Role for central ghrelin in food intake and secretion profile of stomach ghrelin in rats

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Abstract

Ghrelin, a 28-amino-acid peptide, has recently been isolated from the rat stomach as an endogenous ligand for the GH secretagogue receptor. We have reported previously that central or peripheral administration of ghrelin stimulates food intake, and the secretion of GH and gastric acid in rats. In the present study, we investigated how much endogenous centrally released ghrelin is involved in the control of food intake and body weight gain. We also examined the profile of ghrelin secretion from the stomach by RIA using two kinds of anti-ghrelin antiserum, one raised against the N-terminal ([Cys^{12}]-ghrelin[1–11]) region and one raised against the C-terminal ([Cys^{0}]-ghrelin [13–28]) region of the peptide. The former antibody recognizes specifically ghrelin with *n*-octanoylated Ser 3 (acyl ghrelin), and does not recognize des-acyl ghrelin. The latter also recognizes des-acyl ghrelin (i.e. total ghrelin). Intracerebroventricular treatment with the anti-ghrelin antiserum against the N-terminal region twice a day for 5 days decreased significantly both daily food intake and body weight. Des-acyl ghrelin levels were significantly higher in the gastric vein than in the trunk. Either fasting for 12 h, administration of gastrin or cholecystokinin resulted in increase of both acyl and des-acyl ghrelin levels. The ghrelin levels exhibited a diurnal pattern, with the bimodal peaks occurring before dark and light periods. These two peaks were consistent with maximum and minimum volumes of gastric content respectively. These results suggest that (1) endogenous centrally released ghrelin participates in the regulation of food intake and body weight, (2) acyl ghrelin is secreted from the stomach, (3) intestinal hormones stimulate ghrelin release from the stomach, and (4) regulation of the diurnal rhythm of ghrelin is complex, since ghrelin secretion is augmented under conditions of both gastric emptying and filling.

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Introduction

The peptide ghrelin has recently been purified from rat stomach, and has been identified as an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R) (Kojima *et al.* 1999). Ghrelin consists of 28 amino acids, including an O-*n*-octanoylated Ser-3 residue, which is essential for GH release. An analogue of ghrelin, des-Gln14-ghrelin, has also been purified from rat stomach, and is a second endogenous ligand for GHS-R (Hosoda *et al.* 2000). Immunohistochemical studies have revealed that ghrelin is also found in the hypothalamic arcuate nucleus of the brain (Kojima *et al.* 1999). It has been demonstrated that intracerebroventricular (i.c.v.) or intravenous administration of ghrelin stimulates the secretion of GH (Kojima *et al.* 1999, Date *et al.* 2000*b*, Wren *et al.* 2000), insulin, gastrin (Lee *et al.* 2002) and gastric acid (Date *et al.* 2001), and increases food intake and body weight gain in rodents (Wren *et al.* 2000, Asakawa *et al.* 2001, Nakazato *et al.* 2001). In addition to a single injection, daily i.c.v. treatments with ghrelin also increase food intake and body weight gain in rats (Tschop *et al.* 2000). These orexigenic actions of ghrelin are mediated by hypothalamic neuropeptide Y and agouti-related protein (Asakawa *et al.* 2001, Kamegai *et al.* 2001, Nakazato *et al.* 2001, Shintani *et al.* 2001). These results suggest that ghrelin plays an important role in energy balance and digestive function. However, it is not clear how much endogenous centrally released ghrelin is involved in the control of food intake or body weight gain. In the study presented here, we examined food intake and body weight gain in rats that had been administered anti-ghrelin antiserum i.c.v. twice a day for 5 days.

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Immunohistochemical studies, including *in situ* hybridization, have revealed that in the gastrointestinal tract, ghrelin is produced mainly from the neck to the base of the oxyntic glands, especially in the X/A-like cells whose physiological role was previously unknown (Date *et al.* 2000*a*). Although it is likely that ghrelin is secreted from the stomach to the gastric vein, no detailed profile has yet been demonstrated. Analysis by reverse-phase HPLC (RP-HPLC) has revealed that both acyl and des-acyl ghrelin exist in the rat gastric fundus at a ratio of 1·3:1 (des-acyl ghrelin : acyl ghrelin) (Toshinai *et al.* 2001). However, it is not clear whether they are secreted from the stomach. Furthermore, there is little information concerning the regulation of ghrelin secretion from the stomach. Plasma ghrelin levels, as determined by RIA using a ghrelin antiserum against the C-terminal fragment (residues 13–28), were increased by vagotomy, and treatment with thyroxine (T_4) and leptin did not influence plasma ghrelin levels (Lee *et al.* 2002). Although in these cases plasma ghrelin was measured after chronic endocrine manipulation that was performed two or three times per day for 3–4 days, it does appear at least that ghrelin secretion is influenced by some gastrointestinal hormones. In a preliminary experiment, plasma ghrelin levels were measured by RIA using two types of anti-ghrelin antiserum (one raised against the N-terminal region and the other against the C-terminal region). The antibody raised against the N-terminal region recognized acyl ghrelin, but not des-acyl ghrelin; that raised against the C-terminal region recognized both acylated and des-acyl ghrelin. These two antibodies provided values that were quite different. In the present study, we compared the plasma levels of acylated and total ghrelin in the gastric vein and trunk. In addition, we examined the acute effects of administration of gastrin and cholecystokinin (CCK), and electrical stimulation of the vagus nerve on ghrelin secretion from the stomach in rats.

Materials and Methods

Animals and experimental schedule

Adult male Wistar rats (Charles River Japan Inc., Shiga, Japan) were kept under a light regimen of 12 h light : 12 h darkness (light on at 0700 h) and a temperature of 23 ± 1 °C. Animals were supplied with standard laboratory chow and water and allowed to feed *ad libitum*. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care. All experiments were repeated twice.

In the first experiment, we used 15-week-old rats weighing about 400 g. The body weight at this age has almost reached maximum. Each of four rats were administered a 10 μl saline solution of purified anti-ghrelin IgG (0·1 and 1·0 μg) or 1·0 μg pre-immune serum IgG twice a day (at 0845 and 1845 h) for 5 days. The anti-ghrelin antiserum used in this study was the type raised against the N-terminal region of ghrelin (*n*-octanoylated rat [1–11] ghrelin). Food intake and body weight were measured every morning for 5 days. The method of preparation of anti-ghrelin IgG is described elsewhere (Nakazato *et al.* 2001).

In a second experiment, each of four adult male rats weighing about 300 g were used for measurements of plasma ghrelin levels in the gastric vein and trunk. Rats were anesthetized with pentobarbital at 1400 h, after which the blood was collected from the gastric vein (Toshinai *et al.* 2001), and then from the heart puncture. Each 1 ml of truncal or gastric vein blood was drawn into chilled polypropylene tubes containing disodium EDTA (EDTA-2Na; 1 mg/ml blood), aprotinin (500 units/ml blood) and Pefabloc SC (Roche, Nutley, NJ, USA; 2·4 mg/ml blood), and then centrifuged. The plasma was stored at − 80 °C until extraction of ghrelin.

In a third experiment, blood was collected from the gastric vein of each of four rats after 12 h of fasting (from 1900 to 0700 h), and 40 min after an i.p. injection of either CCK (20 μg/kg body weight; Sigma Chemical Co., St Louis, MO, USA), gastrin (2 μg/kg body weight, Sigma) or saline. In addition, eight rats were anesthetized using pentobarbital, and the left cervical vagus nerve was stimulated electrically for 10 min. This electrical stimulation was performed using bipolar electrodes, with stimulation at a frequency, intensity and duration of 20 Hz, 10 V and 1 ms respectively, each for 30 s, and at 30-s intervals. It has been shown that this manipulation increases gastric acid secretion by over fivefold compared with the no-treatment condition. In four of the eight rats, blood samples were collected from the gastric vein 30 and 90 min after stimulation.

In a fourth experiment, to examine the diurnal pattern of plasma ghrelin levels, three rats were killed by decapitation at 3-h intervals for 24 h. The stomach contents were weighed. A sample of blood was taken for glucose measurement using a commercial assay kit (Nakarai Chemical Co., Tokyo, Japan). The remaining blood, which was taken for ghrelin assay, was treated as described earlier.

RIA

After the plasma had been diluted with an equal volume of 0·9% saline, it was applied to a Sep-Pak C-18 cartridge that had been pre-equilibrated with 0·9% saline. The cartridge was washed with saline and 10% CH_3CN solution containing 0·1% trifluoroacetic acid (TFA). The absorbed peptides were eluted with 60% CH_3CN solution containing 0·1% TFA, lyophilized, then subjected to RIA. Two polyclonal antibodies, one against the N-terminal fragment of ghrelin (*n*-octanoylated rat [1–11] ghrelin) and the other against the C-terminal fragment of ghrelin (rat [13–28] ghrelin), were produced in a rabbit. Details of the

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method of preparation and characterization of this antibody have been described elsewhere (Kojima *et al.* 1999). The anti-rat ghrelin [1–11] antiserum #G606 specifically recognized ghrelin with an *n*-octanoylated Ser 3 and did not recognize des-acyl ghrelin. Synthetic rat [Tyr^{29}]-ghrelin[1–28] was radioiodinated by the lactoperoxidase method. The ^{125}I-labeled peptide was then purified by RP-HPLC on TSK ODS SIL 120A column (Tosoh Co. Ltd, Tokyo, Japan). The RIA incubation buffer was 50 mM sodium phosphate (pH 7·4) containing 0·5% bovine serum albumin (BSA; treated with N-ethylmaleimide), 80 mM NaCl, 25 mM EDTA-2Na, 0·05% NaN_3 and 0·5% Triton X-100. For the assay, a diluted sample or standard solution (100 µl) was incubated for 24 h with 100 µl anti-ghrelin antiserum (final dilution 1/620 000). The tracer solution (16 000 c.p.m. in 100 µl) was added, and the mixture was incubated for a further 24 h. The bound and free ligands were separated by a second antibody (200 µl). All procedures were carried out at 4 °C, and all plasma samples were assayed in duplicate. The half-maximum inhibition level produced by rat ghrelin [1–28] on the standard RIA curve was 3·8 fmol/tube with anti-ghrelin [1–11] antiserum (#606) and 80 fmol with anti-ghrelin [13–28] antiserum (#G107). The intra- and inter-assay coefficients of variation of the RIA for the ghrelin N-terminal fragment (#G606) at 50% binding were 3·5% and 3·2% respectively, while those of RIA for the ghrelin C-terminal fragment (#G107) were 3·7% and 3·3% respectively. The mean (± S.E.M.) recoveries of rat ghrelin [1–28] (1 ng) and ^{125}I-rat ghrelin [1–28] (5000 c.p.m.), which were added to the rat plasma then extracted using Sep-Pak C-18 cartridges (Waters, Milford, MA, USA), were 92·2 ± 0·4% and 88·9 ± 0·6% respectively.

The statistical analysis was carried out by analysis of variance with the *post hoc* Fisher test. $P<0·05$ was considered significant.

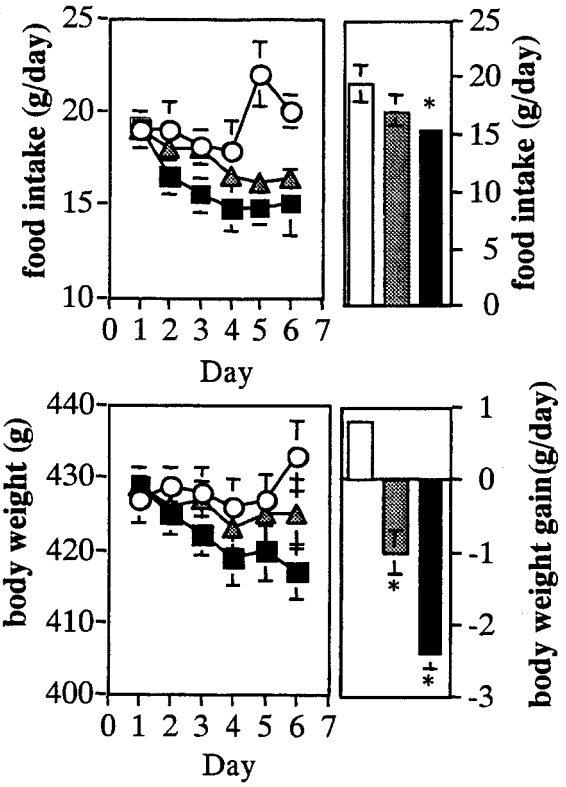

Figure 1 Effect of daily i.c.v. treatment with anti-ghrelin IgG on daily food intake and body weight gain: 1 µg/10 µl pre-immune IgG (open circle and open bar), 0·1 µg/10 µl (gray triangle and gray bar) or 1 µg/10 µl (black square and black bar) anti-ghrelin IgG raised against the N-terminal region of ghrelin were injected twice a day (at 0845 h and 1845 h) for 5 days. The left panels represent the daily change of food intake and body weight. The right panels represent the average food intake and body weight gain per day. The symbols and bars represent mean values, the vertical lines are ± S.E.M. ($n=8$), and the asterisks indicate significant differences ($P<0·05$ vs pre-immune IgG alone).

Results

Daily i.c.v. injections of 0·1 and 1 µg anti-ghrelin IgG gradually decreased both daily food intake and body weight in a dose-dependent manner (Fig. 1). The average of daily food intake was significantly reduced by 1 µg anti-ghrelin IgG. The average body weight gain per day was significantly reduced by both 0·1 µg and 1 µg anti-ghrelin IgG. The effect of over 2 µg of IgG could not be estimated because the same level of pre-immune IgG also decreased food intake and body weight gain. Since levels of acyl ghrelin and total ghrelin were measured by RIA using the anti-ghrelin antiserum against the N-terminal region (rat [1–11] ghrelin) and that against the C-terminal region (rat [13–28] ghrelin) respectively, expected des-acyl ghrelin levels may be calculated using the following equation: des-acyl ghrelin = total ghrelin − acyl ghrelin. Both acyl ghrelin and total ghrelin levels in the gastric vein were higher than those in the trunk (Fig. 2). Total ghrelin was higher by over fivefold compared with acyl ghrelin in the gastric vein and trunk. However, there was no significant difference in calculated des-acyl ghrelin between the gastric vein and trunk (gastric vein: 0·62 ± 0·08 pmol/ml vs trunk: 0·51 ± 0·06 pmol/ml). The 12 h of fasting of dark-phase feeding significantly increased both acyl and total ghrelin levels in the gastric vein. Similarly, a single i.p. administration of CCK or gastrin increased significantly both acylated and total ghrelin levels in the gastric vein (Fig. 3). The expected des-acyl ghrelin levels were as follows: saline 1·15 ± 0·16 pmol/ml, fasting 1·57 ± 0·24 pmol/ml, gastrin 1·89 ± 0·25 pmol/ml ($P<0·05$ vs saline), and CCK 3·38 ± 0·042 pmol/ml ($P<0·05$ vs saline). However, electrical stimulation of the left cervical vagus nerve did not

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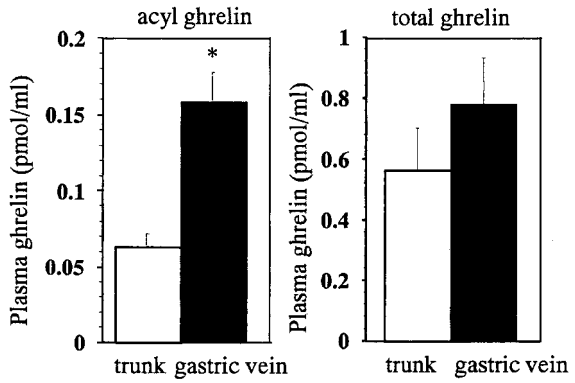


Figure 2 Comparison of plasma acyl and total ghrelin levels between the gastric vein and trunk. Acyl ghrelin and total ghrelin were measured by RIA using the anti-ghrelin antiserum raised against N-terminal (*n*-octanoylated, rat [1–11] ghrelin) and that raised against the C-terminal (rat [13–28] ghrelin) region respectively. The bars and vertical lines represent mean values ± S.E.M. ($n=8$). *$P<0{\cdot}05$ vs trunk.

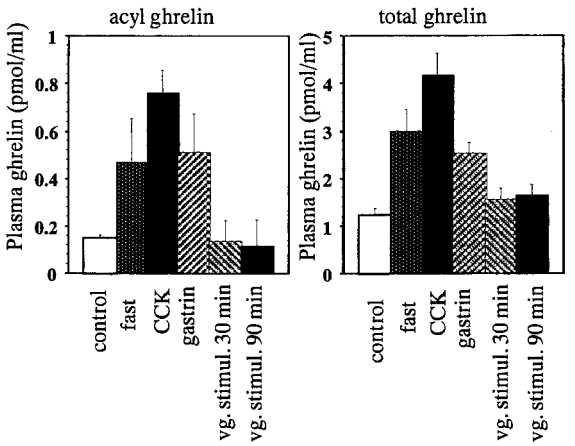
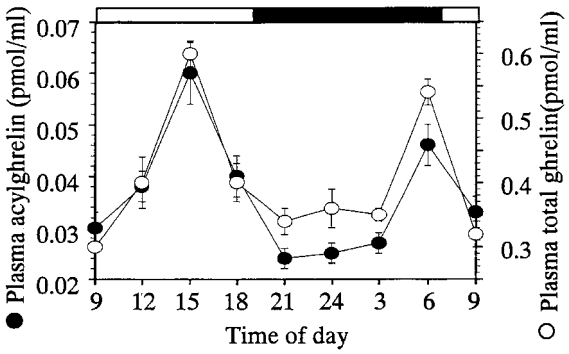


Figure 3 Effect of 12 h of fasting, an i.p. injection of gastrin or CCK, and electrical stimulation of the vagus nerve on plasma ghrelin levels. Blood samples were collected from the gastric vein and from the heart 12 h (1900–0700 h) after fasting, and 40 min after an i.p. injection of CCK (20 μg/kg body weight), gastrin (2 μg/kg body weight) or saline, and 30 or 90 min after electrical stimulation of the left cervical vagus nerve (vg. stimul.). The bars and vertical lines represent mean values ± S.E.M. ($n=8$).



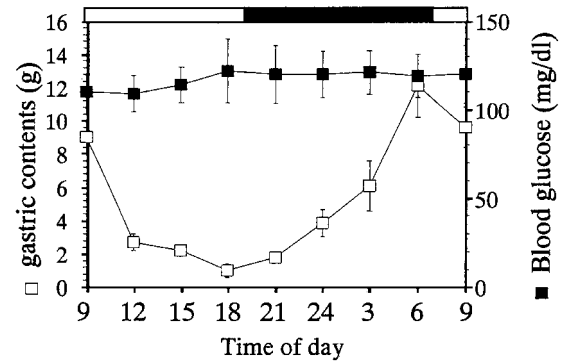


Figure 4 Diurnal pattern of plasma ghrelin levels, blood glucose levels and volume of gastric contents. The blood was collected by decapitation. The open and closed circles represent the total ghrelin and acyl levels respectively. Please note the scale difference. The open and closed squares represent the volume of gastric contents and blood glucose respectively. The symbols and vertical lines represent mean values ± S.E.M. ($n=8$). The upper white and dark bars indicate the light and dark periods respectively.

influence either the acyl or total ghrelin levels in the gastric vein (Fig. 3). Both the acyl and total ghrelin levels exhibited a diurnal pattern of release, with bimodal peaks occurring in free-feeding rats. The first and second peaks were observed at 1500 h into the light period and at 0600 h into the dark period respectively (Fig. 4). Although blood glucose levels were almost constant during the day, the volume of gastric contents exhibited a diurnal rhythm, with the increase and decrease occurring during the dark and light period respectively.

Discussion

It has been discovered recently that ghrelin is an endogenous ligand for GHS-R in rat and human stomachs (Kojima *et al.* 1999). Immunohistochemical studies have revealed that in addition to the stomach, ghrelin may be found in the hypothalamic arcuate nucleus (Kojima *et al.* 1999). Accumulating data on the physiological role of ghrelin indicate that it may play an important role in the secretion of GH (Kojima *et al.* 1999, Date *et al.* 2000*b*, Wren *et al.* 2000), gastric acid (Date *et al.* 2001), insulin, gastrin (Lee *et al.* 2002) and luteinizing hormone (Furuta *et al.* 2001), and in the control of food intake (Tschop *et al.* 2000, Asakawa *et al.* 2001, Nakazato *et al.* 2001). In the present study, daily i.c.v. administrations of anti-ghrelin antiserum to neutralize the centrally released ghrelin decreased significantly daily food intake and body weight gain, supporting the hypothesis that endogenous centrally released ghrelin may be involved in the control of daily food intake. However, contrary to our expectation, the

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suppressing effect of daily food intake by anti-ghrelin antiserum was small, since the i.c.v. injection of even small doses (10 pmol) of ghrelin was shown to significantly stimulate food intake (Nakazato *et al.* 2001). Since, as we observed in a previous report, a single injection of 0·5 μg of anti-ghrelin IgG suppressed food intake to half or less in 8-h fasted rats, it is unlikely that the low level of suppression effected by anti-ghrelin antiserum is the result of insufficient anti-ghrelin. Therefore, the participation of centrally released ghrelin in controlling daily food intake may not be physiologically predominant. On the other hand, it has been reported recently that in mice, vagotomy inhibits the food intake induced by peripheral administration of ghrelin, suggesting that endogenous peripherally released ghrelin stimulates food intake via a mainly peripheral action (Lee *et al.* 2002). Further study is required to elucidate the mechanism of action of ghrelin in the control of food intake.

From their morphology and distribution, the cells in the stomach that were immunostained with the anti-serum against the N-terminal region of the peptide were considered to be X/A-like cells whose hormonal product was previously unclear (Date *et al.* 2000). Analysis by RP-HPLC has revealed that both acyl and des-acyl ghrelin exist in the rat gastric fundus at a ratio of 1·3:1 (des-acyl ghrelin : acyl ghrelin) (Toshinai *et al.* 2001). These results indicate the possibility that both acyl and des-acyl ghrelin are secreted from X/A-like cells. In the present study, plasma ghrelin levels were measured using two kinds of anti-ghrelin antiserum. The antiserum raised against the N-terminal region of ghrelin recognized acylated ghrelin only, which seems to be actively involved in GH secretion. The antiserum raised against the C-terminal region of ghrelin recognized both acylated and des-acylated ghrelin (total ghrelin). The fact that des-acyl ghrelin levels were about fourfold higher than acyl ghrelin suggests that the des-acyl form of ghrelin is dominant in the blood. In a comparison between gastric vein and trunk levels, however, levels of acyl ghrelin were higher in the gastric vein than in the trunk, indicating that acyl ghrelin is the predominantly secreted form in the stomach. Furthermore, these results suggest that endogenous ghrelin is easily and rapidly decomposed after secretion from the stomach, or that the decomposed fragments are retained in the blood for a long period of time. These observations were consistent with the preliminary results obtained by analysis by RP-HPLC (data not shown). The 12-h fast stimulated acyl ghrelin secretion from the stomach. This result is partially consistent with previous reports showing that 48 or 72 h of fasting increases plasma ghrelin levels, and re-feeding decreases these levels (Tschop *et al.* 2000, Toshinai *et al.* 2001, Lee *et al.* 2002). However, this study indicates that even half a day of emptiness can stimulate ghrelin secretion from the stomach. Under empty conditions, vagus nerve activity is reduced. In the present study, electrical stimulation of the vagus nerve did not increase acyl ghrelin secretion, rather it showed a tendency to decrease. Stimulation of the vagus nerve is thought to have similar effects to high activity of the vagus nerve under fully fed conditions. Therefore, these results may indicate that activity of the vagus nerve suppresses ghrelin secretion. This speculation is supported by the fact that vagotomy increases plasma ghrelin levels (Lee *et al.* 2002).

The i.p. administration of gastrin and CCK increased ghrelin secretion from the stomach. Both acyl and des-acyl ghrelin levels were increased in the gastric vein within 40 min of administration, suggesting that gastrin and CCK rapidly stimulate ghrelin secretion. However, the interpretation of these results is difficult, since gastrin and CCK are usually secreted after feeding and, conversely, their levels are reduced by fasting. In addition, CCK increases vagus nerve activity. Therefore, it should be assumed that, in general, gastrin and CCK reduce ghrelin secretion. On the other hand, ghrelin has been shown to stimulate gastric acid secretion via stimulation of the vagus nerve. Therefore, gastrin and CCK may stimulate contemporary ghrelin to stimulate gastric acid secretion.

We observed diurnal change in plasma ghrelin levels in free-feeding rats. Both acyl and des-acyl ghrelin express the same pattern with two peaks, one at 1500 h into the light period and one at 0600 h into the dark period. Although the physiological significance of these peaks is unknown, there was no correlation with blood glucose levels. On the other hand, the first peak of ghrelin at 1500 h into the light period and the second peak at 0600 h into the dark period are consistent with the lowest volume and greatest volume of gastric contents respectively. This indicates that ghrelin secretion is increased under conditions of both gastric emptying and filling. Therefore, one possible explanation is that the function of the peak in ghrelin levels at 1500 h into the light period is to stimulate food intake, and that the peak at 0600 h into the dark period is to stimulate gastric acid secretion. If this is the case, gastrin and CCK may participate in the later peak.

In conclusion, although ghrelin plays an important role in the control of food intake, the site of action may be different between centrally and peripherally released ghrelin. Acyl ghrelin is secreted from the stomach into the gastric vein; the regulation of this secretion seems to be complex, because peripheral ghrelin levels are highest under both gastric emptying and filling conditions. These bimodal peaks, which occur under contradictory conditions, may each play different physiological roles. Further studies are required to elucidate the regulatory mechanism underlying ghrelin secretion, and its physiological roles.

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