

## Differential Importance of Glucose-Dependent Insulinotropic Polypeptide vs Glucagon-Like Peptide 1 Receptor Signaling for Beta Cell Survival in Mice

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See editorial on page 1891.

**BACKGROUND & AIMS:** Glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) activate pathways involved in beta cell survival and proliferation *in vitro*; we compared the relative importance of exogenous and endogenous GIP receptor (GIPR) and GLP-1 receptor (GLP-1R) activation for beta cell cytoprotection in mice. **METHODS:** The effects of incretin hormone receptor signaling on beta cell regeneration and survival were assessed in mice following administration of streptozotocin in the absence or presence of the GIPR agonist [D-Ala<sup>2</sup>]-GIP (D-GIP), the GLP-1R agonist exendin-4, or the dipeptidyl peptidase-4 inhibitor sitagliptin. Beta cell survival was assessed in *Gipr*<sup>-/-</sup> mice given streptozotocin and by gene expression profiling of RNA from islets isolated from *Glp1r*<sup>-/-</sup> and *Gipr*<sup>-/-</sup> mice. The antiapoptotic actions of sitagliptin were assessed in wild-type and dual incretin receptor knockout (DIRKO) mice. **RESULTS:** Administration of exendin-4 for 7 or 60 days improved blood glucose and insulin levels, reduced islet cell apoptosis, and increased pancreatic insulin content and beta cell mass. In contrast, D-GIP was less effective at improving these parameters under identical experimental conditions. Furthermore, *Gipr*<sup>-/-</sup> mice did not exhibit increased sensitivity to streptozotocin-induced diabetes. Sitagliptin reduced hemoglobin A<sub>1c</sub> levels and increased plasma and pancreatic levels of insulin after streptozotocin administration to wild-type mice. Sitagliptin reduced the levels of activated caspase-3 in wild-type islets but not in beta cells from DIRKO mice. **CONCLUSIONS:** There are functionally important differences in the pharmacologic and physiologic roles of incretin receptors in beta cells. GLP-1R signaling exerts more robust control of beta cell survival, relative to GIPR activation or dipeptidylpeptidase-4 inhibition in mice *in vivo*.

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The gastrointestinal tract plays a critical role in the sensing, absorption, and disposal of ingested nutrients via multiple relay mechanisms that involve neural

communication as well as complex endocrine systems converging on target organs regulating glucose homeostasis.<sup>1</sup> A classic role for gut endocrine cells in the facilitation of nutrient disposal is illustrated by the incretin effect, the augmentation of insulin secretion that occurs following enteral nutrient ingestion. Two principal gut hormones account for the majority of the incretin effect: glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1).

GIP and GLP-1 exert their actions through structurally related G protein-coupled receptors, which exhibit considerable amino acid homology and utilize overlapping signal transduction pathways in islet beta cells.<sup>2</sup> Although GIP and GLP-1 both stimulate glucose-dependent insulin secretion, they exert different activities beyond the beta cell. GIP promotes lipid accretion and resistin secretion from adipocytes, leading to progressive impairment of insulin action following long-term GIP administration in rodents fed a high-fat diet.<sup>3,4</sup> Conversely, transient or genetic elimination of GIP action in rodents leads to reduced fat accumulation, resistance to diet-induced obesity, and preservation of insulin sensitivity.<sup>5,6</sup>

In contrast, GLP-1, but not GIP, inhibits glucagon secretion and reduces the rate of gastric emptying. Moreover, GLP-1 induces satiety, and sustained GLP-1 receptor (GLP-1R) activation is associated with weight loss in both preclinical and clinical studies.<sup>7,8</sup> GLP-1 enhances glucose competence and promotes restoration of glucose sensing in diabetic beta cells.<sup>9</sup> Moreover, GLP-1 stimulates beta cell replication, leading to expansion of beta cell mass in rodents with glucose intolerance or experimental diabetes.<sup>10,11</sup> GLP-1 also enhances cell survival in beta cell lines, isolated rodent and human islets, and models of beta cell apop-

*Abbreviations used in this paper:* AUC, area under the curve; D-GIP, [D-Ala<sup>2</sup>] glucose-dependent insulinotropic polypeptide; DIRKO, dual incretin receptor knockout; DPP-4, dipeptidylpeptidase-4; EGFR, epidermal growth factor receptor; Ex-4, exendin-4; GIP, glucose-dependent insulinotropic polypeptide; GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP-1, glucagon-like peptide 1; GLP-1R, glucagon-like peptide 1 receptor; PARP, poly(adenosine diphosphate ribose) polymerase; S-GLP-1, [Ser<sup>8</sup>] GLP-1(7-36)NH<sub>2</sub>; STZ, streptozotocin.

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sis.<sup>12-15</sup> Conversely, elimination of GLP-1R signaling leads to a reduced number of large islets and enhanced susceptibility to beta cell apoptosis in mice.<sup>15,16</sup>

GIP also promotes beta cell proliferation and inhibits apoptosis in islet cell lines and diabetic rodents.<sup>17-19</sup> In contrast to the importance of endogenous GLP-1R signaling for beta cell proliferation and survival,<sup>15,16</sup> whether the GIP receptor (GIPR) is similarly important for maintenance of beta cell mass remains unclear. Unexpectedly, genetic disruption of the GIPR gene in mice was associated with increased<sup>20</sup> or normal<sup>3</sup> islet size, findings inconsistent with an essential role for GIP in the control of beta cell growth or survival. We have now compared the efficacy of pharmacologic activation of single incretin receptors using GLP-1R and GIP agonists versus activation of incretin receptors using the dipeptidylpeptidase-4 (DPP-4) inhibitor sitagliptin to promote beta cell survival in mice. In complementary experiments, we assessed the importance of the endogenous GIPR for beta cell survival in studies using *Gipr*<sup>-/-</sup> mice. Our studies illuminate differences in the biology of incretin receptor signaling for beta cell survival in murine islets.

## Materials and Methods

### Peptides and Reagents

Peptides were reconstituted in phosphate-buffered saline (PBS), aliquoted, and stored at  $-80^{\circ}\text{C}$ . Exendin-4 (Ex-4; purity, 99.9%; peptide content, 82%), [D-Ala<sup>2</sup>]GIP (D-GIP; purity, 99.6%; peptide content, 79%), and native human GIP (purity, 99%; peptide content, 77%) were from California Peptide Research Inc (Napa, CA). [Ser<sup>8</sup>]GLP-1(7-36)NH<sub>2</sub> (S-GLP-1; purity, >95%; peptide content, 81%) and native GLP-1(7-36)NH<sub>2</sub> (purity, >95%; peptide content, 75%) were from Bachem (Torrance, CA). Liraglutide was from Novo Nordisk (Bagsvaerd, Denmark). Peptide doses were corrected for peptide purity and content. Tissue culture reagents were from Invitrogen (San Diego, CA) and streptozotocin (STZ), collagenase V, Hank's balanced salt solution and Histopaque, TRI reagent, and formalin and bovine serum albumin were from Sigma (St Louis, MO). Rabbit anti-IRS2 antisera was from Upstate (Lake Placid, NY), rabbit anti-epidermal growth factor receptor (EGFR) was from Rockland (Gilbertsville, PA), and mouse anti-Hsp90 was from BD Transduction Labs (San Jose, CA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were both from GE Healthcare UK Ltd (Little Chalfont, Buckinghamshire, England).

### Animal Experiments

All animal experiments were performed according to the protocols and procedures outlined by the Toronto Gen-

eral and Mount Sinai Hospital Animal Care Committees. Mice were housed (3-5 per cage) under specific pathogen-free conditions in microisolator cages and maintained on a 12-hour light (7 AM)/dark (7 PM) cycle with free access to food and water unless otherwise noted. Male C57BL/6 mice aged 7-8 weeks (Taconic Farms, Germantown, NY) were allowed to acclimatize for at least 1 week before experimental procedures. Unless otherwise noted, mice were fed a standard rodent diet (LM-485; Harlan Teklad, Madison, WI). Blood glucose level was measured in the morning via a tail vein blood sample, taken before peptide injections, using a blood glucose meter (Bayer, Toronto, Ontario, Canada). Studies in *Gipr*<sup>-/-</sup>, *Glp1r*<sup>-/-</sup>, and dual incretin receptor knockout (DIRKO) mice were conducted in mice aged 10-14 weeks on a C57BL/6 genetic background as described.<sup>21</sup> Littermates were used in all experiments for comparisons between wild-type and single incretin receptor knockout mice. For STZ studies, mice were fasted for 5 hours (8 AM to 1 PM) before STZ injection (50 mg · kg<sup>-1</sup> · day<sup>-1</sup>) or vehicle (0.1 mol/L sodium citrate, pH 5) for 5 consecutive days as described.<sup>15</sup> C57BL/6 mice were randomized to receive 24 nmol/kg Ex-4, 24 nmol/kg D-GIP, or PBS twice daily at approximately 8 AM and 6 PM by intraperitoneal injection. The doses of D-GIP and Ex-4 were chosen based on previous studies in STZ-treated mice<sup>15,22</sup> and on dose-response experiments (Supplementary Figure 1C) demonstrating comparable efficacy of specific peptide doses in reducing blood glucose levels. In experiments conducted using S-GLP-1 and liraglutide, twice-daily doses of 24 nmol/kg and 100 μg/kg (27 nmol/kg) were used, respectively. Control nondiabetic mice received injections of the appropriate saline vehicle. In studies involving sitagliptin, C57BL/6 mice were fed a control diet containing 10% kcal fat (D12450B; Research Diets, New Brunswick, NJ) during acclimatization, following which mice were randomized to receive the same diet with or without sitagliptin for 1 week before the onset of and during STZ administration. In treatment A, mice on the sitagliptin diet were returned to control diet 3 days after the end of STZ injections to determine the effect of transient DPP-4 inhibition on STZ-induced diabetes. In treatment B, mice were kept on the sitagliptin-containing diet for an additional 55 days after STZ administration. Sitagliptin, supplied by Merck Research Laboratories (Rahway, NJ), was incorporated at a concentration of 4 g drug per kilogram diet. This concentration of drug produced ~90% inhibition of plasma DPP-4 activity in both normal mice and STZ diabetic mice.

### Glucose Tolerance Testing

Mice were fasted for 15 hours (6 PM to 9 AM) with free access to water before glucose loading (1 g/kg for diabetic mice or 1.5 g/kg for normal mice) for the intraperitoneal or oral glucose tolerance tests. Blood samples for glucose determinations were drawn from the tail vein

at -15 (15 minutes before glucose loading), 0, 10, 20, 30, 60, and 120 minutes following glucose administration.

### Experimental End Points

Islet apoptosis and pancreatic gene expression was measured in pancreases from cohorts of mice killed ~24 hours after the last STZ injection. In mice killed several weeks following the termination of STZ injections, glycated hemoglobin level was measured in a 5- $\mu$ L blood sample collected from the tail vein using the Bayer DCA 2000+ Analyzer. Cardiac blood was collected and mixed with 10% (vol/vol) solution of 5000 KIU/mL aprotinin and 1.2 mg/mL EDTA; plasma was separated by centrifugation at 4°C and stored at -80°C until assayed. The pancreas was rapidly excised, and 5 mm of the splenic tail was immediately homogenized in cold TRI reagent and stored at -80°C until RNA isolation. An adjacent 5-mm fragment was removed and snap frozen in liquid N<sub>2</sub> for determination of pancreatic insulin content. The remainder of the pancreas was cut into 8–10 similarly sized pieces, fixed in a 10% neutral buffered formalin solution for 48 hours, and embedded in paraffin. Pancreatic insulin content, plasma insulin levels, and quantification of cleaved caspase-3 immunopositivity were measured as described.<sup>3,22,23</sup> For assessment of beta cell mass, pancreatic sections were fixed and immunostained for insulin and scanned using the ScanScope CS system (Aperio Technologies, Vista, CA) at 20 $\times$  magnification.<sup>22</sup> Digital images were analyzed with ScanScope software (Aperio Technologies). Beta cell mass was calculated as the product of the total cross-sectional area of beta cells/total pancreas area using the weight of the pancreas before fixation. Plasma DPP-4 activity was determined using an enzymatic assay.<sup>24</sup>

### Islet Isolation

After the mice were killed using CO<sub>2</sub>, the pancreas was inflated via the pancreatic duct with collagenase type V (0.7 mg/mL in Hank's balanced salt solution), excised, and digested at 37°C for 10–15 minutes. The resulting digest was washed twice with cold Hank's balanced salt solution (containing 0.25% wt/vol bovine serum albumin) and islets were separated using a Histopaque density gradient. The interface-containing islets was removed and washed with Hank's balanced salt solution plus bovine serum albumin, and the islets were resuspended in Ham's F-10 containing 2 mmol/L L-glutamine, 10 mmol/L glucose, 50  $\mu$ mol/L isobutylmethylxanthine, 1% bovine serum albumin, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. Following 4 hours of incubation at 37°C, islets were hand picked into fresh media, washed twice in PBS, following which islets were either lysed for RNA extraction using the RNeasy Micro Kit (Qiagen, Mississauga, Canada) or for Western blot analysis.

### INS-1 832/3 Cell Culture and Insulin Secretion

INS-1 832/3 cells were a kind gift from Dr Christopher Newgard (Duke University, Raleigh, NC). Culture conditions and insulin secretion experiments were conducted as described.<sup>23</sup> Insulin secretion per well was corrected for total cellular protein using the bicinchoninic acid protein assay (Pierce, Rockford, IL) and expressed relative to secretion in 3 mmol/L glucose.

### Western Blot Analysis

An aliquot of islet lysate was reserved for protein determination using the bicinchoninic acid method, and the remainder was boiled for 5 minutes in sample buffer containing  $\beta$ -mercaptoethanol. Islet protein (10–15  $\mu$ g) was resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes overnight at 4°C, and blocked in 5% milk in TBS-T for 1 hour before being incubated with primary antibodies overnight according to the manufacturers' suggested dilutions. After incubation with secondary antibodies, bands were visualized on BioMax film using enhanced chemiluminescence (GE Healthcare, Buckinghamshire, England) and densitometry quantified using Scion Image (Scion Corp, Frederick, MD).

### Complementary DNA Synthesis

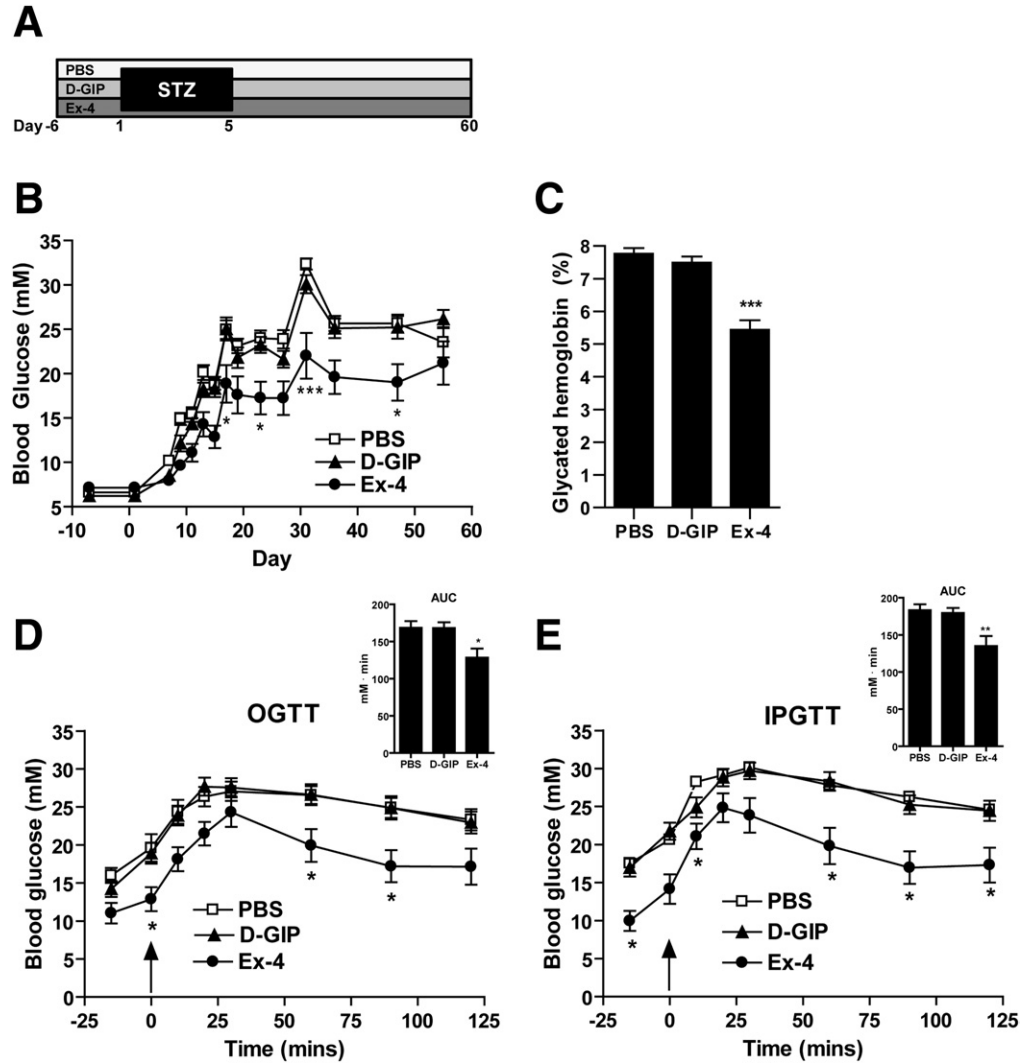
Following RNA isolation according to the respective manufacturer's instructions, first-strand complementary DNA was synthesized from total RNA using the SuperScript III reverse transcriptase synthesis system (Invitrogen, Carlsbad, CA) and random hexamers. Real-time polymerase chain reaction was performed with the ABI Prism 7900 Sequence Detection System using TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Levels of messenger RNA (mRNA) transcripts were normalized to peptidyl-propyl isomerase A (cyclophilin).

### Statistical Analysis

Results are presented as mean  $\pm$  SEM. Statistical significance was determined using 1-way or 2-way analysis of variance with Bonferroni post hoc tests or *t* tests (as appropriate) using GraphPad Prism 4.0 (GraphPad Software Inc, La Jolla, CA). Statistical significance was noted when *P* < .05.

### Results

To determine if GIPR activation promotes beta cell survival, we treated C57BL/6 mice with the potent DPP-4-resistant analogue D-GIP<sup>25,26</sup> or the GLP-1R agonist Ex-4 as a control before and concomitant with administration of STZ 50 mg  $\cdot$  kg<sup>-1</sup>  $\cdot$  day<sup>-1</sup> for 5 days, with Ex-4 and D-GIP administration continuing for 55 days after the last dose of STZ (Figure 1A). Consistent



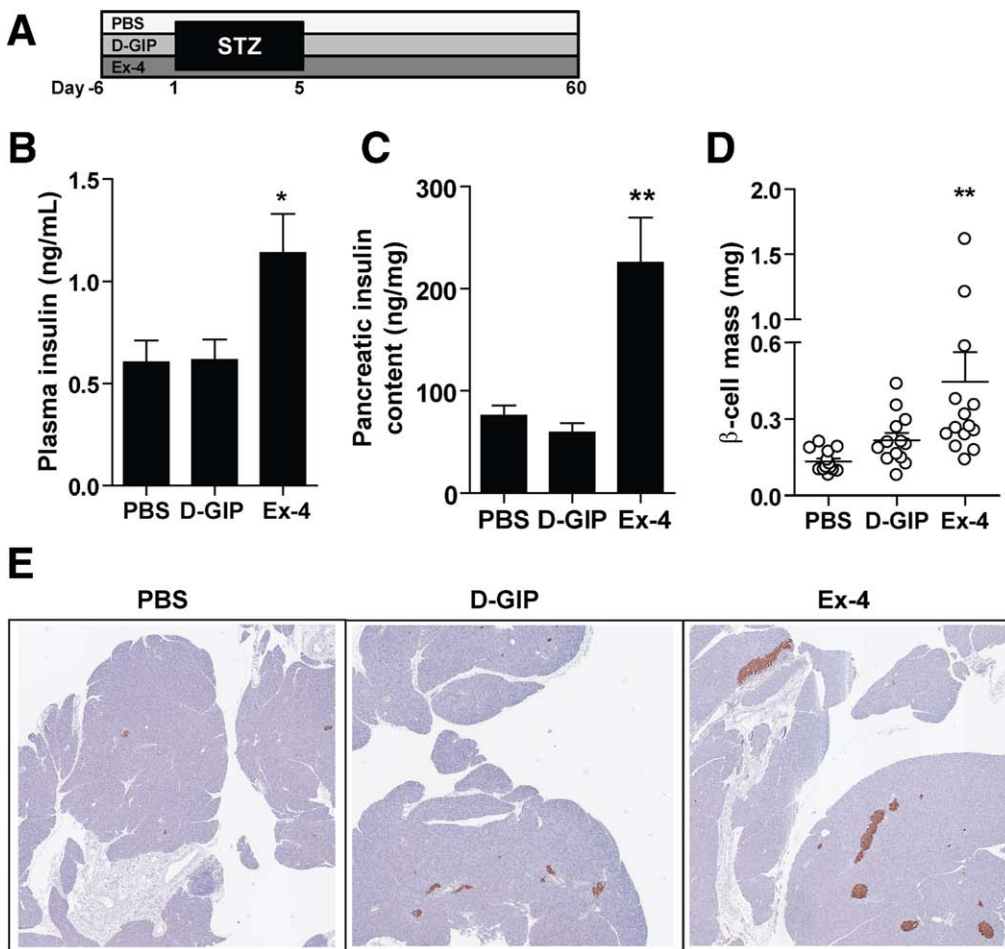
**Figure 1.** Prolonged GIPR agonist administration does not result in preserved or regenerated beta cell mass or function following STZ. (A) Incretin receptor agonists D-GIP, Ex-4, or PBS were administered twice daily (at 24 nmol/kg per injection) commencing 1 week before, during, and for 55 days following STZ administration to C57BL/6 male mice. Incretin treatment resulted in differential improvement in (B) random fed blood glucose levels throughout the study and (C) in glycated hemoglobin measured at day 60 (n = 13–14). (D) Oral and (E) intraperitoneal glucose tolerance tests (oral glucose tolerance test, day 31; intraperitoneal glucose tolerance test, day 37) illustrated that only mice receiving Ex-4 treatment displayed improved fasting glucose and glucose tolerance relative to PBS-treated controls (n = 13–14). For glucose tolerance tests, mice received last peptide/PBS injections 16 hours before glucose administration (1 g/kg, denoted by an arrow). \**P* < .05, \*\**P* < .01, \*\*\**P* < .001 vs PBS-treated STZ group in all cases except in B, where asterisks denote differences between Ex-4- and D-GIP-treated mice.

with previous findings,<sup>25</sup> D-GIP and native GIP were equipotent in vitro, as assessed by stimulation of insulin secretion (Supplementary Figure 1A). However, D-GIP displayed superior glucose-lowering effects in an oral glucose tolerance test in vivo (Supplementary Figure 1B). The doses of D-GIP and Ex-4 were chosen based on similar glucose-lowering efficacies in oral glucose tolerance testing (Supplementary Figure 1C).

Treatment with the GLP-1R agonist Ex-4, but not the GIPR agonist D-GIP, significantly reduced the extent of hyperglycemia following administration of STZ (Figure 1B; area under the curve [AUC] for glucose was 841 vs 522 mmol/L per day for PBS- vs Ex-4-treated mice, respectively; *P* < .001) as well as the level of glycated hemoglobin (7.8% vs 5.4% for PBS- vs Ex-4-treated mice, respectively; *P* < .001; Figure 1C). Moreover, mice treated with Ex-4, but not D-GIP, exhibited significantly lower fasting glucose levels as well as improved oral and intra-peritoneal glucose tolerance relative to PBS-treated mice (Figure 1D and E). After a total of 66 days of treatment

(Figure 2A), fed plasma insulin levels (Figure 2B) and pancreatic insulin content (Figure 2C) were significantly higher in mice treated with Ex-4 but not in mice treated with D-GIP. Furthermore, although beta cell mass tended to be higher in the D-GIP-treated group, it was significantly increased only in mice treated with Ex-4 (Figure 2D and E).

The results of these studies show that twice-daily administration of a potent GIP analogue for ~8 weeks is unable to improve glucose homeostasis or regenerate beta cell mass following STZ administration. Because D-GIP retains its ability to acutely lower blood glucose levels shortly after STZ administration (Supplementary Figure 2), we assessed whether a brief period of D-GIP treatment exerts cytoprotective actions on the murine islet. Wild-type C57BL/6 mice were treated with either Ex-4 or D-GIP before, during, and for 2 days after the administration of STZ, for a total of 13 days. Separate groups of mice were killed for analysis of beta cell apoptosis 24 hours after the final STZ injection or moni-



**Figure 2.** Parameters of beta cell function and beta cell mass after treatment with Ex-4 or D-GIP in STZ-treated mice. (A) Incretin receptor agonists D-GIP, Ex-4, or PBS were administered twice daily (at 24 nmol/kg per injection) (see Figure 1A). Analysis of (B) plasma insulin, (C) pancreatic insulin content, and (D and E) beta cell mass in fed mice killed at day 60 revealed that treatment with Ex-4, but not D-GIP, was associated in improvements in these parameters ( $n = 13-14$ ). (E) Representative images of insulin-immunostained pancreases were obtained at 2 $\times$  magnification. \* $P < .05$ , \*\* $P < .01$  vs PBS-treated STZ group.

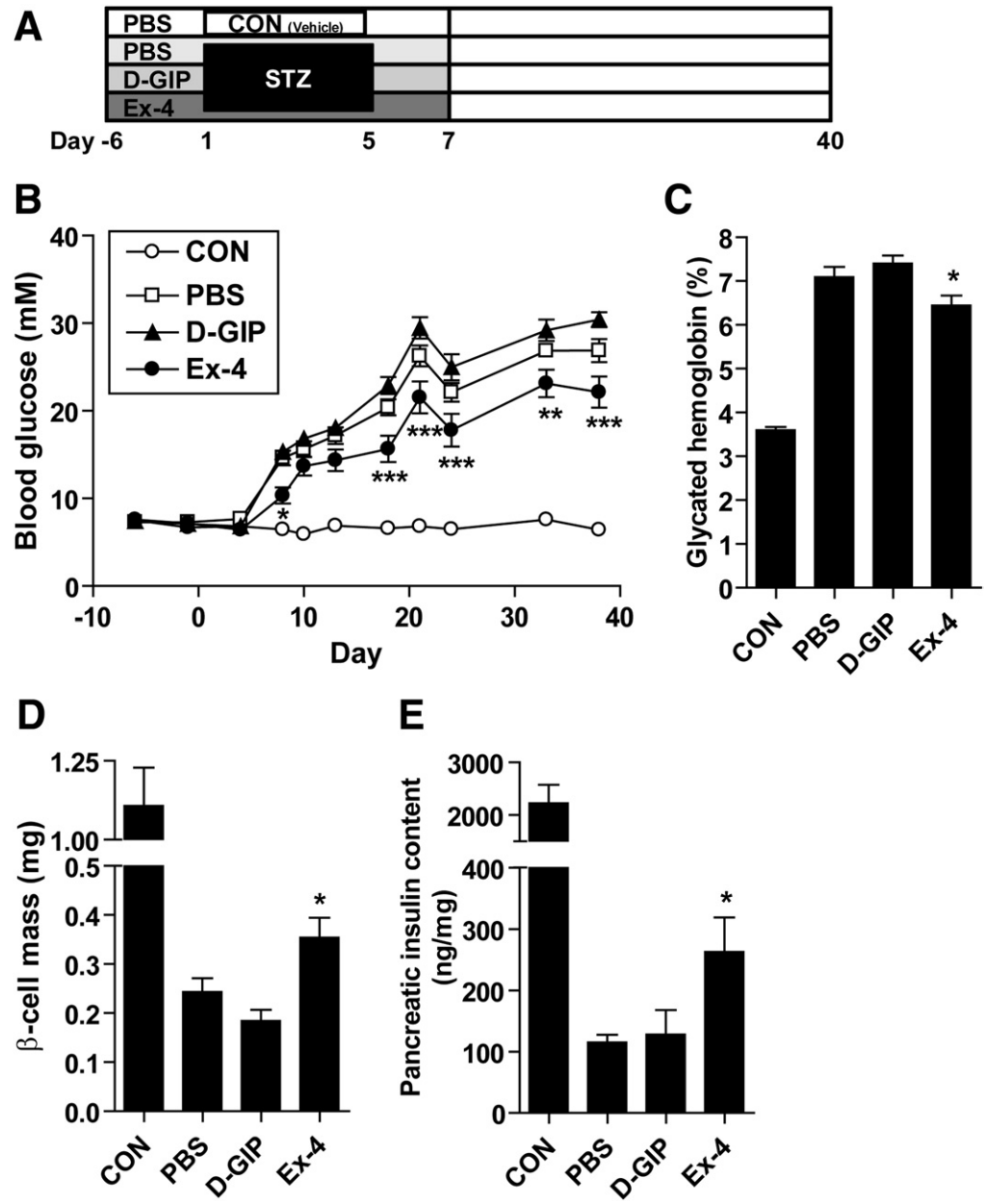
tored for an additional 35 days after the last dose of STZ (Figure 3A). Transient treatment with Ex-4 attenuated the extent of hyperglycemia (AUC for glucose was 493 vs 356 mmol/L per day for PBS vs Ex-4, respectively;  $P < .05$ ; Figure 3B) and reduced levels of glycated hemoglobin at day 40 (Figure 3C; 7.1% vs 6.4% for PBS vs Ex-4, respectively;  $P < .05$ ). In contrast, twice-daily administration of D-GIP for 13 days did not diminish the extent of hyperglycemia or reduce levels of glycated hemoglobin (Figure 3B and C).

To determine whether a brief period of Ex-4 or D-GIP administration increased pancreatic insulin content or beta cell mass in the context of STZ administration, we assessed these parameters 33 days after the last peptide injection. Mice treated with D-GIP did not exhibit increased beta cell mass or pancreatic insulin content (Figure 3D and E). In contrast, Ex-4-treated mice exhibited significantly increased beta cell mass and pancreatic insulin content more than 4 weeks after cessation of Ex-4 administration (Figure 3D and E).

To delineate mechanisms underlying the differential effects of Ex-4 versus D-GIP on beta cell mass, we assessed beta cell apoptosis immediately following STZ

administration. Both Ex-4 and D-GIP significantly reduced levels of cleaved caspase-3 in beta cells (Figure 4A). Moreover, we compared the cytoprotective action of D-GIP with 2 structurally distinct DPP-4-resistant analogues of human GLP-1: S-GLP-1 and liraglutide. Both S-GLP-1 and liraglutide significantly reduced levels of STZ-induced islet apoptosis relative to PBS-treated mice; treatment with D-GIP also reduced beta cell apoptosis, but the difference relative to control was not statistically significant (Figure 4B). Blood glucose monitoring illustrated that mice exposed to S-GLP-1, but not D-GIP, had lower AUC random glucose levels relative to PBS-treated animals (Supplementary Figure 3B).

We next assessed the effects of Ex-4 versus D-GIP on expression of genes important for beta cell function and survival (Supplementary Figure 4). While neither incretin receptor agonist produced broad changes in islet gene expression relative to STZ controls, Ex-4-treated mice exhibited significant reductions in levels of EGFR, epidermal growth factor, and poly(adenosine diphosphate ribose) polymerase (PARP), the latter a key mediator of STZ-induced apoptosis in mice<sup>27</sup> (Supplementary Figure 4A-C). In contrast, mice treated with D-GIP exhibited



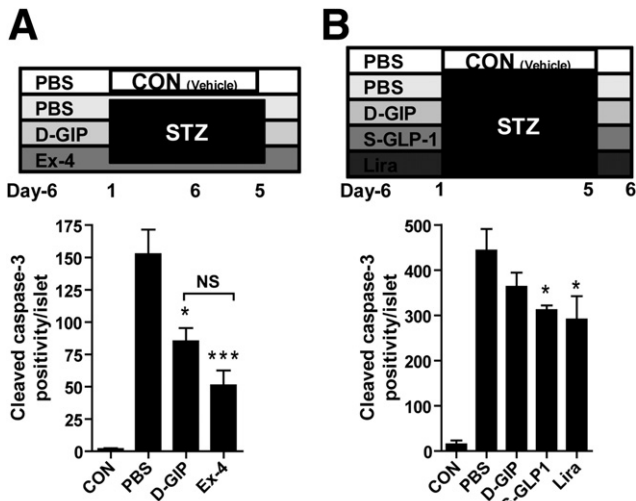
**Figure 3.** Ex-4, but not D-GIP, confers partial protection against STZ-induced diabetes in wild-type mice. (A) C57BL/6 male mice were randomized to one of 4 treatment groups. Control (CON) mice served as a non-diabetic vehicle control group and did not receive STZ; the remaining mice were treated with D-GIP or Ex-4 (each twice daily at 24 nmol/kg per injection) or PBS for 1 week before, during, and 2 days after STZ administration. (B) Random fed blood glucose levels were measured throughout the study, and (C) glycated hemoglobin, (D) beta cell mass, and (E) pancreatic insulin content were assessed in samples taken at day 40 (n = 10–11). (B) \*\**P* < .05, \*\*\**P* < .001 Ex-4 vs D-GIP; (C–E) \**P* < .05 for Ex-4 vs PBS.

higher levels of pancreatic glucagon, insulin receptor, Akt, and PARP mRNA relative to the Ex-4-treated cohort (Supplementary Figure 4A–C).

We next ascertained whether DPP-4 inhibition, which reduces N-terminal degradation of endogenously produced intact GIP and GLP-1, would engage survival or regenerative pathways in murine beta cells. Sitagliptin treatment for 60 days significantly reduced plasma DPP-4 activity by 87%–95% in normal and STZ diabetic mice, respectively (Supplementary Figure 5A). Transient (13 days) or sustained (60 days) sitagliptin administration (Figure 5A) did not modify the extent of hyperglycemia induced by STZ (Figure 5B); however, levels of hemoglobin A<sub>1c</sub> were significantly lower in mice receiving STZ

who were treated with sitagliptin for 60 days (Figure 5C). Moreover, prolonged sitagliptin treatment was associated with significantly higher levels of fed plasma insulin (Figure 5D) and pancreatic insulin content (Figure 5E). While beta cell mass tended to be higher (Figure 5F), the percentage of beta cell area was significantly increased in sitagliptin-treated mice (0.12% ± 0.01% vs 0.20% ± 0.03%, *P* < .05, vehicle vs sitagliptin, respectively). The effect of sitagliptin on beta cell mass versus beta cell area could partially be explained by the slightly lower pancreas weight in sitagliptin-treated animals (259 ± 6 mg vs 219 ± 6 mg; *P* < .001 for STZ vs STZ plus sitagliptin, respectively). Interestingly, sitagliptin therapy was associated with significantly lower levels of cleaved caspase-3

BASIC-LIVER,  
PANCREAS, AND  
BILIARY TRACT



**Figure 4.** Incretin receptor agonists reduce apoptosis in STZ-treated mice. (A, top) Male C57BL/6 mice were treated with either D-GIP or Ex-4 (24 nmol/kg per injection) or PBS twice daily for 7 days before and during 5 consecutive days of STZ administration. (A, bottom) Cleaved caspase-3 positivity within insulin-positive islets was assessed in mice killed on day 6, ~24 hours after the final STZ injection. (B, top and bottom) In a separate cohort of mice, the efficacy of D-GIP in STZ-induced apoptosis was compared with S-GLP-1 (given twice daily at 24 nmol/kg) and liraglutide (Lira, given twice daily at 27 nmol/kg) or PBS. Control nondiabetic mice received injections of the appropriate saline vehicle. Approximately 100 islets per mouse were analyzed ( $n = 7-8$  mice); \* $P < .05$ , \*\*\* $P < .001$  vs PBS-treated mice.

immunopositivity within islets of STZ-treated wild-type mice (Figure 6A). In contrast, sitagliptin failed to reduce levels of cleaved caspase-3 immunopositivity in islets of STZ-treated DIRKO mice which harbor genetic inactivation of both incretin receptors (Figure 6B).

Analysis of pancreatic gene expression profiles in mice killed at day 6, 24 hours following the final STZ injection, showed that sitagliptin treatment was associated with increased levels of mRNA transcripts for Pdx-1 and the insulin receptor (Supplementary Figure 5B-D) and reduced levels of glucokinase. Furthermore, sitagliptin increased pancreatic mRNA levels for IGF-1 and Akt-1 (Supplementary Figure 5B-D), genes important for beta cell survival.<sup>28</sup>

Because  $Glp1r^{-/-}$  mice exhibit increased hyperglycemia and reduced beta cell survival after administration of STZ,<sup>15</sup> we assessed the corresponding susceptibility of  $Gipr^{-/-}$  mice to STZ. Levels of blood glucose were comparable in STZ-treated  $Gipr^{-/-}$  versus  $Gipr^{+/+}$  mice (Figure 7A and B). Moreover, fed plasma insulin levels, beta cell mass, and pancreatic insulin content were reduced to similar levels in  $Gipr^{-/-}$  versus  $Gipr^{+/+}$  mice after administration of STZ (Figure 7C-E) and levels of activated caspase-3 immunopositivity were similar in islets from STZ-treated  $Gipr^{-/-}$  versus  $Gipr^{+/+}$  mice (Figure 7F). To elucidate mechanisms underlying the differential sensitivity of  $Gipr^{-/-}$  versus  $Glp1r^{-/-}$  beta cells to STZ, we

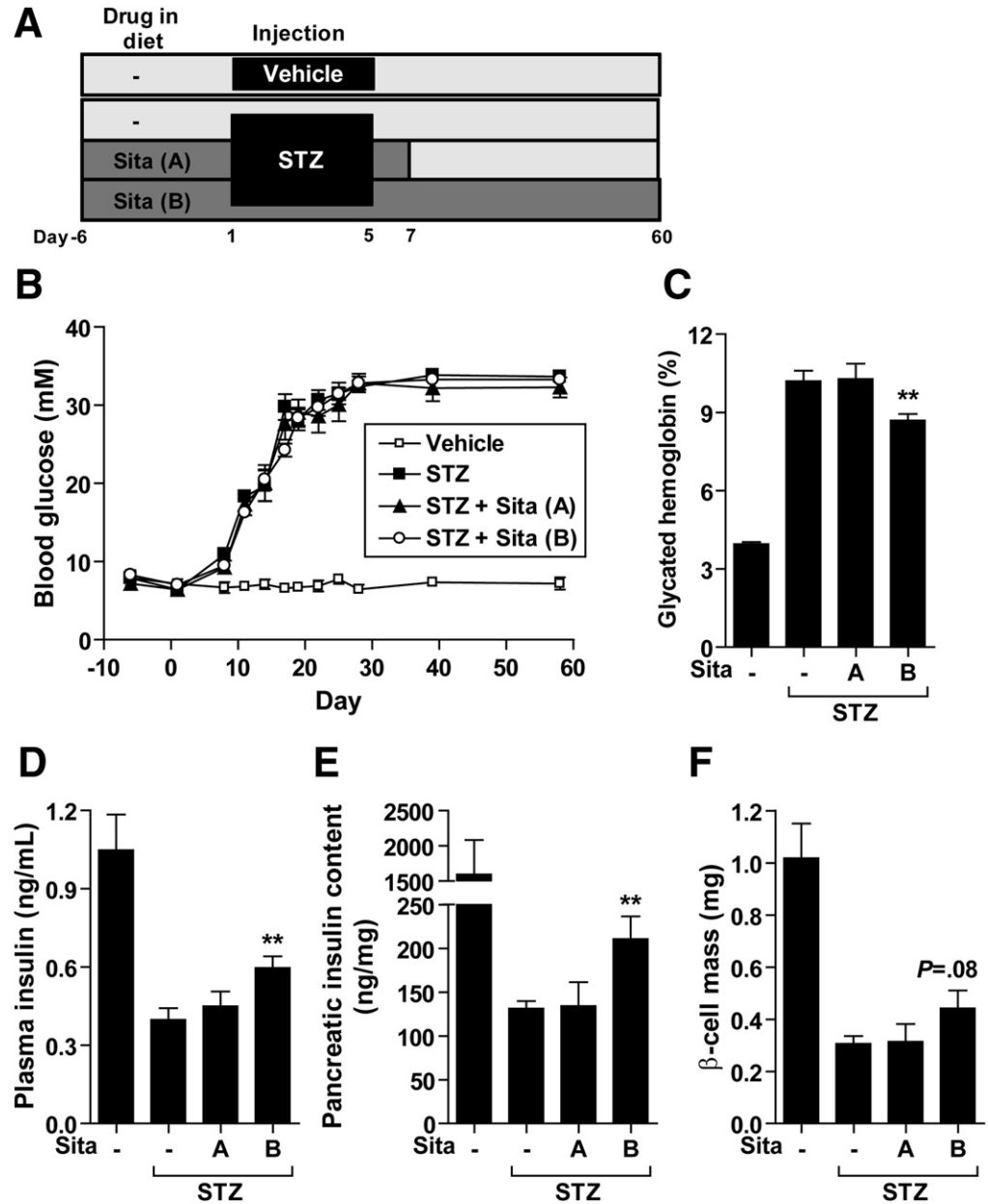
analyzed basal levels of mRNA transcripts in islets from wild-type,  $Glp1r^{-/-}$ , and  $Gipr^{-/-}$  mice. No differences were detected in the levels of mRNAs encoding the insulin receptor, insulin-like growth factor receptors 1 and 2, Akt, Bcl-xL, Bcl-2, Pdx-1, GLUT2, PARP, SOD, Socs-3, nuclear factor  $\kappa$ B, and Creb in littermate wild-type,  $Gipr^{-/-}$ , and  $Glp1r^{-/-}$  islets (Supplementary Figure 6). In contrast, levels of mRNA transcripts and protein for Irs-2 and Egfr were significantly reduced in  $Glp1r^{-/-}$  relative to  $Gipr^{-/-}$  islets (Figure 8A-C).

## Discussion

The incretin hormones GIP and GLP-1 enhance glucose-stimulated insulin secretion in nondiabetic animals and human subjects. However, in patients with type 2 diabetes mellitus, the insulinotropic activities of GIP, and to a lesser extent GLP-1, are diminished, with the GIP defect most noticeable during the late-phase insulin response.<sup>29,30</sup> In contrast, much less is known about the comparative cytoprotective and regenerative actions of GIP and GLP-1, because few experiments have directly compared the ability of these incretins to protect or regenerate beta cells in head-to-head studies. Our experiments using degradation-resistant incretin receptor agonists clearly show that sustained administration of the GLP-1R agonist Ex-4 produces more robust reductions in levels of glycemia, in association with increased levels of plasma insulin, pancreatic insulin content, and beta cell mass. Moreover, although both Ex-4 and D-GIP reduced apoptosis in murine islets immediately after STZ administration, transient administration of Ex-4, but not D-GIP, resulted in lower blood glucose levels and increased insulin content and beta cell mass more than 4 weeks after cessation of incretin therapy (Figure 3).

Treatment with D-GIP tended to increase beta cell mass; in contrast to the effects of Ex-4, D-GIP did not improve plasma or pancreatic insulin levels or glucose tolerance (Figures 1 and 2). Because the doses of Ex-4 and D-GIP used in our experiments were equipotent in glucose reduction at the start of our studies, these findings imply that activation of the GLP-1 receptor using Ex-4 leads to more robust cytoprotection and enhancement of beta cell mass than that seen following activation of the cognate GIPR using D-GIP. Notably, D-GIP administration did reduce levels of islet apoptosis and tended to increase beta cell mass; hence, GIPR activation is able to couple to proliferative and antiapoptotic pathways in diabetic murine beta cells.

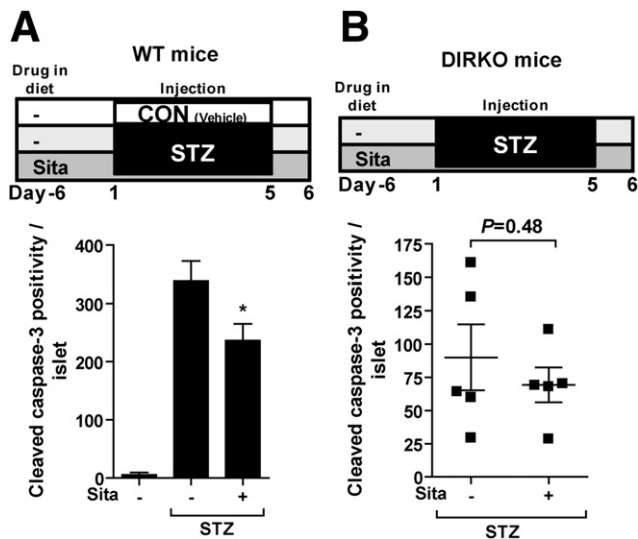
It is important to consider several aspects of the experimental design and choice of reagents that influence interpretation of our data. First, Ex-4 and D-GIP, while producing comparable degrees of acute glucose regulation, may exhibit different pharmacokinetic properties following sustained administration, leading to differential activation of the GLP-1 versus GIP receptors, respectively.



**Figure 5.** DPP-4 inhibition with sitagliptin improves parameters of beta cell function in STZ-treated wild-type mice. (A) In sitagliptin treatment A, mice on the sitagliptin diet were returned to control diet 3 days following the end of STZ injections. In treatment B, mice were kept on the sitagliptin-containing diet through to the experimental end point at day 60. (B) Random fed blood glucose levels were measured in the morning throughout the study. At day 60, samples were taken for measurement of (C) glycated hemoglobin, (D) fed plasma insulin, (E) pancreatic insulin content, and (F) beta cell mass. n = 8–18; \*\*P < .01 vs STZ-treated controls.

Hence, an ideal assessment of the pharmacologic consequences of sustained incretin receptor activation would utilize equipotent GIP and GLP-1R agonists that produce identical pharmacokinetic profiles. It remains possible that more potent GIP analogues would be associated with enhanced proliferative and antiapoptotic actions on islet beta cells in vivo. Moreover, recent reports indicate that the capacity of beta cells to proliferate in response to partial pancreatectomy, STZ, or Ex-4 declines sharply after adolescence in mice.<sup>31,32</sup> Because our experiments were conducted in young adult mice (8–14 weeks of age), whether the antiapoptotic actions of GLP-1R or GIPR agonists or DPP-4 inhibitors are also influenced by aging or the duration of diabetes is an important question that merits further exploration.

The finding by many groups that GLP-1 receptor activation leads to beta cell cytoprotection<sup>8</sup> is consistent with data that *Glp1r*<sup>-/-</sup> mice exhibit increased beta cell apoptosis following STZ administration.<sup>15</sup> In contrast, our data show that unlike *Glp1r*<sup>-/-</sup> mice, *Gipr*<sup>-/-</sup> mice do not exhibit increased susceptibility to beta cell apoptosis following STZ administration. These findings were unexpected given that numerous reports have shown antiapoptotic actions of GIP both in vitro and in vivo.<sup>19,33,34</sup> Potential explanations for these findings include differences in genetic background or a compensatory increase in GLP-1 responsivity, as described in previous studies of *Gipr*<sup>-/-</sup> mice,<sup>20</sup> that might produce enhanced beta cell protection in *Gipr*<sup>-/-</sup> mice. Surprisingly, however, although *Glp1r*<sup>-/-</sup> mice exhibit a compensatory increase in



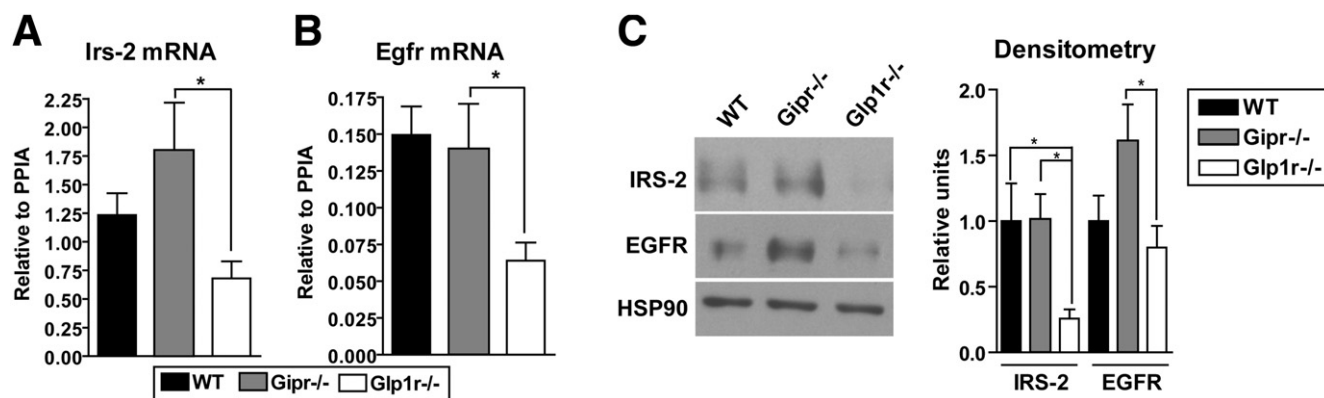
**Figure 6.** DPP-4 inhibition reduces levels of islet apoptosis in STZ-treated wild-type mice but not in mice lacking incretin receptors. Separate cohorts of (A) wild-type and (B) DIRKO ( $Glp1r^{-/-} Gipr^{-/-}$ ) mice were fed either a control diet or the same diet containing sitagliptin for 7 days before and during STZ (A and B, top). Mice were killed ~24 hours following the final STZ injection for quantification of levels of cleaved caspase-3 immunopositivity in beta cells ( $n = 5$ ) (A and B, bottom). For quantification of islet apoptosis in normal mice, the group of mice on control diet in A was injected with citrate buffer vehicle. \* $P < .05$  vs STZ-treated controls.

plasma levels of GIP and enhanced GIP insulinotropic activity,<sup>35</sup> these mice nonetheless remain more susceptible to STZ-induced diabetes.<sup>15</sup>

Although levels of RNA transcripts for the majority of cytoprotective molecules and components of the apoptotic machinery were similar in  $Gipr^{-/-}$  versus  $Glp1r^{-/-}$  islets, levels of the EGFR and Irs-2 were significantly lower in  $Glp1r^{-/-}$  islets (Figure 8A-C). These findings may partially explain the enhanced apoptotic susceptibility to apoptosis and extend previous observations showing that GLP-1 may exert its actions on the islet beta cell in part through EGFR transactivation.<sup>34,36,37</sup> Moreover, Irs-2 has been shown to be critical for the cytoprotective and regenerative effects of Ex-4,<sup>38</sup> and we recently showed that Ex-4, but not D-GIP, increased levels of pancreatic mRNA transcripts for EGFR and Irs-2 in nondiabetic mice fed a high-fat diet.<sup>22</sup> Taken together, our findings in STZ-treated mice imply that the structurally related GLP-1 and GIP receptors exhibit significant differences in their ability to engage downstream molecules important for beta cell survival and regeneration.

Because DPP-4 inhibitors (sitagliptin and vildagliptin) and GLP-1R agonists (Ex-4 and liraglutide) are used to treat type 2 diabetes mellitus, there is active interest in understanding whether these agents regulate beta cell protection and/or regeneration. Considerable data show antiapoptotic and proliferative actions of GLP-1R agonists<sup>8,39</sup>; however, there is less information on cytoprotective or regenerative properties of DPP-4 inhibitors.<sup>22,40-44</sup> Although random fed glycemia was not improved by sitagliptin in STZ-

**Figure 7.**  $Gipr^{-/-}$  and  $Gipr^{+/+}$  mice exhibit similar susceptibility to STZ-induced diabetes and beta cell injury. (A) Fed blood glucose levels were monitored during and after 5 consecutive days of STZ administration ( $50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  intraperitoneally) as indicated by the arrows ( $n = 7-16$ ). (B) AUC for fed blood glucose was calculated for days 16-28. At day 30, mice were killed for determination of (C) fed plasma insulin level, (D) beta cell mass ( $n = 7-15$ ), and (E) pancreatic insulin content ( $n = 7-11$ ). (F) Levels of islet apoptosis were quantified in pancreatic sections from a cohort of mice killed at day 6, ~24 hours after the last injection of STZ ( $n = 5-7$  mice). Cleaved caspase-3 positivity was quantified in a minimum of 30 islets per mouse pancreas. \*\*\* $P < .001$ .



**Figure 8.** Islets from *Gip-1r<sup>-/-</sup>* but not *Gipr<sup>-/-</sup>* mice are deficient in EGFR and IRS-2. Islets were isolated from *Gipr<sup>-/-</sup>* and *Gip1r<sup>-/-</sup>* and wild-type littermates for either real-time polymerase chain reaction assessment of basal levels of (A and B) transcripts or (C) proteins involved in beta cell survival. Levels of each transcript were normalized to the internal control peptidyl-propyl isomerase A (PPIA, also known as cyclophilin). Proteins levels were quantified by densitometry and normalized to levels of Hsp90. n = 7–8 mice per genotype. \**P* < .05.

treated mice, circulating levels of glycated hemoglobin were reduced and pancreatic insulin content and beta cell area and pancreatic IGF-1 and Akt mRNA transcripts were increased following sitagliptin administration. Hence, even modest increases in levels of intact incretin hormones may enhance beta cell survival in mice. Moreover, sitagliptin reduced the extent of caspase-3 activation following STZ administration in wild-type but not in DIRKO mice, showing that incretin receptors are essential transducers of the antiapoptotic actions of DPP-4 inhibitors.

In summary, our studies provide new information on the importance of basal GIPR action for beta cell survival and on how different mechanisms for enhancing incretin receptor activation impact beta cell regeneration in mice. Moreover, unlike the importance of the GLP-1 receptor for beta cell function, elimination of endogenous GIPR signaling does not modify susceptibility to beta cell injury and is not associated with perturbation in levels of key signaling molecules important for beta cell growth and survival. These findings extend our understanding of the relative importance and mechanisms of incretin action for beta cell survival and may have implications for strategies designed to optimize beta cell growth or survival in patients with type 2 diabetes mellitus.

### Supplementary Data

Note: To access the supplementary material accompanying this article visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: [10.1053/j.gastro.2009.09.004](https://doi.org/10.1053/j.gastro.2009.09.004).

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#### Conflicts of interest

The authors disclose the following: Dr Drucker has served as an advisor or consultant within the past 12 months to Amylin

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