ghrelin levels in AN patients with habitual binge/purge behaviour (AN-BP) when compared to restricting behaviour (AN-R) despite similar body mass indeces (Tanaka *et al.*, 2003b). The normal weight of a patient with bulimia nervosa with habitual binge-eating and purging has also been found to increase the ghrelin level (Tanaka *et al.*, 2002). Moreover, carbohydrate metabolism and insulin secretion in eating disorders are considered to be related to nutritional status and eating patterns (Casper *et al.*, 1988; Johnson *et al.*, 1994). These findings suggest that differences in eating behaviour may influence secretion of ghrelin and insulin in AN. Therefore in this study, we measured ghrelin and insulin responses to oral glucose loads in all subjects in order to examine the effect of acute feeding states on ghrelin and insulin secretion, and compared the states between the subtypes of AN patients and healthy controls for the purpose of clarifying the pathophysiology of eating behaviour.

Research design and methods

Subjects

Eleven female AN-R patients, nine female AN-BP patients who met DSM-IV guidelines and 10 age-matched apparently healthy female volunteers (controls) were the subjects in this study. Patients were admitted to our hospitals for inpatient treatment, and were excluded if they had a history of alcohol or substance abuse, or gastrointestinal disease. AN-BP patients had habitual binge-eating and vomiting at least twice a week over the preceding 3 months. Written informed consent was obtained from all participants before starting the study, which proceeded in accordance with the principles of the Declaration of Helsinki. Controls were recruited by advertisement in the local community and were paid for their participation. They had no history of psychiatric illness and metabolic diseases, ate normal diets and were within 10% of ideal body weight.

Protocol

Subjects were given a 75-g/225-ml glucose solution orally at 08·00 h after an overnight fast. A butterfly needle was inserted into a forearm vein and the catheter was kept patent by a saline infusion in order to collect blood samples efficiently. Blood was collected 0, 30, 60, 120 and 180 min after oral administration. During testing, all subjects remained in a recumbent position and no activity or eating was permitted. We measured body weight at the time blood samples were obtained. The AN patients were assayed within 1 week after admission and before the initiation of active treatment including medications such as psychotropics. The Institutional Committee of Kagoshima University approved the protocol.

Measurements

Plasma glucose was measured by the glucose oxidase method. Serum insulin was determined by an EIA kit (SRL, Inc., Tokyo, Japan). Blood was drawn into chilled tubes containing EDTA2Na (1 mg/ml) and aprotinin (500 U/ml). Plasma ghrelin was measured using radioimmunoassay (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 haemocyanin. The antiserum recognized acylated ghrelin and nonacylated 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, USA) 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 ± 4·0% ($n = 6$).

Statistics

The subject groups (mean ± SEM) were compared using analysis of variance (ANOVA) and a posthoc Scheffé test, when data were normally distributed. The Kruskal–Wallis one-way ANOVA with a chi-square statistic was used to test group differences for the subject characteristic variables because the data distributions were skewed. A P-value of < 0·05 was considered statistically significant.

Results

Physiological characteristics for the subject groups are shown in Table 1. The mean body mass index ($P < 0·01$) and basal serum insulin level ($P < 0·05$) in both AN-R and AN-BP were significantly lower than those in controls. Basal plasma glucose levels in AN-R were significantly lower ($P < 0·05$) as compared to the control group. Basal plasma ghrelin in both AN-R and AN-BP were significantly higher ($P < 0·01$) as compared to controls (Table 1).

Table 1 Physiological characteristics (mean ± SEM) of subject groups

	AN-R $n = 11$	AN-BP $n = 9$	control $n = 10$	Kruskal–Wallis*	P
Age (years)	18·5 ± 1·4	20·9 ± 1·4	21·0 ± 0·6	4·2	0·13
Duration of illness (years)	2·2 ± 0·5	5·2 ± 1·3	–	–	–
Body mass index (kg/m^2)	13·3 ± 0·4†	13·8 ± 0·5†	21·4 ± 0·4	–	–
Basal plasma ghrelin (pmol/l)	233·8 ± 39·3	347·4 ± 49·2	123·4 ± 6·6	15·5	< 0·01
Basal plasma glucose (pmol/l)	4·2 ± 0·1†	4·5 ± 0·1	4·7 ± 0·1	–	–
Basal serum insulin (pmol/l)	27·2 ± 3·8	28·7 ± 4·6	50·6 ± 6·6	7·4	0.03

*The Kruskal–Wallis one-way ANOVA was used to test because the data distributions were skewed. †$P < 0·05$ v.s. control, using ANOVA and a posthoc Scheffé test.

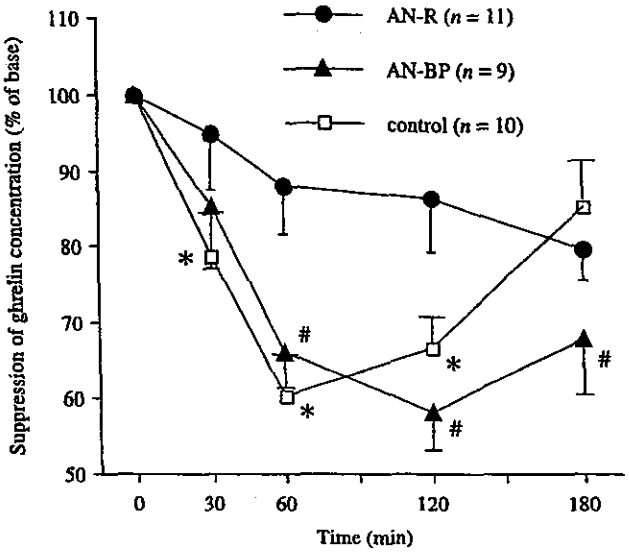

Fig. 1 Plasma ghrelin responses to an oral glucose tolerance test in female anorexia nervosa patients with restricting type (●, AN-R) and binge-eating/purging type (△, AN-BP), and age-matched controls (□). *$P < 0·05$ in control *vs.* 0 min, #$P < 0·05$ in AN-BP *vs.* 0 min.

The mean plasma ghrelin concentrations in both controls and AN-BP patients decreased after administration of an oral glucose load, reaching nadirs of 60·2% (74·3 ± 7·9 pmol/l, mean ± SEM) and 58·1% (204·9 ± 34·3 pmol/l) of basal levels, respectively, 60 min and 120 min after the glucose load, and increasing thereafter. However, the plasma ghrelin level in AN-R patients constantly decreased without reaching the nadir level for 180 minutes (80·0%, 182·4 ± 31·5 pmol/l; Fig. 1).

The plasma glucose level 180 min after the glucose load in AN-R was higher than that in controls ($P < 0·05$; Fig. 2a). Though the peak glucose level occurred 60 min after the glucose load in both AN-BP and controls, the peak in AN-R occurred 120 min after the glucose load (Fig. 2a). After glucose loads in controls, the peak insulin level was found to occur at 60 min (509·2 ± 88·8 pmol/l) while the peaks in AN-BP and AN-R were, respectively, 120 min (319·3 ± 88·8 pmol/l) and 180 min (418·9 ± 68·4 pmol/l) after the glucose load (Fig. 2b).

Discussion

AN-R patients exhibited a constant decreased ghrelin level without reaching a nadir during the test and peaks were delayed for both glucose and insulin as compared to AN-BP and controls. Plasma ghrelin concentrations in healthy humans have been found to decrease significantly after oral and intravenous glucose administration (Shiiya *et al.*, 2002), and it has been suggested that there is delayed glucose absorption due to gastric and duodenal dysmotility in AN-R patients (Stacher *et al.*, 1986; Buchman *et al.*, 1994). However, these results might be caused by impaired regulation of ghrelin secretion as recent studies have shown that plasma ghrelin is not regulated by i.v. administration of glucose, or the combination of glucose and insulin (Caixás *et al.*, 2002; Schaller *et al.*, 2003). A previous study of carbohydrate metabolism (Nozaki *et al.*, 1994) also showed that initial insulin secretion was decreased in AN patients that had glucose level peaking 90 min or later in response to both oral and intravenous glucose. Because our study found that the glucose peak level in AN-R was 120 min, these findings suggest that AN-R patients might also have β-cell dysfunction in acute feeding states.

AN-BP patients had increased basal ghrelin, decreased basal insulin and a delayed nadir for ghrelin and the peak insulin as compared to controls, although the times for peak glucose were similar to controls. Our recent research (Tanaka *et al.*, 2003a) suggests that binge-eating with vomiting rather than binge-eating without vomiting may influence ghrelin levels in eating disorders. In bulimia nervosa patients with unstable weight and with binge-eating and frequent vomiting, a blunted insulin response to a glucose load was found. On the other hand, in these patients who were treated successfully by abstaining from binge-eating and vomiting for 4 weeks, there was a similar insulin response to that seen in normal controls (Russell *et al.*, 1996). These findings suggest that both abnormal eating behaviour and nutritional

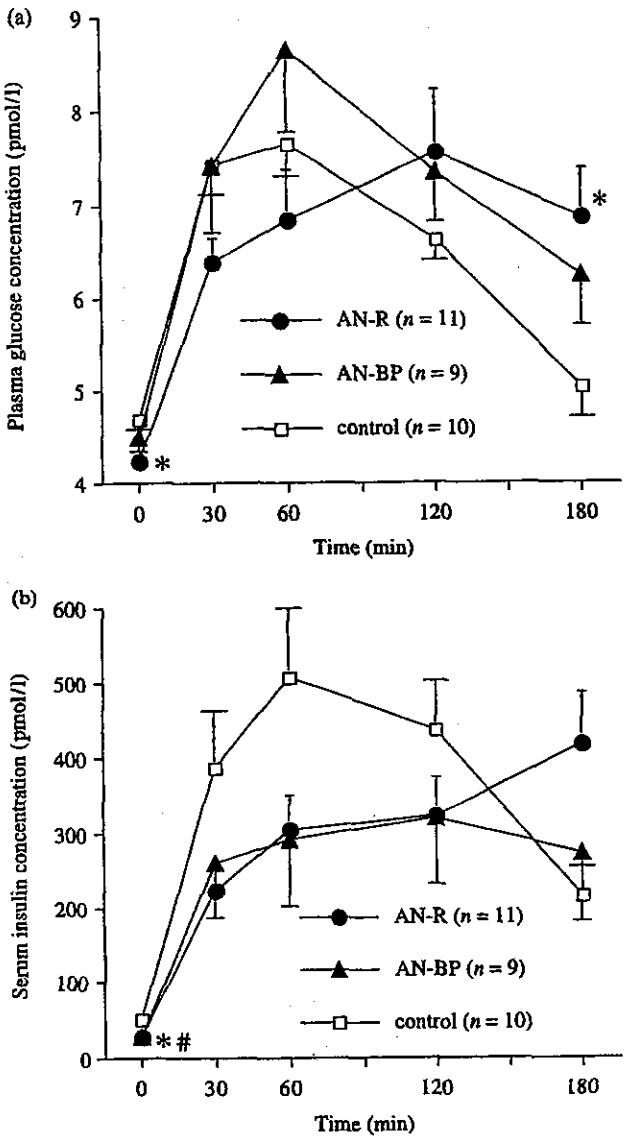

Fig. 2 (a) Comparison of plasma glucose concentration responses to an oral glucose tolerance test among AN-R (●), AN-BP (△) and controls (□). (b) Comparison of serum insulin concentration responses to an oral glucose tolerance test among AN-R, AN-BP and controls. *$P < 0{\cdot}05$ in AN-R *vs.* control, #$P < 0{\cdot}05$ in AN-BP *vs.* control.

depletion may be the cause of the results found in AN-BP, and that abstaining from binge-eating and vomiting may be important for inpatient treatment of AN-BP patients.

The present study suggests that differences in eating behaviour may influence the effect of oral glucose on both ghrelin and insulin secretion in AN patients. A few studies (Stordy *et al.*, 1977; Neuberger *et al.*, 1995) have shown that oral energy intake with nutritional rehabilitation required for weight gain is significantly different between AN subtypes. In particular, AN-R patients required 30–50% more energy intake than AN-BP (Kaye *et al.*, 1986). These findings suggest that impaired regulations of both ghrelin and insulin seen with restrictive eating patterns may be the cause of the higher oral energy intake required for weight gain rather than the actual AN-BP behaviour.

Ghrelin, one of the gastric and orexigenic peptides, is found to inhibit the insulin response to the secretagogues glucose, arginine and carbachol (Egido *et al.*, 2002). In contrast, insulin is one of the anorexigenic peptides and has been shown to decrease plasma ghrelin in humans (Saad *et al.*, 2002). Ghrelin has also been documented to be present in α-cells, and increase the cytosolic free Ca^{2+} concentration in β-cells and stimulate insulin secretion (Date *et al.*, 2002b). These peptides, which are related to energy metabolism, have been suggested to be regulated through the vagal system (Herath *et al.*, 1999; Masuda *et al.*, 2000; Blat & Malbert, 2001; Date *et al.*, 2002a). Therefore, we consider the abnormal eating behaviour and nutritional change in AN to have some influence on the relationship between ghrelin and insulin secretion through the vagal system.

Finally, our study documents that both the time at the nadir of the plasma ghrelin level and the peak serum insulin level are delayed in AN patients, especially in AN-R as compared to controls. The present study suggests that differences in eating behaviour may influence the effect of oral glucose on both ghrelin and insulin secretion in AN patients before active treatment. Thus, alterations in both nutritional status and eating pattern may induce ghrelin and insulin metabolic changes in both the acute and chronic feeding state. Furthermore, these metabolic changes in the restrictive eating patterns may be related to the pathophysiology of small quantitative meal intake in AN-R patients.

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Upregulation of Ghrelin Expression in Cachectic Nude Mice Bearing Human Melanoma Cells

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Ghrelin is a gastrointestinal peptide that stimulates food intake and growth hormone (GH) secretion. We studied the biosynthesis and secretion of ghrelin in a cancer cachexia mouse model. G361, a human melanoma cell line, was inoculated into nude mice. The body weight was reduced and the plasma concentration of interleukin-1β (IL-1β) was markedly higher in tumor-inoculated mice compared with vehicle-treated mice. Furthermore, white adipose tissue (WAT) weight, blood sugar level, and plasma concentrations of leptin and nonesterified fatty acids (NEFA) were significantly lower in tumor-inoculated mice. The plasma concentration of ghrelin increased with the progression of cachexia. The levels of both ghrelin peptide and mRNA in the stomach were also upregulated in tumor-inoculated mice. This study demonstrates that both ghrelin biosynthesis and secretion are stimulated in the long-term negative energy balance of tumor-inoculated cachectic mice. These findings suggest the involvement of ghrelin in the regulation of energy homeostasis in cancer cachexia.

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, a strong independent risk factor for mortality.[3,4] The cachexia cannot be attributed solely to appetite loss, and nutritional supplementation alone is unable to reverse the wasting process. Numerous cytokines produced by tumor cells, including leukemia-inhibitory factor (LIF), tumor necrosis factor-α, interleukin-1β (IL-1β), IL-6, and interferon γ, mediate the cachectic effect of cancer.[2,5] A variety of neuropeptides, including neuropeptide Y (NPY), melanin-concentrating hormone, orexin, melanocortin, cholecystokinin, and corticotropin-releasing hormone, regulate energy balance and metabolic signaling.[3] Alterations of these neuropeptide networks may be responsible for the development of cachectic syndrome.

Ghrelin, initially discovered from rat and human stomach as an endogenous ligand for the growth hormone (GH) secretagogue receptor,[6] is an enteric peptide that stimulates food intake[7-9] and induces adiposity.[10] Daily subcutaneous administration of ghrelin causes body weight gain and increases fat mass in mice and rats. Upon fasting, plasma ghrelin concentrations are increased in rats and humans; these levels are decreased after meals.[11-14] Ghrelin serves as an anabolic hormone produced in the stomach and may contribute to energy homeostasis in cancer cachexia. We previously demonstrated that G361, a human melanoma cell line that produces IL-1β and LIF, causes severe body weight loss in nude mice upon inoculation.[15] To investigate the involvement of ghrelin in cancer cachexia, we have studied ghrelin expression in the stomach of G361-inoculated nude mice. We also determined the gastric content of ghrelin and its plasma concentration in the early and end stages of cancer cachexia.

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MATERIALS AND METHODS

Animals

Five-week-old female BALB/c-*nu/nu* mice weighing 15 to 17 g (Charles River Japan, Atsugi, Japan) were adapted to laboratory conditions for 1 week before the start of experiments. Mice were housed individually in plastic cages containing wood chips in a temperature-controlled 23°C ± 2°C room under 12:12-hour light:dark cycles (light on at 7 AM). 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 and approved by the Miyazaki Medical College animal care committee.

Tumor Inoculation

Mice were allocated into 3 groups. Each mouse in groups 1 and 2 was inoculated subcutaneously in both flanks with either 1×10^7 G361 human melanoma cells (hereafter referred to as "G361-1 and G361-2 mice") or vehicle alone (hereafter referred to as "vehicle mice"). Their body weights were measured every day at 9 AM. G361-1 mice (n = 10) were euthanized with sodium pentobarbital anesthesia 7 days after inoculation, and G361-2 (n = 7) and vehicle mice (n = 8) were killed 13 days after inoculation. Blood was collected from the heart into polypropylene tubes containing EDTA·2Na. The blood sugar level was measured using ANTSENSE II (DAIKIN, Tokyo, Japan). The stomach was removed and divided into 2 portions; one half was utilized for peptide quantification and the other was immediately homogenized with TRIzol (Life Technologies, Frederick, MD) for RNA isolation. The tumor and white adipose tissue (WAT) surrounding the kidney and uterus were removed and weighed.

Plasma Analyses

Plasma concentrations of IL-1β, leptin, and nonesterified fatty acid (NEFA) were measured with a human IL-1β immunoassay kit (R&D Systems, Minneapolis, MN), a Leptin/Mice enzyme-linked immunosorbent assay (ELISA) kit (SEIKAGAKU, Tokyo, Japan), and a

Determiner NEFA assay kit (Kyowa Medex, Tokyo, Japan), respectively.

Ghrelin Quantification in Mice

Stomach. Ghrelin content in the stomach was measured by radioimmunoassay (RIA) specific for ghrelin as described.[16] In brief, approximately 50 mg of mouse stomach was boiled for 10 minutes in a 100-fold volume of water to inactive intrinsic proteases. After cooling to 4°C, CH_3COOH and hydrochloric acid (HCl) were added to final concentrations of 1 mol/L and 20 mmol/L, respectively. The stomach was then homogenized in a Polytron for 1 minute. 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 hours with 100 μL antighrelin (13–28) antiserum (final dilution 1/20,000). Following addition of a tracer solution ([^{125}I-Tyr^0]ghrelin (13–28), 17,000 cpm/100 μL), the mixture was again incubated for 24 hours. Bound and free ligand were separated using a secondary antibody (200 μL). Samples were assayed in duplicate; all procedures were performed at 4°C. The antiserum recognized acylated and nonacylated ghrelin on an equimolar basis. The RIA system specifically detected both ghrelin molecules, a finding that was confirmed by high-performance liquid chromatography coupled with the RIA.[16] The limit of detection of the assay was 12 fmol/tube for mouse ghrelin. The respective intra- and interassay coefficients of variation were 3.7% and 3.3% at 50% binding. More than 95% of ghrelin was recovered from the stomach extract.

Plasma. Plasma from each of the 3 mouse groups was pooled group wise. The pooled plasma samples (0.4 mL) were diluted with equal volumes of 0.9% saline and then applied to a Sep-Pak C_{18} cartridge (Waters, Milford, MA) equilibrated with 0.9% saline. The cartridge was washed with 0.9% saline and 10% acetonitrile (CH_3CN) solution containing 0.1% trifluoroacetic acid (TFA). Absorbed peptides were eluted with 60% CH_3CN solution containing 0.1% TFA, lyophilized, and then subjected to RIA. The recovery of ghrelin added to the mouse plasma sample in the extraction done with a Sep-Pak C_{18} cartridge was more than 92%.

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 were denatured using 2 mol/L 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 recognize full-length ghrelin cDNA and a 0.2-kb cDNA fragment of glycerol-3-phosphate dehydrogenase (G3PDH).[12] Membranes were first hybridized for 1.5 hours at 42°C in 6 × SSPE (900 mmol/L NaCl, 60 mmol/L NaH_2PO_4, 7 mmol/L 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 hours at 42°C in a solution containing the two ^{32}P-labeled cDNA probes. The RNA blot was immersed in 2 × SSC (300 mmol/L NaCl, 30 mmol/L sodium citrate, pH 7.0)/0.1% SDS for 20 minutes at 58°C, followed by an incubation in 1 × SSC/0.1% SDS for 40 minutes. Hybridized signals were 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 < .05 were considered significant.

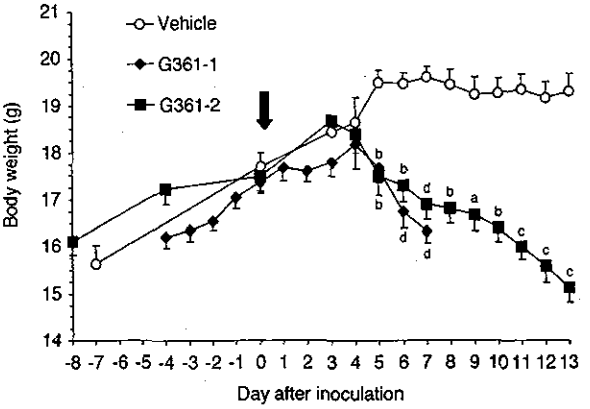

Fig 1. Body weight of G361- (G361-1 and G361-2) or vehicle-inoculated nude mice. G361-1 mice were euthanized 7 days after inoculation, and G361-2 and vehicle mice were euthanized 13 days after inoculation. An arrow indicates the day when either tumor cells or vehicle was inoculated. Data represents means ± SEM. $^aP < .01$, $^bP < .001$, $^cP < .0001$, $^dP < .00001$ *v* vehicle mice.

RESULTS

Body Weight of Tumor-Inoculated Mice

The mean body weights of G361-1 and G361-2 mice began to decrease 5 and 4 days, respectively, after tumor inoculation (Fig 1). Five days after inoculation, the body weights of both G361-1 and G361-2 mice were significantly lower than that of vehicle-treated control mice. There was no significant difference in body weight between G361-1 and G361-2 mice. The ratios of the body weight at sacrifice to the peak body weight were 88% for G361-1 mice and 80% for G361-2 mice, respectively. The individual tumor weights in all mice were less than 0.2 g each, and there was no significant difference in the tumor weight between G361-1 and G361-2 mice. The plasma concentrations of IL-1β in both G361-1 and G361-2 mice were markedly higher than that of vehicle mice (Fig 2).

WAT Weight, Blood Sugar, and Plasma Concentrations of Leptin and NEFA

WAT weight, blood sugar level, and plasma concentrations of leptin and NEFA are shown in Table 1. In both G361-1 and G361-2 mice, the values of all measurements were significantly lower than those in vehicle mice. In G361-2 mice, the mean values of all measurements were lower than those in G361-1 mice; in particular, the blood sugar level was significantly lower.

Ghrelin Peptide and mRNA Levels

Plasma from control vehicle, G361-1, and G361-2 mice was pooled groupwise before assays were performed because the plasma volume from 1 mouse was too small to be subjected to ghrelin RIA. The plasma concentrations of ghrelin in G361-2 and G361-1 mice were higher than that of vehicle mice (Fig 3). The plasma concentration of ghrelin in G361-2 was 2.4-fold higher than that of G361-1 mice. Similarly, the ghrelin content in the stomachs of G361-2 mice was significantly higher than

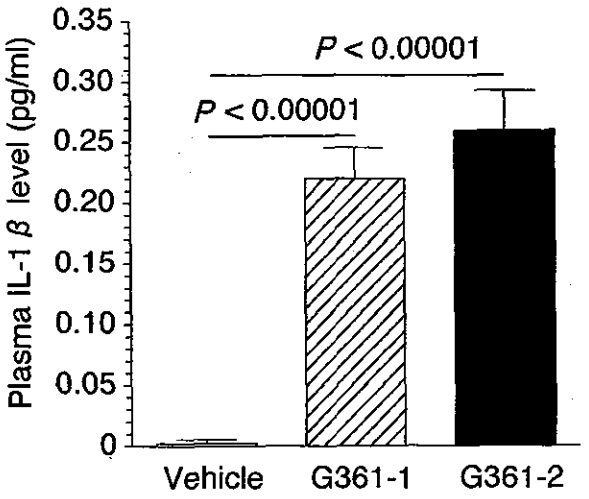

Fig 2. Plasma concentrations of IL-1β.

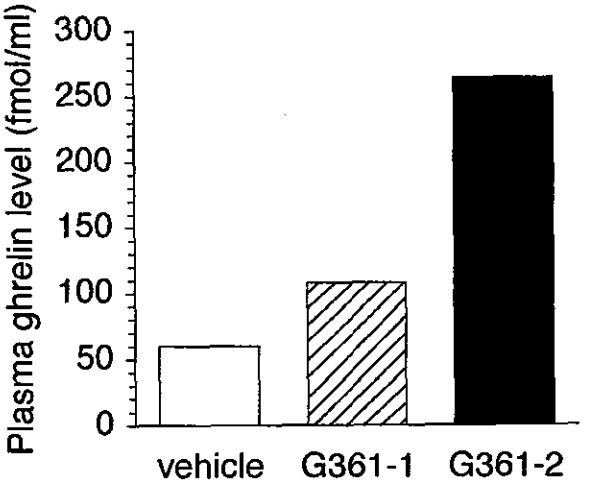

Fig 3. Plasma concentrations of ghrelin.

those of vehicle and G361-1 mice (Fig 4A), and the gastric ghrelin content of G361-1 mice tended to be higher than that of vehicle mice (P = .08). Ghrelin mRNA levels of both G361-1 and G361-2 mice were significantly higher than that of vehicle mice, and the mRNA level of G361-2 mice was significantly higher than that of G361-1 mice (Fig 4B).

DISCUSSION

Ghrelin is the first neuroenteric peptide shown to act as a starvation-signaling molecule in the periphery, stimulating feeding after peripheral administration.[6-14] Ghrelin secretion is upregulated under conditions of negative energy balance and downregulated in the setting of positive energy balance. Plasma ghrelin concentration is increased in cancer anorexia model rats bearing adenocarcinoma cell,[17] human patients with anorexia nervosa,[14,18-20] and cachectic patients with cardiac heart failure.[21] However, little is known regarding alterations of ghrelin biosynthesis and secretion in a state of long-term negative energy balance, such as a cachexia-anorexia syndrome. In this study, a G361-inoculated cachexia mouse model was used to investigate the alteration of ghrelin expression and secretion with the progress of cachexia during its early (7 days after inoculation) and late (13 days) stages.

G361 cells induce severe cachexia by producing several cytokines, including IL-1β and LIF.[15] In the G361-inoculated mice, plasma concentration of IL-1β was elevated compared with that of the vehicle-treated group. The increased level of IL-1β might be one of the causes of cachexia, for IL-1β is known to induce severe weight loss.[22,23] As cachexia worsened, the symptoms of negative energy balance, such as fat loss and hypoglycemia, developed. Plasma leptin concentration correlates with the amount of adipose tissue.[24] The decrease in plasma leptin concentration in tumor-inoculated mice might be due to a reduction in the amount of adipose tissue, which produces leptin. Plasma NEFA concentration usually increases in the initial phase of anorexia because of lipolytic compensation for energy deficiency,[25] but in this study, it was decreased in both early- (G361-1) and late-stage (G361-2) G361-inoculated cachectic mice. The weight of adipose tissue was extremely low in the 2 groups of G361-inoculated mice. Lower plasma NEFA concentration in these mice may be due to decreased adipose tissue mass.

We established 2 ghrelin-specific RIAs; one recognizes the octanoyl-modified portion and another the C-terminal portion of ghrelin.[26] The antibody raised against ghrelin [13-28] equally measures desoctanoylated and octanoylated ghrelin, and it does not distinguish how much of each molecular form is present in the samples. We measured total immunoreactivity of ghrelin molecules by using the ghrelin RIA recognizing its C-terminal portion. Plasma ghrelin concentration, gastric ghrelin content, and ghrelin mRNA level all increased in both early- and late-stages G361-inoculated cachectic mice. These increments may result from stimulation of both biosynthesis and secretion of ghrelin in a long-term negative energy balance state, such as cachexia. In addition, ghrelin levels in the stomach and plasma were higher in late-stage G361-inoculated mice than in early-stage animals, suggesting that ghrelin biosynthesis and secretion became elevated as cachectic symptoms worsened. Increased ghrelin levels in cachectic mice might reflect a physiologic adaptation to negative energy balance. Body weight in cachectic mice decreased, despite elevated ghrelin levels in the stomach and plasma. This may be due to the

Table 1. White Adipose Tissue Weight, Blood Sugar, and Plasma Concentrations of Leptin and Nonesterified Fatty Acid in Mice

	WAT (mg)	BS (mg/dL)	Leptin (pg/mL)	NEFA (μEq/L)
Vehicle (n = 8)	121 ± 32	201 ± 9	1,593 ± 264	427 ± 42
G361-1 (n = 10)	16 ± 2*	163 ± 9*	618 ± 168*	226 ± 30†
G361-2 (n = 7)	7 ± 5*	89 ± 8‡§	361 ± 54†	133 ± 39†

NOTE. Data represent the mean ± SEM.

*P < .01, †P < .001, ‡P < .000001 *v* vehicle mice and §P < .0001 *v* G361-1 mice.

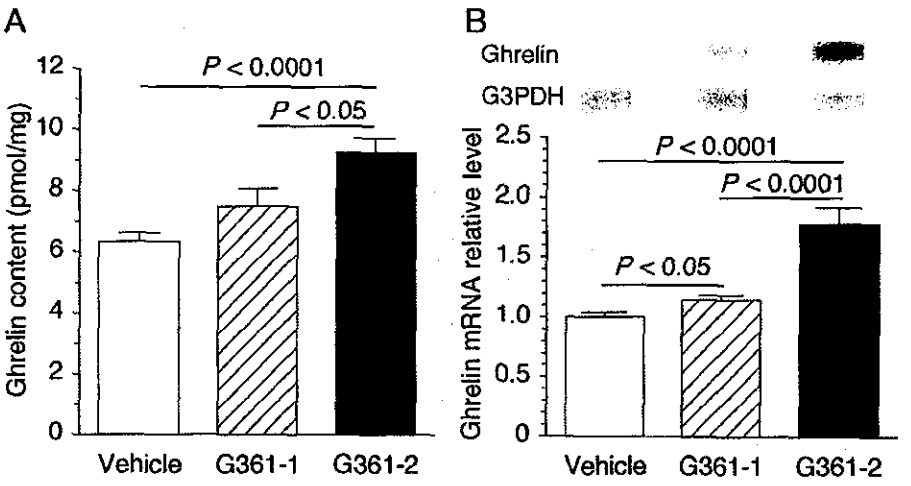

Fig 4. (A) Ghrelin content in the stomach. (B) Upper: representative Northern blot analysis patterns of ghrelin mRNA in the stomach. Lower: ghrelin mRNA levels relative to G3PDH levels.

effects of cachexia-inducing factors produced by G361 melanoma cells that outweigh the anabolic effect of ghrelin.

The stomach is a major source of circulating ghrelin in humans and rats.[6,16,18] Ghrelin-producing endocrine cells, which are most abundant in the oxyntic mucosa of both species, account for about 20% of the oxyntic gland endocrine cell population.[16,27] Ghrelin expression and secretion are affected by energy imbalance.[10-14,17-20] Taken together, there may be a system in ghrelin-producing cells in the stomach that responds to alterations of energy homeostasis. The molecular signals that regulate ghrelin biosynthesis and secretion need to be elucidated.

In summary, the present study demonstrated that ghrelin biosynthesis and secretion are upregulated with the progression of cachexia in tumor-bearing mice. Considering ghrelin's effect on positive energy balance, elevated ghrelin may represent a compensatory mechanism under catabolic-anabolic imbalance in cancer cachexia.

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ORIGINAL ARTICLE

Altered ghrelin and peptide YY responses to meals in bulimia nervosa

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Summary

Objective In recent years great advances have been made in our understanding of the peripheral signals produced within the gastrointestinal tract that regulate appetite, such as ghrelin and peptide YY (PYY). While ghrelin elicites hunger signals, PYY elicites satiety. Therefore, alterations in hormone physiology may play a role in the pathogenesis of bulimia nervosa (BN). In this study, we investigated the postprandial profile of ghrelin and PYY levels in patients with BN.

Design and patients Postprandial plasma ghrelin and PYY levels and insulin and glucose responses were measured in 10 patients with BN and 12 control patients in response to a standard 400 kcal meal.

Results Basal ghrelin levels present in BN subjects (265·0 ± 25·5 pmol/l) were significantly higher than those in healthy controls (199·3 ± 18·4 pmol/l, $P < 0·05$), while basal PYY levels were equivalent in BN (14·6 ± 1·3 pmol/l) and control (12·8 ± 1·1 pmol/l, $P = 0·30$) subjects. Postprandial ghrelin suppression (decremental ghrelin area under the curve) was significantly attenuated in BN patients, compared to controls (−96·3 ± 26·8 pmol/l × 3 h *vs.* −178·2 ± 25·7 pmol/l × 3 h, $P < 0·05$). After a meal, the incremental PYY area under the curve in BN patients was significantly blunted from that observed in controls (9·2 ± 2·6 pmol/l × 3 h *vs.* 26·8 ± 3·2 pmol/l × 3 h, $P < 0·01$). Glucose and insulin responses to meals were similar between the two groups.

Conclusions BN patients exhibit elevated ghrelin levels before meals with reduced ghrelin suppression after eating. In bulimia nervosa subjects, the rise in PYY levels after meals is also blunted. A gut–hypothalamic pathway involving peripheral signals, such as ghrelin and PYY, may be involved in the pathophysiology of BN.

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Bulimia nervosa (BN) is an eating disorder characterized by binge-eating episodes and loss of self-control in eating behaviour. This disorder affects 2–3% of young women.[1] The cause and pathogenesis of BN, however, remain unknown. The phenomenon of binge eating, the consumption of large amounts of food accompanied by a feeling of loss of control, suggests a deficit in the normal mechanisms that turn off eating. Thus, these patients may exhibit corresponding abnormalities in either production or activity of the biological mediators of hunger and satiety.

In recent years, our understanding of the peripheral signals that regulate appetite have increased significantly with the discovery of small peptide signalling molecules secreted from the gastrointestinal (GI) tract. The hormones ghrelin and peptide YY (PYY), secreted from the GI tract in response to changes to nutritional status, are critical regulators of appetite. Ghrelin, a 28-amino acid peptide with an n-octanoyl modification indispensable for its activity, was originally discovered in the rat and human stomach as an endogenous ligand for the GH secretagogue receptor.[2] Ghrelin administration increases food intake and body weight gain.[3–7] In addition, fasting plasma ghrelin concentrations in humans are negatively correlated with body mass index (BMI),[8] percentage body fat and fasting leptin and insulin concentrations.[9] Ghrelin also plays an important role in the pathophysiology of eating disorders.[8,10,11] Recently, our studies have revealed that BN patients who are habitual purgers exhibit elevated ghrelin levels in comparison with those who are not.[12]

PYY is a 36 amino acid peptide secreted following meals from ileal L-cells.[13] PYY belongs to the neuropeptide Y family of peptides, which also includes NPY and pancreatic polypeptide. PYY 3–36, the major form of PYY in the circulation,[14] has a strong affinity for the NPY Y2 receptor.[15] This peptide reduces food intake in rodents and humans.[16] In addition, obese subjects display lower basal fasting PYY levels and exhibit smaller increases in postprandial levels.[17] Additional studies investigating PYY levels in the cerebrospinal fluid

(CSF) of BN patients determined that PYY CSF concentrations were elevated in normal-weight bulimic patients abstinent from pathological eating behaviours for a month.[18–20] Few studies, however, have examined circulating and postprandial PYY levels in BN patients.

The GI hormones ghrelin and PYY act on the arcuate nucleus (ARC) to regulate appetite.[21] As ghrelin elicits hunger signals and PYY elicits satiety, alterations in hormone secretion or activity may function in the pathogenesis of eating disorders such as BN. In this study, we examine the possibility that the postprandial profiles of these hormones are abnormal in BN patients by assessing plasma ghrelin and PYY profiles in BN patients and healthy subjects after the consumption of a standardized meal.

Subjects and methods

Subjects

Ten female BN patients (all of the purging subtype) who met the criteria defined in the the Diagnostic and Statistical Manual of Mental Disorders[19] and 12 age-matched healthy female volunteers (controls) were admitted to our hospital for inpatient treatment. Subjects with any history of alcohol or substance abuse or GI disease were excluded from the study. Control subjects were recruited by advertisement and assessed by clinical interview; all were found to be mentally and physically healthy; none of the control patients had any history of psychiatric illness or metabolic disease. These subjects ate normal diets and were within 10% of ideal body weight.

Subjects were fed a standard breakfast meal at 08·00 h after an overnight (12-h) fasting period. A butterfly needle was inserted into a forearm vein. The catheter was kept patent by saline infusion to collect blood samples efficiently. Blood was collected at 0, 30, 60, 120 and 180 min after ingestion of a standard breakfast (400 kcal), consisting of two slices of bread (90 g), strawberry jam (13 g), milk (200 ml), eggs (50 g) and fruit (40 g; 22% fat, 20% protein and 58% carbohydrate). During testing, all subjects remained in a recumbent position; no activity or additional eating was permitted. BN patients were studied within 1 week of admission, before the initiation of active treatment with medications including psychotropics.

The Institutional Committee of Kagoshima University approved the experimental protocol. All subjects provided written informed consent before participation in this study.

Measurements

Plasma samples for ghrelin and PYY measurement were collected in tubes with EDTA·2Na (1 g/l) and aprotinin (500 U/ml). Plasma ghrelin was measured using a previously described radioimmunoassay (RIA).[8] Inter- and intra-assay variation was < 8 and < 6%, respectively. Plasma PYY levels were analysed by RIA using a commercially available PYY antiserum (Peninsula Laboratories, Inc., San Carlos, CA, USA). For this assay, human PYY3-36 was radio-iodinated by the lactoperoxidase method. Inter- and intra-assay variation was < 9% and < 5%, respectively. Two millilitres of plasma were diluted in 2 ml 0·9% saline and applied to a Sep-Pak C-18 cartridge (Waters Corp., Milford, MA, USA) pre-equilibrated with 0·9% saline. After washing in saline, the cartridge was washed with a 0·1% trifluoroacetic acid solution. Peptides were then eluted in 60% acetonitrile (CH_3CN) containing 0·1% trifluoroacetic acid. The eluate was evaporated, reconstituted in RIA buffer, and subjected to RIA analysis. A standard peptide solution or the diluted samples (100 µl) were incubated for 24 h with 100 µl antiserum. After addition of the tracer solution (16 000 cpm/100 µl), bound and free ligands were separated using the second antibody method. All procedures were performed at 4 °C. Plasma glucose levels were measured by the glucose oxidase method. Plasma insulin was determined by EIA (SRL, Inc., Tokyo, Japan).

Statistics analysis

All values are expressed as means ± SEM. Subject groups were compared using the Student's *t*-test. The postprandial hormonal responses are expressed as delta values compared to baseline values. Differences in postprandial responses were compared by two-way repeated measure ANOVA. When significant differences were found, a statistical comparison between groups at each time point was assessed by Student's *t*-test. *P*-values less than 0·05 were considered significant. The incremental or decremental area under the curve (AUC) for each hormone profile was calculated using the trapezoid method after subtracting the basal AUC from the calculated AUC. Correlation analysis was performed using Pearson's test.

Results

The characteristics of each subject group are shown in Table 1. The subject groups did not differ significantly in age, BMI, or percentage body fat.

Ghrelin responses to a meal are illustrated in Fig. 1. The basal ghrelin levels present in BN subjects (265·0 ± 25·5 pmol/l) were

Table 1. Characteristics (mean ± SEM) of BN patients and controls

	BN $n = 10$	Control $n = 12$	*P*-value*
Age (years)	24·7 ± 1·5	24·8 ± 0·8	0·93
Body mass index (kg/m^2)	20·0 ± 0·6	20·2 ± 0·5	0·88
Percentage body fat (%)	23·3 ± 1·7	24·5 ± 1·0	0·52
Duration of illness (years)	4·8 ± 1·1	–	–

*Student's *t*-test between BN and control subjects.

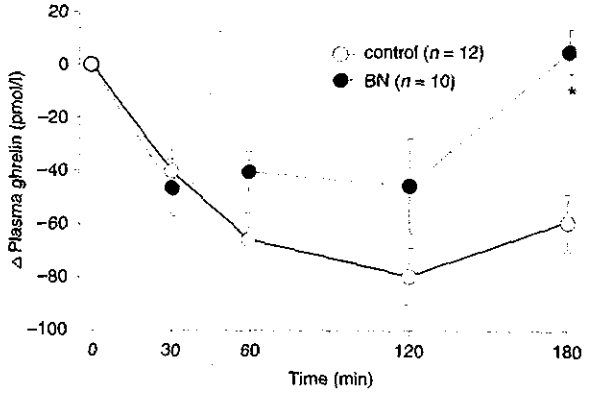

Fig. 1 Comparison of plasma ghrelin responses between BN patients (●) and controls (○) following a meal. *$P < 0·01$ *vs.* control.

Table 2. Comparison of the glucose, insulin, PYY and ghrelin level response to a meal (mean ± SEM) in BN patients and controls

	BN	Control	*P*-value*
Ghrelin			
Basal (pmol/l)	265·0 ± 25·5	199·3 ± 18·4	0·04
Decremental AUC (pmol/l × 3 h)	−96·3 ± 26·8	−178·2 ± 25·7	0·04
PYY			
Basal (pmol/l)	14·6 ± 1·3	12·8 ± 1·1	0·30
Incremental AUC (pmol/l × 3 h)	9·2 ± 2·6	26·8 ± 3·2	< 0·01
Glucose			
Basal (mmol/l)	4·6 ± 0·1	4·8 ± 0·9	0·16
Incremental AUC (mmol/l × 3 h)	1·9 ± 0·5	1·3 ± 0·6	0·50
Insulin			
Basal (pmol/l)	23·9 ± 3·3	41·7 ± 5·0	< 0·01
Incremental AUC (pmol/l × 3 h)	422·5 ± 56·8	637·1 ± 62·1	0·02

*Student's *t*-test between BN and control subjects.

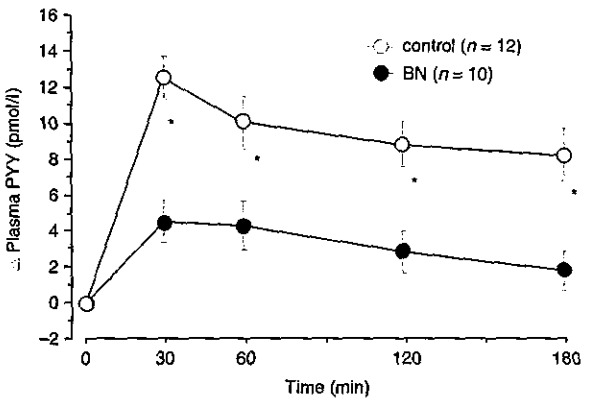

Fig. 2 Comparison of plasma PYY responses between BN patients (●) and controls (○) following a meal. *$P < 0·01$ *vs.* control.

significantly higher than those in controls (199·3 ± 18·4 pmol/l, $P < 0·05$; Table 2). Two-way ANOVA with repeated measures analysis of ghrelin levels demonstrated significant differences between the control and BN groups ($F_{1,20} = 5·12$, $P < 0·05$) evident over time ($F_{4,80} = 22·00$, $P < 0·001$). In addition, we observed a significant group × time interaction ($F_{4,80} = 7·49$, $P < 0·001$). These results indicate that postprandial ghrelin levels are altered in the BN group. The Student's *t*-test analysing these group differences indicated that BN patients had significantly higher ghrelin levels at 180 ($P < 0·001$) minutes after meal from those observed in the control group. Postprandial ghrelin suppression (decremental AUC for ghrelin) between 0 and 180 min after the meal was significantly smaller in BN subjects (−96·3 ± 26·8 pmol/l × 3 h) in comparison to controls (−178·2 ± 25·7 pmol/l × 3 h, $P < 0·05$; Table 2).

The PYY responses to a meal, illustrated in Fig. 2. Basal PYY levels were similar in the BN (14·6 ± 1·3 pmol/l) and controls (12·8 ± 1·1 pmol/l, $P = 0·30$; Table 2). Two-way ANOVA with repeated measures analysis of PYY levels were significantly different between the experimental groups ($F_{1,20} = 18·70$, $P < 0·001$) over time ($F_{4,80} = 26·90$, $P < 0·001$), demonstrating a significant group × time interaction ($F_{4,80} = 5·96$, $P < 0·001$). These results indicate that the postprandial

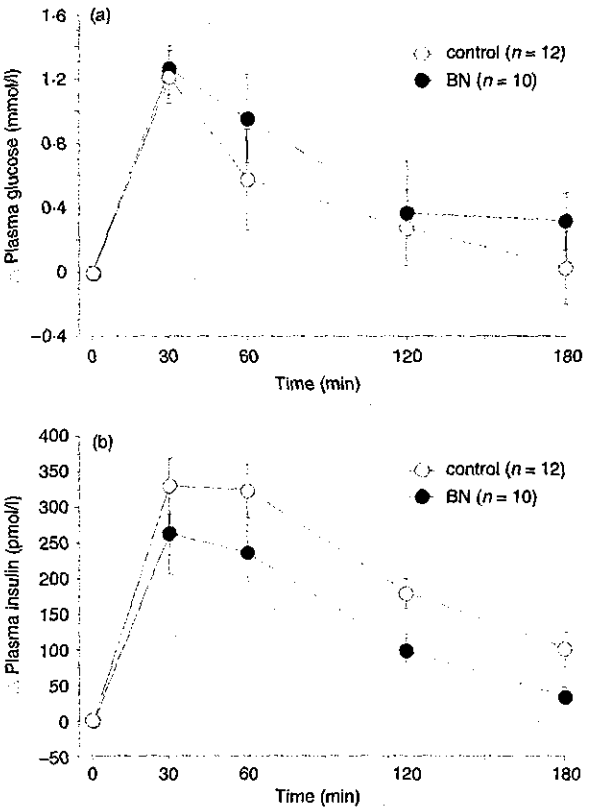

Fig. 3 (a) Comparison of plasma glucose responses between BN patients (●) and controls (○) following a meal. (b) Comparison of plasma insulin responses between BN patients (●) and controls (○) following a meal.

PYY responses in BN subjects differ from that of control subjects. Student's *t*-test analysis of these group differences indicated that BN patients had significantly lower PYY levels than controls at 30 ($P < 0·01$), 60 ($P < 0·01$), 120 ($P < 0·01$) and 180 ($P < 0·01$) min after a meal. The incremental AUC of plasma PYY in BN patients (9·2 ± 2·6 pmol/l × 3 h) was significantly lower than that observed in controls (26·8 ± 3·2 pmol/l × 3 h, $P < 0·01$; Table 2), demonstrating reduced PYY secretion in subjects with BN.

Glucose and insulin responses to a meal are shown in Fig. 3. Basal glucose levels were similar in the BN (4·6 ± 0·1 mmol/l) and control (4·8 ± 0·9 mmol/l) groups (Table 2). Two-way ANOVA with repeated measures on the glucose levels revealed a significant main effect of time on glucose levels ($F_{4,80} = 16·60$, $P < 0·01$), but no significant differences between the groups ($F_{1,20} = 0·546$, $P = 0·47$) or group × time interactions ($F_{4,80} = 0·44$, $P = 0·78$). These results indicate that glucose responses to a meal did not differ significantly between two groups. The incremental AUC of plasma glucose levels after a meal was equivalent in the two groups (1·9 ± 0·5 mmol/l × 3 h in BN subjects *vs.* 1·3 ± 0·6 mmol/l × 3 h in controls, $P = 0·50$; Table 2).

Basal insulin levels in BN subjects (23·9 ± 3·3 pmol/l) were significantly lower than in the controls (41·7 ± 5·0 pmol/l, $P < 0·001$). Two-way ANOVA with repeated measures examination of insulin levels demonstrated a significant effect for time ($F_{4,80} = 44·50$, $P < 0·001$) and for group ($F_{1,20} = 5·30$, $P < 0·05$), but no group × time interaction ($F_{4,80} = 0·81$, $P = 0·53$). These data indicate that, while the incremental

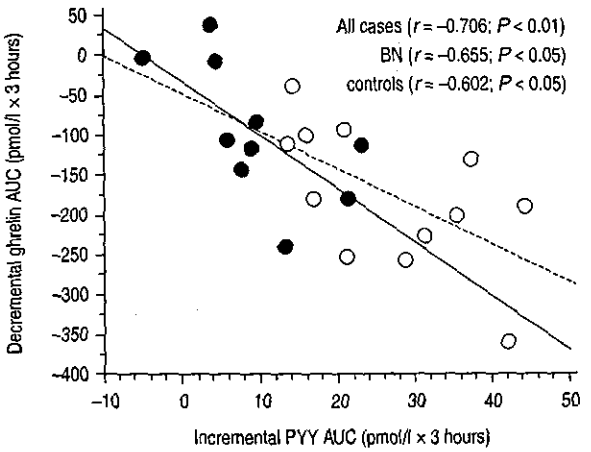

Fig. 4 Correlation between decremental AUC for ghrelin and incremental AUC for PYY in BN patients (●, continuous line) and control subjects (○, dotted line).

AUC of plasma insulin in BN patients (422·5 ± 56·8 pmol/l × 3 h) was significantly lower than that observed in controls (637·2 ± 62·1 pmol/l × 3 h, $P < 0·05$; Table 2), insulin responses to meals were parallel between the two groups.

We observed a significant inverse correlation between decremental AUC for ghrelin and incremental AUC for PYY ($r = -0·706$, $P < 0·01$; Fig. 4). We also identified a significant inverse correlation between decremental AUC for ghrelin and incremental AUC for insulin ($r = -0·450$, $P < 0·05$).

Discussion

We observed reduced postprandial ghrelin suppression and blunted postprandial PYY responses in BN patients, despite parallel postprandial glucose and insulin responses between the two groups. In addition, we also observed a significant correlation between the magnitude of ghrelin suppression and the integrated PYY response.

Similar to the reduced ghrelin suppression after eating observed in another study,[23] we have demonstrated that postprandial ghrelin suppression was inhibited significantly in BN patients. Peripheral ghrelin administration in humans enhances appetite and increases food consumption.[7] Ghrelin may also play an important role in hunger and meal initiation.[24] The fall in plasma ghrelin concentrations may represent suppression of a hunger signal. The reduced ghrelin suppression after a meal may contribute to the impaired satiety and binge eating observed in BN patients.

The mechanisms regulating the suppression of postprandial ghrelin remains unclear. Glucose and insulin have been proposed as the major effectors of ghrelin suppression.[25–29] In this study, we observed parallel postprandial plasma glucose and insulin profiles between BN patients and controls, although insulin AUC values were lower in BN patients. Russell *et al.*[30] reported that while patients with BN who binge and vomit frequently exhibited blunted insulin responses to a glucose load, these responses normalized after treatment. The present study suggests that abnormal eating behaviours may influence postprandial insulin levels; reduced insulin levels may be related to reduced ghrelin suppression in BN patients.

Elevation of ghrelin levels has been observed during weight loss resulting from caloric restriction,[24] cancer anorexia,[31] cardiac cachexia,[32] anorexia nervosa[8,10] and BN.[12] In this study, BN patients displayed significantly increased basal ghrelin levels in comparison to controls. While these results agree with our previous studies,[12] however, Monteleone *et al.*[23] reported that preprandial ghrelin levels did not differ significantly between BN patients and healthy controls. Further studies will be necessary to clarify the relationship between elevated ghrelin levels and the pathophysiology of BN.

We also observed lower postprandial PYY levels in patients with BN. To our knowledge, this is the first investigation exploring postprandial PYY in BN patients. BN patients exhibit impaired cholecystokinin (CCK) secretion.[33] CCK, a satiety factor, is a stimulant of PYY secretion.[34] Therefore, depressed PYY levels may result from reduced CCK secretion. PYY is released by the distal intestine into the circulation in response to meal ingestion, with plasma levels peaking 60–90 min after meal initiation.[35] Postprandial elevation of plasma PYY may act on the ARC NPY Y2 receptor to inhibit feeding in a gut–hypothalamic pathway. Infusions of PYY into humans reduced appetite ratings and decreased food intake.[16] Interestingly, obese subjects exhibit smaller rises in postprandial levels, suggesting that PYY deficiency may contribute to impaired postprandial satiety and the pathogenesis of obesity.[17] Several studies have demonstrated that patients with BN have disturbances in the development of satiety.[36–39] Postprandial PYY deficiencies may contribute to the impaired satiety and increased binge-eating, perpetuating the pathological condition. While it is unclear whether low PYY release is the cause or result of BN, we speculate that such a defect contributes to the pathophysiology of BN.

Little is known about the interactions between PYY and ghrelin production. Both hormones act on the ARC of the hypothalamus to regulate appetite. Konturek *et al.*[40] suggested that a negative interaction between PYY and ghrelin occurs at the levels of the vagal afferents, the nodose ganglion and the ARC of the hypothalamus. In this study, we observed a correlation between incremental AUC for PYY and decremental AUC for ghrelin, suggesting a negative interaction of PYY with ghrelin. In support of this possibility, PYY infusion significantly decreases plasma ghrelin levels.[17] Peripheral interactions between ghrelin and PYY may play an important role in the short-term regulation of food intake. Further studies will be necessary to determine the mechanism of PYY and ghrelin interaction.

In summary, we have demonstrated that reduced ghrelin suppression and blunted PYY responses after eating in bulimia nervosa patients. We speculate that a gut–hypothalamic pathway involving peripheral hormonal signals, such as ghrelin and PYY, may be related to the pathophysiology of bulimia nervosa.

Acknowledgements

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Morphological analysis of ghrelin and its receptor distribution in the rat pancreas

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Abstract

Ghrelin, a novel peptide isolated from stomach tissue of rats and humans, has been identified as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R). In addition to its secretion from the stomach, ghrelin is also expressed in the hypothalamic arcuate nucleus, intestine, kidney, placenta, and pancreas. GHS-R mRNA, on the other hand, is expressed in the hypothalamus, pituitary, heart, lung, liver, pancreas, stomach, intestine, and adipose tissue. Ghrelin is considered to have important roles in feeding regulation and energy metabolism as well as in the release of growth hormone (GH). Recent physiological experiments on the pancreas have shown that ghrelin regulates insulin secretion. However, sites of action of ghrelin in the pancreas are yet to be identified. In this study, to gain insight into the role of ghrelin in rat pancreatic islets, we used immunohistochemistry to determine the localization of ghrelin and GHS-R in islet cells. Double fluorescence immunohistochemistry revealed that weak GHS-R-like immunoreactivity was found in B cells containing insulin. GHS-R immunoreactivity overlapped that of glucagon-like immunoreactive cells. Moreover, both ghrelin and GHS-R-like immunoreactivities were detected mostly in the same cells in the periphery of the islets of Langerhans. These observations suggest that ghrelin is synthesized and secreted from A cells, and acts back on A cells in an autocrine and/or paracrine manner. In addition, ghrelin may act on B cells via GHS-R to regulate insulin secretion.

Keywords: Ghrelin; Growth hormone secretagogue receptor (GHS-R); Pancreas; A cell

1. Introduction

Growth hormone secretagogues (GHSs) belong to a group of chemical compounds that stimulate growth hormone (GH) release in vitro and in vivo [1]. The GHS receptor (GHS-R) is a member of the guanine nucleotide-binding protein (G protein) -coupled receptor superfamily with seven transmembrane domains [2,3]. This receptor promotes calcium release from the endoplasmic reticulum and transduces the GH-releasing activity [4]. GHS-R is mainly localized to the pituitary and hypothalamus in porcine, rat, and humans [5]. Literature reports demonstrate that GHS-R mRNA is expressed in the heart, lung, liver, pancreas, stomach, intestine, and adipose tissue [4,6–8].

Ghrelin, a 28-amino-acid peptide which was isolated from rat's and human's stomach tissue, acts as an endogenous ligand for GHS-R [4]. This novel peptide has an *n*-octanoylated serine residue at the third N-terminal position, whose acylation is essential for ghrelin

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bioactivity. Ghrelin mainly is produced in and secreted from A/X-like endocrine cells of the stomach of rat [9]. Apart from stomach tissue, ghrelin mRNA is expressed in the intestine [9], heart [8], lung [8], pancreas [7], kidney [10], placenta [11], pituitary, and hypothalamus [6]. Administration of ghrelin stimulates the secretion of growth hormone (GH) and increases food intake in rats [12–14]. Circulating ghrelin levels depend on the feeding and metabolic state of the body; they are increased by fasting [15–17] and are decreased by feeding [15,17,18]. It has been reported that circulating ghrelin levels are depressed in cases of obesity in humans [18,19]. These results suggest that ghrelin may play an important role in regulating feeding behavior and energy balance.

Ghrelin is produced not only in enteroendocrine cells but also in pancreatic endocrine cells. Recently, ghrelin was reported to regulate exocrine pancreatic function [20]. As significant as this finding, the sites of action of ghrelin in the pancreas are yet to be identified. In this study, therefore, fluorescence immunohistochemistry has been used in an attempt to identify the sites of action of ghrelin in the pancreas by determining the localization of GHS-R in pancreatic islet cells.

2. Materials and methods

2.1. Preparation of anti-GHS-R antiserum

[Cys0]-rat GHS-R [342–364] was synthesized by the Fmoc solid-phase method using a peptide synthesizer (433A, Applied Biosystems, Foster City, CA, USA), and then purified by reverse-phase HPLC. The synthesized GHS-R peptide (10 mg) was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH, Pierce, Rockford, IL, USA; 6 mg) in conjugation buffer (Pierce). The conjugate was emulsified with an equal volume of Freund's complete adjuvant and was used to immunize New Zealand white rabbits. Animals were boostered every 2 weeks and were bled 7 days after each injection. Specificity of this antiserum was confirmed by the detection of immunoreactivity in GHS-R-expressing cells (CHO–GHSR62 cells) or control cells (CHO cells).

2.2. Animal procedures

Male Sprague–Dawley rats were purchased from Saitama Experimental Animal Supply (Saitama, Japan). They were maintained on a 12-h light/12-h dark cycle under controlled temperature and humidity, and were allowed free access to water and standard rat chow. All protocols used in these studies were reviewed and approved by the Institutional Animal Care and Use Committee of Showa University (#03057).

2.3. Immunohistochemistry

2.3.1. Preparation of sections

Rats were deeply anaesthetized by overdose with an intraperitoneal injection of sodium pentobarbital (50 mg/kg, Dainippon Pharmaceutical) and perfused with 50 ml of saline (37 °C), followed by 250~300 ml of 2% paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 min. In each case, the pancreas was removed, trimmed, and immersed in the same fixative for 12 h at 4 °C. After washing with 0.1 M PB, the fixed pancreas was transferred for 2 days at 4 °C into 0.1 M PB solution containing 20% sucrose. The tissue was then embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and immediately frozen in liquid nitrogen-cooled isopentane and stored at −80 °C.

Immunofluorescence was performed on 6-μm-thick serial cryosections cut from frozen pancreas on a cryostat (MICROM HM 500; MICROM, Heiderberg, Germany) at −30 °C.

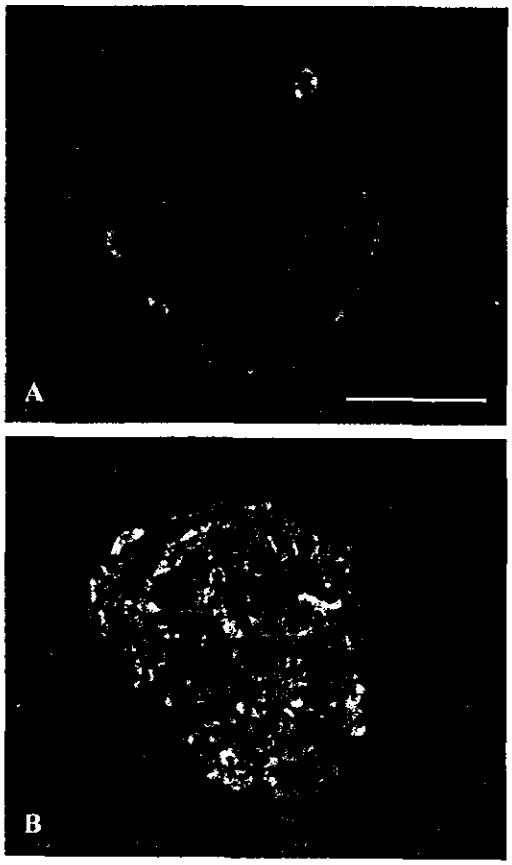

Fig. 1. Localization of growth hormone secretagogue receptor (GHS-R)-like immunoreactive cells and insulin like-immunoreactive cells in rat pancreatic islets. Immunofluorescence staining is shown for GHS-R (red, A) and insulin (green, B). Scale bar is 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3.2. Double staining fluorescence immunohistochemistry

Sections were blocked with 10% normal horse serum in PBS for 1 h. For detecting GHS-R-like immunoreactivity, the sections were incubated with rabbit anti-GHS-R antibody (1:2000) for 48 h at 4 °C and then with Alexa Fluor 546-labeled goat anti-rabbit IgG (1:400, Molecular Probes, OR, USA) for 1.5 h at room temperature. Next, in order to demonstrate simultaneously the presence of glucagon-like immunoreactivity in the same sections as those in which GHS-R-like immunoreactivity was detected, a double immunofluorescence technique was used. The sections were incubated with guinea pig anti-glucagon antibody (1:1 000 000, Linco Research, MO, USA) for 48 h at 4 °C, followed by incubation with Alexa Fluor 488-labeled goat anti-guinea pig IgG (1:400, Molecular Probes) for 1.5 h at 4 °C. The double immunolabelling was detected with the aid of a fluorescence microscope (AX-70, Olympus, Tokyo, Japan). Control experiments were carried out by preabsorption of the 1:2000 diluted anti-GHS-R antibody with a synthesized antigen at a concentration of 10 ng/ml.

2.3.3. Immunofluorescence

Sections were blocked with 10% normal horse serum in PBS for 1 h. For detection of GHS-R-like immunoreactivity, the sections were incubated with rabbit anti-GHS-R antibody (1:2000) for 48 h at 4 °C and then incubated with Alexa Fluor 546-labeled goat anti-rabbit IgG (1:400, Molecular Probes) for 1.5 h at room temperature. For the detection of ghrelin-like immunoreactivity or insulin-like immunoreactivity, sections were incubated with rabbit anti-ghrelin antibody (1:5000, Phoenix Pharmaceuticals) or guinea pig anti-insulin antibody (1:5000, Phoenix Pharmaceuticals) for 48 h at 4 °C and then incubated with Cy3-labeled donkey anti-rabbit IgG (1:400, Chemicon, OR, USA) or Alexa Fluor 488-labeled goat anti-guinea pig IgG (1:400, Molecular Probes) for 1.5 h at room temperature. Immunoreactivities were detected with the aid of a fluorescence microscope (AX-70, Olympus).

3. Results

In control experiments, immunostaining was completely abolished by absorbing the anti-GHS-R antibody with antigen. Weak GHS-R-like immunoreactivity was found in the center of the islets (Fig. 1). Cells positive for GHS-R-like immunoreactivity exhibited a characteristic distribution pattern *different from* that of insulin-like immunoreactive cells.

Both GHS-R-like immunoreactivity (Fig. 2A,D) and glucagon-like immunoreactivity (Fig. 2B,E) were present in the periphery of the islets of Langerhans where the cell population is primarily A cells. GHS-R-like immunoreactive cells exhibited a characteristic distribution pattern identical to those for the glucagon-like immunoreactive cells (Fig. 2C,F).

Fig. 3 indicates the localization of ghrelin-like immunoreactivity (Fig. 3A) or GHS-R-like immunoreactivity (Fig. 3B) in pancreatic islets. Both ghrelin- and GHS-R-like immunoreactivity were located in the mantle area of the pancreatic islets as single cells or small clusters of cells.

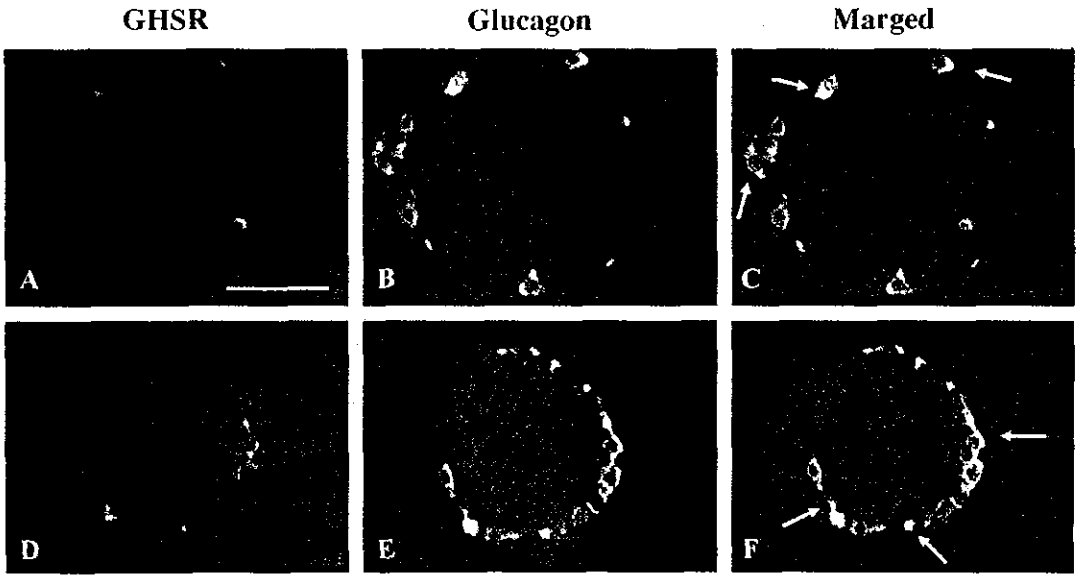

Fig. 2. Localization of growth hormone secretagogue receptor (GHS-R)-like immunoreactive cells in rat islets. Immunofluorescence staining is shown for GHS-R (red, A and D) and glucagon (green, B and E). A marged image of panels A and B is given in panel C. A marged image of panels D and E is given in panel F. Co-localization of GHS-R- and glucagon-like immunoreactivity is indicated in yellow. Arrow indicates the typical colocalization of GHS-R-like immunoreactive cells and glucagon-like immunoreactive cells. Scale bar is 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

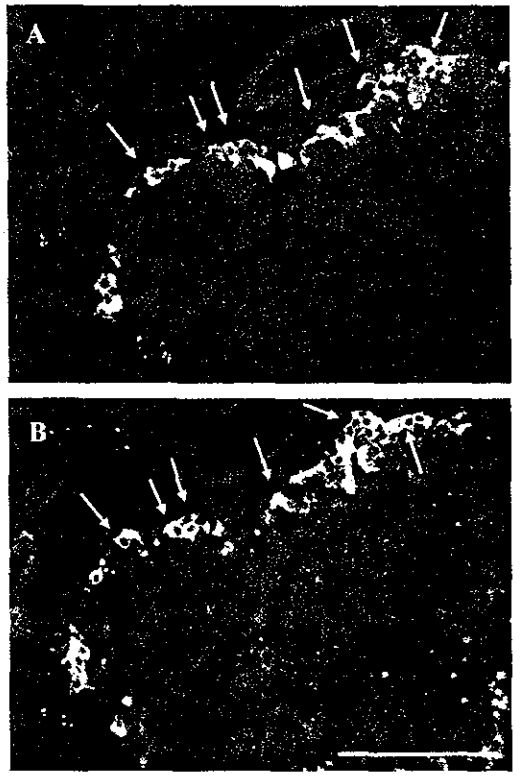

Fig. 3. Localization of growth hormone secretagogue receptor (GHS-R)-like immunoreactive cells and ghrelin-like immunoreactive cells in rat pancreatic islets. Immunofluorescence staining for GHS-R (A) and ghrelin (B). Arrow indicates the colocalization of GHS-R-like immunoreactive cells and ghrelin-like immunoreactive cells. Scale bar is 100 μm.

Furthermore, GHS-R-like immunoreactive cells exhibited a characteristic distribution pattern identical to that of ghrelin-like immunoreactive cells. Immunoreactivity to neither GHS-R nor ghrelin was detected in exocrine acini or centroacinar cells.

4. Discussion

Ghrelin is considered to bind to the growth hormone secretagogue receptor (GHS-R), and then affects GH release, gastrointestinal function, feeding behavior, and energy metabolism. The islets of Langerhans contain at least four different types of endocrine cells, referred to as insulin-secreting B cells (65–80% of the total islet cell population), glucagon-secreting A cells (10–15%), somatostatin-producing D cells (5%), and the pancreatic polypeptide-containing PP cells (10–15%). While few reports have considered the localization of GHS-R in the pancreas, an understanding of its distribution is important in order to understand the action of ghrelin in the pancreas. In the present study, we demonstrated that GHS-R-like immunoreactivity mainly colocalized with glucagon-like immunoreactivity in rat pancreatic islets and that GHS-R-like immunoreactivity was also localized in some of pancreatic B cells. In addition, GHS-R-like immunoreactive cells exhibited a similar distribution pattern identical to those for the glucagon-like immunoreactive cells.

Ghrelin facilitates the release of insulin from isolated rat B cells under high glucose conditions (at 8.3 nmol/l) [7]. An increase in the cytosolic-free Ca^{2+} concentration was observed when ghrelin was added to these cells [7]. Intravenously injected ghrelin also increased circulating insulin in rats [17]. However, ghrelin has been shown to inhibit insulin secretion in perfused pancreas of the rat [21] and mouse [22], and in humans [23,24]. These conflicting results between experiments performed in vitro and in vivo may be explained by species differences and by the use of different experimental protocols. Based on these results, the secretion of insulin could be regulated via direct and/or indirect actions of ghrelin on pancreatic islet cells, with B cells, in particular, being a target of ghrelin. We found that GHS-R was weakly expressed in islet B cells, suggesting that ghrelin might directly regulate insulin secretion in B cells. Although ghrelin is produced in B cells in humans [25], rat B cells have not as yet been identified as ghrelin-producing cells.

There are two possibilities concerning the regulation of B cells by ghrelin. First, ghrelin derived from the stomach and other tissue may be transported in the blood and bind to GHS-R on B cells (endocrine manner). Second, ghrelin derived from A cells may interact with B cells in a fashion independent of microvascular blood flow from B cells to D cells via A cells. These cells may make contact via a form of gap junctional communication between cells within the islets, given that A cells are often located in the immediate vicinity of B cells.

Messenger RNA for GHS-R, the ghrelin receptor, has been detected in the pancreas by means of RNA protection assay [5] and RT-PCR analysis [8,25]. Furthermore, in situ hybridization and immunohistochemistry studies have demonstrated that ghrelin is localized to pancreatic A cells and B cells in humans and to A cells in rats [7,25]. Immunohistochemical studies here revealed that GHS-R was mostly localized to A cells which mainly produce and secrete glucagon. Ghrelin was colocalized with GHS-R in the same cells in the periphery of the islets of Langerhans. From this, we postulate that ghrelin may be involved in the glucagon secretion by an autocrine and/or paracrine action. In a recent study, ghrelin failed to stimulate or inhibit secretion of glucagon from the isolated normal pancreas [26], suggesting that neither ghrelin nor GHS-R is involved in glucagon secretion in vitro. Thus, although the mechanism of action of ghrelin via GHS-R in A cells remains unclear, expression of ghrelin and GHS-R in A cells would suggest that ghrelin is involved in the regulation of pancreatic A cells. Further study of the action of ghrelin in A cells is required.

In conclusion, we have provided evidence here that the GHS-R is located not only on A cells but also on B cells in rat pancreatic islets. These observations suggest that ghrelin

may exert its action on A cells in an autocrine manner. This system may be influenced by a feedback mechanism that controls the actions of ghrelin. In addition, ghrelin may directly regulate the secretion of insulin from B cells via its interaction with GHS-R.

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