

Stomach Is a Major Source of Circulating Ghrelin, and Feeding State Determines Plasma Ghrelin-Like Immunoreactivity Levels in Humans

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Ghrelin, an endogenous ligand for the GH secretagogue receptor, was isolated from rat stomach and is involved in a novel system for regulating GH release. Although previous studies in rodents suggest that ghrelin is also involved in energy homeostasis and that ghrelin secretion is influenced by feeding, little is known about plasma ghrelin in humans. To address this issue, we studied plasma ghrelin-like immunoreactivity levels and elucidated the source of circulating ghrelin and the effects of feeding state on plasma ghrelin-like immunoreactivity levels in humans. The plasma ghrelin-like immunoreactivity concentration in normal humans measured by a specific RIA was 166.0 ± 10.1 fmol/ml. Northern blot analysis of various human tissues identified ghrelin mRNA

found most abundantly in the stomach and plasma ghrelin-like immunoreactivity levels in totally gastrectomized patients were reduced to 35% of those in normal controls. Plasma ghrelin-like immunoreactivity levels were increased by 31% after 12-h fasting and reduced by 22% immediately after habitual feeding. In patients with anorexia nervosa, plasma ghrelin-like immunoreactivity levels were markedly elevated compared with those in normal controls (401.2 ± 58.4 vs. 192.8 ± 19.4 fmol/ml) and were negatively correlated with body mass indexes. We conclude that the stomach is a major source of circulating ghrelin and that plasma ghrelin-like immunoreactivity levels reflect acute and chronic feeding states in humans. (*J Clin Endocrinol Metab* 86: 4753–4758, 2001)

GH SECRETAGOGUES (GHSs) are synthetic compounds that stimulate GH release from the pituitary in animals and humans through a specific G protein-coupled receptor (GHS-R) (1, 2). Recently, an endogenous ligand for the GHS-R, ghrelin, was identified from rat stomach and was revealed to be an acylated peptide of 28 amino acids in which the serine-3 residue is *n*-octanoylated (3, 4). This *n*-octanoylation is indispensable for biological activity of ghrelin. Synthetic ghrelin stimulates GH release in rat primary pituitary cell cultures, and iv administration of ghrelin results in an increase in serum GH in rats and humans (3, 5–7). The stimulatory effect of ghrelin on GH secretion is more potent than that of GHRH (7). These data indicate that ghrelin is involved in a novel GH-regulating system along with GHRH and somatostatin. The sites of action of GH release by ghrelin remain unclear, although they seem to be at both hypothalamic and pituitary levels (1, 8).

Previous studies show that ghrelin acts as an orexigenic peptide in rodents. Intracerebroventricular administration of ghrelin induces food intake and increases mRNA expression of hypothalamic neuropeptide Y, a potent stimulator of food intake (9–11). It is also shown that peripheral daily admin-

istration of ghrelin causes weight gain by reducing fat utilization without a significant change in food intake in rodents (12). These data suggest that ghrelin has actions involved in energy homeostasis in animals. Meanwhile, a few studies on rodents have been made on the source of circulating ghrelin and determinant factors of plasma ghrelin levels. Northern blot analysis shows that ghrelin mRNA is expressed most abundantly in the stomach (3, 13), and circulating ghrelin levels are increased by fasting and reduced by feeding (12). Taken together, in animals it is likely that circulating ghrelin is derived from the stomach and influenced by feeding state. However, little is known about plasma ghrelin in humans.

The aim of this study was to estimate plasma ghrelin-like immunoreactivity (ghrelin-LI) levels in humans and to elucidate the source of circulating ghrelin and the effects of fasting and feeding on them. Here we show that ghrelin-LI is detected in considerable amounts in plasma, and a major source of circulating ghrelin is the stomach in humans. Moreover, we show that plasma ghrelin-LI levels reflect acute and chronic feeding states.

Materials and Methods

The studies conform to the policy of the ethical committee on human research of Kyoto University Graduate School of Medicine, and all subjects gave their written informed consent.

Abbreviations: BMI, Body mass index; ghrelin-LI, ghrelin-like immunoreactivity; GHS, GH secretagogue; GHS-R, GH secretagogue receptor.

Northern blot analysis

Northern blot analysis for human tissues was performed as previously described (14) by using MTN blots (CLONTECH Laboratories, Inc., Palo Alto, CA) and the ^{32}P -labeled full-length human ghrelin cDNA fragment (3) as a probe. The membranes contain 2 μg polyadenylated RNA from specific human tissues in each lane. The membranes were hybridized at 68 C in ExpressHyb (CLONTECH Laboratories, Inc.) for 1 h and were washed at 65 C in $0.1 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl and 15 mM sodium citrate, pH 7.0) twice. Imaging was performed using image analyzer BAS 2500 (Fuji Photo Film Co., Ltd., Tokyo, Japan). The probe was stripped, and the membranes were rehybridized with a human β -actin genomic probe (Wako Pure Chemical, Osaka, Japan) to confirm the integrity of the RNA samples on the membranes used. The mRNA levels were quantitated by using Image Reader (Fuji Photo Film Co., Ltd., Tokyo, Japan), and human ghrelin mRNA levels were normalized to β -actin mRNA levels among the tissues.

Subjects and study protocol

Plasma ghrelin-LI levels in normal subjects. Normal volunteers (26 men and 35 women) with no apparent medical illness were recruited. Their age and body mass index (BMI) were 26 ± 1 yr and 20.7 ± 0.3 kg/m 2 , respectively (mean \pm SD). Blood samples for basal plasma ghrelin-LI levels were drawn between 0800–1000 h after overnight fasting.

Gastrectomized patients. Thirteen patients, aged 68 ± 4 yr, who underwent total gastrectomy due to gastric cancer or perforated gastric ulcer, were studied. They consisted of eight men and five women, and their mean BMI was 23.3 ± 2.8 kg/m 2 . They underwent the gastrectomy 1–8 yr before this study. Apparent medical illnesses were excluded, and they took no medications. Thirteen sex- and age-matched control subjects, aged 70 ± 4 yr, were also examined. They had no apparent medical illness, and their BMI was 23.5 ± 1.5 kg/m 2 . Blood samples were drawn between 0800–1000 h after overnight fasting. Changes in the plasma ghrelin-LI level before and after total gastrectomy were also studied in two male patients, aged 71 and 65 yr, who underwent total gastrectomy due to gastric cancer (Table 1). Blood samples were drawn immediately before and 30 min after the gastrectomy.

Effects of 12-h fasting and habitual feeding. Ten normal subjects were studied. They consisted of five men and five women, aged 27 ± 3 yr, and their mean BMI was 21.3 ± 2.6 kg/m 2 . The subjects fasted for total of 12 h (only free access to water was allowed) initiated at 2100 h on d 1 and terminated at 0900 h on d 2; then they consumed breakfast and lunch at 0900 and 1200 h on d 2, respectively. They were instructed to maintain a balanced diet and to avoid drinking alcohol. Blood samples were drawn at 2100 h on d 1 and at 0900 h (after 12-h overnight fasting), 1000 h (1 h after breakfast), 1200 h (before lunch), and 1300 h (1 h after lunch) on d 2.

Patients with anorexia nervosa. The study population consisted of 31 women, aged 25 ± 1 yr. All patients met the criteria of the Diagnostic and Statistical Manual of Mental Disorders (fourth edition, revised) for anorexia nervosa (15) and had BMIs less than 17.5 kg/m 2 . They took no medications. The control subjects were 35 age-matched healthy female volunteers of normal weight (BMI, 20.4 ± 0.4 kg/m 2) with no medical illness. Changes in the plasma ghrelin-LI level examined in 3 female patients, aged 24, 27, and 29 yr, were shown in Table 2. They were hospitalized for nutritional treatment, and blood samples were drawn before and after 1 and 2 month of treatment. Two men with anorexia nervosa, aged 25 and 19 yr (BMIs, 16.3 and 16.5 kg/m 2 , respectively), were also studied to examine possible gender effect on the plasma ghrelin-LI level in patients with anorexia nervosa. All blood samples were drawn between 0800–1000 h after overnight fasting.

TABLE 1. Plasma ghrelin-LI levels before and 30 min after total gastrectomy in two patients

| Patient | Age (yr)/sex | Plasma ghrelin-LI (fmol/ml) | |
|---------|--------------|-----------------------------|-------------------|
| | | Before gastrectomy | After gastrectomy |
| A | 71/M | 102.1 | 34.9 |
| B | 65/M | 105.5 | 70.2 |

Measurements. Polyclonal antibody was raised against the carboxyl-terminal (Gln 13 -Arg 28) of human ghrelin, which is identical with that of rat ghrelin, in rabbits as previously described (3, 16). Blood samples were immediately transferred to chilled polypropylene tubes, containing Na $_2$ EDTA (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Inc., Kyoto, Japan; 1,000 kallikrein inactivator U/ml) and centrifuged at 4 C. One milliliter of the separated plasma was diluted with an equal volume of 0.9% NaCl and loaded onto a Sep-Pak C $_{18}$ cartridge (Waters, Milford, MA) preequilibrated with 0.9% NaCl. The cartridge was washed with 3.0 ml 5% CH $_3$ CN/0.1% trifluoroacetic acid and eluted with 3.0 ml 60% CH $_3$ CN/0.1% trifluoroacetic acid. The eluate was evaporated, lyophilized, and dissolved in RIA buffer [50 mM sodium phosphate buffer (pH 7.4), 0.5% BSA, 0.5% Triton X-100, 80 mM NaCl, 25 mM EDTA-2Na, and 0.05% NaN $_3$]. The RIA was carried out as previously described (3, 16, 17). A tracer ligand, [Tyr 0]human ghrelin-(13–28) for antihuman ghrelin-(13–28) was synthesized. The ligand was radioiodinated using lactoperoxidase methods (16). After radioiodination, monoiodinated ligand was purified by reverse phase HPLC on a μ Bondasphere C $_{18}$ column (3.9×150 mm; Waters Corp., Milford, MA). The tracer was stored at -20 C in 0.1% BSA. Each RIA incubation mixture was composed of 100 μl standard ghrelin or unknown sample, and 200 μl antiserum diluted with RIA buffer containing 0.5% normal rabbit serum. The antihuman ghrelin-(13–28) antiserum was used at final dilution of 1:20,000. After 12-h incubation, 100 μl ^{125}I -labeled tracer (15,000 cpm) were added. After an additional 36-h incubation, 100 μl antirabbit IgG goat serum were added. Free and bound tracer were separated after incubation for 24 h by centrifugation at 3,000 rpm for 30 min. After aspiration of supernatant, radioactivity in the pellet was counted with a γ -counter (ARC-600, Aloka, Tokyo, Japan). The minimal detectable quantity of the RIA was 5.0 fmol/tube, and the intra- and interassay coefficients of variation were 6.0% and 9.0%, respectively.

Statistical analysis

Data are expressed as the mean \pm SE. Comparisons between groups were performed with unpaired *t* test. Changes in the plasma ghrelin-LI level after fasting and feeding were compared using paired *t* test. Simple linear regression analysis was used to evaluate correlation between plasma ghrelin-LI levels and BMIs. Probabilities less than 0.05 were considered statistically significant.

Results

Tissue distribution of ghrelin mRNA

Northern blot analysis identified two ghrelin mRNA bands of 0.60 kb (major) and 1.10 kb (minor) in size (Fig. 1). They were found most abundantly in the stomach, followed by the duodenum, jejunum, and lung. Only one major ghrelin mRNA band of 0.60 kb in size was detected in the jejunum and lung. The rank order of the normalized ghrelin mRNA levels in human tissues was stomach $>$ duodenum \gg jejunum \geq lung, and the amounts of ghrelin mRNA in arbitrary units were 100, 18, 1.0, and 0.6, respectively. No significant amount of ghrelin mRNA was detected in the esophagus, ileum, ileocecum, cecum, colon, rectum, liver, brain, heart, skeletal muscle, thymus, spleen, kidney, placenta, or leukocyte.

Plasma ghrelin-LI levels in normal subjects and gastrectomized patients

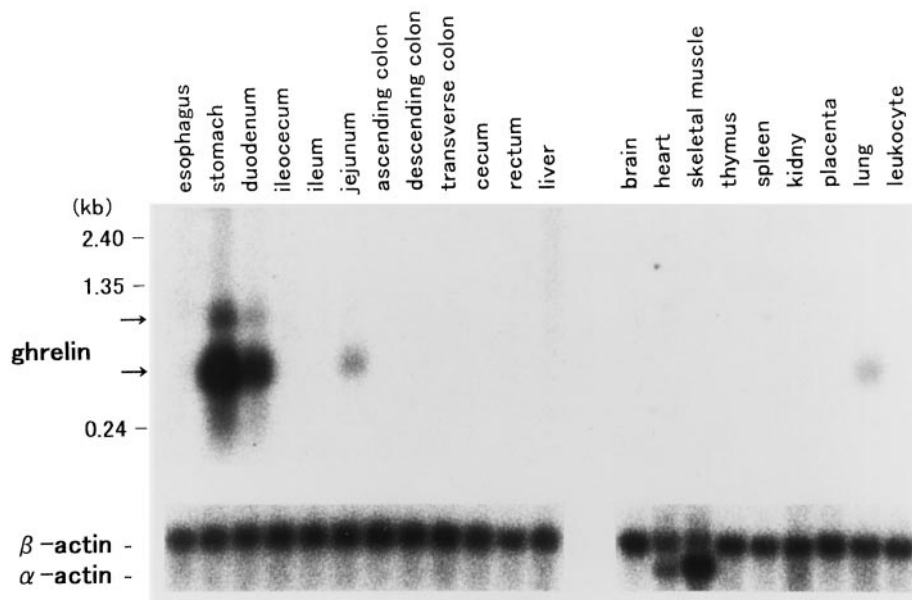
The plasma ghrelin-LI concentration in normal human subjects ($n = 61$) was 166.0 ± 10.1 fmol/ml. To assess the relative contribution of the stomach to circulating ghrelin, plasma ghrelin-LI levels in totally gastrectomized patients were compared with those in sex- and age-matched control subjects. Plasma ghrelin-LI concentrations in the gastrectomized patients and control subjects were 58.7 ± 9.0 and

TABLE 2. Plasma ghrelin-LI levels before and after nutritional treatment in three patients with anorexia nervosa

| Patient | Age (yr)/sex | Plasma ghrelin-LI (fmol/ml) | | | BMI (kg/m ²) | | |
|---------|--------------|-----------------------------|----------------------|----------------------|--------------------------|----------------------|----------------------|
| | | Before treatment | After 1-mo treatment | After 2-mo treatment | Before treatment | After 1-mo treatment | After 2-mo treatment |
| A | 24/F | 826.4 | 525.4 | 337.6 | 12.2 | 10.6 | 12.6 |
| B | 27/F | 1439.0 | 991.2 | 485.9 | 10.7 | 10.7 | 11.3 |
| C | 29/F | 1361.7 | 222.2 | 199.4 | 9.9 | 10.7 | 11.7 |

mo, Month.

FIG. 1. Northern blot analysis of human ghrelin mRNA. Polyadenylated RNA (2 μ g/lane) from various human tissues was analyzed by using the full-length human ghrelin cDNA fragment as a probe. The lower panel indicates hybridization with a β -actin probe as an internal control.



165.4 \pm 28.2 fmol/ml, respectively (Fig. 2). That is, plasma ghrelin-LI levels in the gastrectomized patients were reduced to 35% of those in the control subjects. The difference was statistically significant ($P < 0.005$). There was no significant correlation between the duration after gastrectomy and the plasma ghrelin-LI levels. Plasma ghrelin-LI levels in two patients examined 30 min after total gastrectomy were promptly reduced compared with those immediately before gastrectomy (Table 1).

Effects of fasting and feeding on plasma ghrelin-LI levels

Individual responses of plasma ghrelin-LI levels in 10 normal subjects before and after 12-h fasting are illustrated in Fig. 3A. Nine of 10 subjects showed elevated plasma ghrelin-LI levels after fasting. Plasma ghrelin-LI concentrations before and after 12-h fasting were 114.1 \pm 18.7 and 145.1 \pm 21.5 fmol/ml, respectively (Fig. 3B), and the percent increase was 30.9 \pm 10.2%. The difference was statistically significant ($P < 0.05$). Figure 3, C and D, illustrate responses of plasma ghrelin-LI levels to habitual feeding. Acute 20.3% and 23.6% declines in the plasma ghrelin-LI level occurred 1 h after habitual breakfast and lunch, respectively. The differences were statistically significant ($P < 0.05$ for breakfast and $P < 0.01$ for lunch). We also examined the time course of the plasma ghrelin-LI levels after breakfast and lunch. The nadir of the plasma ghrelin-LI level was 1 or 1.5 h after meals (data not shown).

Plasma ghrelin-LI levels in patients with anorexia nervosa

Plasma ghrelin-LI concentrations in patients with anorexia nervosa and sex- and age-matched control subjects were 401.2 \pm 58.4 and 194.3 \pm 19.4 fmol/ml, respectively (Fig. 4A). The difference was statistically significant ($P < 0.0001$). Figure 4B illustrates the correlation between plasma ghrelin-LI levels and BMIs in the patients with anorexia nervosa and control subjects. There was a significant negative correlation between them ($r = -0.57$; $P < 0.0001$). Plasma ghrelin-LI concentrations in two severely affected patients, whose BMIs were less than 11.0 kg/m², reached 1439.0 and 1361.7 fmol/ml (Fig. 4B). Changes in the plasma ghrelin-LI level in three patients, including the two who were hospitalized and underwent nutritional treatment, are summarized in Table 2. They showed remarkable declines in the plasma ghrelin-LI level after 1- or 2-month treatment despite minimal increases in BMIs. Plasma ghrelin-LI levels in two men with anorexia nervosa were 240.3 and 474.3 fmol/ml.

Discussion

The present study demonstrates that ghrelin-LI is detected at a considerable concentration (166.0 \pm 10.1 fmol/ml) in human plasma. The plasma ghrelin-LI levels were comparable with those in a previous preliminary study in humans (3). Although we observed a difference in the plasma ghrelin-LI level between men and women, with high values in men (men, 130.0 \pm 10.3; women, 194.3 \pm 19.4 fmol/ml), this may be due to difference in BMIs as mentioned below. The

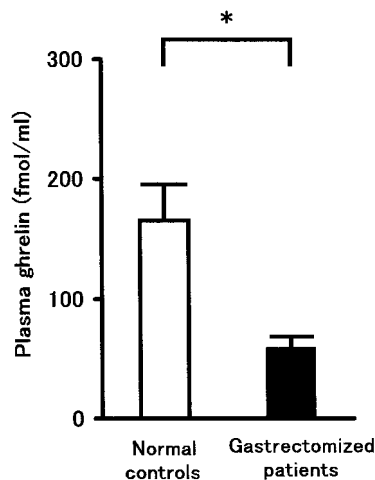


FIG. 2. Plasma ghrelin-LI levels in 13 gastrectomized patients (8 men and 5 women) and in sex- and age-matched normal controls. *, $P < 0.005$.

present study also elucidated tissue distribution of ghrelin gene expression and plasma ghrelin-LI levels in gastrectomized patients to assess the source of circulating ghrelin in humans. Ghrelin mRNA was most highly expressed in the stomach among various human tissues. In addition, plasma ghrelin-LI levels were reduced by 65% in gastrectomized patients. These results indicate that the stomach is a major source of circulating ghrelin in humans. Changes in feeding and/or nutritional states after the gastrectomy might result in the decreased plasma ghrelin-LI levels in these patients. This possibility was ruled out, however, by the observation that plasma ghrelin-LI levels were reduced promptly after gastrectomy in two patients. In addition, there was no significant difference in BMIs between the gastrectomized patients and the control subjects, consistent with the absence of difference in feeding and/or nutritional states between these groups. It should be pointed out, however, that plasma ghrelin-LI levels in the gastrectomized patients still remain 35% of those in normal subjects, suggesting that tissues other than the stomach, such as the duodenum, jejunum, and lung, contribute to a certain amount of circulating ghrelin. A previous study showed that ghrelin mRNA is expressed in human placenta in contrast to the present study (18). This discrepancy, however, can be explained by the difference in methods, as they used a more sensitive method, RT-PCR analysis. It follows from their findings combined with the present data that human placenta may contain only a small amount of ghrelin mRNA, suggesting a paracrine or autocrine mechanism of ghrelin action in the placenta.

As the stomach is a major source of circulating ghrelin in humans, we examined the effects of fasting and feeding on the plasma ghrelin-LI level. The present study demonstrates that plasma ghrelin-LI levels are significantly elevated after 12-h fasting in humans. Moreover, the present study shows that a rapid decline in the plasma ghrelin-LI level occurs within 1 h after habitual feeding. These observations suggest that the plasma ghrelin-LI level reflects the acute feeding state and may serve as an indicator of short-term energy balance when we take into account that stomach expansion does not result in a decline in circulating ghrelin levels (12).

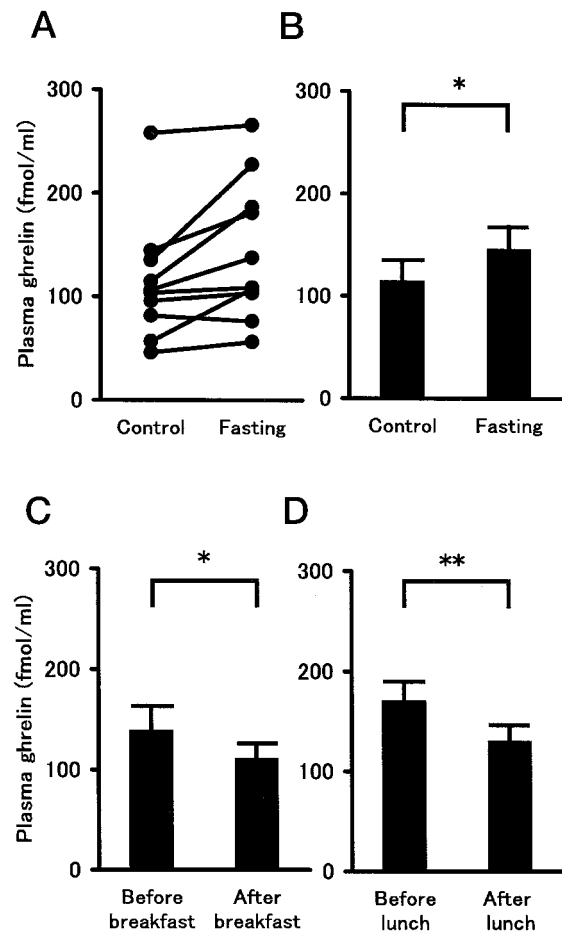


FIG. 3. A, Individual responses of plasma ghrelin-LI levels to 12-h fasting in 10 normal subjects (5 men and 5 women). B, Plasma ghrelin-LI levels before and after 12-h fasting in 10 normal subjects (5 men and 5 women). *, $P < 0.05$. C, Plasma ghrelin-LI levels before and 1 h after breakfast in 10 normal subjects (5 men and 5 women). *, $P < 0.05$. D, Plasma ghrelin-LI levels before and 1 h after lunch in 10 normal subjects (5 men and 5 women). **, $P < 0.01$.

Consequently, we examined plasma ghrelin-LI levels in a group of women with anorexia nervosa to investigate the effects of a chronic feeding state. Plasma ghrelin-LI levels were markedly elevated in these patients. It should be emphasized that plasma ghrelin-LI levels in these patients are extremely high compared with those after 12-h fasting in normal subjects, and that two of these patients showed 7-fold higher plasma ghrelin-LI levels compared with control subjects. These values are the highest we have ever measured in humans. In addition, plasma ghrelin-LI levels were negatively correlated with BMIs. This observation raises the idea that ghrelin is a starvation-related hormone. It is unlikely that altered sex hormone profiles lead to the elevated plasma ghrelin-LI levels in these female patients, as we could also observe elevated plasma ghrelin-LI levels in male patients. It is intriguing to note that remarkable declines in the plasma ghrelin-LI level occurred after nutritional treatment in three patients, when they gained only minimal increases in BMI, indicating that plasma ghrelin-LI levels appear to sensitively reflect current energy balance even in the extreme state of undernutrition. It should be also noted, however, that we

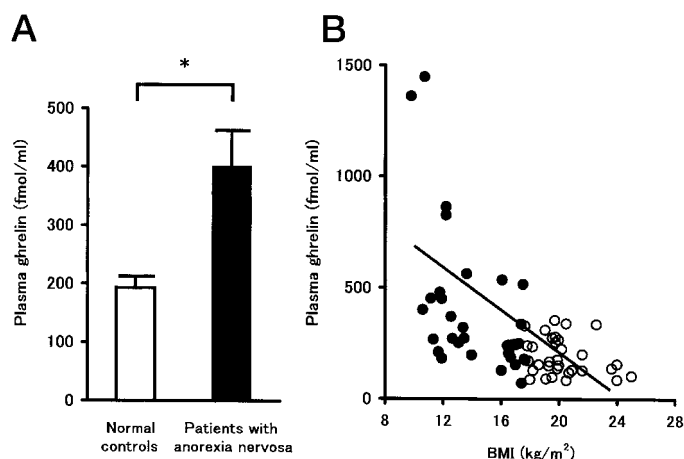


FIG. 4. A, Plasma ghrelin-LI levels in 31 female patients with anorexia nervosa and in 35 female age-matched normal controls. *, $P < 0.0001$. B, Correlation between BMIs and plasma ghrelin-LI levels in 31 female patients with anorexia nervosa (●) and 35 female age-matched normal controls (○). $r = -0.57$ and $P < 0.0001$.

used an RIA for the carboxyl-terminal of ghrelin, as we have observed that acylated ghrelin is too unstable to be measured in stored plasma. On the other hand, des-acyl ghrelin is of high stability even in stored plasma, with a high recovery ratio of approximately 95% (our unpublished data). Further study is needed concerning the molecular forms of circulating ghrelin in states such as those discussed above.

The specific signals that regulate ghrelin secretion from the stomach are not known. Among the known alterations during fasting are the decline in glucose levels, the rise in fatty acid levels, the production of ketones, and several hormonal changes, including the decline in insulin levels and the rise in glucagon, catecholamines, cortisol, and GH levels (19). Although the previous study demonstrated that sugar intake decreases circulating ghrelin levels in rodents (12), little is known about other factors that affect ghrelin secretion from the stomach. Leptin, the product of the *ob* gene (20), is another candidate when we consider that plasma ghrelin-LI levels are negatively correlated with BMIs. Ghrelin needs to be studied further to clarify the mechanism underlying the regulation of secretion.

Some GHSs are reported to stimulate food intake (21–23). Recently, we and others showed that intracerebroventricular administration of ghrelin induces food intake via NPY (9, 10) and Fos protein in the hypothalamic arcuate nucleus (24). In addition, previous studies showed that GHS-R mRNA is expressed in a large population of arcuate neurons containing NPY (25). Meanwhile, peripheral daily administration of ghrelin induces adiposity without increasing food intake in rodents (12). Taken together, it is likely that ghrelin is involved in food intake and energy homeostasis as well as in the regulation of GH secretion. The findings in the present study that the plasma ghrelin-LI level reflects the feeding state combined with previously reported data suggest that ghrelin may signal hypothalamic regulatory centers controlling energy balance in fasting and undernutrition.

The neuroendocrine mechanism regulating GH secretion appears to be exquisitely sensitive and responsive to nutritional state in humans. Fasting stimulates GH release in

humans (26), and patients with anorexia nervosa characteristically show elevated basal levels of GH (27). It has been unclear, however, what factors specifically activate this system in fasting and/or undernutrition. The present study suggests that the augmented ghrelin level may consequently lead to the elevated GH level in these states, as ghrelin has a potent GH-releasing activity (3, 7). Indeed, we observed elevated GH levels in the patients with anorexia nervosa in the present study, and the most severely affected patients showed the highest serum GH levels in accordance with the highest plasma ghrelin-LI levels (unpublished data), consistent with this hypothesis. Ghrelin as well as GH may play important roles in energy homeostasis in these states, as mentioned above.

In summary, the present study clearly demonstrates that the stomach is a major source of circulating ghrelin, and the feeding state determines plasma ghrelin-LI levels in humans, suggesting the involvement of ghrelin in food intake and the regulation of energy homeostasis as well as in the regulation of GH release in humans.

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