Table 2. Correlations between plasma ghrelin concentrations and various parameters in healthy subjects

| | All (n = 36) | | Male (n = 16) | | Female (n = 20) | |
|---|---|---|---|---|---|---|
| | r | p | r | p | r | p |
| Active ghrelin | | | | | | |
| Age | -0.256 | 0.132 | -0.219 | 0.421 | -0.232 | 0.331 |
| BMI | -0.387 | **0.019** | -0.195 | 0.476 | -0.193 | 0.421 |
| GH | 0.567 | **0.0002** | 0.402 | 0.125 | 0.470 | **0.036** |
| IGF-1 | 0.085 | 0.624 | -0.031 | 0.910 | 0.085 | 0.624 |
| Insulin | -0.370 | **0.026** | -0.314 | 0.242 | -0.442 | 0.050 |
| Glucose | -0.535 | **0.0006** | -0.175 | 0.525 | -0.450 | **0.046** |
| HOMA-IR | -0.401 | **0.015** | -0.327 | 0.221 | -0.463 | **0.039** |
| Leptin | -0.053 | 0.760 | -0.065 | 0.814 | -0.053 | 0.760 |
| NEFA | 0.251 | 0.140 | -0.082 | 0.766 | 0.171 | 0.476 |
| Desacyl ghrelin | | | | | | |
| Age | -0.178 | 0.302 | -0.112 | 0.684 | -0.155 | 0.519 |
| BMI | -0.364 | **0.028** | -0.189 | 0.490 | -0.324 | 0.224 |
| GH | 0.446 | **0.0058** | 0.364 | 0.169 | 0.337 | 0.148 |
| IGF-1 | 0.149 | 0.390 | 0.104 | 0.706 | 0.149 | 0.390 |
| Insulin | -0.381 | **0.021** | -0.348 | 0.191 | -0.488 | **0.028** |
| Glucose | -0.508 | **0.0013** | -0.090 | 0.744 | -0.495 | **0.025** |
| HOMA-IR | -0.398 | **0.015** | -0.350 | 0.188 | -0.504 | **0.022** |
| Leptin | -0.076 | 0.663 | -0.155 | 0.572 | -0.076 | 0.663 |
| NEFA | 0.174 | 0.312 | 0.069 | 0.803 | 0.044 | 0.857 |

r: correlation coefficient. Bold values: $P < 0.05$

2.0 to 9.4 ± 0.8%, previously determined by RIA (16, 24). As RIA measuring total ghrelin is measured using an antiserum against the C-terminal region of ghrelin (human ghrelin-(13-28)), these findings suggest that a fragmented form of ghrelin lacking the N-terminal region may naturally exist in human plasma or may be artificially produced during the RIA procedure. If so, then approximately 40-60% of the total ghrelin measured by RIA is likely fragmented. Alternatively, the differences in these measurements may result from the use of different antibodies in the ELISA and RIA systems. The measured values obtained with Linco total ghrelin RIA are approximately 10-fold higher than those obtained with Phoenix RIA (14). Desacyl Ghrelin ELISA-kit gave similar values to Phoenix RIA and our C-RIA, but not Linco total ghrelin RIA. In addition, the values obtained with Active Ghrelin ELISA-kit were similar to those obtained with our N-RIA, but were approximately one-order lower than those with Linco active ghrelin RIA (18). Thus, only Linco's kits give approximately 10-fold higher values. We agree that an adjustment to one reference standard will be required to solve this discrepancy (14).

Although there is an excellent correlation between acylated and desacyl ghrelin levels, the A/D and A/[A + D] ratios significantly correlated with acylated, but not desacyl, ghrelin quantities. This finding suggests that the activity of acylation enzyme is either increased in hyperghrelinemia or decreased in hypoghrelinemia. Although this result is difficult to interpret from a physiological viewpoint, a similar phenomenon is observed in T3 thyrotoxicosis. Frequently occurring in Graves' disease, type I deiodinase activity, which catalyzes the formation of T3 (active form of thyroid hormone) from T4 (inactive form of thyroid hormone), is increased in hyperthyroidism.

Plasma levels of both acylated and desacyl ghrelin in female subjects were significantly higher than those in male subjects. The significant gender difference in plasma acylated, but not in desacyl, ghrelin levels persisted even when the comparison was normalized to subject BMI. This finding agrees with a report from Chan *et al.*, who demonstrated significantly higher total ghrelin levels in females. This result, however, was no longer significant after normalization to BMI (25). Higher GH levels in females may be partially explained by the elevated acylated ghrelin levels. Although glucose levels were all within normal limits, they are slightly higher in females than in males, requiring the careful interpretation of these results.

By correlation analysis, BMI, serum insulin levels, and HOMA-IR negatively correlated with plasma levels of both acylated and desacyl ghrelin. These results are in line with previous reports of a negative correlation of total ghrelin levels in plasma with BMI (5-8, 23), insulin (6, 9, 26), and insulin resistance (9, 26). Here, we confirmed that this correlation holds true for acylated ghrelin levels as well. GH correlated positively with both acylated and desacyl ghrelin levels. Although we could not identify a correlation with IGF-1 levels, the positive correlation of total ghrelin levels in plasma with serum GH levels has been previously reported (16). The stronger correlation with GH of acylated ghrelin, rather than desacyl ghrelin, suggests the physiological role of ghrelin in GH regulation.

In summary, we measured plasma levels of both acylated and desacyl ghrelin in healthy subjects using novel direct ELISA systems. The comparison of these values to those obtained in our previous RIA systems suggests the presence of ghrelin peptide fragments in plasma. Although acylated ghrelin levels in plasma correlated well with desacyl ghrelin, A/D and A/[A + D] ratios could only be correlated to acylated ghrelin levels. The levels of acylated, but not desacyl, ghrelin in plasma was higher in female volunteers than in males after

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BMI adjustment. BMI, serum insulin levels, and HOMA-IR negatively correlated with both plasma acylated and desacyl ghrelin levels. Serum GH levels correlated closely with plasma acylated, rather than desacyl, ghrelin levels. These findings indicate that the separate measurement of the two forms of plasma ghrelin is useful; plasma acylated ghrelin levels may better reflect the physiological states or alterations in patients more than measurement of plasma desacyl or total ghrelin levels.

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LETTER TO THE EDITOR

# Short-term secretory regulation of the active form of ghrelin and total ghrelin during an oral glucose tolerance test in patients with anorexia nervosa

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Ghrelin is an acylated peptide whose octanoyl modification is essential for its biological activities (1). Antibodies used in previous works to measure ghrelin concentrations in human blood do not distinguish between the active form of ghrelin (active ghrelin) and desacyl ghrelin with no biological activities (2, 3). In our previous study, high levels of active ghrelin were present in patients with restricting-type anorexia nervosa (AN-R) and they decreased significantly during an oral glucose tolerance test (OGTT) in these patients as well as in normal controls (4). As a continuation of the previous study and using the same subjects

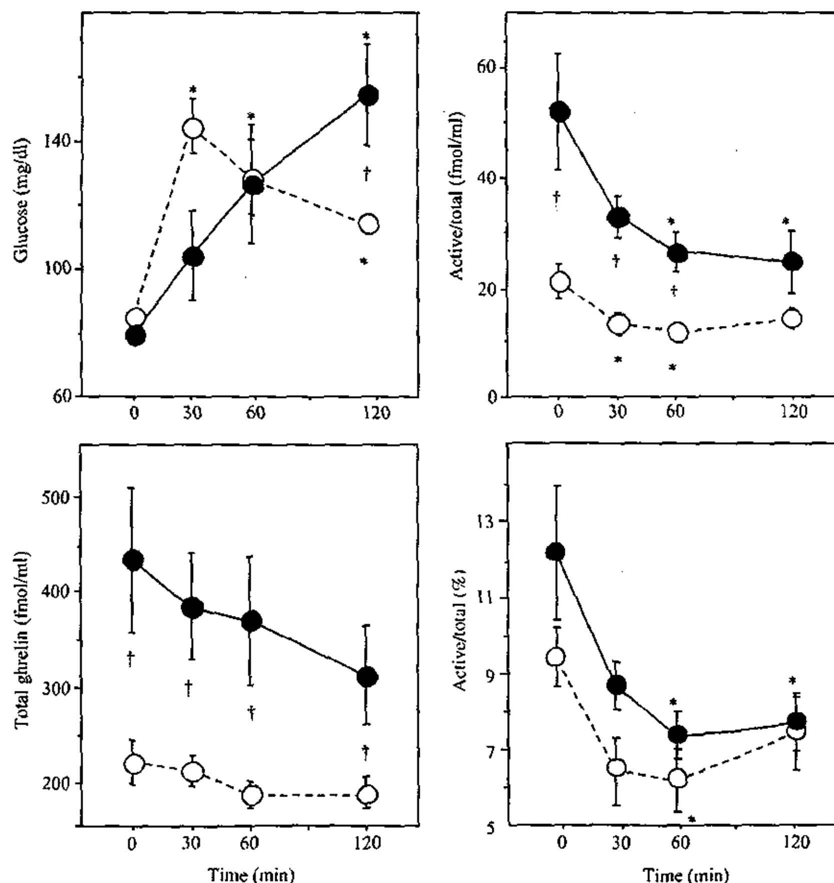


**Figure 1** Plasma levels of glucose, total ghrelin and active ghrelin and the ratios of active ghrelin to total ghrelin (active/total) during an OGTT in five patients with AN-R (●) and seven normal controls (○). Data are shown as means±S.E.M. *$P < 0.01$ vs basal values at 0 min; †$P < 0.01$ vs normal controls.

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(4), we studied short-term secretory regulation of active ghrelin as well as total ghrelin during OGTT using two ghrelin-specific radioimmunoassays (5). The details of the study subjects and methods have been described previously (4, 5). All results are expressed as means± S.E.M. Statistical analysis was performed with the Mann–Whitney U test or the repeated measures ANOVA and subsequently with Dunnett's test. Probabilities of less than 0.05 were considered statistically significant.

Figure 1 shows the plasma levels of glucose, total ghrelin and active ghrelin and the ratios of active ghrelin to total ghrelin during OGTT in patients with AN-R and in controls. Mean basal ratios of active ghrelin to total ghrelin were 12.2±1.7% in anorectic patients and 9.4±0.8% in controls respectively. These results suggest that desacyl ghrelin predominates over active ghrelin in the circulation in anorectic patients as well as in controls.

Mean levels of plasma total ghrelin did not decrease significantly during OGTT in patients with AN-R and in controls. However, mean levels of plasma active ghrelin decreased significantly during OGTT in anorectic patients and in controls. Furthermore, the mean ratios of active ghrelin to total ghrelin also decreased significantly in these patients and in controls (Fig. 1). These results suggested that active ghrelin disappears from the circulation more rapidly than total ghrelin during OGTT in patients with AN-R and in controls.

Although the plasma levels of both active ghrelin and total ghrelin were significantly higher in anorectic patients compared with normal controls, the difference of the ratios of active ghrelin to total ghrelin was not statistically significant at 0, 30, 60 and 120 min during OGTT between the two groups (Fig. 1). These results suggest that the short-term secretory regulation of ghrelin during OGTT in patients with AN-R was similar to that in normal controls, despite the high plasma levels of active ghrelin and total ghrelin in these patients compared with normal controls.

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# Anti-cachectic effect of ghrelin in nude mice bearing human melanoma cells

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**Abstract**

Ghrelin is a novel brain-gut peptide that stimulates food intake and body weight gain. We studied the anabolic effect of ghrelin in a cancer cachexia mouse model. SEKI, a human melanoma cell line, was inoculated into nude mice to examine the effects of ghrelin on food intake and body weight. The intraperitoneal administration of ghrelin twice a day (6 nmol/mice/day) for 6 days suppressed weight loss in SEKI-inoculated mice and increased the rate of weight gain in vehicle-treated nude mice. Ghrelin administration also increased food intake in both SEKI- and vehicle-treated mice. Both the weight of white adipose tissue and the plasma leptin concentration were reduced in tumor-inoculated mice compared with vehicle-treated mice; these factors increased following ghrelin administration. The levels of both ghrelin peptide and mRNA in the stomach were upregulated in tumor-inoculated mice. The anabolic effect of ghrelin efficiently reverses the cachexia in mice bearing SEKI human melanoma. Ghrelin therefore may have a therapeutic ability to ameliorate cancer cachexia.
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*Keywords:* Ghrelin; Cachexia; Body weight gain; Food intake

Cancer cachexia, a catabolic state characterized by weight loss and muscle wasting, occurs frequently in patients with end-stage neoplastic disease [1,2]. Approximately half of all cancer patients suffer from cachexia [3,4], a strong independent risk factor for mortality. Patients with cachexia often experience a shorter survival period and a reduced response to anticancer therapy when compared with those without cachexia [5]. The treatment or amelioration of cachexia should increase both the effectiveness of cancer therapy and the quality of life, decreasing cancer-associated morbidity and mortality. Numerous cytokines, including leukemia-inhibitory factor (LIF), tumor necrosis factor-α, interleukin 1β, interleukin 6, and interferon γ, produced by tumor cells mediate the cachectic effect of cancer [2,6–10].

Body mass is determined by a balance of food intake and energy expenditure. A variety of neuropeptides, including neuropeptide Y, melanin-concentrating hormone, orexin, melanocortin, cholecytokinin, and corticotropin-releasing hormone, regulate energy homeostasis [3]. Ghrelin, initially discovered as an endogenous ligand for the growth-hormone secretagogue receptor in rat and human stomach [11], is a brain-gut peptide that stimulates food intake [12–14] and induces adiposity [15]. Daily subcutaneous administration of ghrelin causes body weight gain and increases fat mass in mice and rats. Ghrelin administration also stimulates appetite and increases food intake in humans [16]. Upon

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fasting, plasma ghrelin concentrations increased in rats and humans; these levels decreased after meals [17–20]. These findings suggest that ghrelin is critical in the regulation of energy homeostasis.

Peripheral administration of ghrelin attenuated body weight loss in a rat model of cachexia with chronic heart failure [21] and in mice given interleukin 1β [22]. The anabolic effects of ghrelin may be promising in the treatment of cachexia. SEKI, a human melanoma cell line that produces LIF, causes severe body weight loss in nude mice upon inoculation [23,24]. In this study, we demonstrated the improvement of cancer cachexia in nude mice bearing SEKI cells following ghrelin administration. We also investigated ghrelin expression in the stomach of cachectic mice.

## Materials and methods

*Animals.* Five-week-old female BALB/c-*nu/nu* mice weighing 14–16 g (Charles River Japan, Atsugi, Japan) were adapted to laboratory conditions for 1 week prior to experiments. Mice were housed individually in plastic cages containing wood chips, in a temperature-controlled 23 ± 2 °C room under 12:12-h light–dark cycles (light on at 07:00 h). Animals were maintained on tap water and a breeding diet (CRF-1, Oriental Yeast, Tokyo, Japan) placed on the ground. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

*Tumor inoculation.* Mice were subcutaneously inoculated in both flanks with either each $1 \times 10^7$ of SEKI (hereafter referred to as "SEKI mice," $n = 12$) human melanoma cells or vehicle alone (hereafter referred to as "vehicle mice," $n = 18$). Animal body weight and the major and minor axes of the tumor were measured every day at 09:00 h. The tumor weight was estimated using the following equation; tumor weight (g) = {major axis (cm) × (minor axis (cm))$^2$}/2 [7]. Single day food intake was measured by subtracting the amount of uneaten food from the initial amount given.

*Ghrelin administration.* Human ghrelin was synthesized as described [25]. Human ghrelin was administered after the mean body weight of tumor-inoculated mice began to decline. Vehicle mice were administered ghrelin according to the same schedule as SEKI mice. Mice were allocated into two groups such that the average body weight between the two groups prior to ghrelin administration was similar. One group of mice was then administered ghrelin (3 nmol/100 µl saline/mice) intraperitoneally twice a day (10:00 and 19:00 h) for 6 days. The other group of mice was administered 100 µl saline. Fourteen hours after the final administration, mice were euthanized. Blood was collected from the heart into polypropylene tubes containing EDTA · 2Na. The stomach was removed and divided into two portions; one-half was utilized for peptide quantification and the other was immediately homogenized with TRIzol (Life Technologies, Fredrick, MD) to isolate RNA. The tumor and white adipose tissue surrounding the kidney and uterus were removed and weighed.

*Plasma analyses.* Plasma concentrations of LIF and leptin were measured using a human LIF immunoassay kit (R&D Systems, Minneapolis, MN) and a Leptin/Mice enzyme-linked immunosorbent assay (ELISA) kit (SEIKAGAKU, Tokyo, Japan), respectively.

*Ghrelin quantification in the stomach.* Ghrelin content in the stomach was measured by radioimmunoassay (RIA) specific for ghrelin as described [26]. In brief, approximately 50 mg of mouse stomach was boiled for 10 min in a 100-fold volume of water to inactivate intrinsic proteases. After cooling to 4 °C, $CH_3COOH$ and HCl were added to final concentrations of 1 M and 20 mM, respectively. The stomach was then homogenized in a Polytron for 1 min. After centrifugation, the supernatant, equivalent to 3 mg wet weight, was lyophilized and subjected to RIA. Both a standard peptide solution and the diluted sample (100 µl) were incubated for 24 h with 100 µl anti-ghrelin-(13–28) antiserum (final dilution 1/20,000). Following addition of the tracer solution ([$^{125}$I-Tyr$^0$]ghrelin-(13–28), 17,000 cpm/100 µl), the mixture was again incubated for 24 h. Bound and free ligands were separated using secondary antibody (200 µl). Samples were assayed in duplicate; all procedures were performed at 4 °C. The antiserum recognizes acylated and non-acylated ghrelin on an equal molar basis. The limit of detection of the assay is 12 fmol/tube for mouse and human ghrelin. The respective intra- and interassay coefficients of variation were 3.7% and 3.3% at 50% binding.

*Northern blot analysis.* Total RNA was extracted from mouse stomach using TRIzol. Ghrelin mRNA was quantified by Northern blot analysis. Ten micrograms of total RNA was denatured using 2 M glyoxal and 50% dimethyl sulfoxide. Following 1.2% agarose gel electrophoresis, the sample was transferred to a Zeta Probe membrane (Bio-Rad Laboratories, Richmond, CA). The probes used for hybridization recognized full-length ghrelin cDNA and a 0.2-kb cDNA fragment of glycerol-3-phosphate dehydrogenase [18]. Membranes were first hybridized for 1.5 h at 42 °C in 900 mM NaCl, 60 mM $NaH_2PO_4$, and 7 mM EDTA, pH 7.4, containing 40% formamide, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 0.1 mg/ml denatured salmon sperm DNA. Membranes were then hybridized for 24 h at 42 °C in a solution containing the two $^{32}$P-labeled cDNA probes. The RNA blot was immersed in 300 mM NaCl, 30 mM sodium citrate, pH 7.0/0.1% SDS for 20 min at 58 °C, followed by an incubation in 150 mM NaCl, 15 mM sodium citrate, pH 7.0/0.1% SDS for 40 min. Hybridized signals were visualized and quantified using a Fuji Bio-image analyzer (BAS 2000, Fuji Film, Tokyo, Japan).

*Statistical analysis.* All data are presented as means ± SEM. Comparisons between groups were performed using the unpaired *t* test. *P* values of <0.05 are considered significant.

## Results

### Body weight and food intake of tumor-inoculated mice

The mean body weight of SEKI mice began to decrease 12 days after inoculation of the tumor. Intraperitoneal administration of ghrelin twice a day began 14 days after tumor inoculation. Ghrelin administration increased the body weight of vehicle-treated and tumor-inoculated mice when compared with the corresponding saline-administered mice (Fig. 1A). In both SEKI and vehicle mice, a 6-day course of ghrelin administration increased body weight as compared to those observed following saline administration (Fig. 1B). No significant difference in the tumor size was observed between the saline- and ghrelin-administered groups of SEKI mice (2.03 ± 0.35 and 2.41 ± 0.36 g for saline- and ghrelin-administered groups at day 6, respectively, $P = 0.47$). Ghrelin administration increased average single-day food intake in both SEKI and vehicle mice when compared with the corresponding saline-administered groups (Fig. 2).

White adipose tissues were not visible in the vicinity of kidney or uterus in SEKI mice, even in those given

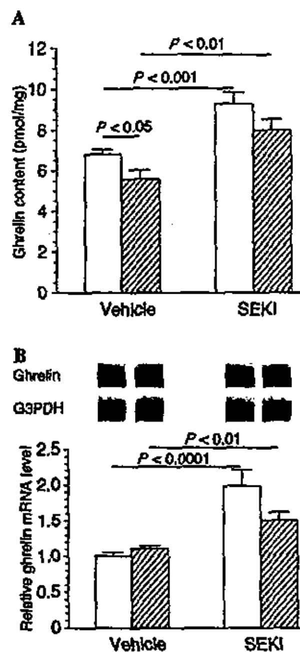


Fig. 5. (A) Ghrelin content in the stomach. (B) Upper: representative Northern blot analysis patterns of ghrelin mRNA in the stomach. Lower: ghrelin mRNA levels relative to glycerol-3-phosphate dehydrogenase (G3PDH) levels. Diagonal striped bars, ghrelin-administered groups; white bars, saline-administered groups.

## Discussion

The basal metabolic rate in chronic starvation decreases as the body adapts to conserve tissues and energy in a low-protein–low-calorie environment. However, cancer patients often have an elevated resting energy expenditure even before the onset of weight loss [2]. This metabolic change probably contributes to cachectic symptoms, expressing fat loss and muscle wasting. We reported that nude mice bearing SEKI human melanoma cells produced LIF and had a severe weight loss and decreased food intake [23]. Ghrelin is an orexigenic peptide hormone secreted from distinct endocrine cells in the stomach with a role in the regulation of energy homeostasis [12,13,15,26]. In this study, SEKI-inoculated cachexia mouse model was used to examine the therapeutic potential of the anabolic effect of ghrelin.

We first demonstrated that peripheral administration of ghrelin increased both food intake and body weight gain in normal nude mice. Ghrelin administration also stimulated food intake and ameliorated weight loss in SEKI mice. Ghrelin did not alter tumor size or plasma LIF concentration in SEKI mice. These findings suggest that the anti-cachectic effect of ghrelin results from the orexigenic activity of ghrelin.

Chronic subcutaneous administration of high doses of ghrelin increased fat mass in normal mice [15]. Ghrelin administration in this study also increased white adipose tissue weight in control nude mice. Plasma leptin concentration positively correlates with quantities of adipose tissue in mice and humans [27]. Plasma leptin concentration was markedly lower in SEKI mice, but increased 2.2-fold following ghrelin administration. This increment suggests that although an increase of white adipose tissue weight was not visible with the naked eye, SEKI mice might possess increased overall white adipose tissue. Ghrelin has been reported to enhance secretion of growth hormone which stimulates growth of the muscle and bone in not only normal rodents but also cachectic rats with chronic heart failure [11,21]. Ghrelin is thought to have a potential to increase the lean body mass in SEKI mice.

Ghrelin synthesis within the stomach increases upon fasting and decreases after meals [17,18]. Ghrelin secretion is also upregulated under conditions of negative energy balance [20]. In this study, ghrelin mRNA expression was upregulated in the stomach of SEKI mice, likely reflecting a physiological adaptation to negative energy balance. In addition, neuropeptide Y mRNA level in the hypothalamus of SEKI mice increases [24]. Since intraperitoneal administration of ghrelin augmented hypothalamic neuropeptide Y mRNA expression [22], the upregulation of neuropeptide Y may correlate with increased ghrelin synthesis in this model of cancer cachexia. Food intake and body weight of SEKI mice were reduced as the tumor grew. The effects of cachexia-inducing factors produced by SEKI melanoma cells probably outweigh the effects of endogenous anabolic factors including ghrelin and neuropeptide Y.

In summary, we demonstrated that ghrelin administration suppresses body weight loss in cachectic nude mice inoculated with human melanoma cells. Ghrelin is effective at counteracting cachexia in a rat model with chronic heart failure [21]. As cachexia is a strong independent risk factor for mortality in cancer patients, additional supplementation of ghrelin may attenuate the development of cachexia and increase the quality of life in cancer patients.

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# Habitual binge/purge behavior influences circulating ghrelin levels in eating disorders

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**Abstract**

Previous studies have reported that fasting plasma ghrelin concentrations play an important role in the pathophysiology of eating disorders. The purpose of this study was to examine the relationship between plasma ghrelin levels and frequency of abnormal eating behaviors, nutritional parameters in eating disorders. Fasting blood samples were obtained in 40 female anorexia nervosa (AN) patients, 21 restricting type (AN-R) and 19 binge-eating/purging type (AN-BP), in 31 bulimia nervosa (BN) patients, 18 purging type (BN-P) and 13 nonpurging type (BN-NP), in 15 female healthy volunteers (control) before the initiation of active treatment. The fasting plasma ghrelin concentrations in all subjects were negatively correlated with nutritional parameters such as body mass index, percent body fat and serum cholinesterase concentration. The mean plasma ghrelin level in BN-P was higher than that in both BN-NP and controls despite similar nutritional parameters. The plasma ghrelin levels in both AN-R and AN-BP did not differ from BN-P despite difference of nutritional parameters. For both AN-BP and BN-P patients with habitual binge/purge behavior, there were significant correlations among plasma ghrelin values, frequencies of binge/purge cycles and serum amylase values. In BN-NP, there were no significant correlations among plasma ghrelin values, frequencies of binge-eating episodes and serum amylase values. These results suggest that habitual binge/purge behavior may have some influence on circulating plasma ghrelin levels in both BN-P and AN-BP. Habitual binge/purge cycles with vomiting as opposed to binge-eating episodes without vomiting may have a greater influence on fasting plasma ghrelin concentration in eating disorders.

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*Keywords:* Ghrelin; Eating disorders; Binge/purge cycles; Vomiting; Amylase

## 1. Introduction

Ghrelin, a 28-amino acid peptide with an *n*-octanoylated serine 3, was originally discovered in the rat and human stomach, and stimulates growth hormone secretion (Kojima et al., 1999) and increases food intake and body weight in rodents (Tschöp et al., 2000; Wren et al., 2001b). This peptide is an orexigenic peptide that antagonizes leptin action has a role in the regulation of feeding behavior and energy metabolism in the central nervous system (Shintani et al., 2001; Nakazato et al., 2001). Regarding humans, ghrelin also stimulates growth hormone (Takaya et al., 2000) and enhances appetite and increases food intake (Wren et al., 2001a). It has been reported that fasting plasma ghrelin concentrations from humans are negatively correlated with body mass index (BMI) (Shiiya et al., 2002), percent body fat, fasting leptin and insulin concentrations (Tschöp et al., 2001).

The eating disorders are characterized by habitual abnormal eating behaviors and are divided into anorexia nervosa (AN) and bulimia nervosa (BN). Both AN (Otto et al., 2001) and BN (Tanaka et al., 2002) patients have been reported to increase fasting plasma ghrelin concentrations and decrease elevated plasma ghrelin of AN patients by weight gain (Otto et al., 2001). According to the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition (DSM-IV) (APA, 1994), AN are classified into two subtypes on the basis of the presence (AN-BP) or absence (AN-R) of habitual binge/purge

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behavior, and BN also can be classified into two subtypes on the basis of the presence (BN-P) or absence (BN-NP) of habitual purging behavior. Since our previous study (Tanaka et al., in press) suggested that differences in eating behavior might influence circulating plasma ghrelin, we therefore in this study measured fasting plasma ghrelin concentrations from both AN and BN subtypes in order to examine furthermore the relationship between plasma ghrelin levels and nutritional parameters, and frequencies of abnormal eating behaviors such as binge/purge cycles.

## 2. Methods

### 2.1. Subjects

Twenty-one female AN-R patients, 19 female AN-BP patients, 18 female BN-P patients, 13 female BN-NP patients meeting DSM-IV and 15 sex-matched healthy volunteers (control) were the subjects in this study. Controls had no history of psychiatric illness or gastrointestinal disease. Patients were all outpatients who were examined to the Kagoshima University Hospital between August 2001 and April 2002, and excluded if they had a history of alcohol or substance abuse, or gastrointestinal disease, and assayed before the initiation of active treatment. To determine the neuroendocrinological aspect of eating behavior, both AN-BP and BN-P patients had habitual vomiting at least twice a week over the preceding 3 months and were excluded if they had only purging behavior without vomiting.

### 2.2. Procedures

We collected blood samples from all subjects between 8.00 and 9.00 a.m. after an overnight fast. Subjects were interviewed to assess psychiatric and medical history and eating behavioral ratings in detail. We measured body weight and percent body fat and serum cholinesterase as nutritional parameters, when samples were obtained. Percent body fat was obtained by bioelectrical impedance analysis (Bolanowski and Nilson, 2001). To determine the relationship between frequencies of abnormal eating behaviors and plasma ghrelin levels, we further measured total serum amylase concentrations (Metzger et al., 1999; Kinzl et al., 1993; Walsh et al., 1990) and serum potassium (Wolfe et al., 2001). The Institutional Committee of Kagoshima University approved the protocol, and all subjects provided written informed consent before participation.

### 2.3. Laboratory methods

Serum amylase, potassium and cholinesterase concentrations were determined in our laboratory. Blood was drawn into chilled tubes containing EDTA·2Na (1 mg/ml) and aprotinin (500 U/ml). Plasma ghrelin was measured using a RIA as described elsewhere (Shiiya et al., 2002). In brief, antiserum against the C-terminal region of human ghrelin was raised in New Zealand white rabbits immunized against synthetic human ghrelin [13-28] that had been coupled with maleimide-activated mariculture keyhole limpet hemocyanin. The antiserum recognized acylated ghrelin and non-acylated ghrelin equally on a molar basis. Human $Tyr^0$-ghrelin [13-28] was radioiodinated by the lactoperoxidase method for use in the assay. Inter- and intra-assay variation was $<8$ and $<6\%$, respectively. The limit of detection of this assay is 12 fmol/tube of human ghrelin. Two milliliters of plasma was diluted with 2 ml of 0.9% saline and applied to a Sep-Pak C-18 cartridge (Waters, Milford, MA) pre-equilibrated with 0.9% saline. The cartridge was washed first with saline and then with a 0.1% trifluoroacetic acid (TFA) solution, and peptides were eluted with a 60% acetonitrile ($CH_3CN$) solution containing 0.1% TFA. The eluate was evaporated, reconstituted with RIA buffer, and subjected to RIA analysis. A diluted sample or a standard peptide solution (100 μl) was incubated for 24 h with 100 μl of the antiserum diluent (final dilution 1/20,000). The tracer solution (16,000 cpm/100 μl) was added, and the mixture incubated for 24 h. Bound and free ligands were separated by the second antibody method. All procedures were done at 4 °C. Recovery of human ghrelin added to the plasma was $90.7\pm4.0\%$ ($n=6$).

### 2.4. Statistical methods

Correction coefficients were calculated by linear regression analysis. The subject groups (mean ± SD) were compared using ANOVA and a post-hoc Fisher's test. $P$ value of $<0.05$ was considered statistically significant.

## 3. Results

The fasting plasma ghrelin concentrations in all subjects were negatively correlated with nutritional parameters such as BMI, percent body fat and serum cholinesterase concentration. There was positive correlation between plasma ghrelin value and serum amylase concentration in all subjects (Table 1).

Physiological characteristics of subject groups were showed in Table 2. The frequencies of binge/purge cycles were $6.2\pm4.3$/week in AN-BP group, $6.2\pm3.8$/week in BN-P group. The frequency of binge-eating episodes was $4.9\pm2.3$/week in BN-NP group. There were no significant differences in age and serum potassium concentration. The mean BMI, percent body fat and serum cholinesterase value in both AN-R and AN-BP groups were lower than those in controls

Table 1
Correlations of ghrelin values and physiological data

| | r | P |
|---|---|---|
| Age (years) | 0.04 | 0.69 |
| Duration of illness (years) | 0.15 | 0.22 |
| Body mass index (kg/m²) | −0.49[a] | <0.0001 |
| Percent body fat (%) | 0.46[a] | <0.0001 |
| Serum amylase (U/l) | 0.57[a] | <0.0001 |
| Serum potassium (mmol/l) | −0.02 | 0.89 |
| Serum cholinesterase (U/l) | −0.31[a] | <0.005 |

[a] Significant correlations ($P<0.05$).

respectively. Mean serum amylase level in AN-BP group was higher than that in AN-R, BN-NP and control groups (Table 2).

The mean plasma ghrelin levels in AN-R (256.5±118.6 pM, mean±S.D.), AN-BP (345.5±151.4 pM) and BN-P (286.9±146.4 pM) were significantly higher than those in both BN-NP (121.2±64.0 pM) and controls (120.8±20.4 pM). Mean plasma ghrelin level in AN-BP was significantly higher than that in AN-R. The plasma ghrelin levels in both AN-R and AN-BP did not significantly differ from that in BN-P. There were no differences on plasma ghrelin levels between BN-NP and controls (Fig. 1).

In both AN-BP and BN-P groups, the frequencies of binge/purge cycles were positively correlated with plasma ghrelin concentration (Fig. 2), and serum amylase value (AN-BP: $r=0.56$, $P<0.05$; BN-P: $r=0.65$, $P<0.005$). There was significant positive correlation between plasma ghrelin value and serum amylase value (Fig. 3).

In BN-NP group, there were no significant correlations among frequencies of binge-eating episodes, plasma ghrelin concentrations and serum amylase values ($P<0.2$). Serum cholinesterase value was significantly correlated with plasma ghrelin value ($r=-0.57$, $P<0.05$).

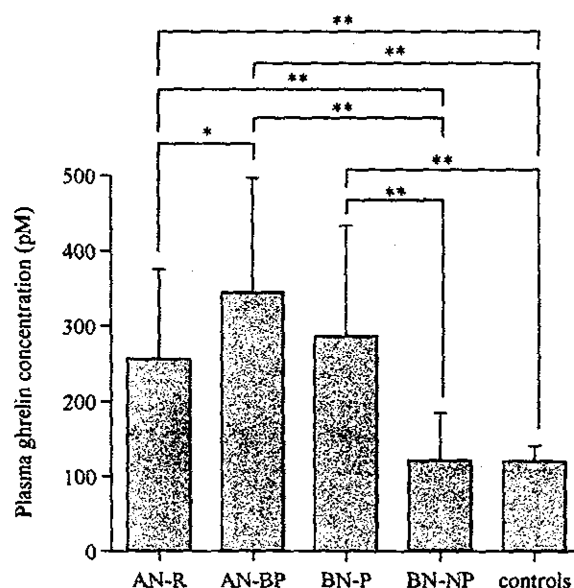


Fig. 1. Comparison of plasma ghrelin concentration in female anorexia nervosa patients, restricting type (AN-R) and binge-eating/purging type (AN-BP), in female bulimia nervosa patients, purging type (BN-P) and nonpurging type (BN-NP), and in sex-matched healthy volunteers (controls). $*P<0.05$, $**P<0.01$.

## 4. Discussion

The fasting plasma ghrelin concentrations in all subjects were negatively correlated with BMI and percent body fat. The mean plasma ghrelin levels in both AN-R and AN-BP patients were higher than healthy volunteers. These findings are in agreement with previous human studies (Shiiya et al., 2002; Tschöp et al., 2001; Otto et al., 2001). First, we have demonstrated that there was positive correlation between plasma ghrelin concentration and total serum amylase value in all subjects. Second, there was significantly difference on plasma ghrelin levels between BN subtypes. Third, there were significant correlations among plasma ghrelin concentrations, frequencies of binge/purge cycles and

Table 2
Physiological characteristics of subject groups

| | AN-R (N=21) | | AN-BP (N=19) | | BN-P (N=18) | | BN-NP (N=13) | | Control (N=15) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. |
| Age (years) | 21.8 | 8.9 | 24.6 | 5.5 | 22.7 | 5.0 | 22.7 | 6.5 | 22.1 | 3.4 |
| Duration of illness (years) | 2.6[b] | 2.4 | 4.6 | 3.9 | 3.0 | 2.4 | 1.8[b] | 1.2 | – | – |
| Body mass index (kg/m²) | 13.9 | 1.9 | 14.4 | 2.1 | 20.0[ab] | 2.1 | 21.2[ab] | 3.9 | 21.4[ab] | 11 |
| Percent body fat (%) | 8.3 | 3.4 | 10.3 | 4.4 | 23.3[ab] | 4.7 | 23.5[ab] | 7.9 | 26.5[ab] | 2.5 |
| Serum amylase (U/l) | 96.9[b] | 31.8 | 132.7 | 73.4 | 109.2 | 26.4 | 85.9[b] | 23.1 | 85.5[b] | 9.6 |
| Serum potassium (mmol/l) | 4.1 | 0.6 | 4.0 | 0.4 | 4.2 | 0.3 | 4.2 | 0.2 | 4.2 | 0.2 |
| Serum cholinesterase (U/l) | 197.5 | 58.1 | 210.1 | 68.1 | 237.7[a] | 43.6 | 272.5[ab] | 75.7 | 269.3[ab] | 56 |

[a] $P<0.05$ vs. AN-R.
[b] $P<0.05$ vs. AN-BP, using ANOVA and post hoc Fisher's test.

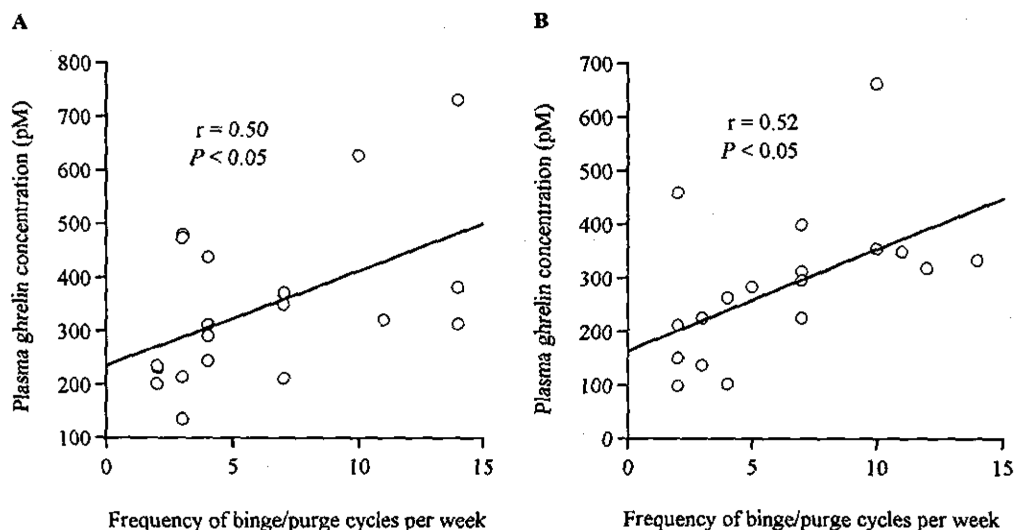


Fig. 2. (A) Relationship of plasma ghrelin concentration and frequency of binge/purge cycles for anorexia nervosa patients with binge-eating/purging type (AN-BP). (B) Relationship of plasma ghrelin concentration and frequency of binge/purge cycles for bulimia nervosa patients with purging type (BN-P).

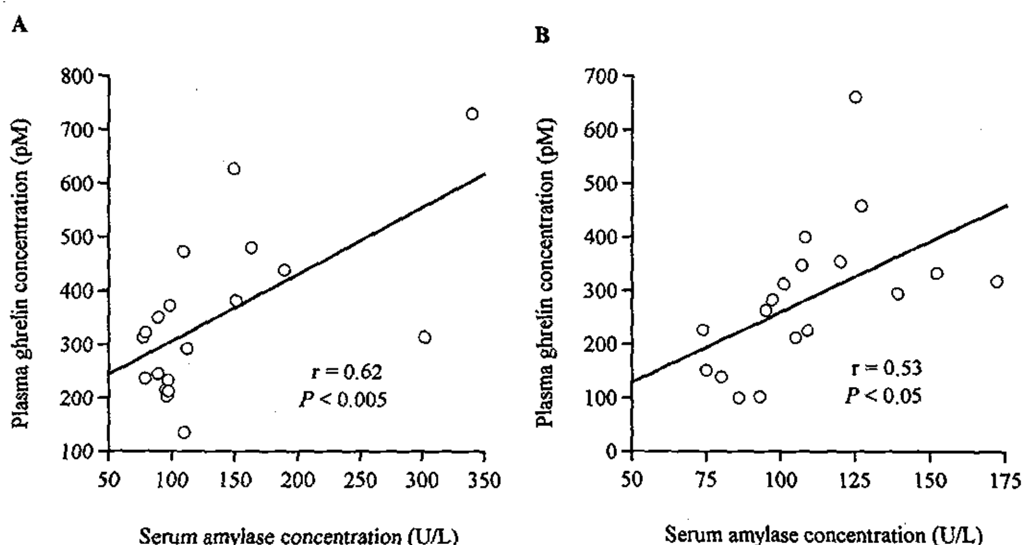


Fig. 3. (A) Relationship of plasma ghrelin concentration and serum amylase concentration for anorexia nervosa patients with binge-eating/purging type (AN-BP). (B) Relationship of plasma ghrelin concentration and serum amylase concentration for bulimia nervosa patients with purging type (BN-P).

serum amylase values in both AN-BP and BN-P patients.

The mean plasma ghrelin in BN-P patients was higher than that found in BN-NP, though mean nutritional parameters between the groups were not significantly different. Eating behavior such as habitual purging with vomiting can only be used to discriminate the subtypes of BN patients. Mean plasma ghrelin in AN-BP patients was higher than that in AN-R despite similar nutritional parameters, as has been found in our previous research (Tanaka et al., in press). Also, habitual binge/purge cycles can only be used to discriminate the subtypes of AN patients. There were no significant differences between BN-P and subtypes of AN despite difference of BMI. Thus, we deem that habitual binge/purge behavior may have some influence on circulating plasma ghrelin levels.

For both AN-BP and BN-P patients with habitual binge/purge behavior, there were significant correlations among plasma ghrelin values and frequencies of binge/purge cycles, and serum amylase values. However, there were no significant correlations among frequencies of

binge-eating episodes, plasma ghrelin concentrations and serum amylase values in BN-NP. It has previously been reported that total serum amylase levels in BN patients were significantly correlated with frequencies of binge eating and vomiting (Metzger et al., 1999; Walsh et al., 1990) and furthermore vomiting repeatedly rather than binge-eating behavior that may increase amylase (Robertson and Millar, 1999). These findings suggest that habitual binge/purge cycles with vomiting as opposed to binge eating episodes without vomiting may have a greater influence on fasting plasma ghrelin concentration.

Ghrelin is one of the gastrointestinal peptides and when administered intracerebroventricularly, stimulates gastric acid secretion by activating the vagus system (Date et al., 2001). As the regulation mechanism between gastrointestinal peptides and the vagus system has been reported to play a role (El-Salhy et al., 2000; Uvnäs-Moberg, 1983), circulating ghrelin levels may act as a feedback system via the vagal system. Several lines of evidence (Halmi and Sunday, 1991; Faris et al., 2000) have led us to postulate that afferent vagal hyperactivity could be an important factor in the pathophysiology of BN. Since BN has been reported to be due to hypervagal activity (Kennedy and Heslegrave, 1989), afferent vagal hyperactivity may increase circulating levels of plasma ghrelin in patients with habitual binge/purge behavior.

In the present study, plasma ghrelin levels in the five subjects were demonstrated to vary even though serum potassium did not differ. Previous study has reported that total body potassium may be depleted even when serum potassium levels are normal (Powers et al., 1995). Another study concluded that routine electrolyte measurement was a poor screening method for purging behavior as the frequency of hypokalemia in the patients with eating disorders was found to occur in only 4.6% of the subjects (Greenfeld et al., 1995). Though hypokalemia in eating disorders may be an important as physiological factor, it is possible that plasma ghrelin concentration might be a better parameter of occult or denied binge/purge behavior with vomiting in eating disorders.

Finally, habitual binge/purge behavior may influence circulating plasma ghrelin levels in both BN-P and AN-BP. Furthermore, habitual binge/purge cycles with vomiting as opposed to binge eating episodes without vomiting may have a greater influence on fasting plasma ghrelin concentration in eating disorders.

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# Somatostatin suppresses ghrelin secretion from the rat stomach☆

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**Abstract**

Ghrelin is an acylated peptide that stimulates food intake and the secretion of growth hormone. While ghrelin is predominantly synthesized in a subset of endocrine cells in the oxyntic gland of the human and rat stomach, the mechanism regulating ghrelin secretion remains unknown. Somatostatin, a peptide produced in the gastric oxyntic mucosa, is known to suppress secretion of several gastrointestinal peptides in a paracrine fashion. By double immunohistochemistry, we demonstrated that somatostatin-immunoreactive cells contact ghrelin-immunoreactive cells. A single intravenous injection of somatostatin reduced the systemic plasma concentration of ghrelin in rats. Continuous infusion of somatostatin into the gastric artery of the vascularly perfused rat stomach suppressed ghrelin secretion in both dose- and time-dependent manner. These findings indicate that ghrelin secretion from the stomach is regulated by gastric somatostatin.
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*Keywords:* Ghrelin; Somatostatin; Vascular perfusion; Immunohistochemistry

Ghrelin, a 28-amino acid peptide with an *n*-octanoylation modification indispensable for activity, was originally discovered in human and rat stomach as an endogenous ligand for growth hormone (GH) secretagogue receptor [1]. At present, ghrelin homologs have been identified in fish, amphibians, birds, and many mammals. Ghrelin is predominantly produced in a subset of endocrine cells within the oxyntic gland of the stomach [2]. In these cells, ghrelin is found within round, compact, electron-dense granules. Ghrelin stimulates GH secretion, food intake, and body weight gain when administered centrally or peripherally [1,3–7]. Plasma concentrations of ghrelin are upregulated under conditions of negative energy balance, including starvation, insulin-induced hypoglycemia, cachexia, and anorexia nervosa; levels are downregulated during positive energy balance, such as feeding and obesity [8–12].

Somatostatin, a peptide produced in the brain, gastrointestinal tract, and pancreas, exerts a broad spectrum of biological activities through paracrine, endocrine, and neuroendocrine mechanisms [13–16]. Somatostatin in gastric D cells functions in a paracrine fashion to suppress the secretion of histamine and gastrin from enterochromaffin-like cells and G cells, respectively [17,18]. Recently, somatostatin infusion into normal human subjects has been reported to reduce plasma ghrelin levels to 70–80% of normal control values [19,20]. Subcutaneous administration of octreotide, a long-lasting somatostatin analog [21], also lowered plasma ghrelin levels in patients with acromegaly. These findings, however, may reflect an indirect effect of somatostatin on ghrelin secretion, as systemic administration of somatostatin

☆ *Abbreviations:* AUC, area under the curve; $CH_3CN$, acetonitrile; GH, growth hormone; ir, immunoreactive; KRBB, Krebs–Ringer bicarbonate buffer; RIA, radioimmunoassay; RP-HPLC, reverse-phase high performance liquid chromatography; TFA, trifluoroacetic acid.

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suppresses the secretion of a wide range of hormones, such as GH, insulin, glucagon, and other gastroentero-pancreatic hormones. Little is known about the direct regulation of ghrelin release from the stomach. Here, we examined the morphological relationship between ghrelin and somatostatin by immunohistochemistry. We examined the effect of intravenous somatostatin administration on the plasma ghrelin level in rats. We used vascularly perfused rat stomachs to investigate the direct effect of somatostatin on ghrelin secretion.

## Materials and methods

*Animals.* Male Wistar rats weighing 300–350 g (Charles River Japan, Inc., Shiga, Japan) were used in all experiments. Rats, housed individually in plastic cages at constant room temperature in a 12-h light (0700–1900)/12-h dark cycle, were given standard laboratory food and water ad libitum. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care. The protocol was approved by the Miyazaki Medical College Animal Care Research Committee.

*Immunohistochemistry.* Three Wistar rats were transcardially perfused with 100 mM phosphate buffer (pH 7.4), followed by 4% paraformaldehyde in 100 mM phosphate buffer. Stomachs were resected and then sectioned into 12 μm-thick slices at −20 °C with a cryostat. Sections were incubated overnight at 4 °C with either mouse monoclonal anti-ghrelin antibody (final dilution 1/5000) or rabbit anti-somatostatin antiserum (Nichirei, Tokyo, Japan; dilution 1/500). Slides were stained by the EnVision method (Dako, Carpintera, CA) as described previously [2]. For ghrelin and somatostatin double staining, samples were first stained for ghrelin with EnVision+ Peroxidase Mouse (Dako) and then with anti-somatostatin antiserum with EnVision+ Peroxidase Rabbit (Dako). These specimens were allowed to react for 3 h and then visualized for 3 min at room temperature with 20 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, MO) and 0.006% hydrogen peroxide in 50 mM Tris.

*Intravenous administration of somatostatin.* Male Wistar rats were examined after an overnight fast. Following anesthesia with pentobarbital (Nembutal, Abbott Lab., North Chicago, IL), rats received a single intravenous injection of rat somatostatin (2 nmol, Peptide Institute, Osaka, Japan) in 0.9% saline (200 μl) through the femoral vein. Rats were sacrificed at the specified time points ranging from 0 to 60 min after the somatostatin injection ($n = 4$–8/group). Truncal blood (4 ml) obtained by decapitation was collected into cooled polypropylene tubes containing EDTA·2Na (1 mg/ml blood) and aprotinin (500 U/ml blood). After centrifugation, plasma was diluted 1:1 with 0.9% saline and applied to a Sep-Pak C-18 (Waters, Milford, MA) cartridge pre-equilibrated with 0.9% saline. The cartridge was washed first with 0.9% saline and then with 10% acetonitrile ($CH_3CN$) containing 0.1% trifluoroacetic acid (TFA). Adsorbed peptides were eluted with 60% $CH_3CN$ containing 0.1% TFA and then subjected to ghrelin C-RIA as described below.

*Gastric perfusion.* A midline incision opened the abdomens of Wistar rats under anesthesia with Nembutal. After ligation, a cannula was inserted into the portal vein. After ligation of the abdominal aorta just above the celiac artery branch, a cannula was inserted into the celiac artery via an incision on the opposite side of the aorta. A cannula was also inserted into the lumen of the stomach via a pylorus ring to facilitate outflow of the gastric contents throughout experimentation. The esophagus was ligated at the esophago-gastric junction. Schematic illustration of this perfusion system is shown in Fig. 3. The system was constantly perfused at a flow rate of 2.0 ml/min with modified Krebs–Ringer biocarbonate buffer (KRBB: 117.5 mM NaCl, 4.7 mM KCl, 2.4 mM $CaCl_2$, 1.1 mM $MgCl_2$, 1.1 mM $NaH_2PO_4$, 25 mM $NaHCO_3$, 11.1 mM glucose, and 0.1% bovine serum albumin, pH 7.4) bubbled with a mixture of 95% $O_2$ and 5% $CO_2$ and maintained at 37 °C [22]. After a 15 min equilibration period, a $10^{-5}$–$10^{-11}$ M octreotide (Sandostatin, Novartis Pharma AG, Basel, Switzerland) solution ($n = 4$–6/group) was added to the perfusion medium for 5 min. One minute effluents isolated from the portal vein were collected over a 40 min period into tubes that contained 12% acetic acid. These samples were then boiled at 100 °C for 3 min and applied to Sep-Pak cartridges. The eluates were submitted to ghrelin N-RIA described below. A portion of the Sep-Pak eluates obtained from the pre-perfusion period was subjected to reverse phase-high performance liquid chromatography (RP-HPLC) on a TSK ODS SIL 120A column (4.6 × 150 mm, Tosoh, Tokyo, Japan). RP-HPLC was performed for 40 min at 1.0 ml/min with a linear gradient of $CH_3CN$ (10–60%) in 0.1% TFA. All HPLC fractions were quantified by both N- and C-RIAs for ghrelin. Plasma glucose was measured by the glucose oxidase method. Plasma insulin was measured by enzyme immunoassay (Morinaga, Yokohama, Japan). The rat gastric fundus was homogenized and centrifuged as described [8]. The sample supernatants were applied to Sep-Pak C-18 cartridges. Eluates were then subjected to RP-HPLC as described above. All HPLC fractions were quantified by both N- and C-RIAs for ghrelin.

*Radioimmunoassays for ghrelin.* Ghrelin content was measured by two types of radioimmunoassays (RIA) recognizing either *n*-octanoylated ghrelin or total ghrelin [23], designated N-RIA and C-RIA, respectively. To generate anti-ghrelin antisera, synthetic [$Cys^{12}$]-ghrelin [1–11] and [$Cys^0$]-ghrelin [13–28] peptides were conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH; Pierce, Rockford, IL). These antigenic conjugate solutions were administered to three New Zealand white rabbits. The anti-rat ghrelin [1–11] antiserum (#G606) specifically recognized *n*-octanoylated ghrelin, while it did not recognize des-acyl ghrelin. Anti-rat ghrelin [13–28] antiserum (#G107) equally recognized *n*-octanoyl modified and des-acyl ghrelin. Synthetic rat [$Tyr^{29}$]-ghrelin [1–28] and [$Tyr^0$]-ghrelin [13–28] were radioiodinated using the lactoperoxidase method. $^{125}I$-labeled peptides were purified on a TSK ODS SIL 120A column by RP-HPLC. Diluted samples or standard peptide solutions (100 μl) were incubated for 24 h with 100 μl diluted antiserum (final dilutions: anti-ghrelin [1–11] antiserum, 1/620,000; anti-ghrelin [13–28] antiserum, 1/20,000). Following addition of the tracer solution (16,000 cpm in 100 μl), mixtures were incubated for 24 h. Samples were assayed in duplicate; all procedures were done at 4 °C. The limits of detection by rat ghrelin [1–28] on the standard RIA curve were 0.5 fmol and 8 fmol per tube in N-RIA and C-RIA, respectively. The respective intra- and interassay coefficients of variation at 50% binding for N-RIA were 3.5% and 3.2% and for C-RIA were 3.7% and 3.3%. The recoveries of rat ghrelin [1–28] (1 ng) and $^{125}I$-rat ghrelin [1–28] (5000 cpm) added to the plasma samples in the extraction with Sep-Pak C-18 cartridges were $92.2 \pm 0.4\%$ (SEM) and $88.9 \pm 0.6\%$ (SEM), respectively.

*Statistics.* All data are expressed as means ± SEM. The area-under-the-curve (AUC) depicting plasma ghrelin concentration from 0 to 20 min (AUC20) after the start of octreotide infusion was calculated with a planimeter. Data groups were compared using ANOVA and a post hoc Fisher's test. Differences were considered significant at $P$ values less than 0.05.

## Results

### Immunohistochemistry

Ghrelin-immunoreactive cells were abundant from the neck to base in the rat oxyntic glands (Figs. 1A and C). Ghrelin-expressing cells were either round basket-shaped

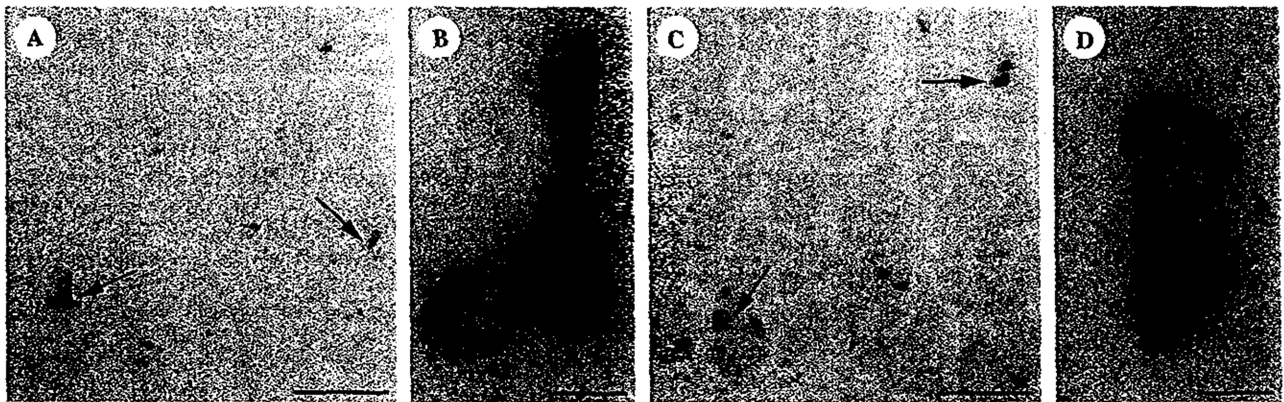


Fig. 1. Localization of ghrelin-immunoreactive and somatostatin-immunoreactive cells in the rat stomach. Ghrelin-immunoreactive and somatostatin-immunoreactive cells are present in the oxyntic glands (A,C). Large magnification (B,D). A subset of ghrelin-immunoreactive cells directly contact somatostatin-immunoreactive cells, as indicated by arrows. Ghrelin is stained in *red* and somatostatin is shown in *brown*. Bar, 50 μm in (A,C); 5 μm in (B,D).

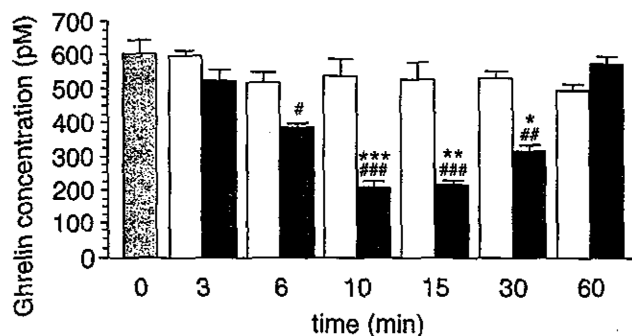


Fig. 2. Effect of intravenous administration of somatostatin on plasma ghrelin concentration. White bars indicate saline-treated groups and black bars indicate somatostatin-treated groups. C-RIA for ghrelin determines ghrelin concentration. Values are means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.005$; and $^{***}P < 0.0005$ vs respective saline groups. $^{\#}P < 0.05$; $^{\#\#}P < 0.005$; and $^{\#\#\#}P < 0.0005$ vs value at 0 min.

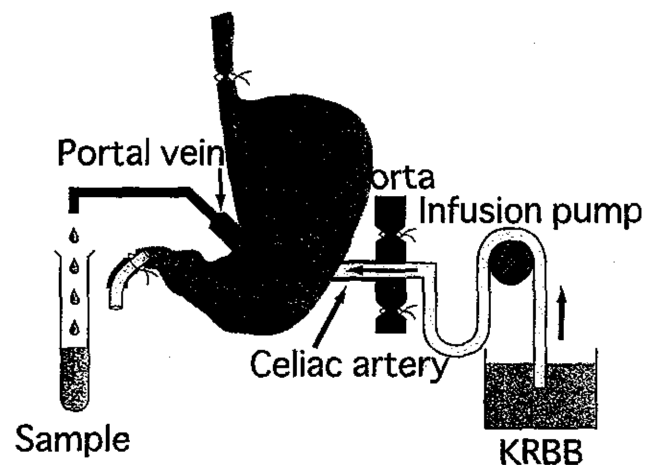


Fig. 3. Schematic illustration of the preparation of vascularly perfused rat stomach.

or tall flask-shaped cells with a dense accumulation of ghrelin within the basal cytoplasm. Somatostatin-immunoreactive cells were also present in the oxyntic glands (Figs. 1A and C), exhibiting a long, thin basal process immunoreactive for somatostatin. A subset of ghrelin-immunoreactive cells contacted somatostatin-immunoreactive cells (Figs. 1B and D). No immunoreactivity was detected in the tissues following incubation

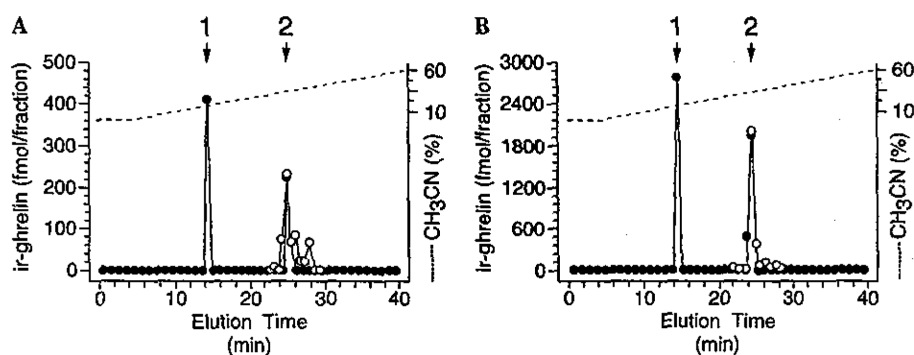


Fig. 4. Representative RP-HPLC profiles of ghrelin immunoreactivity in the rat stomach perfusates (A) and stomach tissue extracts (B). One milliliter portal vein effluent and 1 mg wet weight of rat stomach were analyzed. Open circles indicate immunoreactivity for the octanoyl-modified form of ghrelin and closed circles demonstrate immunoreactivity for the C-terminal portion of ghrelin. Arrows indicate the elution positions of (1) des-acyl ghrelin and (2) *n*-octanoylated ghrelin.

with either normal rabbit serum or specific antiserum pre-absorbed with excessive ghrelin or somatostatin (data not shown).

*Effect of systemic administration of somatostatin on plasma ghrelin level*

The basal plasma concentration of ghrelin in the truncal vein determined by C-RIA was 602.2 ± 50.9 pM. The mean plasma ghrelin concentration gradually decreased after an intravenous administration of somatostatin, reaching a nadir of 36.4% (219.4 ± 14.9 pM) of basal levels 10 min after the injection (Fig. 2). Ghrelin levels increased thereafter, returning to the baseline 60 min after somatostatin administration. Normal responses were observed for plasma glucose (data not shown) and insulin (data not shown) levels. The plasma concentrations of ghrelin did not change in control rats administered saline alone (Fig. 2).

*HPLC characterization of immunoreactive-ghrelin molecules in the stomach perfusate*

RP-HPLC coupled with C- and N-RIAs was used to analyze immunoreactive-ghrelin molecules in portal vein effluents from the vascularly perfused stomach. A large

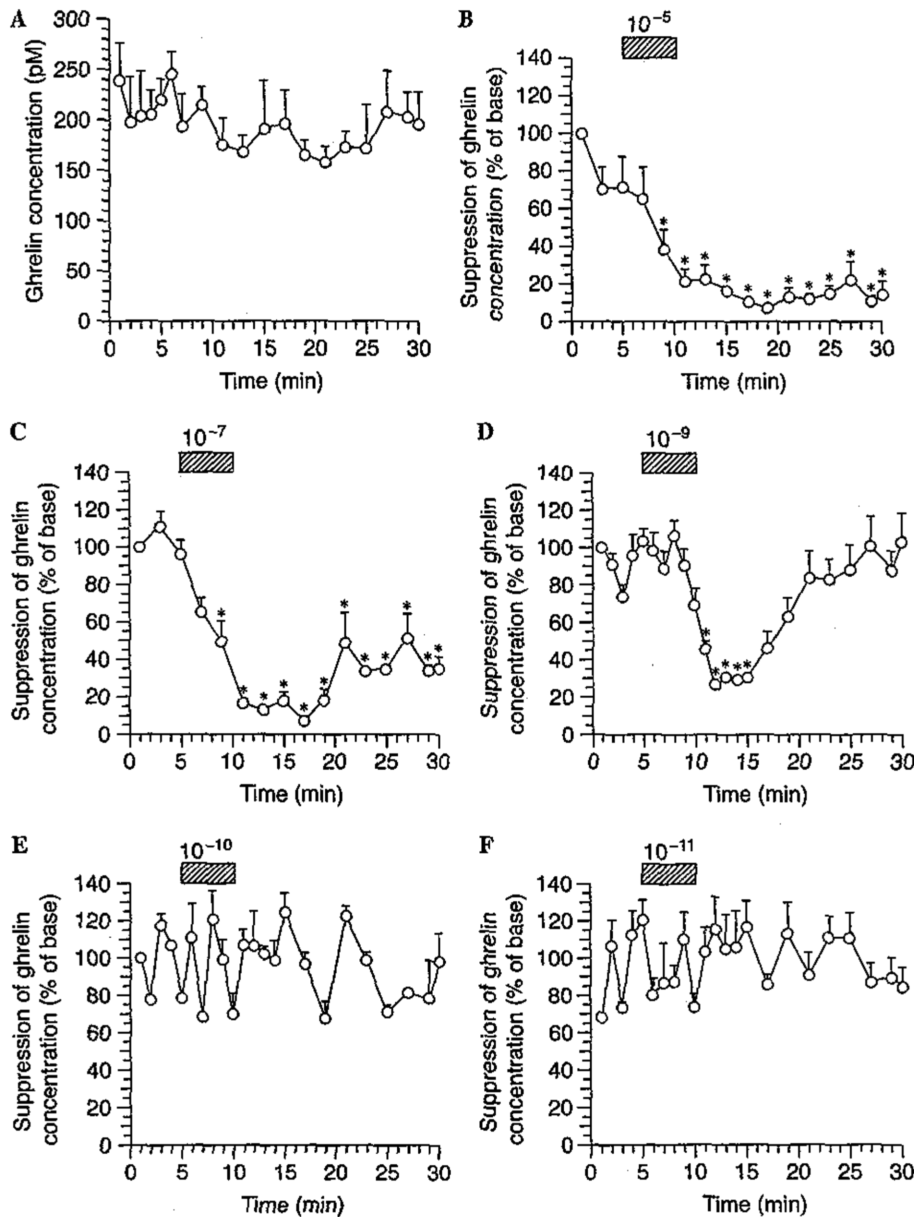


Fig. 5. Effect of octreotide on ghrelin release from vascularly perfused rat stomach. (A) Baseline ghrelin release withoutsomatostatin infusion. (B–F) Octreotide ($10^{-5}$–$10^{-11}$) was perfused for 5 min following a pre-perfusion period (15 min), indicated by shaded bars. Data in B–F are expressed as a percentage of the suppression of ghrelin release compared with pre-perfusion values. N-RIA for ghrelin was used. Values are means ± SEM. $^*P < 0.05$ vs pre-perfusion values.

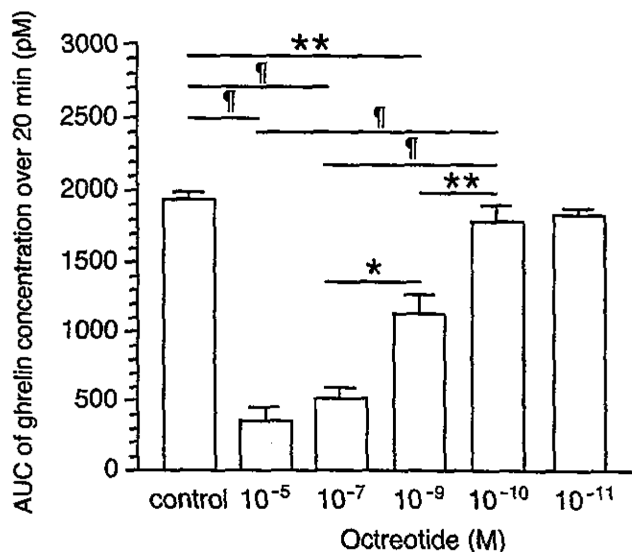


Fig. 6. Dose-response effect of octreotide on ghrelin release from perfused rat stomach. The AUC representing ghrelin concentration from 0 to 20 min after the start of octreotide infusion in Fig. 5 is shown. Values are means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{¶}P < 0.0005$.

ghrelin-immunoreactive peak (peak 1 in Fig. 4) eluted at the position of des-acyl ghrelin. Another major peak (peak 2 in Fig. 4) eluted at the position of *n*-octanoylated ghrelin. The molar ratio of des-acyl ghrelin to acylated ghrelin was 2:1 (Fig. 4A), similar to the ratio seen in gastric tissue extract (Fig. 4B).

*Gastric perfusion experiment*

The portal vein effluents were measured by N-RIA for ghrelin. Ghrelin concentrations in the control vehicle group from 0 to 30 min ranged from 159.9 to 246.7 pM (means ± SEM, 195.9 ± 29.1) (Fig. 5). Octreotide administration at low levels, ranging from $10^{-10}$ to $10^{-11}$ M, did not affect ghrelin concentrations. Infusion of octreotide at $10^{-5}$–$10^{-9}$ M for 5 min following 15 min pre-perfusion, however, rapidly decreased ghrelin concentrations in dose- and time-dependent manner. Ghrelin concentration returned to the pre-perfusion level approximately 10 min after a $10^{-9}$ M octreotide infusion, while it did not return to pre-perfusion levels even at 20 min after $10^{-5}$ and $10^{-7}$ M infusion.

The AUCs of ghrelin concentration from 0 to 20 min after the start of $10^{-5}$–$10^{-9}$ M octreotide infusion were significantly lower than that seen in the control vehicle group (Fig. 6). Infusion of octreotide at $10^{-10}$ or $10^{-11}$ M did not change ghrelin concentration AUC.

## Discussion

Somatostatin in the rat gastrointestinal tract is most abundant in the mucosa of the stomach and duodenum. In the gastric fundus, primarily the 14 amino acid form of somatostatin is produced in endocrine D cells [24], which extend long cytoplasmic processes to contact adjacent cells, delivering somatostatin to these cells in a paracrine manner. The effects of somatostatin on gastrointestinal functions are exclusively inhibitory, acting in opposition to the effects of other gastrointestinal peptides and affecting acid secretion, motility, growth, and hormone secretion. We demonstrated that some somatostatin cells make direct cellular contacts with ghrelin-producing cells in the gastric fundus. Ghrelin cells in the oxyntic mucosa are closed-type cells that have no continuity with the lumen, suggesting that they respond to chemical stimuli originating from the basolateral site. Cytoplasmic processes characteristic of somatostatin cells directly contact ghrelin cells, suggesting a regulatory interaction between somatostatin and ghrelin. We next examined ghrelin levels following both administrations of somatostatin into the systemic vein and into the gastric artery in a vascular perfusion model of rat stomach.

Two ghrelin-specific RIAs have been established: one recognizing the octanoyl-modified, biologically active form of ghrelin [1] and the other recognizing C-terminal portion of all ghrelin forms. We utilized the latter assay to quantify ghrelin in the systemic blood; as acylated ghrelin is significantly less stable than the des-acylated counterpart, measurement of acylated ghrelin from stored plasma samples is unreliable [23]. The former assay was used in the quantification of ghrelin in the effluent samples, as these could be treated with acetic acid immediately after collection.

Intravenous administration of somatostatin significantly reduced the plasma ghrelin level. The effect of a single injection of somatostatin lasted for 30 min. While changes in insulin and glucose concentrations affect ghrelin secretion [8,11], the lack of changes in insulin or glucose concentrations suggests a direct regulation of ghrelin by somatostatin. Somatostatin injection into the gastric artery of the vascularly perfused stomach suppressed ghrelin secretion in dose- and time-dependent manner. In this perfusion system, somatostatin was infused in only the stomach, since the portal vein and abdominal aorta were ligated. Cholecystokinin, gastrin, acidification of the gastric lumen, and all major classes of nutrients stimulate somatostatin release from gastric D cells [25]. Food ingestion and cholecystokinin also reduce plasma ghrelin levels [8,11,12,26]. We have recently verified that stomach-derived ghrelin signals for appetite control and GH secretion are relayed to the brain by means of the vagus afferent nerve, which ultimately interact with the hypothalamus [27]. Ghrelin is the first neuroenteric peptide that acts as a starvation-signaling molecule in the periphery and stimulates feeding after peripheral administration. The regulation of ghrelin secretion by somatostatin emphasizes a

paracrine role of gastric somatostatin in overall nutrient homeostasis. This study extends our understanding of how ghrelin secretion from the stomach is regulated.

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