

Glucose-dependent insulintropic polypeptide receptor null mice exhibit compensatory changes in the enteroinsular axis

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Pamir, Nathalie, Francis C. Lynn, Alison M. J. Buchan, Jan Ehse, Simon A. Hinke, J. Andrew Pospisilik, Kazumasa Miyawaki, Yuichiro Yamada, Yutaka Seino, Christopher H. S. McIntosh, and Raymond A. Pederson. Glucose-dependent insulintropic polypeptide receptor null mice exhibit compensatory changes in the enteroinsular axis. *Am J Physiol Endocrinol Metab* 284: E931–E939, 2003. First published January 21, 2003; 10.1152/ajpendo.00270.2002.—The incretins glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are gut hormones that act via the enteroinsular axis to potentiate insulin secretion from the pancreas in a glucose-dependent manner. Both GLP-1 receptor and GIP receptor knockout mice (*GLP-1R*^{-/-} and *GIPR*^{-/-}, respectively) have been generated to investigate the physiological importance of this axis. Although reduced GIP action is a component of type 2 diabetes, *GIPR*-deficient mice exhibit only moderately impaired glucose tolerance. The present study was directed at investigating possible compensatory mechanisms that take place within the enteroinsular axis in the absence of GIP action. Although serum total GLP-1 levels in *GIPR* knockout mice were unaltered, insulin responses to GLP-1 from pancreas perfusions and static islet incubations were significantly greater (40–60%) in *GIPR*^{-/-} than in wild-type (*GIPR*^{+/+}) mice. Furthermore, GLP-1-induced cAMP production was also elevated twofold in the islets of the knockout animals. Pancreatic insulin content and gene expression were reduced in *GIPR*^{-/-} mice compared with *GIPR*^{+/+} mice. Paradoxically, immunocytochemical studies showed a significant increase in β -cell area in the *GIPR*-null mice but with less intense staining for insulin. In conclusion, *GIPR*^{-/-} mice exhibit altered islet structure and topography and increased islet sensitivity to GLP-1 despite a decrease in pancreatic insulin content and gene expression.

insulin; GLP-1; pancreas perfusion; cAMP; incretin

THE TERM ENTEROINSULAR AXIS was first proposed by Unger and Eisentraut (42) to describe the nutrient, neural, and endocrine interactions between the gut and the endocrine pancreas that amplify insulin secretion. The increased insulin release in response to oral glucose vs. intravenous administration is called the incre-

tin effect. The two most important incretins that act via the enteroinsular axis are glucose-dependent insulintropic polypeptide [GIP-(1–42)] and truncated forms of glucagon-like peptide-1 (GLP-1): GLP-1-(7–36NH₂) and GLP-1-(7–37) (15, 34).

These peptides account for nearly 50% of postprandial insulin release and therefore play a major role in glucose homeostasis (30). The insulintropic effects of GIP and GLP-1 are transduced through specific G protein-coupled receptors on the β -cell, resulting in stimulation of adenylyl cyclase (5, 46) and phospholipase A₂ (9) and increased levels of intracellular Ca²⁺ (20, 25). In addition to their insulintropic effects, both incretins stimulate insulin gene expression and biosynthesis in the β -cell (5, 11). Non- β -cell-mediated effects of GLP-1, including inhibition of gastric emptying and glucagon secretion, also contribute to its blood glucose-lowering effects (3, 47). The possible involvement of GIP and GLP-1 in the etiology of diabetes is still controversial. Studies have shown that, although GIP action is diminished, GLP-1 activity is preserved in type 2 diabetes (28, 29). A recent study from Lynn et al. (24) linked decreased GIP action to reduced GIP receptor levels in the Vancouver Diabetic Zucker (VDF) animal model of type 2 diabetes. Although GLP-1 secretion has been reported to be normal (13, 29, 44), increased (16, 32), or decreased (43) in type 2 diabetes due to its preserved insulintropic activity in the diabetic state, GLP-1 has been widely studied as a possible therapeutic agent (17).

The relative contribution of the incretins to the enteroinsular axis is still under investigation. Mice that are deficient in functional GIP or GLP-1 receptors (*GIPR*^{-/-} and *GLP-1R*^{-/-} mice, respectively) provide unique models for the study of incretin physiology. Both mouse models have impaired glucose tolerance but normal feeding behavior and body weight (26, 37). Different fasting plasma glucose levels have been reported in different colonies. We report that the *GLP-1R*^{-/-} colony exhibits normal glucose levels, whereas others have reported increased fasting levels (33, 37).

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In addition, *GLP-1R*^{-/-} mice have elevated plasma GIP levels and increased β -cell sensitivity to GIP, demonstrating that disruption of one component of the enteroinsular axis may be compensated for by another (33). We hypothesized that this compensatory mechanism is due to a physiological balance between the incretins, allowing for maintenance of glucose homeostasis, and that a similar compensation (enhanced GLP-1/insulin axis) would be observed in the *GIPR*^{-/-} mouse. In the present study, we have tested this hypothesis and report that the sensitivity of the β -cell to GLP-1 is indeed enhanced in the *GIPR*^{-/-} mouse along with reduced pancreatic insulin content and altered islet topography.

MATERIALS AND METHODS

Animals. The background and generation of *GIPR*-deficient C57BL/6 mice used in this study have been previously described (26). All animal experiments were performed in accordance with the guidelines put forth by the University of British Columbia Committee on Animal Care and the Canadian Council on Animal Care. For all the experiments, only 9- to 14-wk-old male *GIPR*^{-/-} and C57BL/6 control mice were used.

Oral glucose tolerance test and hormone radioimmunoassays. After an overnight fast, control and *GIPR*^{-/-} mice were administered 1 g/kg glucose (as a 10% solution) via gavage. Blood glucose levels were measured individually for each mouse by use of a handheld glucometer (Surestep; Lifescan, Burnaby, BC, Canada). Because only a limited volume of blood could be removed from each mouse and it was necessary to measure plasma GIP, GLP-1, and insulin levels in each sample, blood from 5–6 animals was pooled at $t = 0$ and $t = 20$ min after an oral glucose tolerance test (OGTT). Plasma was then separated by centrifugation at 12,000 g for 15 min at 4°C and stored at -20°C until hormone radioimmunoassay (RIA). The GIP RIA utilized a COOH-terminally directed antibody (rabbit anti-human) that has been extensively used for measuring total GIP immunoreactivity [GIP-(1–42) and GIP-(3–42)] (33). Total GLP-1 concentrations were measured by using the rat total GLP-1 kit from Linco (St. Charles, MO) as directed by the manufacturer. The GLP-1 antibody recognizes total immunoreactive GLP-1 (GLP-1-(7–36NH₂), GLP-1-(7–37), and NH₂-terminally truncated forms) and has a detection limit of 3 pM. Insulin was assayed by using a sensitive rat insulin kit (Linco) with an antibody that cross-reacts 100% with mouse insulin. Plasma dipeptidyl peptidase IV (DP IV) activity (μ U/ml) was measured colorimetrically (405 nm) through enzymatic release of *p*-nitroanilide (*p*-NA) from H-gly-pro-*p*-NA (35).

Insulin tolerance test. *GIPR*^{+/+} and *GIPR*^{-/-} mice were fasted for 3 h and given 0.01 U/g insulin (Humulin R, 100 U/ml; Lilly, Toronto, ON, Canada) by intraperitoneal injection. Blood glucose was measured with a handheld glucometer.

In vitro perfused pancreas. *GIPR*^{+/+} and *GIPR*^{-/-} mice were fasted overnight and anesthetized by intraperitoneal injection of 80 mg/kg pentobarbital sodium (Somnotol; MTC Pharmaceuticals, Cambridge, ON, Canada) before surgery. The surgical procedure and the pancreas perfusion protocol are described in Pederson et al. (33). Briefly, the abdominal aorta and the portal vein were cannulated with PE-50 tubing (Cole-Palmer, Chicago, IL). The perfusate consisted of a Krebs-Ringer bicarbonate buffer supplemented with 3% dextran (Sigma, Oakville, ON, Canada) and 0.2% bovine serum

albumin (BSA, fraction V, RIA grade; Sigma) gassed with 5% CO₂ to achieve and maintain pH 7.4. The flow rate was maintained at 1 ml/min, and the outflow was collected at 1-min intervals. GIP and GLP-1 (both from Probiadrug, Hala, Germany) were delivered by sidearm infusion in the presence of 16.7 mM glucose, resulting in a 1 nM final concentration, whereas arginine (10 mM; Sigma) was mixed directly into the perfusate containing 8.8 mM glucose. Insulin was measured by RIA as previously described (33).

Isolation and culture of pancreatic islets. Fed mice were anesthetized with Somnotol, and islets of Langerhans were isolated by collagenase digestion. Collagenase (type V, 2 mg/ml; Sigma) in Hanks' balanced salt solution (HBSS; GIBCO-Life Technologies, Burlington, ON, Canada) supplemented with 10 mM HEPES (GIBCO), 2 mM L-glutamine (Sigma), and 0.2% BSA was injected into the common bile duct to inflate the pancreas. The pancreas was then removed and digested at 37°C for 10 min. The pancreas was then dispersed mechanically with a siliconized Pasteur pipette, washed, filtered through a 1-mm nylon screen, and washed again. Islets were separated by centrifugation at 1,800 g for 20 min in a discontinuous dextran gradient. Hand-picked islets were cultured overnight in RPMI 1640 (Sigma) with 8.8 mM glucose, 10% fetal calf serum (Cansera, Rexdale, ON, Canada), 50 U/ml each of penicillin G (Sigma) and streptomycin (Sigma), 0.07% human serum albumin (Sigma), 0.0025% human apotransferrin (GIBCO), 25 pM sodium selenite (VWR, Mississauga, ON, Canada) and 10 μ M ethanolamine (VWR).

Islet cAMP and insulin measurements. After overnight culture, 15–18 healthy islets were selected, washed twice with 0.5 ml of Krebs-Ringer supplemented with 0.2% BSA and 4.4 mM glucose, and incubated at 37°C for 30 min. Thereafter, islets were incubated in the presence or absence of 10 nM GIP or GLP-1 or 10 μ M forskolin (Sigma) in the same buffer with 0.5 mM 3-isobutyl-1-methylxanthine (Research Biochemicals International, Natick, MA) and 16.7 mM glucose. After a 30-min incubation, islets were lysed by boiling for 3 min in 0.05 N HCl. Samples were subsequently dried by vacuum centrifugation (Speed-Vac; Sorvall, Farmingdale, NY) and stored at -20°C. cAMP levels were assayed by using a kit according to the manufacturer's instructions (Biomedical Technologies, Stoughton, MA). For insulin measurements, islets were collected after overnight culture, washed twice with Krebs-Ringer, and incubated for 45 min in 4.4 mM glucose containing Krebs-Ringer supplemented with 0.1% BSA. After a short centrifugation, the medium was replaced with either 16.7 mM glucose alone or 16.7 mM glucose plus 10 nM GIP or GLP-1. After 45 min, islets were lysed by boiling for 5 min in 1 M acetic acid and centrifuged, and supernatants were assayed for insulin (24).

Measurement of pancreatic insulin content. Animals were rendered unconscious with CO₂ and exsanguinated. Pancreata were removed, homogenized in 2 M acetic acid, and then boiled for 5 min. Homogenates were centrifuged at 15,000 g for 15 min, and the supernatant was stored at -70°C. Total protein levels were measured with a bicinchoninic acid kit (Pierce, Rockford, IL), and insulin values (measured as described) were normalized to total protein content (ng/ μ g protein) (33).

Isolation and measurement of islet insulin and GLP-1 receptor mRNA by reverse transcriptase real-time polymerase chain reaction (PCR). Freshly isolated islets were washed twice with HBSS and solubilized in 1 ml TRIzol (GIBCO) and kept at -70°C. After isolation, 50 ng of total RNA were subjected to reverse transcription (RT) in a volume of 10 μ l containing 0.5 mM deoxynucleotide triphosphates, 15 pmol

specific primers targeted at the carboxy termini of the mouse GLP-1 receptor open reading frame (5'-ACC AAC AGG GAG GAC CGG-3') or the mouse insulin II gene (5'-GTA GTT CTC CAG CTG GTA GAG GG-3') 100 U Superscript II RNase H⁻ reverse transcriptase (GIBCO), 10 U RNase inhibitor (RNA Guard; Amersham Pharmacia, Piscataway, NJ), 1 mM dithiothreitol, 50 mM Tris·HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl₂. After RT, 10 ng (2 μl) of mouse cDNA were used in the real-time PCR reaction to measure insulin and GLP-1 receptor expression. The PCR reaction mix consisted of 1× TaqMan Buffer A (PE Applied Biosystems, Foster City, CA), 10 mM MgCl₂, 200 μM each dATP, dCTP, and dGTP, 400 μM dUTP, 200 nM mouse GLP-1 receptor 5' forward primer (5'-CAG GGC TTG ATG GTG GCT ATC-3') or mouse insulin II 5' forward primer (5'-TGG AGG CCC GGG AGC-3'), 200 nM mouse GLP-1 receptor 3' reverse primer (5'-CGC TCC CAG CAT TTC CG-3') or mouse insulin II 3' reverse primer (5'-ATC TAC AAT GCC ACG CTT CTG C-3'), and 100 nM GLP-1 receptor probe colabeled with the fluorescent dyes VIC and 6-carboxy-tetramethylrhodamine (TAMRA) (5'-ACT GCT TTG TCA ACA ATG AGG TCC AGA TGG-3') or insulin II probe colabeled with fluorescent dyes TET and TAMRA (5'-ACC TTC AGA CCT TGG CAC TGG AGG TG-3'), 0.01 U/μl AmpErase-uracil *N*-glycosylase (UNG; PE Applied Biosystems), and 0.025 U/μl AmpliTaq Gold (PE Applied Biosystems). PCR reactions were carried out in triplicate in the PE Applied Biosystems 7700 sequence detection system. The reaction profile included a 10-min preincubation at 50°C to allow the UNG to degrade any uracil-containing nucleic acids and a further 10-min incubation at 94°C to activate the AmpliTaq Gold. After these preincubations, a two-step PCR protocol was carried out, which included a denaturation step at 94°C for 15 s followed by a 1-min annealing/extension step at 60°C. Fluorescence was measured during the annealing/extension steps over 40 cycles and used to calculate a cycle threshold (Ct), i.e., the point at which the reaction is in the exponential phase and is detectable by the hardware. All reactions followed the typical sigmoidal reaction profile, and Ct was used as a measure of amplicon abundance (12). The results were normalized over total wild-type mRNA levels.

Immunocytochemistry. Mice were fasted overnight before being killed. Pancreata from wild-type and knockout mice ($n = 5$ each) were fixed separately in Bouin's solution (75% picric acid, 8% formaldehyde, 5% glacial acetic acid) for 1 h at room temperature and washed thoroughly with 70% ethanol. The tissue was embedded in paraffin wax, and three consecutive 5-μm sections were taken 300 μm apart and mounted on glass slides. The study was carried out blind: slides were coded to prevent identification of +/+ and -/- tissues before quantification. The sections were dewaxed in xylene and cleared in petroleum ether (Sigma). The sections were then rehydrated in PBS (80 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 2.86 mM KCl, 137 mM NaCl). To control for intra-immunostain variability, all of the sections were incubated in the same batch of solutions and stained simultaneously. The β-cells were detected by overnight incubation with a polyclonal rabbit anti-mouse insulin antibody (1:100, Santa Cruz Biotechnologies, Santa Cruz, CA), followed by a 90-min incubation with a biotinylated goat anti-rabbit secondary antibody (1:300, Jackson Labs, West Grove, PA) at room temperature. Sections were then washed and incubated with avidin-biotin peroxidase complex (Vector Labs, Burlington, ON, Canada) at a dilution of 1:1,000 in PBS supplemented with 5% horse serum (Sigma). The peroxidase reaction was developed with 2% diaminobenzidine tetrahydrochloride in 0.05 M Tris (pH 7.5) with 0.2% H₂O₂. After being counterstained with hematoxylin for 5 min, the sections were dehy-

drated through graded alcohol, and coverslips were applied with Permount (Fisher Scientific, Nepean, ON, Canada). The sections were analyzed using the NIH Image software (<http://rsb.info.nih.gov/nih-image/>), and data were analyzed as islet area over total pancreatic area. Five separate fields of view (under ×10 magnification) per section were randomly chosen, the periphery of the islets in this area was outlined, and area was determined by using the analysis software. The islet area from the five random samples was then normalized to the total pancreatic area in those five fields. Once the quantification had been completed, the source of the sections (*GIPR*^{+/+} or *GIPR*^{-/-}) was identified and statistical significance was assessed.

Data analysis. All data are expressed as means ± SE. An unpaired Student's *t*-test and a Mann-Whitney *U*-test (exclusively for immunocytochemistry) were used to compare the control values with *GIPR*^{-/-} mouse values, where $P < 0.05$ was considered statistically significant. The data analysis and area under the curve calculations were carried out by using graphic analysis software (Graphpad, Prism, San Diego, CA).

RESULTS

OGTT. The OGTT profile of *GIPR*^{-/-} mice over 120 min is very similar to that of the wild-type control animals (Fig. 1A). The 15- and 30-min blood glucose were moderately (18%) but significantly greater in *GIPR*^{-/-} mice. To allow for concurrent measurement of insulin and incretin levels, basal and 20-min blood samples from another OGTT were pooled. This time point was chosen on the basis of preliminary studies to achieve near-peak incretin levels (data not shown). Fasting glucose and insulin levels were both comparable in *GIPR*^{-/-} and wild-type mice (Fig. 1, B and C). However, after a glucose challenge, the 20-min plasma glucose levels were significantly higher in *GIPR*^{-/-} mice than in wild-type mice ($P < 0.05$), and insulin levels were 45% lower in *GIPR*^{-/-} mice than in wild-type mice (Fig. 1, B and C). To further assess the hormonal components of the enteroinsular axis in *GIPR*^{-/-} mice, fasting and 20-min total plasma immunoreactive GLP-1 and immunoreactive GIP levels were determined (Fig. 1, D and E). Total GLP-1 levels did not differ in *GIPR*^{-/-} mice compared with wild-type (Fig. 1D, $P > 0.05$). However, fasting plasma GIP levels were elevated, whereas the 20-min levels were lowered by 25% in *GIPR*^{-/-} compared with *GIPR*^{+/+} mice (Fig. 1E, $P < 0.05$). Plasma DP IV activity was unaltered between groups: values for fasting were 10.5 ± 0.9 vs. 10.2 ± 0.8 μU/ml and 9.1 ± 1.5 vs. 8.7 ± 0.6 μU/ml for 20-min in *GIPR*^{+/+} and *GIPR*^{-/-} mice, respectively.

To explain the alterations in plasma GIP levels, the duodeno-jejunal GIP content was also measured in fed mice. A reduction of 15% in *GIPR*^{-/-} mice was observed compared with control animals (67.5 ± 8.2 pg/ml, $n = 6$, in *GIPR*^{+/+} vs. 59.7 ± 4.8 pg/ml, $n = 7$, in *GIPR*^{-/-} mice, $P < 0.05$).

Insulin tolerance test. To investigate the insulin sensitivity of the *GIPR*^{-/-} mice, blood glucose levels were determined after an insulin challenge. Blood glucose profile over 1 h did not differ in *GIPR*^{-/-} mice compared with wild-type control mice (Fig. 2).

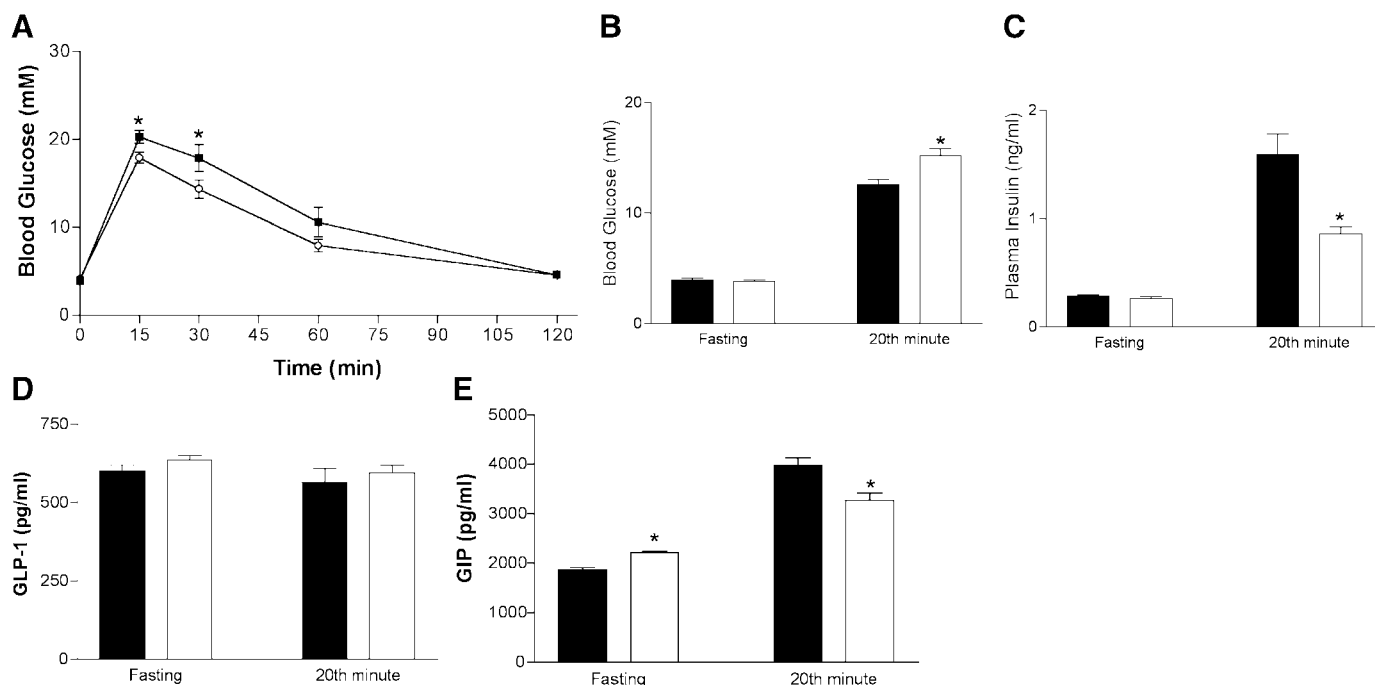


Fig. 1. Oral glucose tolerance test (OGTT) and fasting and 20th-min OGTT plasma total immunoreactive (ir) glucagon-like peptide (GLP)-1, and glucose-dependent insulinotropic polypeptide (GIP) levels in wild-type (WT) and $GIPR^{-/-}$ mice. A: blood glucose levels in WT (\circ , $n = 7$) and $GIPR^{-/-}$ (\blacksquare , $n = 11$) mice during an OGTT. B: blood glucose levels for fasting and 20th min of an OGTT in WT (filled bars, $n = 21$) and $GIPR^{-/-}$ (empty bars, $n = 23$) animals. C: plasma fasting and 20th-min insulin levels in WT (filled bars, $n = 4$, pooled from 21 animals) and $GIPR^{-/-}$ (empty bars, $n = 4$, pooled from 23 animals). Plasma total irGLP-1 (D) and irGIP (E) levels in WT and $GIPR^{-/-}$. Values are means \pm SE (* $P < 0.05$).

In vitro insulin responses to GIP, glucose, arginine, and GLP-1. To verify that GIP was unable to stimulate insulin secretion from the pancreata of $GIPR^{-/-}$ animals, *in vitro* perfusions were carried out (Fig. 3A). As expected, 1 nM GIP generated no insulin response from the perfused knockout pancreata. Subsequently, low (4.4 mM) and high (16.7 mM) glucose perfusions were also performed, demonstrating that glucose-stimulated insulin secretions in $GIPR^{-/-}$ and wild-type mice were comparable (Fig. 3B). In type 2 diabetic patients, insulin responses to secretagogues such as high glucose, sulfonylurea, and arginine are blunted (45). To assess the insulin response of $GIPR^{-/-}$ islets to a stimulant

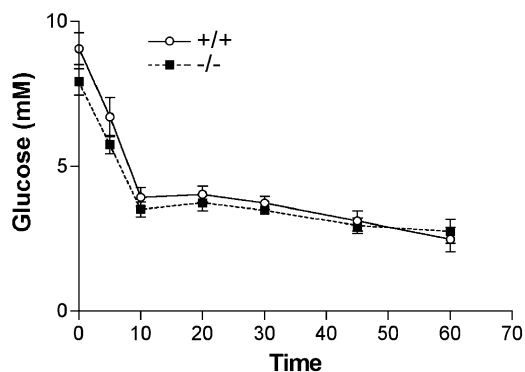


Fig. 2. Insulin tolerance test. Blood glucose was measured after insulin challenge in WT control (+/+; \circ , $n = 8$) and in $GIPR^{-/-}$ (-/-; \blacksquare , $n = 8$) mice. Values are means \pm SE.

other than glucose, high-dose arginine (10 mM) perfusions were performed in the presence of 8.8 mM glucose (Fig. 3C). The results showed no significant differences in insulin secretion between $GIPR^{-/-}$ and control animals. However, $GIPR^{-/-}$ mice exhibited a higher peak and sustained insulin release in response to GLP-1 perfusion (Fig. 3D). The integrated insulin response to perfusion with 1 nM GLP-1 was 60% greater in $GIPR^{-/-}$ mice compared with wild-type mice (Fig. 3E). To determine whether these results were due to an inherent change in islet physiology, islets were isolated, cultured overnight, and stimulated with low (4.4 mM) and high (16.7 mM) glucose alone or in the presence of either 10 nM GIP or GLP-1 (Fig. 4A). The insulin secreted over 45 min was 40% greater in response to GLP-1 stimulation for $GIPR^{-/-}$ vs. $GIPR^{+/+}$ islets ($P < 0.05$), consistent with the data from perfusion experiments. Additionally, GIP did not stimulate insulin release from $GIPR^{-/-}$ islets, and the insulin response to 16.7 mM glucose was comparable in both groups.

Intracellular cAMP production in isolated islets. To correlate GLP-1-stimulated insulin release with receptor activation, we measured cAMP production in response to GLP-1 in isolated islets (Fig. 4B). Forskolin was used as a positive control to assess maximal cAMP production and thus allow normalization of responses to account for discrepancies in islet size and number. Interestingly, basal (16.7 mM glucose) cAMP levels

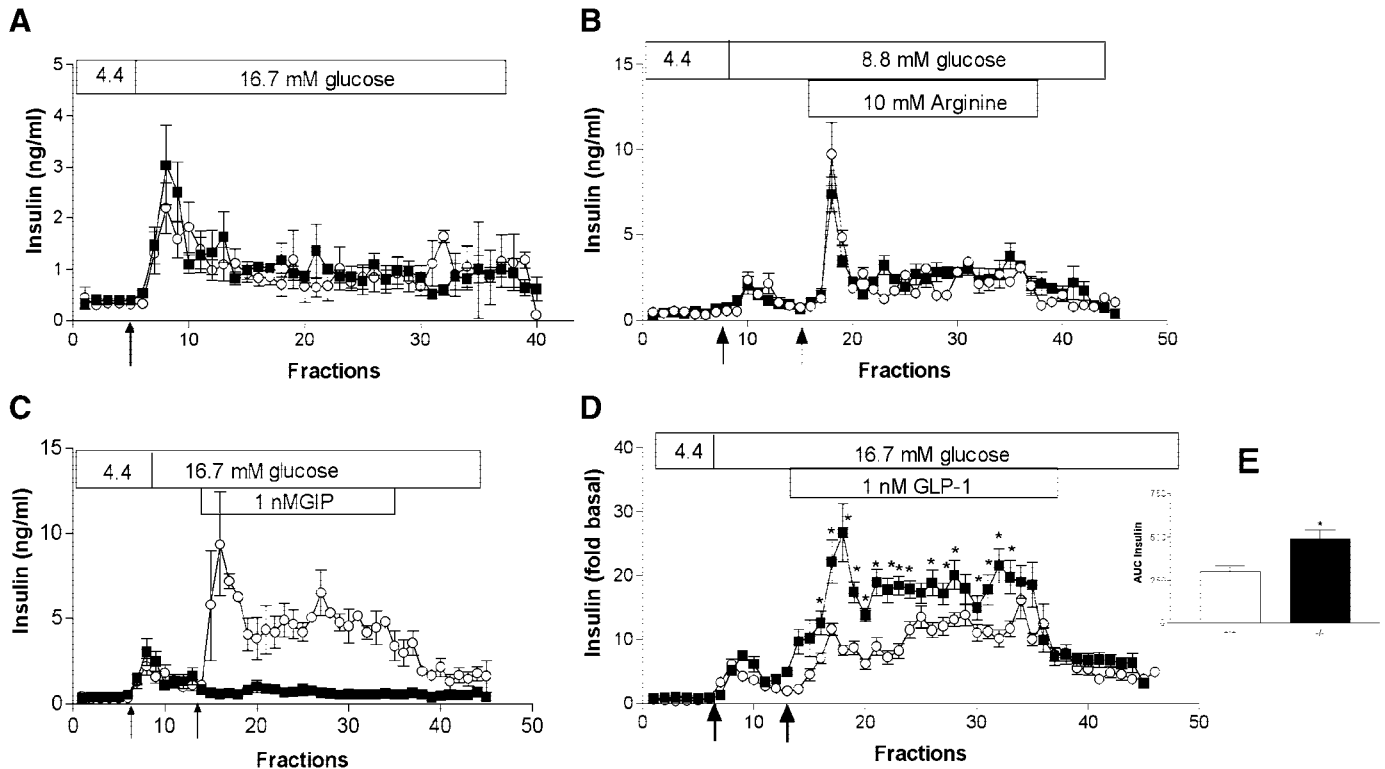


Fig. 3. Insulin secretion from perfused pancreas in response to GIP, glucose alone, arginine, and GLP-1 in WT and *GIPR*^{-/-} mice. Insulin secretion from the perfused pancreas in response to: 1 nM GIP in WT control (○, *n* = 3) and *GIPR*^{-/-} (■, *n* = 4) mice (A); 4.4 and 16.7 mM glucose in WT control and *GIPR*^{-/-} mice (*n* = 4; B); and 10 mM arginine in WT control and *GIPR*^{-/-} mice (*n* = 4; C). D: 1 nM GLP-1, presented as fold of basal, in control and *GIPR*^{-/-} mice (*n* = 6). E: mean integrated insulin response for *GIPR*^{+/+} and *GIPR*^{-/-} mice (**P* < 0.05). AUC, area under the curve. Arrows indicate switching between conditions. Values are means ± SE.

were significantly increased in *GIPR*^{-/-} mice compared with wild-type mice. GLP-1-stimulated cAMP production was also significantly increased in *GIPR*^{-/-} animals vs. wild-type mice (*P* < 0.05), implying that β-cell sensitivity to GLP-1 was increased. Thus these findings are also consistent with the perfusion and static islet stimulation experiments.

Pancreatic insulin content and islet insulin and GLP-1 mRNA content. Both GIP and GLP-1 stimulate insulin gene transcription and protein synthesis in the β-cell (11). Thus absence of GIP action may lead to alterations in insulin gene transcription and, therefore, pancreatic insulin content. The total insulin content from fed mice pancreata was significantly lower (~40%) in *GIPR*^{-/-} than in *GIPR*^{+/+} mice (Fig. 6A; *P* < 0.05). Furthermore, these data are supported by the finding that insulin mRNA levels were significantly reduced (~40%) in isolated islets of *GIPR*^{-/-} mice compared with controls (Fig. 5A, *P* < 0.05). Finally, assessment of GLP-1 receptor mRNA levels revealed that, despite an increase in GLP-1 sensitivity, there was no increase in GLP-1 receptor mRNA levels in the islets of *GIPR*^{-/-} mice (Fig. 5B).

Immunocytochemistry. Immunocytochemical studies were carried out to assess the effect of GIP receptor deficiency on islet and pancreas morphology. β-Cell area as a percentage of total pancreatic area was significantly increased (~45%) in knockout vs. wild-type

mice (Fig. 6B; *P* < 0.05). Additionally, when stained under identical experimental conditions, the staining intensity for insulin was reduced in *GIPR*^{-/-} islets (Fig. 6, C and D). The whole pancreas weight was not different between groups: 1.6 ± 0.4 vs. 1.7 ± 0.3 g in *GIPR*^{+/+} and *GIPR*^{-/-}, respectively.

DISCUSSION

The reduced effect of GIP on insulin secretion in type 2 diabetic patients has been described (21, 29). It has been suggested that this might be due to reduced receptor expression in the β-cell, resulting in its lowered sensitivity to GIP (18). Recently, this possibility was addressed in a model of type 2 diabetes, the VDF rat, in which it was demonstrated that GIP receptor expression was reduced (24). Therefore, mice with a targeted disruption of the *GIPR* gene (*GIPR*^{-/-}) may provide a useful model for studying the potential implications of a lack of GIP signaling on glucose homeostasis and the development of type 2 diabetes. Miyawaki et al. (26) have shown that these mice exhibit modest glucose intolerance along with a 50% reduction in insulin secretion in response to oral glucose, whereas weight gain was reported to remain unchanged under both normal and high-fat diet conditions. It was the hypothesis of the present study that these mice would exhibit compensatory

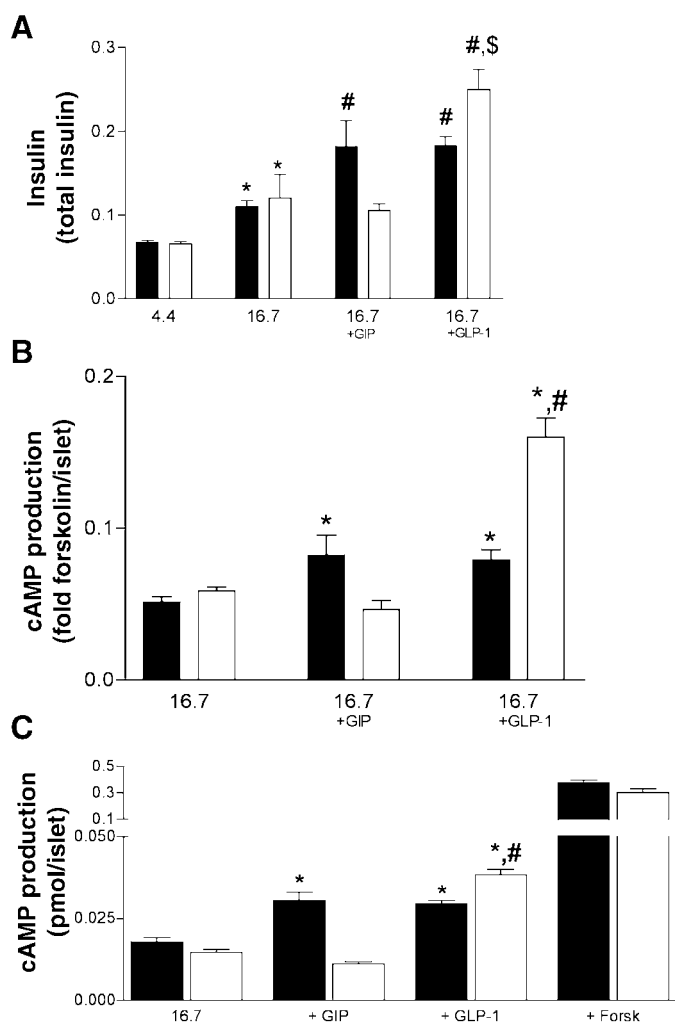


Fig. 4. Static stimulation of isolated pancreatic islets from WT and *GIPR*^{-/-} mice. **A**: insulin secretion in response to GIP ($n = 4$) and GLP-1 ($n = 6$) in WT control (filled bars) and *GIPR*^{-/-} (empty bars) mice. ($\$P < 0.05$ WT vs. *GIPR*^{-/-}; $*P < 0.05$ 16.7 vs. 4.4 mM glucose; $\#P < 0.05$ vs. 16.7 mM glucose alone). Values are means \pm SE. **B**: cAMP production in response to GIP and GLP-1 stimulation in isolated islets from WT control (filled bars) and *GIPR*^{-/-} (empty bars) mice. Data have been normalized to forskolin and are presented as %forskolin. **C**: cAMP production presented with the real values without normalization to forskolin. For all conditions, $n = 5-8$; $\#P < 0.05$ WT vs. *GIPR*^{-/-}; $*P < 0.05$: vs. basal. Values are means \pm SE.

changes in the enteroinsular axis to overcome the absence of GIP action.

Immunoneutralization studies examining the relative contribution of GIP to the enteroinsular axis were first reported in the early 1980s (6–8). In more recent studies, the treatment of rats with a GIP antagonist, GIP-(7–30NH₂), has been shown to result in a 72% decrease in postprandial insulin release along with normal glucose levels (40). Later, the same group provided evidence that GIP-(7–30NH₂) inhibits glucose transport from the small intestine, which might in part explain the relatively small rise in serum glucose levels after oral glucose despite a profound decrease in postprandial insulin levels (41). Studies using a GIPR

antibody have suggested that GIP acts as an anticipatory signal to the β -cell to potentiate insulin release, which in turn primes the periphery for glucose disposal (22). The same antibody was used on *GLP-1R*^{-/-} and *GLP-1R*^{+/+} mice, and it was concluded by Baggio et al. (2) that GIP had a restricted role in the regulation of glucose homeostasis. These studies involved relatively short-term antagonist administration and therefore may not be reflective of the consequences of the chronic absence of the GIP action. The use of GIPR-null mice provides a new approach to the investigation of the effects of GIP.

Despite the loss of GIP receptors, the 2-h blood glucose profiles for *GIPR*^{-/-} and wild-type mice are remarkably similar (Fig. 1A). Blood glucose reached peak values for both groups of animals at the 15-min time point, and the curves merged after 40 min. The subtle increase (18%) in plasma glucose levels at the 20th min of the OGTT does not correlate with the more profound reduction (55%) in plasma insulin levels in the *GIPR*^{-/-} animals (Fig. 1, B and C), suggesting a possible change in insulin sensitivity or glucose disposal. Controversy exists as to whether GLP-1 is capable of exerting insulin-like effects on peripheral tissue in addition to the well-studied insulinotropic effects. Studies have shown that GLP-1 improves glucose disposal in type 2 diabetes by enhancing insulin-stimulated glucose utilization (36), and these effects have been shown to be independent of the amount of insulin secreted (4, 14). Recently, Ahren and Pacini (1) re-

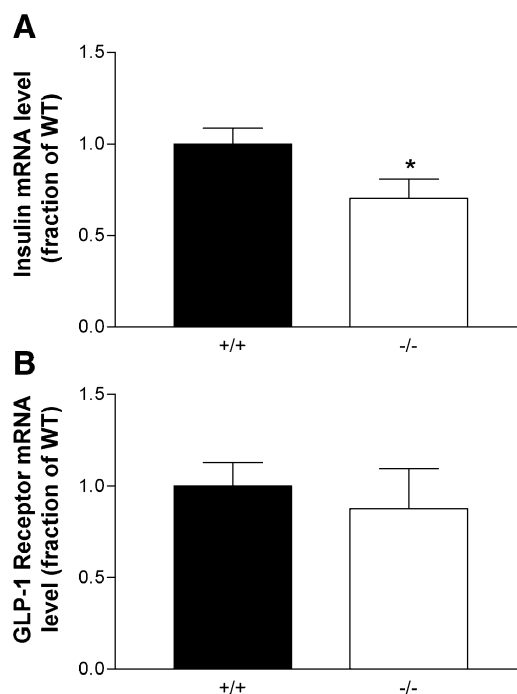


Fig. 5. Isolated pancreatic islet insulin and GLP-1 receptor mRNA content. **A**: islet insulin mRNA levels in *GIPR*^{+/+} (filled bars, $n = 10$) and *GIPR*^{-/-} (empty bars, $n = 9$) mice. **B**: islet GLP-1 receptor mRNA levels in *GIPR*^{+/+} ($n = 12$) and *GIPR*^{-/-} ($n = 12$). Values are presented relative to WT mRNA represented as 1.0 and are means \pm SE ($*P < 0.05$).

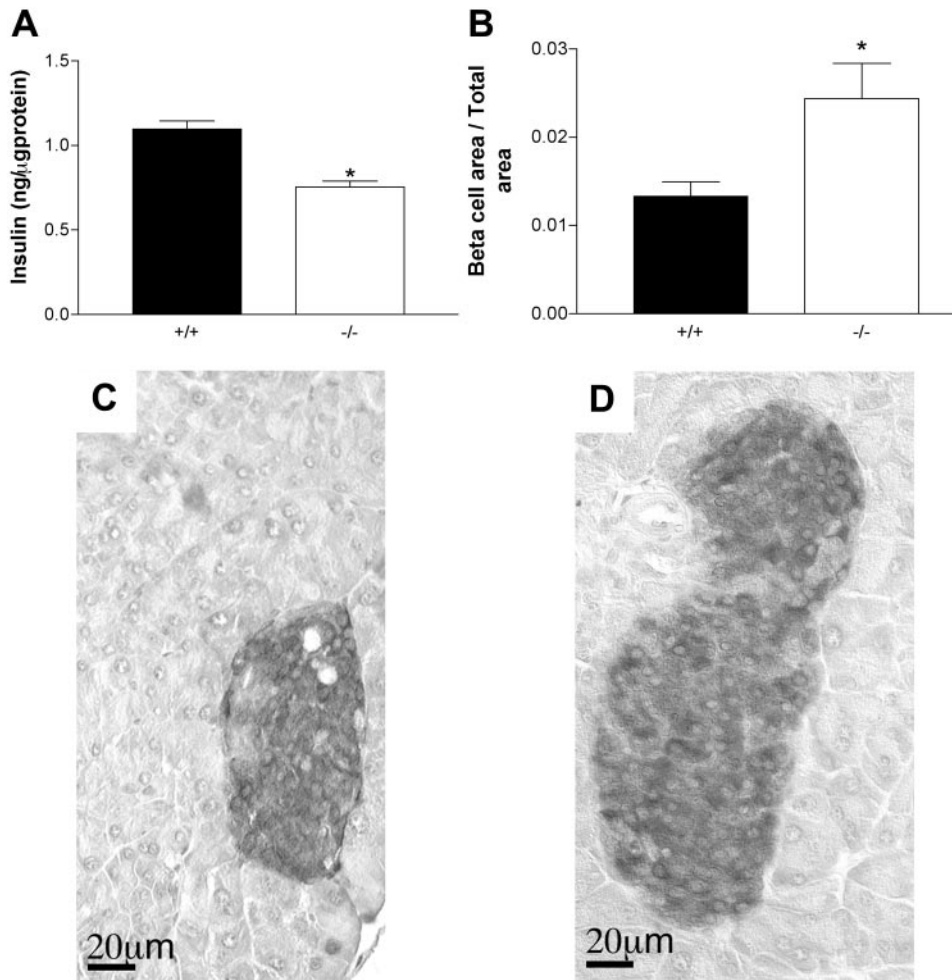


Fig. 6. Total pancreatic insulin content and pancreatic β -cell area. **A**: total pancreatic insulin content in fed *GIPR*^{+/+} (filled bars, $n = 5$) and *GIPR*^{-/-} (empty bars, $n = 5$) mice. Insulin content was normalized for total protein content. **B**: relative area occupied by β -cells (fold total area) in *GIPR*^{+/+} and *GIPR*^{-/-} mice (* $P < 0.05$). Peroxidase staining for insulin in *GIPR*^{+/+} (**C**) and *GIPR*^{-/-} mice (**D**) shows that the area occupied by the β -cells in the *GIPR*^{+/+} mice is smaller than in *GIPR*^{-/-} mice.

ported that, in mice, the effects of GLP-1 on glucose homeostasis were mainly insulin mediated and the use of a relatively specific GLP-1 receptor antagonist, exendin-(9–39), reversed these actions. It remains unclear whether an increased extrapancreatic sensitivity to GLP-1 might play a role in the alteration in glucose clearance in *GIPR*^{-/-} mice. We report normal sensitivity to insulin in *GIPR*^{-/-} mice (Fig. 2); therefore, it appears that enhanced GLP-1 action is not insulin mediated. However, GLP-1 is able to potentiate insulin action (36), and it is possible that, in *GIPR*^{-/-} mice, enhanced GLP-1 action ameliorates insulin action. Furthermore, it is also possible that, in these mice, the enhanced glucose disposal (relative to the insulin levels present) could be a result of the delayed gastric emptying (caused by GLP-1) (31) due to increased peripheral tissue GLP-1 sensitivity.

Similar to *GIPR*^{-/-} mice, *GLP-1R*^{-/-} mice have been shown to exhibit modest glucose intolerance, with upregulation, in this case, of the GIP component of the enteroinsular axis (33). The present study was designed in part to test whether the converse was true in *GIPR*^{-/-} animals. Miyawaki et al. (26) showed that, when administered an intraperitoneal glucose challenge (thus bypassing the enteroinsular axis), *GIPR*^{-/-} mice exhibited no alteration in glucose disposal relative to

wild-type animals. In the present study, pancreas perfusion (Fig. 3), static islet stimulation (Fig. 4A), and cAMP production (Fig. 4B) data clearly showed that the GLP-1 component of the enteroinsular axis in *GIPR*^{-/-} mice was upregulated. These data agree with the evidenced alteration in oral glucose tolerance in the face of unchanged intraperitoneal glucose tolerance. The combined findings that in vitro insulin responses to high glucose and arginine are similar and that islet GLP-1 receptor mRNA levels in *GIPR*^{-/-} and in *GIPR*^{+/+} mice are comparable suggest that compensation occurs distally to the GLP-1 receptor on the β -cell. Because both incretins act through G protein-coupled receptors and signal via the adenylyl cyclase-cAMP system (5, 27, 46), it could be hypothesized that the permanent absence of GIP receptors leads to a compensatory increase in coupling efficiency of GLP-1 receptors. Although the GLP-1 receptor mRNA levels are comparable, we do not have information about the protein synthesis. Hence, despite similar gene expression, the protein synthesis might be enhanced, leading to increased sensitivity to GLP-1. The possibility of upregulation of GLP-1 secretion appears unlikely, since no changes in GLP-1 levels were observed 20 min after an oral glucose challenge (Fig. 1D), nor were there changes in activity of plasma DP IV, the primary

inactivating enzyme for GIP and GLP-1 (35). Together these data suggest that the majority of the compensatory changes in the GLP-1 axis of *GIPR*^{-/-} mice lay at a postreceptor level in the β -cell. That said, the observed reduction in circulating insulin levels and modest increase in blood glucose show that compensation by the GLP-1 axis is not complete and that, although the functions of GIP and GLP-1 overlap, both are required for proper glycemic control.

GIPR^{-/-} mice were shown to have a 40% reduction in pancreatic insulin content and gene expression concomitant with a twofold increase in β -cell area (Fig. 6, A and B), suggesting that insulin gene expression and content were reduced in the *GIPR*-null mice on a cellular level. Because both GIP and GLP-1 stimulate insulin gene expression (5, 11), it might be predicted that an increase in β -cell GLP-1 sensitivity in the absence of GIP action could protect against a decrease in islet insulin mRNA and protein levels. The decrease in insulin mRNA and protein synthesis in *GIPR*^{-/-} mice is comparable to the 35% decrease that was shown by Pederson et al. (33) in *GLP-1R*^{-/-} mice. Hence, absence of either of the incretins results in abnormalities within the β -cell, leading to impaired insulin content. Thus the compensation by GLP-1 or by GIP at the β -cell level, in *GIPR*^{-/-} and in *GLP-1R*^{-/-} mice, respectively, seems not to extend as far as insulin biosynthesis.

In addition to stimulation of insulin production on the cellular level, GLP-1 has also been shown to be involved in the morphological development of the islets of Langerhans. In immunocytochemical studies, Ling et al. (23) showed α - and δ -cell migration toward the islet core and a reduction in islet size in *GLP-1R*^{-/-} mouse pancreata. Examination of pancreatic sections from *GIPR*^{-/-} mice with the same objective showed no such changes in endocrine cell distribution, only in β -cell area (Fig. 6). Reduced insulin gene expression and insulin content correlated well with less intensely stained islets in *GIPR*^{-/-} mice (Fig. 6). Immunostaining for glucagon and somatostatin showed normal topology and distribution with no indication of migration toward the islet core (data not shown). Very recently, it has been shown that GLP-1 has growth hormone-like effects on pancreatic islets and on β -cells (19). Thus the enhanced GLP-1 action on the *GIPR*^{-/-} mouse pancreas, indicated by pancreas perfusions and static islet stimulation, was consistent with the observed increase in β -cell area. Recently, studies have shown on β TC3 insulin-secreting tumors that GIP is a regulator of upstream kinases of apoptosis cascades. In this regard, our finding of increased β -cell area in *GIPR*^{-/-} mice is not fully understood (10, 38, 39). Further studies are required to examine the role of GIP on islet growth and insulin gene expression to clarify these findings. To date, there have been no conclusive studies that have examined the effects of GIP on islet/ β -cell development and survival in vivo.

In summary, we have demonstrated that disruption of the GIP component of the enteroinsular axis in mice results in decreased insulin gene transcription and

protein biosynthesis, increased islet sensitivity to GLP-1, and changes in islet structure. We report that compensation for the absence of a functional GIP receptor occurs, in part, by upregulation of the GLP-1 component of the enteroinsular axis. The physiological changes that take place in both the *GIPR*^{-/-} and the *GLP-1R*^{-/-} strains of knockout mice suggest that the incretins act in concert to maintain glucose homeostasis and that a balance between the two is required for proper function of the enteroinsular axis. Further experiments, targeted at clarifying the molecular changes that occur within the β -cell, are required to extend our understanding of GIP physiology.

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