wave rectified and averaged over 1 s intervals. Renal sympathetic nerve discharge, AP and HR were stored and analysed using a CED data acquisition system and Spike-2 software (Cambridge Electronic Design, Cambridge, UK).

Blood vessel myography

Arteries were dissected from rats that were killed by inhalation of CO₂, with the concentration of CO₂ being gradually increased. Once dissected, the tissues were maintained in

buffered physiological saline with the following composition (in mM): Na⁺, 150.6; K⁺, 4.7; Ca²⁺, 2; Mg²⁺, 1.2; Cl⁻, 144.1; H₂PO₄²⁻, 1.3; HCO₃⁻, 16.3; glucose, 7.8. This solution was gassed with 95% O₂ plus 5% CO₂.

Segments (~1.5 mm long) of second-order mesenteric arteries (~250 µm lumen diameter) supplying the distal ileum and segments of thoracic aorta were mounted isometrically between stainless steel wires (40 µm diameter) in a four-chamber myograph (Multi Myograph 610 M, Danish Myo Technology, Aarhus, Denmark). Each myograph chamber contained 6 mL of physiological saline maintained at ~36.5°C that was exchanged at regular intervals. To normalize the basal conditions, Laplace's equation was used to convert the measured force to the effective transmural pressure (Mulvany and Halpern, 1977). Initially, the vessels were stretched until an effective transmural pressure of $13.3 \times 10^3 \text{ N}\cdot\text{m}^{-2}$ (100 mmHg) was reached. The circumference was adjusted to 90% of that determined at $13.3 \times 10^3 \text{ N}\cdot\text{m}^{-2}$. After equilibration for 30 min, vessels were activated with phenylephrine (1 µM for aortas; 10 µM for mesenteric arteries) and after the contraction had plateaued, the test agonist (ghrelin, UAG, ulimorelin, capromorelin, CP464709 or AZP351) was applied and left in contact with the tissue for at least 5 min or until the vaso-relaxation it produced had plateaued. In all tissues, the viability of the endothelium was confirmed by recording relaxation to the muscarinic agonist, carbachol (1 µM) applied at the end of the experiment after the test agent. For ulimorelin, the effects of pretreating the tissues with L-N^G-nitroarginine methyl ester (L-NAME) (0.1 mM) or the GHSR1a antagonists YIL781 (1 µM) and JMV2959 (1 µM) were assessed; these agents were applied 10 min before phenylephrine. For mesenteric arteries, the effects of mechanically denuding the endothelium on responses to ulimorelin were also assessed. The output from the myograph was recorded and analysed using a PowerLab data acquisition system and the program Chart (ADInstruments). The percentage changes in amplitude of phenylephrine-induced contraction produced by the test agents were assessed.

Intracellular calcium measurements using GHSR1a transfected HEK cells

We used cell lines stably expressing rat GHSR1a that have been previously described and characterized (Callaghan *et al.*, 2012). They were maintained in DMEM (Sigma Aldrich, Sydney, Australia) supplemented with 10% FBS and hygromycin (100 µg·mL⁻¹). Intracellular calcium levels in cell populations were measured by fluorescence using a Flexstation (Molecular Devices, Sunnyvale, CA, USA). HEK293 cells expressing rat GHSR1a were plated in 96-well plates (Corning Incorporated, Corning, NY, USA) at an approximate density of 40 000 cells per well and grown for 48 h. Receptor expression was induced by adding 5 µg·mL⁻¹ tetracycline 4 h prior to assay. Cells were loaded with 2 µM Fura-2 AM for 1 h in the presence of 2.5 mM probenecid and 0.01% pluronic F-127 (all from Invitrogen, Melbourne, Australia) at 37°C. Loading and experiments were performed in HEPES buffer (138 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, 10 mM HEPES; pH 7.4). After washing twice with HEPES buffer, 80 µL HEPES buffer was added to each well. The fluorescence was measured over 100 s using excitation wavelengths of 340 and 380 nm and emission of 520 nm. At

approximately 15 s, 20 µL of agonist was added to cells at five times the final concentration. Antagonists were incubated 15 min before the addition of the agonist. The mean of the peak fluorescence ratio after agonist injection minus the basal ratio was used for plotting concentration response curves.

Data analysis

Data are presented as means ± SEM. Means were compared by two-tailed paired or unpaired Student's *t*-test. A *P*-value of <0.05 was considered significant. For assays of responses of transfected cells, EC₅₀ values were determined by nonlinear regression and expressed as pEC₅₀ values and pA₂ values calculated from concentrations of antagonists that did not depress the maximum agonist response using the Gaddum/Schild EC₅₀ shift analysis using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

Materials

The following compounds were used: CP464709, synthesized by Dr Jason Witherington; capromorelin tartrate (CP424391) from Raqualia Pharmaceuticals, Nagoya, Japan and synthesized by us (KB and JB); ulimorelin from Tranzyme Pharma; JMV2959 synthesized by us (KB and JB); GSK894490 from GlaxoSmithKline, Harlow, UK; YIL781 (Tocris, Bristol, UK); AZP531 (ChinaPeptides, Shanghai, China); carbachol chloride, hexamethonium bromide, L-NAME and phenylephrine hydrochloride (from Sigma Aldrich); rat ghrelin (Auspep, Melbourne, Victoria, Australia and ChinaPeptides); rat UAG (des-acyl ghrelin; GL Biochem, Shanghai, China and ChinaPeptides).

Doses of inhibitors are based on those previously determined to be effective in the rat, *in vivo*: hexamethonium (Bogeski *et al.*, 2005), L-NAME (Ferens *et al.*, 2005), YIL781 (Esler *et al.*, 2007) and JMV2959 (Salomé *et al.*, 2009).

Results

Effects of GHSR1a receptor ligands on HEK cells with heterologous rat GHSR1a transfection

Ulimorelin, capromorelin, CP464709, GSK894490, ghrelin and UAG were all agonists at the rat GHSR1a (Figure 1A). The pEC₅₀ values were -9.06 ± 0.12 (874 pM) for ulimorelin, -9.72 ± 0.21 (190 pM) for capromorelin, -10.06 ± 0.11 (86 pM) for CP464709, -9.54 ± 0.11 (286 pM) for GSK894490 and -8.59 ± 0.28 (2.6 nM) for ghrelin. UAG was considerably less potent, the EC₅₀ being 2.4 µM, as reported previously (Gauna *et al.*, 2007). JMV2959 caused a dose-dependent rightwards shift in the dose response curve for ulimorelin (Figure 1B) with a calculated pA₂ of 6.55 (6.37–6.72). YIL 781 also antagonized the response to ulimorelin (Figure 1C) with a pA₂ of 7.832 (7.454–8.209). Ulimorelin, capromorelin, CP464709 and GSK894490 and ghrelin had no effect in untransfected cells.

Effects on BP, colorectal propulsion and sympathetic nerve activity

Each of the four synthetic ghrelin receptor agonists (ulimorelin, capromorelin, CP464709 and GSK894490), given *i.v.*, caused BP changes, but the patterns of change differed

Figure 1

(A) Responses of HEK293 cells transfected with rat GHSR1a to capromorelin, CP464709, GSK894490, ulimorelin, ghrelin and unacylated ghrelin (UAG). Increases in intracellular Ca^{2+} were measured using a Flexstation assay. (B) Rightward shift in the concentration-response curve for ulimorelin with increasing concentrations of JMV2959. (C) Rightward shift in the ulimorelin concentration-response curve caused by YIL781.

between agonists as detailed below. Three types of BP change were identified: an early hypotensive effect that peaked at 10–30 s; a delayed pressure drop that had its peak later than 1 min; and a slowly developing BP increase that lasted several minutes (Figure 2). The relative amplitudes of these components differed between agonists.

Early hypotensive action and comparison of its pharmacology to the pharmacology of colorectal stimulation by ghrelin receptor agonists in vivo

Intravenous injection of ulimorelin (0.1 to 5 $\text{mg}\cdot\text{kg}^{-1}$) or CP464709 (2.5, 5 and 10 $\text{mg}\cdot\text{kg}^{-1}$) caused an early drop in BP (Figure 2). Ulimorelin, capromorelin, CP464709 and GSK894490, given i.v., all elicited propulsive contractions of the colorectum (Figure 2). The early BP response to ulimorelin reached a peak 9–20 s after its onset. The times from onset to peak response for CP464709 (5 $\text{mg}\cdot\text{kg}^{-1}$) were 8.5–17.5 s. Ulimorelin elicited maximum BP decreases with doses of 1 or 3 $\text{mg}\cdot\text{kg}^{-1}$ (Figure 2), which on average reduced BP by about 25% (from 79.7 ± 4 to 60.2 ± 3.6 mmHg, $n = 21$, for 1 $\text{mg}\cdot\text{kg}^{-1}$ and 82.6 ± 4.4 to 61.7 ± 2.9 mmHg, $n = 18$, for 3 $\text{mg}\cdot\text{kg}^{-1}$ ulimorelin). The threshold dose to reduce BP was 0.1 $\text{mg}\cdot\text{kg}^{-1}$, whereas the threshold to initiate colorectal propulsion was 3 $\text{mg}\cdot\text{kg}^{-1}$. The BP and colorectal responses to ulimorelin did not desensitize with repeated application of the drug. For the first application of 5 $\text{mg}\cdot\text{kg}^{-1}$ ulimorelin, the BP drop was $11 \pm 1\%$ and the number of colorectal contractions ≥ 6 mmHg was 47 ± 15 ; for the second application of the same dose, the BP drop was $15 \pm 4\%$ ($P = 0.24$) and the number of contractions ≥ 6 mmHg was 54 ± 8 ($P = 0.66$; $n = 3$). Dose-dependent desensitization of successive responses has been observed for other GHSR1a agonists (Shimizu *et al.*, 2006; Ferens *et al.*, 2010).

CP464709 also caused a dose-dependent early decrease in BP, and stimulated colorectal activity at slightly higher doses (Figure 2). Capromorelin caused only a very small early decrease in BP. However, a distinct early hypotensive effect was revealed in the presence of the antagonist, JMV2959 (Figure 3). JMV2959 was administered as a 5 $\text{mg}\cdot\text{kg}^{-1}$ i.v. bolus

followed by continuous infusion of 0.5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$, which were doses that inhibited colorectal stimulation (see below). GSK894490 at doses that had strong effects on colorectal propulsion (0.5 and 1.5 $\text{mg}\cdot\text{kg}^{-1}$) had very minor hypotensive effects (Figure 2). The UAG receptor agonist, AZP531 (Julien *et al.*, 2012), up to 1 $\text{mg}\cdot\text{kg}^{-1}$ ($n = 6$), ghrelin and UAG (10, 100 and 300 $\mu\text{g}\cdot\text{kg}^{-1}$; $n = 5$) did not cause an early fall in BP.

Delayed decreases in BP and sympathetic nerve discharge

A second, slower BP decrease of 5–10 mmHg in response to ulimorelin was observed (Figure 2). The amplitude of this component varied considerably between experiments, probably because several influences are superimposed at this time (see Discussion). Ulimorelin (0.3 $\text{mg}\cdot\text{kg}^{-1}$) caused a decrease in renal sympathetic nerve activity (RSNA) of $23.6 \pm 9.0\%$ at the time of the second slower decrease in BP (Figure 4). Inhibition of RSNA had its onset at 4.2 ± 1.2 min post-infusion, and the maximal inhibitory response was at 7.2 ± 1.6 min. A small delayed hypotensive response was observed at this time (Figure 4). At this dose, ulimorelin did not induce a pressor effect. Inhibition of RSNA occurred after the early hypotensive effect of ulimorelin (0.3 $\text{mg}\cdot\text{kg}^{-1}$), which in this series of experiments in which RSNA was recorded had a latency of 30–85 s after i.v. injection, a maximum BP drop at 64 ± 8 s after its onset and an amplitude of $22 \pm 3\%$ reduction in BP (23 ± 3 mmHg, $n = 7$). The time course of the early hypotensive effect was slower in rats that were anaesthetized with isoflurane to optimize the renal sympathetic nerve recording, compared with anaesthesia with α -chloralose plus ketamine. Ulimorelin also induced a bradycardic response (-16 ± 2 bpm), the nadir of which coincided with that of the early hypotensive response. As previously described (Callaghan *et al.*, 2012), ghrelin caused a decrease in BP that began slowly, 1–2 min after the peptide was injected, and which persisted for about 5 min. The decrease in BP caused by ghrelin was also accompanied by a decrease in sympathetic nerve discharge (Callaghan *et al.*, 2012).

The GHSR1a antagonist, YIL781 (3 $\text{mg}\cdot\text{kg}^{-1}$ i.v. bolus plus 5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ maintenance dose) reversed the effect of ulimo-

Figure 2

Effects of GHSR1a receptor agonists on BP and colorectal motility in anaesthetized rats. (A) Ulimorelin, 1 mg·kg⁻¹, i.v., caused BP changes that could be resolved into three components: (a) an early phasic BP decrease; (b) a delayed decrease in BP; and (c) a slowly developing prolonged BP increase. Following the agonist, there was also an increase in the numbers of phasic pressure increases in the colorectum and a transient decrease of HR. (B to E) Quantitative data (means ± SEM) on the early BP decreases (red) and increases in colorectal motility (blue) caused by four different low MW GHSR1a agonists. It is notable that the relative doses to decrease BP and to activate colorectal contractile activity differ between agonists. Numbers of experiments are indicated above the histogram bars in B to E.

reltin (0.3 mg·kg⁻¹) on RSNA. Prior to the antagonist, ulimorelin reduced RSNA by $10.3 \pm 2.7\%$ at 5.4 ± 0.8 min after the ulimorelin bolus, whereas in the presence of the antagonist, RSNA measured at the same time points after injection was $6.6 \pm 4.9\%$ above the pre ulimorelin value. The effect of YIL781 was significant ($P < 0.02$, $n = 7$).

Slowly developing BP increase

Ulimorelin, capromorelin, CP464709 and GSK894490 all caused slowly developing increases in BP. Previous studies showed that the BP increases caused by i.v. CP464709 and GSK894490 were mimicked by direct, intrathecal, application of these agonists or ghrelin to lower thoracic regions of the spinal cord, and that the increases in BP were blocked by hexamethonium (Ferens *et al.*, 2010). AZP531 (i.v.) also increased BP by 11% (0.5 mg·kg⁻¹; $n = 5$) and 15% (1 mg·kg⁻¹; $n = 2$).

Actions of GHSR1a antagonists on early hypotensive and colorectal effects

Two GHSR1a antagonists, JMV2959 (Salomé *et al.*, 2009) and YIL781 (Esler *et al.*, 2007), were used at doses based on the original publications. Following application of JMV2959 (5 mg·kg⁻¹ i.v. bolus and 0.5 mg·kg⁻¹·h⁻¹ maintenance dose), the stimulation of colorectal propulsive activity by ulimorelin was significantly reduced (Figure 5), indicating that JMV2959 is effective *in vivo*, including at sites within the CNS. JMV2959 itself had no effect on colorectal activity, but it caused an immediate short-lasting decrease in BP ($29 \pm 4\%$) followed by a small increase ($8.6 \pm 2\%$) that was maintained for 20 min ($n = 16$). The early hypotensive effect of ulimorelin (1 and 3 mg·kg⁻¹) was not reduced when applied 20 min after the JMV2959 i.v. bolus ($n = 7$; Figure 5).

Unlike the effects described for ulimorelin, the BP and colorectal propulsive activity in response to capromorelin

Figure 3

The effect of the GHSR1a receptor antagonist, (JMV2959) on responses to capromorelin (4 mg·kg⁻¹, i.v.). Experiments were conducted in the absence and in the presence of JMV2959, administered as an i.v. bolus dose (5 mg·kg⁻¹) followed by continuous infusion of 0.5 mg·kg⁻¹·h⁻¹. The numbers of colorectal propulsive contractions caused by capromorelin and the delayed increase in BP were significantly reduced after GHSR1a blockade, but the early decrease in BP to capromorelin was increased. In the capromorelin alone experimental group, the resting BP was 75.7 ± 3.5 mmHg and capromorelin caused a slight reduction to 73.2 ± 3 mmHg. In the JMV2959 plus capromorelin experimental group, the average BP was 73.2 ± 4.7 mmHg in the presence of JMV2959 and capromorelin caused a decrease to 56 ± 0.7 mmHg. Numbers of experiments are indicated adjacent to the bars.

Figure 4

Effects of i.v. ulimorelin (0.3 mg·kg⁻¹) on BP (upper trace), renal sympathetic nerve activity (middle trace) and HR (lower trace) in an anaesthetized rat. The early decrease in BP precedes the decrease in renal sympathetic nerve activity.

desensitize. Therefore, we compared the response to the first application of capromorelin (4 mg·kg⁻¹) in animals pre-exposed to JMV2959 to the responses to the same dose of capromorelin alone in another set of animals. Capromorelin alone decreased the BP of 6 of 15 animals tested; in the six animals, the reduction was 6.3 ± 3.8% from an average BP before application of 68.8 ± 5.2 mmHg (*n* = 6). In the presence of JMV2959, the early hypotensive response to capromorelin was significantly larger than it was in the absence of this GHSR1a antagonist (*P* < 0.05; Figure 3). The opposite occurred for the stimulation of colorectal propulsion, which was blocked by JMV2959. The delayed slow BP increase caused by capromorelin was significantly reduced by JMV2959 (*P* < 0.005; Figure 3).

YIL781 (3 mg·kg⁻¹ bolus plus 5 mg·kg⁻¹·h⁻¹ maintenance dose) decreased resting BP by 3 ± 2%, to an average of

86 ± 3 mmHg. Before treatment with YIL781, ulimorelin (1 mg·kg⁻¹) caused an early decrease in BP by 25 ± 3% and this effect was not significantly altered by the presence of YIL781 (decreased in BP by 31 ± 3%; *n* = 4). YIL781 also antagonized the colorectal stimulating effect of ulimorelin (3 mg·kg⁻¹). The number of colonic contractions ≥6 mmHg per 20 min in response to 3 mg·kg⁻¹ ulimorelin reduced from 12.3 ± 4.4 before YIL781 to 1.4 ± 0.6 (*P* < 0.02, *n* = 7) in the presence of the antagonist.

Effect of sino-aortic denervation

In four rats, the carotid sinus and aortic depressor nerves were cut bilaterally (sino-aortic denervation) between applications of ulimorelin (1 mg·kg⁻¹). Ulimorelin induced an early BP decrease from baseline of 32 ± 3.8% (71.0 ± 4.0 to 48.3 ± 4.2 mmHg) that was significantly (*P* > 0.05) more pro-

Figure 5

Effects of the GHSR1a receptor antagonist JMV2959 on the actions of ulimorelin. JMV2959 (5 mg·kg⁻¹ i.v. bolus followed by continuous infusion of 0.5 mg·kg⁻¹·h⁻¹) significantly reduced the numbers of colorectal contractile events of greater than 6 mmHg (propulsive contractions) that were observed in the 20 min after i.v. injection of ulimorelin (1 mg·kg⁻¹). In the same experiments, there were no changes in the early BP decrease caused by ulimorelin. Before the addition of JMV2959, ulimorelin (1 mg·kg⁻¹) reduced BP from 76.9 ± 7.8 to 57.8 ± 6.6 mmHg and in the presence of JMV2959 ulimorelin reduced the BP from 78 ± 4.8 to 55.9 ± 3.3 mmHg. Ulimorelin (3 mg·kg⁻¹) reduced BP from 81.3 ± 8.5 to 57.6 ± 4.5 mmHg; in the presence of JMV2959, ulimorelin reduced the BP 74.7 ± 5.6 to 55.6 ± 2.8 mmHg. Data presented as means ± SEM. * = significant difference ($P < 0.01$, $n = 7$).

Figure 6

Effects of baroreceptor denervation (sino-aortic denervation; SAD) on BP decreases caused by ulimorelin (1 mg·kg⁻¹). (A) BP records from a rat before and after SAD. After SAD, the BP drop was larger and lasted longer. The BP was also less regular. (B) Quantitative data (means ± SEM). The BP drop was greater after SAD. The GHSR1a antagonist, JMV2959, did not antagonize that hypotensive effect of ulimorelin in sino-aortically denervated rats ($n = 4$).

nounced after sino-aortic denervation, being a decrease of 46 ± 7.5% (79.8 ± 5.0 to 43.0 ± 8.3 mmHg). The decreases were from different baselines: sino-aortic denervation caused BP to increase from 81 ± 4 mmHg to 90 ± 5 mmHg. Moreover, the early BP decrease was also prolonged after sino-aortic denervation (Figure 6). The decrease in BP caused by ulimorelin (1 mg·kg⁻¹) after sino-aortic denervation and in the presence of JMV2959 was 65 ± 7%, to 30 ± 9 mmHg ($n = 4$; Figure 6). The HR increased from 278 ± 28 bpm before to 360 ± 8 bpm ($n = 4$) after sino-aortic denervation ($P < 0.05$).

The slowly developing BP increase in response to ulimorelin (1 mg·kg⁻¹) was still observed after sino-aortic denerva-

tion. Prior to denervation, the peak of this BP increase was 90 ± 1 mmHg and after denervation, it was 101 ± 11 mmHg (not significantly different).

Actions of hexamethonium

Hexamethonium (10 mg·kg⁻¹ bolus and 15 mg·kg⁻¹·h⁻¹ maintenance dose) decreased resting BP by 24 ± 8%, to an average of 65 ± 6 mmHg. BP was restored by infusion of 90–100 µg·kg⁻¹·h⁻¹ of PE. BP was slightly less stable after hexamethonium and PE infusion. The rate of infusion of PE was adjusted so that BP in the presence of hexamethonium was similar to that prior to giving hexamethonium. In the

Figure 7

The effect of a dose of hexamethonium that blocks excitatory transmission in autonomic ganglia (10 mg·kg⁻¹ bolus followed by 15 mg·kg⁻¹·h⁻¹ infusion) on the early BP decrease caused by ulimorelin (1 mg·kg⁻¹). Hexamethonium alone caused a fall in BP to about the same level as the response to ulimorelin. Therefore, the BP in the presence of hexamethonium was restored by infusion of phenylephrine before the second application of ulimorelin. Data are means ± SEM. Hexamethonium did not reduce the fall in BP caused by ulimorelin ($n = 5$).

presence of this dose of hexamethonium, which blocks nicotinic transmission at autonomic ganglionic synapses in rats (Collister and Osborn, 1999; Ferens *et al.*, 2005), there was no reduction in the early hypotensive effect cause by ulimorelin, 0.3 or 1 mg·kg⁻¹ (Figure 7). Prior to hexamethonium the BP decreased by $24 \pm 3\%$ (82.8 ± 3.3 to 63.3 ± 4.2 mmHg), whereas in the presence of hexamethonium and BP restoration, the decrease was $35 \pm 5\%$ (94.5 ± 6.8 to 61.2 ± 5.0 mmHg), the difference not being significant ($P > 0.05$). There was a significant increase in the duration of the hypotensive effect after hexamethonium, from a duration of 35 ± 21 s (at half amplitude) to 123 ± 21 s ($P < 0.05$).

Effect of L-NAME

BP is regulated in part through production of the vasodilator, NO, by NOS activity in vascular endothelial cells. Intravenous injection of the NOS inhibitor, L-NAME (10 mg·kg⁻¹ bolus and 3 mg·kg⁻¹·h⁻¹ maintenance dose) increased BP to 120 ± 10 mmHg a significant increase of $46 \pm 6\%$ ($P < 0.005$). The early hypotensive effect of ulimorelin was reduced in the presence of L-NAME. Prior to L-NAME, ulimorelin (1 mg·kg⁻¹) decreased the BP by $23 \pm 6\%$ (75.7 ± 4.6 to 58.1 ± 5.9 mmHg), while in the presence of L-NAME, a significantly smaller decrease in BP, $12 \pm 3\%$, occurred (120 ± 10 to 106 ± 14 mmHg; $P = 0.01$, $n = 5$).

Effects of GHSR1a receptor ligands on isolated mesenteric artery and aorta

Wall tension of isolated segments of mesenteric artery that were precontracted with phenylephrine (10 μ M) was reduced by ulimorelin, with a half-maximal effect at about 0.1 μ M and near full relaxation with 1 μ M (Figure 8A and C). In vessels in which the endothelium was removed and carbachol

Figure 8

Effects of ulimorelin on wall tension in segments of mesenteric artery that were precontracted with phenylephrine (10 μ M). (A) Responses prior to application of L-NAME (100 μ M). Ulimorelin (1 μ M) caused a prompt relaxation, similar to that caused by carbachol. (B) Responses to ulimorelin in the presence of L-NAME (100 μ M) were similar to those in its absence (c.f. A and B). (C) Quantitative data (means ± SEM) in the absence of L-NAME. The relaxations to ulimorelin were similar in the absence and in the presence of L-NAME.

produced minimal relaxation (<10% of phenylephrine contraction when applied in the absence of ulimorelin), the percentage relaxation to 1 μ M ulimorelin was greatly reduced ($28 \pm 6\%$, $n = 5$). By contrast, inhibition of endothelial NOS with the inhibitor, L-NAME (100 μ M) did not reduce the relaxation caused by ulimorelin (Figure 8B and C). Ulimorelin also relaxed the rat aorta precontracted with phenylephrine (1 μ M), but the percentage relaxation produced by 0.1 μ M ($15 \pm 5\%$, $n = 5$) and 1 μ M ($52 \pm 15\%$, $n = 5$) was less than in mesenteric artery (Figure 8C). In the presence of L-NAME, the percentage relaxation of the aorta produced by 1 μ M ulimorelin was substantially reduced ($16 \pm 7\%$, $n = 5$). Capromorelin also relaxed the mesenteric artery segments. The relaxation was observed with 10 μ M capromorelin, but not with 1 μ M. Capromorelin (up to 10 μ M) did not dilate the segments of aorta.

The GHSR1a antagonists, YIL781 and JMV2959, both 1 μ M, had no effect on the relaxation of mesenteric arteries caused by ulimorelin (0.1 and 1 μ M). The UAG agonist, AZP531 (1 μ M) did not mimic the effect of ulimorelin. It caused a slowly developing relaxation, with a latency of about 2 min and variable amplitude, from 20 to 70% of the constriction caused by phenylephrine.

Discussion and conclusions

Four potent synthetic ghrelin receptor agonists caused a BP decrease in anaesthetized rats after a brief latency. While capromorelin and CP464709 are structurally related pyrazolinone-piperidine dipeptides (Carpino *et al.*, 2003), ulimorelin is a dissimilar tripeptide cyclised using a non-peptide tethering compound (Hoveyda *et al.*, 2011) and GSK894490 is an unrelated non-peptide small-molecule agonist (Witherington *et al.*, 2008). The most potent was ulimorelin, and most of the experiments to analyse sites and mechanisms of action were therefore conducted using ulimorelin.

Site of action for early hypotensive effect

Three sets of evidence indicated that the early BP decrease caused by the ghrelin receptor agonists was not an indirect consequence of reduction of sympathetic nerve activity, which could feasibly be caused by activation of baroreceptor afferents or by actions in the medulla oblongata. First, the early hypotensive effect was unchanged after severing the baroreceptor afferents of the carotid sinus and aorta (sino-aortic denervation). Second, the early hypotensive effect was still observed after autonomic ganglionic transmission was blocked with hexamethonium. Third, although ulimorelin caused a decrease in sympathetic nerve activity, the onset of this decrease was 4.2 ± 1.2 min, compared with a latency of 30–85 s for the early hypotensive effect in the same experiments. Together, these results suggest that ulimorelin causes an early decrease in BP, that does not involve a decrease in sympathetic nerve activity, plus a delayed decrease (discussed below) that is associated with a decrease in sympathetic nerve activity.

The implication of the *in vivo* experiments, that the synthetic ghrelin receptor agonists acted directly on blood vessels, was supported by the *in vitro* experiments. These

showed that ulimorelin relaxed mesenteric arteries with an EC_{50} value of ~ 0.1 μ M (Figure 8). This dilatation was reduced in endothelium denuded vessels. However, there was no significant reduction in the dilatation when endothelial NOS was blocked with L-NAME. This suggests that the relaxation in mesenteric arteries is due to the release of an endothelium-derived relaxing factor other than NO from the endothelial lining (Myers *et al.*, 1990; Ross *et al.*, 1991). By contrast, in aorta, where the relaxation to muscarinic stimulation is primarily dependent on activity of NOS (Shimokawa *et al.*, 1996), the relaxation to ulimorelin was reduced by L-NAME indicating involvement of endothelial NOS. L-NAME reduced the early hypotensive effect of ulimorelin, *in vivo*, by about 50%. This is likely to be due to actions at other vascular beds, such as the femoral bed where vasodilatation is largely dependent on NO in the rat (Zygmunt *et al.*, 1995). Capromorelin was a less potent vasodilator than ulimorelin and at 10 μ M produced $\sim 50\%$ relaxation of mesenteric arteries but did not dilate the aorta.

Receptor pharmacology of the early hypotensive effect

Using HEK cells that were heterologously transfected with rat GHSR1a, we confirmed previous data (Carpino *et al.*, 2003; Hoveyda *et al.*, 2011) that both ulimorelin and capromorelin are effective agonists of GHSR1a. Moreover, we showed that two GHSR1a antagonists, JMV2959 (Salomé *et al.*, 2009) and YIL781 (Esler *et al.*, 2007) blocked GHSR1a activation by ulimorelin. However, neither JMV2959 nor YIL781 caused any reduction of the early hypotensive effect of ulimorelin *in vivo* or the vasodilator effect of this agonist in isolated mesenteric arteries. In contrast, both antagonists substantially reduced the stimulation of colorectal propulsive activity by ulimorelin *in vivo*. The stimulation of colorectal propulsive activity by GHSR1a agonists, including ghrelin, is caused by activation of the defecation control centres in the lumbo-sacral spinal cord (Shimizu *et al.*, 2006; Hirayama *et al.*, 2010; Ferens *et al.*, 2011). Autonomic preganglionic neurons located at the defecation centres express GHSR1a, suggesting that the effects are mediated through GHSR1a. Thus, the experiments demonstrate that ulimorelin, capromorelin, CP464709 and GSK894490 cross the blood–CNS barrier and activate ghrelin receptors. Moreover, the effects of ulimorelin on colorectal activity were substantially reduced by JMV2959 and YIL781. This shows that both antagonists are effective *in vivo* and enter the CNS. The endogenous GHSR1a agonist, ghrelin, did not cause an early hypotensive effect, although it is effective *in vivo*, causing a slowly developing hypotension that is accompanied by reduced sympathetic nerve activity (Nagaya *et al.*, 2001; Okumura *et al.*, 2002; Callaghan *et al.*, 2012). The failure of JMV2959 and YIL781 to cause any reduction in the early hypotensive effect and the failure of ghrelin to mimic this effect indicates that the early onset BP decrease is not caused by activation of GHSR1a. Comparison of *in vitro* effects, using mesenteric artery segments and GHSR1a transfected cells suggests that the vascular receptors are about 100-fold less sensitive to ulimorelin than is GHSR1a.

Many studies indicate that there are unidentified receptors that bind or are activated by ligands of GHSR1a (Camiña, 2006; Seim *et al.*, 2011; Delhanty *et al.*, 2012). These include receptors for ghrelin and UAG that occur where no GHSR1a

expression can be demonstrated (Thompson *et al.*, 2004) and receptors for UAG in GHSR1a knockout animals (Toshinai *et al.*, 2006). The present work indicates that a vascular receptor or receptors exists whose activation dilates blood vessels and lowers BP. This receptor is activated by low MW GHSR1a agonists, but is not activated by ghrelin. The identity of the off-target receptor remains to be determined.

The relative doses of agonists that cause an early drop in BP, that cause later changes in BP and that provoke colorectal contractile activity differ between agonists. For example, the threshold dose of ulimorelin for the early hypotension was 30 times less than the colorectal stimulating dose, whereas GSK984490 was more effective in stimulating colorectal motility than it was in reducing BP. The observations could be due to differences in access to the different sites or to differences in efficacy at different receptor types, or to a combination of these factors. The true relative effects at the receptors are difficult to resolve while the receptor for the early hypotensive effect is unknown, there is no antagonist identified for this effect and the distributions of the agonists *in vivo*, in relation to the different receptor populations, are unknown.

The transient hypotensive effects of i.v. injection of the GHSR1a agonists is probably not relevant to the clinical use of the compounds that are administered orally or by slow infusion at lower doses (Ejskjaer *et al.*, 2010). No adverse cardiovascular effects have been reported.

Other cardiovascular effects of ghrelin receptor ligands

We have also observed a more slowly developing hypotensive effect and a hypertensive effect of ghrelin receptor ligands. These are similar to BP changes that have been previously described. We also observed a brief decrease in HR that we are yet to study in detail.

A slowly developing hypotensive effect of ghrelin has been previously reported in human and in animals (Nagaya *et al.*, 2001; Okumura *et al.*, 2002; Callaghan *et al.*, 2012). The decrease in BP caused by ghrelin occurs at the same time as a decrease in sympathetic nerve activity, comparable to the coincidence of hypotension and decreased renal nerve activity observed with ulimorelin in the current study. Furthermore, ghrelin does not cause a dilation of rat arteries, *in vitro* (Callaghan *et al.*, 2012). It has therefore been suggested that the slower drop in BP could be exerted at the level of the brainstem. This is consistent with observations that ghrelin and UAG applied in the brainstem lower BP and reduce sympathetic nerve activity (Matsumura *et al.*, 2002; Tsubota *et al.*, 2005).

A late, slow increase in BP has only been reported for the systemic application of centrally penetrant GHSR1a agonists (Shimizu *et al.*, 2006; Ferens *et al.*, 2010). Peripheral administration of ghrelin does not have this effect, but ghrelin or low MW GHSR1a agonists cause BP rises when they are applied directly to the lower thoracic spinal cord (Ferens *et al.*, 2010). The physiological relevance of GHSR1a ghrelin receptor expression in the spinal cord is currently obscure, as ghrelin is not present in the spinal cord (Furness *et al.*, 2011).

Clinical implications

A number of ghrelin mimetics have been used clinically, including capromorelin (White *et al.*, 2009) and ulimorelin

(Ejskjaer *et al.*, 2010) that we investigated in this study, and anamorelin (Garcia *et al.*, 2013), which has some structural similarity to capromorelin. No serious off-target effects have been identified. Nevertheless, there may be patient groups where off-target effects could be important. Moreover, it is now apparent that there exist several, so far molecularly undefined, receptors other than GHSR1a at which ghrelin mimetics have effects (Gauna *et al.*, 2005; Camiña, 2006; Delhanty *et al.*, 2007; Seim *et al.*, 2011; Granata *et al.*, 2012). It is quite likely that new compounds that are targeted to GHSR1a will have off-target effects that differ in unpredictable ways from those of existing compounds. Careful assessment of the off-target effects will be required.

In conclusion, in this series of experiments, we have demonstrated a rapid hypotensive effect and vasodilator action of a number of chemically distinct ghrelin receptor agonists that were not induced by ghrelin itself or by UAG.

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Conflict of interest

None of the authors have any conflicts of interest to disclose relating to this submission.

References

- Akamizu T, Kangawa K (2012). The physiological significance and potential clinical applications of ghrelin. *Eur J Int Med* 23: 197–202.
- Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013). The concise guide to PHARMACOLOGY 2013/14: G protein-coupled receptors. *Br J Pharmacol* 170: 1459–1581.
- Andrews ZB (2011). The extra-hypothalamic actions of ghrelin on neuronal function. *Trends Neurosci* 34: 31–40.
- Bogeski G, Shafton AD, Kitchener PD, Ferens DM, Furness JB (2005). A quantitative approach to recording peristaltic activity from segments of rat small intestine *in vivo*. *Neurogastroenterol Motil* 17: 262–272.
- Callaghan B, Hunne B, Hirayama H, Sartor DM, Nguyen TV, Abogadie FC *et al.* (2012). Sites of action of ghrelin receptor ligands in cardiovascular control. *Am J Physiol* 303: H1011–H1021.
- Camiña JP (2006). Cell biology of the ghrelin receptor. *J Neuroendocrinol* 18: 65–76.
- Carpino PA, Lefker BA, Toler SM, Pan LC, Hadcock JR, Cook ER *et al.* (2003). Pyrazolinone-piperidine dipeptide growth hormone secretagogues (GHSs): discovery of capromorelin. *Bioorg Med Chem* 11: 581–590.

- Chollet C, Meyer K, Beck-Sickinger AG (2009). Ghrelin – a novel generation of anti-obesity drug: design, pharmacomodulation and biological activity of ghrelin analogues. *J Pept Sci* 15: 711–730.
- Chollet C, Bergmann R, Pietzsch J, Beck Sickinger AG (2012). Design, evaluation and comparison of ghrelin receptor agonists and inverse agonists as suitable radiotracers for PET imaging. *Bioconj Chem* 23: 771–784.
- Chopin L, Walpole C, Seim I, Cunningham P, Murray R, Whiteside E *et al.* (2011). Ghrelin and cancer. *Mol Cell Endocrinol* 340: 65–69.
- Collister JP, Osborn JW (1999). The chronic infusion of hexamethonium and phenylephrine to effectively clamp sympathetic vasomotor tone: a novel approach. *J Pharmacol Toxicol Methods* 42: 135–147.
- DeBoer MD (2011). Ghrelin and cachexia: will treatment with GHSR-1a agonists make a difference for patients suffering from chronic wasting syndromes? *Mol Cell Endocrinol* 340: 97–105.
- Delhanty PJD, van Koetsveld PM, Gauna C, van de Zande B, Vitale G, Hofland LJ *et al.* (2007). Ghrelin and its unacylated isoform stimulate the growth of adrenocortical tumor cells via an anti-apoptotic pathway. *Am J Physiol* 293: E302–E309.
- Delhanty PJD, Neggers SJ, van der Lely AJ (2012). Ghrelin: the differences between acyl- and des-acyl ghrelin. *Eur J Endocrinol* 167: 601–608.
- Ejskjaer N, Dimcevski G, Wo J, Hellström PM, Gormsen LC, Sarosiek I *et al.* (2010). Safety and efficacy of ghrelin agonist TZP-101 in relieving symptoms in patients with diabetic gastroparesis: a randomized, placebo-controlled study. *Neurogastroenterol Motil* 22: 1069–e281.
- Esler WP, Rudolph J, Claus TH, Tang W, Barucci N, Brown S-E *et al.* (2007). Small-molecule ghrelin receptor antagonists improve glucose tolerance, suppress appetite, and promote weight loss. *Endocrinology* 148: 5175–5185.
- Ferens DM, Chang EC, Bogeski G, Shafton AD, Kitchener PD, Furness JB (2005). Motor patterns and propulsion in the rat intestine *in vivo* recorded by spatio-temporal maps. *Neurogastroenterol Motil* 17: 714–720.
- Ferens DM, Yin L, Bron R, Hunne B, Ohashi-Doi K, Sanger GJ *et al.* (2010). Functional and *in situ* hybridisation evidence that preganglionic sympathetic vasoconstrictor neurons express ghrelin receptors. *Neuroscience* 166: 671–679.
- Ferens DM, Habgood MD, Saunders NR, Tan YH, Brown DJ, Brock JA *et al.* (2011). Stimulation of defecation in spinal cord-injured rats by a centrally acting ghrelin receptor agonist. *Spinal Cord* 49: 1036–1041.
- Furness JB, Hunne B, Matsuda N, Yin L, Russo D, Kato I *et al.* (2011). Investigation of the presence of ghrelin in the central nervous system of the rat and mouse. *Neuroscience* 193: 1–9.
- Garcia JM, Friend J, Allen S (2013). Therapeutic potential of anamorelin, a novel, oral ghrelin mimetic, in patients with cancer-related cachexia: a multicenter, randomized, double-blind, crossover, pilot study. *Support Care Cancer* 21: 129–137.
- Gauna C, Delhanty PJD, Hofland LJ, Janssen JAMJL, Broglio F, Ross RJM *et al.* (2005). Ghrelin stimulates, whereas des-octanoyl ghrelin inhibits, glucose output by primary hepatocytes. *J Clin Endocrinol Metab* 90: 1055–1060.
- Gauna C, van de Zande B, van Kerkwijk A, Themmen APN, van der Lely AJ, Delhanty PJD (2007). Unacylated ghrelin is not a functional antagonist but a full agonist of the type 1a growth hormone secretagogue receptor (GHS-R). *Mol Cell Endocrinol* 274: 30–34.
- Granata R, Settanni F, Julien M, Nano R, Togliatto G, Trombetta A *et al.* (2012). Des-acyl ghrelin fragments and analogues promote survival of pancreatic β -cells and human pancreatic islets and prevent diabetes in streptozotocin-treated rats. *J Med Chem* 55: 2585–2596.
- Hassouna R, Labarthe A, Zizzari P, Videau C, Culler M, Epelbaum J *et al.* (2013). Actions of agonists and antagonists of the ghrelin/GHS-R pathway on GH secretion, appetite, and cFos activity. *Front Endocrinol* 4: 1–9.
- Hirayama H, Shiina T, Shima T, Kuramoto H, Takewaki T, Furness JB *et al.* (2010). Contrasting effects of ghrelin and des-acyl ghrelin on the lumbo-sacral defecation center and regulation of colorectal motility in rats. *Neurogastroenterol Motil* 22: 1124–1131.
- Hoveyda HR, Marsault E, Gagnon R, Mathieu AP, Vézina M, Landry A *et al.* (2011). Optimization of the potency and pharmacokinetic properties of a macrocyclic ghrelin receptor agonist (Part I): development of ulimorelin (TZP-101) from hit to clinic. *J Med Chem* 54: 8305–8320.
- How JMY, Pumpa TJ, Sartor DM (2013). The circulatory and renal sympathoinhibitory effects of gastric leptin are altered by a high fat diet and obesity. *Auton Neurosci* 117: 95–100.
- Isgaard J, Granata R (2011). Ghrelin in cardiovascular disease and atherogenesis. *Mol Cell Endocrinol* 340: 59–64.
- Julien M, Kay RG, Delhanty PJD, Allas S, Granata R, Barton C *et al.* (2012). *In vitro* and *in vivo* stability and pharmacokinetic profile of unacylated ghrelin (UAG) analogues. *Eur J Pharm Sci* 47: 625–635.
- Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: Reporting *in vivo* experiments: the ARRIVE guidelines. *Br J Pharmacol* 160: 1577–1579.
- Kojima M, Kangawa K (2010). Ghrelin: more than endogenous growth hormone secretagogue. *Ann N Y Acad Sci* 1200: 140–148.
- Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402: 656–660.
- Li L, Zhang L-K, Pang Y-Z, Pan C-S, Qi Y-F, Chen L *et al.* (2006). Cardioprotective effects of ghrelin and des-octanoyl ghrelin on myocardial injury induced by isoproterenol in rats. *Acta Pharmacol Sin* 27: 527–535.
- McDonald DM (1983). Morphology of the rat carotid sinus nerve. I. Course, connections, dimensions and ultrastructure. *J Neurocytol* 12: 345–372.
- McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. *Br J Pharmacol* 160: 1573–1576.
- Matsumura K, Tsuchihashi T, Fujii K, Abe I, Iida M (2002). Central ghrelin modulates sympathetic activity in conscious rabbits. *Hypertension* 40: 694–699.
- Mulvany MJ, Halpern W (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res* 41: 19–26.
- Myers PR, Minor RL, Jr, Guerra R, Jr, Bates JN, Harrison DG (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. *Nature* 345: 161–163.
- Nagaya N, Kojima M, Uematsu M, Yamagishi M, Hosoda H, Oya H *et al.* (2001). Hemodynamic and hormonal effects of human ghrelin in healthy volunteers. *Am J Physiol* 280: R1483–R1487.
- Nass R, Gaylinn BD, Thorner MO (2011). The ghrelin axis in disease: potential therapeutic indications. *Mol Cell Endocrinol* 340: 106–110.

- Okumura H, Nagaya N, Enomoto M, Nakagawa E, Oya H, Kangawa K (2002). Vasodilatory effect of ghrelin, an endogenous peptide from the stomach. *J Cardiovasc Pharmacol* 39: 779–783.
- Ross G, Chaudhuri G, Ignarro LJ, Chyu KY (1991). Acetylcholine vasodilation of resistance vessels *in vivo* may not entirely depend on newly synthesized nitric oxide. *Eur J Pharmacol* 195: 291–293.
- Salomé N, Haage D, Perrissoud D, Moulin A, Demange L, Egcioglu E *et al.* (2009). Anorexigenic and electrophysiological actions of novel ghrelin receptor (GHS-R1A) antagonists in rats. *Eur J Pharmacol* 612: 167–173.
- Seim I, Josh P, Cunningham P, Herington A, Chopin L (2011). Ghrelin axis genes, peptides and receptors: recent findings and future challenges. *Mol Cell Endocrinol* 340: 3–9.
- Shimizu Y, Chang EC, Shafon AD, Ferens DM, Sanger GJ, Witherington J *et al.* (2006). Evidence that stimulation of ghrelin receptors in the spinal cord initiates propulsive activity in the colon of the rat. *J Physiol (Lond)* 576: 329–338.
- Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y *et al.* (1996). The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J Cardiovasc Pharmacol* 28: 703–711.
- Thompson NM, Gill DAS, Davies R, Loveridge N, Houston PA, Robinson ICAF *et al.* (2004). Ghrelin and des-octanoyl ghrelin promote adipogenesis directly *in vivo* by a mechanism independent of the type 1a growth hormone secretagogue receptor. *Endocrinology* 145: 234–242.
- Toshinai K, Yamaguchi H, Sun Y, Smith RG, Yamanaka A, Sakurai T *et al.* (2006). Des-acyl ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor. *Endocrinology* 147: 2306–2314.
- Tsubota Y, Owada-Makabe K, Yukawa K, Maeda M (2005). Hypotensive effect of des-acyl ghrelin at nucleus tractus solitarius of rat. *Neuroreport* 16: 163–166.
- Van Der Lely AJ, Tschöp M, Heiman ML, Ghigo E (2004). Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocr Rev* 25: 426–457.
- Warzecha Z, Dembinski A (2012). Protective and therapeutic effects of ghrelin in the gut. *Curr Med Chem* 19: 118–125.
- White HK, Petrie CD, Landschulz W, MacLean D, Taylor A, Lyles K *et al.* (2009). Effects of an oral growth hormone secretagogue in older adults. *J Clin Endocrinol Metab* 94: 1198–1206.
- Witherington J, Abberley L, Briggs MA, Collis K, Dean DK, Gaiba A *et al.* (2008). Potent achiral agonists of the growth hormone secretagogue (ghrelin) receptor. Part 2: lead optimisation. *Bioorg Med Chem Lett* 18: 2203–2205.
- Zygmunt PM, Ryman T, Högestätt ED (1995). Regional differences in endothelium-dependent relaxation in the rat: contribution of nitric oxide and nitric oxide-independent mechanisms. *Acta Physiol Scand* 155: 257–266.