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

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Ghrelin acts on the dorsal vagal complex to stimulate pancreatic protein secretion. Am J Physiol Gastrointest Liver Physiol 290: G1350–G1358, 2006. First published February 9, 2006; doi:10.1152/ajpgi.00493.2005.—Ghrelin receptors are present in the central nervous system. We hypothesized that ghrelin released from the stomach acts as an endocrine substance and stimulates brain stem vagovagal circuitry to evoke pancreatic secretion. In an in vivo anesthetized rat model, an intravenous infusion of ghrelin at doses of 5, 10, and 25 nmol increased pancreatic protein secretion from a basal level of 125 ± 6 to 186 ± 8, 295 ± 12, and 356 ± 11 mg/h, respectively. Pretreatment with atropine or hexamethonium or an acute vagotomy, but not a perivagal application of capsaicin, completely abolished pancreatic protein secretion responses to ghrelin. In conscious rats, an 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 an intravenous infusion of ghrelin in sham-lesion 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 brain stem vagovagal circuitry via the area postrema, which represents the primary target on which peripheral ghrelin may act as an endocrine substance to stimulate pancreatic secretion.

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). An intravenous infusion of ghrelin in rats induces growth hormone release by stimulating GHS-R at the pituitary (21). An intracerebroventricular 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). An intracerebroventricular 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). A further study (33) has suggested that an intravenous administration of ghrelin stimulated gastric acid secretion and increased motility by acting through the vagus nerve pathway; however, the 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, which 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 brain stem vagovagal circuitry to evoke pancreatic secretion. The effects of intravenous infusions of graded doses of ghrelin on pancreatic enzyme secretion were examined in anesthetized rats. Furthermore, 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 intravenous 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 brain stem neurocircuitry to stimulate pancreatic secretion. Immunohistochemical staining showing that intravenous 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 serotonin (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 (PE) catheters were inserted into the right jugular vein for the intravenous infusion of ghrelin.

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were placed in the external jugular veins for intravenous infusion with a syringe-driven pump. A PE-10 cannula (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 rats were maintained at 37°C with a heating pad.

**Pancreatic Secretion Study**

After a 30-min stabilization period, 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 before its entrance into the pancreas. Collections were made under both basal and intracerebroventricular 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⁻¹·h⁻¹) was infused 30 min before the 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⁻¹·h⁻¹ 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 an intravenous infusion of ghrelin acts by stimulating 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 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 Atropine and hexamethonium infusion studies. Six rats were tested for each group.

**Perivagal application of capsaicin.** To investigate the role of the vagal afferent pathway in the mediation of ghrelin’s action, we examined the effects of a perivagal application of capsaicin. After the animals were anesthetized, the abdominal vagal trunks were exposed. A small piece of gauze soaked in 0.1 capsaicin solution (0.1 ml/rat) was 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 an intracerebroventricular infusion of ghrelin (0.1 or 0.5 nmol/10 µl) was examined in six rats. In separate studies, pure bile juices were collected, and the volume and protein were measured. Artificial cerebrospinal fluids were intracerebroventriculally infused as a vehicle and as the medium for aliquots of ghrelin. Four rats were tested for each group. In another group of rats, an acute vagotomy was performed before the intracerebroventricular infusion of ghrelin. Proper placement of the cannulas was verified at the end of each experiment by the administration of dye (31). Six rats were tested for each group.

**Surgical Procedure to Ablate the AP**

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. Sham-lesioned controls were subjected to the same surgical procedure without application of the swab. Animals were given 6–7 wk to recover from AP lesion or sham lesion with ad libitum access to food and water, and pancreatic surgery was then performed. Pancreatic secretion studies were conducted in conscious rats 1 wk 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 of recovery was less than that of sham-lesioned rats (312 ± 5 vs. 345 ± 7 g, P < 0.01). The significant difference in the postoperative weights of AP-lesioned and sham-lesioned animals corresponded to results of a previous report (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, rats were anesthetized deeply with pentobarbital sodium 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. Brains were postfixed in formalin for 24 h and 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. Sections were stained with 0.5% (wt/vol) cresyl violet using a standard procedure and then examined by light microscopy to determine the extent of the lesion.

**Effect of AP Lesion on Pancreatic Secretion Induced by Intravenous 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 with 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 an intravenous infusion of ghrelin and diversion of bile-pancreatic juice in conscious rats. The surgical procedure has been described previously (30). Briefly, after the animals were anesthetized, a PE-10 catheter 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 the 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 the intravenous 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. After an overnight fast, rats were lightly restrained in Bollman cages, and pancreatic secretion studies in response to an intravenous infusion of ghrelin (10 nmol) or vehicle (saline) were performed in eight AP-lesioned and five sham-lesioned rats. Secretion studies were completed in all rats of these two groups. In a separate group of eight AP-lesioned and five 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 two rats: one AP lesioned and one sham lesioned. At the end of the experiment, anesthetized rats were killed by decapitation.

**Immunohistochemistry**

Immunohistochemical studies were performed in eight AP-lesioned and five 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 nonfasted rats between 10:00 AM and 12:00 PM. Rats were injected with either vehicle (saline) or ghrelin (10 nmol iv). Rats were euthanized with an overdose of pentobarbital sodium (60 mg/ml ip) 1 h after the termination of the ghrelin infusion. A transcardiacal perfusion through the heart or ascending aorta was administered 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 before being cryosectioned. Frozen sections (30 µm) were cut in a precision cryostat (Leica Microsystems) separated into five series, thaw mounted on gelatin-chromium slides, and stored at −70°C until being further processed.

c-Fos immunocytochemistry. Immunohistochemical staining of c-Fos was performed using the avidin-biotin peroxidase complex (ABC) technique with 3,3’-diaminobenzidine as the chromagen. 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. 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. 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, 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, sections were incubated for 90 min in ABC (Vectorstain 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- to 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 cell nuclei. For controls, the primary antiserum was omitted, and sections were incubated in normal goat serum. Antibody specificity was verified by a 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 Axioscope ×20 objective and a SPOT RT digital camera. Regions of interest were delineated, and numbers 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 are expressed as means ± SE per section for all ganglia and were 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 ± SE. Multivariate ANOVA 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 Intravenous 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). An infusion of ghrelin at 0.5 nmol had no effect on pancreatic protein output. An 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 an intravenous infusion of CCK-8 at doses of 20–80 pmol·kg⁻¹·h⁻¹ (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 an intravenous 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 an intravenous infusion of ghrelin (Fig. 2, A and B), 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 an intravenous infusion of ghrelin (Fig. 2B). As a control, we showed that pancreatic protein secretion in response to CCK-8 (40 pmol·kg\(^{-1}\)·h\(^{-1}\)) was completely abolished in the same group of rats treated with a perivagal application of capsaicin (data not shown). These observations suggest that ghrelin administered systemically acts on the vagal efferent cholinergic pathway but not on capsaicin-sensitive vagal afferent fibers to stimulate pancreatic secretion.

Effects of Intracerebroventricular Administration of Ghrelin on Pancreatic Secretion

To determine whether ghrelin acts centrally to stimulate pancreatic protein secretion, we performed an intracerebroventricular infusion study. An intracerebroventricular infusion of ghrelin resulted in a marked increase in protein secretion (Fig. 3). Note that the intracerebroventricular infusion of ghrelin at a dose of 0.1 nmol produced a similar increase in protein output as that stimulated by the intravenous 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, and the volume and protein were mea-

![Fig. 1. Pancreatic protein secretion in response to an intravenous (IV) infusion of ghrelin in anesthetized rats. A: intravenous infusion of ghrelin at 0.5 nmol had no effect on pancreatic protein output. Infusions 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, 10, 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.](http://ajpgi.physiology.org/)

![Fig. 2. Effects of administration of atropine or hexamethonium, vagotomy, and perivagal application of capsaicin on ghrelin-induced pancreatic secretion](http://ajpgi.physiology.org/)

Fig. 2. Effects of administration of atropine or hexamethonium, vagotomy, and perivagal application of capsaicin on pancreatic protein secretion stimulated by ghrelin. Neither intravenous 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 an intravenous infusion of ghrelin (10 nmol). Values are means ± SE for 6 rats for each time point. *P < 0.05.
sured. 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 an intracerebroventricular injection of ghrelin at a 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 Intravenous 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 vs. 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 an intravenous 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 five sham-lesioned and four 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, an intravenous injection of saline did not increase Fos immunoreactivity in either sham-lesioned or AP-lesioned rats. In sham-lesioned rats, an infusion of ghrelin (10 nmol) increased the number of c-Fos-positive neurons in the NTS and DMV (Fig. 5, A and B) from a basal of 6–11 ± 2–3 to 91 ± 21, 80 ± 23, and 60 ± 19 neurons/section, in three levels, respectively. Compared with AP-lesioned rats (Fig. 5C), there
or into the brain, stimulates gastric acid secretion (7, 33) and a variety of effects on the stomach. Ghrelin, given peripherally, stimulates digestive functions (4). In fact, ghrelin has been shown to have a variety of effects on the stomach. Ghrelin, given peripherally, stimulates digestive functions (4). In fact, ghrelin has been shown to have intestinal enterochromaffin cells acts as a paracrine substance to mediate luminal non-CCK-stimulated pancreatic secretion (29). 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 vagovagal reflexes within the brain stem, research has demonstrated that these reflexes may also be modulated by input from higher centers, 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 superphysiological 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 because 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 (36) reported that centrally administered ghrelin stimulates pancreatic exocrine secretion through vagal efferent pathways in conscious rats. Ghrelin and ghrelin receptor have been shown to be consistently expressed in the rat pancreatic exocrine tumor AR42J cell line (22). Fluorescence imaging showed that ligand stimulation of the ghrelin receptor led to biphasic intracellular Ca\(^{2+}\) concentration mobilization in these cells (22). Moreover, the 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 ghrelin in pancreatic secretion warrants further evaluation.

**Histological Analysis**

A total of 23 AP-lesioned rats and 14 sham-lesioned rats was 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 eight rats, four rats had lesions extending to the underlying medial NTS or the commissural subnucleus, two rats had subtotal lesions sparing part of the caudal AP, and two rats had nonselective lesions with major damage extending into the gracile and hypoglossal nuclei. Pancreatic secretion measurements from these eight 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 vagovagal 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. 5-HT released from intestinal enterochromaffin cells acts as a paracrine substance to mediate luminal non-CCK-stimulated pancreatic secretion (42). 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 vagovagal reflexes within the brain stem, research has demonstrated that these reflexes may also be modulated by input from higher centers, 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 superphysiological 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 because 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 (36) reported that centrally administered ghrelin stimulates pancreatic exocrine secretion through vagal efferent pathways in conscious rats. Ghrelin and ghrelin receptor have been shown to be consistently expressed in the rat pancreatic exocrine tumor AR42J cell line (22). Fluorescence imaging showed that ligand stimulation of the ghrelin receptor led to biphasic intracellular Ca\(^{2+}\) concentration mobilization in these cells (22). Moreover, the 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 ghrelin in pancreatic secretion warrants further investigation.

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Fig. 5. Intravenous ghrelin induced c-Fos expression in neurons of the rat dorsal vagal complex. A: photomicrograph of a section of the dorsal medulla – 13.8 mm posterior to the bregma showing that an intravenous injection of ghrelin (10 nmol) induced c-Fos expression in the 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 boxed area in A. Scale bar = 20 μm. C: in contrast, AP lesion eliminated the induction of c-Fos expression in response to an intravenous injection of ghrelin in the dorsal vagal complex. CC, central canal. Scale bar = 100 μm.
in investigation. In the present 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 which reproduced the concentration occurring in blood after a 24-h fast (2.83 ± 0.13 pmol/ml at 60 min postinjection vs. 2.79 ± 0.32 pmol/ml after a 24-h fast) (41). We show that intravenous bolus infusions 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 intravenous infusions of CCK-8 at doses of 20–80 pmol·kg⁻¹·h⁻¹ (27). We did not measure the plasma concentration after intravenous administrations of ghrelin; however, it may not be within physiological ranges. Because atropine and hexamethonium each completely abolished pancreatic enzyme responses to an intravenous infusion of ghrelin, this 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 whether the intravenous infusion of ghrelin exerts its action via an afferent vagal pathway, we examined the effect of perivagal 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 a perivagal 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 whether ghrelin acts centrally to stimulate pancreatic protein secretion, we examined the effect of an intracerebroventricular infusion of ghrelin on pancreatic protein secretion. We showed that an intracerebroventricular 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 a perivagal application of capsaicin to prevent the increase in pancreatic protein secretion stimulated by an intravenous infusion of ghrelin, which acts via a vagal cholinergic efferent pathway, prompted us to examine alternate route(s) by which ghrelin reaches the central nervous system to exert its functions. A recent study (46) using in situ hybridization histochemistry demonstrated that all three components of the dorsal vagal complex of the rat contain GHS-R mRNA. The highest expression was within the AP, with a moderately dense signal in the NTS and a low-density signal in the DMV. It has been reported that ghrelin injected intravenously induces Fos expression in the rat arcuate nucleus (15). In the mouse, ghrelin (10 μg/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). A previous study (40) has shown binding sites for peptide YY1–36 and pancreatic polypeptide in the dorsal vagal complex when these peptides were given peripherally. The most prominent functions ascribed to the AP have been its roles in mediating emesis (3) and other afferent 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 brain stem regulating the vagovagal 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 present 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 an intravenous 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 a diversion of bile-pancreatic juice. Previous research has demonstrated that diversion of bile-pancreatic juice from the 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 pathways (8, 28). These observations provide an important positive control because CCK acts via vagal afferent pathways to stimulate pancreatic secretion.

Furthermore, the c-Fos expression study showed that an intravenous 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 neurocircuity in the DMV to modulate vagal efferent signals to the pancreas (44). We showed that AP lesion prevents c-Fos expression in response to intravenous 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 brain stem 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 central nervous system (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 contributes 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 remain to be determined.

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