Obestatin inhibits motor activity in the antrum and duodenum in the fed state of conscious rats

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Ataka K, Inui A, Asakawa A, Kato I, Fujimiya M. Obestatin inhibits motor activity in the antrum and duodenum in the fed state of conscious rats. Am J Physiol Gastrointest Liver Physiol 294: G1210–G1218, 2008. First published March 6, 2008; doi:10.1152/ajpgi.00549.2007—Obestatin is a novel peptide encoded by the ghrelin precursor gene; however, its effects on gastrointestinal motility remain controversial. Here we have examined the effects of obestatin on fed and fasted motor activities in the stomach and duodenum of freely moving conscious rats. We examined the effects of intravenous (IV) injection of obestatin on the percentage motor index (%MI) and phase III-like contractions in the antrum and duodenum. The brain mechanism mediating the action of obestatin on gastroduodenal motility and the involvement of vagal afferent pathway were also examined. Between 30 and 90 min after IV injection, obestatin decreased the %MI in the antrum and prolonged the time taken to return to fasted motility in the duodenum in fed rats given 3 g of chow after 18 h of fasting. Immunohistochemical analysis demonstrated that corticotropin-releasing factor- and urocortin-2-containing neurons in the paraventricular nucleus in the hypothalamus were activated by IV injection of obestatin. Intracerebroventricular injection of CRF type 1 and type 2 receptor antagonists prevented the effects of obestatin on gastroduodenal motility. Capsaicin treatment blocked the effects of obestatin on duodenal motility but not on antral motility. Obestatin failed to antagonize ghrelin-induced stimulation of gastroduodenal motility. These results suggest that, in the fed state, obestatin inhibits motor activity in the antrum and duodenum and that CRF type 1 and type 2 receptors in the brain might be involved in these effects of obestatin on gastroduodenal motility.

Manometry; CRF type 1; CRF type 2
Implantation of catheters for IVC injection. Implantation of catheters for IVC injection in rats was performed by a method described in previous studies (6, 10). Animals were anesthetized with an IP injection of pentobarbital sodium (50 mg/kg Nembutal; Abbott Laboratories, North Chicago, IL). A chronic guide cannula (25-gauge; Eicom, Kyoto, Japan) was implanted into the right lateral brain ventricle 0.8 mm posterior to the bregma, 1.5 mm right lateral to the midline, and 4 mm below the outer surface of the skull using a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA). When IVC injections were performed in conscious rats, the dummy cannula was replaced by a microinjection cannula (AMI-5; Eicom) 0.5 mm longer than the guide cannula. At the end of the experiments, the correct location of the cannula in the lateral ventricle was verified by a 10-μl injection of dye (0.05% cresyl violet).

Implantation of catheters for manometric recordings and IV injection or infusion. Seven days after brain surgery, the rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and treated with atropine (1 mg/kg ip) to reduce the effects of capsaicin on the cardiovascular and respiratory systems. The left and right vagal nerves were exposed through a midline neck incision. A small piece of cotton soaked in capsaicin (10 mg/ml Nacalai Tesque, Kyoto, Japan) or vehicle (10% Tween 80 in olive oil) was placed on the left vagal nerve for 30 min, and then the treatment area was thoroughly rinsed with saline. The procedure was repeated on the right vagal nerve, and the neck incision was closed with sutures. The validity of this method was confirmed by the lack of c-Fos expression in the nucleus of the solitary tract (NTS) stimulated by gastric distension in conscious rats (17).

Manometric recordings. Gastroduodenal motility was measured by manometric methods described in previous studies (6, 9, 10, 15). After 18 h of fasting, two manometric catheters from each rat were connected to an infusion swivel (dual type, 20-gauge; Instech Laboratories, Plymouth Meeting, PA) to allow free movement, and each catheter was connected to a pressure transducer (TP-400T; Nihon Koden Kogyo, Tokyo, Japan). The catheters were continuously infused with degassed distilled water at a rate of 1.5 ml/h by a heavy-duty pump (CVF-3100; Nihon Koden). Pressure signals from the transducers were recorded and stored by a PowerLab system (AD Instruments, Colorado Springs, CO).

Peptide synthesis and preparation of drugs. Rat obestatin (FNAPFDVGIKLSGQAQQHQGRAL-NH2) was synthesized by a solid-phase fluorenlymethylxycarbonyl-based strategy with the use of an automated peptide synthesizer (Model Pioneer; Applied Biosystems, Foster, CA) and purified by a reverse-phase column, Develosil ODS-HG-5 (2 × 25 cm) (Nomura Chemical, Seto, Japan) on a Delta 600 HPLC System (Waters, Milford, MA). The homogeneity of the purified peptide was confirmed by analytical HPLC, MALDI-TOF MS, and sequence analysis. After purification, obestatin was dissolved in saline containing heparin. CRF type 2 antagonist, Antisauvagine-30 (Phoenix Pharmaceutical, Belmont, CA), and CRF type 1 receptor antagonist, NBI 27914 (Sigma, St. Louis, MO), were dissolved in saline and a mixture of 12.5% dimethyl sulfoxide and 87.5% saline, respectively.

Experimental design. Manometric measurement of pressure waves was started after 1 h of stabilization and continued for 4 h. After a typical pattern of fasted motility was observed, rats were given 3 g of laboratory chow, which was eaten within 20 min. To examine the effects of obestatin on fasted rat duodenal motility, vehicle (200 μl saline) or obestatin (30 nmol in 200 μl saline) was administered IV to rats in the fasted state. To examine the effects of obestatin on fed rat duodenal motility, vehicle (200 μl saline) or obestatin (7.5, 15, or 30 nmol in 200 μl saline, corresponding to 18.9 μg, 37.8 μg, and 75.6 μg/rat) was administered IV 30–40 min after eating. In some experiments, vehicle (5 μl saline), antisauvagine-30 (2.5 nmol in 5 μl saline), or NBI 27914 (100 nmol in 5 μl saline) was administered ICV 10 min before the IV administration of vehicle or obestatin (15 nmol in 200 μl saline). In capsaicin-treated rats, vehicle (200 μl saline) or obestatin (15 nmol in 200 μl saline) was administered IV 30 min after eating. In other experiments, ghrelin (0.3 nmol in 200 μl saline) alone was administered IV 40 min after eating, and/or obestatin (30 nmol in 200 μl saline) was administered IV 10 min before ghrelin administration. Phase III-like contractions were defined as clusters of strong contractions with an amplitude of more than 10 cm H2O (9). The frequency of the fasted motility was obtained from the average of the onset of the phase III-like contractions per hour. In the duodenum of rats in the fed state, the time between the initiation of phase III-like contractions and obestatin/vehicle injection was measured. The %MI for a 10-min period in the antrum was calculated by %MI = (area under the manometric trace for each 10-min period after obestatin, ghrelin, or vehicle injection)/(area under the manometric trace for the 10-min period before obestatin or vehicle injection) × 100. Changes in the mean value of the %MI for each 10-min period between 0–30, 30–60, 60–90, and 90–120 min after administration of obestatin were compared in experiments involving a single injection of obestatin or vehicle; in other experiments, however, only the %MI between 30–60 min after administration was compared.

c-Fos immunohistochemistry and fluorescence overlap staining. On the day of the study, rats were administered vehicle (200 μl saline) or obestatin (30 nmol in 200 μl saline) IV. Subsequently, 120 min after injection, the rat brain was fixed and processed for c-Fos immunohistochemistry with DAB-nickel reactions, as described previously (6). c-Fos-positive cells in the paravenricular nucleus (PVN), arcuate nucleus (ARC), and NTS were observed under a light microscope. Quantitative analysis of the number of c-Fos-positive cells was performed as follows. The total number of c-Fos-positive cells was counted in each brain nucleus of the unilateral side of a brain section under a light microscope at ×200 magnification. The mean value of each brain nucleus was determined by sampling from five randomly selected sections, and the results are expressed as the mean ± SE from three animals. For double staining of c-Fos and corticotropin-releasing factor (CRF) or urocortin 2, sections cut through the PVN from obestatin-injected rats were incubated with a mixture of c-Fos antibody (rabbit polyclonal, Ab5; Oncogene, San Diego, CA) and CRF antibody (guinea-pig polyclonal; Peninsula Laboratory, San Carlos, CA), or a mixture of c-Fos antibody (sc-52G, goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) and urocortin 2 antibody (RY1647, rabbit polyclonal; Yanaihara Instruments, Shizuoka, Japan) diluted 1:5,000. The sections were then reacted with a mixture of FITC-labeled anti-guinea pig IgG (Chemicon, Temecula, CA) and Cy3-labeled anti-rabbit IgG (Chemicon) or a mixture of FITC-labeled anti-rabbit IgG (Chemicon) and Cy3-labeled anti-goat IgG (Chemicon) diluted 1:1,000 and observed under a confocal laser scanning microscope (LSM 510, Carl Zeiss Japan, Tokyo, Japan). To examine the specificity of the positive reaction for CRF and urocortin 2, sections were exposed to the fluorescence-labeled second antibody without the first antibody. The proportion of CRF-positive or urocortin 2-positive neurons that were also c-Fos-positive neurons was determined in the PVN by sampling from five randomly selected...
sections, and the results are expressed as the means ± SE from three animals.

**Statistical analysis.** Data are expressed as the means ± SE. Comparisons between groups were performed by using one-way ANOVA, followed by a Dunnett multiple comparison test (Fig. 2), a Newman-Keuls multiple comparisons test (Figs. 4, 6), or Student’s t-test (Figs. 3, 5). Differences were considered significant at *P* < 0.05.

**RESULTS**

**Effects of obestatin on fasted motor activity in the antrum and duodenum.** In fasted rats, cyclic changes in pressure waves were detected in both the antrum and duodenum, including a quiescent period (phase I-like contractions), followed by a cluster of strong contractions (phase II-like contractions, indicated by arrowheads, Fig. 1). The frequency of phase III-like contractions was not altered in either the antrum or the duodenum by IV injection of obestatin (6.7 ± 0.4/h, *n* = 6 in the antrum; 6.2 ± 0.4/h, *n* = 6 in the duodenum) compared with saline-injected controls (6.3 ± 0.5/h, *n* = 4 in the antrum; 6.0 ± 0.6/h, *n* = 4 in the duodenum).

**Effects of obestatin on fed motor activity in the antrum and duodenum.** When rats were given 3 g of chow, which was consumed within 20 min, the fasted motor pattern was disrupted immediately after feeding and replaced by the fed motor pattern (Fig. 2A). When obestatin was injected IV, the time between the initiation of phase III-like contractions and injection was prolonged dose dependently in the duodenum compared with saline-injected controls (Fig. 2A). In the antrum, sequence changes in the %MI after injection of obestatin were compared with those in saline-injected controls (Fig. 2A). IV injection of obestatin dose dependently decreased the %MI during the 30–90-min period after injection of obestatin (Fig. 2C).

**Activation of specific neuropeptides in the brain induced by IV injection of obestatin.** To elucidate the brain mechanism mediating the action of obestatin on gastroduodenal motility, c-Fos expression in brain nuclei including the PVN, ARC, and NTS was examined. IV injection of obestatin induced a significant increase in the density of c-Fos-positive cells in the PVN, ARC, and NTS (Fig. 3, A and B). Immunofluorescence overlap staining showed that CRF-positive or urocortin-2-positive immunoreactivity was present in the cytoplasm of neuronal cell bodies located in the PVN (green color in Fig. 3C), whereas c-Fos-positive immunoreactivity was found in the nuclei of PVN neurons (red color in Fig. 3C). The specificity of the positive reaction for CRF and urocortin 2 was confirmed by immunofluorescence control study (Fig. 3C, a and d). Results showed that c-Fos-positive neurons overlapped with CRF-positive or urocortin 2-positive neurons in the PVN (arrows in Fig. 3C, c and f). CRF-positive neurons occupied 55 ± 7% (*n* = 3), whereas urocortin-2-positive neurons occupied 43 ± 6% (*n* = 3) of c-Fos-positive neurons in the PVN.

**Blockade of CRF type 1 and type 2 receptors in the brain.** Because morphological findings showed that IV injection of obestatin activated CRF- and urocortin-2-containing neurons in the PVN, we examined the involvement of CRF receptor subtypes in mediating the action of IV-injected obestatin on gastroduodenal motility. The elongation of the time between injection of obestatin and initiation of phase III-like contractions in the duodenum induced by obestatin was reversed by ICV injection of the CRF type 1 antagonist NBI 27914 (5) and the CRF type 2 antagonist antisauvagine-30 (20) (Fig. 4, A–C). The decrease in %MI that was observed 30–60 min after IV injection of obestatin was also reversed by ICV injection of NBI 27914 and antisauvagine-30 (Fig. 4, A, B, and D).

**Effects of vagal afferent nerve blockade on obestatin-induced suppression of gastroduodenal motility.** To investigate the pathways mediating the action of IV-injected obestatin on gastroduodenal motility, rats were subjected to selective vagal afferent nerve blockade by capsaicin. The effect of obestatin on duodenal motility was blocked by capsaicin treatment, whereas the effect of obestatin on antral motility was not altered by this treatment (Fig. 5, A–C).

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**Fig. 1.** Effects of intravenous (IV) injection of obestatin (30 nmol) on fasted motor activity in the antrum and duodenum. Shown is a representative tracing of antral and duodenal motility in fasted rats treated with IV injection of obestatin or saline. Arrowheads indicate phase III-like contractions during fasted motility.
Effects of obestatin on gastroduodenal motility induced by ghrelin. Because ghrelin is known to increase the %MI in the antrum and to induce fasted motor activity in the duodenum when given to fed rats (10), the effects of obestatin on ghrelin-induced changes in gastroduodenal motility were examined. IV injection of ghrelin increased the %MI in the antrum and shortened the time between the injection of ghrelin and the initiation of phase III-like contractions in the duodenum (Fig. 6, A–C). IV injection of obestatin did not alter the increase in the %MI in the antrum induced by ghrelin, nor did it alter the decrease in the time between injection of ghrelin and the initiation of phase III-like contractions in the duodenum induced by ghrelin (Fig. 6, A–C).

DISCUSSION

The present results show that motor activity in the antrum and duodenum is inhibited when obestatin is given IV to conscious rats in the fed state but not when it is given to conscious rats in the fasted state. IV injection of obestatin decreased the %MI of fed motility in the antrum and prolonged the time before the return of fasted motility in the duodenum. Such inhibitory actions are the opposite of those obtained with ghrelin: IV injection of ghrelin stimulates the %MI of fed motor activity in the antrum and induces fasted motility in the duodenum when given in the fed state (10). Inhibition of fed motor activity in the antrum, as measured in the present study, is comparable to the inhibition of gastric emptying measured in previous studies. Zhang et al. (23) showed that obestatin (315 and 2,517 g/kg mouse ip) inhibited gastric emptying 1 to 2 h after food intake but not within 30 min (23), consistent with our data. On the other hand, Gourcerol et al. (11, 12) showed that obestatin affected neither gastric emptying (300 g/kg rat ip) nor gastric intraluminal pressure (300 g/kg rat iv); however, they measured gastric emptying for only 20 min after obestatin administration. De Smet et al. (8) monitored gastric emptying for 4 h by the $^{14}$CO$_2$ octanoic breath test and found that obestatin at various doses (151, 315, and 630 g/kg mouse ip) failed to alter gastric emptying. The doses of obestatin found to inhibit gastroduodenal motility in our study (151 and 302 g/kg rat iv) were consistent with those in previous studies. Our results show that the inhibitory action of obestatin appears 30–90 min after IV injection, which is consistent with the timing of the effects of IV injection of ghrelin (30 min) on gastroduodenal motility (10). Although a previous study has
shown that plasma concentrations of IV-injected obestatin are reduced to 27% within 30 min of injection (24), the time taken for the effects to become apparent does not always seem to be relevant to the plasma concentration of peptides. The present results show that, in the duodenum, obestatin prolongs the time required for return of the fasting pattern when given in the fed state but does not affect the frequency of phase III-like contractions when given in the fasted state. Although it has been
Fig. 4. Effects of intercerebroventricular (ICV) injection of a CRF type 1 receptor antagonist, NBI 27914, or a CRF type 2 antagonist, antisauvagine-30, on gastroduodenal motility induced by obestatin. A and B: representative tracing of antral and duodenal motility in fed rats treated with IV injection of obestatin (15 nmol) or saline combined with ICV injection of NBI 27914 (100 nmol) (A) or antisauvagine-30 (5 nmol) (B). C: effect of antagonists on the time between obestatin injection (15 nmol) and the initiation of phase III-like contraction in the duodenum. **P < 0.01 compared with vehicle-injected controls. D: effect of antagonists on the change in the %MI in the 30–60-min period after obestatin injection (15 nmol). *P < 0.05 compared with vehicle-injected controls. Values are means ± SE (n = 5).
shown that obestatin does not affect the frequency of the MMC in the jejunum of conscious rats (1). In that study, the effect of obestatin was evaluated only in the fasted state and not in the fed state. The present results indicate that obestatin exerts inhibitory effects on the gastroduodenal motility in the fed state but not in the fasted state of animals. We should point out that, in the fed state experiment, rats were given a restricted amount of chow (3 g) after 18 h of fasting; however, rats would normally eat continuously as long as food is available, in contrast to humans and dogs, which consume relatively fixed scheduled meals. In future studies, the effects of obestatin should be examined in continuously fed rats.

As described above, the present results suggest that obestatin may act as an opponent of ghrelin on gastrointestinal motility. Because the effects of ghrelin on gastroduodenal motility are mediated by neuropeptide Y (NPY) neurons in the brain (10), we examined the brain neuropeptides mediating the effects of obestatin on gastroduodenal motility. IV injection of obestatin induces a significant increase in the number of c-Fos-positive cells in the PVN and ARC compared with saline-injected controls, suggesting that neuropeptides located in the PVN and ARC might be activated by obestatin. The orexigenic peptides neuropeptide Y/Agouti-related peptide, as well as the anorexigenic peptides proopiomelanocortin/cocaine- and amphetamine-regulated transcript, are located in the ARC, and these primary neurons may project axons toward secondary neurons located in the PVN and then exert functions on food intake and gastrointestinal motility (14). It is well known that CRF type 1 and type 2 receptors in the brain are involved in the regulation of gastrointestinal motility: CRF is a selective ligand for CRF type 1 receptors, and urocortin 2 is a selective ligand for CRF type 2 receptors (3, 7). For this reason, among the many neuropeptides located in the PVN, we chose to focus on CRF and urocortin 2, and we examined whether neurons expressing these peptides might be activated by peripheral injection of obestatin by using immunofluorescence double staining for neuropeptides and c-Fos. The present results show that the PVN neurons activated by IV injection of obestatin contain CRF or urocortin 2. We further examined the involvement of CRF type 1 and type 2 receptors in the effects of obestatin on gastroduodenal motility by CRF type 1 and type 2 receptor blockade in the brain.

Our results show that the inhibitory action of obestatin on motor activity in the antrum and duodenum is blocked by ICV...
injection of CRF type 1 and type 2 receptor antagonists, suggesting that both types of CRF receptor in the brain may mediate the action of peripherally injected obestatin on gastroduodenal motility. Three peptides, obestatin, ghrelin, and des-acyl ghrelin, are derived from a common prohormone precursor (23); however, des-acyl ghrelin and obestatin exert inhibitory actions, whereas ghrelin exerts stimulatory actions on gastrointestinal motility. Des-acyl ghrelin exerts inhibitory actions in the fasted state but not in the fed state of animals (6), whereas obestatin exerts inhibitory actions in the fed state but not in the fasted state of animals. CRF type 2 receptors in the brain mediate the action of des-acyl ghrelin (6), whereas both CRF type 1 and type 2 receptors in the brain mediate the action of obestatin.

Our results show that vagal afferent nerve blockade by capsaicin reverses the inhibitory effects of obestatin on duodenal motility but does not alter the inhibitory effects of obestatin on antral motility. These results suggest that vagal afferent pathways might be involved partially, but not entirely, in the action of obestatin in regulating gastrointestinal motor activity. Involvement of vagal afferent pathways was confirmed by our observation that c-Fos expression in the NTS is activated by IV injection of obestatin. Although the involvement of vagal afferent pathways in the action of obestatin has been examined previously, the effects of obestatin on vagal afferent nerve activity were found to be very weak or negative compared with those of CCK (11). Taking the results of present and previous studies together, not only vagal afferent...
but also other pathways seem to be involved in the action of obestatin on gastrointestinal motility. In addition to vagal afferent pathways, it is possible that circulating obestatin acts on brain targets directly by crossing the blood-brain barrier (BBB) because a previous study has shown that there is a rapid influx of IV-injected 125I-labeled obestatin from the blood to the brain (19). Therefore, the lack of effects of obestatin on antral motility during capsaicin treatment might be explained by a direct action of peripherally injected obestatin on brain targets by crossing the BBB, similar to what has been observed for Des-acyl ghrelin (6).

We further examined whether obestatin can antagonize the stimulatory effects of ghrelin on gastrointestinal motility. We found that obestatin failed to antagonize the ability of ghrelin either to stimulate the %MI in the antrum or to accelerate the initiation of fasting motor activity in the duodenum when administered in the fed state. These results are consistent with previous studies in which obestatin failed to antagonize the ability of ghrelin to stimulate gastric emptying (8) or to shorten the MMC cycle time (1).

GPR39 was initially proposed as the receptor for obestatin (23), and GPR39 expression has been detected in peripheral organs such as the duodenum and kidney but not in the pituitary or hypothalamus (13). However, more recent publications indicate that obestatin is unlikely to be the endogenous ligand for GPR39 on the basis of a lack of specific binding of obestatin to GPR39 receptor-expressing cells (4, 13, 21, 22). Nevertheless, although binding of obestatin to the receptor GPR39 remains controversial, the functional effect of obestatin on gastrointestinal motility has been clearly demonstrated in the present study.

In conclusion, our findings indicate that obestatin inhibits gastrointestinal motility in the fed state but not in the fasted state of conscious rats. In the brain, CRF- and urocortin 2-containing neurons might be activated by IV injection of obestatin, and, at the receptor level, CRF type 1 and type 2 receptors might be involved in the inhibitory action of obestatin on antral and duodenal motility. Last, vagal afferent pathways might be involved partially, but not entirely, in these actions of obestatin.

REFERENCES


