Pancreatic β-cell overexpression of the glucagon receptor gene results in enhanced β-cell function and mass

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Gelling RW, Vuguin PM, Du XQ, Cui L, Rømer J, Pederson RA, Leiser M, Sørensen H, Holst JJ, Fledelius C, Johansen PB, Fleischer N, McIntosh CH, Nishimura E, Charron MJ. Pancreatic β-cell overexpression of the glucagon receptor gene results in enhanced β-cell function and mass. Am J Physiol Endocrinol Metab 297: E695–E707, 2009. First published July 14, 2009; doi:10.1152/ajpendo.00082.2009.—In addition to its primary role in regulating glucose production from the liver, glucagon has many other actions, reflected by the wide tissue distribution of the glucagon receptor (Gcgr). To investigate the role of glucagon in the regulation of insulin secretion and whole body glucose homeostasis in vivo, we generated mice overexpressing the Gcgr specifically on pancreatic β-cells (RIP-Gcgr). In vivo and in vitro insulin secretion in response to glucagon and glucose was increased 1.7- to 3.9-fold in RIP-Gcgr mice compared with controls. Consistent with the observed increase in insulin release in response to glucagon and glucose, the glucose excursion resulting from both a glucagon challenge and intraperitoneal glucose tolerance test (IPGTT) was significantly reduced in RIP-Gcgr mice compared with controls. However, RIP-Gcgr mice display similar glucose responses to an insulin challenge. β-cell mass and pancreatic insulin content were also increased (20 and 50%, respectively) in RIP-Gcgr mice compared with controls. When fed a high-fat diet (HFD), both control and RIP-Gcgr mice developed similar degrees of obesity and insulin resistance. However, the severity of both fasting hyperglycemia and impaired glucose tolerance (IGT) were reduced in RIP-Gcgr mice compared with controls. Furthermore, the insulin response of RIP-Gcgr mice to an IPGTT was twice that of controls when fed the HFD. These data indicate that increased pancreatic β-cell expression of the Gcgr increased insulin secretion, pancreatic insulin content, β-cell mass, and, when mice were fed a HFD, partially protected against hyperglycemia and IGT.

counterregulatory hormone to insulin, stimulating hepatic glucose production by increasing glycogenolysis and gluconeogenesis, while inhibiting glycogen synthesis. In addition, glucagon has many other extrahepatic effects, including a possible role in intraislet hormone regulation by potentiating glucose-induced insulin secretion from pancreatic β-cells, stimulating somatostatin release from pancreatic δ-cells, and inhibiting its own release from pancreatic α-cells (5, 28). However, there is some controversy as to the physiological relevance of these actions (8), given that in many studies pharmacological levels were used. Furthermore, it is unclear if the direction of blood flow through the islets would expose the β-cell core to hormones (such as glucagon) released by the outer peripheral non-β-cells (3, 4, 23).

The glucagon receptor (Gcgr) specifically mediates all actions of glucagon. The Gcgr genes cloned from rat (24), human (35), and mouse (6) have been found to belong to the superfamily of G-protein-coupled receptors (GPCRs). Sequence homology analysis indicates that the Gcgr falls into a smaller subset of the GPCRs known as the vaso-intestinal peptide/gluca gon/secretin receptor family (49). Although all of these receptors activate adenylyl cyclase, many have also been shown to stimulate alternative intracellular second messenger pathways such as the stimulation of phospholipase C and the release of intracellular Ca2+ stores (17, 20, 34).

Hyperglucagonemia and increased basal hepatic glucose production are characteristics of type 2 diabetes. In fact, hyperglucagonemia was found to be a good predictive measurement of the development of glucose intolerance in a study of postmenopausal women (32). However, the cause of this hyperglucagonemia has not been determined (33), and it is also unclear how elevated glucagon levels present in type 2 diabetic individuals may affect other glucagon-regulated tissues. Interestingly, a single point mutation (gly40ser) that has been associated with diabetes in Sardinian and French populations (18) has been shown by us to result in a receptor with decreased glucagon responsiveness in both signaling and insulin secretion (19). This paradoxical observation suggests that defective Gcgr signaling in the pancreatic β-cell may be a contributing factor to the diabetic state.

In the present study, the effect of increasing β-cell sensitivity to glucagon was examined in mice overexpressing the Gcgr

GLUCAGON IS A 29-amino-acid pancreatic hormone that is derived from tissue-specific processing of proglucagon such that glucagon is produced in the α-cells of the islets of Langerhans, whereas in the intestinal L cells the structurally related peptides glucagon-like peptide-1 and -2 (GLP-1 and -2) are the major biological product of the same precursor (5, 10, 33). Glucagon is secreted from the pancreatic α-cells in the portal blood supply in response to hypoglycemia and acts as the major

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gene under the regulation of the β-cell-specific rat insulin promoter (RIP-Gcgr). This approach allowed us to closely examine the role of increased glucagon action on insulin secretion and content, β-cells mass, and glucose homeostasis. Finally, we determined whether increased pancreatic islet Gcgr expression affected the ability of a high-fat diet (HFD) to induce diabetes in mice.

MATERIALS AND METHODS

Construction of the RIP-Gcgr transgene and generation of transgenic mice. To obtain the coding region of the mouse Gcgr from a 11.4-kb genomic clone previously described (6), a 649-bp Pst I/Nsi I fragment containing exons one and two was ligated to a 3,569-bp Nsi I/Hind III fragment containing exons 3–13 in pGEM9Z + and designated pL7. The 695-bp Bam HI/Xba I fragment of the rat insulin II promoter (12) from RIP-I/AP (kind gift from Dr. S. Efrat, Albert Einstein College of Medicine) was ligated into the same sites of pGEM11Z + (designated pGEM11-RIP). The entire coding region of the Gcgr was then ligated 3' of the RIP promoter as a Not I/Hind III fragment in the same sites in pGEM11-RIP (designated pL88). A hybrid SV40 intron/SV40 polyadenylation signal sequence (48) was subcloned into the Hind III/Not I sites of pGEM9Z +. This allowed the RIP promoter Gcgr 5'I/Hind III fragment from pL88 to be ligated 3’ of the SV40 intron/polycadenylation signal. The resulting construct was designated pRIP-Gcgr-pA. The transgene was liberated from the plasmid backbone by digestion with Sal I/Spe I and injected in the pronucleus of fertilized C57BL/6/J × CBA F2 mouse eggs. Ten transmitting transgenic founders were identified by PCR as previously described (48). All animals were fed ad libitum and maintained in a murine hepatitis virus-free barrier facility on a 14:10-h light-dark cycle. In most of the studies described here, F2 and F3 mice backcrossed on the C57BL/6 × CBA F2 mouse background were used, whereas mice used for the HFD study were backcrossed at least eight times on the C57BL/6/J background. All studies were carried out with sex- and age-matched RIP-Gcgr transgenics and RIP-Gcgr-mice. The samples were normalized to ACTB and Hprt1 gene expression using the ΔΔCt value method (51).

Intraperitoneal glucose tolerance test, insulin tolerance test, glucagon, and intravenous arginine challenge. After a 6-h [insulin tolerance test (ITT) and glucagon challenge (GC)] or 12- to 16-h fast [intraperitoneal glucose tolerance test (IPGTT)], conscious mice were challenged with either 16 μg/kg of human glucagon, 0.75 U/kg of porcine insulin, or a glucose load of 1.5 g/kg, administered intraperitoneally. For the arginine challenge, 0.25 g/kg of arginine in PBS was administered intravenously via the tail vein. Tail blood was taken at the times indicated, and blood glucose levels were determined using a OneTouch II glucose meter (LifeScan, Milpitas, CA). In experiments involving anesthesia (glucagon and arginine challenge), mice were fasted for 12–16 h and then received 12.5 mg/kg dormicum (Hoffman-LaRoche) and 25 mg/kg fluanison and 0.78 mg/kg fentanyl citrate (Hypnorm; Janssen) intraperitoneally. Blood (30–50 μl) was drawn at designated times from the retro-orbital sinus using microcapillary tubes and spun at 5,000 g to obtain plasma.

Analysis of serum metabolites and hormones. Blood samples from fed animals were obtained between 12:00 and 2:00 AM, while blood samples from fasted animals were obtained 12 h following food removal. Blood was drawn from the retro-orbital sinus using microcapillary tubes and spun at 5,000 g to obtain plasma. Plasma lactate was measured using a kit from Sigma Chemical. Free fatty acid levels were determined using a kit from Wako Chemicals. Clinical chemistries were run on a Synchron CX5 Autoanalyzer (Beckman Instruments). Triglycerides (TG), cholesterol, and high-density lipoprotein (HDL) were determined using standard human kits. Low-density lipoprotein (LDL) was analyzed by a user-defined method (Diagnostic Chemicals). Immunoreactive insulin and leptin levels were determined by ELISA (Crystal Chemicals, Chicago, IL). Serum levels of glucagon were determined using an RIA kit from Linco Research (St. Charles, MO). Daily blood glucose profiles were obtained from tail blood samples taken at the indicated times and determined using a handheld glucose meter (LifeScan).
transferred to a small glass/glass homogenizer to which was added 2.5 ml of trifluoroacetic acid in distilled water (0.5%). After homogenization, the suspension was left to stand for 1 h, rehomogenized, and centrifuged in a microfuge at 10,000 rpm. The supernatants were then applied to SepPak C18 cartridges (Waters, Milford, MA) that were activated according to the manufacturer’s instructions and eluted with 70% (vol/vol) ethanol containing 0.1% trifluoroacetic acid. After evaporation in a vacuum centrifuge, the eluates were reconstituted in assay buffer (PBS supplemented with 0.1% human serum albumin and 0.6 mmol/l timosal) and assayed for hormone content.

**Isolated perfused mouse pancreas.** Pancreases of fasted mice were surgically isolated and perfused with a modified Krebs-Ringer buffer with 3% Dextran, 0.2% BSA (RIA grade) and gassed with 95% O2-5% CO2 to achieve a pH of 7.4. Perfusion rate was 1 ml/min, with 0.6 mmol/l timosal (Eli Lilly). Each condition was assayed in triplicate. Secreted insulin was assayed for hormone content.

**Immunocytochemistry.** Entire pancreases were isolated for histological and morphometric analysis. Pancreases were fixed overnight in 4% buffered formalin at 4°C, cut in 2 mm sections, dehydrated, and embedded in paraffin. Paraffin sections (3 μm) were cut according to the fractionator principle (2), deparaffinated, and rehydrated, and the endogenous peroxidase was blocked by 0.5% H2O2 in 99% ethanol. Antigen retrieval was accomplished by microwave treatment in citrate buffer (0.01 M, pH 6.0) at 90°C for 3 × 5 min. Before immunohistochemical staining for glucagon and somatostatin, sections were blocked with avidin and then with biotin (DAKO, Copenhagen, Denmark). Insulin was detected with guinea pig anti-insulin (ICN); glucagon was detected with monoclonal mouse anti-glucagon (Glut01; Novo Nordisk) (1), and somatostatin was detected with rabbit anti-somatostatin (DAKO). A peroxidase-coupled rabbit anti-guinea pig IgG secondary antibody was used with diamobenzidine (DAB) to visualize insulin staining. Secondary antibodies, biotinylated goat anti-rabbit IgG (DAKO) and DAB to visualize insulin staining. Secondary antibodies, biotinylated goat anti-rabbit IgG (DAKO) and DAB to visualize glucagon and somatostatin positive cells. All sections were lightly counterstained with Mayer’s hematoxylin (Bie & Berntsen, Copenhagen, Denmark).

**Immunofluorescence.** Adult mice were perfused through the heart with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer and postfixed for several hours (51). Fixed tissues (3 animals/strain) were cut according to the fractionator principle (2), deparaffinated, rehydrated, and embedded in paraffin. Paraffin sections (3 μm) were cut according to the fractionator principle (2), deparaffinated, rehydrated, and the endogenous peroxidase was blocked by 0.5% H2O2 in 99% ethanol. Antigen retrieval was accomplished by microwave treatment in citrate buffer (0.01 M, pH 6.0) at 90°C for 3 × 5 min. Before immunohistochemical staining for glucagon and somatostatin, sections were blocked with avidin and then with biotin (DAKO, Copenhagen, Denmark). Insulin was detected with guinea pig anti-insulin (ICN); glucagon was detected with monoclonal mouse anti-glucagon (Glut01; Novo Nordisk) (1), and somatostatin was detected with rabbit anti-somatostatin (DAKO). A peroxidase-coupled rabbit anti-guinea pig IgG secondary antibody was used with diamobenzidine (DAB) to visualize insulin staining. Secondary antibodies, biotinylated donkey anti-mouse IgG, and biotinylated goat anti-rabbit IgG (Jackson Laboratories) were used with streptavidin peroxidase (DAKO) and DAB to visualize glucagon and somatostatin positive cells. All sections were lightly counterstained with Mayer’s hematoxylin (Bie & Berntsen, Copenhagen, Denmark).

**Results**

The RIP-Gcgr transgene (Fig. 1A) was injected in fertilized eggs, and 10 transmitter-transgenic founder mice were obtained. Quantitative RT-PCR analysis of total pancreatic RNA indicated that 8 of the 10 lines established from transgenic founders expressed high levels (20- to 200-fold increased compared with controls) of Gcgr mRNA (data not shown). Two lines, RIP-Gcgr3 and RIP-Gcgr6, which displayed 21.7 ± 0.3- and 193 ± 44-fold increases in islet Gcgr mRNA levels compared with controls (P < 0.001, n = 3; Fig. 1B), were chosen for further analysis. As several recent studies have shown that the RIP-driven transgene may be expressed in nonislet cells (44, 50), we determined whether RIP expression...
could be found outside islets in RIP-Gcgr mice. Using Gcgr expression in liver from WT mice as a positive control, Gcgr expression in liver, white adipose tissue, and heart was unchanged (data not shown). A small level of Gcgr expression was detected within the brain (cortex and hypothalamus) of RIP-Gcgr mice (0.02- to 0.04-fold of Gcgr expression levels of WT liver, $P < 0.001$, $n = 3$). RIP-Gcgr3 and RIP-Gcgr6 mice, maintained heterozygous in a mixed C57BL/6/ CBA background, transmitted the transgene in a Mendelian fashion. Both males and females appeared to be healthy, fertile, and displayed normal growth rates. In addition, RIP-Gcgr transgenic mice did not differ from controls in their fasting or fed blood glucose, plasma insulin and glucagon levels, nor in any of the serum metabolites examined (Table 1). However, tail blood samples from RIP-Gcgr3 mice throughout the day indicated that RIP-Gcgr3 males maintained lower blood glucose levels in the postprandial state compared with littermate controls (Fig. 1C), while RIP-Gcgr3 females displayed lower blood glucose level throughout most of the day (Fig. 1D). Similar data were obtained for both male and female RIP-Gcgr6 mice (Supplemental Fig. S1 [Supplemental data for this article may be found on the American Journal of Physiology: Endocrinology and Metabolism website].)

To examine whether the RIP-Gcgr transgene increased glucagon-stimulated insulin release, islets were isolated from RIP-Gcgr6 mice and incubated in 10 mM glucose in the presence or absence of 0.3 nM glucagon concentrations (Fig. 2A). Glucose alone stimulated similar insulin secretion from both transgenic and control islet preparations (RIP-Gcgr6, 2.10 ± 0.32 ng/ml, vs. controls, 1.98 ± 0.21 ng/ml, $n = 4$; Fig. 2A). Insulin release from control islets in response to 10 mM glucose + 0.3 nM glucagon did not differ from that seen in control islets exposed to 10 mM glucose alone (10 mM, 1.98 ± 0.32 vs. 10 mM + 0.3 nM glucagon, 1.95 ± 0.30 ng/ml, $P = n$; $n = 4$; group; Fig. 2A), indicating that this dose of glucagon was subthreshold for stimulating insulin release from control islet preparations. In contrast, RIP-Gcgr6 islets displayed a significant increase in insulin secretion in response to 0.3 nM glucagon compared with that for RIP-Gcgr3 islets incubated with glucose alone (10 mM, 2.10 ± 0.32 vs. 10 mM + 0.3 nM glucagon, 3.47 ± 0.34 ng/ml, $P < 0.05$; $n = 4$; group; Fig. 2A) or control islets incubated with glucose and 0.3 nM glucagon (RIP-Gcgr6, 3.47 ± 0.34 ng/ml, $P < 0.01$, $n = 4$; controls, 1.95 ± 0.30 ng/ml, $P < 0.01$, $n = 4$; group; Fig. 2A). This suggests that insulin release may be associated with the number of glucagon binding sites on β-cells. However, the insulinotropic action of glucagon appears to be saturatable, since as the response of RIP-Gcgr6 islets to 20 nM glucagon was similar to that seen with 0.3 nM. Although stimulation of both RIP-Gcgr6 and control islet preparations with 20 nM glucagon resulted in increased insulin release, there was a nonsignificant trend toward increased insulin release in the RIP-Gcgr6 islets compared with that of control islets (RIP-Gcgr6, 3.07 ± 0.49 ng/ml, vs. controls, 2.42 ± 0.09 ng/ml, $P = NS$, $n = 4$; Fig. 2A).

We next examined the response to glucagon in vivo by challenging anesthetized mice with 16 μg/kg of glucagon intraperitoneally. Peak insulin responses to glucagon in 15- to 20-wk-old RIP-Gcgr3 (Fig. 2B) and RIP-Gcgr6 mice were significantly increased compared with controls (males: RIP-Gcgr3, 3.12 ± 0.31 ng/ml vs. controls, 1.87 ± 0.32 ng/ml, $P < 0.05$, $n = 4$; and RIP-Gcgr6, 4.36 ± 0.59 ng/ml vs. controls, 2.61 ± 0.36 ng/ml, $P < 0.05$, $n = 7$–8; females: RIP-Gcgr6, 2.87 ± 0.31 vs. controls, 1.69 ± 0.27 ng/ml, $P < 0.05$, $n = 8$).

As expected, given the observed increase in insulin release, peak glucose levels and duration of glucose excursion stimulated by intraperitoneal glucagon were decreased in both anesthetized (Fig. 2C) and conscious ad libitum-fed RIP-Gcgr
Table 1. Fed and fasted serum hormone and metabolite levels in RIP-Gcgr3 transgenic and littermate control mice

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<th>Control Female</th>
<th>Control Male</th>
<th>RIP-Gcgr3 Female</th>
<th>RIP-Gcgr3 Male</th>
<th>RIP-Gcgr6 Female</th>
<th>RIP-Gcgr6 Male</th>
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<tr>
<td>Glucose, mM</td>
<td>6.9 ± 0.2 (16)</td>
<td>6.1 ± 0.1 (14)</td>
<td>6.0 ± 0.1 (19)</td>
<td>6.2 ± 0.1 (26)</td>
<td>7.2 ± 0.2 (20)</td>
<td>6.8 ± 0.2 (18)</td>
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<tr>
<td>Fasting</td>
<td>6.0 ± 0.8 (18)</td>
<td>5.8 ± 0.4 (12)</td>
<td>6.0 ± 0.6 (13)</td>
<td>6.0 ± 0.6 (14)</td>
<td>6.2 ± 0.4 (15)</td>
<td>6.0 ± 0.5 (13)</td>
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<tr>
<td>Fed</td>
<td>7.8 ± 0.9 (13)</td>
<td>8.2 ± 0.4 (16)</td>
<td>7.8 ± 0.9 (19)</td>
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<tr>
<td>Fasting</td>
<td>6.0 ± 0.3 (11)</td>
<td>5.0 ± 0.1 (9)</td>
<td>7.8 ± 0.9 (19)</td>
<td>6.0 ± 0.3 (11)</td>
<td>6.2 ± 0.4 (12)</td>
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<td>Fed</td>
<td>7.8 ± 0.9 (13)</td>
<td>7.8 ± 0.9 (13)</td>
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Values are means ± SE with the no. of experiments indicated in parentheses. RIP, rat insulin promoter; Gcgr, glucagon receptor; FFA, free fatty acid.

In situ perfused pancreas experiments were carried out to examine glucose-stimulated insulin release in RIP-Gcgr mice. Although basal insulin secretion in response to 4.4 mM glucose did not differ between the two groups, both the first phase (RIP-Gcgr3, 3.40 ± 0.11 ng/ml, vs. controls, 2.20 ± 0.15 ng/ml, P < 0.05, n = 3–4 and RIP-Gcgr6, 3.27 ± 0.38 ng/ml, vs. controls, 2.16 ± 0.17, P < 0.05) and second phase (RIP-Gcgr3, 2.14 ± 0.17 ng/ml, vs. controls, 0.76 ± 0.05 ng/ml, P < 0.05, n = 3–4 and RIP-Gcgr6, 1.93 ± 0.25 ng/ml, vs. controls, 0.73 ± 0.07 ng/ml, P < 0.05, n = 4) peak insulin levels were higher in RIP-Gcgr mice (Fig. 3, A and C). The integrated insulin response [area under the curve (AUC)] was approximately two times that seen in control mice (Fig. 3, B and D) (ng of insulin over 40 min: RIP-Gcgr3, 356 ± 35 ng, vs. controls, 163 ± 12 ng, P < 0.05, n = 4 and RIP-Gcgr6, 339 ± 33 ng, vs. controls, 153 ± 13 ng, P < 0.05, n = 4). RIP-Gcgr3 females displayed a similar increase in insulin release in perfused pancreas experiments (data not shown).

We hypothesized that the enhanced glucose- and glucagon-stimulated insulin release observed in RIP-Gcgr mice could improve glucose tolerance. To test this hypothesis, IPGTTs were conducted. Both RIP-Gcgr3 males and RIP-Gcgr6 females displayed enhanced glucose tolerance compared with controls (Fig. 4, A and C). Peak glucose levels (males: RIP-Gcgr3, 11.9 ± 1.2 mM, vs. controls, 16 ± 1.1 mM, P < 0.05, n = 4–5; females; RIP-Gcgr6, 10.6 ± 1.3 mM, vs. controls, 16.2 ± 1.3 mM, P < 0.05, n = 5) and the magnitude of glucose excursion (Fig. 4, B and D) were decreased in RIP-Gcgr mice compared with littermate controls (AUC in mM of glucose over 90 min: Males: RIP-Gcgr3, 799 ± 59, vs. controls, 1,027 ± 68, P < 0.05, n = 4–5, and RIP-Gcgr6, 712 ± 34 vs. controls, 1,027 ± 59, P < 0.05, n = 5). However, RIP-Gcgr mice displayed similar insulin tolerance when challenged with 0.75 U/kg of porcine insulin administered intraperitoneally (Fig. 4, E and F), suggesting the improved glucose tolerance was not due to a change in insulin sensitivity.

Pancreases from transgenic and control animals were extracted for islet hormone determination. Total pancreatic insulin content of RIP-Gcgr3 males was increased to 1.5 times that seen in control animals (Fig. 5A). In addition, pancreatic somatostatin levels were decreased in the transgenic mice compared with controls, whereas no difference was observed in islet glucagon levels.

Immunocytochemistry studies of pancreases did not indicate any gross differences in islet architecture or islet cell organization of RIP-Gcgr mice compared with controls (Fig. 5, C and D). However, quantification of the islet cell population by stereology indicated a small, but significant, increase (~20%) in β-cell volume in 16- to 17-wk-old male RIP-Gcgr3 compared with littermate controls (Fig. 5B). In contrast to the extract data, no difference in either glucagon or somatostatin pancreatic volumes was observed in RIP-Gcgr3 mice. A similar increase in the β-cell volume was observed in RIP-Gcgr6 females (RIP-Gcgr6, 0.76 ± 0.3, vs. controls, 0.62 ± 0.01 volume fraction, P < 0.001, n = 8).
Given the enhanced glucose homeostasis displayed by RIP-Gcgr mice, we hypothesized that β-cell overexpression of the Gcgr may protect against the induction of diabetes when the mice were fed a HFD. RIP-Gcgr and littermate controls fed a HFD displayed similar increases in body weight and were significantly heavier than either control or transgenic mice fed the low-fat diet (LFD) by 6–7 wk (Fig. 6A and Table 2). Over the course of the 12 wk, total caloric intake did not differ between the four groups studied (Table 2). DEXA analysis indicated that control and Rip-Gcgr3 male transgenic mice. Anesthetized mice were challenged with 16 μg/kg glucagon ip after a 6-h fast or 0.25 g/kg arginine iv after a 12-h fast. Data represent means ± SE; n = 7–13. *P < 0.05.

Fig. 2. A: glucagon-stimulated insulin release from isolated islets. Islets from RIP-Gcgr mice responded to a dose of glucagon (0.3 nM) that was subthreshold for that of control islets, whereas 20 nM glucagon increased insulin release from both RIP-Gcgr and control islets. Data represent means ± SE; n = 4 mice. **P < 0.01. Glucagon-stimulated insulin response (B) and plasma glucose (C) and arginine-stimulated insulin release (D) from control and RIP-Gcgr3 male transgenic mice. Anesthetized mice were challenged with 16 μg/kg glucagon ip after a 6-h fast or 0.25 g/kg arginine iv after a 12-h fast. Data represent means ± SE; n = 7–13. *P < 0.05.
As was predicted, fasting blood glucose levels of RIP-Gcgr3 mice, while elevated compared with LFD-fed transgenic and littermate controls, were significantly lower than control mice fed the HFD after 10 and 12 wk on the diet (Fig. 6B). Fasting insulin levels increased over time in both HFD groups of animals compared with LFD-fed control and transgenic mice (Fig. 6C). However, although fasting insulin levels were significantly higher in RIP-Gcgr3 HFD compared with control HFD mice after 8 wk, no significant difference was evident at weeks 10 and 12. As would be predicted, given the similar increases in adiposity, both HFD-fed RIP-Gcgr3 and controls displayed similar increases in plasma leptin levels (Fig. 6B).

Whereas glucagon levels were significantly increased in both HFD groups compared with controls, the increase was greater in RIP-Gcgr mice than that seen in the HFD-fed controls (Table 3).

Further analysis of serum metabolites collected upon death demonstrated no differences in circulating LDL levels in all four groups after 12 wk on the diets (Table 3). Both control HFD and RIP-Gcgr HFD mice displayed similar increases in cholesterol and HDL, while RIP-Gcgr displayed a small increase in TG levels compared with control mice fed the LFD (Table 3). However, as would be predicted given the improvement in blood glucose levels of RIP-Gcgr3 HFD mice, Hemoglobin A1c levels, while increased compared with LFD-fed RIP-Gcgr3 mice, were significantly lower than that seen in the control HFD group (Table 3).

We next sought to determine if the improved glycemic control observed in the RIP-Gcgr HFD group was due to either increased insulin sensitivity or enhanced glucose-stimulated insulin secretion. Both groups of mice fed the HFD displayed similar decreases in insulin sensitivity compared with control groups after 12 wk on the diets (Fig. 7, A and B). When challenged with an IPGTT, both the RIP-Gcgr3 and littermate controls fed the HFD displayed impaired glucose tolerance compared with LFD-fed control and RIP-Gcgr3 mice (Fig. 7, C and D). However, pancreatic islet overexpression of Gcgr resulted in a 34 ± 3% decrease in the glucose excursion in RIP-Gcgr mice fed the HFD compared with controls fed the same diet (Fig. 7, C and D). Insulin levels determined from an anesthetized IPGTT experiment indicated that, similar to data from the perfused pancreas experiments, RIP-Gcgr3 mice fed...
the LFD had increased insulin secretion compared with control LFD animals (Fig. 7, E and F). Although HFD-fed controls displayed a similar increase in glucose-stimulated insulin release compared with LFD-fed RIP-Gcgr, it did not reach statistical significance. In contrast, HFD-fed RIP-Gcgr mice displayed an increase in insulin release in response to glucose that was greater than that seen in the other three groups. The insulin content of RIP-Gcgr mice fed a LFD displayed a near-identical increase in pancreatic insulin content to that seen previously, in the less congenic RIP-Gcgr mice (Fig. 5A). In contrast, the pancreatic insulin content of RIP-Gcgr and control mice fed the HFD did not differ from that of controls on the LFD (insulin content: control LFD, 6.1 ± 0.3 vs. RIP-Gcgr3 LFD, 8.7 ± 0.3 vs. control HFD, 6.5 ± 6 vs. RIP-Gcgr3 HFD, 5.7 ± 0.6 pmol/mg protein, P < 0.01 compared with control LFD, n = 7–8).

DISCUSSION

In the present study, we generated mice overexpressing the Gcgr in pancreatic β-cells to examine the effects of increased glucagon action on β-cell function, and the resulting effects on whole body glucose homeostasis. Insulin secretion was increased by 1.7- to 2.9-fold in RIP-Gcgr mice compared with controls in four separate experimental paradigms: in vitro (Fig. 2A) and in vivo GC (Fig. 2B), perfused pancreas (Fig. 3, A–D), and IPGTT (Fig. 7, E and F) experiments. The enhanced insulin secretion observed in RIP-Gcgr mice resulted in reduced glucose excursions in response to intraperitoneal glucagon (Fig. 2C) and intraperitoneal glucose (Figs. 4, A–D, and 7, C and D). RIP-Gcgr and control mice displayed similar insulin sensitivity as measured by ITT (Fig. 4, E and F), suggesting that the enhanced glucose homeostasis of RIP-Gcgr mice did not involve an improvement in peripheral insulin sensitivity. These data indicate that both glucagon- and glucose-stimulated insulin release are increased in RIP-Gcgr mice. This likely reflects increased insulin-dependent inhibition of hepatic glucose production and increased peripheral glucose uptake in RIP-Gcgr compared with control mice. Furthermore, overexpression of Gcgr in pancreatic β-cells increased both insulin content and β-cell mass, but did not affect islet cell organization or architecture (Fig. 5, A–D). Importantly, when fed a HFD, the development of hyperglycemia and impaired glucose tolerance were partially attenuated in RIP-Gcgr mice due to, at least in part, an increase in the insulin secretory response. These findings are consistent with a model where glucagon action at the level of the β-cell is an important regulator of insulin release, synthesis, and β-cell mass.

Basal insulin levels in RIP-Gcgr mice did not differ from those seen in control mice, suggesting that glucose sensing in β-cells of RIP-Gcgr mice is normal (Fig. 3, A and B). In keeping with this observation, fasting insulin and glucose levels of male and female RIP-Gcgr mice were not different from those seen in controls (Table 1). However, when more detailed blood glucose profiles were obtained, it became apparent that RIP-Gcgr blood glucose was lower than that of controls at different times throughout the day (Fig. 1, C and D). This suggests that, even when given a mixed meal, RIP-Gcgr mice regulate blood glucose levels more tightly than control mice. The difference was more predominant in the postprandial state in males while it was seen throughout most of the day in RIP-Gcgr females (Fig. 1, C and D). This may reflect the greater insulin sensitivity of female compared with male mice (27).
The insulin promoter is frequently used to drive β-cell-specific transgene expression in the β-cell. In RIP-Gcgr mice, Gcgr expression was mainly detected in islet cells. Despite 10-fold higher expression of Gcgr mRNA in the islets of RIP-Gcgr6 compared with that of RIP-Gcgr3 mice, there was no obvious gene-dose effect in any of the parameters examined. This suggests that the effect of islet Gcgr overexpression reaches a saturation point above which no further improvement is obtainable. In support of this hypothesis is the observation that transgenic islets were hyperresponsive to a low dose of glucagon (Fig. 2A), but no further increase in insulin release was observed when transgenic islets were stimulated with 20 nM glucagon. In addition, liver membranes from mice lacking one copy of the Gcgr gene display a 50% reduction in glucagon binding but no defects in glucagon-stimulated cAMP production (16).

Table 2. Total caloric intake and body composition analysis of RIP-Gcgr3 transgenic and littermate control mice fed either LFD or HFD for 12 wk.

<table>
<thead>
<tr>
<th></th>
<th>Control LFD</th>
<th>RIP-Gcgr3 LFD</th>
<th>Control HFD</th>
<th>RIP-Gcgr3 HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL, kcal/mouse</td>
<td>1,145±33</td>
<td>1,109±25</td>
<td>1,162±21</td>
<td>1,184±24</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>34.5±0.4</td>
<td>32.3±0.7</td>
<td>43.5±1.2*</td>
<td>46.6±0.9*</td>
</tr>
<tr>
<td>Lean mass, g</td>
<td>30.6±0.8</td>
<td>27.9±0.9</td>
<td>22.99±0.8**</td>
<td>24.6±4*</td>
</tr>
<tr>
<td>Lean mass, %</td>
<td>89.2±1.8</td>
<td>87.3±1.8</td>
<td>52.7±1.4**</td>
<td>52.3±1.6**</td>
</tr>
<tr>
<td>Fat mass, g</td>
<td>3.03±0.6</td>
<td>3.42±0.6</td>
<td>19.75±0.9**</td>
<td>21.34±0.8**</td>
</tr>
<tr>
<td>Fat mass, %</td>
<td>8.8±1.7</td>
<td>10.7±1.8</td>
<td>45.2±1.4**</td>
<td>45.7±1.6**</td>
</tr>
</tbody>
</table>

Values are means ± SE. LFD, low-fat diet; HFD, high-fat diet. Total caloric intake was calculated from the total food intake (FI)/mouse for each cage (n = 4 cages/group). Lean and fat mass were determined by DEXA at the end of 12 wk on the diets (n = 8/group). P < 0.01 compared with control LFD diet (*) and compared with RIP-Gcgr3 LFD (†).

Similar to previous studies utilizing the RIP promoter, low-level extrapancreatic expression was present in the cortex and the hypothalamus (15, 43). Although glucagon is found in the central nervous system, whether central glucagon signaling plays a role in β-cell phenotype and function has not been determined (25, 37). One action of central glucagon is to activate the sympathetic nervous system (31). As sympathetic innervation of the pancreas inhibits insulin release (29) it seems unlikely that increased central signaling by glucagon would contribute to the phenotype of RIP-Gcgr mice. An interesting finding of our study is that, in contrast to in vivo insulin release, glucose-stimulated insulin secretion from isolated islets was similar between the WT and the RIP-Gcgr mice. This observation raises the possibility that loss of a central signal exacerbated the insulin response to glucose in...
isolated islets. In contrast, sympathetic innervation may have contributed to the enhanced insulin response in vivo. Thus it will be important to determine the role central glucagon plays in β-cell function.

Although Gcgr expression in β-cells has been demonstrated both at the mRNA (22, 30, 36) and protein (17, 22, 30, 34, 36) levels, and glucagon has been shown in humans (22) and rodents (30, 38) to stimulate insulin secretion, controversy still exists as to whether glucagon actually plays a physiological role in the regulation of insulin release (8). This is largely based on studies of the microcirculation in rodent islets (3, 23). It has been reported that, in rodents, afferent arterioles enter the islet β-cell mass before breaking into capillaries that traverse the β-cell core, passing through the non-β-cell mantle of α-, δ-, and pancreatic polypeptide cells before collecting into a network that surrounds the islet mantle. This blood flow pattern suggests that high levels of glucagon most likely do not reach the β-cell mass via the microcirculation (3). However, there is evidence supporting two other models of islet microcirculation where either the non-β-cell mantle is perfused before the β-cell core or a gated portal pattern where no set order of perfusion can be predicted (4). In humans β-cells are aligned along blood vessels with no particular order or arrangement, indicating that islet microcirculation likely does not determine the order of paracrine interactions (26). The data presented here strongly suggest that glucagon has access to β-cells via the circulation and can act via its receptor to influence β-cell function.

Indeed, there is a growing body of evidence that indicates glucagon signaling is required for normal β-cell function. Glucagon-rich islets from the dorsal pancreas secrete more insulin in response to glucose than islets with lower glucagon content found in the more ventral lobes of the pancreas (47). Impaired glucose-stimulated insulin release from nearly pure rat β-cell preparations could be reversed by the addition of nanomolar concentrations of glucagon (40). More recently,
Huypens and colleagues (22) demonstrated that the Gcgr specific antagonist des-His^5-[Glu^6]glucagon-amide inhibited glucose-stimulated insulin release in dispersed human islets, supporting a role for glucagon in the maintenance of glucose competence in humans. Finally, the insulin response in isolated islets from mice lacking the Gcgr (Gcgr^-/-) was significantly reduced in response to glucose and a number of other secretagogues (42). In addition, while the in vivo insulin response to intravenous glucose was enhanced (apparently due to a compensatory increase in circulating GLP-1 and an increase in peripheral insulin sensitivity), the response to the glucose-independent secretagogues cholecystokinin and arginine were reduced in Gcgr^-/- mice (42). That loss of glucagon signaling results in multiple defects in insulin secretion, and such a robust compensatory response implies its importance to glucose homeostasis.

Glucagon has previously been shown to influence pancreatic β-cell growth/differentiation, since rats with increased circulating glucagon levels due to transplantation of glucagonoma cells displayed regression of α-cell mass (1). We previously have shown that glucagon signaling via its own receptor is an important determinant of α-cell mass, since global deletion of the Gcgr gene resulted in a marked α-cell hyperplasia and organomegaly of the pancreas (16, 51). Furthermore, ablation of Gcgr affected β-cell number per islet and β-cell phenotype (51). Gcgr^-/- mice had a significant decrease in the percentage of β-cells per islet compared with control animals. Immunohistochemical and quantitative PCR analysis revealed that the level of expression of Pdx-1, membrane-localized glucose transporter 2 and MafA, a transcription factor proposed to be involved in the glucose-mediated increase in insulin gene transcription, was lower in β-cells of Gcgr^-/- than in control mice (51). The results of this study further confirm that glucagon is involved in the regulation of the β-cell mass and insulin gene expression. Although the underlying cause of the increase in β-cell mass has not yet been fully investigated, it has previously been shown that GLP-1 and the GLP-1 receptor agonist, exendin-4, both increase the β-cell mass in vitro (7, 54) and in vivo (39, 46, 53). In addition, GLP-1 and gastric inhibitory glucagon-dependent polypeptide (GIP) have been shown to increase insulin gene expression (11, 13, 14, 52). Assuming that the Gcgr activates the same signaling pathways as GLP-1, the increased β-cell mass and insulin content could reflect stimulation of the same or similar pathways (21, 41).

One question raised by these studies is to what degree the increased β-cell mass vs. increased β-cell competence contributes to the improved glucose homeostasis observed in RIP-Gcgr mice. Although it is likely that both of these components of the β-cell response contribute, we hypothesize that the increase in β-cell competence is likely to predominate. This is based on the observation that isolated islets from RIP-Gcgr mice responded to a subthreshold dose of glucagon and that an intravenous arginine challenge resulted in only a small increase in insulin release in RIP-Gcgr mice compared with control. Further studies are required to quantify the contribution of both β-cell mass and β-cell competence to the increased insulin release observed in RIP-Gcgr mice in vivo.

Pancreatic β-cell overexpression of the Gcgr did not protect mice from the development of obesity and dislipidemia when fed HFD. However, as we predicted, the RIP-Gcgr mice did exhibit less severe hyperglycemia (reduced by 20% of control fed HFD) and impairment of glucose tolerance compared with control animals fed the same diet (Figs. 6 and 7). The improved glycemic control appears to have extended throughout the experiment, since RIP-Gcgr mice had decreased glycosylated hemoglobin levels compared with control animals fed the same HFD. This apparently was due to the enhanced insulin secretory response of RIP-Gcgr mice that becomes even more evident when the animals are rendered insulin resistant by the HFD. Interestingly, serum glucagon levels taken at the time of death were elevated to a greater extent in the RIP-Gcgr HFD group compared with controls fed the same diet (Table 3). Although the mechanism for this enhanced release of glucagon remains to be determined, the enhanced insulin release may in part be due to increased activation of β-cell Gcgr signaling.

In conclusion, overexpression of the Gcgr in β-cells resulted in increased glucagon and glucose-stimulated insulin secretion, an increase in insulin content and β-cell mass, as well as attenuated hyperglycemia and impaired glucose tolerance associated with mice fed a HFD. These data suggest that increased glucagon action at the level of the β-cell, similar to GLP-1 and GIP, plays a role both in regulating β-cell mass and in potentiating glucose-stimulated insulin secretion, by increasing β-cell competency.

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β-CELL OVEREXPRESSION OF THE GLUCAGON RECEPTOR


