Disruption of G Protein-Coupled Receptor 39 Impairs Insulin Secretion *in Vivo*

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GPR39 is a G protein-coupled receptor expressed in liver, gastrointestinal tract, adipose tissue, and pancreas. We have recently shown that young GPR39$^{-/-}$ mice have normal body weight, food intake, and fasting glucose and insulin levels. In this study, we examined the role of GPR39 in aging and diet-induced obese mice. Body weight and food intake were similar in wild-type and GPR39$^{-/-}$ mice as they aged from 12 to 52 wk or when fed a low-fat/high-sucrose or high-fat/high-sucrose diet. Fifty-two-week-old GPR39$^{-/-}$ mice showed a trend toward decreased insulin levels after oral glucose challenge. When fed either a low-fat/high-sucrose or high-fat/high-sucrose diet, GPR39$^{-/-}$ mice had increased fed glucose levels and showed decreased serum insulin levels during an oral glucose tolerance test in the face of unchanged insulin tolerance. Pancreas morphology and glucose-stimulated insulin secretion in isolated islets from wild-type and GPR39$^{-/-}$ mice were comparable, suggesting that GPR39 is not required for pancreas development or *ex vivo* insulin secretion. Small interfering RNA-mediated knockdown of GPR39 in clonal NIT-1 β-cells revealed that GPR39 regulates the expression of insulin receptor substrate-2 and pancreatic and duodenal homeobox-1 in a cell-autonomous manner; insulin receptor substrate-2 mRNA was also significantly decreased in the pancreas of GPR39$^{-/-}$ mice. Taken together, our data indicate that GPR39 is required for the increased insulin secretion *in vivo* under conditions of increased demand, *i.e.* on development of age-dependent and diet-induced insulin resistance. Thus, GPR39 agonists may have potential for the treatment of type 2 diabetes. (***Endocrinology*** **150: 2586–2595, 2009)**

GPR39 was first identified as a G protein-coupled receptor (GPCR) related to GH secretagogue receptor (also known as ghrelin receptor) and neurotensin receptor (1). Similar to other receptors in this family, GPR39 shows significant constitutive activity; functional studies *in vitro* have shown that overexpression of GPR39 increases inositol phosphate turnover as well as cAMP response element- and serum response element-driven transcription in a ligand-independent manner (2). Considerable interest in GPR39 was generated by the observation that obestatin, a putative satiety peptide encoded by the ghrelin gene, is able to activate GPR39 (3). Although some level of controversy still exists (3–6), the majority of studies to date indicate that obestatin is not a major modulator of food intake and does not bind to or activate GPR39 (7–14).

A candidate agonist ligand of GPR39 is Zn^{2+}. Multiple studies have shown that micromolar concentrations of Zn^{2+} stimulate mouse, rat and human GPR39 signaling *in vitro* (10, 15, 16). Recently the Zn^{2+}-binding site of GPR39 has been mapped using mutagenesis, and two histidines in the N-terminal, cytoplasmic domain of GPR39 were identified as critical for the agonist activity of Zn^{2+} (15). It is currently unclear whether Zn^{2+} is a physiological agonist ligand of GPR39 or whether it acts as an agoallosteric modulator to enhance the function of a yet-to-be-identified GPR39 ligand. Interestingly, Zn^{2+} has been reported to be an allosteric agonist for the melanocortin-1 and melanocortin-4 receptors (17), indicating that activation by Zn^{2+} is not unique to GPR39 and can occur even in the presence of an agonist ligand.

The physiological role of GPR39 is still unclear. GPR39 mRNA is highly expressed in the gastrointestinal tract, kidney, liver, adipose tissue, and pancreas and to a lower extent in spleen, thyroid, lung, and heart (1, 18, 19). Expression of a splice variant

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Abbreviations: AUC, Area under the curve; GLP, glucagon-like peptide-1; GPCR, G protein-coupled receptor; GSIS, glucose-stimulated insulin secretion; HBSS, Hanks' balanced salt solution; HFHS, high-fat/high-sucrose; IRS, insulin receptor substrate; LFHS, low-fat/high-sucrose; OGTT, oral glucose tolerance test; PDX-1, pancreatic and duodenal homeobox-1; RNAi, RNA interference; siRNA, small interfering RNA; WT, wild type.

encoding a truncated but not full-length form of GPR39 has also been detected in various regions of the brain (18). Analyses of GPR39 knockout mice under normal conditions have shown relatively minor phenotypes: Moechars *et al.* (19) found that mice in which GPR39 has been disrupted by the insertion of the β-galactosidase (LacZ) gene ($GPR39^{LacZ/LacZ}$) had enhanced gastric fluid secretion, enhanced gastric emptying, and increased gastrointestinal transit time. However, body weight and food intake as well as glucose and insulin levels were comparable between wild-type (WT) and $GPR39^{LacZ/LacZ}$ mice up to 20 wk of age (19). Upon aging, $GPR39^{LacZ/LacZ}$ mice gained more weight than their WT counterparts, despite reduced fasting-induced hyperphagia (19). Using an independently generated line of GPR39 knockout mice, we also did not observe any differences in body weight, food intake, insulin, or glucose levels in young (<24 wk old) $GPR39^{-/-}$ mice when fed a standard chow diet (13). No studies to date have addressed the possible role of GPR39 in the pancreas. Because modulation of GPCR signaling in β-cells, in particular alterations in Ca^{2+} signaling and cAMP levels, can have profound effects on insulin secretion and β-cell survival (20), it might be expected that β-cell function is altered in $GPR39^{-/-}$ mice.

In this study, we explored the possibility that GPR39 modulates glucose metabolism under conditions of increased demand for insulin secretion, *i.e.* on aging and during diet-induced obesity. We also examined islet morphology and insulin secretion from animals exposed to different diets.

Materials and Methods

Animals

$GPR39^{-/-}$ mice were generated by homologous recombination at Deltagen (San Mateo, CA). The GPR39 targeted allele was generated by replacing nucleotides 278–647 of the open reading frame with a cassette encoding the neomycin resistance marker using homologous recombination in a 129/OlaHsd ES cell line (schematically illustrated in supplemental Fig. 1A, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). The targeting event was confirmed by Southern blotting using probes directed against chromosomal DNA flanking the targeting vector at both the 5′ and the 3′ end. Mice carrying the mutated allele were backcrossed into the C57BL/6 background for six generations and maintained as heterozygous. WT littermates from heterozygous mating pairs were used as controls. All mice were singly housed in a temperature-controlled facility with a 12-h light, 12-h dark cycle and had free access to water and a normal chow diet (PharmServ, Framingham, MA). All studies were performed in male mice. For diet-induced obesity studies, 16-wk-old mice were fed either a low-fat/high-sucrose (LFHS; 11% kilocalories derived from fat; 4.07 kcal/g; catalog no. D12329) or high-fat/high-sucrose (HFHS) diet (58% kilocalories derived from fat; 5.56 kcal/g; catalog no. D12331; Research Diets, New Brunswick, NJ) for 22 wk. All experimental work was conducted in accordance with the humane guidelines for ethical and sensitive care of the Institutional Animal Care and Use Committee of the National Institutes of Health.

Glucose and insulin tolerance tests

Glucose tolerance was measured in fasted (16 h) mice after oral gavage or ip injection of 2 g glucose/kg body weight. Insulin tolerance was measured in fasted (6 h) animals after ip injection of 1.5 U insulin per kilogram body weight. Blood was taken from the tail vein before and 15, 30, 60, and 120 min after glucose or insulin administration. Blood glucose was monitored with a glucometer (Ascencia Elite; Bayer, Mishawaka, IN), and serum insulin was measured using an ultrasensitive rat ELISA kit with 100% cross-reactivity with mouse insulin (Crystal Chem, Downers, IL).

Serum chemistry

Serum glucose, triglyceride, and free fatty acid levels in random-fed mice were measured using a 912 automatic analyzer (Roche, Indianapolis, IN). Serum insulin levels were determined as described above.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated (for glucagon, insulin, Ki67, and cleaved caspase-3 staining). Sections used for Ki67 and cleaved caspase-3 detection were retrieved using antigen Decloaker solution and Decloaker chamber (Biocare Medical, Concord, CA). All immunohistochemistry labeling was performed on Nemesis 7200 autostainer (Biocare Medical). Tris-buffered saline containing Tween 20 was used for washes between each step (except after the protein block). Endogenous peroxidase was quenched with 3% hydrogen peroxide followed by protein block with 2.5% normal horse serum. Each section was incubated with primary antibody or IgG control sera as follow: guinea pig antiinsulin (1:150, catalog no. 0564; Dako, Carpinteria, CA); rabbit antiglucagon (1:75, Dako catalog no. A0565); rabbit monoclonal anti-Ki67 (1:200, catalog no. 9106; Lab Vision, Fremont, CA); rabbit monoclonal anticleaved caspase-3 (1:200, catalog no. 9664; Cell Signaling Technology, Danvers, MA); or rabbit IgG negative control (Dako catalog no. X0903). Rabbit IgG-horseradish peroxidase polymer (Rabbit ImmPRESS, catalog no. MP-7401; Vector Laboratories, Burlingame, CA) was used to detect the primary antibodies. Liquid diaminobenzidine (Dako catalog no. K3468) substrate chromogen was used followed by hematoxylin counterstaining. For rat antimouse CD31/platelet endothelial cell adhesion molecule-1 immunohistochemistry, snap-frozen tissue crysosections were fixed in zinc fixative (BD Biosciences, San Jose, CA) and then incubated with rat antimouse CD31, MEC13.3 (1:50), or isotype control (rat IgG2aκ). For detection of the primary antibody, antirat Ig-horseradish peroxidase detection kit (BD Biosciences catalog no. 551013) was used according to the manufacturer's recommendation. Sections were dehydrated, cleared, and coverslipped using mounting medium.

Histomorphometrical analysis

All histomorphometrical analysis were conducted using 8-bit RGB color photomicrographs at 1388 × 1040 resolution taken using a Axio Imager A1 microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) and a Zeiss AxioCam HRc digital microscope camera or with 1 GB color digital images of scanned slides obtained from a MedMicro digital slide module slide scanning system (Carl Zeiss MicroImaging Inc., Thornwood, NY). Total area of the pancreas, islet and β-cell (*i.e.* insulin-positive cells) was determined morphometrically in pancreas sections stained for insulin. The number of cell nuclei in insulin-stained areas of each islet, interpreted as the number of β-cells, was manually counted. Total number of nuclei exhibiting positive staining for Ki67 in islets was counted manually from pancreas sections. Total area of positive staining for CD31, interpreted as the total area of the vasculature within the islet, was determined morphometrically in pancreas sections.

Islet isolation and culture

Islets were isolated by a modified version of the collagenase digestion technique (21). Briefly, 3 ml of cold Hanks' balanced salt solution (HBSS), supplemented with 1.35 U/ml collagenase (collagenase P, specific activity 1.52 U/mg; Roche), glucose, BSA, and sodium bicarbonate (pH 7.4) (22), was injected into the common bile duct to fill the pancreas. The pancreas was subsequently removed and incubated for 20 min at 37 C before addition of ice-cold HBSS to stop the digestion. After washing

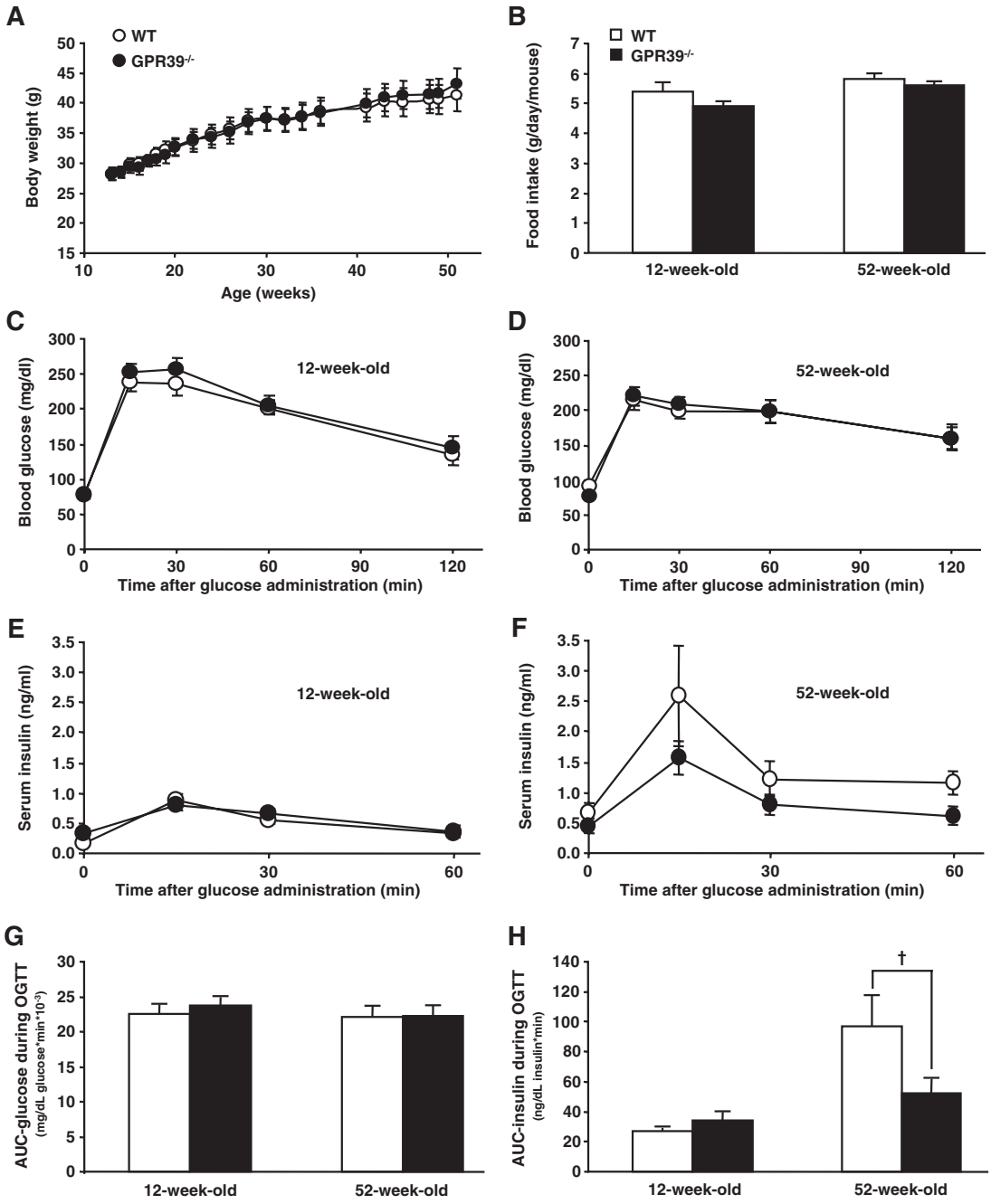

FIG. 1. Characterization of GPR39$^{-/-}$ mice during aging. Body weight (A) and food intake (B) were measured in WT and GPR39$^{-/-}$ male mice as they aged from 12 to 52 wk old. Glucose (2 g/kg) was administered orally to 12- (C and E) or 52-wk-old (D and F) fasted (16 h) mice. Blood glucose (C and D) and serum insulin (E and F) were monitored for 120 and 60 min, respectively. The AUC for glucose (G) and insulin (H) after glucose administration was calculated from glucose (C and D) and insulin (E and F) excursion curves. The means ± SEM of eight (WT) and nine (GPR39$^{-/-}$) animals are shown. †, $P = 0.07$.

three times with HBSS, islets were isolated from the exocrine tissue by centrifugation in Histopaque 1077 (Ficoll gradient; Sigma, St. Louis, MO) at 3000 × *g* for 20 min. Islets were removed from the interface; washed twice with HBSS; transferred into RPMI 1640 supplemented with 11 mM glucose, 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin; and cultured overnight in a humidified atmosphere of 5% CO_2 at 37 C.

Insulin secretion in isolated islets

Batches of five to 10 islets per tube were washed twice and preincubated in Krebs-Ringer buffer [119 mM NaCl, 4.6 mM KCl, 1 mM $MgSO_4$, 0.15 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 20 mM HEPES (pH 7.4), 0.05% (wt/vol) BSA (fraction V; Sigma) and 2.8 mM glucose] for 30 min at 37 C. Islets (in quadruplicate for each condition) were then incubated in 200 μl of Krebs-Ringer buffer containing either 2.8 or 11 mM glucose for 30 min at 37 C and then placed on ice to stop secretion. In some cases, islets were also treated with 50 or 250 μM zinc chloride, 0.4 mM palmitate complexed with 0.67% BSA, 20 nM glucagon-like peptide-1 (GLP)-1, or 10 mM arginine during the 30 min incubation. One hundred fifty microliters of each sample were transferred to a new tube for insulin measurement by homogeneous time-resolved fluorescence (CIS Bio, St. Louis, MO). Islets were solubilized in Krebs-Ringer buffer containing 25 mM NaOH and 0.1% Triton X-100

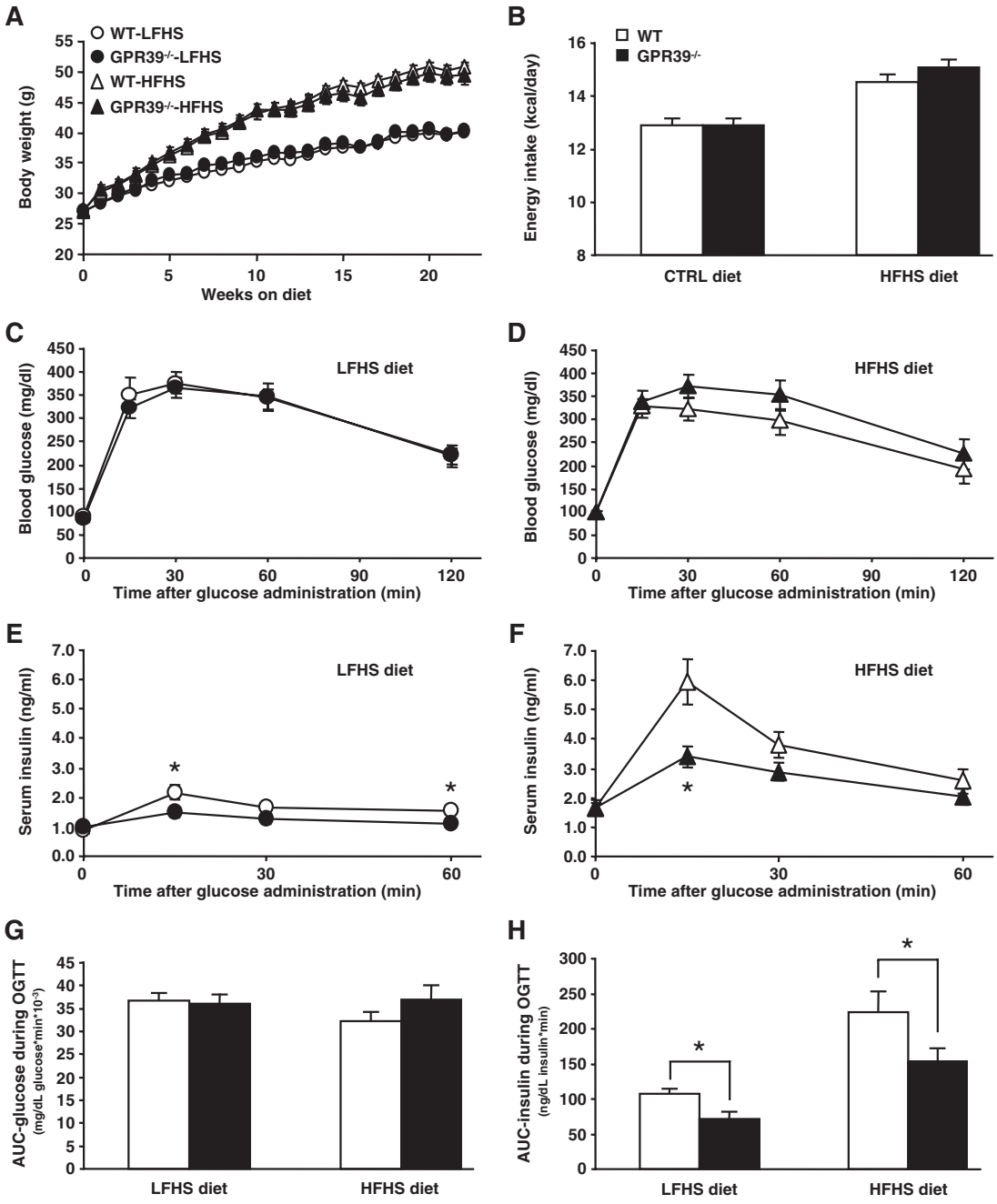

FIG. 2. Characterization of GPR39$^{-/-}$ mice fed a LFHS or HFHS diet. Body weight (A) and food intake (B) were measured in WT and GPR39$^{-/-}$ male mice that were fed either a LFHS or HFHS. Glucose (2 g/kg) was administered orally to fasted (16 h) mice fed a LFHS (C and E) or HFHS (D and F) diet. Blood glucose (C and D) and serum insulin (E and F) were monitored for 120 and 60 min, respectively. The AUC for glucose (G) and insulin (H) after glucose administration was calculated from glucose (C and D) and insulin (E and F) excursion curves. The means ± SEM of 10 animals/group are shown. *, $P < 0.05$ *vs.* corresponding WT.

for measurement of total insulin by homogeneous time-resolved fluorescence or protein content using the bicinchoninic acid method (Pierce, Rockford, IL).

RNA interference (RNAi)

The clonal NIT-1 β-cells were seeded at a density of 3×10^5 cells/well in a 6-well plate and grown for 18–24 h in antibiotic-free DMEM containing 10% fetal bovine serum. Cells were transfected with a control small interfering RNA (siRNA) or siRNAs against mouse GPR39 (final concentration 50 nM) using Lipofectamine RNAiMAX according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Negative control and predesigned GPR39 siRNAs [catalog no. S89441 (no. 1) and s89442 (no. 2)] were obtained from Ambion (Austin, TX). Six hours after transfection, cells were placed in Ham's F12 medium containing 10% dialyzed fetal bovine serum and incubated for 72 h. Cells were rinsed once with PBS, snap frozen with liquid nitrogen, and stored at −80 C until processed for RNA extraction as described below.

RNA extraction and Taqman real-time PCR

Freshly isolated pancreas from mice fed a HFHS diet were homogenized in Trizol (Invitrogen). Total RNA from pancreas and NIT-1 β-cells was isolated and purified using the RNeasy Plus kit (QIAGEN, Valencia, CA). Taqman quantitative PCR was performed on a 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA) using 18S as an endogenous control. Predesigned mouse primers and probe were obtained from Applied Biosystems.

Statistical analysis

Results are expressed as mean ± SEM. Differences between groups were determined by using unpaired two-tailed student's *t* test and considered to be statistically significant at $P < 0.05$.

Results

Characterization of GPR39$^{-/-}$ mice during aging

We and others have previously reported that young (<24 wk old) GPR39$^{-/-}$ mice are metabolically indistinguishable from WT littermates (13, 19). To determine whether GPR39 plays a role in energy and/or glucose metabolism upon aging, we followed a cohort of WT and GPR39$^{-/-}$ male mice for a period of up to 1 yr of age. Mice with a targeted deletion of the GPR39 gene were used (see *Materials and Methods* and supplemental Fig. 1A). We confirmed the absence of GPR39 transcripts in tissues isolated from knockout animals (supplemental Fig. 1B). Body weight (Fig. 1A) and food intake (Fig. 1B) were virtually identical between WT and GPR39$^{-/-}$ mice as they aged from 12 to 52 wk. Prandial serum glucose, insulin, and triglyceride levels were not significantly different between WT and GPR39$^{-/-}$ mice at 52 wk of age (data not shown). To further examine the role of GPR39 in glucose metabolism, an oral glucose tolerance test (OGTT) was performed in 12- and 52-wk-old WT and GPR39$^{-/-}$ mice. Glucose levels during OGTT were similar between WT and GPR39$^{-/-}$ mice at both 12 (Fig. 1C) and 52 (Fig. 1D) wk of age. Measurement of the area under the curve (AUC) revealed that glucose excursion during OGTT was affected by neither genotype nor age, at least until 1 yr of age (Fig. 1G). In younger animals, serum insulin levels were modestly elevated after glucose administration and were not significantly different between WT and GPR39$^{-/-}$ mice (Fig. 1E). Serum insulin levels after glucose challenge were significantly higher in 52-wk-old compared with younger mice (compare Fig. 1, F and E), indicating the development of insulin resistance during aging. Aged GPR39$^{-/-}$ mice showed a trend toward lower serum insulin levels in response to glucose compared with WT littermates (Fig. 1, F and H); however, this trend did not reach statistical significance ($P = 0.07$ when comparing AUC values). Together, our data suggest no gross abnormality in body weight or glucose metabolism in GPR39$^{-/-}$ mice upon aging. The observed trend toward a decreased insulin excursion on glucose challenge prompted us to examine glucose metabolism in GPR39$^{-/-}$ mice during diet-induced insulin resistance and obesity.

Characterization of GPR39$^{-/-}$ mice fed a HFHS diet

Diet-induced obesity in mice is known to cause metabolic abnormalities that encompass common features of abdominal obesity in humans (23). At 16 wk of age, WT and GPR39$^{-/-}$ mice were randomly assigned to a LFHS or HFHS diet for 22 wk. As expected, animals fed a HFHS diet gained twice as much weight as those fed the LFHS diet, but no genotype-dependent differences were noted on either diet (Fig. 2A). Energy intake was higher in animals fed a HFHS diet but was not different between WT and GPR39$^{-/-}$ mice (Fig. 2B). To further delineate the role of GPR39 in diet-induced obese mice, an OGTT was performed where both glucose and insulin levels were monitored. The glycemic response observed after an oral glucose challenge was similar between WT and GPR39$^{-/-}$ mice fed a LFHS diet (Fig. 2C) and was slightly but not significantly higher in the knockout mice fed a HFHS diet (Fig. 2D). Despite no significant changes in glucose tolerance *per se*, circulating insulin levels were decreased by about 30–40% ($P < 0.05$ *vs.* WT) 15 min after glucose administration in GPR39$^{-/-}$ mice fed either a LFHS (Fig. 2E) or HFHS diet (Fig. 2F). AUC measurements confirmed a statistically significant decrease in insulin excursion in GPR39$^{-/-}$ mice fed either diet (Fig. 2H). In contrast, no significant genotype-dependent changes in the AUC for glucose were found (Fig. 2G). Although fasting glucose and insulin levels were unchanged in GPR39$^{-/-}$ mice (see time 0 min in Fig. 2, C–F), disruption of GPR39 resulted in elevated glucose levels in the fed state (Fig. 3A), irrespective of the diet, and there was a trend ($P = 0.07$) for lower circulating insulin levels in obese GPR39$^{-/-}$ mice when compared with their WT counterparts fed a HFHS diet (Fig. 3B). These data suggest that GPR39$^{-/-}$ mice have reduced insulin secretion rates under condition of increased demand and that this reduction can lead to prandial hyperglycemia.

Insulin tolerance in GPR39$^{-/-}$ mice fed a HFHS diet

To confirm that the changes observed after glucose challenge represent indeed changes in insulin secretion, rather than altered insulin sensitivity, we performed insulin tolerance tests in WT and GPR39$^{-/-}$ mice fed either a LFHS or a HFHS diet. Injection of insulin resulted in a marked reduction in blood glucose levels in WT mice fed a LFHS or

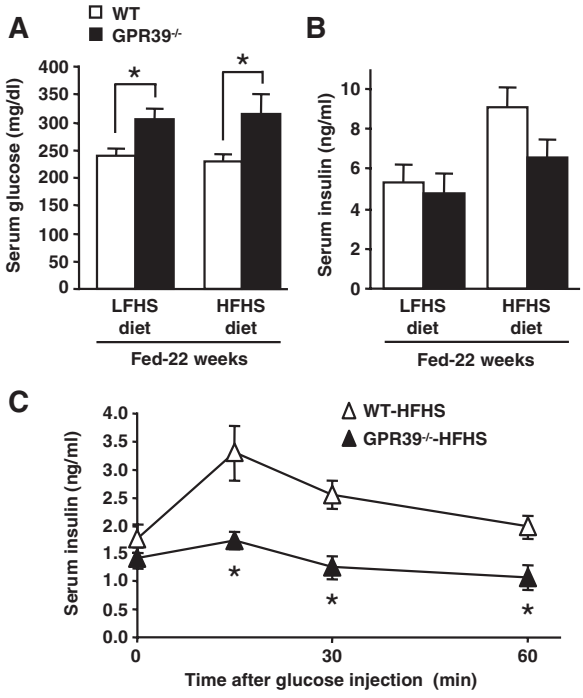

FIG. 3. Serum glucose and insulin levels in GPR39$^{-/-}$ mice fed a LFHS or HFHS diet. Serum glucose (A) and insulin (B) levels in the fed state were measured in WT and GPR39$^{-/-}$ male mice that were fed either a LFHS or HFHS. The means ± SEM of 14 (WT-LFHS), 13 (GPR39$^{-/-}$-LFHS), 14 (WT-HFHS), and 13 (GPR39$^{-/-}$-HFHS) animals are shown. C, Glucose (2 g/kg) was administered ip to overnight fasted WT and GPR39$^{-/-}$ male mice that were fed a HFHS diet. Serum insulin was monitored for 60 min. The means ± SEM of 10 animals/group are shown. *, $P <$ 0.05 *vs.* corresponding WT.

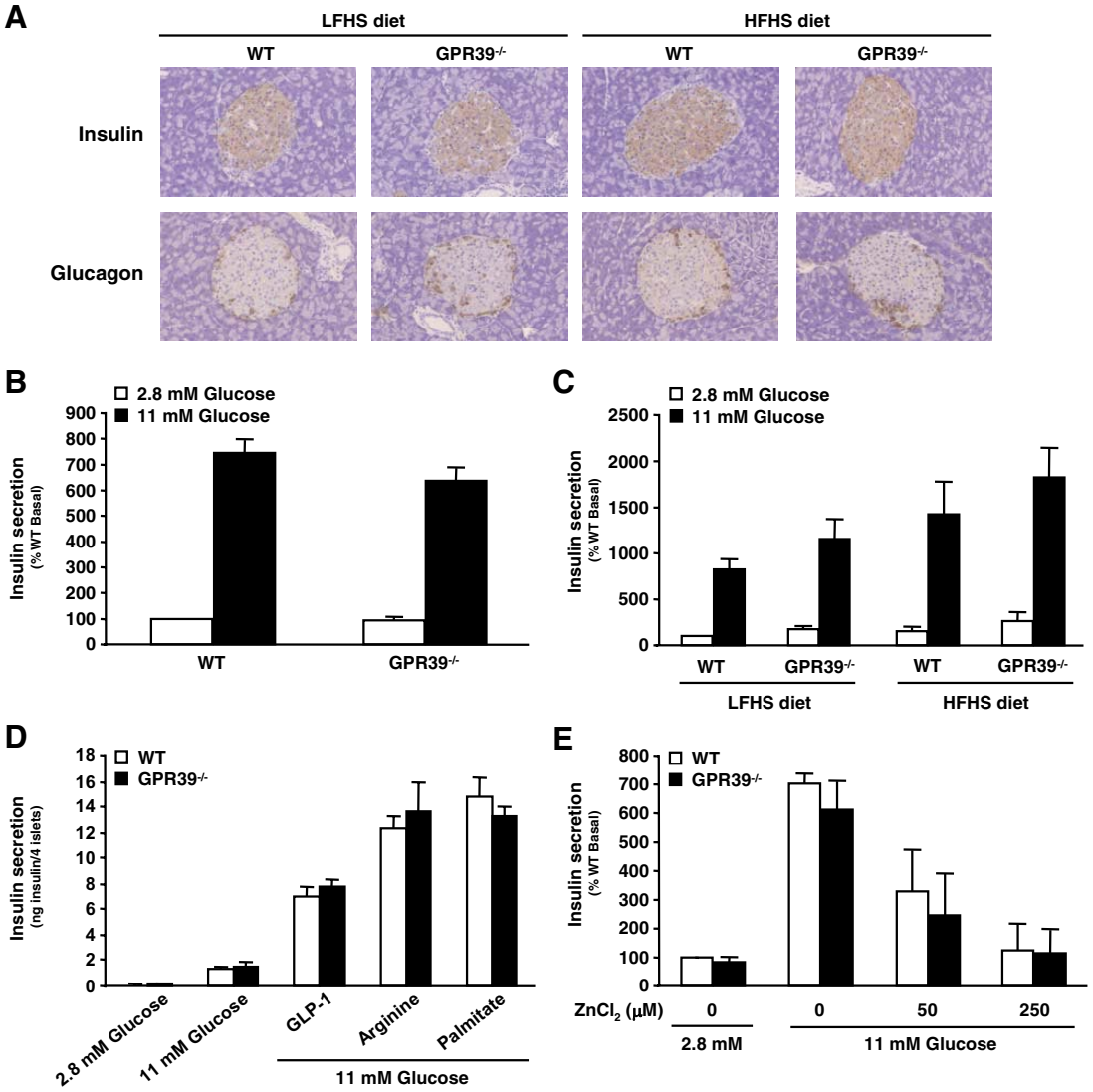

FIG. 4. Glucose-stimulated insulin secretion in islets isolated from GPR39$^{-/-}$ mice. A, Expression of insulin and glucagon was determined by immunohistochemistry in pancreatic sections obtained from WT and GPR39$^{-/-}$ male mice that were fed either a LFHS or HFHS diet. Insulin secretion in response to low (2.8 mM) or high (11 mM) glucose concentrations was measured in isolated islets from WT and GPR39$^{-/-}$ mice fed a standard chow diet (B) or a LFHS or HFHS diet (C). Insulin secretion in islets incubated *ex vivo* with low (2.8 mM) or high (11 mM) glucose concentrations in the presence or absence of 20 nM GLP-1 10 mM arginine or 0.4 mM palmitate (D), and 50 or 250 μM $ZnCl_2$ (E). The means ± SEM of four to five independent experiments (one animal/group for each experiment) performed in quadruplicate are shown.

HFHS diet (supplemental Fig. 2, A and B). The hypoglycemic effect of acute insulin administration was more pronounced in LFHS compared with HFHS mice, but no difference between WT and GPR39$^{-/-}$ mice was observed on either diet (supplemental Fig. 2, A and B). These data suggest that deletion of GPR39 does not affect peripheral insulin sensitivity, at least under the conditions used in this study.

Decreased insulin secretion *in vivo* after ip injection of glucose in GPR39$^{-/-}$ mice fed a HFHS diet

Insulin secretion in response to oral administration of glucose is partly mediated by the release of gut hormones that act as insulin secretagogues known as the incretin effect (24). Because GPR39 is highly expressed in the gastrointestinal tract, it is possible that GPR39 modulates insulin secretion by affecting the release of incretins. To evaluate insulin secretion in the absence of an incretin effect, we measured insulin levels after administering glucose ip. After the injection of glucose in the ip cavity, insulin levels increased significantly in WT mice fed a HFHS diet (peak insulin level 3.3 ng/ml; see Fig. 3C), although the increase was less pronounced than that observed after oral administration of glucose (peak insulin level 6.0 ng/ml; see Fig. 2F). Similar to what was observed after oral glucose challenge, the increase in insulin after ip injection of glucose was significantly blunted in GPR39$^{-/-}$ mice fed a HFHS diet (Fig. 3C). Changes in AUC levels for insulin after oral [WT: 224 ± 27; GPR39$^{-/-}$: 154 ± 17 (differential of 70 AUC units)] *vs.* ip injection [WT: 150 ± 16; GPR39$^{-/-}$: 81 ± 10 (differential of 69 AUC units)] of glucose (2 g/kg body weight) were similar, indicating that the decrease in insulin secretion in GPR39$^{-/-}$ mice fed a HFHS diet does not involve major alterations in the incretin effect.

Normal glucose responsiveness in islets isolated from GPR39$^{-/-}$ mice

Because GPR39 is expressed in the pancreas, it is possible that this GPCR directly modulates islet and/or β-cell functions. In

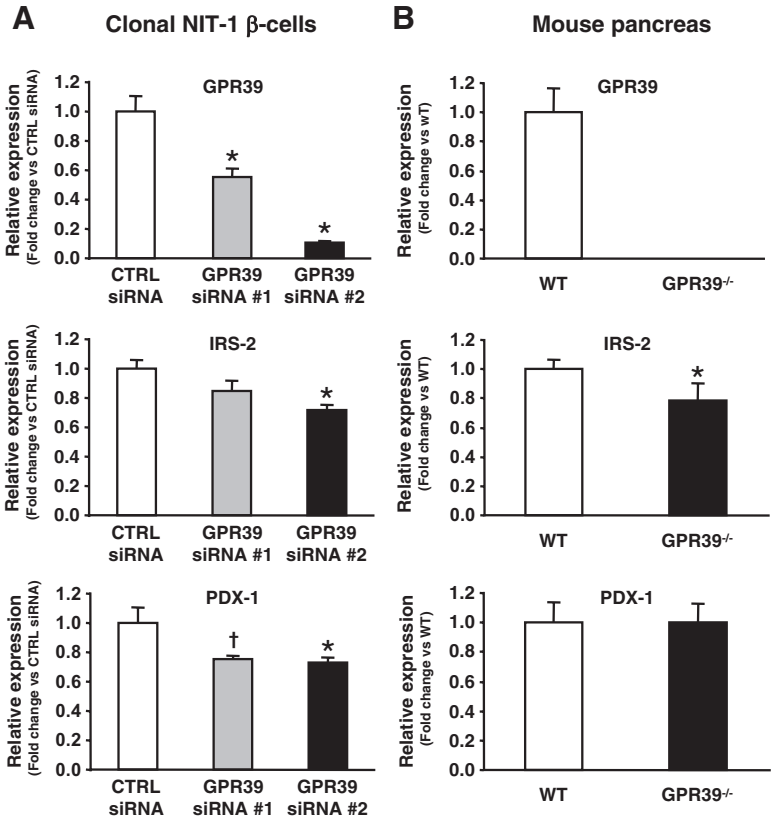

FIG. 5. Silencing of GPR39 by RNAi in clonal NIT-1 β-cells: comparison with pancreas from GPR39$^{-/-}$ mice. A, Clonal NIT-1 β-cells were transfected with a control (CTRL) siRNA or two separate siRNA against mouse GPR39. Expression of GPR39, IRS-2, and PDX-1 was determined by real-time Taqman PCR 72 h later. B, Expression of GPR39, IRS-2 and PDX-1 was determined by real-time Taqman PCR in pancreas from WT and GPR39$^{-/-}$ male mice that were fed a HFHS diet. *, $P < 0.05$ *vs.* CTRL siRNA (A) or WT (B). †, $P = 0.06$ *vs.* CTRL siRNA.

agreement with previous reports (18, 19), we found that GPR39 is found not only in pancreas but also pancreatic islets isolated from WT but not GPR39$^{-/-}$ mice (supplemental Fig. 1B). Furthermore, high levels of GPR39 mRNA were detectable in mouse NIT-1 and rat INS-1 cells, two immortalized clonal β-cell lines (data not shown). Immunohistochemistry of pancreatic sections from WT and GPR39$^{-/-}$ mice showed normal islet morphology and staining patterns for insulin and glucagon in mice fed either a LFHS or HFHS diet (Fig. 4A). Thus, deletion of GPR39 does not result in gross abnormalities in islet architecture. A detailed histomorphometrical analysis showed no significant differences (two-way ANOVA) in β-cell size, β-cell density, islet size, and islet density between WT and GPR39$^{-/-}$ mice fed either a LFHS or HFHS diet (Table 1). We noted, however, that islet size and islet density were increased by about 2- to 2.5-fold in WT mice fed a HFHS diet compared with their LFHS-fed counterparts (Table 1). In contrast, pancreas sections from GPR39$^{-/-}$ mice showed no increase in islet size or islet density in response to a HFHS diet (Table 1), suggesting a possible impairment in islet hyperplasia in diet-induced obese GPR39$^{-/-}$mice. No obvious differences in β-cell proliferation, as shown by anti-Ki67 staining, was observed (Table 1). We also tried to measure β-cell apoptosis by staining sections with an antibody that recognizes the cleaved form of caspase-3 but were unable to detect any specific signals within islets despite evidence of staining of nuclei in lymph nodes included in some pancreas sections and lymphoid aggregates within the pancreatic tissues (data not shown).

To directly evaluate the role of GPR39 in insulin secretion, we measured glucose-stimulated insulin secretion (GSIS) in islets from WT and GPR39$^{-/-}$ mice. Islets isolated from GPR39$^{-/-}$ mice maintained on a standard chow diet for 12–16 wk showed no significant differences in their ability to secrete insulin under both low and high glucose conditions (Fig. 4B). Insulin secretion was similarly unaffected by GPR39 gene deletion in islets from mice fed either a LFHS or HFHS diet (Fig. 4C). We also tested the ability of WT and GPR39$^{-/-}$ islets to respond to known insulin secretagogues. GSIS was markedly potentiated by GLP-1, arginine, or palmitate when measured in isolated islets from WT mice (Fig. 4D). The effect of these three agents was preserved in GPR39$^{-/-}$ islets *ex vivo*, indicating that the secretory response to glucose and other insulin secretagogues is normal in islets isolated from the knockout animals (Fig. 4D).

Because changes in insulin secretion *ex vivo* might be difficult to observe in the absence of exogenous GPR39 activation, we also analyzed GSIS in the presence of Zn^{2+}, a known GPR39 agonist (10, 15, 16). However, consistent with previous reports (25–27), we found that Zn^{2+} strongly decreased GSIS in isolated islets (Fig. 4E). The Zn^{2+}-induced decrease in insulin secretion was independent of the presence of GPR39 and occurred at con-

TABLE 1. Histomorphometrical analysis of pancreas sections

	LFHS diet		HFHS diet	
	WT	GPR39$^{-/-}$	WT	GPR39$^{-/-}$
β-Cell size (μm^2)	250 ± 10	264 ± 23	307 ± 23	264 ± 16
β-Cell density (percent of islet area)	82.7 ± 1.4	80.5 ± 2.6	84.9 ± 1.5	83.9 ± 1.5
Islet size (μm^2)	11198 ± 2251	18832 ± 4969	22171 ± 5989	15722 ± 4696
Islet density (percent of pancreas area)	0.9 ± 0.2	1.7 ± 0.6	2.6 ± 0.9	1.5 ± 0.5
Ki67-positive nuclei (percent of islet area)	0.027 ± 0.004	0.016 ± 0.002	0.022 ± 0.004	0.023 ± 0.006
CD31-positive cells (percent of islet area)	9.7 ± 0.5	6.6 ± 0.4	7.4 ± 0.9	5.4 ± 0.2

Each value represents the means ± SEM of six to seven mice (two to five mice for CD31 positive cells).

centrations of Zn^{2+} that have been shown to activate GPR39 in transiently transfected cells (50 μM; Fig. 4E). Thus, although Zn^{2+} acts as a GPR39 agonist in recombinant overexpression systems, its GPR39-independent effects on islets prevented us from using this agonist to evaluate GPR39 function in isolated islets. More specific GPR39 agonists will be needed to examine the function of GPR39 in isolated islets.

Silencing of GPR39 by RNAi in NIT-1 β-cells

To determine whether GPR39 can regulate β-cell function in a cell-autonomous fashion, we used the clonal β-cell line NIT-1, which express endogenous levels of GPR39 (Fig. 5A). Silencing of GPR39 in NIT-1 cells using two separate siRNA resulted in knockdown efficiency of 50% (GPR39 siRNA no. 1) and 90% (GPR39 siRNA no. 2) when compared with cells transfected with a control siRNA (Fig. 5A). Importantly, the expression of insulin receptor substrate (IRS)-2 and pancreatic and duodenal homeobox-1 (PDX-1), two important regulators of β-cell function (28–31), was significantly decreased in GPR39 knockdown cells (Fig. 5A). Moreover, the expression of IRS-2, but not that of PDX-1, was reduced in pancreas from $GPR39^{-/-}$ mice fed a HFHS diet (Fig. 5B). Cellular ATP levels, a measure of cell viability, were similar between control and GPR39 knockdown cells (relative ATP levels: control siRNA, 1.00 ± 0.11; GPR39 siRNA no. 1, 0.99 ± 0.10; GPR39 siRNA no. 2, 1.14 ± 0.07; P = NS).

Discussion

GPCRs expressed in pancreatic islets play key roles in insulin secretion by integrating nutritional cues from the diet and hormonal signals from peripheral organs (20). Here we show that under conditions of increased insulin resistance, GPR39 is required for optimal glucose homeostasis and that the primary defect in $GPR39^{-/-}$ mice appears to be an impaired insulin secretion in response to glucose. We examined the effect of GPR39 deletion in three models of insulin resistance: aging and exposure to a LFHS diet for 22 wk induced moderate insulin resistance, whereas a HFHS diet induced more severe insulin resistance accompanied by obesity and dyslipidemia. No significant differences were observed for aged $GPR39^{-/-}$ mice, although a trend toward decreased prandial and glucose-induced insulin levels was noted. Furthermore, $GPR39^{-/-}$ mice fed either a LFHS or HFHS diet develop prandial hyperglycemia and show significantly decreased insulin levels after a glucose challenge. It is noteworthy that we examined only male mice. Interestingly, in an accompanying paper, Holst and colleagues (39) showed that female $GPR39^{-/-}$ mice are more sensitive than their male counterparts in developing glucose intolerance, indicating potential gender-specific differences in glucose homeostasis in mice lacking GPR39. Our data and those of Holst *et al.* (39) are reminiscent of the phenotype described for GLP-1 receptor knockout mice in which both female and male knockout mice showed a significantly blunted insulin response during a glucose tolerance test, but only female mice displayed robust glucose intolerance (32). Defects in insulin secretion were observed only after challenge with glucose, and fasting glucose and insulin levels were similar in GLP-1 receptor knockout mice (32), similar to what was observed for $GPR39^{-/-}$ mice in this study. Taken together, our data are consistent with an insulin secretion defect in $GPR39^{-/-}$ mice that is uncovered during conditions of increased demand, *i.e.* during insulin resistance.

Our conclusion is further supported by the absence of an effect of GPR39 deletion on peripheral insulin sensitivity, as measured by insulin tolerance tests. It should be noted, however, that subtle changes in insulin sensitivity between WT and $GPR39^{-/-}$ mice might be uncovered by performing euglycemic-hyperinsulinemic clamps. Because the decrease in the insulin response in $GPR39^{-/-}$ mice was similar, whether glucose was injected ip or administered orally, it is unlikely that the impaired rise in insulin in $GPR39^{-/-}$ mice reflects an impaired incretin response or is a consequence of altered gastrointestinal motility in these mice.

An important question is how GPR39 modulates insulin secretion *in vivo*. Because GPR39 is highly expressed in pancreatic β-cells, we hypothesized that GPR39 may modulate insulin secretion directly. However, we found that glucose-stimulated insulin secretion was comparable in islets from young and old as well as diet-induced obese $GPR39^{-/-}$ mice. One possible explanation is that addition of GPR39 ligands is required to unmask a defect in insulin secretion *ex vivo*. This phenomenon has been observed for multiple GPCRs that are known to modulate insulin secretion. For example, islets isolated from GLP-1 receptor (33), GPR40 (34, 35) and GPR119 (36) knockout mice all show completely normal insulin secretion in the absence of stimulation with their respective ligands; indeed, the importance of these receptors in insulin secretion is only uncovered after addition of their respective ligands *ex vivo* or when glucose homeostasis is assessed *in vivo*. Interestingly, several of these knockout mice also show a significantly more pronounced insulin secretion phenotype upon ligand administration *in vivo* (32, 36, 37). It is likely that a specific natural or pharmacological ligand for GPR39 would provide an important tool to further understand the role of this GPCR in islet biology; unfortunately, no such ligand is currently available. It is possible that GPR39, owing to its high constitutive activity, can modulate insulin secretion to some extent even in the absence of a ligand. Using different methodologies, Holst and coworkers (39) showed that glucose-stimulated insulin secretion, especially the first phase, is impaired in isolated islets from $GPR39^{-/-}$ mice. Some of the differences between our study and the one by Holst *et al.* (39) are their use of longer incubation periods, perifusion, and islets from female as well as male mice. It is worth noting that although the knockout mice used in both studies were obtained from the same source, our mice were rederived before use, resulting in one additional cross with C57BL/6J females.

An alternative explanation is that GPR39 affects β-cell function independent of a direct role in insulin secretion. Our histomorphometrical analysis of islet and β-cell size and density suggests that disruption of GPR39 in mice may prevent islet hyperplasia associated with the development of diet-induced insulin resistance. Whereas the precise mechanism is still un-

known, we provide evidence that GPR39 regulates IRS-2 and PDX-1 expression in a cell-autonomous manner in β-cells. In addition, the expression of IRS-2 was also reduced in pancreas from obese GPR39$^{-/-}$ mice. Interestingly, disruption of IRS-2 in mice causes type 2 diabetes by preventing β-cell compensation during the development of insulin resistance as the animals aged (30). Thus, it is possible that GPR39 signaling is required for the full expression of IRS-2 and the subsequent increase in β-cell mass needed to counteract the development of age-dependent and diet-induced insulin resistance. It is also interesting to note that decreased levels of the transcription factor PDX1, which is important for β-cell differentiation and function (29), results in an *in vivo* phenotype similar to that of GPR39$^{-/-}$ mice in which insulin secretion is defective *in vivo* but not when measured in isolated islets *ex vivo* (28). Indeed, islets isolated from PDX1$^{+/-}$ mice show enhanced rather than decreased insulin secretion (28) but were more susceptible to apoptosis, resulting in reduced β-cell mass and islet number in older PDX1$^{+/-}$ mice (28). Although PDX-1 was not reduced in pancreas from GPR39$^{-/-}$ mice, RNAi experiments in NIT-1 β-cells indicate that GPR39 regulates, either directly or indirectly, PDX-1 expression. It is thus possible that GPR39 regulates PDX-1 expression *in vivo* in earlier phases of pancreas development and growth. Alternatively, GPR39 may regulate PDX-1 expression and/or function via posttranscriptional mechanism(s).

We also considered other possible explanations for the *in vivo* insulin secretion defect, such as a change in islet vascularization. For example, mice with islet-specific deletion of vascular endothelial growth factor-A show impaired insulin secretion *in vivo* but enhanced insulin secretion from isolated islets (38). Whereas we found no significant difference in CD31 staining between knockout and WT mice, it is possible that we may have missed a significant difference due to limited sample numbers. Importantly, however, CD31 mRNA levels were not significantly different between WT and GPR39$^{-/-}$ mice (data not shown). Clearly, further investigations will be required to determine whether GPR39 plays a role in islet vascularization.

In summary, we have shown that disruption of GPR39 impairs insulin secretion *in vivo*. Whereas the exact mechanism by which GPR39 exerts its action remains to be determined, our findings add GPR39 to the growing list of islet GPCRs that affect metabolic function and raise the possibility that GPR39 activators may be beneficial for the treatment of type 2 diabetes. It will be interesting to determine the effects of GPR39 deficiency and overactivation in models of β-cell failure and/or islet deficiency such as db/db mice and streptozotocin-treated animals. It will also be important to determine whether GPR39 modulates insulin secretion in humans. The isolation of endogenous or pharmacological agonists of GPR39 will be required to further examine its physiological role.

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References

1. **McKee KK, Tan CP, Palyha OC, Liu J, Feighner SD, Hreniuk DL, Smith RG, Howard AD, Van der Ploeg LH** 1997 Cloning and characterization of two human G protein-coupled receptor genes (GPR38 and GPR39) related to the growth hormone secretagogue and neurotensin receptors. Genomics 46:426–434
2. **Holst B, Holliday ND, Bach A, Elling CE, Cox HM, Schwartz TW** 2004 Common structural basis for constitutive activity of the ghrelin receptor family. J Biol Chem 279:53806–53817
3. **Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, Hsueh AJ** 2005 Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science 310:996–999
4. **Bresciani E, Rapetti D, Donà F, Bulgarelli I, Tamiazzo L, Locatelli V, Torsello A** 2006 Obestatin inhibits feeding but does not modulate GH and corticosterone secretion in the rat. J Endocrinol Invest 29:RC16–RC18
5. **Lagaud GJ, Young A, Acena A, Morton MF, Barrett TD, Shankley NP** 2007 Obestatin reduces food intake and suppresses body weight gain in rodents. Biochem Biophys Res Commun 357:264–269
6. **Zhang JV, Jahr H, Luo CW, Klein C, Van Kolen K, Ver Donck L, De A, Baart E, Li J, Moechars D, Hsueh AJ** 2008 Obestatin induction of early-response gene expression in gastrointestinal and adipose tissues and the mediatory role of G protein-coupled receptor, GPR39. Mol Endocrinol 22:1464–1475
7. **Chartrel N, Alvear-Perez R, Leprince J, Iturrioz X, Reaux-Le Goazigo A, Audinot V, Chomarat P, Coge F, Nosjean O, Rodriguez M, Galizzi JP, Boutin JA, Vaudry H, Llorens-Cortes C** 2007 Comment on "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake." Science 315:766
8. **Depoortere I, Thijs T, Moechars D, De Smet B, Ver Donck L, Peeters TL** 2008 Effect of peripheral obestatin on food intake and gastric emptying in ghrelin-knockout mice. Br J Pharmacol 153:1550–1557
9. **Gourcerol G, St-Pierre DH, Taché Y** 2007 Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)? Regul Pept 141:1–7
10. **Holst B, Egerod KL, Schild E, Vickers SP, Cheetham S, Gerlach LO, Storjohann L, Stidsen CE, Jones R, Beck-Sickinger AG, Schwartz TW** 2007 GPR39 signaling is stimulated by zinc ions but not by obestatin. Endocrinology 148:13–20
11. **Kobelt P, Wisser AS, Stengel A, Goebel M, Bannert N, Gourcerol G, Inhoff T, Noetzel S, Wiedenmann B, Klapp BF, Taché Y, Mönnikes H** 2008 Peripheral obestatin has no effect on feeding behavior and brain Fos expression in rodents. Peptides 29:1018–1027
12. **Nogueiras R, Pfluger P, Tovar S, Arnold M, Mitchell S, Morris A, Perez-Tilve D, Vázquez MJ, Wiedmer P, Castañeda TR, DiMarchi R, Tschöp M, Schurmann A, Joost HG, Williams LM, Langhans W, Diéguez C** 2007 Effects of obestatin on energy balance and growth hormone secretion in rodents. Endocrinology 148:21–26
13. **Tremblay F, Perreault M, Klaman LD, Tobin JF, Smith E, Gimeno RE** 2007 Normal food intake and body weight in mice lacking the G protein-coupled receptor GPR39. Endocrinology 148:501–506
14. **Unniappan S, Speck M, Kieffer TJ** 2008 Metabolic effects of chronic obestatin infusion in rats. Peptides 29:1354–1361
15. **Storjohann L, Holst B, Schwartz TW** 2008 Molecular mechanism of Zn(2+) agonism in the extracellular domain of GPR39. FEBS Lett 582:2583–2588
16. **Yasuda S, Miyazaki T, Munechika K, Yamashita M, Ikeda Y, Kamizono A** 2007 Isolation of Zn2+ as an endogenous agonist of GPR39 from fetal bovine serum. J Recept Signal Transduct Res 27:235–246
17. **Holst B, Elling CE, Schwartz TW** 2002 Metal ion-mediated agonism and agonist enhancement in melanocortin MC1 and MC4 receptors. J Biol Chem 277:47662–47670
18. **Egerod KL, Holst B, Petersen PS, Hansen JB, Mulder J, Hökfelt T, Schwartz TW** 2007 GPR39 splice variants versus antisense gene LYPD1: expression and

regulation in gastrointestinal tract, endocrine pancreas, liver, and white adipose tissue. Mol Endocrinol 21:1685–1698
19. **Moechars D, Depoortere I, Moreaux B, de Smet B, Goris I, Hoskens L, Daneels G, Kass S, Ver Donck L, Peeters T, Coulie B** 2006 Altered gastrointestinal and metabolic function in the GPR39-obestatin receptor-knockout mouse. Gastroenterology 131:1131–1141
20. **Winzell MS, Ahrén B** 2007 G-protein-coupled receptors and islet function-implications for treatment of type 2 diabetes. Pharmacol Ther 116:437–448
21. **Winzell MS, Svensson H, Arner P, Ahrén B, Holm C** 2001 The expression of hormone-sensitive lipase in clonal β-cells and rat islets is induced by long-term exposure to high glucose. Diabetes 50:2225–2230
22. **Longo EA, Tornheim K, Deeney JT, Varnum BA, Tillotson D, Prentki M, Corkey BE** 1991 Oscillations in cytosolic free Ca2+, oxygen consumption, and insulin secretion in glucose-stimulated rat pancreatic islets. J Biol Chem 266:9314–9319
23. **Després JP, Lemieux I** 2006 Abdominal obesity and metabolic syndrome. Nature 444:881–887
24. **Drucker DJ** 2007 The role of gut hormones in glucose homeostasis. J Clin Invest 117:24–32
25. **Aspinwall CA, Brooks SA, Kennedy RT, Lakey JR** 1997 Effects of intravesicular H+ and extracellular H+ and Zn2+ on insulin secretion in pancreatic β cells. J Biol Chem 272:31308–31314
26. **Ferrer R, Soria B, Dawson CM, Atwater I, Rojas E** 1984 Effects of Zn2+ on glucose-induced electrical activity and insulin release from mouse pancreatic islets. Am J Physiol 246:C520–C527
27. **Ghafghazi T, McDaniel ML, Lacy PE** 1981 Zinc-induced inhibition of insulin secretion from isolated rat islets of Langerhans. Diabetes 30:341–345
28. **Johnson JD, Ahmed NT, Luciani DS, Han Z, Tran H, Fujita J, Misler S, Edlund H, Polonsky KS** 2003 Increased islet apoptosis in $Pdx1^{+/-}$ mice. J Clin Invest 111:1147–1160
29. **Melloul D** 2004 Transcription factors in islet development and physiology: role of PDX-1 in β-cell function. Ann NY Acad Sci 1014:28–37
30. **Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF** 1998 Disruption of IRS-2 causes type 2 diabetes in mice. Nature 391:900–904
31. **Withers DJ, Burks DJ, Towery HH, Altamuro SL, Flint CL, White MF** 1999 Irs-2 coordinates Igf-1 receptor-mediated β-cell development and peripheral insulin signalling. Nat Genet 23:32–40
32. **Preitner F, Ibberson M, Franklin I, Binnert C, Pende M, Gjinovci A, Hansotia T, Drucker DJ, Wollheim C, Burcelin R, Thorens B** 2004 Gluco-incretins control insulin secretion at multiple levels as revealed in mice lacking GLP-1 and GIP receptors. J Clin Invest 113:635–645
33. **Flamez D, Van Breusegem A, Scrocchi LA, Quartier E, Pipeleers D, Drucker DJ, Schuit F** 1998 Mouse pancreatic β-cells exhibit preserved glucose competence after disruption of the glucagon-like peptide-1 receptor gene. Diabetes 47:646–652
34. **Latour MG, Alquier T, Oseid E, Tremblay C, Jetton TL, Luo J, Lin DC, Poitout V** 2007 GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion *in vivo*. Diabetes 56:1087–1094
35. **Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD, Edlund H** 2005 The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. Cell Metab 1:245–258
36. **Chu ZL, Jones RM, He H, Carroll C, Gutierrez V, Lucman A, Moloney M, Gao H, Mondala H, Bagnol D, Unett D, Liang Y, Demarest K, Semple G, Behan DP, Leonard J** 2007 A role for β-cell-expressed G protein-coupled receptor 119 in glycemic control by enhancing glucose-dependent insulin release. Endocrinology 148:2601–2609
37. **Miyawaki K, Yamada Y, Yano H, Niwa H, Ban N, Ihara Y, Kubota A, Fujimoto S, Kajikawa M, Kuroe A, Tsuda K, Hashimoto H, Yamashita T, Jomori T, Tashiro F, Miyazaki J, Seino Y** 1999 Glucose intolerance caused by a defect in the entero-insular axis: a study in gastric inhibitory polypeptide receptor knockout mice. Proc Natl Acad Sci USA 96:14843–14847
38. **Iwashita N, Uchida T, Choi JB, Azuma K, Ogihara T, Ferrara N, Gerber H, Kawamori R, Inoue M, Watada H** 2007 Impaired insulin secretion *in vivo* but enhanced insulin secretion from isolated islets in pancreatic β cell-specific vascular endothelial growth factor-A knock-out mice. Diabetologia 50:380–389
39. **Holst B, Egerod KL, Jin C, Petersen PS, Østergaard MV, Hald J, Sprinkel AME, Størling J, Mandrup-Poulsen T, Holst JJ, Thams P, Ørskov C, Wierup N, Sundler F, Madsen OD, Schwartz TW** 2009 G protein-coupled receptor 39 deficiency is associated with pancreatic islet dysfunction. Endocrinology 150:2577–2585