Sites of action of ghrelin receptor ligands in cardiovascular control

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Circulating ghrelin reduces blood pressure, but the mechanism for this action is unknown. This study investigated whether ghrelin has direct vasodilator effects mediated through the growth hormone secretagogue receptor 1a (GHSR1a) and whether ghrelin reduces sympathetic nerve activity. Mice expressing enhanced green fluorescent protein under control of the promoter for growth hormone secretagogue receptor (GHSR) and RT-PCR were used to locate sites of receptor expression. Effects of ghrelin and the nonpeptide GHSR1a agonist capromorelin on rat arteries and on transmission in sympathetic ganglia were measured in vitro. In addition, rat blood pressure and sympathetic nerve activity responses to ghrelin were determined in vivo. In reporter mice, expression of GHSR was revealed at sites where it has been previously demonstrated (hypothalamic neurons, renal tubules, sympathetic preganglionic neurons) but not in any artery studied, including mesenteric, cerebral, and coronary arteries. In rat, RT-PCR detected GHSR1a mRNA expression in spinal cord and kidney but not in the aorta or in mesenteric arteries. Moreover, the aorta and mesenteric arteries from rats were not dilated by ghrelin or capromorelin at concentrations >100 times their EC50 determined in cells transfected with human or rat GHSR1a. These agonists did not affect transmission from preganglionic sympathetic neurons that express GHSR1a. Intravenous application of ghrelin lowered blood pressure and decreased splanchic nerve activity. It is concluded that the blood pressure reduction to ghrelin occurs concomitantly with a decrease in sympathetic nerve activity and is not caused by direct actions on blood vessels or by inhibition of transmission in sympathetic ganglia.

Ghrelin, a 28-amino acid acylated peptide hormone, was purified and identified from rat stomach in 1999 (23). The ghrelin-producing cells contain ghrelin O-acyltransferase, which acylates the peptide before its secretion (16, 43, 53, 59). Nevertheless, both ghrelin and its unacylated form, desacyl ghrelin, are found in the circulation. The only identified receptor for ghrelin is the growth hormone secretagogue receptor (GHSR), which does not respond to desacyl ghrelin (23), although other ghrelin receptors probably exist (49). GHSRS were identified before the discovery of ghrelin and its unacylated form, desacyl ghrelin (23), although other ghrelin receptors probably exist (49). The GHSR receptor was cloned from the pituitary and hypothalamus of swine and human with two cDNAs being identified, one that encoded 366 amino acids for a classical full-length G protein-coupled receptor with seven transmembrane domains (GHSR1a) and an unspliced transcript that encodes a protein (GHSR1b) that terminates after the fifth transmembrane domain (18). Although acyl ghrelin, but not desacyl ghrelin, is an agonist at GHSR1a expressed in growth hormone-secreting cells and at cells transfected with GHSR1a (23), there is convincing evidence that desacyl ghrelin is a bioactive compound at other sites (2, 4, 54, 55). The desacyl ghrelin receptor has not been identified.

In addition to its roles in stimulating growth hormone release and in energy balance, ghrelin appears to have beneficial cardiovascular effects, with plasma concentrations in humans being negatively associated with both systolic and diastolic blood pressure (41). Ghrelin that is administered into the peripheral circulation decreases blood pressure (35, 36), but the mechanism of this depressor effect is not known. At least four possibilities can be considered: a direct vasodilator effect on blood vessels, an inhibition of transmission in vasoconstrictor pathways, an effect on afferent nerve endings of blood pressure control pathways, or an effect at central nervous system (CNS) sites. In relation to a direct effect on blood vessels, some authors have reported that ghrelin, desacyl ghrelin, or ghrelin receptor ligands are potent dilators of blood vessels in vitro (22, 33, 50, 57), whereas others have reported vasoconstriction in response to ghrelin (8, 9, 15, 40). GHSR1a receptors are expressed by preganglionic neurons of vasoconstrictor pathways, but not by postganglionic neurons (10, 12). Thus ghrelin could lower blood pressure if it inhibited transmission from preganglionic neurons, a possibility that does not appear to have been previously investigated. Other gut-derived hormones, such as cholecystokinin and leptin, lower blood pressure by acting on afferent nerve endings (44, 46). Stimulation of cardiac afferents by ghrelin has been suggested to explain how this agent prevents the rise in cardiac sympathetic nerve activity that is produced by cardiac infarction (52). Furthermore, ghrelin administered directly into the brain stem lowers blood pressure and reduces activity of sympathetic nerves (29, 31); access of circulating ghrelin to these brain stem sites could explain the lowering of blood pressure.

In view of the contrasting effects reported in the literature regarding the cardiovascular actions of ghrelin on blood vessels, we have reinvestigated a possible role of direct actions on blood vessels in lowering blood pressure and have examined GHSR expression in blood vessels. We have also investigated...
whether the hypotensive effect of peripherally administered ghrelin may be explained by a reduction in ongoing sympathetic nerve activity or by inhibition of transmission through sympathetic ganglia.

**MATERIALS AND METHODS**

Male 8- to 9-wk-old Sprague-Dawley rats, mice with a reporter protein gene inserted upstream of the coding sequence for GHSR, and wild-type C57BL/6 mice were used. All procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee and by the Austin Health Animal Ethics Committee. All experimental procedures performed abided by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

**Blood Vessel Responses**

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 physiological saline with the following composition (in mM): 150.6 Na⁺, 4.7 K⁺, 2 Ca²⁺, 1.2 Mg²⁺, 144.1 Cl⁻, 1.3 HPO₄²⁻, 16.3 HCO₃⁻, and 7.8 glucose. This solution was gassed with 95% O₂ plus 5% CO₂.

**Wire myography.** Segments of mesenteric artery or thoracic aorta (~1.5 mm long) were mounted isometrically between stainless steel wires (40 μm diameter) in a four-chamber myograph (Multi Myograph 610M; Danish Myo Technology). 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 (34). Initially, the vessels were stretched until an effective transmural pressure of 13.3 kPa (34). After equilibration for 30 min, vessels were activated with phenylephrine (PE; 1 μM for aortas; 10 μM for mesenteric arteries) and, after the contraction had plateaued, ghrelin or capromorelin was applied. In all tissues, the level of stretch was not changed, whereas in mesenteric arteries the circumference was adjusted to 90% of that determined at 13.3 kPa (34). After equilibration for 30 min, vessels were activated with phenylephrine (PE; 1 μM for aortas; 10 μM for mesenteric arteries) and, after the contraction had plateaued, ghrelin or capromorelin was applied. In all tissues, the viability of the endothelium was confirmed by relaxation to the muscarinic agonist, carbachol (1 μM), that was applied at the end of the experiment, after ghrelin or capromorelin. The output from the myograph was recorded and analyzed using a PowerLab data acquisition system and the program Chart (ADInstruments, Bella Vista, NSW, Australia). The percentage changes in amplitude of PE-induced contraction produced by ghrelin or capromorelin were compared with those measured at the same time points in arteries treated with PE alone.

**Pressure myography.** Segments of mesenteric arteries (~5 mm in length) were mounted in a pressure myograph, with the proximal end attached to a glass cannula containing physiological saline and, after filling the vessel with this solution, its distal end was tied off. The vessels were superfused with physiological saline at ~36°C, and the internal pressure was controlled with a servo-controlled pump (Living Systems, Burlington, VT). The myograph was mounted on an inverted microscope and viewed on a monitor screen via a 10× objective and a video camera, and the internal diameter was measured. After confirming that there were no leaks, the pressure was slowly raised to 80 mmHg, and the vessels were then allowed to equilibrate for 30 min. Arteries were activated with PE (1 μM) applied to the superfusing solution, and in the continued presence of this agent responses to ghrelin or capromorelin, also added to the superfusing solution, were assessed. In all vessels, the viability of the endothelium was confirmed by vasodilation to carbachol (1 μM). The percentage change in the PE-induced constriction produced by each of the agents was calculated.

**Perfused mesenteric vascular bed.** The abdominal cavity of the rat was opened, and the proximal end of the superior mesenteric artery was identified, cleaned of connective tissue, and cannulated. After its removal from the abdominal cavity, the mesenteric vascular bed was separated from the gut by carefully cutting close to the intestinal wall. The preparation was then placed in a humid chamber warmed to ~36°C and perfused at a constant flow rate of 4.5 ml/min with physiological saline containing 0.1% BSA. Vascular responses were measured as changes in perfusion pressure (mmHg) detected by a pressure transducer attached to the infusion line and recorded using a PowerLab data acquisition system. After allowing the preparation to equilibrate for 30 min, it was perfused with a solution containing PE (80 μM) and, when the increase in perfusion pressure had plateaued, ghrelin, desacyl ghrelin, or capromorelin were added to the perfusate at 3- to 4-min intervals. After testing these agents, carbachol (1 μM) was added to the perfusate to confirm that the endothelium was viable. The percentage change in the PE-induced pressor response produced by the various agents was calculated.

**Cloning, Cell Culture, and Transfection of HEK Cells**

cDNA for human GHSR1α (accession no. NM_032075) was synthesized (Genescript), and cDNA for rat GHSR1α (NM_198407) was obtained from the Missouri S&T cDNA resource centre. These cDNAs were cloned into pcDNA5FRT/TO (Invitrogen, Melbourne, Australia). This plasmid contains an operator sequence, that binds a tetracycline-sensitive repressor, between the promoter and the cDNA cloning site. Receptors were stably cloned into the HEK-TRex cell line (which express a tetracycline-sensitive repressor) according to the manufacturer’s instructions (Invitrogen) by cotransfection with plasmid pOG44 and selection in hygromycin (100 μg/ml) for 3 wk. Stable cell lines expressing human GHSR1α or rat GHSR1α were maintained in Dulbecco’s modified Eagle’s medium (Sigma Aldrich, Sydney, Australia) supplemented with 10% FBS and hygromycin (100 μg/ml).

**Intracellular Ca²⁺ Measurements**

Intracellular Ca²⁺ levels in cell populations were measured by fluorescence using a Flexstation (Molecular Devices, Sunnyvale, CA). HEK293 cells expressing rat GHSR1α or human GHSR1α were plated in 96-well plates (Corning, NY) at an approximate density of 40,000 cells/well and grown for 48 h. Receptor expression was induced by adding 5 μg/ml tetracycline 4 h before 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) at 37°C. Loading and experiments were performed in HEPES buffer (in mM: 138 NaCl, 5 KCl, 1.2 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES; pH 7.4). After being washed two times with assay buffer, 80 μl assay buffer were 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 ~15 s, 20 μl of agonist were added to cells at five times the final concentration. EC₅₀ values were determined by nonlinear regression using Prism 5.0 software (GraphPad Software, San Diego, CA).

**Arterial Pressure and Splanchnic Nerve Recording**

Experiments were carried out in anesthetized and artificially ventilated male Sprague-Dawley rats (300–400 g), as previously described (46). The jugular vein was cannulated for intravenous administration of drugs and the left carotid artery for arterial pressure (AP) and heart rate (HR) measurement. For sympathetic nerve recordings, the left splanchnic nerve was isolated via a retroperitoneal approach, placed on a silver wire electrode, and embedded in silicone sealant. The incision was closed, and the wires were externalized. Nerve signals were amplified (30–3,000 Hz bandpass), full-wave rectified, and averaged over 1-s intervals. Splanchnic sympathetic nerve discharge, AP, and HR were stored and analyzed using a CED data acquisition system and Spike-2 software (Cambridge Electronic Design, Cambridge, UK).
Detection of Ghrelin Receptor Gene Expression

Total RNA was extracted from freshly dissected rat spinal cord, kidney, aorta, mesentery, and HEK293 cells expressing rGHSR1a using the RNeasy Mini Kit (Qiagen, Melbourne, Australia) and was reverse transcribed using Superscript III reverse transcriptase (Invitrogen). Rat GHSR1a mRNA was amplified by polymerase chain reaction (PCR) using 5PRIME MasterMix (5PRIME, Hamburg, Germany) with intron-spanning primers. The forward primer for rat GHSR1a (accession no. NM 032075) was TGGAAACGCGACCC-CCAGCGA, and the reverse primer was AGCAGAGGATGAA-GTCGTCGTCGACAACG-GCTC and the reverse CAAACATGATCTGGGTCATCTTCTC, producing a band of 353 bp. Touchdown amplification was performed with an initial step of 95°C for 5 min, followed by 15 cycles of 95°C for 40 s, annealing temperatures starting at 70°C for 30 s (decreasing by 1°C/cycle), and 72°C for 45 s for extension. This step was followed by 35 cycles of 95°C for 40 s, 50°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min. Reactions were also conducted without reverse transcriptase, which confirmed the absence of genomic DNA.

Localization Studies

Reporter mice, which have been previously validated for localizing sites of expression of the GHSR protein in the hypothalamus, spinal cord, and kidney (13, 56), were used. These GHSR reporter mice were obtained from the Mutant Mouse Regional Resource Center (MMRRC) at the University of California, Davis (www.mmrrc.org/strains/30942/030942.html). The mice are described by the Gene Expression Nervous System Atlas (GENSAT; www.gensat.org). They express enhanced green fluorescent protein (EGFP) under control of the promoter for GHSR. The EGFP reporter gene is inserted immediately upstream of the coding sequence for the receptor gene. Mice were genotyped using tail snips. Genomic DNA was isolated by standard methods. The EGFP gene was identified by PCR using 5PRIME MasterMix (5PRIME) with forward primer GGACCTCCTC-CCAGCGA, and the reverse primer was AGCAGAGGATGAA-GTCGTCGTCGACAACG-GCTC and the reverse CAAACATGATCTGGGTCATCTTCTC, producing a band of 330-bp product.

To prepare tissue for immunohistochemistry, the mice were anesthetized with a mixture of ketamine hydrochloride (100 mg/kg) and xylazine (20 mg/kg), both from Troy Laboratories (Sydney, Australia), and a perfusion needle was inserted in the left ventricle. The right atrium was cut open, and the animal was perfused with a mixture of 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.2. After perfusion was completed, the specimens were postfixed in the same fixative overnight at 4°C. Fixative was washed out with by 6 × 10 min washes in PBS (0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2), and tissues were placed in PBS-sucrose-azide (PBS containing 30% sucrose, as a cryoprotectant, plus 0.1% sodium azide) and stored at 4°C overnight. The following day, tissue was transferred to a mixture of PBS-sucrose and optimum cutting temperature (OCT) compound (Tissue Tek, Elkhart, IN) in a ratio of 1:1 for a further 24 h before being embedded in 100% OCT. The next day, cryostat sections of 12 μm thickness were cut. Sections were then incubated with goat anti-GFP raised against the full-length GHSR protein and a mixture of 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium azide) and stored at 4°C overnight, before application of labeled secondary antibodies for 1 h. Sections of celiac ganglion were double stained for neuromedin Y, as previously described (12). Fluorescence was visualized using a Zeiss AxioImager microscope or a Zeiss Meta laser confocal microscope. Differential interference contrast images were taken using the AxioImager microscope.

Electrophysiological Recording

Wild-type mice (C57BL6, male) were killed by dislocating the cervical spine and severing their carotid arteries. The celiac ganglia with attached splanchnic nerves were dissected in oxygenated (95% O₂–5% CO₂) physiological saline of the following composition (in mM): 118 NaCl, 4.8 KCl, 25 NaHCO₃, 1.0 NaH₂PO₄, 1.2 MgSO₄, 11.1 glucose, and 2.5 CaCl₂. The current-clamp technique was used for intracellular recording from neurons in the right lobe of the celiac ganglia. Glass microelectrodes used for recording were filled with 1.0 M KCl and had resistances in the range of 100–150 MΩ. Signals were recorded with an AxoClamp 900A amplifier (Axon Instruments, Foster City, CA), were digitized at 10 or 20 kHz with an Axon Digitida 1440A, and stored using PC-based data acquisition software (Clampex 10.2; Axon Instruments).

The right splanchnic nerve was stimulated via a suction stimulating electrode. Electrical stimuli were generated and controlled by a Grass S8800 stimulator that was combined with a Grass SIU5 stimulus isolation unit. Amplitudes of stimuli were from 10 to 50 V, 0.1 ms pulse width, delivered at 1 stimulus/min.

Reagents

The following compounds were used: capromorelin (CP424391) from Pfizer Pharmaceuticals (Sandwich, UK) and synthesized by us: carbachol (carbamylcholine chloride), PE hydrochloride (from Sigma Aldrich), rat ghrelin (Auspex, Melbourne, Victoria, Australia), rat desacyl ghrelin (GL Biochem), and UK-14304 (Tocris, Bristol, UK).

Statistics

Data are presented as means ± SE. Means were compared by two-tailed paired or unpaired Student’s t-tests. A P value of <0.05 was considered significant. Concentration-response relationships were curve fitted by nonlinear regression analysis using GraphPad Prism 5.0 (GraphPad Software).

RESULTS

Ghrelin Receptor Ligands did not Inhibit Mechanical Responses of Arterial Vessels

To investigate the actions of ghrelin receptor ligands on vascular responses, we first examined the effects of ghrelin and capromorelin on isometrically mounted segments of rat mesenteric arteries contracted with PE (10 μM). Compared with vessels treated with PE alone, neither ghrelin (0.01–1 μM) nor capromorelin (0.1–1 μM) significantly changed the PE-induced contraction (Fig. 1, A–C). This was the case even during prolonged periods of application (see Fig. 1D). The functional integrity of the endothelium in these vessels was confirmed because carbachol (1 μM) produced a >80% reduction in the PE-induced contraction. The concentration of PE used to constrict the vessels (10 μM) caused close to maximum constriction. To ensure that this was not an insurmountable constriction, the experiments were repeated using PE at 1 μM. Ghrelin also had no vasodilator effect on rat mesenteric arteries preconstricted with this lower concentration of PE (constriction in the presence of 0.1 μM ghrelin plus PE was 95 ± 7% of 1 μM PE alone, n = 3). Capromorelin (1 μM) had no effect on rat mesenteric arteries preconstricted with 1 μM PE (n = 3).

Because expression of GHSR1a using intron-spanning primers has been reported in cultured aortic smooth muscle cells from humans (42), we also examined the effects of ghrelin on...
Fig. 1. Ghrelin and the nonpeptide ghrelin agonist capromorelin (Capro) do not elicit vascular responses in rat mesenteric arteries or aorta mounted in wire myographs. A: representative trace showing the contractile response to phenylephrine (PE; 10 μM) and the lack of effect of ghrelin (0.1 μM), Bars indicate the time of addition and duration of the exposures to PE and ghrelin. B: percentage change in PE-induced contractions measured in mesenteric arteries between the start and end of the exposures to ghrelin (0.01–1 μM) and between the same time points in vessels treated with PE alone (time control). The vessels relaxed in response to carbachol (Carbac). C: percentage change in the PE-induced contraction produced by capromorelin (0.1–10 μM) in mesenteric arteries. D: percentage change in the PE-induced contraction produced by ghrelin (0.1 and 1 μM) in segments of aorta. In B, C, and D, the no. in parentheses above each data group is the no. of experiments for that group.

Ghrelin and Capromorelin Mobilize Intracellular Ca$^{2+}$ in Transfected Cells

Ca$^{2+}$ mobilization is the best-characterized intracellular change associated with GHSR1a activation (39). Therefore, to test the effectiveness of the compounds that were used without effect in the experiments on isolated blood vessels, their activity was assessed using Ca$^{2+}$ mobilization assays in HEK293 cells expressing human or rat GHSR1a. Ghrelin stimulated intracellular Ca$^{2+}$ mobilization with EC$_{50}$ values of 6.2 and 3.5 nM in cells expressing human or rat GHSR1a, respectively (Fig. 3, A and C). These EC$_{50}$ values are in good agreement with those previously published for ghrelin at human GHSR1a expressed in CHO cells (23). Capromorelin was a more potent agonist than ghrelin for mobilizing Ca$^{2+}$ (EC$_{50}$ was 0.46 nM in the human GHSR1a cell line and 0.13 nM in the rat GHSR1a cell line; Fig. 3, B and D).

GHSR1a Receptor Agonists did not Inhibit Transmission in Sympathetic Ganglia

GHSR1a is expressed by sympathetic preganglionic neurons of vasoconstrictor pathways but not by postganglionic vasomotor neurons (Fig. 4B, see DISCUSSION). Thus a possible peripheral action that could lower blood pressure is inhibition of transmitter release from the preganglionic nerve endings. We tested this possibility by recording from neurons in the lateral parts of the celiac ganglia, where the cell bodies of postganglionic neurons that supply the splanchnic vasculature are concentrated.
Ghrelin Reduced Both Sympathetic Nerve Activity and Blood Pressure In Vivo

Ghrelin, 10 µg/kg, infused into the jugular vein of anesthetized rats over a 60-s period, compared with a saline infusion, caused a significant reduction in integrated sympathetic nerve activity by 11.7 ± 4.2% (P < 0.05, paired t-test) and mean AP by 5.1 ± 1.9 mmHg (P < 0.05, paired t-test) compared with saline infusion (1.5 ± 3.0% and −1.1 ± 1.8 mmHg, respectively). Traces from these experiments are shown in Fig. 5. HR was reduced by 4.8 ± 2.3% (15.4 ± 7.2 beats/min), but this was not statistically significant when compared with saline infusion (0.2 ± 2.4%; −0.5 ± 7.9 beats/min). Blood pressure and nerve activity decreased with similar time courses, beginning about 1 min after ghrelin administration. The decrease continued for 10–20 min after the bolus of ghrelin was injected.

**GHSR Expression was not Detected in Blood Vessels but Occurred in Other Tissues from Reporter Mice**

Sections through the renal vessels (Fig. 6, A and B), cerebral vessels (Fig. 6C), coronary arteries (Fig. 6D), aorta (Fig. 7A), mesenteric arteries (Fig. 7B), cutaneous vessels, femoral artery, and muscle arteries of the hindlimb failed to reveal GHSR expression in any component of the vascular wall, including lack of receptor in vascular endothelium and smooth muscle cells. Nevertheless, GHSR expression was revealed in other tissue elements. Visceral nerve bundles containing immunoreactive nerve fibers were observed adjacent to the aorta (Fig. 7A). Positive labeling was also observed in distal tubules of the kidney in the same microscope fields as renal arteries and in nerve cells in the hypothalamus in sections that included cerebral arteries (Fig. 6). Each of these is a known site of GHSR expression.

Stimulation of presynaptic inputs elicited fast excitatory postsynaptic potentials (EPSPs) in all neurons. To study the effect of GHSR1a receptor agonists on synaptic transmission, a negative current was injected to hold the cell membrane around −100 mV, to better reveal the fast cholinergic EPSPs, without them initiating action potentials. Capromorelin was used at concentrations of 1, 10, and 100 nM and ghrelin at 10 and 100 nM. There were no changes in shapes or amplitudes of fast EPSP during exposure to the agonists at any concentration, or after the agonists were washed out (Fig. 4, A and C). α2-Adrenoreceptor agonists inhibit excitatory transmitter release in sympathetic ganglia (32). Therefore, as a positive control, we used the agonist of α2-adrenoreceptors, UK-14304, 1 µM. The amplitudes of fast EPSPs before UK-14304 was added were 26.1 ± 5.3 mV and in the presence of UK-14304 amplitudes were 15.0 ± 4.8 mV, a significant difference (P < 0.01, t-test, 2-tail, n = 5). For all neurons tested, UK-14304 reduced fast EPSP amplitude.

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Another site of expression that was discovered was endothelial cells within the heart (Fig. 6E). These were prominent on the mitral, tricuspid, aortic, and pulmonary valves and in the lining of the right atrium. Positive nerve fibers were found in cardiac ganglia (Fig. 6F). These nerve fibers are probably of vagal origin; neurons in the nucleus ambiguus, where cell bodies of vagal neurons to the heart are located, contain GHSR1a message (60), whereas cardiac sympathetic postganglionic neurons do not express the receptor (12).

**GHRS1a Receptor Expression Detected by RT-PCR was not Found in Rat Arteries**

GHRS1a mRNA was detected using intron-spanning primers in tissue extracts from rat spinal cord and kidney (Fig. 8). HEK cells expressing rat GHRS1a were used as a positive control for the GHRS1a primers. The products had the predicted molecular sizes and were not observed in control reactions with reverse transcriptase omitted. There was no detectable GHRS1a expression in tissue extracts.
were conducted for each analysis (Fig. 8). At least three independently collected samples of rat tissue were used for each study. PCR products also failed to detect GHSR1a. At all rat samples, indicating the quality of the starting template was good.

We recorded was about one-half that observed in response to iv vehicle (saline) or ghrelin (10 μg/kg). Arrows indicate the times of injection of vehicle (A) or ghrelin (B). Ghrelin significantly reduced both sympathetic nerve discharge and blood pressure. From rat aorta or mesenteric vessels. β-Actin was detected in all rat samples, indicating the quality of the starting template was good. Reamplification of a 10-fold dilution of aorta and mesenteric PCR products also failed to detect GHSR1a. At least three independently collected samples of rat tissue were conducted for each analysis (Fig. 8).

**DISCUSSION**

It is consistently observed that ghrelin administered by a peripheral route to humans (24–26, 35, 36) or to animals (20, 27, 31, 51) lowers blood pressure, an effect that we have confirmed in the current study. The decrease in blood pressure that we recorded was about one-half that observed in response to similar doses of ghrelin in conscious unrestrained Sprague-Dawley rats (20), indicating that responses were blunted by anesthesia.

There are four obvious sites at which peripherally administered ghrelin might act to lower blood pressure. It could act directly on blood vessels, either on the smooth muscle or on the vascular endothelium; it could act on pre- or postganglionic sympathetic vasoconstrictor neurons; it could act on autonomic neurons of vasomotor control circuits, similar to other gastrointestinal hormones that lower blood pressure; or it could cross the blood-brain barrier to act at central cardiovascular control centers. Each of these is considered below.

**Effects of Ghrelin and Ghrelin Mimetics on Blood Vessels**

We have used three methods to investigate whether ghrelin causes mesenteric arteries of the rat to dilate: wire myography, pressure myography, and perfusion of the entire mesenteric arterial vascular bed. We found no evidence that ghrelin caused vasodilatation of mesenteric arteries at concentrations up to 1 μM. Similarly, we found that ghrelin did not dilate isometrically mounted aortas. Despite this lack of responsiveness to ghrelin, the vessels dilated rapidly and strongly to the muscarinic agonist, carbachol, which indicates that the endothelium was functionally intact in our experiments. This failure to detect an effect of ghrelin cannot be attributed to the sample of ghrelin used because at 10 nM it near maximally activated both rat and human GHSR1a in cells transfected with these receptors. Thus the blood vessels are at least 100 times less responsive to ghrelin than is the authentic GHSR1a. The circulating concentration of ghrelin in humans is around 3 nM in the fasted state and 500 pM in the fed state (38), and in rats with free access to food it is about 100 pM (13). Therefore, it seems unlikely that arterial vessels are responsive to circulating concentrations of ghrelin. We also found that a small molecule GHSR1a agonist, capromorelin (3), was potent at GHSR1a but ineffective on arteries.

Two groups have reported vasodilator effects of ghrelin on isolated arteries. One group described a slowly developing vasodilation of endothelium-denuded, endothelin-constricted human internal mammary arteries (22, 57). However, both guinea pig renal arteries and human mesenteric arteries that were also constricted with endothelin responded to ghrelin with further constriction (8, 9). This excitatory effect of ghrelin did not depend on the presence of an intact endothelium, but it did depend on the contractile agent used to preconstrict the artery; ghrelin in concentrations up to 10 μM did not affect guinea pig renal arteries constricted with norepinephrine or with spontaneous tone (9). Coronary artery blood flow in rat and porcine heart is reduced by ghrelin, an effect that was also attributed to vasoconstriction, which was recorded directly from isolated coronary arteries (15, 40). The second group to report vasodilation investigated perfused rat mesenteric arteries constricted with PE (33). We have used the same technique as Moazed et al. (33), and two other techniques, and have failed to observe dilation of rat mesenteric arteries in response to ghrelin using concentrations up to 1 μM, whereas Moazed et al. (33) reported a rapid endothelium-dependent dilation in response to concentrations as low as 10 pM.

In the rat mesenteric artery, Moazed et al. (33) reported that desacyl ghrelin and the unacylated NH2-terminal tripeptide of ghrelin had a similar vasodilator potency to ghrelin. Similarly, desacyl ghrelin had similar vasodilator potency to ghrelin in human internal mammary arteries (22). Desacyl ghrelin does not activate GHSR1a, either in somatotrophs or in transfected cells (23), and the minimum sequence of ghrelin required to activate Chinese hamster ovary cells expressing GHSR1a is the acylated NH2-terminal tetrapeptide, which is about 500-fold less potent than ghrelin; the NH2-terminal tripeptide had no effect (30). Therefore, where described, the vasodilator effects of ghrelin do not appear to be mediated through activation of GHSR1a and may involve an as yet unknown ghrelin receptor. In the perfused mesenteric vascular bed, we demonstrated that desacyl ghrelin, like ghrelin, was without effect, and together our functional studies suggest that the vessels we tested lack functional ghrelin receptors.

Consistent with lack of functional evidence, we found no evidence for GHSR1a expression in arteries of any major vascular bed using a mouse with a fluorescent protein linked to GHSR expression, or by RT-PCR applied to mRNA from rat arteries. Other studies have described variable GHSR1a mRNA expression in vascular tissue and cultured vascular smooth muscle and endothelial cell lines using RT-PCR (14,
19, 58) and Western blot (28). However, many RT-PCR studies of GHSR1a gene expression have used non-intron-spanning primers that cannot distinguish between genomic DNA and cDNA for GHSR1a and GHSR1b as discussed by Gnanapavan et al. (14). Additionally, cultured smooth muscle and endothelial cells undergo phenotypic changes compared with freshly isolated cells (1). Therefore, expression in cultured cells does not necessarily reflect expression levels in intact...
vessels. The GHSR reporter revealed cells that have been shown to express functional receptors: sympathetic preganglionic neurons, distal renal tubules, and hypothalamic neurons, as previously published (12, 56), and the RT-PCR also revealed expression where functional GHSR1α is known to occur, spinal cord and kidney.

We conclude that ghrelin acting at GHSR1α or at a novel receptor does not cause dilation of rat blood vessels and that vasodilation through a direct action on blood vessels is unlikely to contribute to the action of ghrelin to lower blood pressure.

Effects of Ghrelin on Sympathetic Nerve Activity

The decrease in blood pressure produced by intravenously administered ghrelin occurred concomitantly with a decrease in splanchnic sympathetic nerve activity, suggesting that the depressor effect of ghrelin is mediated, at least in part, by a decrease in sympathetic nerve-mediated vasoconstriction. To our knowledge, this is the first demonstration that peripherally administered ghrelin reduces sympathetic nerve activity in normal rats. However, there are several reports that peripherally administered ghrelin reduces the elevated levels of cardiac sympathetic nerve activity that occur in rats following myocardial infarction (47, 48, 52). In humans, the reduction in blood pressure produced by intravenous infusion of ghrelin was associated with an increase in muscle sympathetic vasoconstrictor nerve activity (MSNA) (25). This effect on MSNA is most likely baroreceptor mediated (24), and it remains possible that ghrelin reduces sympathetic nerve activity to other vascular beds in humans. Ghrelin infusion did blunt increases in BP and MSNA produced by stress (25) and, following recovery of blood pressure, MSNA is less than control (24), suggesting that ghrelin does inhibit sympathetic nerve activity in humans.

Inhibition of Transmission in Sympathetic Ganglia

We have not found any published investigation of the effects of ghrelin on sympathetic ganglia. Its receptor, GHSR1α, is expressed by preganglionic sympathetic neurons, including preganglionic neurons of vasomotor pathways (10, 11). In GHSR reporter mice, the endings of these neurons are found in sympathetic ganglia, surrounding nerve cell bodies where they are deduced to form synapses (12). In the celiac ganglion, which we have used in the present study, the terminals appear to surround all neurons, including the cell bodies of postganglionic vasomotor neurons that are found in the lateral parts of the ganglia (12). In the CNS, ghrelin (100 nM) acts on presynaptic receptors to inhibit excitatory transmitter release (5). A similar action in sympathetic ganglia could lower blood pressure. However, no presynaptic effect of ghrelin was found. It thus appears that suppression of transmission in vasomotor pathways is unlikely to explain ghrelin’s effect in lowering blood pressure.

Action on Cardiovascular (Depressor) Afferents

Increased activity in carotid sinus nerves, aortic baroreceptors, and certain abdominal afferents decreases blood pressure. The gut hormones, cholecystokinin and leptin, activate subdiaphragmatic vagal afferents to induce a reflex response, resulting in a lowering of blood pressure and inhibition of splanchnic sympathetic nerve activity (45, 46). If ghrelin, another gut hormone, were to stimulate abdominal vagal endings, this might contribute to the lowering of blood pressure. However, ghrelin inhibits, rather than excites, vagal afferents (6, 37). Whether ghrelin acts on aortic or carotid sinus baroreceptor endings is unknown.

Materials and Methods

Detection of GHSR1α mRNA

Fig. 8. RT-PCR detection of GHSR1α. Detection of GHSR1α mRNA in extracts from spinal cord, kidney, aorta, and mesentery vessels. HEK293 cells expressing GHSR1α were included as a positive control for primer specificity. The bands were of the size predicted by the primers used for amplification, 816 bp. β-Actin was used as a positive control to indicate the integrity of RNA isolated from rat tissues (353-bp product). RT, reverse transcriptase; NTC, no template control; + and −, presence or absence, respectively, of reverse transcriptase.
Action within the CNS

Despite being a large peptide, ghrelin is found in some regions of the CNS after peripheral administration. For example, labeled ghrelin, injected intravenously, is found bound to neurons in the hippocampus, and functional data also indicate effects of peripherally administered ghrelin in the hippocampus (7). Ghrelin also enters the hypothalamus and other regions of cortex (7). Ghrelin that is injected in the cerebral ventricles, or directly into the nucleus tractus solitarius, lowers blood pressure and sympathetic nerve activity (29, 31). Administered at these central sites, it also reduces HR, which is also reported after intravenous administration in some animal studies (27, 31).

Thus access of ghrelin and action at critical sites in the CNS that affect blood pressure may provide the best explanation of the lowering of blood pressure.

An Indirect Effect Through a Renal Action of Ghrelin

In the kidney, the ghrelin receptor is localized to the distal nephron (56). However, an effect at this site to lower blood pressure is unlikely. When ghrelin is applied directly by renal interstitial infusion, distal tubule sodium reabsorption is increased, without affecting glomerular filtration rate or blood pressure (21).

In conclusion, ghrelin, administered peripherally, in most cases intravenously, lowers blood pressure. We have investigated whether this effect can be attributed to a direct action of ghrelin on blood vessels, and if it does act at blood vessels, to an action at GHSR1a receptors. We used three methods to investigate possible vasodilation and two methods to investigate GHSR1a expression in blood vessels. The results were negative, although positive controls confirmed the effectiveness of each method of investigation. Thus we conclude that the blood pressure-lowering effect is not due to a direct vascular action. Significant possibilities are that ghrelin activates baroreceptor afferents or it crosses the blood-brain barrier and acts on centers, such as the nucleus of the solitary tract, that control blood pressure.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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VASCULAR RESPONSES TO GHRELIN


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