Analysis of Rat Insulin II Promoter-Ghrelin Transgenic Mice and Rat Glucagon Promoter-Ghrelin Transgenic Mice*

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We developed and analyzed two types of transgenic mice: rat insulin II promoter-ghrelin transgenic (RIP-G Tg) and rat glucagon promoter-ghrelin transgenic mice (RGP-G Tg). The pancreatic tissue ghrelin concentration measured by C-terminal radioimmunoassay (RIA) and plasma desacyl ghrelin concentration of RIP-G Tg were about 1000 and 3.4 times higher than those of nontransgenic littermates, respectively. The pancreatic tissue n-octanoylated ghrelin concentration measured by N-terminal RIA and plasma n-octanoylated ghrelin concentration of RIP-G Tg were not distinguishable from those of nontransgenic litters. RIP-G Tg showed suppression of glucose-stimulated insulin secretion. Arginine-stimulated insulin secretion, pancreatic insulin mRNA and peptide levels, β cell mass, islet architecture, and GLUT2 and PDX-1 immunoreactivity in RIP-G Tg pancreas were not significantly different from those of nontransgenic litters. Islet batch incubation study did not show suppression of insulin secretion of RIP-G Tg in vitro. The insulin tolerance test showed lower tendency of blood glucose levels in RIP-G Tg. Taking lower tendency of triglyceride level of RIP-G Tg into consideration, these results may indicate that the suppression of insulin secretion is likely due to the effect of desacyl ghrelin on insulin sensitivity. RGP-G Tg, in which the pancreatic tissue ghrelin concentration measured by C-RIA was about 50 times higher than that of nontransgenic litters, showed no significant changes in insulin secretion, glucose metabolism, islet mass, and islet architecture. The present study raises the possibility that desacyl ghrelin may have influence on glucose metabolism.

Ghrelin is a 28-amino acid peptide with unique modification of acylation, which is essential for its biological action (1). Ghrelin was originally identified in rat stomach as an endogenous ligand for an orphan receptor, which has been so far called growth hormone secretagogue receptor (GHS-R)1 (1). Ghrelin expression is detected in the stomach, intestine, hypothalamus, pituitary gland, kidney, placenta, and testis (2–6). Ghrelin is involved in a wide variety of the functions, including the regulation of growth hormone release, food intake, gastric acid secretion, gastric motility, blood pressure, and cardiac output (7–19).

Recently Date et al. (20) reported that ghrelin is present in α cells of normal human and rat pancreatic islets. Volante et al. (21) described ghrelin-expression in β cells of human islet. Wierup et al. and Prado et al. reported that ghrelin-expressing cells are a new islet cell type distinct from α, β, δ, and PP cells in human, rat, and mouse islets (22–24). Although there was no apparent change of plasma insulin levels in ghrelin null mouse (25, 26), which may indicate that ghrelin is not a direct regulator of insulin secretion in the physiological condition, there have been several reports on the effect of pharmacological dose of ghrelin on insulin secretion. Broglio et al., Egidio et al., and Reimer et al. have reported that ghrelin has an inhibitory effect on insulin secretion (27–30). Adeghate et al., Date et al., and Lee et al. have reported that ghrelin stimulates insulin secretion (20, 31, 32). Salehi et al. have reported ghrelin has both inhibitory and stimulatory effects depending on its concentration (33). Therefore, there is still a lot of controversy about the localization of ghrelin in the pancreas and the effects of ghrelin on the insulin secretion. As for the effects of desacyl ghrelin on insulin secretion, Broglio et al. (34) have reported that acute desacyl ghrelin administration has no effect on insulin secretion in human but that it counteracts the inhibitory effect of n-octanoylated ghrelin on insulin secretion when co-administered with n-octanoylated ghrelin (35).

Here we developed and analyzed two types of transgenic mice: rat insulin II promoter-ghrelin transgenic mice (RIP-G Tg) and rat glucagon promoter-ghrelin transgenic mice (RGP-G Tg). The purpose of this study was to clarify the effect of transgenic overexpression of ghrelin cDNA in pancreatic islets.

EXPERIMENTAL PROCEDURES

Generating RIP- and RGP-ghrelin Transgenic Mice—Mouse stomach cDNA library was constructed from 1 μg of mouse stomach poly(A)+

The abbreviations used are: GHS-R, growth hormone secretagogue receptor; RIP-G Tg, rat insulin II promoter-ghrelin transgenic; RGP-G Tg, rat glucagon promoter-ghrelin transgenic mice; RIA, radioimmunoassay; C-RIA, anti-ghrelin [13–28] antisemur; N-RIA, anti-ghrelin [1–11] antiserum; RT, reverse transcription; HDL, high density lipoprotein; PP, pancreatic polypeptide.
RNA with a cDNA synthesis kit (Amersham Biosciences). Mouse ghrelin cDNA was isolated from this library, using rat ghrelin cDNA as a probe. A fusion gene comprising RIP and mouse ghrelin cDNA coding sequences was designed. The purified fragment (10 μg/ml, 352 kDa injected into the pronucleus of fertilized C57/B6J mice (SCL, Shizuoka, Japan) eggs. The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (SCL) using standard techniques. Transgenic founder mice were identified by Southern blot analysis of tail DNAs using the mouse ghrelin cDNA fragment as a probe. RGP-G Tg was generated similarly. Transgenic mice were used as heterozygous. Animals were maintained on standard rat food (CE-2, 352 kcal/ 100 g, Japan CLEA, Tokyo, Japan) on a 12-h light/12-h dark cycle. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

**Immunohistochemistry—** Formalin-fixed, paraffin-embedded tissue sections were immunostained using the avidin-biotin peroxidase complex method (Vector Laboratories, Burlingame, CA) as described previously (36). Serial sections were used, and the thickness of each section was 5 μm. Sections were incubated with anti-C-terminal ghrelin [1–11] (1:2000) (1), which recognizes the n-octanoylated portion of ghrelin, anti-glucagon (1:500), anti-insulin (1:500), anti-somatostatin (1:500), anti-pancreatic polypeptide (PP, 1:500), DAKO, Glostrup, Denmark), anti-glucagon (1:2000, kindly provided by Christoph T. Lorenz, Wright) (37), and anti-GLUT2 (1:200, kindly provided by Bernard Thorens) (38) antisera. Quantification of β cell area was performed in insulin-stained sections by using Axio Vision (Carl Zeiss, Hallbergmoos, Germany) and Scion Image (Scion Corp., Frederick, MD). Ten sections (200-μm interval) for each mouse (n = 5) were analyzed. The percentage of β cell area in the pancreas was determined by dividing the area of all insulin-positive cells in one section by the total area of the section.

**Measurements of Plasma and Tissue Ghrelin Concentrations—** Plasma was sampled from 10-week-old RGP-G Tg and their nontransgenic littermates under ad libitum feeding states considering the promoter activity. From RGP-G Tg and their littermates, it was sampled after overnight fast. Blood was withdrawn from the retroorbital vein or the proximal end of the portal vein under ether anesthesia, immediately trypsinized in acid-ethanol, and then dissolved in 0.1N HCl containing Acetic acid was added to each solution so that the final concentration of HCl was 0.1M and the final concentration of acetic acid was 10% (v/v). 10-fold v/w of water. Acetic acid was added to each solution so that the final concentration of HCl was 0.1M and the final concentration of acetic acid was 10% (v/v).

**Distribution of Ghrelin in Normal Mouse Pancreas—** Most of the ghrelin-positive cells were glucagon-positive by serial sections. Quantitative PCR was performed with ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The following primers and TaqMan probes were used: mouse GH (sense, 5′-CACCAACTCCTCTCTACCTACATGCACATTTGTTGAC-3′); antisense, 5′-GCATGAACTGGAGAGTGTGGG-3′; mouse ghrelin (sense, 5′-CACCATCTGCTTATCTTCTTGAGTGGACATGT-3′); antisense, 5′-TGGTGCGCTTGGATCTTCTGCCT-3′; TaqMan probe, 5′-GGCAGAACGCAGACAGAGCGAGCAAGGGTGCAGG-3′. Lipid Measurements—Blood was collected from the retroorbital vein or the portal vein of 35-week-old RGP-G Tg and their nontransgenic littermates. After separation of serum, total cholesterol, triglyceride, free fatty acid, and HDL-cholesterol levels in serum were determined by Cholesterol E-test (Wako, Tokyo, Japan), Triglyceride E-test Wako, NEFA C-test Wako, and HDL-cholesterol E-test Wako (Wako Pure Chemical Industries, Osaka, Japan).

**Statistical Analysis—** All values were expressed as means ± S.E. Statistical significance of mean values was assessed by repeated measures analysis of variance or Student’s t-test.

**RESULTS**

**Distribution of Ghrelin in Normal Mouse Pancreases—** We first examined which cell type of islet cells expresses ghrelin in mouse by immunohistochemistry using anti-C-terminal ghrelin antisem. In the most of the islets no ghrelin-like immunoreactivity was detected. C-terminal ghrelin-like immunoreactivity was observed in the periphery of minor proportion of islets of wild type mice (Fig. 1A). Most of the ghrelin-positive cells were also glucagon-positive by serial section analysis (Fig. 1B), whereas most of the glucagon-positive cells were not ghrelin-positive.

**Generation of RIP- and RGP-ghrelin Transgenic Mice—** A fusion gene comprising RIP and mouse ghrelin cDNA coding sequences was designed so that ghrelin expression might be targeted to the pancreatic β cells (Fig 2A). The ghrelin mRNA level of RGP-G Tg in pancreas determined by quantitative RT-PCR was about 215 times higher than that of nontransgenic littermates (215.3 ± 40.6 versus 1.0 ± 0.025 arbitrary units, n = 5, p < 0.01). There was also an increase in ghrelin mRNA levels in brain of RGP-G Tg (242.6 ± 17.6 versus 89.1 ± 27.3 arbitrary unit, n = 5, p < 0.01). To confirm the expression of ghrelin transgene in pancreatic β cells, we performed an immunohistochemical analysis using anti-C-terminal ghrelin antisem. C-terminal ghrelin-like immunoreactivity was observed in the near whole area of the islets of the RIP-G Tg (Fig. 2C), whereas it was only seen in the periphery of the islets.
of their nontransgenic littermates (Fig. 1A). Immunohistochemical analysis using anti-N-terminal ghrelin antisera showed the same staining pattern (Fig. 2D), indicating that n-octanoylated ghrelin may be produced in H9252 cells of this transgenic mouse. We also stained the brain section of RIP-G Tg. No ghrelin-like immunoreactivity was detected either with anti-C-terminal or anti-N-terminal ghrelin antisera (data not shown).

The pancreatic tissue ghrelin concentration of RIP-G Tg measured by C-RIA was about 1000 times higher than that of their nontransgenic littermates (1024 ± 108.9 fmol/mg versus 1.2 ± 0.1 fmol/mg, n = 5, p < 0.01). This concentration was about one third of the nontransgenic stomach concentration (3558.1 ± 51.0 fmol/mg, n = 5). The pancreatic tissue ghrelin concentration of RIP-G Tg measured by N-RIA tended to be also higher than that of their nontransgenic littermates (0.054 ± 0.017 fmol/mg versus 0.038 ± 0.006 fmol/mg, n = 5, NS; not significant), but it did not reach statistical significance. Plasma desacyl ghrelin concentration of RIP-G Tg was about 3.4 times higher than that of nontransgenic littermates under the ad libitum feeding states (2805.5 ± 236.4 versus 825.9 ± 244.4 fmol/ml, n = 5, p < 0.01, Fig. 2G). We also measured desacyl ghrelin levels in portal vein of the mice. In the nontransgenic mice, the portal desacyl ghrelin level was significantly higher than that in retroorbital vein (1108.0 ± 257.3 fmol/ml versus 825.9 ± 244.4 fmol/ml, n = 5, p < 0.05, Fig. 2G). The desacyl ghrelin concentration collected from portal vein of RIP-G Tg at the same time was much higher than that of nontransgenic littermates (3671.8 ± 328.6 versus 1108.0 ± 257.3 fmol/ml, n = 5, p < 0.01, Fig. 2G). The step-up of desacyl ghrelin concentration from retroorbital vein to portal vein of RIP-G Tg was significantly higher than that of nontransgenic littermates (866.3 ± 182.2 fmol/ml versus 262.9 ± 59.8 fmol/ml, p < 0.01, Fig. 2H). Plasma n-octanoylated ghrelin levels in retroorbital and portal vein of RIP-G Tg tended to be higher than those of their nontransgenic littermates (retroorbital: 78.5 ± 13.4 versus 66.1 ± 7.1 fmol/ml, n = 5, NS; portal: 104.6 ± 15.3 versus...
71.4 ± 9.0 fmol/ml, n = 5, NS), but it did not reach statistical significance.

We also generated RGP-G Tg, in which ghrelin expression is targeted to the pancreatic α cells (Fig. 2B). The ghrelin mRNA level in pancreas of RGP-G determined by quantitative RT-PCR was about 16 times higher than that of nontransgenic litters (16.3 ± 1.7 versus 1.0 ± 0.24 arbitrary unit, n = 5, p < 0.01). The ghrelin mRNA level in duodenum of RIP-G Tg was not statistically different from that of nontransgenic litters (520.1 ± 111.1 versus 379.1 ± 37.6 arbitrary unit, n = 5, NS). The ghrelin mRNA level in brain of RGP Tg was not distinguishable from that of nontransgenic litters (72.0 ± 6.4 versus 71.8 ± 7.8 arbitrary unit, n = 5, NS). Immunohistochemical analysis showed ghrelin-like immunoreactivity in the periphery of the pancreatic islet of RGP-ghrelin transgenic mouse by both anti-C-terminal ghrelin and anti-N-terminal ghrelin antiserum (Fig. 2E and F). The pancreatic tissue ghrelin concentrations of RGP-G Tg measured by C-RIA were about 50 times higher than those of their nontransgenic litters (48.9 ± 2.5 fmol/mg versus 1.2 ± 0.1 fmol/mg, n = 5, p < 0.01). The pancreatic tissue ghrelin concentration of RGP-G Tg measured by N-RIA tended to be higher than that of their nontransgenic litters (0.076 ± 0.019 fmol/mg versus 0.038 ± 0.006 fmol/mg, n = 5, NS), but it did not reach statistical significance. The plasma desacyl ghrelin concentrations in retroorbital vein were not elevated in RGP-G Tg after overnight fasting compared with nontransgenic litters (661.6 ± 38.0 versus 1024.7 ± 27.1 fmol/ml, n = 5). The portal desacyl ghrelin concentrations of RGP-G Tg were also indistinguishable from those of their nontransgenic litters (1320.6 ± 164.7 versus 1442.9 ± 361.5 fmol/ml, n = 5, NS). Plasma n-octanoylated ghrelin levels in retroorbital and portal vein of RGP-G Tg were indistinguishable from those of their nontransgenic litters (retroorbital: 98.3 ± 18.7 versus 133.5 ± 25.3 fmol/ml, n = 5, NS; portal: 154.3 ± 20.7 versus 198.9 ± 34.9 fmol/ml, n = 5, NS).

Body Weight, Food Consumption, and Percent Body Fat—There was no significant difference in body weight and food intake between RIP-G Tg and their nontransgenic litters (Fig. 3). Percent body fat and visceral/subcutaneous fat ratio of RIP-G Tg were not different from those of nontransgenic litters (Fig. 2, C and D). No significant changes were observed in RGP-G Tg, either (data not shown).

Glucose Metabolism and Insulin Secretion—Although no significant differences in blood glucose levels were noted between RIP-G Tg and their nontransgenic litters on the fasting state and intraperitoneal glucose tolerance tests (Fig. 4, A and C), plasma insulin levels 2 and 30 min after the glucose injection were significantly decreased in RIP-G Tg compared with those in their nontransgenic littermates (Fig. 4D). Suppression of insulin secretion was not observed in RIP-G Tg on intraperitoneal injection of arginine (Fig. 4G). Blood glucose level of RIP-G Tg in the insulin tolerance test tended to be lower than those of their nontransgenic litters, but it did not reach statistical significance (Fig. 4H).

No significant differences in blood glucose or insulin levels were observed between RGP-G Tg and their nontransgenic litters on the fasting state, ad libitum feeding, or intraperitoneal glucose or arginine injection (Fig. 4, B, E, and F, and data not shown). Blood glucose levels on insulin tolerance test showed no differences between RGP-ghrelin and their nontransgenic litters (data not shown).

Islet Architecture and β Cell Mass—We studied the tissue sections of RIP-G Tg to explore the effect of ghrelin on the islet architecture and β cell mass. There were no obvious abnormalities in the intra islet cytoarchitecture and cell number of insulin, glucagon, somatostatin, and PP cells in the islets of the RIP-G Tg (Fig. 5A–D). The intensity of staining of these four islet hormones in the islets of the RIP-G Tg was not apparently different from those of nontransgenic litters. The ratio of the β cell area to whole pancreas was not changed significantly.
We also studied the tissue sections of RGP-G Tg and found no significant differences (Fig. 5, E–H, and J).

Expression of Insulin mRNA and Insulin Content—Because RIP-G Tg showed suppression of insulin secretion, we examined pancreatic mRNA expression and peptide content of insulin in RIP-G Tg and their nontransgenic littermates by Northern blot analysis and RIA. The insulin mRNA in RIP-G Tg did not differ from those of their nontransgenic littermates (Fig. 6, A and B). No significant differences of insulin contents were observed between RIP-G Tg and their nontransgenic littermates (Fig. 6).

PDX-1 and GLUT2 Immunoreactivity—We examined the immunoreactivity of PDX-1 and GLUT2 in RIP-G Tg. The staining intensities of PDX-1 and GLUT2 in the RIP-G Tg (Fig. 7, A

(Fig. 5I). We also studied the tissue sections of RGP-G Tg and found no significant differences (Fig. 5, E–H, and J).

Expression of Insulin mRNA and Insulin Content—Because RIP-G Tg showed suppression of insulin secretion, we examined pancreatic mRNA expression and peptide content of insulin in RIP-G Tg and their nontransgenic littermates by Northern blot analysis and RIA. The insulin mRNA in RIP-G Tg did not differ from those of their nontransgenic littermates (Fig. 6, A and B). No significant differences of insulin contents were observed between RIP-G Tg and their nontransgenic littermates (Fig. 6).

PDX-1 and GLUT2 Immunoreactivity—We examined the immunoreactivity of PDX-1 and GLUT2 in RIP-G Tg. The staining intensities of PDX-1 and GLUT2 in the RIP-G Tg (Fig. 7, A
and C) were not apparently different from those in the nontransgenic littermates (Fig. 7, B and D).

Expression of GHS-R mRNA—To rule out possible downregulation of GHS-R due to chronic exposure to high level ghrelin, we measured the expression level of GHS-R mRNA in pancreas and pituitary by real-time quantitative RT-PCR.

**Fig. 5. Islet morphology and β cell area in RIP-G Tg (A–D) and RGP-G Tg (E–H).** The sections were stained with anti-insulin (A and E), anti-glucagon (B and F), anti-somatostatin (C and G), and anti-PP antiserum (D and H). I and J, the ratio of β cell area to that of whole section in RIP-G Tg (I) and RGP-G Tg (J). non, nontransgenic littermates; Tg, RIP-G Tg; NS, not significant.
There were no significant differences in GHS-R mRNA levels between RIP-G Tg and their nontransgenic littermates either in pancreas (Fig. 8A) or in pituitary (Fig. 8B).

**Batch Incubation of Islets**—The insulin secretion from isolated islet of RIP-G Tg by batch incubation was indistinguishable from that of nontransgenic littermates, in 3.3 or 8.7 or 16.7 mM glucose conditions (Fig. 9).

**Lipid Metabolism**—Plasma total cholesterol level of RIP-G Tg tended to be lower than those of nontransgenic littermates, but it did not reach statistical significance (total cholesterol: 85.4 ± 6.9 versus 79.4 ± 7.5 mg/dl, n = 6, NS). The plasma triglyceride level of RIP-G Tg tended to be lower than that of nontransgenic littermates, but it did not reach statistical significance (154.5 ± 11.0 versus 136.9 ± 10.3 mg/dl, n = 6, NS). Free fatty acid level and HDL-cholesterol level of RIP-G Tg were not significantly different from those of nontransgenic littermates (free fatty acid; 0.44 ± 0.05 versus 0.48 ± 0.07 mEq/liter, n = 6, NS, HDL-cholesterol; 46.1 ± 2.3 versus 44.9 ± 3.4 mg/dl, n = 6, NS).

**DISCUSSION**

In wild-type mice, no ghrelin-like immunoreactivity was detected in most of the islets. C-terminal ghrelin-like immunoreactivity was observed in the periphery of minor proportion of islets of wild type mice, which is consistent with a previous report (24). By the serial section analysis, most of the ghrelin-producing cells also showed glucagon-like immunoreactivity. These findings indicate that ghrelin was expressed in minor proportion of mouse pancreatic β cells. Expression of ghrelin was not detected in pancreatic β cells of wild type mice.

In the present study we developed RIP-G Tg, in which pancreatic ghrelin concentration measured by C-RIA was ~1000 times higher than that of nontransgenic littermates. By immunohistochemistry using anti-C-terminal ghrelin [13–28] antisera we detected C-terminal ghrelin-like immunoreactivity in almost the whole area of islets. Therefore, because ghrelin was not detected in β cells of control mice by immunohistochemistry, ghrelin transgene driven by RIP was considered to be expressed in β cells.

We also found about 3 times higher expression level of ghrelin mRNA in the brain of RIP-G Tg compared with that of nontransgenic littermates, which could not be detected by immunohistochemistry. Although a small amount of ghrelin has been reported to be expressed in brain, which can be detected by immunohistochemistry only after colchicine treatment (1), there have been controversies as to whether this small amount of ghrelin in the brain has a biological role. Because the food intake of RIP-G Tg was not different from that of nontransgenic littermates, the ghrelin produced by transgene in the brain seems not to show bioactive effect of n-octanoylated ghrelin.

By immunohistochemistry using anti-ghrelin [1–11] anti-
The triglyceride levels of our RIP-G Tg only showed lower tendency compared with that of nontransgenic littermates. The lack of small phenotype and milder phenotype of lipid metabolism in RIP-G Tg may result from the fact that plasma desacyl ghrelin level of RIP-G Tg was only 3.4 times higher than those of nontransgenic littermates.

The tissue sections of the pancreas of these transgenic mice showed no apparent disarrangement in the islet architecture and in β cell mass. There have been several reports on the transgenic mice overexpressing humoral factors in the β cells, such as parathyroid hormone-related peptide, hepatocyte growth factor, and insulin-like growth factor-I (46–49). Some of these transgenic mice showed islet hypertrophy or disarrangement of the endocrine cells in the islet (46–49). Our observation showed that desacyl ghrelin might have no apparent effects on the islet architecture and β cell mass.

In the present study plasma insulin levels after the 3.0 g/kg glucose injection were significantly lower in RIP-G Tg than those in nontransgenic littermates, although there was no significant difference in plasma insulin levels between RIP-G Tg and nontransgenic littermates on the fasting state. To rule out the decreased production of insulin caused by exogenous insulin promotor, we measured insulin mRNA level and content in the pancreata of our transgenic mice. The insulin mRNA level and content from the transgenic mice were not significantly different from those from nontransgenic littermates. Therefore, the insulin production might not be disturbed in these mice either in transcriptional or translational levels. The immunoreactivity of PDX-1, which is the master regulator of the pancreas development and essential for insulin transcription, in RIP-G Tg β cell was not different from that in β cells of nontransgenic littermates. These results suggest that the suppression of glucose-stimulated insulin secretion in RIP-G Tg might not be due to the transcriptional deregulation of insulin caused by injection of exogenous insulin promotor.

RIP-G Tg did not show decreased-insulin secretion in response to arginine. Arginine is known to stimulate insulin secretion by the mechanisms that are different from those used by glucose, although the detail remains controversial (50, 51). However, it seems certain that arginine somehow evoked Ca2+ influx into the β cell, and that leads to the exocytosis of insulin-containing vesicles (52, 53). So at least, the decreased insulin secretion in RIP-G Tg might not be due to disorders in exocytosis process. Egido (28) reported that ghrelin inhibits insulin secretion from rat pancreas in response to arginine in vitro, however, there has been no report on the effect of desacyl ghrelin on arginine-induced insulin secretion.

The immunoreactivity of GLUT2, glucose transporter in the pancreatic β cell, in RIP-G Tg β cells, was indistinguishable from that in the β cells of nontransgenic littermates. Although immunohistochemistry is not so suitable for quantitative analysis, at least no apparent decreased expression or disposition of GLUT2 in RIP-G Tg β cell exists. Chronic exposure to the high level of desacyl ghrelin may not influence on GLUT2 expression. We performed a batch incubation study of RIP-G Tg islet. The insulin secretion from isolated islets of RIP-G Tg was indistinguishable from that of nontransgenic littermates. This finding indicates that insulin secretion was not affected by overexpression of ghrelin transgene in vitro but was affected in vivo. The different observations in vitro and in vivo may be explained by dilution of ghrelin produced by transgene with the incubation buffer. Alternatively, suppression of insulin secretion of RIP-G Tg was not due to the effect of desacyl ghrelin on serum that recognizes the n-octanoylated portion of ghrelin, ghrelin-like immunoreactivity was also demonstrated in nearly whole area of islets of RIP-G Tg, indicating the production of n-octanoylated ghrelin in β cells. This finding indicates that the mechanism of acylation may exist not only in pancreatic α cells but also in β cells. This is reasonable, because α and β cells are pancreatic endocrine cells derived from common precursor cells (40). Because the N-RIA/C-RIA ratio of the pancreatic tissue ghrelin concentration of RIP-G Tg was much lower than that of the stomach (0.0053% versus 11.67%, p < 0.01), the ability of acylation in β cell might be lower than that of ghrelin-producing cell in the stomach (X/A-like cell). It is possible that exocrine pancreatic enzymes might interfere with the results, although these were inactivated by boiling before extraction. The other possibility is that because of the formalin fixation of ghrelin in the tissue section the epitope recognized by immunohistochemistry using anti-ghrelin [1–11] antiserum might not be exactly the same as that recognized by N-RIA or enzyme-linked immunosorbent assay. Because the amount of n-octanoylated ghrelin was so little that it could not be detected by RIA if any, we considered that the phenotype of these transgenic mice is due to the effect of desacyl ghrelin. Desacyl ghrelin has been shown not to activate GHS-R (39). There have been several reports saying that desacyl ghrelin has biological activities, such as promoting adipogenesis (41), inhibition of cell proliferation, inhibition of apoptosis (42), and counteracting the effect of n-octanoylated ghrelin (35).

We showed here that the ghrelin level in portal vein is significantly higher than that in retroorbital vein in wild type mouse. Ghrelin has been reported to be mainly synthesized in stomach and intestine. The step-up of plasma ghrelin level in gastric vein has been reported previously (43), but there has been no report showing the step-up of plasma ghrelin level in portal vein as compared with that in systemic circulation. The present study is the first report of the step-up of plasma ghrelin levels in portal vein. Moreover, the step-up of desacyl ghrelin in RIP-G Tg was much higher than that in control littermates, indicating overproduction of desacyl ghrelin by transgene in the pancreas.

The body weight, percent body fat, and food consumption of RIP-G Tg were not significantly different from those of nontransgenic littermates. Recently, we and Asakawa et al. have reported the studies of β-actin promoter ghrelin transgenic mouse (44, 45), in which plasma desacyl ghrelin levels were 30 and 50 times higher than those of their nontransgenic littermates. These transgenic mice were reported to show small phenotype, although some discrepancy of interpretation regarding on etiology exists. Asakawa et al. reported that the triglyceride level of β-actin promoter ghrelin transgenic mouse was lower, but that cholesterol level and free fatty acid level were not changed compared with their nontransgenic littermates. The triglyceride levels of our RIP-G Tg only showed lower tendency compared with that of nontransgenic littermates. The lack of small phenotype and milder phenotype of lipid metabolism in RIP-G Tg may result from the fact that plasma desacyl ghrelin level of RIP-G Tg was only 3.4 times higher than those of nontransgenic littermates.

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RIP-G Tg did not show decreased-insulin secretion in response to arginine. Arginine is known to stimulate insulin secretion by the mechanisms that are different from those used by glucose, although the detail remains controversial (50, 51). However, it seems certain that arginine somehow evoked Ca2+ influx into the β cell, and that leads to the exocytosis of insulin-containing vesicles (52, 53). So at least, the decreased insulin secretion in RIP-G Tg might not be due to disorders in exocytosis process. Egido (28) reported that ghrelin inhibits insulin secretion from rat pancreas in response to arginine in vitro, however, there has been no report on the effect of desacyl ghrelin on arginine-induced insulin secretion.

The immunoreactivity of GLUT2, glucose transporter in the pancreatic β cell, in RIP-G Tg β cells, was indistinguishable from that in the β cells of nontransgenic littermates. Although immunohistochemistry is not so suitable for quantitative analysis, at least no apparent decreased expression or disposition of GLUT2 in RIP-G Tg β cell exists. Chronic exposure to the high level of desacyl ghrelin may not influence on GLUT2 expression. We performed a batch incubation study of RIP-G Tg islet. The insulin secretion from isolated islets of RIP-G Tg was indistinguishable from that of nontransgenic littermates. This finding indicates that insulin secretion was not affected by overexpression of ghrelin transgene in vitro but was affected in vivo. The different observations in vitro and in vivo may be explained by dilution of ghrelin produced by transgene with the incubation buffer. Alternatively, suppression of insulin secretion of RIP-G Tg was not due to the effect of desacyl ghrelin on

![Batch incubation study of isolated islets of RIP-G Tg (Tg) and their nontransgenic littermates (non).](http://www.jbc.org/)

**FIG. 9.** Batch incubation study of isolated islets of RIP-G Tg (Tg) and their nontransgenic littermates (non).
insulin secretion from β cell but on insulin sensitivity. Recently Gauna et al. (55) reported that co-administration of desacyl ghrelin and active ghrelin improves insulin sensitivity in humans (54) and that desacyl ghrelin suppresses glucose output from liver. Although an insulin tolerance test did not show a statistically significant difference in blood glucose levels between RIP-G Tg and their nontransgenic littermates, there was a tendency for lower blood glucose levels of RIP-G Tg. Moreover, plasma triglyceride levels of RIP-G Tg showed lower tendency. Taken together, these results may indicate that desacyl ghrelin may improve insulin sensitivity of RIP-G Tg. The suppression of insulin secretion of RIP-G Tg is likely due to the effect of desacyl ghrelin on insulin sensitivity.

To explore if chronic exposure to high level desacyl ghrelin may influence the expression level of GHS-R, we investigated the mRNA level of GHS-R in the pancreas and pituitary of RIP-G Tg. No significant differences were found in GHS-R mRNA levels in pancreas or in pituitary between RIP-G Tg and their nontransgenic littermates. These findings indicate that chronic exposure to high level desacyl ghrelin might not influence the GHS-R mRNA expression level.

We also developed RGP-G Tg. The pancreatic tissue ghrelin concentrations determined by C-RIA of RGP-G Tg were about 50 times higher than those of their nontransgenic littermates, indicating that ghrelin was overexpressed in RGP-G Tg. However, there was no obvious phenotype regarding insulin secretion and pancreatic morphology. Considering the observation that portal ghrelin levels were not elevated in RGP-G Tg condition and pancreatic morphology. Considering the observation indicating that ghrelin was overexpressed in RGP-G Tg. How-
Analysis of Rat Insulin II Promoter-Ghrelin Transgenic Mice and Rat Glucagon Promoter-Ghrelin Transgenic Mice
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