Enhanced plasma ghrelin levels in patients with functional dyspepsia

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SUMMARY

Background
Ghrelin, growth-hormone-releasing peptide, has been reported to accelerate food intake and gastrointestinal motility.

Aim
The present study was designed to investigate the plasma ghrelin levels in patients with functional dyspepsia (FD).

Patients and Methods
Ninety-seven patients, who showed no evidence of peptic ulcer disease or gastrointestinal cancer on upper gastrointestinal endoscopy, were recruited. Seventeen patients who had no gastrointestinal symptoms were recruited as controls. Forty-seven patients were diagnosed to be suffering from FD, based on the Rome II criteria. The FD patients were further subdivided into those with ulcer-like FD, dysmotility-like FD and non-specific-type FD, based on their Gastrointestinal Symptom Rating Scale (GSRS) scores. Fourteen patients were categorized as having gastro-oesophageal reflux disease, and 19 patients were excluded as having the irritable bowel syndrome, based on the GSRS. The plasma ghrelin levels were measured by radioimmunoassay.

Results
The plasma ghrelin levels were significantly higher in FD patients, especially in those with dysmotility-like FD, as compared with those in controls. The plasma ghrelin levels were also correlated well with the indigestion scores.

Conclusion
Plasma ghrelin levels are significantly higher in patients with dysmotility-like FD, suggesting that this parameter could become useful as a novel supportive marker for the diagnosis of FD.

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INTRODUCTION

Ghrelin, a recently discovered growth-hormone-releasing peptide, was isolated from the rat stomach and has been demonstrated to be localized in the endocrine cells of the stomach and the hypothalamic arcuate nucleus.[1] Regulation of gastric ghrelin secretion is still poorly understood. The plasma concentrations of ghrelin have been shown to increase before meals and decrease postprandially,[2] showing a distinct meal-related diurnal profile. Ghrelin has been reported to play a role in the control of food intake and in energy homeostasis[3] and also to stimulate gastric acid secretion and gastrointestinal motility.[4] The major proportion of circulating ghrelin is produced in the A-like cells of the gastric mucosa.[5]

Functional dyspepsia (FD) refers to a broad range of chronic upper abdominal symptoms associated with food intake (pain or discomfort localized to the upper abdomen, early satiety, fullness, bloated sensation in the upper abdomen, nausea, etc.)[6] and is a frequently encountered condition in clinical practice. It has been reported to occur at a prevalence rate of about 15% in adults, and it has been estimated that about 25% of these patients are receiving some or the other form of treatment.[7] Recently, a multinational consensus document on functional gastrointestinal disorders[8] recommended the following definition for FD (Rome II): 'the following symptoms or conditions occurred for at least 12 weeks, which need not be consecutive, within the preceding 12 months of (1) persistent or recurrent dyspepsia (pain or discomfort localized to the upper abdomen), (2) no evidence of organic disease (including by upper gastrointestinal endoscopic evaluation) that can potentially explain the symptoms and (3) no evidence that the dyspepsia is exclusively relieved by defecation or is associated with a change of stool frequency or stool form.' The wide range of symptoms encountered in patients with dyspepsia has led to the classification of this condition into four types based on the major symptom pattern, namely reflux-like, ulcer-like, dysmotility-like or non-specific-type FD.[9] The Rome II criteria recommended that the reflux-like subgroup be excluded from the classification of FD, as these patients may be categorized as having symptomatic gastro-oesophageal reflux disease (GERD).[8]

The Gastrointestinal Symptom Rating Scale (GSRS) is a specific 15-item questionnaire for patients with gastrointestinal symptoms.[10] The patients are asked to numerically score their subjective symptoms on a scale of 1–7. The sum of the scores for all the 15 items is regarded as the total GSRS score. Furthermore, the scores for five symptom categories, namely the reflux score, abdominal pain score, indigestion score, diarrhoea score and constipation score, are obtained by calculating the mean of the scores in the 15 items for each of the symptom patterns. As each of the 15 questions can be scored from 1 to 7, the minimum score obtainable is 15 and the maximum score obtainable is 105. This is then divided by 15 to obtain the minimum overall GSRS score of 1 and maximum score of 7. The higher the overall score, the more severe the symptoms.

However, no clinical data analyses have been conducted to determine the relationship between the plasma ghrelin levels and the presence of FD. The present study was designed to investigate the fasting plasma levels of ghrelin in patients with dyspeptic symptoms.

PATIENTS AND METHODS

Ninety-seven patients in whom upper gastrointestinal endoscopy did not reveal any evidence of peptic ulcer or gastrointestinal cancer were recruited as the subjects for the present study (62 men, 35 women; age 20–69 years, mean age ± S.E.M. 53.2 ± 9.4; BMI 17.5–26.5, mean BMI ± S.E.M. 22.6 ± 2.3). The GSRS questionnaire was administered to the patients. Of the total, 17 patients who had no gastrointestinal symptoms were categorized as the control group (12 men and 5 women; mean age ± S.E.M. 57.5 ± 12.1 years; mean BMI ± S.E.M. 21.5 ± 2.1. Forty-seven patients were diagnosed to have FD based on the Rome II criteria (33 men and 14 women; mean age ± S.E.M. 51.4 ± 7.7 years; mean BMI ± S.E.M., 22.7 ± 2.1). The FD patients were divided into those with ulcer-like FD, dysmotility-like FD and non-specific-type FD, based on their GSRS scores.[11] The number of patients with ulcer-like FD, dysmotility-like FD and non-specific-type FD were 12, 16 and 19, respectively. Nineteen patients with a constipation or diarrhoea score of more than 3 were excluded as being cases of the irritable bowel syndrome, and 14 patients in whom the reflux score was the highest were categorized as having GERD (Table 1). Peripheral venous blood specimens were collected from the patients after 12 h of fasting, just before upper gastrointestinal endoscopy.

Table 1. The BMI and plasma ghrelin level (fmol/mL) in control patients, patients with FD (dysmotility-like FD, ulcer-like FD and non-specific-type FD), GERD and IBS

	Control	FD	Dysmotility-like	Ulcer-like	Non-specific-type	sGERD	IBS
Number	17	47	16	12	19	14	19
Age	57.5 ± 12.2	51.4 ± 7.7	53.5 ± 8.3	51.3 ± 6.9	49.7 ± 7.2	52.0 ± 10.6	54.9 ± 8.0
Male:female	12:5	33:14	10:6	7:5	16:3	7:7	10:9
BMI	21.5 ± 2.1	22.7 ± 2.1	22.5 ± 2.5	21.9 ± 2.0	23.3 ± 1.3	22.7 ± 1.5	23.6 ± 2.8
Total ghrelin	147.2 ± 30.7	199.0 ± 102.6*	225.1 ± 126.7*	206.5 ± 107.6	172.3 ± 62.1	176.0 ± 64.1	186.1 ± 98.3
Active ghrelin	9.4 ± 3.7	13.4 ± 5.7**	14.9 ± 6.3**	13.7 ± 4.8*	11.9 ± 5.2	9.9 ± 3.1	12.6 ± 8.1

BMI, body mass index; FD, functional dyspepsia; sGERD, symptomatic gastro-oesophageal reflux disease; IBS, irritable bowel syndrome.
*$P < 0.05$ as compared with the value in the control group.
**$P < 0.01$ as compared with the value in the control group.

Ghrelin measurement

Two radioimmunoassays (RIAs) were used to measure the plasma ghrelin levels, as described previously.[12–14] Two polyclonal rabbit antibodies were raised against the N-terminal [1–11] (Gly1-Lys11) and C-terminal [13–28] (Gln13-Arg28) fragments of rat ghrelin.[11] The ghrelin values obtained by N-terminal RIA using anti-ghrelin [1–11] antiserum were considered to represent the values of active, *n*-octanoylated form of ghrelin. The values obtained using C-terminal RIA using anti-ghrelin [13–28] antiserum were considered to represent the total ghrelin concentration, including that of the inactive, des-acyl form of ghrelin. Both antisera were equally cross-reactive with human ghrelin and did not recognize other peptides. The respective intra- and inter-assay coefficients of variation for the N-terminal RIA were 3% and 6%, and those for the C-terminal RIA were 6% and 9%, respectively. The blood samples were drawn into chilled tubes containing EDTA·2Na (1 mg/mL blood) and aprotinin (500 U/mL blood). Each separated plasma sample was treated with a C18 Sep-Pak cartridge (Waters, Milford, MA, USA), and the cartridge was washed and used for the assay.

Endoscopic evaluation of gastric mucosal atrophy

Endoscopic studies have reported that the area of atrophy in patients with chronic atrophic gastritis extends from the antrum to the corpus. Previously, Kimura and Takemoto used endoscopy to divide gastric mucosal atrophy into six stages, based on endoscopic evaluation (C1, C2, C3, O1, O2 and O3).[15] It has been clarified that mucosal atrophy progresses sequentially from C1 to O3, and that the above classification correlates well with the histological stage of gastric atrophy. A recent study also demonstrated that this endoscopic classification was consistent with the Sydney system of classification of gastric atrophy.[16, 17] Therefore, in this study, we assessed the degree of mucosal atrophy according to the endoscopic classification system. We defined C1–C3 as closed-type gastric mucosal atrophy, and O1–O3 as open-type gastric mucosal atrophy. Two endoscopists who were blinded to any information about the patients evaluated the endoscopic findings. Patients with the open-type gastric mucosal atrophy have been reported to secrete less gastric acid than those with the closed-type gastric mucosal atrophy.[18]

Statistical analyses

Groups of data (mean ± S.E.M.) were compared using ANOVA and Sheffe's test. Correction coefficients were calculated by linear regression analysis. $P < 0.05$ was considered to be statistically significant.

RESULTS

Plasma ghrelin levels in functional dyspepsia patients

The plasma levels of total and active ghrelin were significantly higher in FD patients than in the control

group of subjects (total: FD, 199.0 ± 102.6; control, 147.2 ± 30.7; $P < 0.05$). When stratified according to the type of dyspepsia, the plasma levels of total and active ghrelin were significantly higher in the dysmotility-like FD group than in the control group (total: dysmotility-like FD, 225.1 ± 126.7; control, 147.2 ± 30.7; $P < 0.05$). In the ulcer-like FD group, the plasma levels of active ghrelin were significantly higher than those in the control group, and the plasma levels of total ghrelin tended to be higher than those in the control group. There were no significant differences in the plasma ghrelin levels between the control group and the non-specific-type FD group (Figure 1).

Correlations between the plasma ghrelin levels and the GSRS scores

Figure 2 illustrates the relationship between the indigestion scores and the plasma ghrelin levels. There was a significant linear correlation between the indigestion scores and the plasma levels of active ghrelin ($r = 0.34$, $P < 0.001$; Figure 2a). There was also a significant linear correlation between the indigestion scores and the plasma levels of total ghrelin ($r = 0.20$, $P < 0.05$; Figure 2b).

Figure 3 illustrates the relationship between the abdominal pain scores and the plasma ghrelin levels. Although there was no significant correlation between the abdominal pain scores and the plasma levels of total ghrelin, there was a significant linear correlation between the abdominal pain scores and the plasma levels of active ghrelin ($r = 0.27$, $P < 0.01$, Figure 3a,b).

Plasma ghrelin levels in patients with gastric mucosal atrophy

Among the total of 97 patients, 72 were endoscopically diagnosed as having the closed-type gastric mucosal atrophy and the remaining 25 were diagnosed as having the open-type atrophy. Among the 72 patients with the closed-type atrophy, 52 patients had symptoms of indigestion and the remaining 20 had no symptoms of indigestion. Among the 25 patients with the open-type atrophy, 18 patients had symptoms of indigestion and the remaining seven had no symptoms of indigestion. The plasma levels of total ghrelin were significantly higher in the patients with symptoms of indigestion than in those without symptoms of indigestion, but only in the closed-type atrophy group (Figure 4a). In relation to the plasma levels of active ghrelin also, patients with symptoms of indigestion showed significantly higher levels than those without symptoms of indigestion, again only in the closed-type

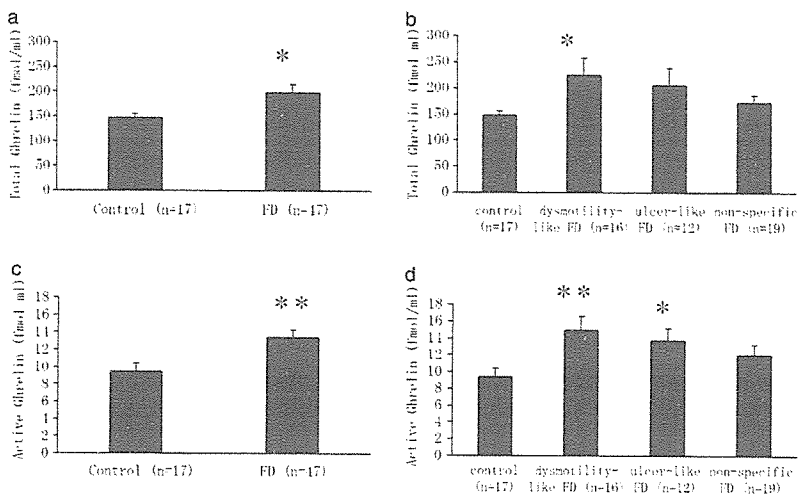

Figure 1. (a) Plasma levels of total ghrelin in the control group ($n = 17$) and functional dyspepsia (FD) patients ($n = 47$). (b) Plasma levels of total ghrelin in the control group ($n = 17$), dysmotility-like FD ($n = 16$), ulcer-like FD ($n = 12$) and non-specific-type FD patients ($n = 19$). (c) Plasma levels of active ghrelin in the control group and FD patients. (d) Plasma levels of active ghrelin in the control group, dysmotility-like FD, ulcer-like FD and non-specific-type FD patients. $^*P < 0.05$ as compared with the value in the control group. $^{**}P < 0.01$ as compared with the value in the control group.

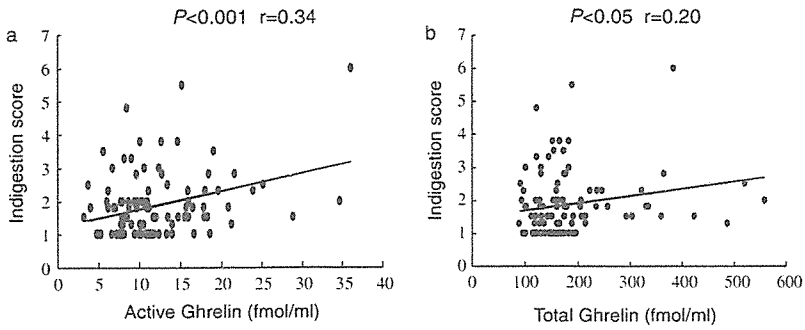

Figure 2. Correlation between the plasma levels of ghrelin and the indigestion scores. (a) Relationship between the plasma levels of active ghrelin (fmol/mL) and the indigestion scores. (b) Relationship between the plasma levels of total ghrelin (fmol/mL) and the indigestion scores.

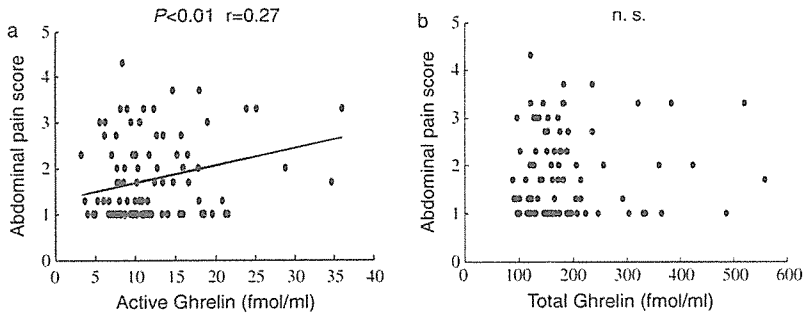

Figure 3. Correlation between the plasma levels of ghrelin and the abdominal pain scores. (a) Relationship between the plasma levels of active ghrelin (fmol/mL) and the abdominal pain scores. (b) Relationship between the plasma levels of total ghrelin (fmol/mL) and the abdominal pain scores.

atrophy group (Figure 4b). There were no significant differences in the plasma levels of total or active ghrelin between patients with and without the symptoms of abdominal pain, reflux, constipation and diarrhoea.

DISCUSSION

The present study has shown for the first time that the plasma levels of ghrelin may be significantly higher in FD patients, especially in those with dysmotility-like FD, than in control subjects without the symptoms referable to the gastrointestinal tract. The plasma levels of ghrelin were also found to be well correlated with the indigestion symptom scores.

Recent studies in animals have shown that ghrelin stimulates upper gastrointestinal motility through the vagus and enteric nervous system,[19] and ghrelin has been shown to accelerate gastric emptying.[20] Delayed gastric emptying has been reported in 30% and 70% of patients with FD,[21] and impaired accommodation, which is due to one of the disturbed functions of gastrointestinal tract, has been shown to be a frequent finding in patients with FD.[21] As a possible explanation for the high level of ghrelin in FD patients, it has been suggested that compensatory secretion of ghrelin is enhanced in FD patients in order to normalize the impaired gastrointestinal motility, and then plasma ghrelin level is increased. As the secretion of ghrelin is lasting continuously in the patients who have the long-lasting and repeated clinical dysmotility-like symptoms, plasma levels of ghrelin would persistently elevate. In addition, there is the possibility that ghrelin secretion is regulated by the autonomic nervous system, especially through the cholinergic projections to the stomach,[22] and patients with FD have been reported to have disturbances of autonomic nervous functions;[23] thus disturbed autonomic functions may be related to the abnormal ghrelin release in FD patients.

Although the plasma levels of ghrelin were significantly higher in patients with symptoms of indigestion than in those without symptoms of indigestion among patients with the closed-type gastric mucosal atrophy, no such differences were seen among patients with the open-type gastric mucosal atrophy. We recently reported that the plasma levels of ghrelin were significantly lower in patients with endoscopically diagnosed open-type atrophy than in those with similarly diagnosed closed-type atrophy,[24] suggesting that the compensatory increase of ghrelin may be attenuated when the mucosal atrophy becomes marked.

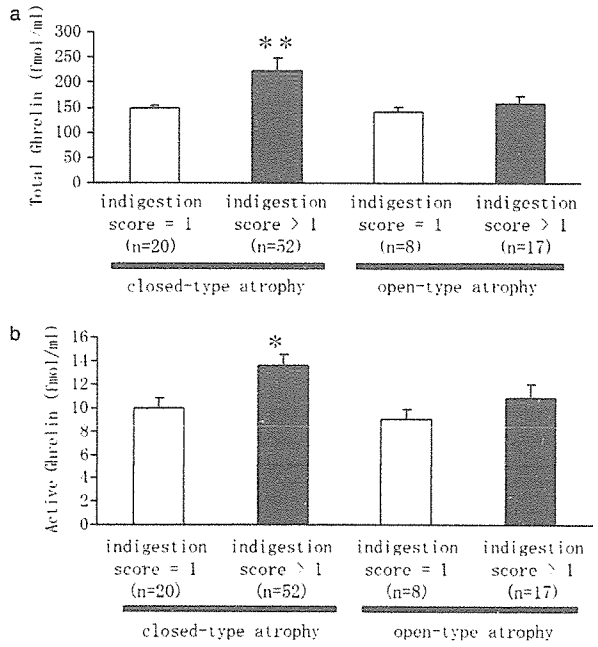

Figure 4. (a) Plasma levels of total ghrelin in patients with and without the symptoms of indigestion among patients with the closed-type atrophy and open-type gastric mucosal atrophy. (b) Plasma levels of active ghrelin in patients with and without the symptoms of indigestion among patients with the closed-type atrophy and open-type gastric mucosal atrophy. The indigestion score of the patients without the symptoms of indigestion is 1. The score of the patients with the symptoms of indigestion is more than 1.

The levels of active ghrelin (*n*-octanoylated form of ghrelin) and total ghrelin (inactive, des-acyl form of ghrelin and active ghrelin) were measured in this study. The *n*-octanoyl modification is essential for the biological function of this hormone such as in growth hormone secretion, as the octanoylation of Ser3 is necessary to bind to growth hormone secretagogue receptor.[25] However, this octanoylated active-form ghrelin is unstable in blood and easily changes to the des-acyl form. This des-acyl form does not possess the hormonal activities such as in growth hormone secretion.[26] Plasma concentration of active ghrelin changes more dynamically than those of total ghrelin,[27] and the similar tendency was observed in this study. However, as the ester bond is chemically unstable, elimination of the octanoyl modification of ghrelin would occur during storage and handling. On the contrary, des-acyl ghrelin is relatively stable. It is suggested that des-acyl ghrelin concentrations may serve as indicator of both ghrelin production and secretion.[27]

In conclusion, plasma ghrelin levels are significantly higher in patients with FD, especially in those with dysmotility-like FD. The results of the present study suggest that the plasma level of ghrelin may come to be used as a novel marker for the diagnosis of FD, particularly dysmotility-like FD. Based on the results of the present study, large-scale clinical trials for examining plasma ghrelin level in FD patients should be conducted.

ACKNOWLEDGEMENTS

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Original Article

Blockade of Pancreatic Islet–Derived Ghrelin Enhances Insulin Secretion to Prevent High-Fat Diet–Induced Glucose Intolerance

Katsuya Dezaki,[1] Hideyuki Sone,[1] Masaru Koizumi,[1,2] Masanori Nakata,[1] Masafumi Kakei,[3] Hideo Nagai,[2] Hiroshi Hosoda,[4] Kenji Kangawa,[4] and Toshihiko Yada[1]

The gastric hormone ghrelin and its receptor, growth hormone secretagogue receptor (GHSR), are expressed in pancreas. Here, we report that ghrelin is released from pancreatic islets to regulate glucose-induced insulin release. Plasma concentrations of ghrelin, as well as insulin, were higher in pancreatic veins than in arteries. GHSR antagonist and immunoneutralization of endogenous ghrelin enhanced glucose-induced insulin release from perfused pancreas, whereas exogenous ghrelin suppressed it. GHSR antagonist increased plasma insulin levels in gastrectomized and normal rats to a similar extent. Ghrelin knockout mice displayed enhanced glucose-induced insulin release from isolated islets, whereas islet density, size, insulin content, and insulin mRNA levels were unaltered. Glucose tolerance tests (GTTs) in ghrelin knockout mice showed increased insulin and decreased glucose responses. Treatment with high-fat diet produced glucose intolerance in GTTs in wild-type mice. In ghrelin knockout mice, the high-fat diet–induced glucose intolerance was largely prevented, whereas insulin responses to GTTs were markedly enhanced. These findings demonstrate that ghrelin originating from pancreatic islets is a physiological regulator of glucose-induced insulin release. Antagonism of the ghrelin function can enhance insulin release to meet increased demand for insulin in high-fat diet–induced obesity and thereby normalize glycemic control, which may provide a potential therapeutic application to counteract the progression of type 2 diabetes. *Diabetes* 55:3486–3493, 2006

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ELISA, enzyme-linked immunosorbent assay; GHRP, growth hormone releasing peptide; GHSR, growth hormone secretagogue receptor; GTT, glucose tolerance test; HKRB, HEPES-added Krebs-Ringer bicarbonate buffer; ITT, insulin tolerance test.

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Ghrelin, an acylated 28–amino acid peptide, was isolated from the stomach as the endogenous ligand (1) for the growth hormone secretagogue receptor (GHSR) (2). Circulating ghrelin is produced predominantly in the stomach (3). Ghrelin potently stimulates growth hormone release and feeding and exhibits positive cardiovascular effects, suggesting a possible clinical application of ghrelin (4). Ghrelin inhibits insulin release in mice, rats, and humans (5–8). Low plasma ghrelin levels are associated with elevated fasting insulin levels and insulin resistance (9,10). These findings suggest both physiological and pathophysiological roles for ghrelin in insulin release.

Although the nutritional, endocrine, and neural regulation of insulin release has been well characterized, much less is known about its autoregulation within islets. Ghrelin and GHSR are also located in pancreatic islets (8,11–15). We previously reported that in isolated islets, GHSR blockade and antiserum against acylated ghrelin markedly enhanced glucose-induced increases in insulin release and cytosolic Ca^{2+} concentration in islets (8). Although exogenous ghrelin suppressed insulin release, this effect required a concentration of 10 nmol/l, which is higher than the circulating ghrelin levels (16,17). These findings suggest that ghrelin at relatively high concentrations achieved within islets, rather than the circulating ghrelin, may regulate insulin secretion. The current study examined whether ghrelin originating from pancreatic islets could regulate insulin release and whether manipulation of ghrelin could affect glucose intolerance associated with obesity. We show here that ghrelin is released from pancreatic islets to downregulate glucose-induced insulin release and that ghrelin knockout mice escape high-fat diet–induced glucose intolerance because of enhanced insulin release.

RESEARCH DESIGN AND METHODS

Male Wistar rats (Japan SLC, Hamamatsu, Japan), ghrelin knockout mice, and wild-type C57BL/6J mice (Charles River Laboratories Japan, Yokohama, Japan) were housed in accordance with our institutional guidelines and with the Japanese Physiological Society's guidelines for animal care. Ghrelin knockout mice were the kind gift of Drs. T. Sato and M. Kojima (Kurume University). In these mice, the whole ghrelin gene sequence has been deleted. Animals were backcrossed with the C57B6/J strain for at least six generations. Proper deletion of the ghrelin gene was confirmed by Southern and Northern blot analysis. Total gastrectomy in 6-week-old male Wistar rats was carried out by resecting the stomach, followed by anastomosis of the cut edge of the

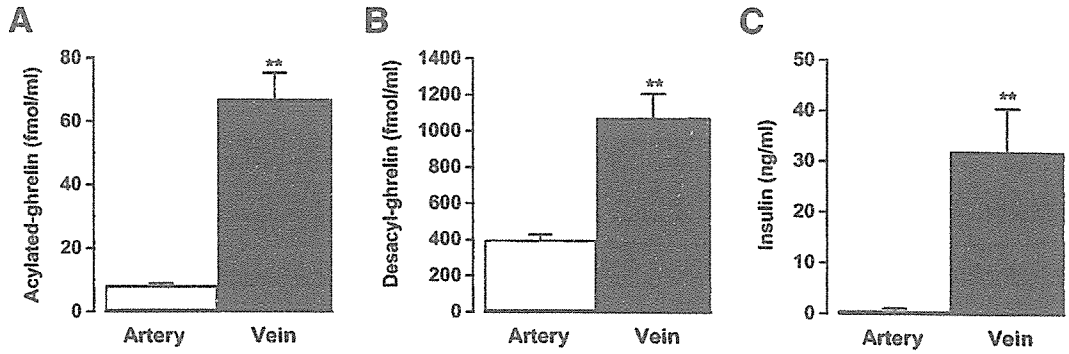

FIG. 1. Acylated ghrelin (*A*) and desacyl-ghrelin (*B*), as well as insulin (*C*), were present at higher concentrations in the pancreatic vein (splenic vein) than in the pancreatic artery (celiac artery) in rats ($n = 8$). **$P < 0.01$ vs. artery.

esophagus and the upper jejunum 4 cm distal to the Treitz ligament. At 2 months after surgery, gastrectomized rats were used for experiments.

Measurements of plasma insulin and ghrelin concentrations. Ghrelin (Peptide Institute, Osaka, Japan) and [D-Lys^3]-growth hormone releasing peptide-6 ([D-Lys^3]GHRP-6; Sigma-Aldrich, St. Louis, MO) were administered intraperitoneally to male Wistar rats (8 weeks old) or gastrectomized rats (3 months old) after overnight fasting. Blood was obtained from tails, and plasma insulin concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Yokohama, Japan). To measure plasma ghrelin concentrations, blood samples were collected from the pancreatic arteries (celiac artery) and veins (splenic vein) and portal veins of anesthetized rats or mice. To avoid inflow of ghrelin from intestine and stomach to the splenic vein, the inferior mesenteric vein and spleen side of the splenic vein—including the short gastric and left gastroomental veins—were ligated. Plasma concentrations of acylated ghrelin and desacyl-ghrelin were measured using ELISA kits (Mitsubishi Kagaku Iatron, Tokyo, Japan).

Morphological analysis of pancreatic islets in wild-type and ghrelin knockout mice. Pancreata from male wild-type and ghrelin knockout mice were fixed in 4% paraformaldehyde, and two to three random sections were generated per mouse pancreas. Three mice were studied in each genotype. The sections were incubated overnight with mouse monoclonal anti-insulin antibodies (Sigma-Aldrich) at dilutions of 1:1,000 at 4°C. Samples were then incubated in Alexa Fluor 488–labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR). Immunofluorescence for insulin was observed with photomultipliers of a multiphoton laser-scanning microscope (FluoView FV300-TP; Olympus, Tokyo, Japan). Islet number per unit area of pancreas and islet size were measured.

Measurements of insulin release in islets. Islets of Langerhans were isolated by collagenase digestion from male ghrelin knockout and wild-type (C57BL/6J) mice as previously reported (8,18), with slight modifications. Animals were anesthetized by intraperitoneal injection of pentobarbitone at 80 mg/kg, and collagenase at 1.05 mg/ml (Sigma-Aldrich) was injected into the common bile duct. Collagenase was dissolved in 5 mmol/l Ca^{2+}-containing HEPES-added Krebs-Ringer bicarbonate buffer (HKRB) solution (in mmol/l: 129 NaCl, 5.0 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 2.0 $CaCl_2$, 1.2 $MgSO_4$, and 10 HEPES, pH 7.4, with 0.1% BSA). Pancreata were dissected and incubated at 37°C for 16 min. Islets were collected and used for insulin release experiments. Groups of 12–15 islets were incubated for 1 h in HKRB at 37°C with 2.8 mmol/l glucose for stabilization, followed by test incubation for 1 h in HKRB with 2.8, 8.3, or 16.7 mmol/l glucose. Insulin concentrations were determined by an ELISA kit (Morinaga Institute of Biological Science).

Real-time RT-PCR analysis. Total RNA of islets was isolated using TRIzol (Invitrogen, Carlsbad, CA) and treated with RQ1-DNase (Promega, Madison, WI) to remove residual contaminations with DNA. First-strand cDNA synthesis was completed using ReverTra Ace (Toyobo, Osaka, Japan). Primers for real-time PCR were first examined by HotStarTaq DNA polymerase (94°C for 15 s, 55°C for 20 s, and 72°C for 20 s × 30 cycles; Qiagen, Hilden, Germany) and agarose gel electrophoresis for correct product size and absence of primer-dimer formation. Using a QuantiTect SYBR Green PCR kit, real-time PCRs (95°C for 15 s, 55°C for 20 s, and 72°C for 20 s × 35 cycles) were performed in an ABI-Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). Product accumulation was measured in real time, and the mean cycle threshold (Ct; the cycle during which product is first detected) was determined for replicate samples ($n = 5$ independent reactions per primer pair and cDNA sample) run on the same plate. Different cDNA samples were normalized using primer sets to the housekeeping gene β-actin. Primers were as follows: β-actin, 5′-TTCCCCTCCATCGTGGGCCGC-3′ and 5′-GATGG CTACGTACATGGCTGG-3′; Insulin 1, 5′TAGTGACCAGCTATAATCAGAG-3′ and 5′-ACGCCAAGGTCTGAAGGTCC-3′; and Insulin 2, 5′-CCCTGCTGGCCC TGCTCTT-3′ and 5′-AGGTCTGAAGGTCACCTGCT-3′.

In vitro perfusion of the pancreas. Pancreata were perfused using a modification of the method of Grodsky and Fanska (19). Pancreata were isolated with segments of the duodenum and spleen. An arterial cannula was introduced into the celiac artery, and a venous cannula was inserted into the portal vein. The baseline perfusate consisted of HKRB (pH 7.4) containing 2.8 mmol/l glucose, 0.5% BSA, and 4% Dextran T70. The perfusate, maintained at 37°C, was continually oxygenated in an atmosphere of 95% O_2/5% CO_2. After a 20-min preincubation period, each pancreas was perfused for 10 min with the baseline perfusate. Pancreata were then perfused for 30 min with 8.3 mmol/l glucose or 8.3 mmol/l glucose with test reagents. The flow rate was maintained at 2.5 ml/min throughout measurements. Fractions, collected in chilled tubes at 1-min intervals, were stored at −20°C until assayed for immunoreactive insulin.

Glucose tolerance tests and insulin tolerance tests. In glucose tolerance test (GTT) studies, 2 g/kg glucose was injected intraperitoneally into mice, followed by blood sampling from the tail vein. In insulin tolerance test (ITT) studies, insulin (0.75 units/kg) was injected intraperitoneally, followed by collection of blood samples from the tail vein. Blood glucose concentrations were measured using a GlucoCard DIA meter (Arkray, Kyoto, Japan), while insulin concentrations were determined using an ELISA kit (Morinaga Institute of Biological Science).

Statistical analysis. Data are the means ± SE. Statistical analyses were performed using Student's *t* test or one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Release of ghrelin from pancreas. Ghrelin is expressed in the pancreatic islets (8,11–15). Release of ghrelin from pancreatic islets was assessed by comparing the ghrelin level in the pancreatic vein (splenic vein) with that in the pancreatic artery (celiac artery) in anesthetized rats. The concentrations of both acylated ghrelin and desacyl-ghrelin in the pancreatic vein were significantly higher (about eight times and three times, respectively) than those in the pancreatic artery in rats (Fig. 1*A* and *B*). The concentration of insulin was significantly higher in the pancreatic vein than in the pancreatic artery (Fig. 1*C*).

Endogenous ghrelin inhibits insulin release in perfused pancreas. Our previous in vitro study showed that GHSR antagonists and anti-ghrelin antiserum enhanced glucose-induced insulin release in isolated rat islets (8), suggesting an insulinostatic action of islet-originated ghrelin. To establish a physiological function of endogenous ghrelin in islets, we studied insulin release using perfused rat pancreas, which retains well intact islet circulation. A rise in the perfusate glucose concentration from 2.8 to 8.3 mmol/l evoked insulin release in a biphasic manner (Fig. 2*A*). The first and second phases of glucose-induced

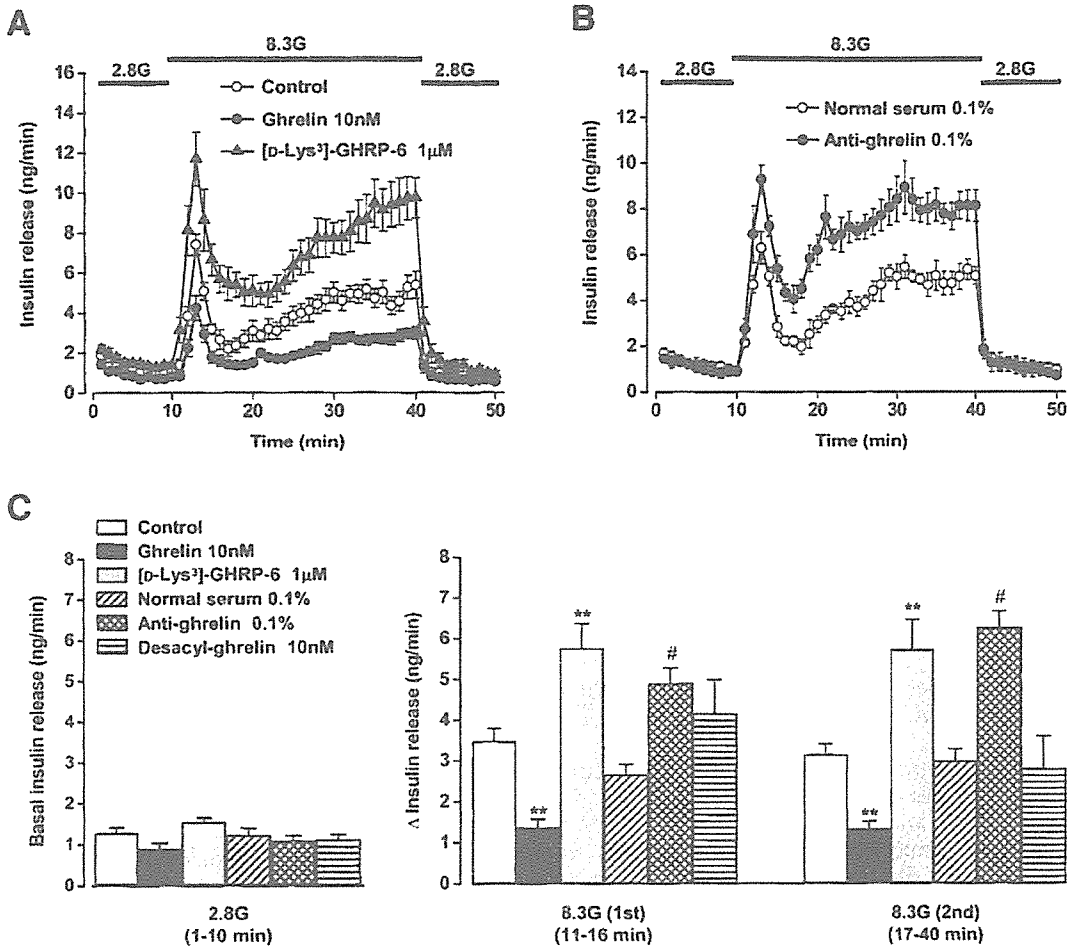

FIG. 2. Insulinostatic effects of endogenous ghrelin in perfused pancreas. *A*: Blockade of GHSR by [D-Lys^3]GHRP-6 (1 μmol/l) enhanced glucose (8.3 mmol/l)-induced insulin release in perfused rat pancreas, whereas exogenous ghrelin (10 nmol/l) administration inhibited it (n = 6–9). *B*: Immunoneutralization of endogenous ghrelin using an anti-ghrelin antiserum (0.1%) enhanced glucose (8.3 mmol/l)-induced insulin release in perfused rat pancreas (n = 3–4). *C*: [D-Lys^3]GHRP-6 (1 μmol/l) and anti-ghrelin antiserum (0.1%) increased, whereas exogenous ghrelin (10 nmol/l) inhibited, both the first and second phases of insulin release. Control nonimmune serum (0.1%) had no effect on insulin release. Desacyl-ghrelin (10 nmol/l), an inactive form of ghrelin incapable of activating GHSR, did not alter insulin release (n = 3–9). None of these treatments affected basal levels of insulin release at 2.8 mmol/l glucose. *P < 0.05; **P < 0.01 vs. control; #P < 0.05 vs. nonimmune normal serum (0.1%). G, glucose.

insulin release were significantly enhanced both by blockade of GHSR with the GHSR antagonist [D-Lys^3]GHRP-6 (1 μmol/l) (Fig. 2*A* and *C*) and by immunoneutralization of endogenous ghrelin with anti-ghrelin antiserum (0.1%) (Fig. 2*B* and *C*). Conversely, administration of exogenous ghrelin (10 nmol/l) suppressed both phases of glucose-induced insulin release (Fig. 2*A* and *C*). Desacyl-ghrelin, which cannot activate GHSR (1,20), did not significantly alter glucose-induced insulin release (Fig. 2*C*). None of these treatments affected basal levels of insulin release at 2.8 mmol/l glucose.

Endogenous ghrelin downregulates plasma insulin concentrations in both normal and gastrectomized rats. Our findings that glucose-induced insulin release from perfused pancreas was enhanced by ghrelin immunoneutralization and GHSR antagonist suggest that ghrelin originating from pancreatic islets suppresses insulin release. GHSR antagonists also increase systemic insulin responses to GTTs (8), which could be attributable to blockade of ghrelin originated from stomach and/or from other tissues, including islets. To examine the contribution of ghrelin from the stomach and other sources, we produced gastrectomized rats lacking stomach-derived ghrelin and examined the effect of intraperitoneal administration of the GHSR antagonist [D-Lys^3]GHRP-6 (10 μmol/kg) on plasma insulin concentrations in gastrectomized and normal rats fasted overnight. In gastrectomized rats, plasma concentrations of acylated ghrelin were markedly reduced (5.2 ± 0.7 vs. 32.5 ± 9.7 fmol/ml in gastrectomized rats vs. normal rats, respectively; P < 0.01, n = 15) (Fig. 3*C*), indicative of a lack of stomach-derived ghrelin. The remaining levels of acylated ghrelin may be derived substantially from the intestine, the second largest source of ghrelin (12,21). Although the remaining circulating acylated ghrelin was dramatically reduced in gastrectomized rats, intraperitoneal injection of GHSR antagonist increased plasma insulin concentrations at 30 min in gastrectomized rats to a similar extent as that in normal rats (P < 0.05, n = 15) (Fig. 3*B* vs. *A*). These results suggest that the effect of GHSR antagonist is not attributable to antagonism of circulating ghrelin but primarily to blockade of local ghrelin, including that in islets. It was con-

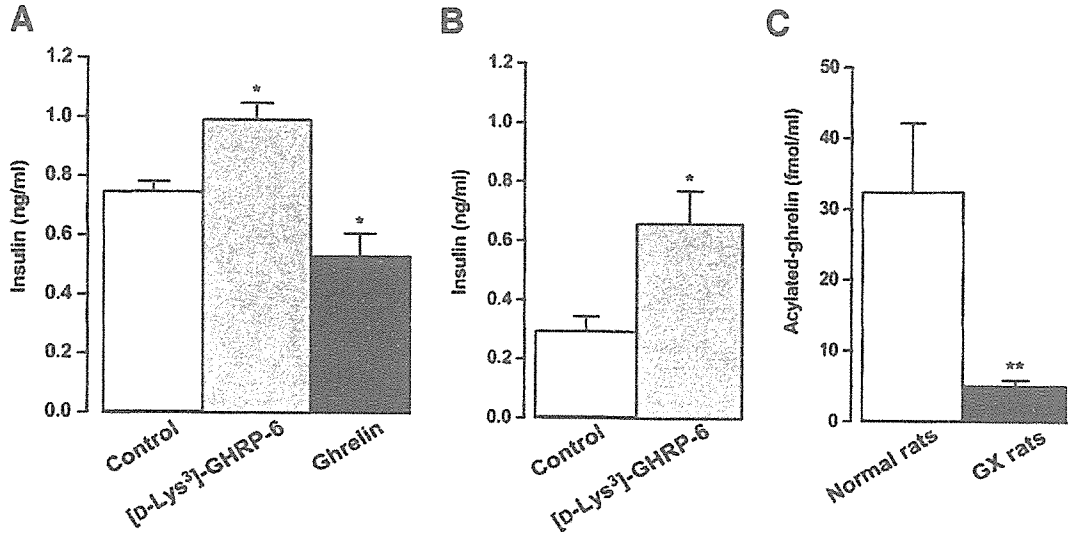

FIG. 3. Endogenous ghrelin decreases plasma insulin concentrations in normal and gastrectomized (GX) rats. *A*: A GHSR antagonist, [D-Lys^3]GHRP-6 (10 μmol/kg i.p.), increased plasma insulin concentrations at 30 min after administration in rats fasted overnight. Conversely, administration of ghrelin (10 nmol/kg i.p.) significantly decreased plasma insulin levels. Data are the means ± SE of 15 rats. *$P < 0.05$ vs. control. *B*: GHSR blockade increased plasma insulin levels in gastrectomized rats ($n = 15$). *$P < 0.05$ vs. control. *C*: Plasma acylated ghrelin levels were reduced in gastrectomized rats ($n = 15$). **$P < 0.01$ vs. normal rats.

firmed that application of exogenous ghrelin (10 nmol/kg i.p.) significantly decreased plasma insulin levels in control rats (Fig. 3*A*).

Ghrelin knockout mice display increased insulin and decreased glucose levels. The effects of GHSR antagonist and anti-ghrelin antiserum in the perfused pancreas and in isolated islets may result from counteraction of the action of endogenous ghrelin in islets. This hypothesis was further examined using ghrelin knockout mice. When fed standard chow, no significant differences between male ghrelin knockout and wild-type (C57BL/6J) mice were observed at 8 weeks of age in body weights (23.4 ± 0.7 vs. 23.5 ± 0.3 g in ghrelin knockout vs. wild-type mice, respectively; $n = 10$), total 24-h food intake (3.51 ± 0.14 vs. 3.54 ± 0.04 g, $n = 10$), and blood glucose levels in fed states (120 ± 3.1 vs. 127 ± 6.0 mg/dl, $n = 10$), confirming previous reports in ghrelin knockout mice (22–25). In ghrelin knockout mice, plasma acylated ghrelin levels were undetectable (Fig. 4*A*). Morphological analysis of pancreatic sections showed that the density and average size of islets were not significantly different between wild-type and ghrelin knockout mice (Fig. 4*B* and *C*). Moreover, the number and size of isolated islets obtained by collagenase digestion were not altered in ghrelin knockout mice (islet number: 138.6 ± 14.3, $n = 5$ mice, vs. 151.2 ± 18.9, $n = 5$, for ghrelin knockout vs. wild-type mice; islet diameter: 165.2 ± 2.3 μm, $n = 714$ islets, vs. 164.7 ± 2.3, $n = 756$). Glucose (8.3 and 16.7 mmol/l)-induced insulin release from isolated islets of ghrelin knockout mice was significantly greater than that of wild-type mice (Fig. 4*D*), whereas basal levels of insulin release at 2.8 mmol/l glucose were not altered. No difference was observed between ghrelin knockout and wild-type mice in insulin content per islet (Fig. 4*E*), mRNA expression of insulin 1, and that of insulin 2 (Fig. 4*F*). These data indicate that the larger amount of insulin release in islets of ghrelin knockout mice results from greater insulin secretory response to glucose, whereas insulin production is unaltered. In GTTs, ghrelin knockout mice exhibited markedly enhanced insulin responses and attenuated glucose responses (Fig. 4*G* and *H*). The profiles of blood glucose levels during ITTs exhibited little differences between ghrelin knockout and wild-type mice (Fig. 4*I*), suggesting that insulin sensitivity was not significantly altered. Thus, the suppressed glycemic responses to GTTs in ghrelin knockout mice may primarily result from enhanced insulin secretion, although possible additional effects of ghrelin on glucose production (26) or insulin sensitivity (27) cannot be disregarded.

High-fat diet–induced glucose intolerance is prevented in ghrelin knockout mice. The enhanced insulin and suppressed glycemic responses to GTTs in ghrelin knockout mice could be beneficial under conditions of increased demand for insulin. We examined this possibility using a model of high-fat diet–induced obesity. Both wild-type and ghrelin knockout mice fed a high-fat diet for 4 weeks displayed moderate increases in body weight (Fig. 5*A*). High-fat diet resulted in moderate increases in blood glucose levels in wild-type mice, whereas this change was not significant in ghrelin knockout mice (Fig. 5*B*). High-fat diet also increased plasma insulin levels, and this change was much greater in ghrelin knockout than wild-type mice (Fig. 5*C*). These results suggest that high-fat diet–induced elevation of blood glucose was corrected by enhanced insulin release in islets of ghrelin knockout mice. A possible impact of this ghrelin knockout mouse islet phenotype on systemic control of glucose and insulin was examined by GTTs. In wild-type mice, increases in blood glucose levels at 15–120 min of the GTT were significantly larger in the high-fat diet group than in the control diet group, exhibiting glucose intolerance (Fig. 5*D*). Insulin response to GTTs at 15 min also tended to be enhanced in the high-fat diet group, although the change was not statistically significant (Fig. 5*E*). In ghrelin knockout mice, by contrast, increases in blood glucose levels at 15–120 min of the GTT in the high-fat diet group were not significantly different from those of the control diet group, and insulin response to GTTs at 15 min was markedly enhanced in the high-fat diet group (Fig. 5*F* and *G*). Thus,

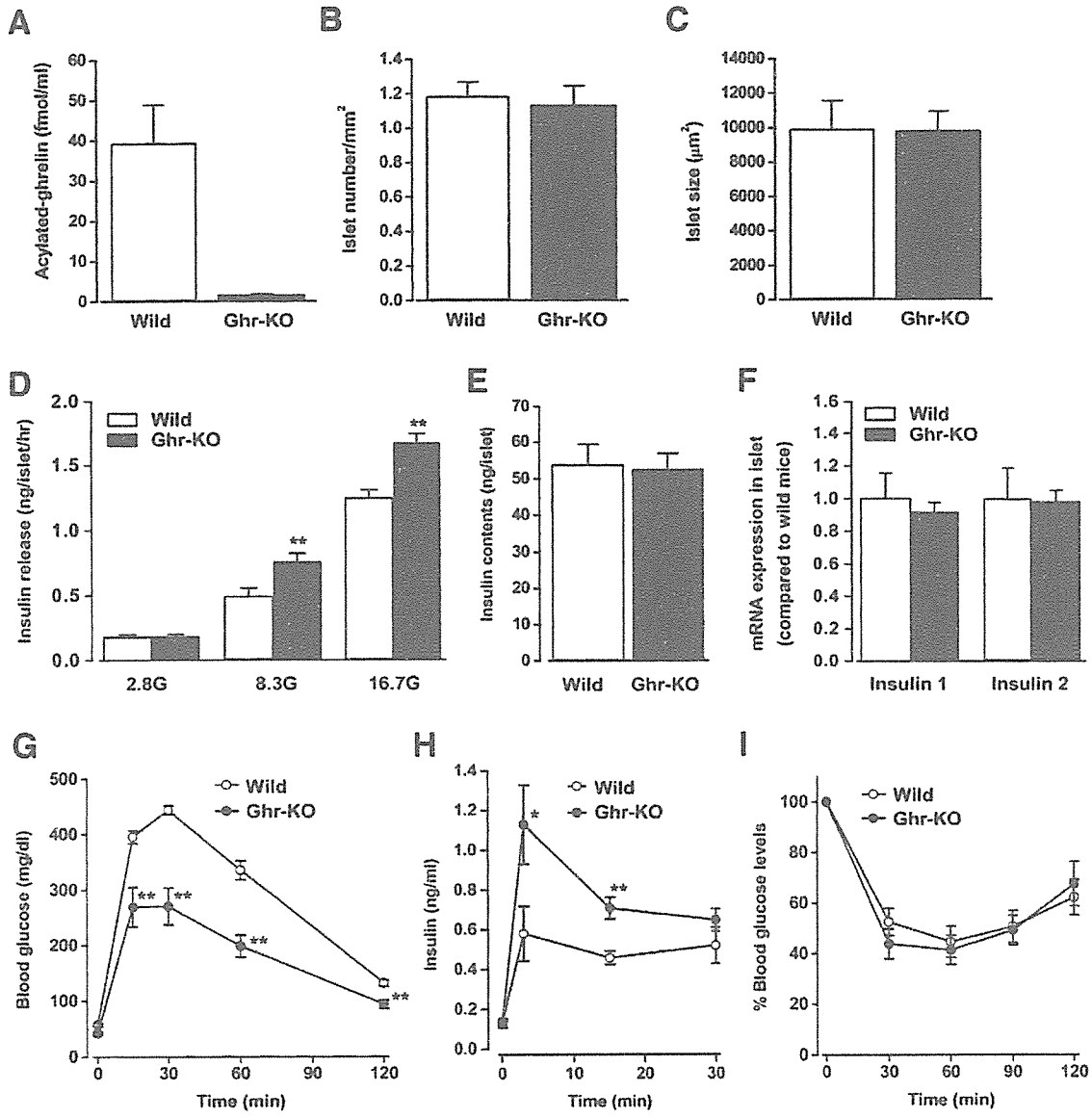

FIG. 4. Ghrelin knockout (Ghr-KO) mice display increased insulin and decreased glucose levels. *A*: Acylated ghrelin was not detected in the plasma of male ghrelin knockout mice (n = 6). *B* and *C*: The number of islets (*B*) in an area unit (1 mm^2, n = 8 slices from three mice) and the size of islets (μm^2, n = 50–51 islets) (*C*) on pancreatic sections were not significantly different between wild-type (Wild) and ghrelin knockout mice at 8 weeks of age. *D*: Glucose (8.3 and 16.7 mmol/l)-induced insulin release was enhanced in ghrelin knockout mouse islets (n = 9–12). $^{**}P <$ 0.01 vs. wild-type mice. *E* and *F*: Islet insulin (*E*) contents (n = 12) and mRNAs expressions (*F*) of insulin 1 and 2 (n = 4–5) were not different between wild-type and ghrelin knockout mice. In GTTs (glucose 2 g/kg i.p.), male ghrelin knockout mice exhibited attenuated elevations of blood glucose (*G*) and enhanced elevations of insulin levels (*H*) in comparison to wild-type mice (n = 9–10). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. wild-type mice. *I*: Profiles of blood glucose levels during the ITT (insulin 0.75 units/kg i.p.) did not differ between ghrelin knockout and wild-type mice (n = 12–15). G, glucose.

ghrelin deficiency promoted insulin release and prevented glucose intolerance in a high-fat diet–induced obese model.

DISCUSSION

In this study, we demonstrated that plasma ghrelin concentrations were significantly higher in the pancreatic vein than in the artery in rats and that glucose-induced insulin release from the perfused pancreas was markedly enhanced by blockade of GHSR and immunoneutralization of endogenous ghrelin. Furthermore, GHSR blockade increased plasma insulin concentrations in gastrectomized and normal rats to a similar extent. In addition, in ghrelin-deficient (ghrelin knockout) mice, glucose-induced insulin release from isolated islets was enhanced, systemic insulin response was increased, and glucose response was attenuated in the GTT. Furthermore, ghrelin deficiency promoted insulin release and prevented glucose intolerance in a high-fat diet–induced obese model.

This study demonstrated that ghrelin knockout mice exhibit decreased glucose responses and increased insulin responses in GTTs. Similar results have recently been reported in another line of ghrelin-deficient mice (28). The results of GTTs and ITTs in our ghrelin knockout mice are similar to those previously observed with pharmacological blockade of ghrelin action (8), reinforcing the concept that endogenous ghrelin serves as a downward regulator of

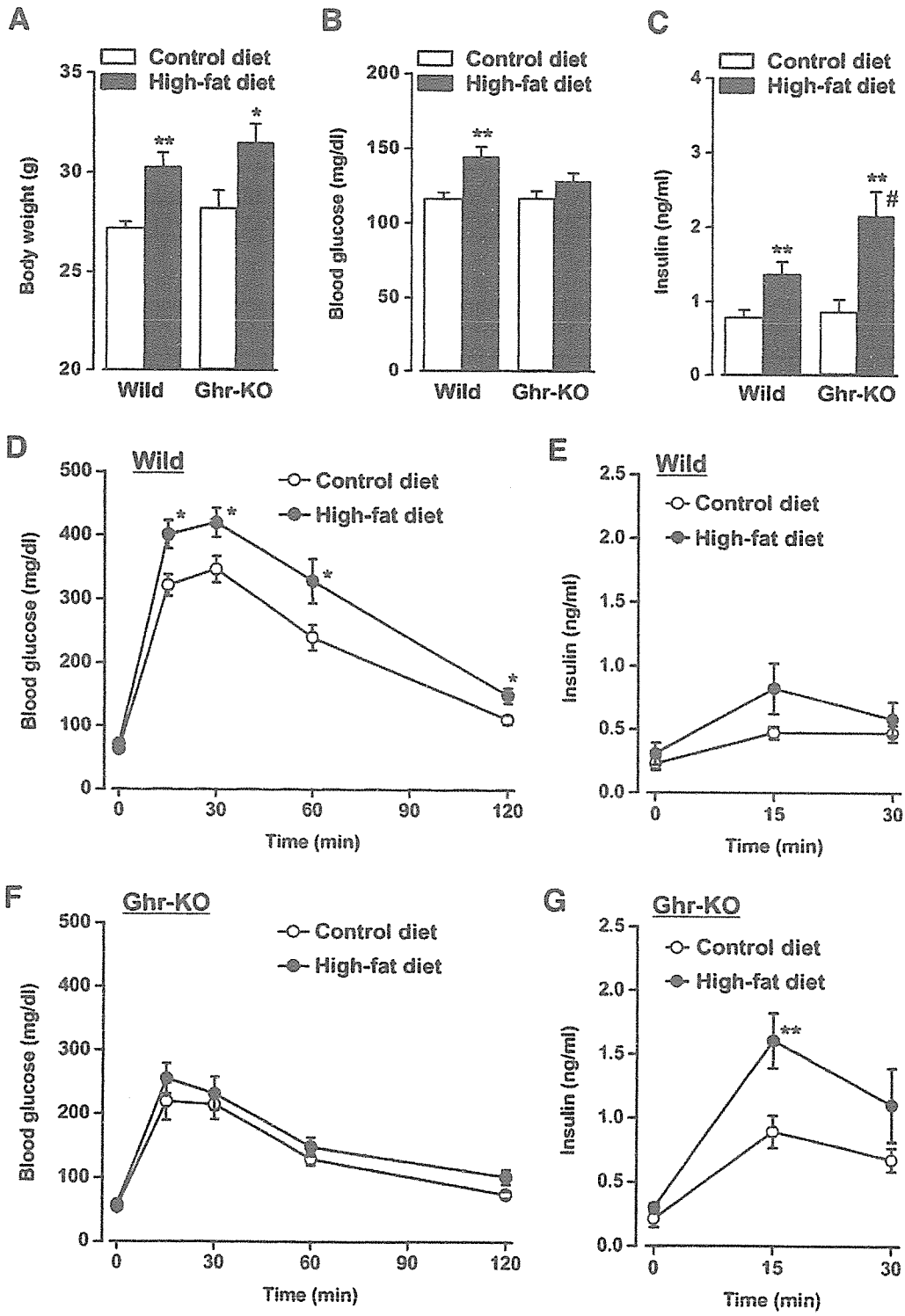

FIG. 5. High-fat diet–induced glucose intolerance is prevented in ghrelin knockout (Ghr-KO) mice. Body weight (*A*), fed blood glucose (*B*), and insulin (*C*) levels in 12-week-old wild-type and ghrelin knockout mice fed a high-fat diet or control diet. The mice were given a high-fat diet or control diet from 8 to 12 weeks old. On a caloric base, high-fat diet consisted of 23% protein, 44.6% carbohydrates, and 32.4% fat (total 17.9 kJ/g), whereas control diet consisted of 29.2% protein, 58.8% carbohydrates, and 12% fat (total 14.5 kJ/g, $n = 9$ per group). In wild-type mice, the high-fat diet group exhibited glucose intolerance (*D*) and slight enhancement of insulin release (*E*) during the GTT (glucose 2 g/kg i.p.). In ghrelin knockout mice, in contrast, glycemic responses to GTTs in the high-fat diet group were not different from those of control diet group (*F*), and insulin response at 15 min was markedly enhanced in the high-fat diet group (*G*). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control diet mice; $^{\#}P < 0.05$ vs. wild-type high-fat diet mice.

insulin release and consequently upward regulator of glycemia. Furthermore, we found that glucose-induced insulin release from isolated islets of ghrelin knockout mice was greater than that of wild-type mice, whereas insulin content per islet was unaltered in ghrelin knockout mice. Consistent with this observation, glucose-induced insulin release from perfused pancreas in normal rats was augmented by GHSR antagonist and ghrelin immunoneutralization. Thus, pharmacological, immunological, and genetic blockade of ghrelin or ghrelin action in pancreatic islets all markedly enhanced glucose-induced insulin release. These findings reveal the insulinostatic function of endogenous ghrelin within islets. It should be noted that the effect of GHSR antagonist could be partly attributable to blockade of the constitutive activity of GHSR (29,30).

This study demonstrated that the ghrelin level is higher in the pancreatic vein than in the artery. Moreover, blockade of ghrelin action with antagonist and antiserum enhanced glucose-induced insulin release from perfused pancreas, which retains well physiologic circulation. Insulin release from isolated islets was similarly enhanced with ghrelin antagonist and antiserum in our previous report (8). These findings may indicate that ghrelin is released from and acting on pancreatic islets, thus serving as an intraislet regulator of insulin release. The importance of islet-originated ghrelin in the regulation of insulin release is supported by the current finding that GHSR antagonist increased plasma insulin concentrations in gastrectomized and normal rats to a similar extent. Regarding the islet cell species that could release ghrelin, multiple experimental systems have shown ghrelin immunoreactivity in α-cells (8,11), β-cells (13), and islet ghrelin cells (14,31,32), including those named ε-cells (15). It was also reported that ghrelin is expressed together with glucagon or pancreatic polypeptide in immature islet cells in rats (31). mRNAs encoding ghrelin and GHSR are expressed in the pancreas of rats and humans (1,11–13) as well as in β-cell lines (31). Thus, ghrelin appears to be expressed by multiple islet cell types. Further studies are required to identify the cell types that produce ghrelin, which could depend on specific conditions and ages of animals/humans.

We have not yet examined the effect of ghrelin on δ-cells, and therefore a possibility that the observed effects require the participation of δ-cells cannot be excluded. Ghrelin reportedly suppresses the release of somatostatin (6). However, the reduction in this insulinostatic hormone does not appear to mediate the action of ghrelin to inhibit insulin release. Moreover, the direction of microcirculation previously reported was against the physiological role of somatostatin in the regulation of insulin release (33). Because ghrelin directly inhibits β-cells (8), the insulinostatic effect of ghrelin is produced, at least partly, via its direct effect on β-cells. In addition to the regulation of insulin release, ghrelin could also serve as a novel medium of communication between β- and non-β-cells: anterograde versus retrograde perfusion with antisera against ghrelin and conventional islet hormones appears to be a promising approach. However, further studies are definitely needed to address this issue.

Low plasma ghrelin levels are associated with elevated insulin levels (9,10). The inverse relationship between plasma levels of ghrelin and insulin may be explained, at least in part, by the inhibition of insulin release by ghrelin. The current study, by using blockade of ghrelin in pancreas and islets, as well as in vivo in gastrectomized rats, demonstrated that ghrelin originating from pancreatic islets plays an essential role in suppression of insulin release. This study also suggested a pathophysiological role for ghrelin. High-fat diet produced glucose intolerance in wild-type mice. By contrast, ghrelin knockout mice fed a high-fat diet showed close to normal glucose responses and markedly enhanced insulin responses to GTTs compared with control ghrelin knockout mice fed a normal diet. As a possible underlying mechanism, lack of ghrelin and its insulinostatic activity may raise the maximal capacity of glucose-induced insulin release and enable islets to secrete more insulin to meet an increased demand associated with high-fat diet–induced obesity, thereby achieving normoglycemia. It has recently been reported that in *ob/ob* mice, a genetic model of obesity attributable to leptin deficiency, ablation of ghrelin in *ob/ob* mice augmented insulin release and thereby markedly reduced hyperglycemia (28). We hypothesize that the ghrelin system in islets, by altering its insulinostatic efficacy, optimizes the amount of insulin release to meet the systemic demand. In early stages of obesity, antagonization of ghrelin function can prevent glucose intolerance, providing a potential therapeutic application to counteract the progression of type 2 diabetes.

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Ghrelin reduction after esophageal substitution and its correlation to postoperative body weight loss in esophageal cancer patients

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Background. *Body weight loss is observed commonly after esophagectomy with gastric tube reconstruction in thoracic esophageal cancer patients. The functional and anatomical alteration of the stomach by this surgery should affect ghrelin secretion, a novel gastric hormone that upregulates body weight through appetite control and metabolic reaction.*

Methods. *Early-phase postoperative alteration of serum ghrelin was measured before and at day 3 and day 7 after surgery in 9 patients. With 26 other patients, who had previously undergone surgery from 3 months to 67 months (mean, 25 months) before the present study period, the late-phase postoperative alteration of serum ghrelin was investigated along with postoperative body weight loss and serum leptin.*

Results. *Serum ghrelin concentration, which was equivalent to the control group before surgery (88.6 fmol/mL vs 97.5 fmol/mL) significantly decreased by half at 3 and 7 days after surgery. Thereafter, the serum ghrelin decline continued in the outpatients within 1 year after surgery (58.8 fmol/mL), while it was marginal in those from 1 to 3 years after surgery (77.2 fmol/mL). Serum ghrelin was significantly higher than the control after 3 years (185.1 fmol/mL). Thus, a significant positive correlation was observed between ghrelin and time after surgery ($P < .0001$). Postoperative body weight loss was significant, averaged as $\Delta BMI - 2.7$ in the outpatients ($P < .0001$). Until 3 years after surgery, a significant correlation between ghrelin and postoperative body weight loss was observed ($P = .0152$), with those having higher serum ghrelin showing less body weight loss. Serum leptin correlated well with body weight ($P = .0144$), but not with postoperative time, the degree of body weight loss, or serum ghrelin concentration.*

Conclusion. *Gastric tube replacement for esophagectomy resulted in temporary reduction of ghrelin production, which is associated with body weight loss after surgery. The decline of ghrelin may play some role in the serious body weight loss after esophagectomy, thus encouraging clinical application of exogenous recombinant ghrelin for these patients. (Surgery 2006;139:797-805.)*

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THE PROGNOSIS of esophageal cancer after surgery has improved gradually with adjuvant therapies, lymphadenectomy, and management of postoperative complications to reach a 5-year survival rate of more than 40%. Surgeons now need to consider the quality of life (QOL) after surgery. The function of the esophagus seems to be simply to send food from the mouth to the stomach; however, esophagectomy with gastric replacement, the most common surgical treatment for thoracic esophageal cancers, leads to various sequelae including dysphagia, gastroesophageal reflux, aspiration, choking, dyspnea, cough, and hoarseness.[1-6] Compared with these symptoms, body weight loss after esophagectomy has not been well discussed, probably because of the long follow-up required for evaluation. However, in general, body weight loss is

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the most reliable indicator of postoperative malnutrition and strongly affects postoperative QOL as well as immune function, infection, and survival,[7,8] and with respect to esophagectomy, significant body weight loss, reported to be 10% to 20% of preoperative body weight,[4,5] is similar to that of total gastrectomy.[9,10] It is of interest that, among the types of surgery for digestive cancers, body weight loss is observed characteristically after operation on the upper digestive tract, such as esophagectomy, gastrectomy, or pancreaticoduodenectomy,[11] but not with that of other sites (eg, liver, colon, and rectum).[12,13] Thus, postoperative body weight loss is a serious problem that needs to be addressed by surgeons dealing with esophageal cancers.

Ghrelin was first identified as an intrinsic ligand for the growth hormone secretagogue (GHS) receptor of the pituitary gland in 1999.[14] However, recent studies have revealed that ghrelin's function is to stimulate not only GH secretion from the pituitary gland but also the appetite signal in the hypothalamus, in opposition to leptin,[15] a well-known appetite-suppressing hormone from fat tissue, and gastrointestinal activity, such as peristalsis, gastric acid secretion, and pancreatic excretion, through the vagal nerve.[16] These pleiotropic effects of ghrelin result in body weight gain, especially associated with fat metabolism.[17] Most of the ghrelin is secreted by the stomach, with only trace amounts from the duodenum, brain, and other organs.[18] The discovery of ghrelin led to the novel concept of body weight regulation by the stomach, which has been investigated in the surgical field, especially in bariatric surgery and gastrectomy.[19–21] Manipulation of the stomach by these surgical procedures reduced ghrelin production, resulting in loss of body weight and appetite. To date, there has been no study investigating ghrelin levels after esophagectomy with gastric tube reconstruction, despite the fact that this procedure including anatomic translocation of the stomach and vagotomy should affect ghrelin production from the stomach. For these reasons, the present study was designed to investigate the association between postoperative body weight loss and ghrelin production by the stomach after esophageal substitution.

PATIENTS AND METHODS

Patients and blood sample collection. Two groups of esophageal cancer patients, who had given their informed consent, were enrolled in this study from January to June 2003. Nine hospital patients were subjected to investigation of the early-phase effect of surgery on ghrelin production, and 26 outpatients were studied for late-phase effects of surgery on ghrelin and leptin production and their effects on postoperative body weight. For the 9 patients in the former group, 10 mL of blood sample was collected before and at 3 and 7 days after surgery, when they had not yet started oral food intake after surgery. For the latter group, a blood sample was collected from each patient at around 9 to 10 AM before breakfast. Clinical data, including height and body weight before and after surgery and the results of routine blood examinations, were obtained from the patient's charts. Body mass index (BMI) was calculated as body weight (kg)/height^2 (m^2). Postoperative body weight loss (ΔBMI) of outpatients was defined as (postoperative BMI at ghrelin measurement) minus (BMI before surgery) (ie, not initial body weight or body weight at diagnosis). The interval after the surgery of 26 outpatients ranged from 3 to 67 months (mean, 25 months), and none of them displayed tumor recurrence by physical examination, chest-abdominal computed tomography scan, abdominal ultrasonography, fiberscopy, or blood tumor markers. Patients exhibiting the following features were excluded from this study: (1) body weight loss of more than 3 kg before surgery attributable to advanced esophageal cancer; (2) serious postoperative complications requiring more than 3 months hospital stay; (3) the use of other operative procedures, such as colon reconstruction, abdominal para-aortic lymph adenectomy or left thoracotomy; (4) inadequate postoperative survey; (5) noncurative surgery, tumor recurrence, or presence of other malignancies; and (6) absence of informed consent. The control group included 22 patients before surgical treatment with early digestive cancers including gastric cancer (10), colorectal cancer (10), and pancreatic cancer (2); they did not show significant body weight change attributable to cancer. Early-phase alteration of serum ghrelin after esophagectomy was observed along with those after total gastrectomy with Roux-en-Y reconstruction (8 patients), distal gastrectomy with Billroth I (5 patients), and colectomy (5 patients including 3 sigmoidectomy and 2 right hemicolectomy).

Surgery and adjuvant treatments. The patients' clinical backgrounds are shown in Table I. All patients had squamous cell carcinoma of the thoracic esophagus and underwent curative surgical treatment, which we previously described in detail[22,23] and summarize here as follows. The thoracic esophagus was removed by right thoracotomy and replaced by the stomach with cervical anastomosis. The gastric tube was reconstructed with the whole stomach, removing 1 to 2 cm of the proximal por-

Table I. Clinical background of 35 esophageal cancer patients enrolled in this study

Gender	
Male/Female	26/9
Age at surgery	
Mean (y [range])	63.3 (48-80)
Clinical TNM stage at initial diagnosis	
I/II/III/IV	6/13/9/7
Gastric tube type	
Whole stomach/great curvature	28/7

tion with the esophagus. Otherwise, the greater curvature gastric tube, approximately 5 cm in diameter without the cardia and lesser curvature portion, was used in 7 patients because of small-sized stomach or gastric ulcer scar in the angle of the stomach. Pyloroplasty was not performed, but finger bougie of the pyloric ring was done for all patients. The posterior mediastinal route was used mostly for the reconstruction. Bilateral truncal vagotomy was done below the bronchial branch. The morbidity rate of these surgical treatments was 35%, including transient recurrent nerve palsy, minor anastomotic leakage, atelectasis, and pneumonia; however, as they were not life threatening, the median hospital stay after surgery was 27 days (range, 23-65 days) in these patients. There were various clinical stages before treatment, according to TNM classification.[24] Fifteen patients received surgical treatment alone, while the remainder received adjuvant therapies, including preoperative chemotherapy, postoperative chemotherapy, and preoperative chemoradiotherapy.[22]

Measurement of serum ghrelin and leptin. The serum was obtained from each blood sample and kept below −70°C until serum ghrelin and leptin were measured with sandwich-type enzyme immunoassay kits for ghrelin (SCETI Co, Ltd, Tokyo, Japan) and leptin (IBL Co, Ltd, Gunma, Japan) according to the manufacturer's instructions. Measurement was done in duplicate for each sample, and the mean value was used. Two different forms of ghrelin, active (acyl) and inactive (desacyl) forms,[25] were measured with different kits from the same company. Since the amount of the active form was very small and unstable in the serum, the active form could not be measured in most serum samples and, at most, was only 3% to 5% of the inactive form, even when measurable. Ghrelin is secreted as an active form and immediately inactivated by elastase and protease in the blood; the proportion of active/inactive ghrelin is relatively constant.[26] Thus, the amount of inactive form was used in this study to represent serum ghrelin. For precise measurement of acylated ghrelin, the plasma sample must be preserved with protease inhibitors.

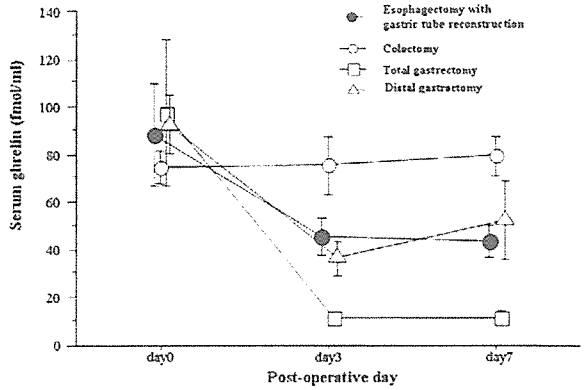

Fig 1. Early-phase effect of esophagectomy and gastrectomy on serum ghrelin. Serum ghrelin was measured before surgery and on days 3 and 7 after surgery, including esophagectomy with gastric tube reconstruction (*closed circles*, 9 patients), total gastrectomy (*open squares*, 8 patients), distal gastrectomy (*open triangles*, 5 patients), and colectomy (*open circles*, 5 patients). The mean of each time point is indicated with a *standard error bar*. Significant decline of serum ghrelin after surgery by Student *t* test ($P < .05$) was observed at day 7 for esophagectomy, at days 3 and 7 for total gastrectomy, and at day 3 for distal gastrectomy patients.

Statistical analysis. The difference in variables including BMI, postoperative change in BMI, and serum ghrelin and leptin concentration were evaluated by the Mann-Whitney *U* test; their multiplicity was adjusted by the Bonferroni method. The relationship among these parameters was investigated by the Pearson correlation analysis. A *P* value of less than .05 was considered to indicate statistical significance. All statistical analyses were performed with the software package StatView version 5.0 (Abacus Concepts, Berkeley, Calif).

RESULTS

Early-phase effect of surgery on ghrelin production. Esophageal cancer patients showed a similar amount of serum ghrelin to the control group before surgery (88.6 fmol/mL vs 97.5 fmol/mL, $P = .5325$). After esophageal replacement by the stomach, serum ghrelin decreased to approximately half of the preoperative level at day 3 (45.5 fmol/mL) and day 7 (44.1 fmol/mL) after surgery, when patients had not started oral food intake or were on enteral nutrition, receiving only about 15 cal/kg/day at day 3 and 30 cal/kg/day at day 7 by intravenous alimentation (Fig 1). Total gastrec-

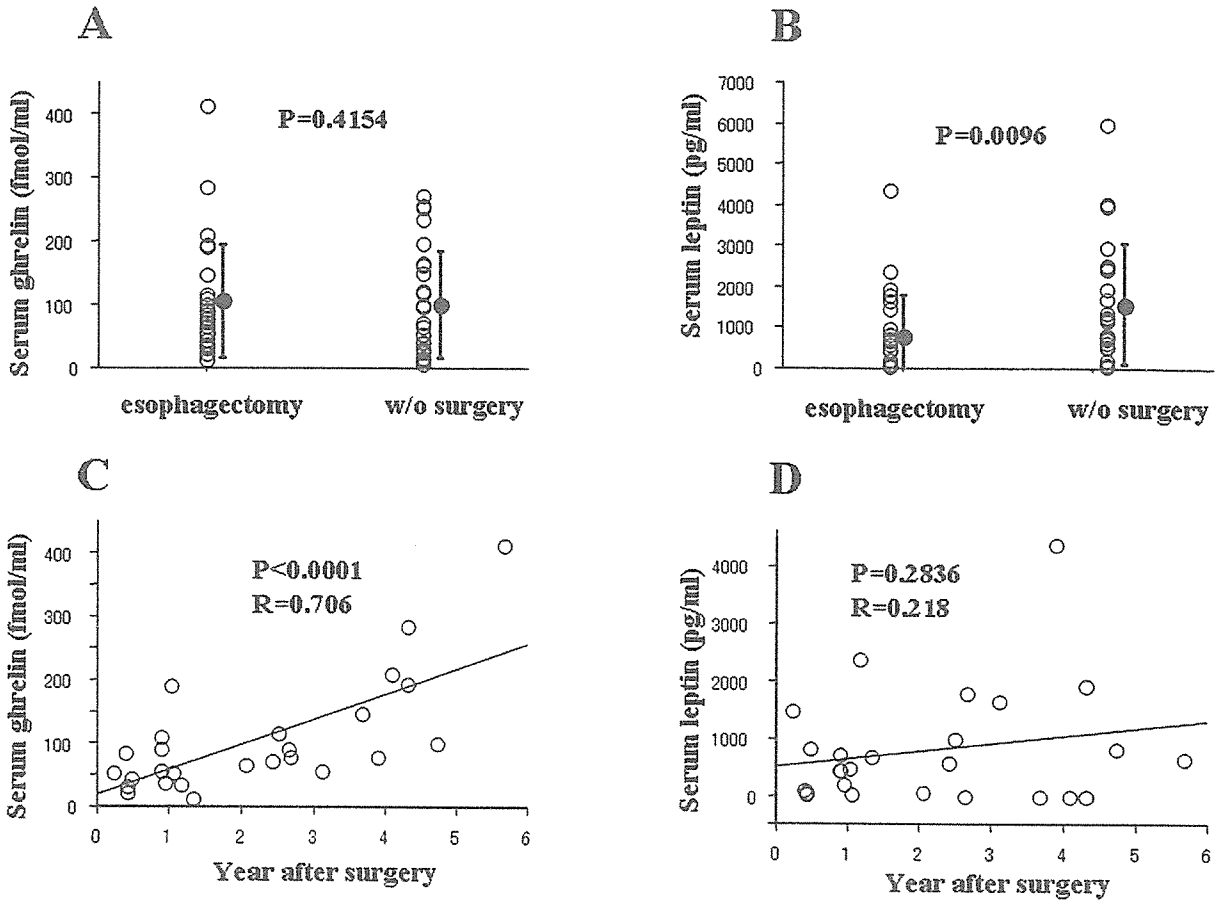

Fig 2. Late-phase effect of esophagectomy with gastric tube reconstruction on serum ghrelin and leptin. Serum ghrelin (A, C) and leptin (B, D), measured for 26 outpatients who had previously undergone esophagectomy with gastric tube reconstruction, were compared with the control group (w/o surgery) (A, B) and correlated with time after surgery (C, D). The value for each subject (*open circles*) and mean (*closed circles*) with standard deviation (*bar*) are indicated. Differences between the patients and controls were evaluated by the Mann-Whitney *U* test, and the correlations with time after surgery were evaluated by a linear regression model along with the regression line.

tomy, serving as a negative control, showed an extreme decline of serum ghrelin (12% of the preoperative level) immediately after surgery. Distal gastrectomy, which removed two thirds of the stomach, showed an approximately 50% reduction of serum ghrelin at days 3 and 7. Colectomy, which did not include gastric manipulation, did not show any ghrelin alteration on either day 3 or day 7 after surgery.

Late-phase effect of surgery on ghrelin production. Patients were discharged from the hospital 3 weeks after the esophagectomy. The ghrelin amount was investigated for 26 outpatients, who had previously undergone esophagectomy from 3 and 70 months before this study. The mean of ghrelin in outpatients was similar to that of the control group (Fig 2, *A;* 104.7 fmol/mL vs 97.5 fmol/mL, $P = .7652$). However, it was of interest that serum ghrelin exhibited a significant positive correlation with postoperative time (Fig 2, *C*): Patients within 1 year showed significantly lower ghrelin levels (58.8 fmol/mL) than those after 3 years (185.1fmol/mL, $P = .0115$); their levels tended to be lower than those in the control group (97.5 fmol/mL, $P = .2764$). Patients after 3 years showed significantly higher ghrelin levels than those at 1 to 3 years (77.2 fmol/mL, $P = .0184$) and those of the control group ($P = .0136$; Table II). The type of gastric tube did not affect the ghrelin level, which was 108.8 fmol/mL for the whole stomach and 95.6 fmol/mL ($P = .0739$) for the greater curvature. Other clinicopathological factors (Table I), such as age, gender, reconstruction route, stage of cancer and adjuvant therapy did not significantly

Table II. Late-phase effect of surgery on body weight and weight control hormones after surgery

	Duration after surgery			
	<1 year (8 cases)	*1-3 years (10 cases)*	*>3 years (8 cases)*	*Control (22 cases)*
Ghrelin (fmol/mL)	58.8 + 32.0	77.2 + 49.1	185.1 + 118.6	97.5 + 85.0
Leptin (pg/mL)	479.3 + 507.1	738.7 + 786.4	1184.7 + 1498.3	1674.8 + 1496.1
BMI (kg/m^2)	18.58 + 2.61	20.47 + 2.25	19.32 + 3.41	22.32 + 3.05
ΔBMI (kg/m^2)	−3.26 + 1.82	−2.37 + 3.25	−2.54 + 2.06	

Significant difference ($P < .05$ by Students *t* test) present in ghrelin (1 year vs > 3 years, 1-3 years vs >3 years, >3 years vs control), leptin (<1 year vs control), and BMI (<1 year vs control, >3 years vs control). Control group was composed of patients without surgery.

affect the serum ghrelin and leptin concentration, and postoperative body weight loss (data not shown).

Post operative body weight loss and ghrelin. Since esophageal cancer patients with significant body weight loss before surgery were excluded from this study, the average of their preoperative body weight in this study did not show significant difference from the control (BMI 22.11 vs 22.32, $P = .7334$; Fig 3, *A*). Among the 26 outpatients, body weight loss after surgery was significant (average ΔBMI, −2.70, $P < .0001$); Figure 3, *A*. Body weight showed a weak tendency to recover with longer postoperative times (Fig 3, *B*); thus, there was a difference between those within 1 year after surgery and from 1 to 3 years, but it was not statistically significant (ΔBMI −3.26 vs −2.37, $P = .499$; Table II). We therefore investigated the relationship between postoperative body weight loss and serum ghrelin. Since postoperative body weight did not recover after more than 3 years after surgery, this relationship was evaluated separately for those up to 3 years and those more than 3 years after surgery. In patients within 3 years after surgery, there was a significant positive correlation between serum ghrelin concentration and postoperative body weight loss (ie, those with high serum ghrelin showed less body weight loss after surgery $P = .0152$; Fig 4, *A*). However, absolute body weight did not correlate with serum ghrelin ($P = .9071$; Fig 4, *B*). In patients after more than 3 years after surgery, loss of absolute body weight did not correlate with serum ghrelin (data not shown). Multivariate analysis of outpatients within 3 years from surgery revealed that serum ghrelin level ($P = .0346$) but not time after surgery ($P = .3040$) was an independent factor affecting the postoperative body weight loss. Nutritional parameters other than the body weight, including serum albumin, hemoglobin, and lymphocyte, did not show significant association with serum ghrelin or leptin (data not shown).

Post operative leptin as a counterpart of ghrelin. Outpatients showed significant reduction of serum leptin, compared with the control (796 pg/mL vs 1581 pg/mL, $P = .0162$; Fig 2, *B*) or esophageal cancer patients before surgery (1422 pg/mL). Serum leptin tended to be higher in those with a long postoperative time (Table II), although the correlation was not statistically significant ($P = .2836$; Fig 2, *D*). Significant correlation was present between postoperative BMI and serum leptin in both patients within 3 years after surgery (Fig 4, *D*) and more than 3 years after surgery (data not shown). However, serum leptin did not correlate with body weight loss after surgery ($P = .9306$; Fig 4, *C*) or serum ghrelin concentration ($P = .5928$).

DISCUSSION

Body weight loss, which is generally observed after surgery of the upper digestive tract, greatly impairs QOL. Few reports have described body weight loss after esophagectomy with reconstruction using the stomach, which amounts to 10% to approximately 20% of preoperative body weight.[4,5] This study, in which we used BMI to standardize the body weight, revealed that, although preoperative BMI (22.3) was identical to the ideal BMI (22), postoperative BMI (mean, 18.6; minimum, 16.4) was significantly lower than the recommended BMI (19.8 to approximately 24.2) for the Japanese people.[27] Thus, body weight loss is one of the serious issues for esophageal cancer patients after esophagectomy reconstruction using the stomach. Swallowing and eating dysfunctions, which are characteristic complications after this surgical treatment, may be attributed to postoperative body weight loss, but researchers could not find significant correlation among these symptoms and body weight loss.[3,4] In addition, clinical trials concerning the route of reconstruction,[28] type of gastric tube (whole or narrow tube),[29] pyloroplasty,[30] and medication for gastric tube motility[31] could reduce these symptoms, but they do not improve calorie intake or reduce postoperative body weight loss.

Ghrelin, which is secreted mainly from A-like cells in the fundic gland of the stomach,[18] increases