Endogenous Ghrelin in Pancreatic Islets Restricts Insulin Release by Attenuating Ca^{2+} Signaling in β-Cells

Implication in the Glycemic Control in Rodents

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Ghrelin, isolated from the human and rat stomach, is the endogenous ligand for the growth hormone (GH) secretagogue receptor, which is expressed in a variety of tissues, including the pancreatic islets. It has been shown that low plasma ghrelin levels correlate with elevated fasting insulin levels and type 2 diabetes. Here we show a physiological role of endogenous ghrelin in the regulation of insulin release and blood glucose in rodents. Acylated ghrelin, the active form of the peptide, was detected in the pancreatic islets. Counteraction of endogenous ghrelin by intraperitoneal injection of specific GH secretagogue receptor antagonists markedly lowered fasting glucose concentrations, attenuated plasma glucose elevation, and enhanced insulin responses during the glucose tolerance test (GTT). Conversely, intraperitoneal exogenous ghrelin GH-independently elevated fasting glucose concentrations, enhanced plasma glucose elevation, and attenuated insulin responses during GTT. Neither GH secretagogue receptor antagonist nor ghrelin affected the profiles of the insulin tolerance test. In isolated islets, GH secretagogue receptor blockade and antiserum against acylated ghrelin markedly enhanced glucose-induced increases in insulin release and intracellular Ca^{2+} concentration ([Ca^{2+}]_i), whereas ghrelin at a relatively high concentration (10 nmol/l) suppressed insulin release. In single β-cells, ghrelin attenuated glucose-induced first-phase and oscillatory [Ca^{2+}]_i increases via the GH secretagogue receptor and in a pertussis toxin-sensitive manner. Ghrelin also increased tetraethylammonium-sensitive delayed outward K+ currents in single β-cells. These findings reveal that endogenous ghrelin in islets acts on β-cells to restrict glucose-induced insulin release at least partly via attenuation of Ca^{2+} signaling, and that this insulinostatic action may be implicated in the upward control of blood glucose. This function of ghrelin, together with inducing GH release and feeding, suggests that ghrelin underlies the integrative regulation of energy homeostasis.

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Ghrelin, a novel acylated 28-amino acid peptide, was isolated from the human and rat stomach as the endogenous ligand (1) for the growth hormone (GH) secretagogue receptor (2). Circulating ghrelin is produced predominantly in the stomach (3). Ghrelin is a potent stimulator of GH release (1) and feeding (4). In addition, the plasma ghrelin level correlates inversely with obesity (5–7). A recent report of a cohort study has shown that low plasma ghrelin is associated with elevated fasting insulin levels, insulin resistance, and type 2 diabetes (8). These findings suggest that ghrelin could be involved in energy and glucose metabolism, in which insulin plays a crucial role. It has been well documented that systemic administration of ghrelin elevates blood glucose (9–11). However, the physiological role of endogenous ghrelin in the regulation of blood glucose remains to be established. This question was addressed in the present study by examining the effects of ghrelin receptor blockade on blood glucose and insulin levels in rodents.

It has been reported that ghrelin (12–16) and its mRNA (1,12,13,17), as well as GH secretagogue receptor mRNAs (1,12,13,17,18), are expressed in the pancreas and islet cells. The present study aimed to explore a role of endogenous ghrelin in islets in the regulation of insulin release. This was achieved by counteraction of the islet ghrelin with specific GH secretagogue receptor antagonists and antiserum against acylated ghrelin, the active form of the peptide (active ghrelin). Furthermore, although ghrelin reportedly influences insulin release in several different systems (12,19–21), the signaling mechanisms of ghrelin in islet β-cells are unknown. We studied ghrelin’s action mechanisms with special attention to the Ca^{2+} signaling in islet β-cells. Based on the fact that the reported effects of ghrelin on GH release and feeding were essentially similar between rats and mice, rats were used for the current in vitro studies because we have long
established the rat islet and β-cell systems for measuring insulin release, intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ), and whole-cell currents, and mice were used for the in vivo studies that required a large amount of ghrelin and antagonists. Here we present evidence to support the hypothesis that endogenous ghrelin in islets directly acts on β-cells to inhibit glucose-induced insulin release by attenuating increases in [Ca²⁺]ᵢ, in which enhancement of delayed outward K⁺ currents is partly involved. The present study also shows that the insulinostatic action of ghrelin is implicated in the upward control of blood glucose.

**RESEARCH DESIGN AND METHODS**

Male ddY mice and Wistar rats (Japan SLC), as well as GH-deficient little mice and wild-type C57BL/6J mice (The Jackson Laboratory) were cared for in accordance with our institutional guidelines.

**Measurements of fasted blood glucose concentrations in mice.** Ghrelin (Peptide Institute), [α-Lys]₃-GHRP-6 (Sigma), [α-Arg, α-Phe, α-Trp, Leu]⁻ substance P (SPA, Sigma), and des-acetyl ghrelin (1) were intraperitoneally administered to male 8-week-old ddY mice, GH-deficient little mice, and wild-type C57BL/6J mice after overnight fasting. Blood was obtained from tails, and glucose concentrations were measured using GlucoCard DIA meter (Arkray).

**Glucose tolerance test and insulin tolerance test in mice.** In glucose tolerance test (GTT) studies, 1 g/kg glucose with or without ghrelin (1–10 nmol/kg) or [α-Lys]₃-GHRP-6 (10 μmol/kg) was either intraperitoneally injected into mice followed by blood sampling from the tail vein for glucose measurements, or it was intravenously injected into jugular veins followed by blood sampling from the hepatic portal vein for insulin measurements. In insulin tolerance test (ITT) studies, insulin (1 unit/kg) with or without ghrelin (10 μmol/kg) or [α-Lys]₃-GHRP-6 (10 μmol/kg) was intraperitoneally injected, and blood samples were collected from the tail vein.

**Preparation of rat pancreatic islets and single β-cells.** Islets of Langerhans were isolated from Wistar rats aged 8–12 weeks by collagenase digestion as reported (22) with slight modification. Animals were anesthetized by injection of pentobarbital at 80 mg/kg i.p., and collagenase (1.05 mg/ml; Sigma) was injected into the common bile duct. The pancreas was dissected and incubated in 5 mmol/l Ca²⁺-containing Krebs-Ringer bicarbonate HEPES buffer (KRHB; in mmol/l: NaCl 129, NaHCO₃ 5.0, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 2.0, MgSO₄ 1.2, and HEPES 10 at pH 7.4, with 0.1% BSA). The pancreas was dissected and incubated in 5 mmol/l Ca²⁺-free KRHB. The single cells were plated sparsely on coverslips and maintained in short-term culture for up to 2 days in Eagle’s minimal essential medium containing 5.6 mmol/l glucose supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37°C.

**Antiserum and radioimmunooassay for ghrelin.** In the present study, we used rabbit polyclonal antiserum raised against COOH-terminally Cys-ex- tended rat ghrelin (position 11–11) in New Zealand white rabbits (#G606). This antiserum was shown to specifically recognize ghrelin with octanoylated Ser 3 (acylated ghrelin) (23).

A ghrelin-specific radioimmunoassay (RIA) based on the antiserum against ghrelin (position 11–11), described previously (23), was used. The bound and free ligands were separated using a second antibody. All the procedures were performed at 4°C.

**Immunohistochemistry.** The rat pancreatic islets were fixed with 4% paraformaldehyde and incubated with mouse monoclonal anti-ghrelin antibody (Sigma) at a dilution of 1:1,000 overnight at 4°C followed by Alexa Fluor 488–labeled goat anti-mouse IgG (Molecular Probes). Next, the islets were incubated with rabbit antiserum against rat ghrelin (position 11–11) at a dilution of 1:5,000 overnight at 4°C followed by Alexa Fluor 568–labeled goat anti-rabbit IgG (Molecular Probes). Control experiments were carried out by omitting the primary antibody and antiserum. Glucagon immunofluorescence and ghrelin immunofluorescence were observed with a photomultiplier of the multiphoton laser scanning microscope (FluoView FV300-TP; Olympus).

**Measurements of insulin and glucagon release.** Measurements of insulin and glucagon release were carried out as previously described (24). Briefly, groups of 12–15 islets were incubated at 37°C for 1 h in KRHB with 2.5 mmol/l glucose and then for 0.5–1 h in KRHB with 2.8, 5.6, or 8.3 mmol/l glucose. Ghrelin, [α-Lys]₃-GHRP-6, SPA, rabbit antiserum against rat ghrelin (position 11–11), and nonimmune rabbit serum were added in KRHB throughout incubation. Insulin and glucagon concentrations were determined using enzyme immunsorbent assay kits (Morinaga) and enzyme-linked immunosorbent assay kits (Wako), respectively.

**Measurements of [Ca²⁺]ᵢ, and NAD(P)H.** Measurements of [Ca²⁺]ᵢ, were carried out by previously described procedures (22,24), with slight modification. Briefly, isolated single islets and single β-cells on coverslips were incubated in an open chamber and superfused in KRHB, [Ca²⁺]ᵢ, in single islets and single β-cells was measured by dual-wavelength fura-2 microfluorimetry with 340/380 nm excitation and 510 nm emission using a cooled charged- coupled device camera, and the ratio image was produced by an Aquacosmos system (Hamamatsu Photonics, Hamamatsu, Japan). Autofluorescence of NAD(P)H in single β-cells was measured by procedures similar to those for [Ca²⁺]ᵢ, measurements, except for the use of 680 nm for excitation and 470 nm for emission. In single-cell experiments, data were taken only from the β-cells confirmed by morphologic and physiological criteria (24).

**Patch-clamp experiments.** A patch-clamp technique (25,26) was used to record perforated whole-cell currents with the pipette solution including nystatin of 150–200 μg/ml dissolved in 0.1% DMSO. The membrane currents were recorded using an amplification (Axopatch 200B; Axon Instruments, Foster, CA) and stored online in the computer with pCLAMP9 software. The voltage clamp in a perforated mode was considered adequate when a series resistance was <20 MΩ. The patch pipettes were pulled from glass tubing purchased from Narishige (Tokyo), and their resistances were between 4 and 7 MΩ when filled with the pipette solution, which contained (in mmol/l): KSO₄ 40, KCl 50, MgCl₂ 5, EGTA 0.5, and HEPES 10 at pH 7.2 with KOH. Cell capacitances recorded from single β-cells were 7.5 ± 1.7 pF (n = 17). β-Cells were voltage-clamped from the holding potential of −70 mV to test potentials of −100 to +50 mV in a 10-mV step by pulses with 100-ms durations and 5- to 8-s intervals. The electrophysiological experiments were performed at room temperature (25°C).

**Statistical analysis.** Data represent the means ± SE or the means (95% CI). Statistical analysis was performed using the Student’s t test. P < 0.05 was considered statistically significant.

**RESULTS**

**Effects of GH secretagogue receptor antagonists on systemic blood glucose and insulin levels.** To assess the role of endogenous ghrelin, the effects of blockade of GH secretagogue receptor (ghrelin receptor) on systemic blood glucose and insulin levels were studied in mice fasted overnight. Intraperitoneal administration of the specific GH secretagogue receptor antagonists (27) [α-Lys]₃-GHRP-6 and SPA significantly reduced fasting blood glucose concentrations by 10–30 mg/ml at 30 and 60 min in a dose-dependent manner (Fig. 1A and B). These results suggest that endogenous ghrelin is involved in the homeostatic regulation of fasting blood glucose. In the GTT, when [α-Lys]₃-GHRP-6 was injected simultaneously with 1 g/kg glucose, increases in plasma glucose at 30 and 60 min were markedly attenuated compared with the corresponding values in saline-injected control mice (Fig. 1C). Concomitantly, the insulin responses in GTT were markedly enhanced at 10 and 15 min (Fig. 1D).

**Effects of exogenous ghrelin on systemic blood glucose and insulin levels.** To further substantiate the role of endogenous ghrelin, we examined whether exogenously administered ghrelin exerts effects opposite to those obtained with blockade of endogenous ghrelin. Intraperitoneal administration of ghrelin concentration-dependently and, at 1 and 10 nmol/kg, significantly (P < 0.05) elevated blood glucose levels at 30 min by ~10–20 mg/dl in mice fasted overnight (Fig. 2A and B). The hyperglycemic effect of ghrelin was completely blocked by [α-Lys]₃-GHRP-6 at 1 μmol/kg (Fig. 2B). Des-acyl ghrelin, an inactive form of ghrelin that does not activate GH secretagogue receptor (1,28), failed to significantly alter blood glucose levels (Fig. 2B). These results indicate that ghrelin increases blood glucose via specific interaction with GH secretagoge receptor. The hyperglycemic effect of ghrelin could...
be mediated by the well-known ability of ghrelin to release GH, a hyperglycemic hormone. However, ghrelin increased blood glucose at 30 min in GH-deficient little mice and control wild mice in a similar manner (Fig. 2). In GTT, when ghrelin at 1 and 10 nmol/kg was simultaneously injected with 1 g/kg glucose, the glucose levels at 30 and 60 min were higher and the insulin levels at 5 and 10 min markedly attenuated in comparison to control values (Fig. 2D and E).

**Effects of GH secretagogue receptor antagonists and ghrelin on ITT profiles.** Injection of 1 unit/kg i.p. insulin lowered blood glucose levels, and the time course and magnitude of the changes were indistinguishable between the ghrelin-injected, the ghrelin receptor antagonist–injected, and control mice (Fig. 2F). The results suggest that neither exogenous nor endogenous ghrelin significantly alter insulin sensitivity.

**Presence of ghrelin and ghrelin-immunoreactivity in islets.** Immunofluorescence double-staining for glucagon and the active form of ghrelin were performed in the rat pancreatic islets, and the fluorescence was measured using a multiphoton laser scanning microscope. Immunohistochemistry with antibody against glucagon demonstrated glucagon immunoreactivity (green) in the islet cells located in the periphery of islets (Fig. 3A). Immunohistochemistry with antiserum against ghrelin (position 1–11) demonstrated the immunoreactivity for acylated ghrelin (active ghrelin) (red) in a fraction of islet cells, which were observed mainly in the periphery of islets. Ghrelin-immunoreactive cells highly overlapped with glucagon-immunoreactive cells, confirming our previous report (12). Some of glucagon-immunoreactive cells were not immunoreactive to ghrelin. Furthermore, ghrelin was detected in islets using RIA; acylated ghrelin measured with antiserum against ghrelin (position 1–11) was 0.0038 ± 0.0002 fmol/islet (n = 3).

**Effects of counteraction of ghrelin by GH secretagogue receptor antagonists and anti-ghrelin antiserum on insulin release and [Ca^{2+}]_i in islets.** In isolated rat islets, [d-Lys^3]-GHRP-6 and SPA markedly increased insulin release in the presence of 5.6 mmol/l glucose, and this response was abolished in the absence of external Ca^{2+} (Fig. 3B), indicative of an appearance of Ca^{2+}-dependent insulin release by the receptor antagonists. Furthermore, antiserum against active ghrelin also increased insulin release, whereas control nonimmune serum had no effect (Fig. 3B). These results suggested that endogenous ghrelin could suppress Ca^{2+}-mediated insulin release. The insulinoostatic action of endogenous ghrelin, thus suggested, became apparent when exogenous ghrelin at relatively high concentration was administered; ghrelin at 10 nmol/l, but not 0.1 nmol/l and 1 pmol/l, attenuated 8.3 mmol/l glucose–induced insulin release in islets, whereas it had no effect at 2.8 mmol/l glucose (Fig. 3C). In contrast, 10 nmol/l ghrelin failed to alter glucagon release at both 2.8 mmol/l glucose (in pg · islet^{-1} · 30 min^{-1}; 84.0 ± 24.4, n = 10, for control vs. 82.1 ± 14.7, n = 10, with ghrelin) and 8.3 mmol/l glucose (54.9 ± 11.3, n = 10, for control vs. 49.1 ± 13.0, n = 10, with ghrelin). Glucagon release at 5.6 mmol/l glucose was not significantly altered by 1 µmol/l [d-Lys^3]-GHRP-6 (in pg · islet^{-1} · 30 min^{-1}; 63.0 ± 9.9, n = 10, for control vs. 61.4 ± 5.6, n = 10, with [d-Lys^3]-GHRP-6).

We next examined whether counteraction of the endog-
Subsequent to ghrelin-induced hyperglycemia at 30 min and its inhibition by [d-Lys³]-GHRP-6 (1 μmol/kg). Des-acyl ghrelin (10 nmol/kg) had no significant effect on blood glucose levels. C: Hyperglycemic effects of ghrelin in control wild-type (Wild) and GH-deficient little (Little) mice at 30 min (n = 8). D and E: Ghrelin-induced increases in blood glucose levels (D) and decreases in insulin levels (E) during GTT in ddY mice (n = 6–10). F: Little effects of ghrelin and GH secretagogue receptor antagonist on the profiles of ITT in mice (n = 7). *P < 0.05; **P < 0.01.

FIG. 2. Exogenously administered ghrelin increases blood glucose levels and decreases insulin levels. A: Time courses of blood glucose concentrations after intraperitoneal injections of ghrelin into male ddY mice that were fasted overnight (n = 10). B: Dose-dependency of ghrelin-induced hyperglycemia at 30 min and its inhibition by [d-Lys³]-GHRP-6 (1 μmol/kg). Des-acyl ghrelin (10 nmol/kg) had no significant effect on blood glucose levels. C: Hyperglycemic effects of ghrelin in control wild-type (Wild) and GH-deficient little (Little) mice at 30 min (n = 8). D and E: Ghrelin-induced increases in blood glucose levels (D) and decreases in insulin levels (E) during GTT in ddY mice (n = 6–10). F: Little effects of ghrelin and GH secretagogue receptor antagonist on the profiles of ITT in mice (n = 7). *P < 0.05; **P < 0.01.

Effects of ghrelin on [Ca²⁺]i and NAD(P)H in single β-cells. The above-mentioned data suggested that ghrelin elevates blood glucose levels mainly via inhibition of insulin release. To elucidate the insulinostatic mechanisms, we studied ghrelin signaling in rat single β-cells with special attention to Ca²⁺ handling. Ghrelin at a relatively high concentration of 10 nmol/l, but not 0.1 nmol/l, markedly suppressed the peak of the first-phase [Ca²⁺]i responses to 8.3 nmol/l glucose, and this effect was blocked by GH secretagogue receptor antagonist (Fig. 4A and B). Ghrelin preincubated with antiserum against active ghrelin (immunoneutralized ghrelin) had no inhibitory effect on the [Ca²⁺]i responses (Fig. 4B), confirming that the antiserum used in the present study neutralizes the activity of ghrelin. Ghrelin at 10 nmol/l (Fig. 4C, upper panel), but not at 0.1 nmol/l (data not shown), also attenuated oscillations of [Ca²⁺]i during the second-phase responses to 8.3 nmol/l glucose in oscillating β-cells. The attenuation of [Ca²⁺]i oscillations by ghrelin was abolished in the presence of GH secretagogue receptor antagonist (Fig. 4C, middle panel). The effect of ghrelin was also abolished in β-cells after exposure to 1 μM pertussis toxin, a specific inhibitor of certain subtypes of trimeric GTP-binding proteins, including Gi and Go (29), that are functional in islet β-cells (30) (Fig. 4C, lower panel). In contrast, 10 nmol/l ghrelin had no significant effect on the [Ca²⁺]i responses to 300 μmol/l tolbutamide (Fig. 4D) and 10 μmol/l acetylcholine at 2.8 mmol/l glucose (Fig. 4E). Thus, the counteracting action of ghrelin appeared to be specific for glucose, suggesting a possibility that ghrelin could interfere with the glucose metabolic pathway in β-cells. However, the increase in NAD(P)H in response to 8.3 nmol/l glucose, as monitored by its autofluorescence, was not altered by 10 nmol/l ghrelin in β-cells (Fig. 4F).
FIG. 3. Ghrelin is present in islets, and blockade of endogenous ghrelin potentiates glucose-induced insulin release and Ca\(^{2+}\) signaling in pancreatic islets. A: Immunofluorescence double-staining for glucagon (green) (left) and the active form of ghrelin (red) (center) in the rat pancreatic islet was observed using a multiphoton laser-scanning microscope. Ghrelin-immunoreactive cells overlapped with glucagon-immunoreactive cells (right). Bar = 50 μm. B: Enhancement of glucose (5.6 mmol/l)-induced insulin release by [D-Lys\(^3\)]GHRP-6 (1 μmol/l), SPA (1 μmol/l) and antiserum against ghrelin (position 1–11, 0.1%), but not nonimmune serum (0.1%), in rat islets. The effect of [D-Lys\(^3\)]GHRP-6 (1 μmol/l) was not observed in the absence of external Ca\(^{2+}\) (Ca 0; 0.1 mmol/l EGTA and no added Ca\(^{2+}\)). Tolbutamide (Tolb) is a positive control. Data represent the means ± SE of 10 experiments (n = 10). C: Inhibition by ghrelin (10 nmol/l) of insulin release in rat islets in the presence of 8.3 mmol/l, but not 2.8 mmol/l glucose (n = 10). D: GH secretagogue receptor antagonist enhanced the first-phase [Ca\(^{2+}\)] responses to 5.6 mmol/l glucose and induced [Ca\(^{2+}\)] oscillations in rat islets. Each recording was obtained from a single islet. E: Relationships for glucose concentrations and peaks of the first-phase [Ca\(^{2+}\)] responses to glucose in rat islets in the presence and absence of [D-Lys\(^3\)]GHRP-6 (1 μmol/l) (n = 18) for each point. F: Enhancement by [D-Lys\(^3\)]GHRP-6 (1 μmol/l) of the integrated [Ca\(^{2+}\)] increase in rat islets for 30 min after stimulation with 8.3 mmol/l glucose, as expressed by the area under the curve (AUC) (n = 17). G: Antiserum against ghrelin (position 1–11, 0.1%) enhanced the first-phase [Ca\(^{2+}\)] responses to 8.3 mmol/l glucose and induced [Ca\(^{2+}\)] oscillations in rat islets. Each recording was obtained from a single islet. H: Enhancement by antiserum against ghrelin (position 1–11, 0.1%) of peaks of the first-phase [Ca\(^{2+}\)] responses to 8.3 mmol/l glucose in rat islets (n = 14). *P < 0.05; **P < 0.01 vs. control; #P < 0.05 vs. nonimmune normal serum (0.1%).
Glucose and tolbutamide superfusion protocol.

**FIG. 4.** Inhibitory effects of ghrelin on glucose-induced [Ca^{2+}]_{i} increases in single rat β-cells. **A:** Effects of ghrelin (10 nmol/l) on the first-phase [Ca^{2+}]_{i} responses to 8.3 mmol/l glucose. Glucose stimulation was repetitively applied to β-cells, in which ghrelin was added 5 min before the second glucose stimulation (lower panel). **B:** The ratio of the peak Ca^{2+} response to the second 8.3 mmol/l glucose stimulation (S2) to that to the first glucose stimulation (S1). Ghrelin at 10 nmol/l, but not 0.1 nmol/l, significantly decreased the S2-to-S1 ratio, indicating inhibition of the first-phase [Ca^{2+}]_{i} responses to glucose. This inhibition of the first-phase [Ca^{2+}]_{i} response was abolished in the presence of [D-Lys^{3}]-GHRP-6 (1 μmol/l). Ghrelin, immunoneutralized by incubation with antiserum against ghrelin (position 1–11) for 1 h, had no inhibitory effect on the [Ca^{2+}]_{i} responses (n = 91–103). **C:** Effects of ghrelin (10 nmol/l) on [Ca^{2+}]_{i} oscillations during the second-phase responses to 8.3 mmol/l glucose. This attenuation of [Ca^{2+}]_{i} oscillations was abolished in the presence of [D-Lys^{3}]-GHRP-6 (1 μmol/l) and in β-cells after exposure to 1 μg/ml pertussis toxin (PTX) overnight. Ghrelin (10 nmol/l) affected neither 300 μmol/l tolbutamide (Tolb) (D) nor 10 μmol/l acetylcholine (ACh)-induced [Ca^{2+}]_{i} responses (E) at 2.8 mmol/l glucose. **F:** Ghrelin (10 nmol/l) had no effect on increases in NAD(P)H in response to 8.3 mmol/l glucose.

DISCUSSION

The present study presents evidence to support a novel role of endogenous ghrelin in islets in the regulation of...
insulin secretion. Acylated ghrelin (active ghrelin) was detected in islets both immunohistochemically and by RIA. Counteraction of the endogenous ghrelin by specific GH secretagogue receptor antagonists and antiserum against active ghrelin markedly enhanced the glucose-induced insulin release and \([\text{Ca}^{2+}]_i\) increase in islets. It was confirmed that the GH secretagogue receptor antagonist and antiserum against active ghrelin used in the present study abolished the effects of exogenously administered ghrelin on \(\beta\)-cells. Therefore, the bioactivity manifested by the GH secretagogue receptor antagonist and antiserum against active ghrelin is most likely due to ghrelin, although possible involvement of additional substances cannot be excluded. These data indicate that ghrelin in islets restricts glucose-induced insulin release presumably via a paracrine and/or autocrine route. The inhibitory action was further confirmed by the finding that administration of 10 nmol/l ghrelin suppressed glucose-induced insulin release and \([\text{Ca}^{2+}]_i\) increase in islets and \(\beta\)-cells. Although this concentration is higher than that of circulating ghrelin, which ranges from 100 pmol/l to 3 nmol/l (7,31), it is generally conceived that the level of hormone working in a paracrine/autocrine manner is higher than that working in an endocrine manner.

Another important finding of the present study is that GH secretagogue receptor antagonists increased and ex-
ogenous ghrelin decreased insulin release in vivo, in which changes in serum insulin were followed by inverse changes in blood glucose. The insulinostatic and hyperglycemic effects of ghrelin were previously reported in humans (9–11). Although only one report has by now examined the systemic effect of GH secretagogue receptor antagonists, it showed that chronic treatment with GH secretagogue receptor antagonists reduced blood glucose and increased insulin levels in ob/ob mice (27). Our results, together with these reports, indicate a systemic role of ghrelin in suppressing serum insulin and consequently elevating glucose levels. These actions may be accounted for by the insulinostatic action of the ghrelin of islet origin shown in this study, and also possibly by circulating ghrelin originating largely from the stomach. Furthermore, regarding the source of circulating ghrelin, gastrectomy in humans does not abolish but reduces circulating ghrelin to a level of 35% (3), suggesting that a minor but substantial portion is originated from organs other than stomach, which could include the pancreatic islets.

The insulin and  
\[ \text{Ca}^{2+} \text{i} \]  responses to glucose in islets were potentiated by immunoneutralization of ghrelin and ghrelin receptor blockade. This finding suggests that glucose-induced insulin release in islets is substantially restricted by endogenous ghrelin, which renovates the long-standing concept that insulin response to glucose in islets is determined largely by glucose per se. Our present and previous (12) studies have shown that ghrelin immunoreactivity is partly localized in α-cells. Studies using different experimental systems have demonstrated ghrelin immunoreactivity in α-cells (12,15), β-cells (13), PP-cells (15), and other islet cells (14,15), including those named e-cells (16).

It is therefore suggested that ghrelin may be expressed and located in different islet cell types depending upon the species, age, and condition of animals/humans. The mRNAs for ghrelin and GH secretagogue receptor are expressed in the pancreas of rats and humans (1,12,13,17) and β-cell lines (15,32). We speculate that ghrelin may exert its insulinostatic activity at least partly via the α-to β-cell paracrine route in the adult rat islets, while other paracrine and/or autocrine routes could also operate.

The present in vivo results suggest that the endogenous ghrelin upwardly controls plasma glucose. It is likely that the hyperglycemic effect of ghrelin is mainly caused by its insulinostatic action for the following reasons. Changes in blood insulin preceded those of blood glucose. In our study, neither GH secretagogue receptor antagonist nor ghrelin significantly affected glucagon release in isolated islets. No effect of ghrelin on glucagon release has also been reported in human subjects and perfused rat pancreas (9,19). Therefore, the effects of ghrelin, its antisemum, and GH secretagogue receptor antagonists on blood glucose and insulin release, both in vivo and in vitro, are not likely to be mediated by glucagon. Intravenous ghrelin administration reportedly increases circulating somatostatin levels in healthy volunteers (33). However, we found that ghrelin increased blood glucose irrespective of the presence and absence of cyclo-somatostatin, a nonspecific somatostatin receptor antagonist (34) (data not shown). Ghrelin induced hyperglycemia in GH-deficient little mice. Our ITT experiments, furthermore, showed that both GH secretagogue receptor antagonist and exogenous ghrelin failed to significantly alter the insulin action.

Taken together, it is likely that ghrelin controls blood glucose levels mainly via inhibition of insulin release, whereas neither the glucagon-, somatostatin-, nor GH-mediated pathway nor alteration of insulin sensitivity appears to have significant contribution, if any.

In apparent contradiction to our finding, no significant change of serum insulin levels in ghrelin knockout mice has recently been reported (35). However, when the data of this publication are carefully examined, there appears to be a tendency of an increase in fasting insulin levels in ghrelin knockout mice as compared with wild-type mice. Moreover, a possibility exists that a yet-unknown compensatory mechanism could have occurred in the knockout mice to maintain homeostatic regulation of insulin release and blood glucose levels because these are the life-saving functions. Conditional knockout of ghrelin specifically in the pancreatic islets and that of GH secretagogue receptor specifically in β-cells at postdevelopmental stages would be appropriate genetic approaches to address the role of ghrelin in islets.

Regarding the effects of exogenously administered ghrelin, the present in vivo hyperglycemic and insulin-lowering effects are consistent with previous studies in humans (9–11). The concentration- and glucose-related inhibitory effects on insulin release in vitro are in accordance with previous reports in perfused rat pancreas (19) and rat (32) and mouse islets (20). We found that ghrelin at 10 nmol/l, but not 1 pmol/l and 0.1 nmol/l, suppressed the [Ca2+], responses to glucose in single β-cells of rats. We previously reported that 1 pmol/l ghrelin mildly potentiated [Ca2+]i increases and insulin release induced by 8.3 nmol/l glucose (12). In this study, therefore, we carefully examined the dose-dependent effects of ghrelin with an increased number of observations and with better caution in the handling of ghrelin. We found that ghrelin at 1 pmol/l and 0.1 nmol/l modestly potentiated glucose-induced [Ca2+]i responses in a little portion of β-cells, but it failed to significantly alter insulin release as shown in Fig. 3C. This observation that ghrelin is inhibitory at relatively high concentrations of ~10 nmol/l, while having little effect at lower concentrations, is consistent with the majority of other reports (15,19,20,32). Thus, we now partly correct our previous report (12) and conclude that ghrelin at 10 nmol/l is insulinostatic, and at lower concentrations it has no significant effect on glucose-induced insulin release.

Our results demonstrate that ghrelin via GH secretagogue receptor and pertussis toxin–sensitive mechanisms suppresses glucose-induced [Ca2+], signaling in islet β-cells, which may at least partly account for the inhibition of insulin release. Thus, pertussis toxin, formerly recognized as the islet-activating protein (29), abolished the ability of ghrelin to inhibit glucose responses. This finding provides a mechanism through which islet-activating protein stimulates islets.

Ghrelin reversibly increased the amplitudes of delayed outward currents conspicuously at membrane potentials over −30 mV. The currents activated by ghrelin were sensitive to TEA and insensitive to tolbutamide. In the presence of TEA, the ability of ghrelin to attenuate glucose-induced [Ca2+], increases in β-cells was partially but significantly reduced. These findings suggest that ghrelin
may attenuate glucose-induced [Ca^{2+}]_i increases partly via activation of voltage-dependent delayed rectifier K^+ channels in association with rapid hyperpolarization of the membrane and consequent shortening of the durations of bursting action potentials. However, yet unknown mechanisms could also underlie the action of ghrelin in islet β-cells, which include interference with excytosis because ghrelin may interact with pertussis toxin–sensitive GTP-binding proteins that have been implicated in inhibition of excytotic processes (30).

In conclusion, this study reveals that endogenous ghrelin in islets restrict insulin release via the following mechanism: ghrelin directly acts on the β-cell GH secretagogue receptor and via pertussis toxin–sensitive mechanisms attenuates glucose-induced [Ca^{2+}]_i signaling partly through enhancement of TEA-sensitive currents. This insulinostatic action of ghrelin of islet origin, possibly together with that of circulating ghrelin, upwardly controls blood glucose levels. This function of ghrelin in regulating glucose metabolism, together with inducing GH release (1) and feeding (4), suggests that ghrelin underlies the integrative regulation of energy homeostasis. Possible manipulations of the ghrelin–GH secretagogue receptor system could provide novel tools to treat patients with hyperinsulinemia, type 2 diabetes, and obesity.

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