Ghrelin acts on the dorsal vagal complex to stimulate pancreatic protein secretion

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**Running head:** Ghrelin stimulates pancreatic secretion

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ABSTRACT

Ghrelin receptors are present in the central nervous system. We hypothesize that ghrelin released from the stomach acts as an endocrine substance and stimulates brainstem vago-vagal circuitry to evoke pancreatic secretion. In an in vivo anesthetized rat model, intravenous infusion of ghrelin at doses of 5, 10, and 25 nmol increased pancreatic protein secretion from a basal level of $125 \pm 6$ to $186 \pm 8$, $295 \pm 12$, and $356 \pm 11$ mg/h, respectively. Pretreatment with atropine or hexamethonium, or acute vagotomy, but not perivagal application of capsaicin, completely abolished pancreatic protein secretion responses to ghrelin. In conscious rats, intravenous infusion of ghrelin at a dose of 10 nmol resulted in a 2.2-fold increase in pancreatic protein secretion over basal volume. Selective ablation of the area postrema abolished pancreatic protein secretion stimulated by intravenous infusion of ghrelin, but did not alter the increase in pancreatic protein secretion evoked by diversion of bile–pancreatic juice. Immunohistochemical staining showed a marked increase in the number of c-Fos–expressing neurons in the area postrema, nucleus of the solitary tract, and dorsal motor nucleus of the vagus after intravenous infusion of ghrelin in sham-lesioned rats; selective ablation of the area postrema eliminated this increase. In conclusion, ghrelin stimulates pancreatic secretion via a vagal cholinergic efferent pathway. Circulating ghrelin gains access to the brainstem vago-vagal circuitry via the area postrema, which represents the primary target on which peripheral ghrelin may act as an endocrine substance to stimulate pancreatic secretion.

Keywords: area postrema, pancreatic exocrine secretion, vagotomy
INTRODUCTION

Ghrelin is a novel acylated peptide localized in endocrine cells of the stomach and neurons of the hypothalamic arcuate nucleus. It acts as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (4, 5, 21). Intravenous (IV) infusion of ghrelin in rats induces growth hormone release by stimulating GHS-R at the pituitary (21). Intracerebroventricular (ICV) administration of ghrelin increases food intake and body weight (38,42). GHS-R is present in various regions of the brain (46), suggesting that ghrelin may have additional as yet undefined physiological functions (2). Evidence of preprandial elevation in ghrelin secretion in humans raised the possibility that ghrelin participates in digestive functions (4). ICV administration of ghrelin (0.6 nmol) has been shown to increase gastric acid secretion in anesthetized rats and at the same time to induce c-Fos expression in the neurons of the nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus (DMV) (7). Further studies suggested that IV administration of ghrelin stimulated gastric acid secretion and increased motility by acting through the vagus nerve pathway (33); however, precise sites of action have not been identified.

It is well known that postprandial pancreatic enzyme secretion is controlled mainly by the vagal-vagal reflex that activates cholinergic postganglionic neurons in the pancreas (24, 25, 27, 28). We hypothesized that ghrelin released from the stomach acts as an endocrine substance and stimulates brainstem vago-vagal circuitry to evoke pancreatic secretion. The effects of IV infusion of graded doses of ghrelin on pancreatic enzyme secretion were examined in anesthetized rats. Further, the mechanisms and sites responsible for the action of circulating ghrelin on pancreatic secretion were investigated. We showed that selective ablation of the area postrema (AP) (3) but not perivagal treatment with the sensory neurotoxin capsaicin abolished pancreatic secretion evoked by IV infusion of ghrelin. This supports the hypothesis that circulating ghrelin acts on the AP, a region in the dorsal vagal complex with an incomplete
blood-brain barrier, allowing ghrelin to enter the dorsal vagal complex and activate the brainstem neurocircuitry to stimulate pancreatic secretion. Immunohistochemical staining showing that IV infusion of ghrelin induced c-Fos expression in the AP, NTS, and DMV, which was eliminated by AP lesioning, further substantiated this hypothesis.

MATERIALS AND METHODS

Materials
Ghrelin, capsaicin, atropine, and hexamethonium were purchased from Sigma-Aldrich (St. Louis, MO); secretin and 5-HT were purchased from Peninsula Laboratories (member of the Bachem group, San Carlos, CA).

Animal preparation
Experiments were performed on adult male Sprague-Dawley rats (250–300 g) that were housed four per plastic cage. Animals were maintained on a 12:12 h light–dark cycle (lights on at 7 AM) and given access to food and water ad libitum. Experimental procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan. After an overnight fast, rats were anesthetized with an intramuscular injection of a mixture of xylazine and ketamine (13 and 87 mg/kg body wt, respectively). One third of the initial dose of anesthetic was given every 90 min to maintain surgical anesthesia. One or more polyethylene catheters were placed in the external jugular veins for IV infusion with a syringe-driven pump. A polyethylene cannula (PE-10, Clay-Adams, Becton Dickinson, Sparks, MD) was inserted through a midline incision into the common bile–pancreatic duct at the sphincter of Oddi. To permit infusion of bile–pancreatic juice, a second cannula was placed into the duodenum,
slightly above the sphincter of Oddi. Abdominal wounds were covered with saline-moistened gauze and the rats were maintained at 37°C with a heating pad.

**Pancreatic secretion study**

After a 30-min stabilization period, the combined biliary–pancreatic secretions were collected every 15 min. The volume was measured and an aliquot was taken and diluted with distilled water for protein determination. The remaining undiluted bile–pancreatic juice was pumped back into the rat through the duodenal cannula during the next collection period at the rate of secretion of the preceding collection period. Spectrophotometric measurements of protein in the bile–pancreatic juice were made using the assay method of Bradford (27). In separate studies, changes in protein output attributed to biliary volume and biliary protein secretion were assessed by cannulating the bile duct prior to its entrance into the pancreas. Collections were made under both basal and ICV ghrelin infusion.

**Ghrelin dose-response studies.** Graded doses of ghrelin (0.5, 5, 10, 25 nmol, bolus) were infused intravenously. Bile–pancreatic juice was collected every 15 min. Six rats were tested for each dose.

**Atropine and hexamethonium infusion studies.** To determine the role of cholinergic pathways in the mediation of ghrelin’s action, atropine (100 µg/kg per hour) was infused 30 min before infusion of ghrelin and administered throughout the remainder of the experiment. Pancreatic protein outputs were compared with those obtained without atropine. Similar studies were performed with hexamethonium (15 mg/kg bolus plus 7.5 mg/kg per hour continuous infusion)
to determine the role of presynaptic cholinergic neurons in the mediation of ghrelin-stimulated pancreatic enzyme secretion. Six rats were tested for each group.

**Bilateral subdiaphragmatic vagotomy.** To show that IV infusion of ghrelin acts by stimulating the vagal pathways, acute bilateral subdiaphragmatic vagotomy was performed. A midline incision was made in the abdominal wall, through which the stomach was carefully manipulated to expose the esophagus. The subdiaphragmatic vagal trunks were exposed halfway between the diaphragm and the gastric cardia. Both anterior and posterior trunks of the vagal nerves were transected (27). Control experiments involved exposing but not cutting the vagal nerves. Ghrelin dose-response studies were performed as described in the previous section. Six rats were tested for each group.

**Perivagal application of capsaicin.** To investigate the role of vagal afferent pathway in the mediation of ghrelin’s action, we examined the effects of perivagal application of capsaicin. After anesthetizing the animals, the abdominal vagal trunks were exposed. A small piece of gauze soaked in 0.1 capsaicin solution (0.1 ml per rat) was left on the vagal trunks for 30 min (27). Vehicle alone was applied to control rats. Ghrelin dose-response studies as described were performed 5 days after surgery in these two groups of rats. Six rats were tested for each group.

**Intracerebroventricular cannulation**

Rats were anesthetized with a mixture of xylazine and ketamine and placed in a stereotaxic head holder with the upper incisor bar placed 3 mm above the interaural line. A small hole was drilled into the skull through which a 15-mm-long stainless steel cannula (24 gauge) was introduced perpendicularly into the middle of the left cerebral ventricle. The cannula was secured with two
screws inserted into the surface of the parietal bone and fixed with cranioplastic powder (Plastics One, Roanoke, VA). Coordinates from the bregma were as follows: anteroposterior, 0.6 mm; lateral, 2 mm; and ventral, 4 mm. After a 5-day recovery period, only animals that showed progressive weight gain after surgery were used in subsequent experiments. Bile-pancreatic secretion in response to ICV infusion of ghrelin (0.1, 0.5 nmol per 10 µl) was examined in 6 rats. In separate studies, pure bile juices were collected, the volume and protein were measured. Artificial cerebrospinal fluids were ICV infused as vehicle and as medium for aliquots of ghrelin. Four rats were tested for each group. In another group of rats, acute vagotomy was performed before ICV infusion of ghrelin. Proper placement of the cannulas was verified at the end of each experiment by administration of dye (31). Six rats were tested for each group.

**Surgical procedure to ablate the area postrema**

Rats were anesthetized with a mixture of xylazine and ketamine and placed in a stereotaxic head holder with the upper incisor bar placed parallel to the interaural line. The AP was exposed through a surgical incision between the occipital crest and second cervical vertebra in the dorsal musculature. The muscles were retracted and the dura over the cisterna magna was excised to expose the dorsal medulla. The foramen magnum was slightly enlarged, exposing the AP. The AP was ablated using a cotton swab drawn to a fine tip. Twisting the tip of the swab stiffened the material. When the AP was touched, tissue adhered to the tip, permitting us to tease the AP from its locus. The sham-lesioned controls were subjected to the same surgical procedure without application of the swab. The animals were given 6 to 7 wk to recover from AP-lesion or sham-lesion with *ad libitum* access to food and water, and then pancreatic surgery was performed. Pancreatic secretion studies were conducted in conscious rats one week after. Body weight and food and water intakes were monitored weekly during the recovery period. All rats with an AP
lesion survived and gained weight, although the average weight for AP-lesioned rats after 6 wk recovery was less than that of sham-lesioned rats (312 ± 5 versus 345 ± 7 g, P < 0.01). The significant difference in the postoperative weights of AP-lesioned and sham-lesioned animals corresponded to previous reports (1) and had no apparent effect on pancreatic secretion. A total of 24 AP-lesioned and 15 sham-lesioned rats were used in the pancreatic secretion studies and c-Fos immunohistochemistry studies.

**Histological analysis**

After testing was completed, the rats were anesthetized deeply with pentobarbital and perfused transcardially with 50 mL ice-cold heparinized PBS (10 mM pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were postfixed in formalin for 24 h, then in 20% (wt/vol) sucrose for a minimum of 24 h. Frozen coronal sections (30 µm) of the hindbrain were cut in a precision cryostat (Leica Microsystems, Bannockburn, IL) beginning 300–400 µm rostral to the AP, continuing through the AP, to a level 300–400 µm caudal to the AP. The sections were stained with 0.5% (wt/vol) cresyl violet using a standard procedure and then were examined by light microscopy to determine the extent of the lesion.

**Effect of AP lesion on pancreatic secretion induced by IV infusion of ghrelin or diversion of bile–pancreatic juice in conscious rats**

Physiological control of pancreatic secretion may be different in anesthetized and conscious rats. We used a mixture of xylazine and ketamine to anesthetize the animals. Ketamine appears to act primarily on the phencyclidine receptor on the N-methyl-D-aspartate receptor complex (16). Some phencyclidines have been shown to interact with the muscarinic receptors. Research has shown that ketamine may inhibit muscarinic receptor signaling in the rat cortex and
hippocampus (11). This effect may explain the significantly lower basal pancreatic protein output in anesthetized rats compared to conscious rats. Therefore, to verify the physiological relevance of our observations in anesthetized rats, we evaluated the effects of AP lesion on pancreatic secretion evoked by IV infusion of ghrelin and diversion of bile–pancreatic juice in conscious rats. The surgical procedure has been described previously (30). Briefly, after anesthetizing the animals, a polyethylene catheter (PE-10) was inserted through a midline abdominal incision into the bile-pancreatic duct. A second catheter (PE-50) was placed in the duodenum slightly above the sphincter of Oddi for intestinal perfusion of bile–pancreatic juice. The catheters were brought through the body wall and pulled through a subcutaneous tunnel to an exit site between the scapulae. Disinfectant (chlorhexidine) was applied to prevent infection. A plastic jacket worn by the animals prevented contact with the catheters. The cannulas were connected between experiments. For IV infusion of ghrelin one catheter was placed into the external jugular vein pulled through a subcutaneous tunnel to the back and covered by the jacket. After recovery from anesthesia, the rats were returned to their home cages. Pancreatic secretion studies were performed 6 days later after the rats had fully recovered from surgery. Following an overnight fast, the rats were lightly restrained in Bollman cages and pancreatic secretion studies in response to IV infusion of ghrelin (10 nmol) or vehicle (saline) were performed in 8 AP-lesioned and 5 sham-lesioned rats. Secretion studies were completed in all rats of these 2 groups. In a separate group of 8 AP-lesioned and 5 sham-lesioned rats, bile–pancreatic juice was diverted after a 45-min basal period and the duodenum was perfused with saline at 1 ml/h for 75 min. Pancreatic juice was collected every 15 min. For the control, pancreatic protein output was measured after bile–pancreatic juice was returned to the duodenum in the same rat. We were not able to complete the protocol in 2 rats: 1 lesioned and 1 sham-lesioned. At the end of the experiment, anesthetized rats were killed by decapitation.
Immunohistochemistry

Immunohistochemical studies were performed in 8 AP-lesioned and 5 sham-lesioned rats to examine c-Fos expression in the dorsal vagal complex in response to ghrelin (10 nmol). All experiments were performed in conscious non-fasted rats between 10:00 A.M. and 12 P.M. Rats were injected with either vehicle (saline) or ghrelin (10 nmol, IV). Rats were euthanized with an overdose of pentobarbital sodium (60 mg/ml, intraperitoneal) 1 h after termination of the ghrelin infusion. A transcardiac perfusion through the heart or the ascending aorta was administrated with 50 mL ice-cold heparinized PBS (10 mM pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer.

Tissue preparation. Brains were removed, blocked, and postfixed in the same fixative overnight. Tissue was immersed in 25% sucrose in 4% paraformaldehyde for 24 h prior to cryosectioning. Frozen sections (30 µm) were cut in a precision cryostat (Leica Microsystems, Bannockburn, IL) separated into five series, thaw-mounted on gelatin-chromium coated slides, and stored at −70ºC until further processing.

c-Fos immunocytochemistry. Immunohistochemical staining of c-Fos was performed using the avidin–biotin peroxidase complex (ABC) technique with 3,3′-diaminobenzidine serving as the chromagen. The sections were pretreated for 20 min in a solution of 1.5% hydrogen peroxide, 20% methanol, and 0.2% Triton X-100 in PBS to inactivate endogenous peroxidase activity, and then rinsed four times in PBS until bubbling ceased. The sections were immersed in a blocking solution (5% normal goat serum, 1% BSA, and 0.5% Triton X-100 in PBS) for 20–30 min to inhibit nonspecific binding. The sections were then incubated in a humid chamber in c-Fos rabbit polyclonal antibody (Ab-5, Oncogene Science, Cambridge, MA) diluted 1:8,500–1:10,000 for
24–48 h at 7°C. After incubation, the sections were washed in PBS-Gel (0.1% gelatin in PBS) and incubated for 2 h at room temperature in biotinylated secondary antibody, goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:500–1:300 dilution. After three rinses, the sections were incubated for 90 min in ABC (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) at half the recommended dilution. Sections were then rinsed three times in PBS. The c-Fos antibody–peroxidase complex was visualized using a metal enhanced 3,3′-diaminobenzidine substrate kit (Pierce Biotechnology, Rockford, IL). After a 2–10 min staining period, during which time reactivity was confirmed with visual microscopy, the reaction was stopped by transferring the tissue into an acetate solution and rinsing it with PBS. Bright-field microscopy was used to confirm the presence of c-Fos antigen, which is revealed as a brown-black precipitate in the cell nuclei. For controls, the primary antiserum was omitted and the sections were incubated in normal goat serum. Antibody specificity was verified by preabsorbed incubation with synthetic c-Fos (10 µmol, c-Fos peptide-2, lot no. 954701, Oncogene Science) for 1 h at room temperature. This procedure blocked all immunocytochemical staining.

**Quantification and statistical analysis.** Ghrelin-induced c-Fos expression in the dorsal vagal complex was quantified using images generated using a Zeiss Axioskop 20× objective and a SPOT RT digital camera. Regions of interest were delineated and the number of c-Fos–immunoreactive cell nuclei fulfilling certain size, shape, and density criteria were counted using Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Initially, cell counts obtained by Image Pro Plus were manually verified through the ocular of the microscope by an independent observer. The dorsal vagal complex was divided into a rostral part (−13.3 to −13.5), a middle part (−13.6 to −13.8), and a caudal part (−14.1 to −14.2). Average counts per 30-µm section
(both hemispheres) were obtained from three to four sections in each part. Data were expressed as means ± SEM per section for all ganglia and evaluated by the appropriate Student paired or unpaired \( t \) test with a Newman-Keuls test when multiple comparisons were made. \( P < 0.05 \) was considered statistically significant.

**Statistical analysis**

Results are expressed as means ± SEM. Multivariant analysis of variance was used to evaluate the effects of repeated measurements over time, the effects of treatment, and the interaction between these two variables. Basal output was determined as the average of two 15-min periods. The increases of protein output were calculated as the average of two 15-min of peak protein output (30 and 45 min after ghrelin infusion) minus basal output. Subsequent comparisons were made with the Newman-Keuls test (InStat Biostatistics 2.01. Graphpad Software, San Diego, CA). \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Effects of IV infusion of ghrelin on pancreatic secretion in anesthetized rats**

In the ketamine-xylazine anesthetized rat model, basal pancreatic secretion was stable, averaging 125 ± 6 mg/h (Fig. 1A). Infusion of ghrelin at 0.5 nmol had no effect on pancreatic protein output. Infusion of ghrelin at 5, 10, and 25 nmol increased protein secretion in a dose-dependent manner to 186 ± 8, 295 ± 12, and 356 ± 18 mg/h, representing a 48%, 136%, and 184% increase over basal, respectively (Fig. 1B). These changes, which were observed soon after the administration of rat ghrelin, peaked in 30 min and then declined gradually over 90 min. These increased outputs were similar to those observed after IV infusion of CCK-8 at doses of 20 to 80 pmol/kg per hour (27).
Effects of atropine, hexamethonium, vagotomy and perivagal application of capsaicin on ghrelin-induced pancreatic secretion

Consistent with the results observed in our earlier study (27), neither IV administration of atropine or hexamethonium nor acute vagotomy significantly affected basal pancreatic secretion in the anesthetized rat. These treatments, however, completely abolished pancreatic responses to IV infusion of ghrelin (Fig. 2A, 2B), suggesting that circulating ghrelin acts at a presynaptic site of the vagal cholinergic pathway to stimulate pancreatic secretion. Perivagal application of capsaicin did not affect basal pancreatic enzyme secretion. In contrast to truncal vagotomy, perivagal application of capsaicin had no effect on pancreatic secretion in response to IV infusion of ghrelin (Fig. 2B). As a control, we showed that pancreatic protein secretion in response to CCK-8 (40 pmol/kg per hour) was completely abolished in the same group of rats treated with perivagal application of capsaicin (data not shown). These observations suggest that ghrelin administered systemically acts on vagal efferent cholinergic pathway but not capsaicin-sensitive vagal afferent fibers to stimulate pancreatic secretion.

Effects of ICV administration of ghrelin on pancreatic secretion

To determine if ghrelin acts centrally to stimulate pancreatic protein secretion we performed an ICV infusion study. ICV infusion of ghrelin resulted in a marked increase in protein secretion (Fig. 3). Note that ICV infusion of ghrelin at a dose of 0.1 nmol produced a similar increase in protein output as that stimulated by IV infusion of ghrelin at a dose of 5 nmol (see Fig. 1). Vagotomy abolished the response (Fig. 3), indicating that ghrelin exerts its stimulatory action at a central vagal site. In separate experiments, pure bile juices were collected, the volume and protein were measured. The mean bile protein output during the basal collection period was 96 ±
7 mg/h (n=4). Bile protein did not increase with ghrelin stimulation. After ICV injection of ghrelin at dose of 0.1 and 0.5 nmol, bile protein outputs were 94 ± 5 and 92 ± 3 mg/h, respectively. These observations confirm that the increase in protein output in the bile–pancreatic juice after ghrelin stimulation reflects protein from the pancreatic source, and protein in the bile does not increase with these stimulations.

Effects of AP lesion on pancreatic secretion induced by IV infusion of ghrelin or diversion of bile–pancreatic juice in conscious rats

Conscious, sham-lesioned rats have relatively high basal pancreatic protein secretion rates, averaging 308 ± 9 mg/h. Basal protein output in AP-lesioned rats averaged 265 ± 12 mg/h, a decrease of 19% compared with basal output in sham-lesioned rats (P < 0.05). In sham-lesioned and AP-lesioned rats, diversion of bile–pancreatic juice significantly stimulated pancreatic protein secretion (Fig. 4B). Although basal protein output was lower in AP-lesioned rats, the net increases in protein output after diversion of bile–pancreatic juice were not significantly different between sham-lesioned controls and AP-lesioned rats (net peak protein output was 351 ± 19 versus 345 ± 22 mg/h in sham-lesioned and AP-lesioned rats, respectively). In contrast to pancreatic secretion induced by diversion of bile–pancreatic juice, AP lesion abolished pancreatic protein secretion stimulated by IV infusion of ghrelin (Fig. 4A).

c-Fos immunoreactive neurons in the dorsal vagal complex

The rostral, middle, and caudal levels of the dorsal vagal complex were examined in 5 sham-lesioned and 4 AP-lesioned rats. Average counts per 30-µm section (both hemispheres) were obtained from three to four sections in each level. Immunohistochemical studies showed that in conscious fasting rats, IV injection of saline did not increase Fos immunoreactivity in either
sham-lesioned or AP-lesioned rats. In sham-lesioned rats, infusion of ghrelin (10 nmol) increased the number of c-Fos–positive neurons in the NTS and DMV (Fig. 5A, B) from a basal of 6–11 ± 2–3 per section to 91 ± 21, 80 ± 23, and 60 ± 19 per section, in three levels, respectively. As compared with AP-lesioned rats (Fig. 5C), there were no increases in c-Fos immunoreactive cells in the NTS and DMV after IV infusion of ghrelin ($P < 0.05$). These observations suggest that the AP is the primary site of action of circulating ghrelin. Peripherally administrated ghrelin acts on the AP and in turn activates the NTS and DMV to stimulate pancreatic secretion.

**Histological analysis**

A total of 23 AP-lesioned rats and 14 sham-lesioned rats were examined. No tissue damage was observed in the sham-lesioned rats. Histological analysis revealed that in 15 of 23 AP-lesioned rats, the lesions appeared to be very specific; damage was confined to the AP with no discernible damage to the NTS. Only data collected from these rats were included in the statistical analyses of the pancreatic secretion responses to stimulation by ghrelin or diversion of bile–pancreatic juice. Of the remaining 8 rats, 4 rats had lesions extending to the underlying medial NTS or the commissural subnucleus; 2 rats had subtotal lesions sparing part of the caudal AP; and 2 had nonselective lesions with major damage extending into the gracile and hypoglossal nuclei. Pancreatic secretion measurements from these 8 rats were excluded from data evaluation.

**DISCUSSION**

Ghrelin is a novel acylated peptide that mediates growth hormone release and energy metabolism. The principal peripheral site of ghrelin synthesis is the gastric oxyntic mucosa, specifically the A-like endocrine cells, which contribute about 80% of circulating ghrelin (4, 5).
Smaller amounts of ghrelin are found in the small intestine (10, 18). Ghrelin levels in the circulation are influenced by the feeding state. Fasting increases gastric fundic ghrelin mRNA expression and plasma levels and there is a postprandial decrease in ghrelin plasma values in rats and humans (4, 41). Existing evidence suggests that ghrelin functions as a gut–brain endocrine hormone to regulate food intake and energy balance, activities independent of its growth hormone–releasing action in rats and humans (21, 42). A preprandial elevation in ghrelin secretion in humans suggests that ghrelin may participate in the mediation of digestive functions (4). In fact, ghrelin has been shown to have a variety of effects on the stomach. Ghrelin, given peripherally or into the brain, stimulates gastric acid secretion (7, 33) and gastric motility in rats (33). Ghrelin stimulates gastric emptying in mice (37).

Ghrelin and its receptors, which are expressed in normal and neoplastic cells in the endocrine pancreas (23), are involved in regulating pancreatic endocrine function (23, 34). Exocrine pancreatic secretion is largely controlled by the autonomic nervous system. We and other investigators have clearly demonstrated that the vago-vagal reflex plays a crucial role in the mediation of postprandial pancreatic enzyme secretion. Previous studies in the rat showed that CCK (27, 28) and luminal non-CCK–dependent factors (29) such as osmolality, disaccharides, and mechanical stimulation evoke pancreatic secretion via intestinal vagal mucosal afferent fibers. Serotonin released from intestinal enterochromaffin cells acts as a paracrine substance to mediate luminal non-CCK–stimulated pancreatic secretion (24, 30). Moreover, somatostatin (26), CGRP (25), peptide YY (8), and pancreatic polypeptide (9) inhibit pancreatic secretion by a central vagal site of action. Although pancreatic secretion is mainly mediated by vago-vagal reflexes within the brainstem, research has demonstrated that these reflexes may also be modulated by input from higher centres, such as the hypothalamus (31, 43). To date, information on the function of ghrelin in the exocrine pancreas has been limited and
discussion of a potential role for ghrelin in pancreatic secretion has provoked controversy. For example, it has been shown that pancreatic exocrine secretion stimulated by super-physiological doses of CCK is inhibited by ghrelin in anesthetized rats and in dispersed rat acinar cells (45). The physiological relevance of this observation is unknown since pharmacological doses of CCK were used to stimulate pancreatic secretion. It has been demonstrated that high doses of CCK directly stimulate pancreatic acini (27). Another study reported that centrally administered ghrelin stimulates pancreatic exocrine secretion through the vagal efferent pathways in conscious rats (36). Ghrelin and ghrelin receptor have been shown to be consistently expressed in rat pancreatic exocrine tumor AR42J cell line (22). Fluorescence imaging showed that ligand stimulation of the ghrelin receptor led to a biphasic \([\text{Ca}^{2+}]_i\) mobilization in these cells (22). Moreover, administration of ghrelin attenuated pancreatic damage in caerulein-induced pancreatitis (12). In view of the complexity involved and the inconsistency of information in the reported literature, the function of the ghrelin in pancreatic secretion warrants further investigation. In the current study we examined the effects of rat ghrelin on pancreatic secretion in anesthetized and conscious rats. A study in rats showed that ghrelin increased food intake when injected intraperitoneally at a dose of 1 nmol that reproduced the concentration occurring in the blood after a 24-h fast (2.83 ± 0.13 pmol/ml at 60 min postinjection vs. 2.79 ± 0.32 pmol/ml after 24-h fast (41). We show that intravenous bolus infusion of ghrelin at 5, 10, and 25 nmol increased protein secretion in a dose-dependent manner to 186 ± 8, 295 ± 12, and 356 ± 11 mg/h, representing a 48%, 136%, and 184% increase over basal, respectively. These increases were similar to those observed after IV infusion of CCK-8 at doses of 20 to 80 pmol/kg per hour (27). We did not measure plasma concentration after IV administration of ghrelin, however, it may not be within the physiological ranges. Since atropine and hexamethonium each completely abolished pancreatic enzyme responses to IV infusion of ghrelin, it suggests that ghrelin is acting
on a presynaptic site along the cholinergic pathway. To further identify the site(s) of action of circulating ghrelin on pancreatic secretion, we showed that similar to atropine and hexamethonium, vagotomy completely abolished pancreatic responses to ghrelin.

To determine if IV infusion of ghrelin exerts its action via an afferent vagal pathway we examined the effect of perivaginal treatment with the sensory neuron toxin capsaicin. This treatment has been shown to abolish pancreatic secretion stimulated by CCK- and 5-HT–dependent luminal factors at physiological conditions (24, 27). We showed that perivaginal application of capsaicin did not affect ghrelin-stimulated pancreatic protein secretion, indicating that the vagal afferent pathway is not the primary site responsible for ghrelin’s action on the pancreas.

To determine if ghrelin acts centrally to stimulate pancreatic protein secretion we examined the effect of ICV infusion of ghrelin on pancreatic protein secretion. We showed that ICV infusion of ghrelin resulted in a marked increase in pancreatic protein secretion. Vagotomy abolished this response, indicating that ghrelin exerts a stimulatory action at a central vagal site.

The failure of perivaginal application of capsaicin to prevent the increase in pancreatic protein secretion stimulated by IV infusion of ghrelin, which acts via vagal cholinergic efferent pathway, prompted us to examine alternate route(s) by which ghrelin reaches the central nervous system to exert its functions. Recent study using in situ hybridization histochemistry demonstrated that all three components of the dorsal vagal complex of the rat contained GHS-R mRNA (46). Highest expression was within the area postrema (AP), with moderately dense signal in the nucleus of the solitary tract (NTS) and low-density signal in the dorsal motor nucleus of the vagus (DMV). It has been reported that ghrelin injected intravenously induces Fos expression in rat arcuate nucleus (15). In mouse, ghrelin (10 µg per mouse) selectively induced c-Fos expression in the ventromedial part of the hypothalamic arcuate nucleus (39). Although
increased c-Fos expression was observed in the AP and NTS areas, the changes were small and not statistically significant (39). The AP is a circumventricular organ situated in the floor of the fourth ventricle. It has highly fenestrated capillaries and provides an incomplete blood-brain barrier that allows small peptide hormones to enter the dorsal vagal complex (35). The AP can monitor blood-borne and cerebrospinal fluid-borne information (3). Previous studies have shown binding sites for peptide YY$_{1-36}$ and pancreatic polypeptide in the dorsal vagal complex when these peptides were given peripherally (40). The most prominent functions ascribed to the AP have been its roles in mediating emesis (3) and other aversive responses to certain toxic stimuli (35). However, the AP and adjacent NTS have also been implicated in the regulation of digestive functions more generally (13, 19). It has been suggested that the AP is one of the central components of the brainstem regulating vago-vagal reflex pathway. Pancreatic polypeptide inhibits basal pancreatic secretion (9) and peptide YY inhibits CCK-stimulated pancreatic secretion through an AP-dependent mechanism (8). In the current study, we showed that selective ablation of the AP caused a 19% decrease in basal protein output in conscious rats. This suggests that the AP may contribute to basal vagal tone to regulate spontaneous pancreatic protein secretion. We showed that AP lesion eliminated pancreatic protein secretion stimulated by IV infusion of ghrelin, indicating that the primary site of circulating ghrelin to stimulate pancreatic secretion is the AP. In contrast, AP lesion did not affect pancreatic secretion induced by diversion of bile–pancreatic juice. Previous researchs demonstrated that diversion of bile–pancreatic juice from duodenum elevated plasma CCK levels (28, 32), and caused more than a twofold increase in pancreatic protein secretion in rats via stimulation of vagal afferent pathway (8, 28). These observations provide an important positive control since CCK acts via vagal afferent pathway to stimulate pancreatic secretion.
Furthermore, the c-Fos expression study showed that IV administration of ghrelin activated neurons in the NTS and DMV of the medulla oblongata. These nuclei are key sites for the regulation of pancreatic secretion in the brain. The NTS receives major inputs from the vagus nerve and sends direct projections to the DMV (14). The DMV integrates visceral sensory input from the NTS and central input arising from the hypothalamus. Vagal motor neurons that innervate the pancreas originate in the DMV (20). Multiple substances act on neurocircuitry in the DMV to modulate vagal efferent signals to the pancreas (44). We showed that AP lesion prevents c-Fos expression in response to IV ghrelin in the NTS and DMV. This observation also supports the hypothesis that the primary site of action of circulating ghrelin is the AP. Peripheral administration of ghrelin acts on the AP and in turn stimulates the NTS and DMV to stimulate pancreatic protein secretion. Given the low concentration of ghrelin in the human circulation (21), further studies are required to determine whether physiologically released ghrelin can reach the central nervous system in sufficient concentrations to interact with the brainstem circuits to regulate pancreatic secretion.

GHS-R has a widespread distribution, suggesting that ghrelin may have diverse biological functions. GHS-R is expressed in several hypothalamic nuclei, many of which have long been recognized as playing roles in body weight and food intake. GHS-R also was found in many parasympathetic preganglionic neurons (46) and vagal afferent neurons (6). However, it is as yet unclear how circulating ghrelin (especially with its unique acetyl modification) would gain access to and influence the activity of neurons in the areas of GHS-R expression that are more distantly situated from circumventricular organs. One possible explanation comes from the observations that ghrelin-producing neurons are found in the CNS (17, 21). In such circumstances, centrally produced ghrelin and not circulating ghrelin would be readily available to engage the GHS-R. The widespread distribution of GHS-R likely contribute differentially to
ghrelin’s actions, including its important roles in inducing varied behavioral, neuroendocrine, and autonomic responses to changes in nutritional availability. However, the source(s) and physiological significance of CNS ghrelin remains to be determined.
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Figure legends

**Fig. 1. Pancreatic protein secretion in response to intravenous (IV) infusion of ghrelin in anesthetized rats.** A: IV infusion of ghrelin at 0.5 nmol had no effect on pancreatic protein output. Infusion of ghrelin at 5, 10, and 25 nmol increased protein secretion in a dose-dependent manner. These changes were observed soon after the administration of rat ghrelin; output peaked in 30 min and then declined gradually over 90 min. B: Ghrelin at doses of 5 nmol, 10 nmol, and 25 nmol caused increases in pancreatic protein secretion of 48%, 136%, and 184%, respectively, over basal. Values are means ± SE for 6 rats for each time point. *$P < 0.05$. 

**Fig. 2. Effects of administration of atropine or hexamethonium, vagotomy, and perivagal application of capsaicin on pancreatic protein secretion stimulated by ghrelin.** Neither IV administration of atropine or acute vagotomy (A), nor administration of hexamethonium or perivagal application of capsaicin (B) had a significant effect on basal pancreatic secretion in the anesthetized rat. However, these treatments, with the exception of capsaicin application, completely abolished pancreatic secretion responses to IV infusion of ghrelin (10 nmol). Values are means ± SE for 6 rats for each time point. *$P < 0.05$. 

**Fig. 3. Effects of intracerebroventricular (ICV) administration of ghrelin on pancreatic protein secretion.** ICV infusion of ghrelin 0.1 and 0.5 nmol resulted in a marked increase in pancreatic protein secretion. Note that ICV infusion of ghrelin at a dose of 0.1 nmol produced a similar increase in protein output as that stimulated by IV infusion of ghrelin at a dose of 5 nmol (see Fig. 1A). Vagotomy abolished this response. Values are means ± SE for 6 rats for each time point. *$P < 0.05$. 
Fig. 4. Effects of ablation of the area postrema (AP) on pancreatic protein secretion stimulated by intravenous (IV) ghrelin administration and bile–pancreatic juice (BPJ) diversion. Conscious rats have relatively high basal pancreatic protein secretion rates, averaging 308 ± 9 mg/h. Basal protein output in AP-lesioned rats was decreased by 19% compared with sham-lesioned rats. A: AP lesion abolished pancreatic protein secretion stimulated by IV infusion of ghrelin (10 nmol). Values are means ± SE for 5 sham-lesioned rats and 6 AP-lesioned rats for each time point. *P < 0.05. B: In contrast to pancreatic protein secretion induced by intravenous infusion of ghrelin in both sham-lesioned and AP-lesioned rats, diversion of BPJ significantly stimulated pancreatic protein secretion. Although basal protein output was lower in the AP-lesioned rats, the net increase in protein output after diversion of BPJ was not significantly different between sham-lesioned controls and AP-lesioned rats. Values are means ± SE for 4 sham-lesioned rats and 5 AP-lesioned rats for each time point. *P < 0.05.

Fig. 5. Intravenous (IV) ghrelin induced c-Fos expression in neurons of rat dorsal vagal complex. A:: Photomicrographs of a section of the dorsal medulla –13.8 mm posterior to bregma showing that IV injection of ghrelin (10 nmol) induced c-Fos expression in the area postrema (AP) and in the medial (med), dorsomedial (dm), and commissural (com) subnuclei of the nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMV) in sham-lesioned rats. Scale bar: 100 µm. B: High magnification of the square area lined in the A. Scale bar: 20 µm. C: In contrast, AP lesion eliminated the induction of c-Fos expression in response to IV injection of ghrelin in the dorsal vagal complex. CC, central canal. Scale bar: 100 µm.
Figure 1
Figure 2
Figure 3
Figure 4