Original Article

Downregulation of GLP-1 and GIP Receptor Expression by Hyperglycemia

Possible Contribution to Impaired Incretin Effects in Diabetes

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Stimulation of insulin secretion by the incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) has been found to be diminished in type 2 diabetes. We hypothesized that this impairment is due to a defect at the receptor level induced by the diabetic state, particularly hyperglycemia. Gene expression of incretin receptors, GLP-1R and GIPR, were significantly decreased in islets of 90% pancreatectomized (Px) hyperglycemic rats, with recovery when glucose levels were normalized by phlorizin. Perifused islets isolated from hyperglycemic Px rats showed reduced insulin responses to GLP-1 and GIP. To examine the acute effect of hyperglycemia on incretin receptor expression, a hyperglycemic clamp study was performed for 96 h with reduction of GLP-1 receptor expression but increase in GIP receptor expression. Similar findings were found when islets were cultured at high glucose concentrations for 48 h. The reduction of GLP-1 receptor expression by high glucose was prevented by dominant-negative protein kinase C (PKC)α overexpression, whereas GLP-1 receptor expression was reduced with wild-type PKCα overexpression. Taken together, GLP-1 and GIP receptor expression is decreased with chronic hyperglycemia, and this decrease likely contributes to the impaired incretin effects found in diabetes. *Diabetes* 56:1551–1558, 2007

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Ad-GFP, adenovirus-expressing green fluorescent protein; cAMP, cyclic AMP; DN-PKCα, dominant-negative PKCα; dNTP, deoxynucleotide triphosphate; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide 1; GSIS, glucose-stimulated insulin secretion; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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The incretin effect causes more insulin to be secreted when glucose is taken orally than when given intravenously, even when blood glucose levels have the same profile (1,2). This effect is thought to be very important for maximizing insulin responses during meals, thereby limiting postprandial glucose excursions. Two incretins have been identified: glucagon-like peptide 1 (GLP-1) (3,4) and glucose-dependent insulinotropic peptide (GIP) (2,5). It is thought that incretins play an important role in glucose homeostasis by promoting insulin secretion immediately on meal ingestion. Their physiological importance has been demonstrated by the finding of glucose intolerance both in GLP-1 (6,7) and GIP (8) receptor knockout mice.

The incretin effect has been extensively studied in type 2 diabetes. Plasma GIP levels after meals have been found in most studies to be normal in type 2 diabetes while GLP-1 levels have been found to be modestly reduced (9,10). Striking abnormalities, however, have been found in the action of incretin hormones in type 2 diabetes, particularly GIP, which, when infused, has almost no effect on insulin secretion (11,12). In contrast, the effects of GLP-1 are partially preserved, which is important for its therapeutic potential, but insulin responses are substantially reduced, especially when studies are done at comparable glucose levels (13,14). When studied in the rat partial pancreatectomized (Px) model of hyperglycemia, GLP-1 effect on insulin secretion was similarly reduced (15). As expected, GLP-1 and GIP receptors are expressed in pancreatic β-cells (16), which raises questions about whether some of the impairment of the incretin effect occurs at the receptor level. An earlier study found a modest reduction of GLP-1 gene expression in rat islets cultured in high glucose concentrations (17). In the present study, this hypothesis is further examined with in vivo and in vitro experiments.

RESEARCH DESIGN AND METHODS

Px model. Male Sprague-Dawley rats (Taconic Farms, Germantown, NY), weighing 90–100 g were submitted to 85–95% Px or sham surgery as described previously (18). Briefly, tissue was removed by gentle abrasion with cotton applicators leaving the pancreas within 1–2 mm of the common pancreatic bile duct and extending from the duct to the first part of the duodenum. The proportion removed was varied to generate 85–95% Px rats that develop different degrees of hyperglycemia. For sham surgery, the pancreatic tissue

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was only lightly rubbed instead of being removed. Animals were kept under conventional conditions with free access to water and standard food. All animal procedures were approved by the Joslin Diabetes Center Animal Care Committee.

Animals were weighed, and blood was obtained in heparinized microcapillary tubes from snipped tails of fed rats (9:00–10:00 A.M.) weekly. Whole blood glucose levels were measured with a portable Medisense Precision QID glucometer (Abbott Laboratories, Bedford, MA). Rats were classified according to their averaged blood glucose levels from 3 weeks after surgery; low Px was assigned to Px rats with blood glucose levels <100 mg/dl, moderate Px between 100 and 150 mg/dl, and high Px >150 mg/dl as described previously (19,20). To reverse hyperglycemia, Px rats were treated with phlorizin for the final 2 weeks of the 4-week study period. Phlorizin was dissolved in 1, 2-propanediol and injected intraperitoneally twice a day (9:00 A.M. and 9:00 P.M.) at a dose of 0.8 $g \cdot kg^{-1} \cdot day^{-1}$. At 4 weeks after the surgery, rats were anesthetized, and their islets from the pancreatic remnant or sham pancreas were isolated by distending the pancreas duct with collagenase. After digestion, the islets were separated on a Histopaque density gradient (Histopaque 1077; Sigma-Aldrich, St. Louis, MO) and further purified by handpicking under a stereomicroscope. Islets of similar size were used for extraction of RNA. In several cases, it was necessary to pool islets from two Px rats with similar glycemic levels to obtain an islet yield sufficient for RNA extraction. Gene expression data for other genes in these animals were previously published (21).

Hyperglycemic clamp study. Catheterized Sprague-Dawley rats (~250 g) were infused for 4 days with glucose (500 g/l hydrated glucose; McGaw, Irvine, CA) or saline (4.5 g/l) as previously described (22,23). The glucose infusion rate was regularly adjusted to maintain the blood glucose level at ~200 mg/dl. At the end of the infusion period, islets were isolated and RNA extracted for RT-PCR analysis; genetic expression data for other genes in these animals were previously published (23).

Islet isolation and culture. Islets isolated from normal Sprague-Dawley rats as above were cultured in RPMI-1640 medium (Cell-gro; Mediatech) containing 11.1 mmol/l glucose supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in standard humidified culture conditions of 5% CO_2 and 95% O_2 air at 37°C. After overnight culture, batches of 200 islets were further cultured in six-well dishes for 48 h under various glucose concentrations.

Immunostaining. Under anesthesia, the pancreas or pancreatic remnant was excised, fixed in 4% paraformaldehyde, and processed for paraffin embedding. Sections (5 μm) were immunostained using rabbit anti–protein kinase C (PKC) isoform antibodies (Santa Cruz) and rabbit anti–GLP-1 receptor (HM-316) (24) and anti-GIP receptor (25) antibodies (kindly provided by Dr. Joel F. Habener, Massachusetts General Hospital, and by Dr. Timothy J. Kieffer, University of British Columbia) and fluorochromes as previously described (26). No signal was present if the primary antibodies were omitted. Images were taken at the same settings for all groups of the same experiment for comparison on a Zeiss 410 LSM confocal microscope in the confocal mode.

Semiquantitative radioactive multiplex PCR. After quantification of total RNA extracted from the rat islets, 500 ng RNA was heated at 85°C for 3 min and then reverse transcribed into cDNA in a final reaction solution of 25 μl containing the following: 1X (5 μl) Superscript first-strand buffer (50 mmol/l Tris-HCl, 75 mmol/l KCl, and 3 mmol/l $MgCl_2$) (Invitrogen), 160 umol/l deoxynucleotide triphosphate (dNTP), 40 units RNAsin (Promega, Madison, WI), 10 mmol/l dithiothreitol, 50 ng random hexamers, and 200 units Superscript II Rnase H- reverse transcriptase (Invitrogen). Reverse transcription reactions were incubated for 10 min at 25°C, 60 min at 42°C, and 10 min at 95°C. Resultant cDNA products were diluted to a concentration corresponding to 10 ng of starting RNA per 1.5 μl. PCRs were carried out in a volume of 25 μl consisting of 10 ng cDNA (1.5 μl), 1 μl GeneAmp PCR Gold buffer (Applied Biosystems, Foster City, CA), 1–2 mmol/l $MgCl_2$, 80–160 μmol/l dNTP, 80–600 nmol oligonucleotide primers (Sigma-Aldrich), 1.25 μCi of [α-^{32}P] dCTP (3,000 Ci/mmol; Perkin-Elmer Life Sciences), and 2.5 units AmpliTaq Gold DNA Polymerase (Applied Biosystems). Reactions were performed in a 9700 Thermocycler (Applied Biosystems) in which samples underwent a 10-min initial denaturing step, followed by the number of amplification cycles (1 min denaturation at 94°C, 1 min at the annealing temperature, and 1 min extension at 72°C). The final extension step was 10 min at 72°C. Amplimers were resolved by 6% PAGE in Tris borate EDTA buffer. The amount of [α-^{32}P] dCTP incorporated into amplimers was measured with a Storm 840 PhosphorImager and quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The average intensity of each product was expressed as relative to the internal control gene. These ratios were then used to calculate the percentage of control expression for each sample in the same RT-PCR. We verified that the multiplex PCR products for each set of primers were linearly amplified. Control experiments were performed to adjust the PCR conditions so that the number of cycles used was in the exponential phase of amplification for all products and that each PCR product in a multiplex reaction increased linearly with the amount of starting material. Thirty cycles were used for both GLP-1 and GIP receptors.

Real-time PCR. Islet RNA samples from obese diabetic *db/db* and littermate lean nondiabetic *db/+* mice were analyzed by real-time PCR; genetic expression data for other genes in these animals were previously published (27). Reactions were carried out in a volume of 10 μl consisting of 1 μl cDNA, 1x LightCycler enzyme and reaction mix (SYBR Green I dye, *Taq*DNA polymerase, dNTP; Roche), 1.5 mmol/l $MgCl_2$, and 600 nmol oligonucleotide primers. All reactions were performed in a LightCycler (Roche) in which samples underwent 40 cycles of PCR with an annealing temperature of 55°C. The following primers were used (forward and reverse, respectively): GGGTCTCT GGCTACATAAGGACAAC and AAGGATGGCTGAAGCGATGAC (GLP-1 receptor), GCGTGCTCTACTGCTTCATCAAC and AACTTTCCAAGACCTCATCCCC (GIP receptor), and TGTGCCAGGGTGGTGACTTTAC and TGGGAACCGTT TGTGTTTGG (cyclophilin). The value obtained for each specific product was normalized to a control gene (cyclophilin) and expressed as a percentage of the value in control *db/+* extracts.

Assessment of insulin secretion by islet perifusion. Insulin secretion from islets in response to GLP-1 was assessed by perifusion in RPMI-1640 with 10% newborn calf serum at a flow rate of 0.5 ml/min at 37°C. At 4–6 weeks after surgery, 50–100 handpicked islets from sham and Px rats were loaded into chambers (Swinnex 13; Millipore, Bedford, MA) and perifused for 1 hour with 2.8 mmol/l glucose, followed by 15 min with 16.7 mmol/l glucose, 25 min with 16.8 mmol/l glucose plus 100 nmol/l GLP-1 or 10 nmol/l GIP (Sigma-Aldrich), and then 20 min at 2.8 mmol/l glucose. Islets from five sham and Px animals each were used as separate samples; the size of the islets was visually measured with an eyepiece micrometer for normalization to islet equivalents (150 μm diameter). Samples were taken at 10-min intervals, except for 1-min intervals during stimulation, and stored at −20°C until radioimmunoassay. Insulin concentrations were measured by radioimmunoassay in the Joslin Diabetes Endocrine Research Center Specialized Assay Core. Stimulation index was calculated as the area under the curve during GLP-1 or GIP stimulation and normalized per islet equivalent.

Preparation of recombinant adenoviruses containing the cDNA encoding each PKC isoform (α, β2, δ, ε, and ζ). Recombinant adenoviruses containing the cDNA encoding each PKC isoform (α, β2, δ, ε, and ζ) were prepared using the AdEasy system (kindly provided by Dr. Bert Vogelstein, Johns Hopkins Oncology Center) (28). In brief, the encoding region of each PKC isoform (α, β2, δ, ε, and ζ) was cloned into a shuttle vector pAdTrack-CMV. To produce homologous recombination, 1.0 μg linearized plasmid containing each PKC isoform and 0.1 μg of the adenoviral backbone plasmid pAdEasy-1 were introduced into electrocompetent *Escherichia coli* BJ5183 cells with electroporation (2,500 V, 200 Ohms). The resultant plasmids were re-transformed into *E. coli* XL-Gold Ultracompetent Cells (Stratagene, La Jolla, CA), linearized with *Pac*I, and then transfected into the adenovirus packaging cell line 293 using LipofectAMINE (Life Technologies, Grand Island, NY). Ten days after transfection, cell lysates were obtained from the 293 cells. The cell lysates were added to 293 cells again, and when most of the cells were killed by the adenovirus infection and detached, cell lysates were again obtained (this process was repeated three times). Control adenovirus-expressing green fluorescent protein (Ad-GFP) was prepared in the same manner. Isolated rat islets (~200 islets) were infected with adenovirus-expressing PKC isoforms (α, β2, δ, ε, or ζ) (with green fluorescent protein) or control Ad-GFP (without PKC isoform), using a 1-h exposure to 30 μl of the adenovirus (1×10^8 plaque forming units/ml). One hour after infection, the islets were cultured for 3 days in 3 ml RPMI-1640 medium.

Statistical analysis. Results were expressed as means ± SE. One-way ANOVA was used for the first determination. Unpaired Student's *t* test (two tailed) followed if there was significance. P value <0.05 was considered statistically significant.

RESULTS

Decreased GLP-1 and GIP receptor expression in islets of 90% Px diabetic rats and obese diabetic *db/db* mice. We first examined the expression of GLP-1 and GIP receptors in islets isolated from 85 to 95% Px diabetic rats that had been exposed to chronic hyperglycemia for 4 weeks. The animals were divided into the following groups according to blood glucose levels by the end of 4 weeks after surgery: Low Px (<100 mg/dl), moderate Px (100–150 mg/dl), and high Px (>150 mg/dl) rats. As shown in Fig. 1*A*, GLP-1 receptor mRNA expression was decreased in a glucose-dependent manner, and

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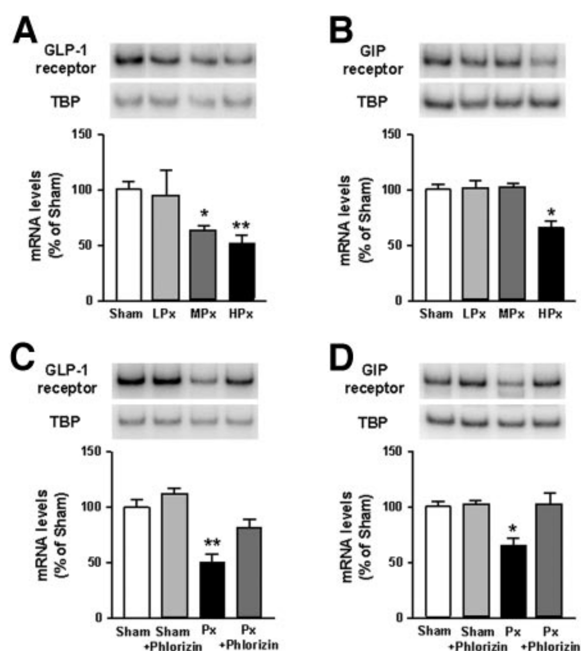

FIG. 1. Decreased GLP-1 and GIP receptor mRNA expression in islets of 85–95% Px diabetic rats. *A* and *B*: Pancreatic islets were isolated from Px rats that were exposed to chronic hyperglycemia for 4 weeks, and GLP-1 and GIP receptor mRNA expression was evaluated. Rats were classified according to their averaged blood glucose levels from 3 weeks after surgery; low Px was assigned to Px rats with blood glucose levels <100 mg/dl, moderate Px between 100 and 150 mg/dl, and high Px >150 mg/dl. *C* and *D*: Phlorizin is known to lower blood glucose levels by preventing reabsorption of glucose from the glomerular filtrate. To examine the effects of hyperglycemia, we treated some high Px rats with phlorizin and evaluated its effect on GLP-1 and GIP receptor mRNA levels. *$P < 0.05$; **$P < 0.01$. $n = 4–6$ in each group.

phlorizin treatment prevented this decrease (Fig. 1*C*). Phlorizin lowers blood glucose levels by preventing glucose reabsorption from the glomerular filtrate in the kidney. These data suggest that hyperglycemia per se leads to decreased GLP-1 receptor expression. In contrast, GIP receptor expression was decreased only in the high Px group (Fig. 1*B*), suggesting that GIP receptor expression is less sensitive to hyperglycemia than that of the GLP-1 receptor. The decrease of GIP receptor expression was also prevented by phlorizin treatment (Fig. 1*D*).

This reduction of receptor expression was confirmed in another well-characterized model of type 2 diabetes, the *db/db* mouse. Islets from obese diabetic *db/db* mice and lean nondiabetic *db/+* littermates show a similar pattern of incretin receptor downregulation: both GLP-1 and GIP receptor mRNA levels were decreased in the islets of long-term diabetic *db/db* mice (GLP-1 receptor mRNA levels: control 100 ± 10 vs. *db/db* 52 ± 7%, $P < 0.01$; GIP receptor: control 100 ± 7 vs. *db/db* 55 ± 15%, $P < 0.01$). Thus, data from the *db/db* mouse model confirm the Px data of downregulation of incretin receptors with long-term diabetes. The *db/db* mouse also shows a similar pattern of β-cell dedifferentiation, which is induced by time-dependent exposure to hyperglycemia (27).

Next, to examine the effect of hyperglycemia on GLP-1 and GIP receptor protein expression in β-cells, we performed double staining for insulin and the GLP-1 or GIP receptor on pancreatic tissue from sham and diabetic Px rats 4 weeks after surgery. As shown in Fig. 2, GLP-1 receptor was mainly localized in β-cells with strong expression in islets from sham-operated rats. The intensity of GLP-1 receptor staining was dramatically decreased in islets from high Px rats compared with those from sham rats; this decrease of intensity was prevented by phlorizin

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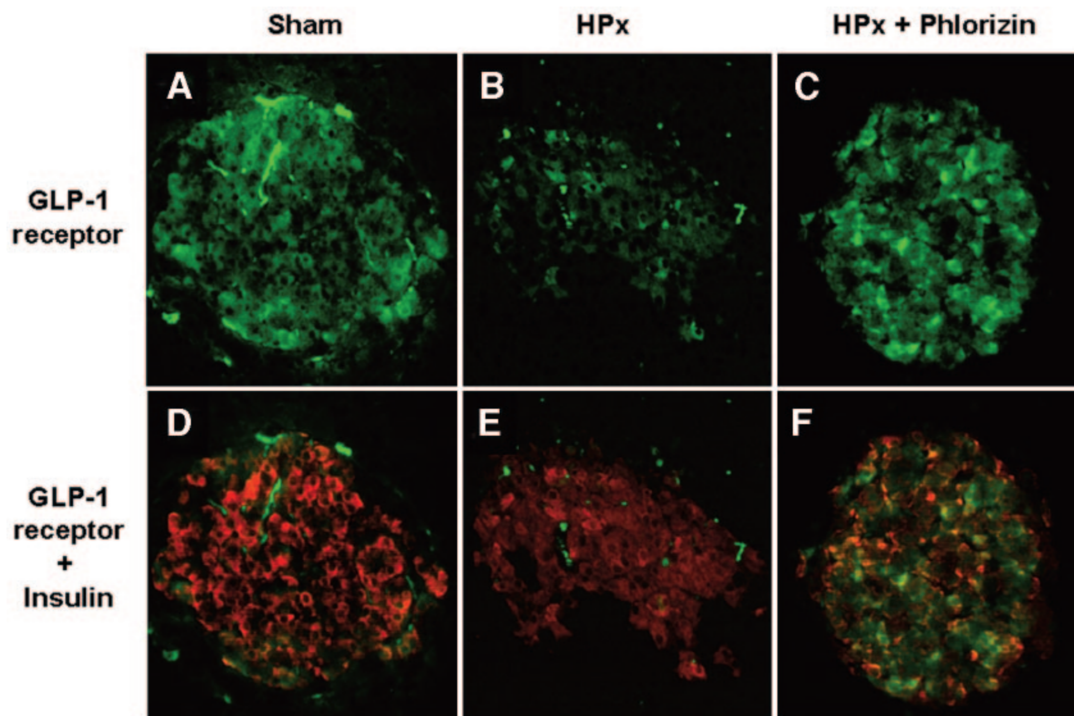

FIG. 2. Decreased GLP-1 receptor protein expression in islets of hyperglycemic Px rats. Sections of pancreas from sham (*A* and *D*) and Px (high Px) (*B* and *E*) rats were immunostained with GLP-1R and insulin antibodies. To examine the effects of hyperglycemia, we treated some high Px rats with phlorizin and evaluated its effect on GLP-1R expression (*C* and *F*). In *A–C*, only GLP-1 staining (green channel) is shown; in *D–F*, the same image with both the red (insulin) and green (GLP-1 receptor) channels is shown. Representative of three evaluations. Magnification bar = 50 μm.

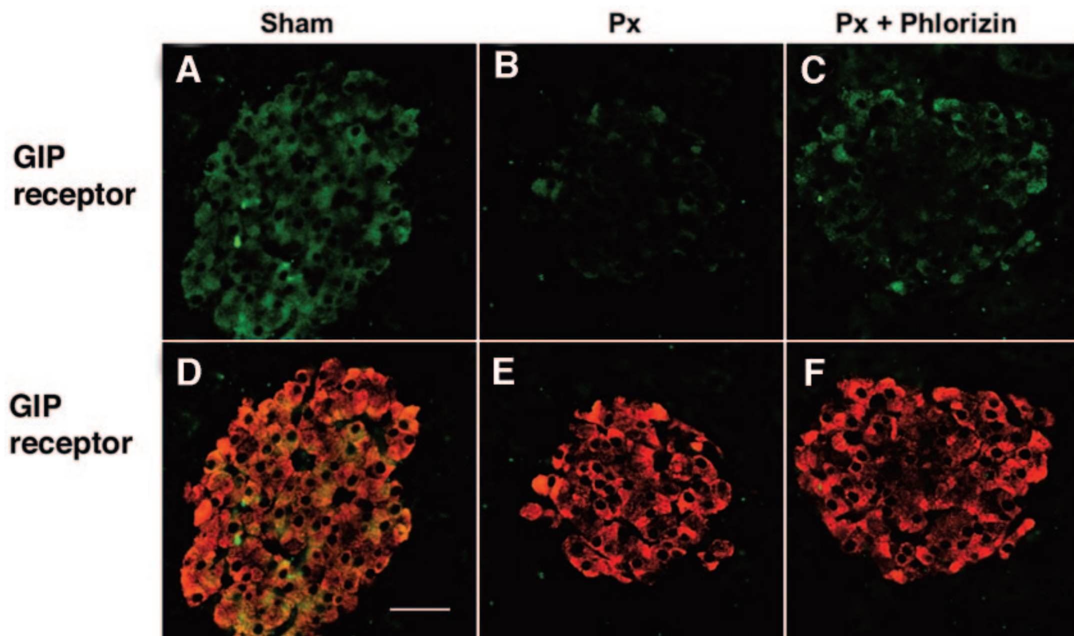

FIG. 3. Decreased GIP receptor protein expression in islets of hyperglycemic Px rats. Sections of pancreas from sham (*A* and *D*) and Px (high Px) (*B* and *E*) rats were immunostained with GIPR and insulin antibodies. To examine the effects of hyperglycemia, we treated some high Px rats with phlorizin and evaluated its effect on GIPR expression (*C* and *F*). In *A–C*, only GIPR (green channel) is shown; in *D–F*, the same image with both the red (insulin) and green (GIPR) channels are shown. Representative of three evaluations. Magnification bar = 50 μm.

treatment associated with normalization of hyperglycemia. As shown in Fig. 3, GIP receptor expression was mainly localized in β-cells with strong expression in islets from sham rats, but the intensity of GIP receptor staining was only moderately decreased in islets from high Px rats compared with those from sham rats.

Impaired insulin secretion in response to GLP-1 or GIP in islets isolated from hyperglycemic Px rats. To assess the possible functional consequence of reduction in GLP-1 and GIP receptor expression, we perifused islets isolated from Px and sham rats 4–6 weeks after surgery. As seen in Fig. 4, the insulin response to GLP-1 was markedly reduced in islets isolated from high Px rats compared with those from sham rats. Similarly, the insulin response to GIP was also reduced in islets isolated from high Px rats compared with those from sham rats (Fig. 5). These results indicate that decreased GLP-1 and GIP receptor expression leads to reduction of β-cell function.

Decreased GLP-1 receptor expression, but not that of GIP receptor, in islets after exposure to high glucose. To further examine the role of hyperglycemia on incretin receptor expression, glucose infusions were performed in conscious rats for 4 days. Intravenous infusion of 50% glucose resulted in blood glucose levels of 150–250 mg/dl throughout the experiment, while the saline-infused controls varied from 117 to 125 mg/dl. As shown in Fig. 6*A*, GLP-1 receptor mRNA levels 4 days after glucose infusion were lower compared with those of saline-infused rats. By contrast, however, GIP receptor mRNA levels 4 days after glucose infusion were higher compared with saline-infused rats (Fig. 6*B*). To further examine the effect of glucose concentration on GLP-1 and GIP receptor expres-

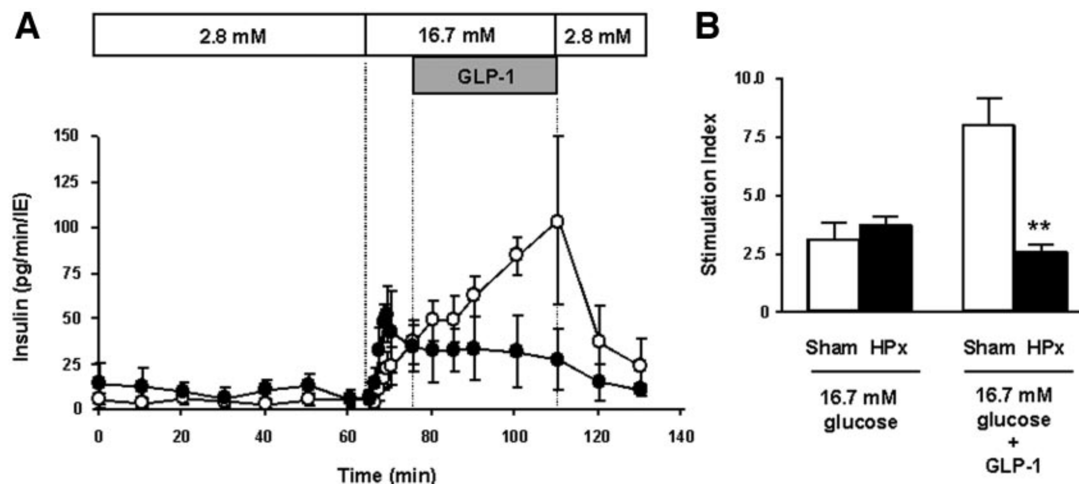

FIG. 4. Impaired insulin secretion in response to GLP-1 in islets isolated from hyperglycemic Px rats. To assess the possible functional consequence of a reduction in expression of GLP-1 receptor, islet perifusion studies were performed using islets isolated from sham and high Px rats. We evaluated the effect of GLP-1 (100 nmol/l) on insulin secretion (*A*) and estimated stimulation index by calculating the area over basal (*B*); $n = 5$ animals per group. Data are means ± SE. ○, sham; ●, high Px.

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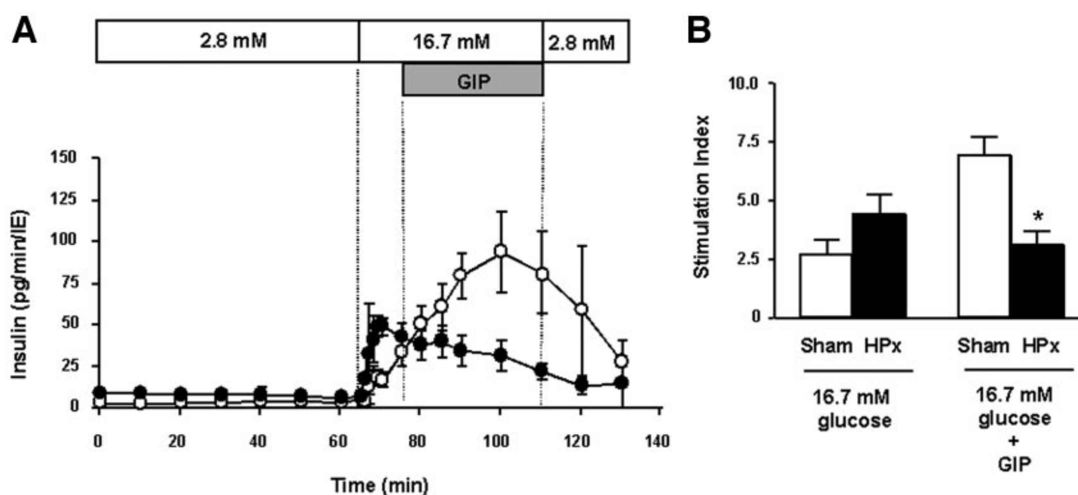

FIG. 5. Impaired insulin secretion in response to GIP in islets isolated from hyperglycemic Px rats. To assess the possible functional consequence of the reduction in expression of GLP-1 receptor, we performed islet perifusion study using islets isolated from sham and high Px rats. We evaluated the effect of GIP (10 nmol/l) on insulin secretion (*A*) and estimated stimulation index by calculating the area over basal (*B*); *n* = 5 animals per group. Data are means ± SE. ○, sham; ●, high Px.

sion, we exposed isolated rat islets to various concentrations of glucose (5–30 mmol/l) for 2 days. Isolated islets were placed in six-well culture plates with 2 ml media containing 5, 10, 20, or 30 mmol/l glucose. In cultured islets, high glucose resulted in decreased GLP-1 receptor expression but increased GIP receptor expression (Fig. 6*C* and *D*).

Involvement of PKCα in decreased GLP-1 receptor by high glucose. Since PKC is activated by high glucose in various cell types, including pancreatic islets (29,30), we hypothesized that PKC activation is involved in the reduction of GLP-1 receptor expression by high glucose. Indeed, GLP-1 receptor expression was decreased after treatment with 100 nmol/l 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of the PKC pathway (control 100 ± 12 vs. 6 h TPA 72 ± 7%, $P < 0.01$; 12 h TPA 85 ± 9%, $P < 0.05$), indicating an involvement of PKC activation in reduction of GLP-1 receptor expression. PKCα, -β2, -δ, -ε, and -ζ have been detected in pancreatic islets (29,30), and PKCα, -β2, -δ, and -ε are activated by high glucose (29,30). First, we examined the distribution of PKC isoforms (PKCα, -β2, -δ, -ε, and -ζ) in rat pancreatic islets by immunostaining (Fig. 7). PKCα, -β2, and -δ were clearly expressed in rat β-cells, but PKCε and -ζ were not detected. Next, we examined the effect of each PKC isoform on GLP-1R mRNA expression in isolated rat islets by adenovirus-mediated overexpression. Three days after exposure to each adenovirus, we examined GLP-1 receptor expression levels. Figure 8*A* shows representative islets 3 days after exposure to control Ad-GFP; many cells in islets were infected with the adenovirus. We have previously found that Ad-GFP infects >50% of the islet cells but does not penetrate to the center of the islet and that glucose-stimulated insulin secretion (GSIS) was not affected by such adenoviral infection (31). GLP-1 receptor mRNA levels were markedly reduced by PKCα overexpression but not by overexpression of the other PKC isoforms (PKCβ2, -δ, -ε, and -ζ) (Fig. 8*B*). To examine an involvement of PKCα in the downregulation of GLP-1 receptor by high glucose, we prepared dominant-negative PKCα (DN-PKCα)-expressing adenovirus (K368R). As shown in Fig. 8*C*, overexpression of DN-PKCα did not result in reduced GLP-1 receptor expression. Together, these results suggest that PKCα is involved in downregulation of GLP-1 receptor by high glucose.

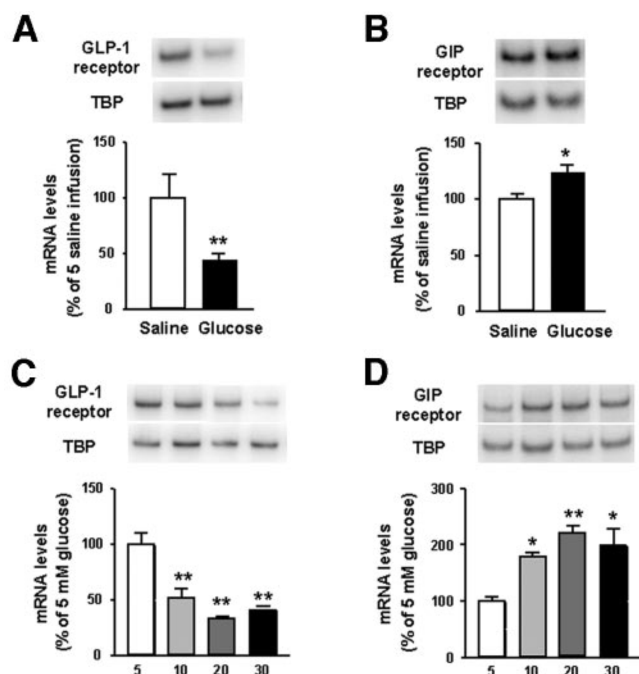

FIG. 6. Decreased GLP-1 receptor expression in islets after exposure to high glucose. *A* and *B*: Glucose or saline was infused in conscious rats for 4 days. GLP-1 and GIP receptor mRNA levels for each animal were examined 4 days after infusion. *C* and *D*: Isolated rat islets were exposed to various concentrations of glucose (5–30 mmol/l) for 2 days, and GLP-1 and GIP receptor mRNA levels were examined (*n* = 4 independent experiments). Representative gels show the receptor and internal control (TBP [TATA-binding protein]) to which the values were normalized in the graphs. *$P < 0.05$; **$P < 0.01$.

DISCUSSION

In this study, it was found that exposure of β-cells to high blood glucose concentrations in vivo and in vitro led to downregulation of the GLP-1 receptor both at the gene expression and protein levels. The gene expression changes were found in rat islets 4 weeks after partial Px, after 4 days of an in vivo glucose clamp, and in isolated

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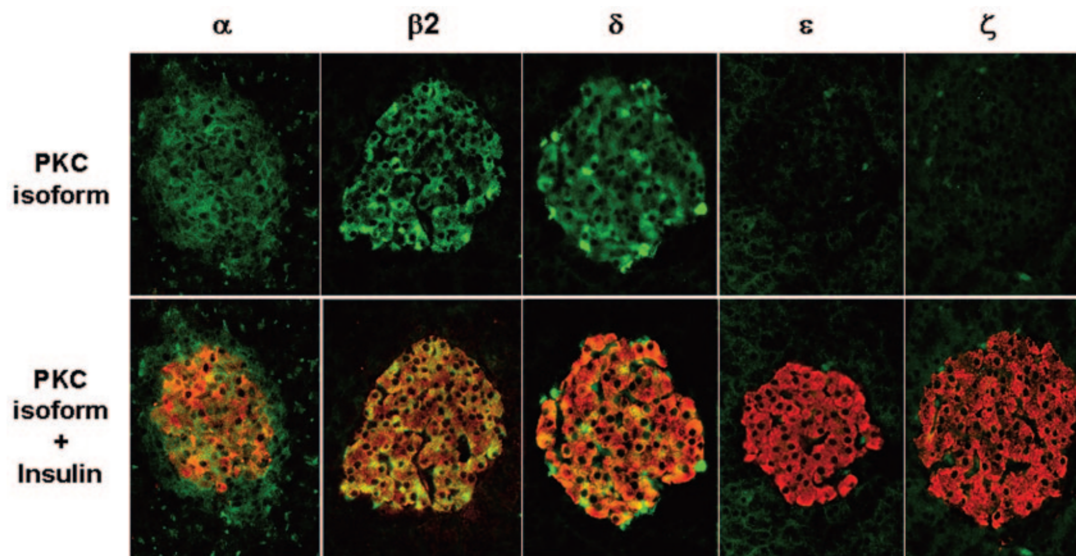

FIG. 7. Immunostaining for PKC isoforms (α, β2, δ, ε, and ζ) in rat pancreatic islets. Pancreatic sections of normal rats were immunostained with each PKC isoform antibody (PKCα, -β2, -δ, -ε, and -ζ) (green; *upper panels*) and insulin (red; *lower panels*). Magnification bar = 50 μm.

islets cultured in the presence of high glucose for 48 h. Supporting the concept that the changes are caused by reversible glucotoxicity effect, the gene expression changes were prevented when glucose levels were normalized with phlorizin, as has previously been found with various other expression changes seen in the Px model (19,21,32). With regard to insulin secretion, in agreement with observations in humans (13,14) and perfused pancreas studies in partially Px rats (15), reduced insulin responses to GLP-1 were found with perifused isolated islets. These findings are consistent with the hypothesis that the unresponsiveness to GLP-1 is in part due to the receptor changes. It would be important to examine whether GLP-1 receptor gene transcription or its mRNA stability is decreased by hyperglycemia, but practically it is very difficult to perform luciferase assay and/or run-off assay with freshly isolated rat islets. It has been reported, however, that GLP-1 receptor mRNA stability is not affected by TPA, an activator of PKC, in β-cell line RINm5F (20). Thus, although not examined in this study, we assume that decrease of GLP-1 receptor mRNA expression was presumably due to decrease of GLP-1 receptor gene transcription itself rather than decrease of its mRNA stability.

There are interesting questions about the relationship of the reduced responsiveness to GLP-1 and the impairment of GSIS consistently found in humans and in animal models of diabetes (33). Earlier studies in the neonatal streptozocin rat model showed that treatment with theophylline, which raises cyclic AMP (cAMP) levels, partially restored defective GSIS secretion (34), raising questions about the degree to which defective cAMP generation from reduction of GLP-1 signaling might contribute to abnormalities in GSIS. It has been postulated that GLP-1 acts as a glucose sensitizer on β-cells (2), which fits with this concept.

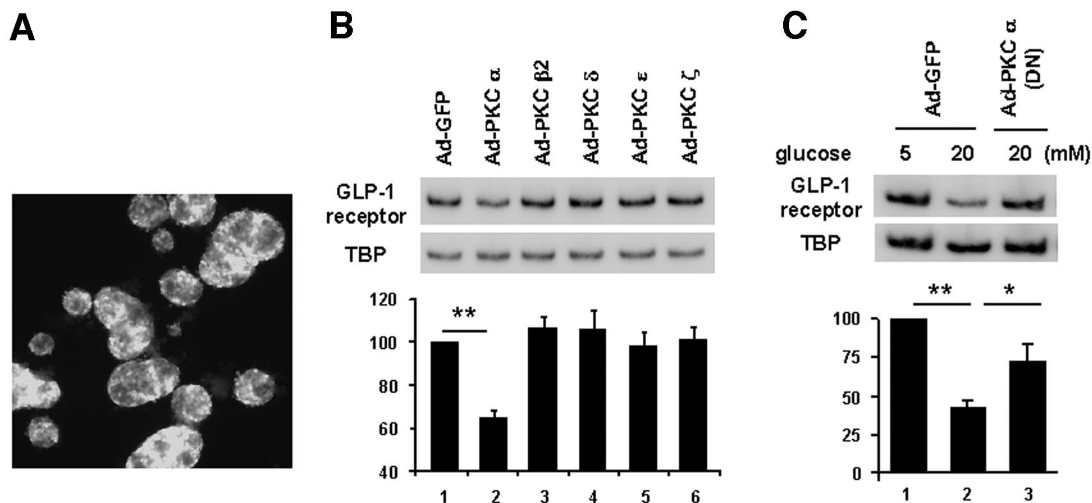

FIG. 8. Involvement of PKCα in downregulation of GLP-1 receptor by high glucose in isolated rat islets. *A*: Isolated rat islets infected with Ad-GFP were cultured for 3 days in RPMI medium. Panel shows representative islets 3 days after exposure to control Ad-GFP. As seen in this fluorescent micrograph, many cells in islets are infected with the adenovirus. Representative of five evaluations. *B*: Isolated rat islets were infected with an adenovirus-expressing PKC isoform (α, β2, δ, ε, or ζ), and 3 days after the infection, GLP-1 mRNA levels were evaluated. *C*: Isolated rat islets were infected with Ad-GFP– or DN-PKCα–expressing adenovirus, and 3 days after the infection the islets were exposed to 5 or 20 mmol/l glucose for 2 days; then GLP-1 mRNA levels were evaluated. $^*P < 0.05$; $^{**}P < 0.01$ ($n = 4$ independent experiments). TBP, TATA-binding protein.

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The changes in GIP receptor gene expression are somewhat different than those found with the GLP-1 receptor. Reduction in expression was found in the Px model, but in the shorter-term glucose infusion model and cultured islets with exposure to high glucose, increases in GIP receptor gene expression were found. These differences in the GIP and GLP-1 receptor gene expression appear to be due to timing and raise interesting questions about the mechanisms underlying these major differing outcomes. It was surprising to find no impairment in the first-phase insulin response in the perifused islets and to find such similarly reduced insulin responses to GIP and GLP-1, considering the studies in humans showing profound reductions in responsiveness to GIP and partially preserved responses to GLP-1 (11,12,14). It is possible that a more complete dose-response study might have brought out differences between GIP and GLP-1 receptors—or perhaps this is a species difference.

The results for GIP receptor are discrepant between the partial Px rats (hyperglycemia for 4 weeks) and the glucose infusion for 4 days. In the first model, GIP receptor expression was reduced, as was in vitro insulin secretion in response to GIP, whereas in the second model, GIP receptor gene expression was increased. The duration of hyperglycemia (acute versus chronic) may account for the difference in the receptor expression. We have previously shown that short-term (4-day) hyperglycemia maintained by glucose clamp induced very little change in β-cell gene expression (22), whereas long-term hyperglycemia after Px induced global alterations (35). These data establish a relationship between the duration of hyperglycemia and the deterioration of the β-cell phenotype with diabetes. The rapid increase in GIP receptor could contribute to the maintenance of insulin output during the elevated metabolic demand induced by hyperglycemia. In fact, the overall capacity of insulin secretion is not diminished after acute hyperglycemia. In contrast, the reduction of the incretin receptors may be, at least partially, due to the dedifferentiation or loss of the specific phenotype of β-cells in Px rats as a result of chronic exposure to hyperglycemia (35). Indeed, when we examined the expression of these genes in another long-term model of type 2 diabetes, the *db/db* mouse, we observed a similar alteration in the pattern of incretin receptor expression: both GLP-1 and GIP receptor expression were decreased in islets of hyperglycemic *db/db* mice.

While GLP-1 is known to stimulate the generation of cAMP and activate protein kinase A, it has recently been appreciated that it can also work through the cAMP-regulated guanine nucleotide exchange factor II (also known as Epac2), which has a variety of targets (36). Stimulation with phorbol esters causing PKC activation has been shown to phosphorylate the GLP-1 receptor with a resultant rapidly diminished signaling (37,38). Since high glucose can activate PKC, hyperglycemia was suggested to act through the PKC pathway to desensitize the GLP-1 receptor without change in expression (38). The present study suggests that the PKCα isoform, which is clearly present in β-cells, is involved and actually decreases the GLP-1 message. Overexpression of PKCα following adenoviral infection led to decrease of GLP-1 message that was comparable with the infection efficiency of β-cells. Then, even more convincing, treatment with an adenovirus containing a dominant-negative form of PKCα partially protected islets against the reduction of GLP-1 receptor expression seen when islets were cultured in a high-glucose concentration.

Taken together, our results show that GLP-1 receptor expression is decreased by hyperglycemia. We suggest that the downregulation of the GLP-1 receptor by hyperglycemia is largely responsible for the impaired incretin effects and thus in part explains the β-cell dysfunction found in diabetes. These findings have important implications for the design of new therapies based on activation of the GLP-1 receptor.

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REFERENCES

1. McIntyre N, Holdsworth CD, Turner DS: Intestinal factors in the control of insulin secretion. *J Clin Endocrinol Metab* 25:1317–1324, 1965
2. Holst JJ, Gromada J: Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans. *Am J Physiol Endocrinol Metab* 287:E199–E206, 2004
3. Mojsov S, Weir GC, Habener JF: Insulinotropin: glucagon-like peptide I (7–37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas. *J Clin Invest* 79:616–619, 1987
4. Kreymann B, Williams G, Ghatei MA, Bloom SR: Glucagon-like peptide-1 7–36: a physiological incretin in man. *Lancet* 2:1300–1303, 1987
5. Brown JC, Dryburgh JR, Ross SA, Dupré J: Identification and actions of gastric inhibitory polypeptide. *Recent Prog Horm Res* 31:487–532, 1975
6. Scorcchi LA, Brown TJ, MaClusky N, Brubaker PL, Auerbach AB, Joyner AL, Drucker DJ: Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. *Nat Med* 2:1254–1258, 1996
7. Baggio L, Kieffer TJ, Drucker DJ: Glucagon-like peptide-1, but not glucose-dependent insulinotropic peptide, regulates fasting glycemia and nonenteral glucose clearance in mice. *Endocrinology* 141:3703–3709, 2000
8. Miyawaki K, Yamada Y, Yano H, Niwa H, Ban N, Ihara Y, Kubota A, Fujimoto S, Kajikawa M, Kuroe A, Tsuda K, Hashimoto H, Yamashita T, Jomori T, Tashiro F, Miyazaki J, Seino Y: Glucose intolerance caused by a defect in the entero-insular axis: a study in gastric inhibitory polypeptide receptor knockout mice. *Proc Natl Acad Sci U S A* 96:14843–14847, 1999
9. Toft-Nielsen MB, Damholt MB, Madsbad S, Hilsted LM, Hughes TE, Michelsen BK, Holst JJ: Determinants of the impaired secretion of glucagon-like peptide-1 in type 2 diabetic patients. *J Clin Endocrinol Metab* 86:3717–3723, 2001
10. Forbes S, Moonan M, Robinson S, Anyaoku V, Patterson M, Murphy KG, Ghatei MA, Bloom SR, Johnston DG: Impaired circulating glucagon-like peptide-1 response to oral glucose in women with previous gestational diabetes. *Clin Endocrinol (Oxf)* 62:51–55, 2005
11. Nauck MA, Heimesaat MM, Orskov C, Holst JJ, Ebert R, Creutzfeldt W: Preserved incretin activity of glucagon-like peptide 1 [7–36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. *J Clin Invest* 91:301–307, 1993
12. Elahi D, McAloon-Dyke M, Fukagawa NK, Meneilly GS, Sclater AL, Minaker KL, Habener JF, Andersen DK: The insulinotropic actions of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (7–37) in normal and diabetic subjects. *Regul Pept* 51:63–74, 1994
13. Fritsche A, Stefan N, Hardt E, Haring H, Stumvoll M: Characterisation of beta-cell dysfunction of impaired glucose tolerance: evidence for impairment of incretin-induced insulin secretion. *Diabetologia* 43:852–858, 2000
14. Kjems LL, Holst JJ, Volund A, Madsbad S: The influence of GLP-1 on glucose-stimulated insulin secretion: effects on β-cell sensitivity in type 2 and nondiabetic subjects. *Diabetes* 52:380–386, 2003
15. Hosokawa YA, Hosokawa H, Chen C, Leahy JL: Mechanism of impaired

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glucose-potentiated insulin secretion in diabetic 90% pancreatectomy rats: study using glucagonlike peptide-1 (7–37). *J Clin Invest* 97:180–186, 1996
16. Moens K, Heimberg H, Flamez D, Huypens P, Quartier E, Ling Z, Pipeleers D, Gremlich S, Thorens B, Schuit F: Expression and functional activity of glucagon, glucagon-like peptide 1, and glucose-dependent insulinotropic peptide receptors in rat pancreatic islet cells. *Diabetes* 45:257–261, 1996
17. Abrahamsen N, Nishimura E: Regulation of glucagon and glucagon-like peptide-1 receptor messenger ribonucleic acid expression in cultured rat pancreatic islets by glucose, cyclic adenosine 3′,5′-monophosphate, and glucocorticoids. *Endocrinol* 136:1572–1578, 1995
18. Bonner-Weir S, Trent DF, Weir GC: Partial pancreatectomy in the rat and subsequent defect in glucose-induced insulin release. *J Clin Invest* 71: 1544–1553, 1983
19. Laybutt DR, Sharma A, Sgroi DC, Gaudet J, Bonner-Weir S, Weir GC: Genetic regulation of metabolic pathways in beta-cells disrupted by hyperglycemia. *J Biol Chem* 277:10912–10921, 2002
20. Fehmann HC, Schweinfurth J, Jiang J, Goke B: Regulation of glucagon-like peptide-I receptor expression and transcription by the protein kinase C pathway. *Res Exp Med (Berl)* 196:219–225, 1996
21. Jonas JC, Sharma A, Hasenkamp W, Ilkova H, Patane G, Laybutt R, Bonner-Weir S, Weir GC: Chronic hyperglycemia triggers loss of pancreatic beta cell differentiation in an animal model of diabetes. *J Biol Chem* 274:14112–14121, 1999
22. Steil GM, Trivedi N, Jonas JC, Hasenkamp WM. Sharma A, Bonner-Weir S, Weir GC: Adaptation of beta-cell mass to substrate oversupply: enhanced function with normal gene expression. *Am J Physiol Endocrinol Metab* 280:E788–E796, 2001
23. Jonas JC, Laybutt DR, Steil GM, Trivedi N, Pertusa JG, Van de Casteele M, Weir GC, Henquin JC: High glucose stimulates early response gene c-Myc expression in rat pancreatic beta cells. *J Biol Chem* 276:35375–35381, 2001
24. Heller RS, Kieffer TJ, Habener JF: Insulinotrophic glucagon-like peptide I receptor expression in glucagon-producing α cells of the rat endocrine pancreas. *Diabetes* 46:785–791, 1997
25. Lewis, JT, Dayanandan B, Habener JF, Kieffer TJ: Glucose-dependent insulinotropic peptide confers early phase insulin release to oral glucose in rats: demonstration by a receptor antagonist. *Endocrinology* 141:3710–3716, 2000
26. Xu G, Stoffers DA, Habener JF, Bonner-Weir S: Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. *Diabetes* 48:2270–2276, 1999
27. Kjorholt C, Akerfeldt MC, Biden TJ, Laybutt DR: Chronic hyperglycemia, independent of plasma lipid levels, is sufficient for the loss of β-cell differentiation and secretory function in the *db/db* mouse model of diabetes. *Diabetes* 54:2755–2763, 2005
28. He TC, Zhou S, Da Costa LT, Yu J, Kinzler KW, Vogelstein B: A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* 95:2509–2514, 1998
29. Kaneto H, Suzuma K, Sharma A, Bonner-Weir S, King GL, Weir GC: Involvement of protein kinase C beta 2 in c-myc induction by high glucose in pancreatic beta-cells. *J Biol Chem* 277:3680–3685, 2002
30. Hoy M, Berggren PO, Gromada J: Involvement of protein kinase C-epsilon in inositol hexakisphosphate-induced exocytosis in mouse pancreatic beta-cells. *J Biol Chem* 278:35168–35171, 2003
31. Kaneto H, Sharma A, Suzuma K, Laybutt DR, Xu G, Bonner-Weir S, Weir GC: Induction of c-Myc expression suppresses insulin gene transcription by inhibiting NeuroD/BETA2-mediated transcriptional activation. *J Biol Chem* 277:12998–13006, 2002
32. Laybutt DR, Kaneto H, Hasenkamp W, Grey S, Jonas JC, Sgroi DC, Groff A, Ferran C, Bonner-Weir S, Sharma A, Weir GC: Increased expression of antioxidant and antiapoptotic genes in islets that may contribute to β-cell survival during chronic hyperglycemia. *Diabetes* 51:413–423, 2002
33. Weir GC, Bonner-Weir S: Five stages of evolving β-cell dysfunction during progression to diabetes. *Diabetes* 53 (Suppl 3):S16–S21, 2004
34. Weir GC, Clore ET, Zmachinski CJ, Bonner-Weir S: Islet secretion in a new experimental model for non-insulin-dependent diabetes. *Diabetes* 30:590–595, 1981
35. Laybutt DR, Glandt M, Xu G, Ahn YB, Trivedi N, Bonner-Weir S, Weir GC: Critical reduction in beta-cell mass results in two distinct outcomes over time: adaptation with impaired glucose tolerance or decompensated diabetes. *J Biol Chem* 278:2997–3005, 2003
36. Holz GG: Epac: A new cAMP-binding protein in support of glucagon-like peptide-1 receptor–mediated signal transduction in the pancreatic β-cell. *Diabetes* 53:5–13, 2004
37. Widmann C, Dolci W, Thorens B: Heterologous desensitization of the glucagon-like peptide-1 receptor by phorbol esters requires phosphorylation of the cytoplasmic tail at four different sites. *J Biol Chem* 271:19957–19963, 1996
38. Widmann C, Dolci W, Thorens B: Internalization and homologous desensitization of the GLP-1 receptor depend on phosphorylation of the receptor carboxyl tail at the same three sites. *Mol Endocrinol* 11:1094–1102, 1997

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