Glucagon-like Peptide-1 Receptor Signaling Modulates β Cell Apoptosis*

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Glucagon-like peptide-1 (GLP-1) stimulates insulin secretion and augments β cell mass via activation of β cell proliferation and islet neogenesis. We examined whether GLP-1 receptor signaling modifies the cellular susceptibility to apoptosis. Mice administered streptozotocin (STZ), an agent known to induce β cell apoptosis, exhibit sustained improvement in glycemic control and increased levels of plasma insulin with concomitant administration of the GLP-1 agonist exendin-4 (Ex-4). Blood glucose remained significantly lower for weeks after cessation of exendin-4. STZ induced β cell apoptosis, which was significantly reduced by co-administration of Ex-4. Conversely, mice with a targeted disruption of the GLP-1 receptor gene exhibited increased β cell apoptosis after STZ administration. Exendin-4 directly reduced cytokine-induced apoptosis in purified rat β cells exposed to interleukin 1β, tumor necrosis fator α, and interferon γ *in vitro*. Furthermore, Ex-4-treated BHK-GLP-1R cells exhibited significantly increased cell viability, reduced caspase activity, and decreased cleavage of β-catenin after treatment with cycloheximide *in vitro*. These findings demonstrate that GLP-1 receptor signaling directly modifies the susceptibility to apoptotic injury, and provides a new potential mechanism linking GLP-1 receptor activation to preservation or enhancement of β cell mass *in vivo*.

Glucagon-like peptide-1 (GLP-1)[1] is derived from posttranslational processing of proglucagon in enteroendocrine L cells (1)

[1] The abbreviations used are: GLP-1, glucagon-like peptide-1; STZ, streptozotocin; Ex-4, exendin-4; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick end labeling; BrdUrd, 5′-bromo-2′-deoxyuridine; ELISA, enzyme-linked immunosorbent assay; BHK, baby hamster kidney; CHX, cycloheximide.

and is secreted from the distal gut after nutrient ingestion (2). The termination of GLP-1 action by the enzyme dipeptidyl peptidase IV occurs within minutes following GLP-1 secretion (3–5), yet GLP-1 exerts several rapid metabolic actions including stimulation and inhibition of insulin and glucagon secretion, respectively (6–10). GLP-1 action is essential for glucose homeostasis, because GLP-1 receptor blockade with the antagonist exendin (9–39) increases blood glucose and decreases levels of circulating insulin in human and rodent studies (11–14).

Activation of GLP-1 receptor signaling leads to enhanced expression of mRNA transcripts for glucokinase, GLUT-2, Pdx-1, and insulin in β cell lines (15–17) and in both normal and diabetic rodents (18–20). Furthermore, GLP-1 and exendin-4 promote differentiation of exocrine cell lines toward a β cell phenotype (21), a process that appears to depend on the expression of Pdx-1 (22, 23).

GLP-1 receptor signaling is also coupled to formation of new β cells through enhanced proliferation of existing β cells (24) and via induction of islet neogenesis (25). The mitogenic actions of GLP-1 are detectable in normal rodents (20, 24) and in the setting of experimental diabetes (19, 25). Administration of GLP-1 or exendin-4 to newborn rats treated with the β cell toxin streptozotocin (STZ) leads to increased β cell mass at postnatal day 7, which persists and remains increased at 2 months of age. The increased β cell mass in the GLP-1/exendin-4 treated rats was attributed to both enhanced β cell proliferation and increased numbers of small budding islets (26). Because STZ is known to induce β cell destruction in part through activation of apoptotic pathways (27–29), we examined whether GLP-1 receptor activation influences β cell mass via regulation of cellular susceptibility to apoptotic cell death.

MATERIALS AND METHODS

Materials—Tissue culture medium, serum, flasks, plates, and antibiotics, including G418, were from Invitrogen. Cycloheximide, forskolin, and protease inhibitor mixture were purchased from Sigma. Exendin-4 was from California Peptide Research (Napa, CA).

Animal Experiments—Male C57BL/6 mice, 8 weeks of age, were used for experiments shown in Figs. 1–3. Age- and sex-matched CD-1 GLP-1R+/+ control mice housed in the same animal facility were used for studies of GLP-1R−/− mice in the CD1 background (8-week-old male mice). All animals were maintained on standard laboratory chow under a 12 h:12 h light-dark schedule, and experiments were conducted according to protocols and guidelines approved by the Toronto General Hospital Animal Care Committee. STZ (Sigma) (50 mg/kg body weight, intraperitoneal injection once daily for 5 days) was administered as a freshly prepared solution in 0.1 mM sodium citrate pH 5.5. Exendin-4 (Ex-4; 24 nmol/kg body weight, a dose selected based on therapeutic efficacy in previous mouse experiments (30)) was administered as a single daily intraperitoneal injection. For studies depicted in Figs. 1 and 2, morning blood glucose was measured periodically throughout the experimental period and an oral glucose tolerance tests was done at day

30. For histological studies of islet apoptosis, Ex-4 administration was commenced either 2 or 7 days before STZ in separate experiments and continued until the last injection of STZ; C57BL/6 mice were sacrificed within ~24 h after the last STZ injection. For studies of apoptosis in GLP-1R−/− mice, wild-type GLP-1R+/+ and GLP-1R−/− mice were divided into separate groups (*n* = 4–6) and administered a slightly lower dose of STZ (40 mg/kg body weight) because of the different sensitivities of CD-1 *versus* C57BL/6 mice to STZ as delineated in preliminary dose-response studies caused by the known species-specific sensitivity to streptozotocin-induced apoptosis (31). After completion of the experiments (~48 h after the last dose of STZ), mice were euthanized by CO_2 anesthesia, blood was collected by cardiac puncture for plasma insulin determinations, and pancreases were removed, fixed in 10% formalin overnight, and embedded in paraffin for histological analyses.

Oral Glucose Tolerance Test and Measurement of Plasma and Pancreatic Insulin Levels—Oral glucose tolerance tests were carried out after an overnight fast as described (14, 32). A blood sample was collected from the tail vein during the 10–20 min time period for measurement of plasma insulin using a rat insulin enzyme-linked immunoassay kit (Crystal Chem. Inc., Chicago, IL) with mouse insulin as a standard (14).

Histological Assessment of Islet Apoptosis and Proliferation—To detect apoptosis, TUNEL (terminal deoxynucleotide transferase-mediated dUTP nick end labeling) staining was performed using ApopTag Peroxidase *in situ* Apoptosis detection kit (S7100) (Intergen Company, Purchase, NY), according to the manufacturer's instructions as described (33). Slides were analyzed with a Leica microscope, and apoptotic rates were calculated as the number of TUNEL-positive cells per islet, *n* = 6–7 pancreases for each experimental group of C57Bl/6 +/+ mice, or *n* = 4–6 pancreases for each group of CD1 GLP-1R+/+ or GLP-1R−/− mice. Analysis of serial consecutive islet sections stained with either insulin or the ApopTag reagent demonstrated that the apoptotic nuclei were localized to insulin-immunopositive β cells.

Islet cell proliferation was assessed by counting the number of 5′-bromo-2′-deoxyuridine-positve (BrdUrd+) islet cells in multiple pancreatic sections from both wild-type C57BL/6 and CD1 and GLP-1R−/− CD1 mice administered BrdUrd (Roche) by intraperitoneal injection, 50 mg/kg body weight, ~ 5 h prior to removal of the pancreas. Immunohistochemical detection of BrdUrd+ cells was carried out using an anti-BrdUrd antibody (CalTag Laboratories, Burlingame, CA). Serial sections were stained for either insulin or BrdUrd, and islet and pancreatic areas were measured using a Leica microscope and Q500MC

software. The vast majority of BrdUrd+ cells were immunopositive for insulin, and hence represented β cells. The relative cross-sectional β cell area, as a percentage of total pancreatic area, was assessed quantitatively as previously described (34–36). The number of BrdUrd-positive cells are expressed both per islet and per $10^5 \ \mu m^2$ β-cell area.

Rat Islets and Sorted β Cells—Islets were isolated from the pancreas of adult male Wistar rats (180–220 g) by collagenase digestion and purified on a gradient of Ficoll (37). The islets were further dissociated into single cells by trypsinization, and β cells were sorted on the basis of their autofluorescence using a FACStar Plus (BD Biosciences) as described (37). The sorted cell population comprises 95% β cells (37). Cells were allowed to recover from the isolation/sorting procedures by culture overnight in Dulbecco's modified Eagle's medium, 10% fetal calf serum, 11.2 mM glucose using plastic dishes to which they did not
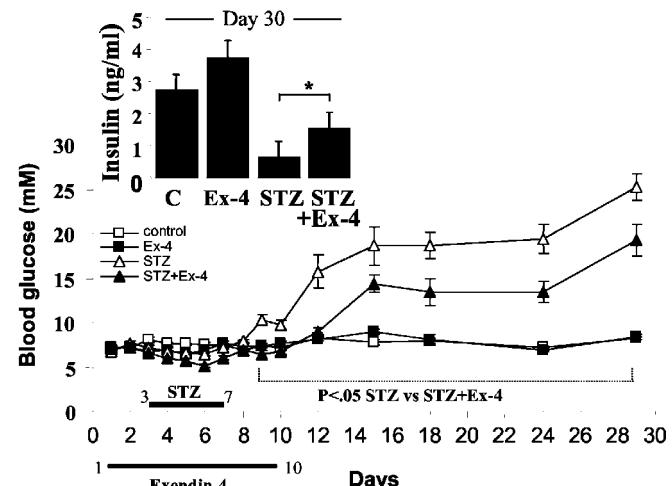

FIG. 1. **Morning-fed blood glucose and plasma insulin in wild-type C57BL/6 mice treated with saline, exendin-4 (Ex-4), STZ, or STZ+Ex-4.** Blood glucose was significantly lower from day 9 to day 29 in STZ+Ex-4 mice compared with glucose in mice receiving STZ alone ($p < 0.05$), whereas fed plasma insulin level measured at day 30 was significantly higher in STZ+Ex-4 mice *versus* STZ alone (*, $p < 0.05$); *n* = 10 mice per each experimental group. *C*, control (saline).

FIG. 2. **Ex-4 reduces hyperglycemia in ST2-treated mice.** *a*, morning-fed blood glucose and plasma insulin levels in wild-type C57BL/6 mice treated with saline (*C*, control), exendin-4 (Ex-4), STZ, or STZ + Ex-4. Morning-fed blood glucose was significantly lower from day 15 to day 52 in Ex-4+STZ mice *versus* STZ-treated mice alone ($p < 0.05$). The levels of fed plasma insulin were significantly increased at day 55 in STZ mice treated with Ex-4 (*, $p < 0.05$ for insulin in STZ *versus* STZ+Ex-4 mice). *n* = 10 mice per group; *, $p < 0.05$. *b*, difference in blood glucose from fasting baseline (Δ*G*) during oral glucose tolerance tests carried out at day 30. *n* = 8 mice per group; *, $p < 0.05$ for differences with and without Ex-4. The area under the curve (*AUC*) for glucose levels from 0 to 20 min was significantly lower in STZ+Ex-4 mice compared with mice receiving STZ alone ($p < 0.05$). Plasma insulin concentrations (*inset*) were measured in blood obtained during the oral glucose tolerance tests between the 10- and 20-min time points. *OGTT*, oral glucose tolerance test.
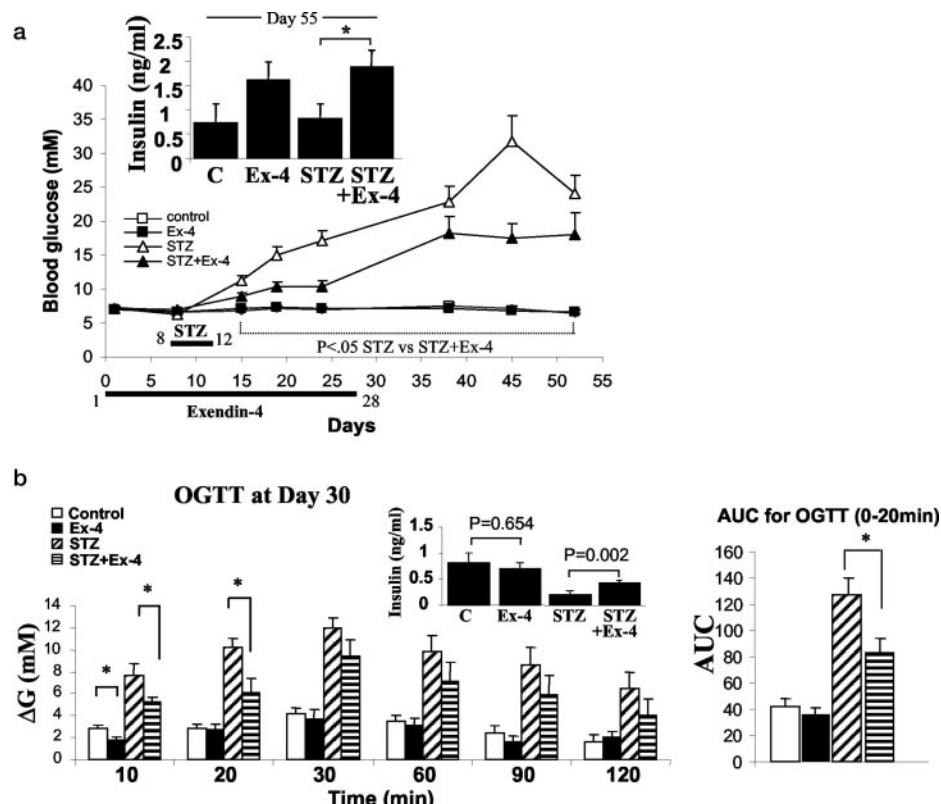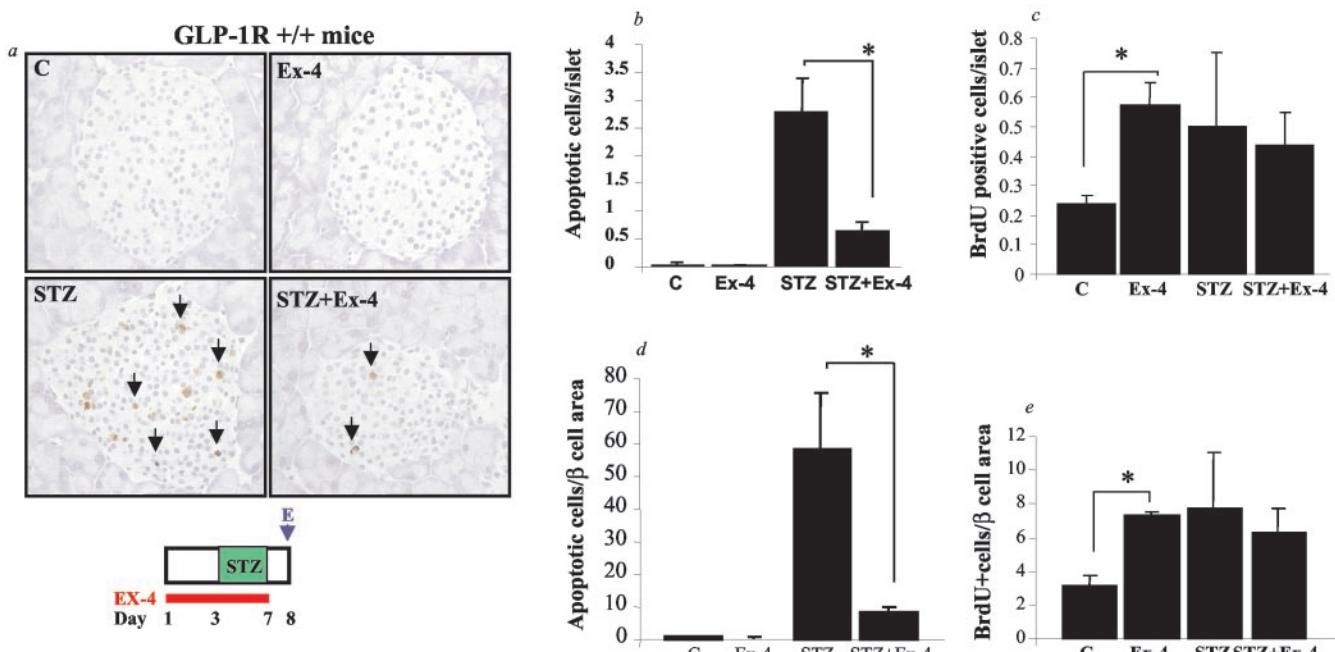
FIG. 3. **Ex-4 reduces islet apoptosis in ST2-treated mice.** *a*, photomicrograph of islet apoptosis (*arrows* denote TUNEL-positive cells) in wild-type control (*C*), Ex-4, STZ, and STZ+Ex-4 C57BL/6 mice ~24 h after 5 daily injections of STZ. *b* and *d*, number of apoptotic cells detected on day 8 at the end (*E*) of the experiment, ~24 h after the last dose of STZ normalized per islet (*b*) or per relative islet area (*d*) was quantified as described under "Materials and Methods." *, $p < 0.05$, STZ *versus* STZ+Ex-4 mice, $n = 6$–7 mice per group, 2 slides analyzed per mouse. The relative extent of basal β-cell apoptosis observed after STZ varies in the different genetic strains of mice in the experiments shown in Fig. 3 (*C57Bl/6*) *versus* Fig. 4 (*CD1*), as previously described (31, 66). Approximately 20 islets per slide were assessed, with a minimum of 2 slides analyzed per mouse. *c* and *e*, number of BrdUrd+ islet cells was quantified in multiple histological sections from the four different experimental groups, and expressed relative to the number of islets (*c*) or normalized to relative islet area (*e*). *, $p < 0.05$ for number of BrdUrd+ cells in exendin-4 (*Ex-4*) *versus* control (*C*) mice.

attach. For measurement of apoptosis by ELISA (see below), cells were seeded (5–8×10^5 cells/ml, Dulbecco's modified Eagle's medium, 11.2 mM glucose, 10% fetal calf serum, 50 μl/well) in 96-well plates pre-coated with extracellular matrix from 804G rat bladder carcinoma cells (Desmos, San Diego, CA) (38). For TUNEL labeling (see below) and labeling with BrdUrd, the sorted β cells were seeded at the same density and in the same medium as 50-μl microdroplets placed at the center of 35-mm-diameter plastic Petri dishes coated with 804G matrix. This allowed for use of an inverted-stage fluorescent microscope to examine the cells (under a coverslip) after fixation. Sorted rat β cells maintained in monolayer culture were exposed to a mixture of three cytokines for 18 h at 8.3 mM glucose. These conditions were established on the basis of preliminary experiments (data not shown) designed to obtain marked augmentation of apoptosis without significant cell necrosis or detachment of cells from the culture vessel. All incubations of cells were in a humidified atmosphere of 5% CO_2 at 37 °C. Exendin-4 was present throughout the 18-h incubation with or without cytokines at a final concentration of 100 nM.

Quantification of Apoptosis and Cell Division in Isolated β Cells— Apoptosis of purified β cells was estimated using Cell Death Detection Elisa^{PLUS} (Roche Biochemicals, Mannheim, Germany) for determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) in cell lysates, a method that correlates well with apoptosis quantification by annexin V staining (39). Alternatively, cells seeded in microdroplets on Petri dishes were processed for estimation of apoptosis using the TUNEL technique according to the manufacturer's instructions ("*in situ* cell death detection kit" from Roche Biochemicals) following fixation for 20 min in 4% paraformaldehyde and permeabilization using 0.5% Triton X-100 for 4 min at room temperature. Cell replication was assessed by incorporation of BrdUrd (Sigma). For this purpose, BrdUrd (10 μM) was included throughout the 18-h incubation with cytokines or exendin. Cells were then fixed, and BrdUrd+ cells were visualized by immunoflueorescence.

*Cell Culture and Apoptosis Experiments—*BHK fibroblasts were grown in Dulbecco's modified Eagle's medium, 4.5g/l glucose supplemented with 5% calf serum. Cells were transfected with cDNAs encoding the rat GLP-1 receptor cloned in the pcDNA3.1 eukaryotic expression vector (Invitrogen, San Diego, CA). Stably transfected cell populations were selected by growth in G418 (Invitrogen) at 0.8 mg/ml for 2 weeks and studies of apoptosis in BHK-GLP-1R cells were done

using pools of G418-resistant clones. For apoptosis experiments, cells were replated in culture medium lacking G418, serum-starved overnight, and treated with cycloheximide in the presence or absence of the indicated peptides or drugs as described (40).

*Cell Viability Assay—*Cells were exposed to either vehicle or cycloheximide in the presence or absence of the indicated drugs, and the number of viable cells was assessed by measuring the bioreduction of a methane thiosulforate tetrazolium salt at 490 nm using the CellTiter 96 aqueous assay (Promega, Madison, WI.)

*SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—*Cell pellets were lysed at 4 °C in radioimmune precipitation assay buffer containing a protease inhibitor mixture, and cleared lysates were boiled in sample buffer containing β-mercaptoethanol and stored at −70 °C. Protein concentration was determined using bovine serum albumin as a standard and equal amounts of cell lysates were separated by discontinuous SDS-polyacrylamide gel electrophoresis under reducing conditions and electrotransferred onto Hybond-C nitrocellulose membrane (Amersham Biosciences). The resultant blot was blocked with 5% skim milk in phosphate-buffered saline containing 0.2% Tween 20 and incubated with the indicated primary antibody overnight at room temperature. Proteins were detected with a secondary antibody conjugated to horseradish peroxidase and an enhanced chemiluminescence commercial kit (Amersham Biosciences). Western blot analyses were carried out using primary antibodies reactive to active caspase-3 p17 subunit (1:1000 dilution; PerkinElmer Life Sciences), cytochrome *c* (1:250 dilution; BIOSOURCE International), porin/VDAC 31HL (1:500 dilution; Calbiochem), Akt (1:1000; Cell Signaling Technology (Beverly, MA)) and actin (1:5,000 dilution; Sigma).

*Statistical Analysis—*All values are presented as means ± S.E. Statistical significance between groups was evaluated by student's *t* test or Bonferroni-corrected analysis of variance.

RESULTS

As treatment of rodents with GLP-1 agonists leads to increased islet mass in association with β cell proliferation and islet neogenesis (19, 25), we hypothesized that GLP-1 might also enhance β cell mass via protection from cellular apoptosis. To test this hypothesis, wild-type C57BL/6 mice were treated with low-dose streptozotocin, a chemical known to induce β-cell
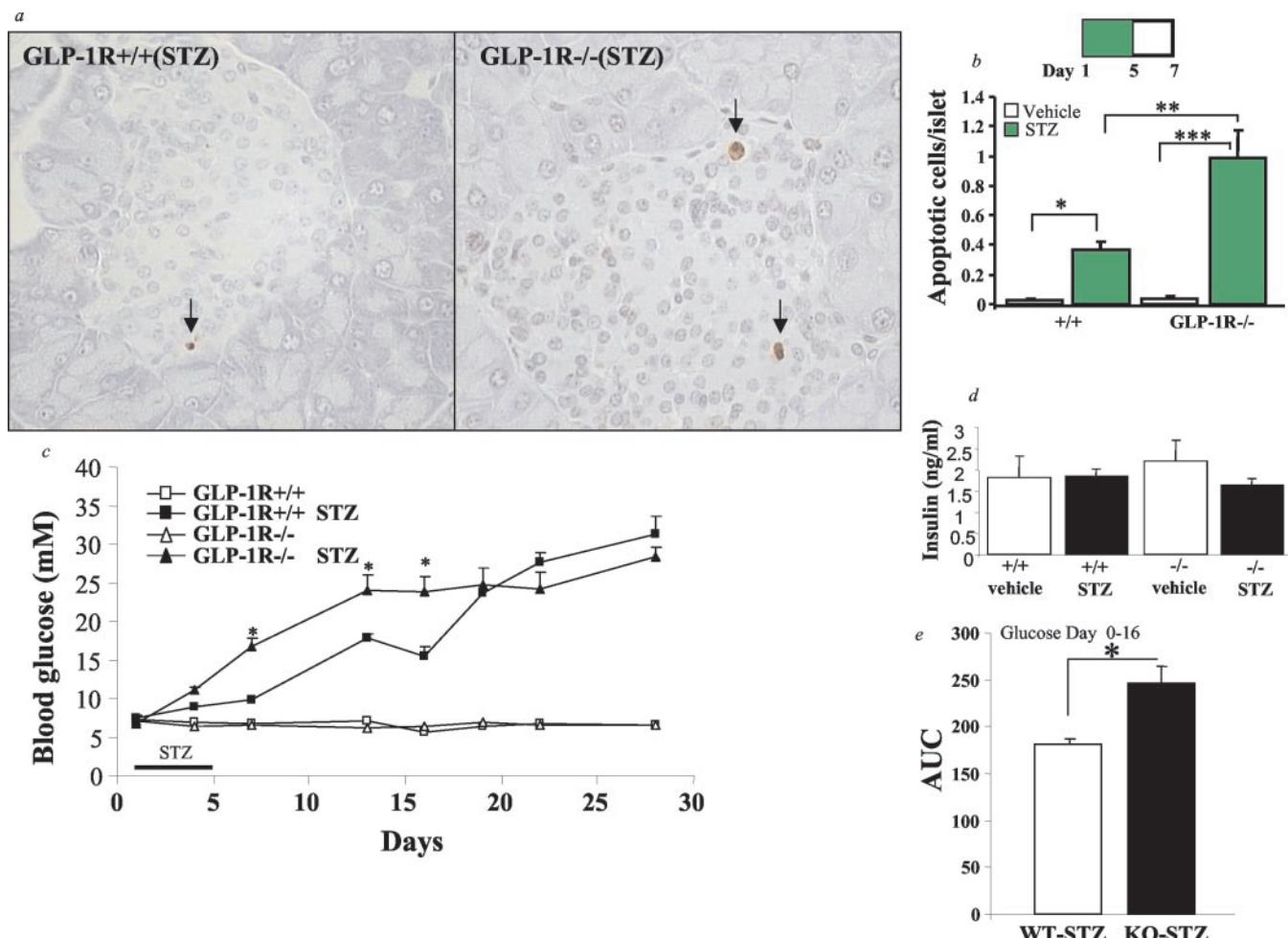
GLP-1 and β Cell Apoptosis

FIG. 4. **Islet apoptosis, glucose, and plasma insulin in GLP-1R+/+ and GLP-1R−/− mice following vehicle (0.1 mM sodium citrate buffered saline) or STZ administration.** Equal volumes of saline (vehicle) or STZ were administered by intraperitoneal injection to groups of mice for 5 days, after which mice were euthanized ~48 h later for histological assessment of islet apoptosis (*a* and *b*) (*arrows* denote apoptotic β cells detected by TUNEL assay) as described under "Materials and Methods." Approximately 30 islets per slide were assessed, with a minimum of 2 slides analyzed per mouse. *, $p < 0.03$, GLP-R+/+ vehicle *versus* STZ; **, $p < 0.0003$ GLP-1R+/+ *versus* GLP-1R−/− both treated with STZ; ***, $p < 0.0001$ GLP-1R−/− vehicle *versus* STZ for rates of islet apoptosis in GLP-1R+/+ *versus* GLP-1R−/− mice with and without STZ. The entire pancreas was cut into multiple pieces and embedded, and two representatives slides from each mouse were analyzed for apoptosis ($n = 4-6$ mice per group). No changes in β-cell mass or β-cell proliferation were detected in GLP-1R+/+ *versus* GLP-1R−/− mice at the end of this experiment (data not shown). *c–e*, fed blood glucose in CD1 control GLP-1R+/+ and CD1GLP-1R−/− mice treated for 5 days with STZ. The area under the curve for blood glucose between days 0 and 16 is shown in *e*. *, $p < 0.05$ for glucose in GLP-1R+/+ *versus* GLP-1R−/− mice treated with STZ in *c* and for AUC glucose (*e*) between days 0 and 16 in wild-type (*WT*) *versus* GLP-1R−/− (*KO*) mice treated with streptozotocin (*STZ*). No significant differences were observed in levels of glucose-stimulated insulin assessed at day 28 (*d*).

apoptosis (29), in the presence or absence of the GLP-1 analog Ex-4, administered for 2 days before STZ, during, and 3 days after STZ (Fig. 1). The pretreatment regimen was selected in part because of observations that pretreatment of mice with the related glucagon-like peptide GLP-2 significantly reduced apoptosis in experimental models of intestinal injury (33, 41). Mice treated with STZ developed progressive hyperglycemia, with levels of blood glucose rising steadily several days after STZ administration. In contrast, mice that received both STZ (5 days) and Ex-4 (10 days) exhibited a significantly delayed onset of hyperglycemia (compare day 9–12 glucose in STZ *versus* STZ+Ex-4 mice, Fig. 1) and blood glucose remained significantly lower even 2 weeks after the last dose of Ex-4 (Fig. 1, $p < 0.05$ for STZ alone *versus* STZ+Ex-4 glucose between day 9–29). Furthermore, levels of circulating insulin at day 30 were significantly greater in STZ+Ex-4 mice, 20 days after the last Ex-4 injection (Fig 1).

A separate experiment was carried out using a different pre-treatment period starting exendin-4 administration 7 days before STZ, and continuing exendin-4 administration for a total

of 28 days, with assessment of oral glucose tolerance and glucose-stimulated insulin at day 30. A similar protective response to Ex-4 was observed in this longer experiment shown in Fig 2. Although hyperglycemia developed in all mice treated with STZ, levels of blood glucose were significantly lower in the STZ+Ex-4 group, even more than 3 weeks after cessation of Ex-4 (Fig. 2*a*; $p < 0.05$ for day 15–52 glucose, STZ *versus* STZ+Ex-4). Oral glucose tolerance testing on day 30, 2 days after the last dose of Ex-4, revealed significantly lower glucose excursion specifically at early time points following oral glucose loading, in association with significantly increased levels of plasma insulin in STZ+Ex-4 mice (Fig. 2*b*; $p < 0.002$). Furthermore, the levels of fed plasma insulin remained significantly greater in the STZ+Ex-4-treated mice and were comparable to levels detected in Ex-4-alone mice that did not received streptozotocin ($p < 0.05$) at day 55, 27 days after the last dose of exendin-4 (Fig. 2*a*).

To ascertain the mechanisms underlying the sustained improvement in levels of glucose and insulin in STZ+Ex-4 mice, we assessed pancreatic histological sections for the presence of
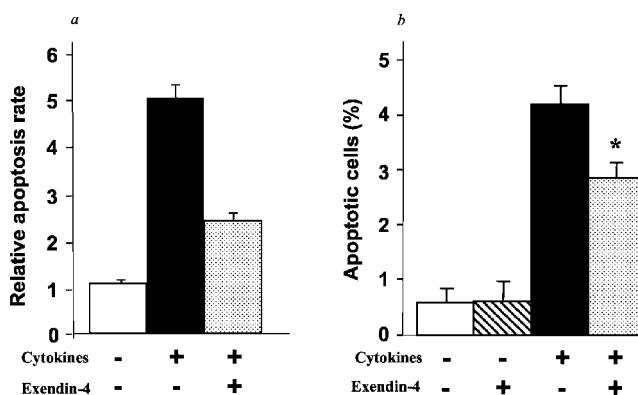
FIG. 5. **The GLP-1 receptor agonist exendin-4 reduces cytokine-induced apoptosis of rat β cells.** β cells were sorted (from adult rat islets) and established in monolayers. The cells were incubated for 18 h with cytokines (1 ng/ml interleukin 1β, 5 ng/ml tumor necrosis factor α, 5 ng/ml interferon γ) with or without exendin 4 (100 nM) as indicated. *a*, apoptosis was quantified by ELISA and normalized to values measured in untreated cells. Data are mean ± S.E. (*n* = 5 per experiment) for one of a total of 3 independent experiments. *p* < 0.001 for differences between all 3 groups. *b*, TUNEL-positive cells were assessed directly by immunohistochemistry. Data (TUNEL-positive cells as percentage of all cells in microscopic field) are mean ± S.E., *n* = 8 fields from 2 independent experiments (4 fields per experiment). The number of cells per field (mean ± S.E., *n* = 8) was 126 ± 6, 120 ± 6, 110 ± 8, and 111 ± 6 for controls, exendin-4 alone, cytokines alone, or exendin-4 plus cytokines, respectively (differences between groups not statistically significant). *, *p* < 0.001 for cytokines alone *versus* cytokines plus exendin-4.

apoptotic β cells in separate groups of mice treated with STZ, with or without Ex-4. Only a rare apoptotic β cell was detectable in histological sections from pancreases of control or Ex-4-treated mice in the absence of STZ (Fig 3*a*). In contrast, morphological features of apoptosis, including pyknotic nuclei, were readily detectable in pancreatic sections from STZ-treated mice. The numbers of TUNEL-positive apoptotic β cells were markedly increased in STZ-treated mice, and significantly reduced (4.5-fold) in mice administered both STZ and Ex-4, whether expressed as the number of apoptotic cells per islet or normalized to relative β cell area (Fig. 3, *b* and *d*; *p* < 0.001, STZ *versus* STZ+Ex-4).

Because GLP-1 agonists have been shown to induce β-cell proliferation (19), we assessed the extent of β-cell proliferation and expansion of islet mass in the same experiment. Wild-type mice treated with Ex-4 alone for 7 days in the absence of STZ exhibited a greater than 2-fold increase in the number of BrdUrd+ β cells, whether expressed as BrdUrd+ cells per islet, or normalized to β cell area (Fig. 3, *c* and *e*, respectively; *p* < 0.05 for control *versus* Ex-4-treated mice). In contrast, we did not detect a significant increase in the number of BrdUrd+ cells in Ex-4-treated mice treated for 5 consecutive days with STZ (Fig. 3, *c* and *e*).

These findings demonstrate that exogenous activation of GLP-1 receptor signaling reduced STZ-associated islet apoptosis in wild-type mice *in vivo*. To ascertain whether basal levels of endogenous GLP-1 receptor signaling protected β cells from external injury, we administered STZ to mice with a targeted disruption of the *Glp-1R* gene (GLP-1R−/− mice (32)). Blood glucose increased more rapidly in GLP-1R−/− *versus* GLP-1R+/+ mice after STZ administration (Fig. 4, *c* and *e*; *p* < .05 for glucose at day 7 in STZ-treated GLP-1R+/+ *versus* GLP-1R−/− mice) and remained significantly greater in STZ-treated GLP-1R−/− from day 7–16 (Fig. 4, *c* and *e*; *p* < 0.05; GLP-1R−/− *versus* control GLP-1R+/+ mice treated with STZ). In contrast, after day 16, the levels of blood glucose in STZ-treated mice remained elevated however no significant differences in glucose (Fig. 4, *c*) were detected in GLP-1R−/−

versus GLP-1R+/+ by day 28, 23 days after the last dose of STZ. Similarly, levels of glucose-stimulated insulin at day 7 were not significantly different. In contrast, the number of apoptotic β cells detected ~48 h after the last dose of STZ was increased in both GLP-1R+/+ and GLP-1R−/− mice and was significantly greater (2.7-fold) in GLP-1R−/− mice treated with identical doses of STZ (Fig. 4, *a* and *b*; *p* < 0.002). Taken together, the data presented in Figs. 1–4 demonstrate that activation or abrogation of GLP-1 receptor signaling regulates the extent of murine β cell apoptosis *in vivo*.

To determine whether GLP-1 agonists exert direct anti-apoptotic effects on islet β cells *in vitro* using a different approach for generation of cytotoxic injury, we induced apoptosis in purified populations of sorted rat β cells using a combination of cytokines (1 ng/ml interleukin 1β, 5 ng/ml tumor necrosis factor α, 5 ng/ml interferon γ) as previously described (42). Incubation with cytokines alone for 18 h produced a 4.9-fold increase in apoptosis, however co-incubation with cytokines and exendin-4 significantly reduced the extent of apoptosis (Fig 5*a*) by 44.0 ± 5.2% compared with the extent of apoptosis with cytokines alone (*p* < 0.001). To assess directly the percentage of apoptotic cells under these various conditions, individual rat β cells were examined for TUNEL staining (Fig. 5*b*). Cytokines increased the percentage of TUNEL-positive β cells from 0.6 to 4.2%. Consistent with results seen by ELISA, exendin-4 significantly reduced the percentage of TUNEL-positive cells compared with values seen with cytokines alone (Fig. 5*b*; *p* < 0.001). Because GLP-1 agonists are known to stimulate β cell proliferation, it was possible that the observed quantitative effects on apoptosis were to some degree modified by increased cell replication. To test this possibility, cell division was estimated by incorporation of BrdUrd (present throughout the 18 h incubation period). As expected for primary adult β cells, the number of dividing cells was extremely low under control conditions. Although there did appear to be a modest increase with exendin-4 treatment, no more than 10 BrdUrd-fluorescent cells were visible in each monolayer culture of 25,000 cells (amounting to less than 0.05%) even under these conditions. In the presence of cytokines, the cells change their morphology making it difficult to observe BrdUrd-positive cells with any accuracy, but there was no evidence for any increase in cell division. Similarly, glucose-stimulated insulin secretion was abolished by treatment with the cytokines and exendin-4 was not able to reverse this impairment (data not shown), in keeping with the known and severe effects of this combination of cytokines on β-cell function (43).

These findings demonstrate that direct activation of the β-cell GLP-1 receptor is coupled to reduction of apoptosis in wild-type mice *in vivo* and in primary rat β-cell cultures *in vitro*. To determine whether GLP-1 receptor activation is sufficient for direct engagement of antiapoptotic pathways in heterologous cells, we introduced the rat GLP-1 receptor into BHK fibroblasts. BHK-GLP-1R cells responded to GLP-1 with a dose-dependent increase in cAMP accumulation, whereas GLP-1 had no effect on wild-type BHK cells (data not shown). After treatment with cycloheximide, BHK cells exhibit morphological features associated with apoptosis including membrane blebbing, cell shrinkage and detachment, and cell fragmentation into apoptotic bodies (40). BHK-GLP-1R cells treated with 80 μM cycloheximide exhibited progressively reduced cell viability, whereas co-incubation of cells with Ex-4 significantly increased cell viability at multiple time points (Fig. 6*a*). The relative activities of caspase-3, caspase-8, and caspase-9 were markedly induced after exposure to cycloheximide and significantly reduced after treatment with Ex-4 in the same experiments (data not shown). Cycloheximide-treated BHK-GLP-1R cells exhib-
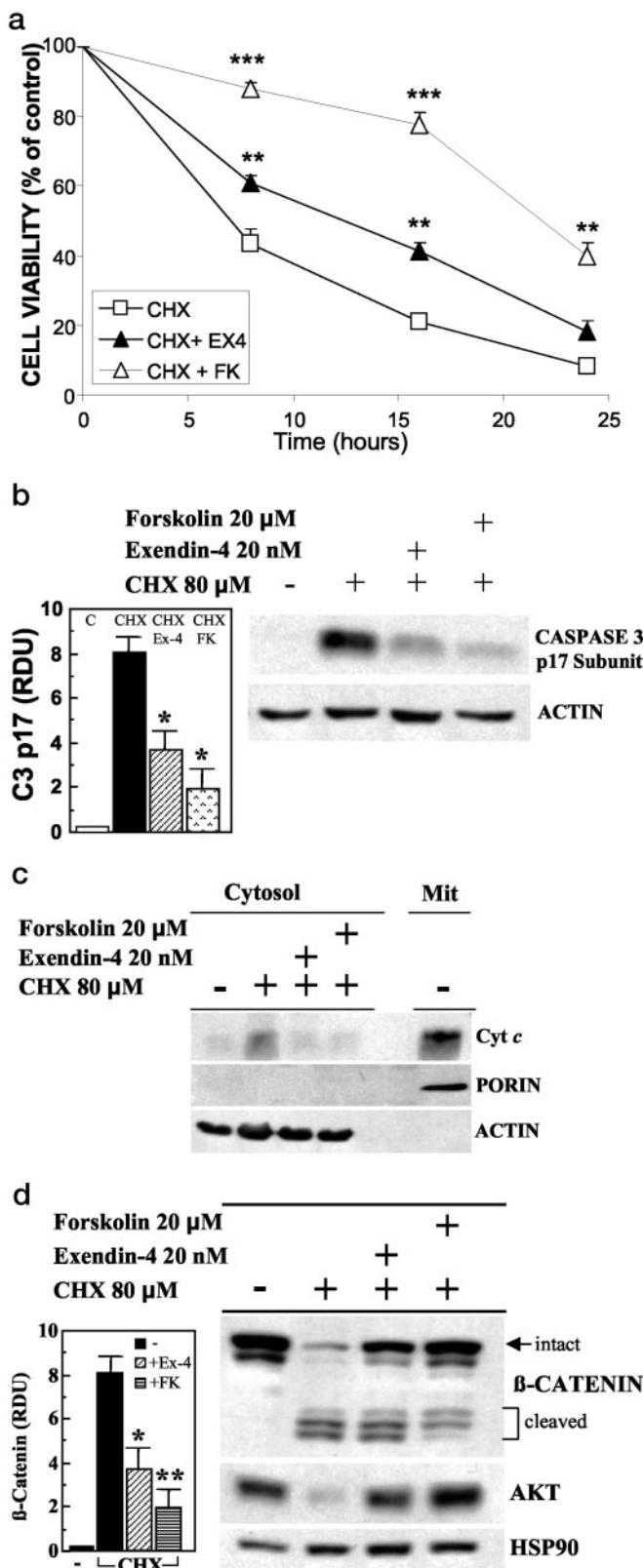
FIG. 6. **Ex-4 protects BHK-GLP-1R cells from cycloheximide-induced apoptosis.** For assessment of cell viability, cells were cultured as described under "Materials and Methods" and treated with 80 μM CHX in the presence or absence of 20 nM Ex-4 or 20 μM forskolin, a concentration previously shown to attenuate cycloheximide- or LY294002-induced apoptosis in BHK cells (40, 67). Dose-response studies demonstrated significant cAMP accumulation in BHK-GLP-1R cells with 10–a, 100 nM exendin-4 (data not shown). a, cell viability was quantified using a tetrazolium salt bioreduction assay and expressed as a percentage of the values obtained from analysis of vehicle alone-treated cultures. No effect on cell survival or cAMP accumulation was

ited increased levels of the active p17 caspase 3 subunit that were markedly diminished by treatment with Ex-4 (Fig. 6*b*). Similarly, Ex-4 reduced the translocation of cytochrome *c* from the mitochondrial compartment to the cytosol in the setting of cycloheximide-induced apoptosis (Fig. 6*c*). Furthermore, the levels of intact β-catenin, a caspase-3 substrate, and the pro-survival kinase Akt, were markedly reduced following exposure to cycloheximide, whereas Ex-4 treatment clearly preserved levels of both intact β-catenin and Akt (Fig. 6*d*).

DISCUSSION

Original concepts of GLP-1 action focused on its role as a gut-derived incretin acting on islet β cells to augment nutrient-stimulated insulin release in the postprandial state (44, 45). The finding that GLP-1 agonists also promote β cell proliferation and islet neogenesis (19, 25), taken together with the defects in islet topography observed in the GLP-1R−/− mouse (35) has broadened the physiological actions of incretin peptides to encompass the regulation of β cell mass. Indeed, recent evidence suggests a potential role for the structurally related incretin, glucose-dependent insulinotropic polypeptide as a second gut-derived regulator of β-cell proliferation (46).

The concept that circulating gut peptides exert cytoprotective actions on distal target tissues is exemplified by the actions of GLP-2, a proglucagon-derived peptide co-secreted from the L cell together with GLP-1 (47, 48). GLP-2 promotes cell proliferation indirectly via activation of a distinct G-protein-coupled receptor expressed in human enteroendocrine cells and murine enteric neurons, leading to expansion of the mucosal epithelium in the small and large bowel (49–53). Although the anti-apoptotic actions of GLP-2 are not readily apparent in the normal epithelial mucosa, induction of experimental intestinal injury is associated with increased crypt apoptosis that is markedly suppressed by exogenous GLP-2 administration (33, 41).

Given the low basal rate of apoptosis in the normal islet (54), induction of islet injury is necessary to unmask the anti-apoptotic effects of GLP-1 on the β cell *in vivo*. Rats treated with

intraperitoneal STZ for 5 days exhibited increased pancreatic levels of prohormone convertase-1, the primary enzyme responsible for liberation of GLP-1 from proglucagon (55). Furthermore, the levels of bioactive GLP-1(7–36 amide) were significantly elevated in the circulation and pancreas of STZ-treated diabetic rats (55). These findings suggest that at least some types of experimental diabetes associated with β-cell injury may be characterized by enhanced liberation of both pancreatic and intestinal GLP-1, which may have physiological implications for protection of vulnerable β cells from further cell death.

A recent report suggests that GLP-1 may exert cytoprotective effects in rat islets incubated with cytokines *in vitro* (56). Because primary cultures of rat islets generally contain a mixture of both islet and contaminating non-islet cell types (57), it is not possible to determine whether the antiapoptotic effects of GLP-1 in mixed islet cultures are exerted through direct or indirect actions on the islet β cell. Furthermore, the GLP-1 receptor has been localized to not only β but also to islet α and δ cells (58); hence even mixed islet endocrine cultures free of exocrine contamination will contain several distinct cell types capable of responding to exogenous GLP-1 *in vitro*.

To determine whether GLP-1 exerts antiapoptotic effects via direct or indirect mechanisms, we used two independent experimental models, highly purified sorted rat β cells and heterologous cells transfected with the GLP-1 receptor. The finding that GLP-1 directly inhibits apoptosis both in populations of islet β cells exposed to cytokines and in BHK-GLP-1R fibroblasts treated with cycloheximide strongly implicates a direct antiapoptotic effect of GLP-1 agonists transduced via the GLP-1 receptor. Similarly, heterologous cells stably transfected with the GLP-2 receptor exhibit enhanced survival after CHX treatment and reduced activation of proapoptotic effector caspases after treatment of cells with GLP-2 (33, 40). Because both GLP-1 and GLP-2 increase the levels of cAMP, and forskolin mimics the antiapoptotic actions of these peptides *in vitro*, activation of downstream cAMP-dependent prosurvival pathways may be an important feature of several G-protein-coupled receptors that regulate cellular apoptosis. Indeed, the vasoactive intestinal peptide and pituitary adenylate cyclose-activating peptide receptors are also expressed on islet β cells (59) and coupled to adenylate cyclase activation and these insulinotropic peptides exert antiapoptotic and cytoprotective actions *in vitro* (60–63). Nevertheless, elevated levels of intracellular cAMP are not always associated with enhanced β-cell survival (64), hence the precise signal-transduction mechanisms linking G-protein-coupled receptor activation to β-cell injury require further clarification.

The majority of actions ascribed to GLP-1 have been deduced following exogenous GLP-1 administration and some, but not all of these effects are physiologically important for metabolic regulation and β-cell function. GLP-1R−/− β cells exhibit reduced levels of cAMP, defects in glucose-stimulated insulin secretion (32), and abnormalities in glucose-stimulated calcium signaling (65). Nevertheless, ob/ob:GLP-1R−/− mice exhibit enhanced islet proliferation and up-regulation of islet mass despite the complete absence of GLP-1R signaling (34). In contrast, the finding that GLP-1R−/− β cells exhibit enhanced susceptibility to STZ-induced apoptosis demonstrates that GLP-1 receptor signaling is an important physiological determinant of β-cell survival following external injury. Because GLP-1 analogues are currently being developed for the treatment of type 2 diabetes, a disease characterized by progressive deterioration and ultimate loss of β cell function, understanding the cytoprotective and proliferative mechanisms activated by GLP-1 in the islet β cell is potentially relevant to the therapy of type 2 diabetes.

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