β-Cell Pdx1 Expression Is Essential for the Glucoregulatory, Proliferative, and Cytoprotective Actions of Glucagon-Like Peptide-1

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Glucagon-like peptide-1 (GLP-1) regulates energy intake, gastrointestinal motility, and nutrient disposal. The relative importance of the islet β-cell for GLP-1 actions remains unclear. We determined the role of the islet β-cell and the pancreatic duodenal homeobox-1 (Pdx1) transcription factor for GLP-1 receptor (GLP-1R)-dependent actions through analysis of mice with β-cell–specific inactivation of the *Pdx1* gene (β-cell$^{Pdx1-/-}$ mice). The GLP-1R agonist exendin-4 (Ex-4) reduced glycemic excursion following intraperitoneal (i.p.) glucose challenge in control littermates (β-cell$^{Pdx1+/+}$ mice) but not in β-cell$^{Pdx1-/-}$ mice. Similarly, Ex-4 failed to increase levels of plasma insulin, pancreatic insulin content, and pancreatic insulin mRNA transcripts in β-cell$^{Pdx1-/-}$ mice. Furthermore, Ex-4 significantly increased β-cell proliferation and reduced β-cell apoptosis in β-cell$^{Pdx1+/+}$ mice but not in β-cell$^{Pdx1-/-}$ mice. Moreover, Ex-4 increased the levels of insulin and amylin mRNA transcripts and augmented glucose-stimulated insulin secretion in islets from β-cell$^{Pdx1+/+}$ mice but not in β-cell$^{Pdx1-/-}$ islets. Surprisingly, Ex-4 failed to reduce levels of plasma glucagon in β-cell$^{Pdx1-/-}$ mice. These findings demonstrate that Pdx1 expression is essential for integrating GLP-1R–dependent signals regulating α-cell glucagon secretion and for the growth, differentiated function, and survival of islet β-cells. *Diabetes* 54:482–491, 2005

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Ex-4, exendin-4; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; IBMX, 3-isobutyl-1-methylxanthine; IPGTT, intraperitoneal glucose tolerance test; KRB, Krebs-Ringer bicarbonate; OGTT, oral glucose tolerance test; Pdx1, pancreatic duodenal homeobox-1.

Glucagon-like peptide 1 (GLP-1) is secreted from enteroendocrine cells following nutrient ingestion and controls blood glucose through pleiotropic effects on the pancreatic islets, central nervous system, and the gastrointestinal tract. The principal glucoregulatory mechanisms activated by GLP-1 receptor (GLP-1R) agonists include stimulation of insulin and inhibition of glucagon secretion, reduction of food intake, and inhibition of gastric emptying. Although the glucose-lowering effects of GLP-1 were originally attributed to its incretin actions on the β-cell, subsequent studies demonstrated that insulin levels are often normal or reduced following treatment with GLP-1R agonists (1). Hence, the control of gastric emptying and inhibition of glucagon secretion have emerged as important mechanisms for GLP-1R–dependent control of glucose homeostasis. As GLP-1R agonists are being developed for the treatment of type 2 diabetes (2–4), understanding the mechanisms for GLP-1R–dependent action has both physiological and clinical importance.

Treatment of islet cells or rodents with GLP-1 is associated with induction of *Pdx1* gene expression, and GLP-1 regulates a subset of genes, including proinsulin, GLUT-2, and glucokinase, that are also known to be transcriptional targets of Pdx1 action (5). These findings, taken together with the importance of both Pdx1 and GLP-1 for proliferation and survival of islet β-cells (6,7), raises the possibility that Pdx1 may be an important downstream mediator of GLP-1 action. Pancreatic duodenal homeobox-1 (Pdx1) is a homeobox transcription factor that is highly conserved across different species and is expressed predominantly in the endocrine pancreas and in subsets of enteroendocrine cells in the duodenum (8). Genetic disruption of the *Pdx1* gene in mice abrogates normal pancreatic development (9,10) and results in selective loss of enteroendocrine cell populations, whereas inactivating mutations of *Pdx1* in human subjects is associated with pancreatic agenesis (11). Hence, *Pdx1* is essential for the development and formation of both the exocrine and endocrine pancreas.

Analysis of heterozygous *Pdx1*+/− mice has demonstrated an important role for Pdx1 in the control of β-cell function. *Pdx1*+/− mice exhibit progressive glucose intolerance, in association with defective glucose-stimulated insulin secretion (6,12). Although the molecular targets of Pdx1 action are incompletely understood, several Pdx1

target genes, including glucokinase and proinsulin, are critically important for the differentiated function of the islet β-cell (13). Moreover, restoration of *Pdx1* expression enhances β-cell function and expands β-cell mass in experimental models of murine diabetes (14). Intriguingly, the pleiotropic actions of Pdx1 in the β-cell overlap the actions of GLP-1. To determine whether Pdx1 is an essential downstream target for GLP-1R–dependent action in the islet β-cell, we studied the consequences of acute or repeated GLP-1R agonist administration in mice with β-cell–specific inactivation of the *Pdx1* gene.

RESEARCH DESIGN AND METHODS

Rip1/Pdx1 mutant mice, renamed here as Pdx1:loxP/loxP;RIP:cre mice and referred to throughout this article as β-cell$^{Pdx1-/-}$ mice, were generated as described before by mating Rip1 promoter-Cre transgenic mice with homozygous Pdx1-loxP mice, thus allowing for Cre recombinase–mediated β-cell–specific inactivation of the mouse *pdx1* gene (15). Homozygous Pdx1:loxP/loxP littermates with intact β-cell Pdx1 expression (β-cell$^{Pdx1+/+}$ mice) were used as controls for all experiments. Most experiments were performed in both 8- to 12-week-old and 10-month-old male and female mice. Animals were maintained on standard laboratory diet under a 12-h light-dark schedule, and experiments were conducted according to protocols approved by the Toronto General Hospital Animal Care Committee.

Immunohistochemistry. Immunohistochemistry was performed by the Histology Core Laboratory in the Pathology Department of the Toronto General Hospital. Rabbit anti-IPF1/Pdx1 antibody was used as described previously for detection of Pdx1 expression (16). Immunohistochemical staining for insulin and glucagon was carried out as described (17,18). Islet β-cell proliferation and apoptosis were assessed by BrdU (5′-bromo-2′-deoxyuridine) staining and TUNEL (terminal deoxynucleotide transferase–mediated dUTP nick-end labeling) analyses, respectively, as described (19). Histological sections from a minimum of five mice per group were analyzed in each experiment.

Glucose tolerance testing. Oral glucose tolerance tests (OGTTs) or intraperitoneal glucose tolerance tests (IPGTTs) were carried out after an overnight (~16-h) fast. Glucose administration was performed as described (19) using 1.5 g/kg body wt glucose in six to eight mice for each group. A blood sample was collected from the tail vein at 10 min for measurement of plasma insulin.

Acute and repeated administration of exendin-4. Synthetic exendin-4 (Ex-4) was dissolved in PBS at a concentration of 4.8 μmol/l. For acute administration, Ex-4 was given at a dose of 24 nmol/kg body wt by intraperitoneal administration 30 min before intraperitoneal glucose loading. For repeated Ex-4 administration, Ex-4 was administrated by intraperitoneal injection twice daily (at 8:00 A.M. and 4:00 P.M.) for 3 days at a dose of 24 nmol/kg body wt. Age-matched saline-injected mice were treated identically and used as controls for the effects of Ex-4 administration. For analysis of cell proliferation, BrdU (100 mg/kg body wt) (Roche) was administrated by intraperitoneal injection 4 h before termination of the experiment. Mice were killed by CO_2 anesthesia, and blood was collected by cardiac puncture for analysis of plasma insulin and glucagon. In some mice, pancreata were removed, a small piece of pancreas from the splenic tail was immediately homogenized in Trizol reagent for RNA extraction, a second piece of pancreas was frozen in liquid nitrogen for pancreatic insulin content measurement, and the rest of the pancreas was fixed in 10% formalin overnight and embedded in paraffin for histological analysis.

Measurement of pancreatic hormone levels. Plasma insulin and pancreatic insulin content were measured using a rat insulin enzyme-linked immunoassay kit (Crystal Chem, Chicago, IL) with mouse insulin as a standard. Plasma glucagon levels were measured using a glucagon radioimmunoassay kit (LINCO Research, St. Charles, MO).

Islet isolation and treatment. Islets from 6- to 8-week-old male β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ mice were isolated by collagenase digestion. β-cell$^{Pdx1-/-}$ mice up to 8 weeks of age yielded reasonable numbers of intact islets, whereas mice older than 8 weeks of age usually yielded fewer islets, consistent with studies of *Pdx1*+/− mice (6). For analysis of glucose- and Ex-4–regulated islet gene expression, islets were collected and incubated at three to five islets per vial in RPMI-1640 medium supplemented with 2 mmol/l glucose and 10% FCS for 2 h before treatment. Islets were then incubated in RPMI-1640 media containing 3.3 mmol/l glucose, 11 mmol/l glucose, or 11 mmol/l glucose + 10 nmol/l Ex-4 for 24 h, after which islets were collected by centrifugation and extracted using Trizol for subsequent RNA isolation. A consistent attempt was made to pick islets of approximately the same size from β-cell$^{Pdx1+/+}$ versus β-cell$^{Pdx1-/-}$ mice. For analysis of basal islet gene expression, 15 islets were collected per vial and incubated in RPMI medium containing 11 mmol/l glucose for 24 h, after which islets were extracted in Trizol for RNA isolation. For analysis of secretagogue-stimulated insulin secretion, islets were isolated from 8-week-old male β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ mice and cultured overnight in RPMI-1640 medium supplemented with 11 mmol/l glucose, 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For insulin secretion experiments, islets were preincubated in Krebs-Ringer bicarbonate (KRB) buffer containing 2 mmol/l glucose, 10 mmol/l HEPES (pH 7.4), and 0.2% BSA for 45 min at 37°C; 5 size-matched islets were pooled and incubated with 2 mmol/l glucose, 11 mmol/l glucose, or 11 mmol/l glucose + 10 nmol/l Ex-4 in KRB buffer for 30 min. Similar experiments were performed using 20 islets per vial, cultured at 11 mmol/l glucose, with or without 10 nmol/l Ex-4, or 10 μmol/l forskolin, in the presence of 3-isobutyl-1-methylxanthine (IBMX) (250 μmol/l). Studies of insulin secretion were also carried out in response to exposure to 30 mmol/l KCl or 100 μmol/l Tolbutamide (Sigma) in the presence of 2 mmol/l glucose. Islet insulin content was determined after islet extraction in 200 μl acid ethanol containing 95% ethanol and 0.1 mol/l HCl.

Real-time PCR. Total RNA from isolated islets was extracted in Trizol reagent, and first-strain cDNA was generated by reverse transcription using SuperScript II reverse transcription system (Invitrogen Canada, Burlington, Canada). Quantitative detection of specific mRNA transcripts was carried out by real-time PCR using an ABI PRISM 7900HT instrument. The sequences of specific primer pairs are available upon request. SYBR green PCR mix (PE Applied Biosystems, Foster City, CA) was used and the total real-time PCR volume was 10 μl in a 384 well–thin wall PCR plate using a final primer concentration of 0.5 μmol/l. All samples for real-time PCR were assayed in triplicate, and data were normalized to the relative levels of β-actin mRNA transcripts in the same experiment and analyzed by ABI PRISM SDS 2.1 software. Oligonucleotide primer sequences are available on request.

Statistical significance. All data are expressed as mean ± SE. Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA), and data were considered significant when $P < 0.05$ using an unpaired t test or ANOVA.

RESULTS

Immunohistochemical analysis of the β-cell$^{Pdx1-/-}$ pancreas. To determine the utility of β-cell$^{Pdx1-/-}$ mice for analysis of GLP-1R–dependent action, we examined islet histology and glucose homeostasis at different ages. β-cell–specific inactivation of the Pdx1 gene becomes increasingly prominent at 3–5 weeks of age, with β-cell$^{Pdx1-/-}$ mice developing progressive diabetes once ~80% of β-cells lose Pdx1 expression (15). Islets from littermate control β-cell$^{Pdx1+/+}$ mice examined at 8 weeks or 10 months of age appeared normal and exhibited the typical central core of insulin-staining β-cells with a peripheral rim of glucagon-immunopositive α-cells (Fig. 1). The majority of insulin-immunopositive β-cells contained strong nuclear Pdx1 immunoreactivity. In contrast, the numbers of insulin-immunopositive β-cells were reduced in 8-week-old β-cell$^{Pdx1-/-}$ mice, whereas the relative number of glucagon-immunopositive α-cells was preserved in 8-week-old and 10-month-old murine β-cell$^{Pdx1-/-}$ islets (Fig. 1). The majority of β-cells from β-cell$^{Pdx1-/-}$ islets failed to exhibit nuclear Pdx1 immunopositivity at 8 weeks of age, with a further loss of β-cell nuclear Pdx1 immunopositivity observed in islets from 10-month-old mice (Fig. 1). These observations demonstrate that β-cell$^{Pdx1-/-}$ mice represent a useful model for analysis of progressive islet β-cell Pdx1 deficiency.

Glucose intolerance and impaired glucose-induced insulin secretion in β-cell$^{Pdx1-/-}$ mice. To determine the consequences of β-cell Pdx1 deficiency for glucose homeostasis, we performed oral glucose tolerance testing in young (8–12 weeks) and in older (10 months) mice (Fig. 1). Remarkably, fasting blood glucose was normal in both male and female β-cell$^{Pdx1-/-}$ mice (Fig. 1). Although

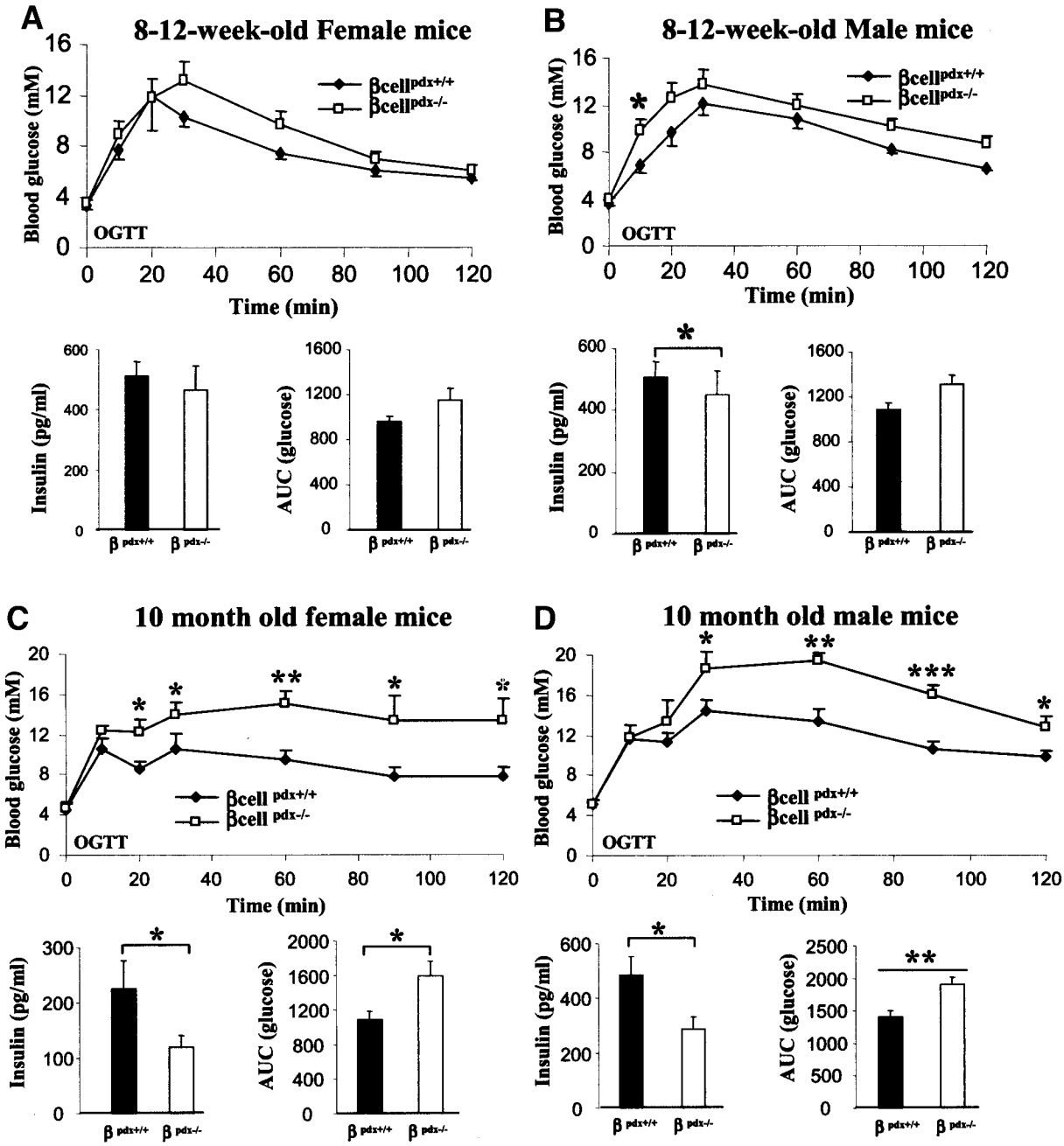

FIG. 1. OGTTs in female (*A* and *C*) and male (*B* and *D*) β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ mice. *A* and *B* depict blood glucose results from 8- to 12-week-old mice (n = 6 female and male β-cell$^{Pdx1+/+}$; n = 8 female and male β-cell$^{Pdx1-/-}$mice). *C* and *D* represent data from 10-month-old mice (n = 7 female β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$; n = 6 male β-cell$^{Pdx1+/+}$; n = 8 male β-cell$^{Pdx1-/-}$). Area under the curve (AUC) was calculated for the glucose excursion during the OGTT from 0 to 120 min (units are mmol · l^{-1} · min^{-1}). Plasma insulin levels were measured at the 10-min time point during OGTT. *P < 0.05; **P < 0.01; ***P < 0.001. *E*: Immunohistochemical analysis of islets from β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ mice. Pancreatic sections were stained with antisera against insulin, glucagon, and Pdx1 in β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ islets from both 8-week-old mice and 10-month-old mice. Magnification ×400.

glycemic excursion and levels of glucose-stimulated insulin secretion were normal in female β-cell$^{Pdx1-/-}$ mice at 8–12 weeks of age (Fig. 1*A*), male β-cell$^{Pdx1-/-}$ mice exhibited significantly higher levels of blood glucose at the 10-min time point of the OGTT accompanied by significantly decreased levels of plasma insulin (Fig. 1*B*). Fasting glucose remained normal even in older mice, but by 10 months of age, both female and male β-cell$^{Pdx1-/-}$ mice exhibited clearly abnormal glycemic excursions following oral glucose loading (Fig. 1*C* and *D*), in association with significant reductions in the levels of glucose-stimulated plasma insulin (Fig. 1*C* and *D*). Hence, genetic elimination of β-cell Pdx1 expression is associated with progressive development of glucose intolerance and impaired glucose-stimulated insulin secretion.

Importance of β-cell Pdx1 expression for GLP-1R–dependent glucose clearance. To determine whether β-cell Pdx1 expression was required for the acute glucose-lowering effects of GLP-1R agonists, we performed OGTTs in 12- to 16-week-old male β-cell$^{Pdx1-/-}$ mice. Although β-cell$^{Pdx1-/-}$ mice exhibited increased glucose excursion compared with control β-cell$^{Pdx1+/+}$ mice, the GLP-1R

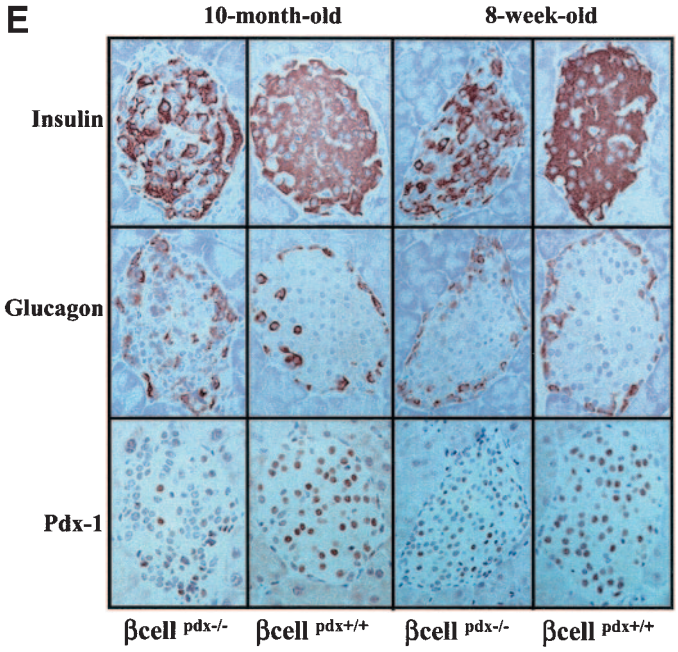

FIG. 1—Continued

agonist Ex-4 significantly reduced glucose excursion in both β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ mice (Fig. 2*A* and *B*). In contrast, however, Ex-4 significantly increased the levels of plasma insulin in β-cell$^{Pdx1+/+}$ mice (Fig. 2*A*) but not in β-cell$^{Pdx1-/-}$ mice (Fig. 2*B*).

To eliminate the contribution of gastric emptying as a locus for Ex-4 action, we administered glucose by intraperitoneal injection. Ex-4 significantly reduced glycemic excursion after intraperitoneal glucose loading and increased levels of plasma insulin in β-cell$^{Pdx1+/+}$ mice (Fig. 2*C*). In contrast, Ex-4 had no effect on glycemic excursion or plasma insulin following intraperitoneal glucose challenge in β-cell$^{Pdx1-/-}$ mice (Fig. 2*D*). Hence, β-cell Pdx1 expression is essential for the acute effects of Ex-4 on glucose-stimulated insulin secretion.

Effects of sustained Ex-4 administration on the murine pancreas in the absence of β-cell Pdx1 expression. GLP-1R activation leads to induction of insulin biosynthesis, β-cell proliferation, and enhanced β-cell survival (20). To ascertain whether β-cell Pdx1 expression is essential for these GLP-1R–dependent actions, we treated mice with Ex-4 twice daily for 3 days (Fig. 3*A*). Remarkably, despite the putative importance of Pdx1 for insulin gene transcription, basal levels of insulin mRNA transcripts were comparable in mice with or without β-cell–specific deletion of Pdx1 (Fig. 3*B*). Ex-4 significantly increased the levels of insulin mRNA transcripts in β-cell$^{Pdx1+/+}$ but not in β-cell$^{Pdx1-/-}$ mice (Fig. 3*B*). Pancreatic insulin content was higher in β-cell$^{Pdx1+/+}$ compared with β-cell$^{Pdx1-/-}$ mice, and Ex-4 significantly increased insulin content in β-cell$^{Pdx1+/+}$ but not in β-cell$^{Pdx1-/-}$ mice (Fig. 3*C*). Similarly, Ex-4 increased the plasma insulin–to–glucose ratio in β-cell$^{Pdx1+/+}$ but not in β-cell$^{Pdx1-/-}$ mice (Fig. 3*D*).

GLP-1R agonists also inhibit glucagon secretion, which is thought to occur either via a direct GLP-1R–dependent action on islet α-cells or indirectly via GLP-1R–mediated stimulation of mediators such as insulin or somatostatin secreted from β- or δ-cells, respectively (4). Although Ex-4 significantly reduced levels of plasma glucagon in β-cell$^{Pdx1+/+}$ mice, no reduction in plasma glucagon was observed in β-cell$^{Pdx1-/-}$ mice (Fig. 3*E*).

GLP-1R agonists stimulate β-cell proliferation and inhibit β-cell apoptosis (20); however, the GLP-1R–activated pathways coupled to cell proliferation and survival remain incompletely understood (21). Ex-4 significantly increased β-cell proliferation in β-cell$^{Pdx1+/+}$ mice, whether expressed as the number of BrdU+ cells per histological section or per islet (Fig. 3*F*). In contrast, Ex-4 had no effect on β-cell proliferation in β-cell$^{Pdx1-/-}$ mice (Fig. 3*F*). A significant increase in the number of TUNEL-positive apoptotic β-cells was detected in β-cell$^{Pdx1-/-}$ mice compared with β-cell$^{Pdx1+/+}$ mice in the absence of Ex-4 (Fig. 3*G*). Ex-4 reduced the rate of β-cell apoptosis in β-cell$^{Pdx1+/+}$ mice but had no effect on β-cell apoptosis in β-cell$^{Pdx1-/-}$ mice (Fig. 3*G*). These results demonstrate that Pdx1 expression is essential for both the proliferative and anti-apoptotic actions of GLP-1R agonists on the islet β-cell.

Pdx1 is required for GLP-1R–dependent regulation of β-cell gene expression. As Pdx1 is a critical determinant of β-cell identity, we examined the relative expression of islet genes important for the control of glucose homeostasis. Total islet RNA isolated from β-cell$^{Pdx1-/-}$ and β-cell$^{Pdx1+/+}$ islets cultured in RPMI supplemented with 11 mmol/l glucose for 24 h was analyzed by real-time PCR (Table 1). The levels of 13 functionally important islet genes were significantly reduced in β-cell$^{Pdx1-/-}$ islet RNA, including mRNA transcripts for insulin, amylin, and glucagon, the transcription factor Nkx6.1, and the kinase Akt1 (Table 1). In contrast, the basal levels of known GLP-1R–activated transcripts such as glucokinase were not reduced in RNA from β-cell$^{Pdx1-/-}$ islets. Although Foxa2 is an essential GLP-1R–regulated activator of Pdx1 expression (22–24), we did not observe a reduction in Foxa2 expression in β-cell$^{Pdx1-/-}$ islets. Similarly, although GLP-1R agonists activate CREB and insulin receptor substrate (IRS)-2 in association with enhanced β-cell growth and survival (25), the levels of CREB and IRS-2 mRNA transcripts were comparable in β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ islets (Table 1).

To determine whether the loss of β-cell Pdx1 expression disrupts GLP-1R–dependent regulation of islet gene expression, we treated cultured islets with Ex-4. The levels of insulin and amylin mRNA transcripts, two genes known to be regulated by both glucose and GLP-1R agonists, were increased following a shift of β-cell$^{Pdx1+/+}$ islets from 3.3 to 11 mmol/l glucose, and Ex-4 significantly augmented the levels of both mRNA transcripts (Fig. 4*A*,B). In contrast, a shift from 3.3 to 11 mmol/l glucose failed to increase insulin or amylin gene expression in β-cell$^{Pdx1-/-}$ islets. Furthermore, Ex-4 had no effect on insulin or amylin gene expression in β-cell$^{Pdx1-/-}$ islets (Fig. 4*A* and *B*). Hence, disruption of β-cell Pdx1 expression is associated with defects in both glucose- and GLP-1R–stimulated islet gene expression.

Insulin secretion is selectively impaired in β-cell$^{Pdx1-/-}$ islets. GLP-1R–dependent potentiation of glucose-stimulated insulin secretion is impaired in β-cell$^{Pdx1-/-}$ mice in vivo (Fig. 3*D*). Despite the genetic

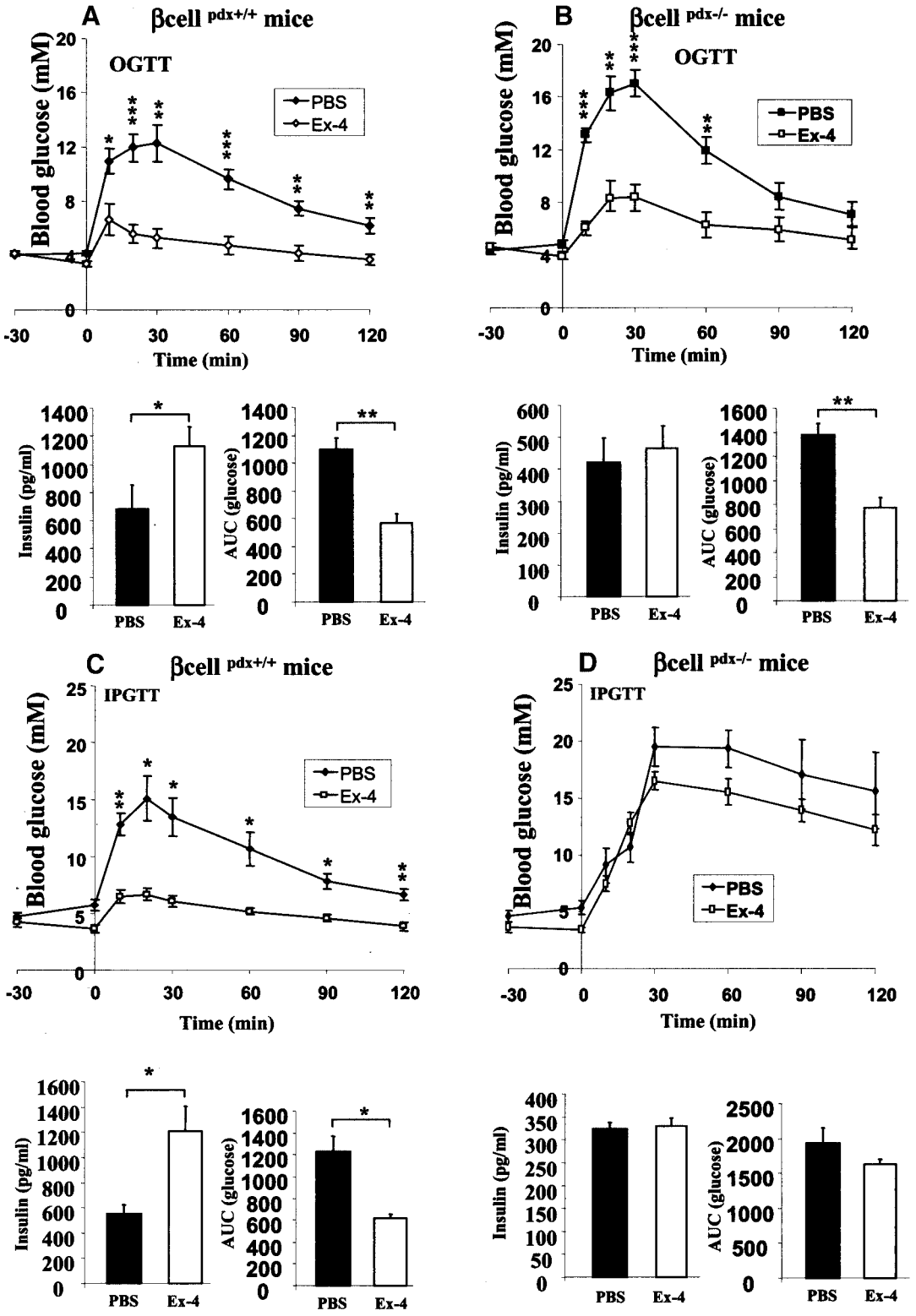

FIG. 2. Acute effects of Ex-4 on glycemic excursion and levels of plasma insulin. OGTTs carried out 30 min after intraperitoneal Ex-4 (24 nmol/kg) injection for 12- to 16-week-old control male mice are shown in *A* (β-cell$^{Pdx1+/+}$ mice, $n = 6$ for PBS; $n = 5$ for Ex-4) and *B* (β-cell$^{Pdx1-/-}$ mice, $n = 6$ for PBS; $n = 5$ for Ex-4). IPGTTs carried out 30 min after intraperitoneal Ex-4 injection for 12- to 16-week-old male mice are shown in *C* (β-cell$^{Pdx1+/+}$ mice, $n = 6$ for PBS; $n = 5$ for Ex-4) and *D* (β-cell$^{Pdx1-/-}$ mice, $n = 5$ for PBS; $n = 8$ for Ex-4). AUC was calculated for the glucose excursion from 0 to 120 min (units are mmol · l^{-1} · min^{-1}). Plasma insulin levels were determined in samples taken from the 10-min time point during OGTT or IPGTT. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

extinction of Pdx1 expression in β-cell$^{Pdx1-/-}$ islets, fasting glucose remained normal and levels of glucose-stimulated insulin were only modestly reduced in 8- to 12-week-old β-cell$^{Pdx1-/-}$ mice (Fig. 2*A*). To assess whether the loss of β-cell Pdx1 expression compromised GLP-1R–dependent stimulation of insulin secretion, we assessed

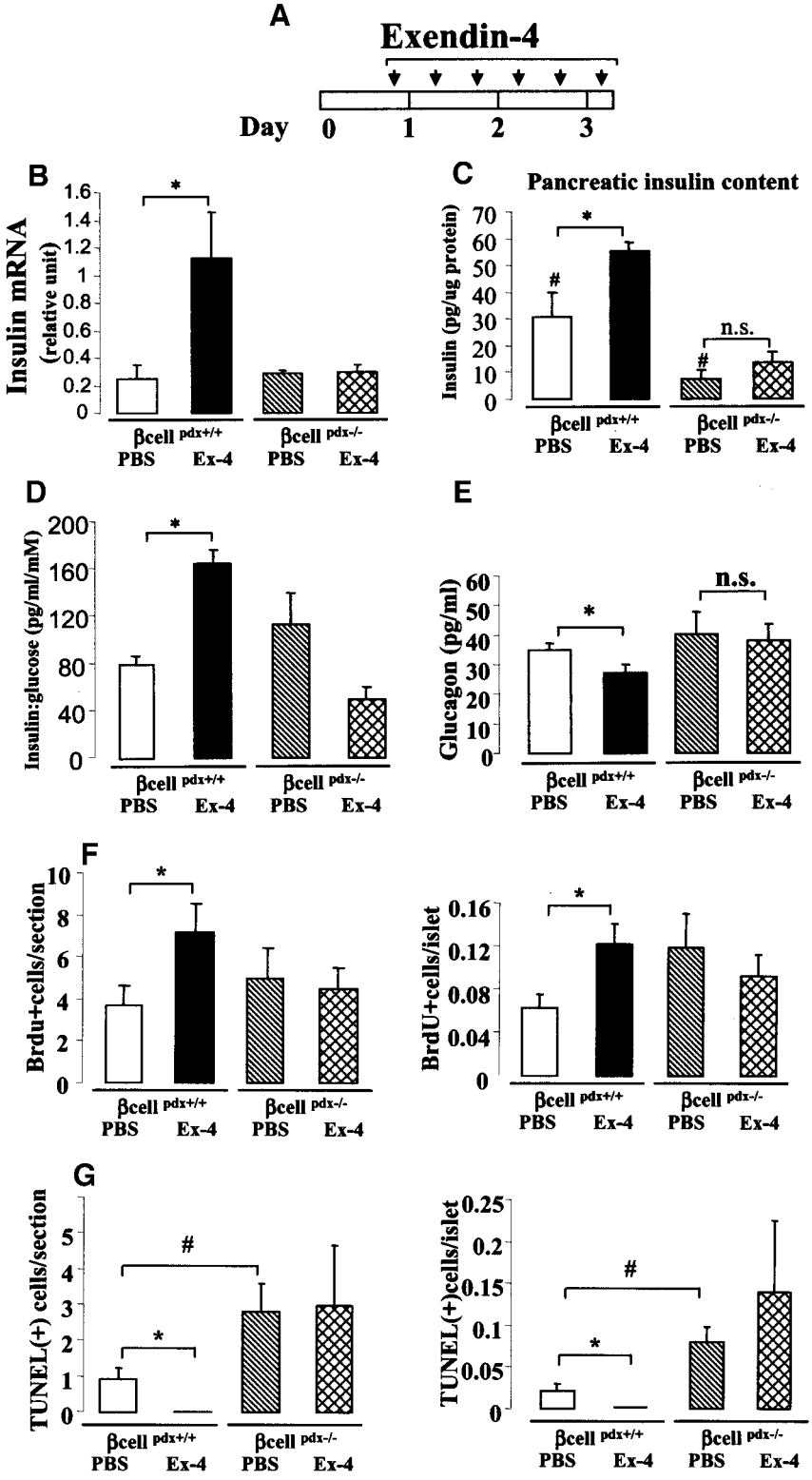

FIG. 3. Importance of Pdx1 for Ex-4 action in the murine pancreas. *A*: Diagram shows the experimental protocol (twice daily Ex-4 or saline [PBS] administration for 3 days); male mice (n = 5 per group) were killed at the end of day 3. *B*: Quantitative representation of data from Northern blot analysis of insulin mRNA transcripts in mouse pancreas. Data are expressed as relative units for levels of insulin mRNA normalized to the level of 18S RNA transcripts from five mice. *C*: Pancreatic insulin content after 3 days of twice-daily saline (PBS) or Ex-4 treatment. *D*: Insulin-to-glucose ratios determined from analysis of glucose and insulin in plasma from fed mice at the end of the 3-day experiment. *E*: Plasma glucagon levels were determined in mice treated with PBS or Ex-4 from plasma samples obtained at the end of the 3-day experiment. *F*: Analysis of β-cell proliferation in mice treated with saline (PBS) or Ex-4. BrdU (100 mg/kg body wt) was given to mice 4 h before the end of the experiments by intrperitoneal injection. β-cell proliferation was evaluated by BrdU staining and the number of BrdU+ β-cells were quantitated per section or per islet. Magnification ×400. *G*: β-cell apoptosis was evaluated in β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ mice treated with saline (PBS) or Ex-4 by determining the number of TUNEL-positive β-cells per section or per islet. For *B–G*, *$P < 0.05$ for PBS vs. Ex-4 β-cell$^{Pdx1+/+}$ mice, and #$P < 0.05$ for PBS-treated β-cell^{Pdx1} vs. β-cell$^{Pdx1-/-}$mice.

TABLE 1
Gene expression profile in islet RNA from β-cell$^{Pdx1-/-}$ relative to β-cell$^{Pdx1+/+}$ islets

	% of wild type	SEM (%)	*P*
Pdx1	27.2	6.7	0.014
Insulin-1	32.1	0.7	0.00000009
Insulin-2	24.6	1.7	0.0000657
IAPP	23.3	1.6	0.0003
GLUT2	18.1	1.6	0.005
Neuronatin	26.6	5.0	0.004
IGRP	33.9	4.5	0.006
Glucagon	40.3	0.6	0.00002
Nkx6.1	42.1	5.0	0.004
Kir6.2	40.4	2.1	0.024
SUR1	46.9	5.8	0.002
GLP-1R	50.9	1.7	0.0002
Pax6	70.0	3.2	0.016
Akt1	64.3	7.7	0.037
GK	91.2	10.2	0.74
IRS2	98.9	8.0	0.138
Nkx2.2	74.0	7.5	0.199
Isl1	84.3	12.6	0.243
CREB	92.6	8.7	0.116
Foxa2	141.1	9.7	0.045

Data show the gene expression profile in β-cell$^{Pdx1-/-}$ islets (pool of 15 islets) incubated in RPMI-1640 media with 11 mmol/l glucose for 24 h (n = 6 for β-cell$^{Pdx1+/+}$ islets and n = 4 for β-cell$^{Pdx1-/-}$ islets). The relative levels of mRNA transcripts were normalized to the levels of β-actin mRNA transcripts in the same experiments. Results for β-cell$^{Pdx1-/-}$ islets are presented as percentage of gene expression levels in β-cell$^{Pdx1+/+}$ islets (WT); $P < 0.05$ is considered a significant difference using an unpaired t test.

insulin secretion in β-cell$^{Pdx1-/-}$ and β-cell$^{Pdx1+/+}$ islets in the presence or absence of Ex-4. A shift from 2 to 11 mmol/l glucose was associated with a significant stimulation of insulin secretion in β-cell$^{Pdx1+/+}$ islets, and coincubation with Ex-4 further augmented insulin secretion from β-cell$^{Pdx1+/+}$ islets (Fig. 5*A*). In contrast, a similar shift from 2 to 11 mmol/l glucose did not significantly stimulate insulin secretion from β-cell$^{Pdx1-/-}$ islets, and Ex-4 had no effect on insulin secretion in β-cell$^{Pdx1-/-}$ islets (Fig. 5*A*). Despite the defective insulin secretory response to glucose and Ex-4 in β-cell$^{Pdx1-/-}$ islets, both KCl and the sulfonylurea tolbutamide robustly stimulated insulin secretion from β-cell$^{Pdx1-/-}$ islets (Fig. 5*B*). Furthermore, the phosphodiesterase inhibitor IBMX also significantly enhanced insulin secretion from both β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ islets (Fig. 5*C*). In contrast, forskolin stimulated insulin secretion from β-cell$^{Pdx1+/+}$ but not β-cell$^{Pdx1-/-}$ islets (Fig. 5*C*). Finally, consistent with the reduction in levels of insulin mRNA transcripts detected in RNA from β-cell$^{Pdx1-/-}$ islets, islet insulin content was significantly reduced in β-cell$^{Pdx1-/-}$ islets (Fig. 5*D*). These findings indicate that β-cell$^{Pdx1-/-}$ islets retain the capacity for regulated insulin secretion yet exhibit selective secretory defects, including loss of the stimulatory response to the GLP-1R agonist Ex-4.

DISCUSSION

Our data demonstrate that progressive loss of β-cell Pdx1 expression does not impair the ability of GLP-1R agonists such as Ex-4 to reduce glycemic excursions after oral glucose loading. Nevertheless, the response to intraperitoneal glucose was abnormal and Ex-4 failed to reduce glycemic excursions or stimulate insulin secretion in β-cell$^{Pdx1-/-}$ mice under these conditions. Hence, during oral nutrient loading, the control of gastric emptying and/or glucagon secretion represent important initial mechanisms for postprandial glucoregulation by GLP-1R agonists, but a functional β-cell remains essential for GLP-1R actions if hyperglycemia persists, such as observed during the IPGTT. These findings suggest a temporal hierarchy of actions of GLP-1 on peripheral tissues and within the islet after food ingestion and nutrient assimilation.

An unexpected observation in the present study was the complete abrogation of GLP-1R–dependent inhibition of glucagon secretion in β-cell$^{Pdx1-/-}$ mice. Current concepts of GLP-1R–dependent inhibition of islet α-cell secretory activity involve both GLP-1R–dependent stimulation of the β- or δ-cell (26,27) liberating indirect mediators, such as insulin or somatostatin, as well as the direct inhibitory action of GLP-1 itself directly on the α-cell (28). The finding that Ex-4 failed to decrease levels of plasma glucagon in β-cell$^{Pdx1-/-}$ mice, taken together with the defect observed in GLP-1R–dependent insulin secretion, strongly implicates a fully functional β-cell as a critical

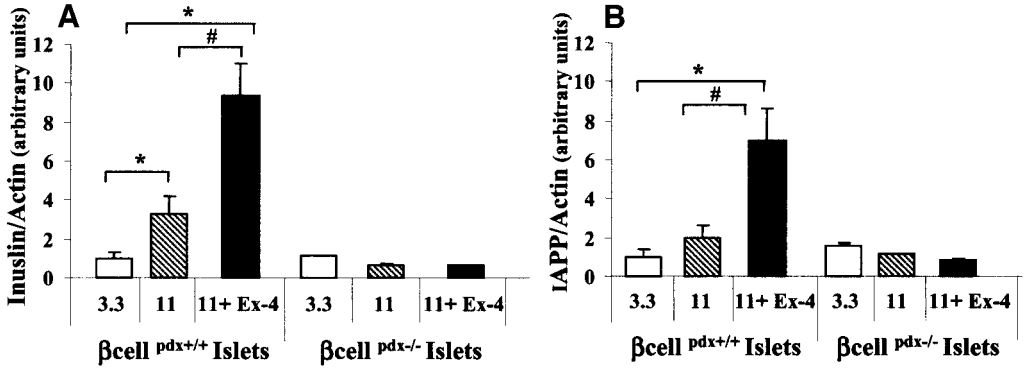

FIG. 4. Real-time PCR analysis of gene expression in isolated murine islets. *A* and *B*: The effects of glucose and Ex-4 on insulin and IAPP gene expression in β-cell$^{Pdx1-/-}$ islets (n = 6 for β-cell$^{Pdx1+/+}$ islets; n = 4 for β-cell$^{Pdx1-/-}$ islets). Islets were incubated in RPMI-1640 media with 3.3 mmol/l glucose, 11 mmol/l glucose, or 11 mmol/l glucose + 10 nmol/l Ex-4 for 24 h. * $P < 0.05$ for different treatments at 3.3 vs. 11 mmol/l glucose; # $P < 0.05$ for different treatments at 11 mmol/l glucose. The relative levels of gene expression were normalized to the levels of β-actin mRNA transcripts in the same experiments.

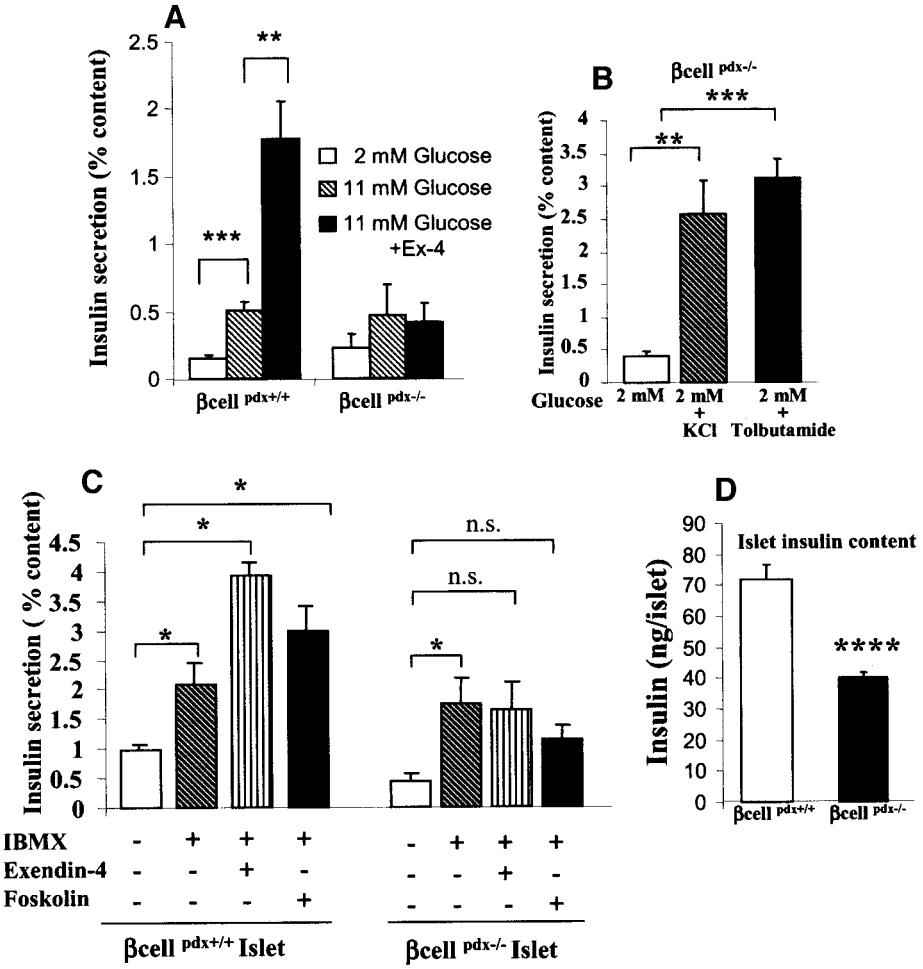

FIG. 5. Insulin secretion and insulin content in isolated murine islets. *A*: Insulin secretion from both β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ islets incubated in KRB buffer containing 2 mmol/l glucose, 11 mmol/l glucose, or 11 mmol/l glucose plus 10 nmol/l Ex-4 for 30 min as indicated; $n = 5$ and 6 for β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ islets, respectively. $^{**}P < 0.02$ and $^{***}P < 0.001$. *B*: Insulin secretion from β-cell$^{Pdx1-/-}$ islets incubated in KRB buffer containing 2 mmol/l glucose, 2 mmol/l glucose plus 30 mmol/l KCl, or 2 mmol/l glucose plus 100 μmol/l tolbutamide for 1 h as indicated; $n = 4$ for each group of islets, and insulin secretion is normalized as a percentage of islet insulin content. $^{**}P < 0.02$ and $^{***}P < 0.002$. *C*: Insulin secretion from both β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ islets incubated in KRB buffer containing 11 mmol/l glucose alone, 11 mmol/l glucose plus 250 μmol/l IBMX with or without 10 nmol/l Ex-4, or 10 μmol/l foskolin for 30 min as indicated; $n = 5$ for each group of islets, and insulin secretion is normalized as a percentage of islet insulin content. $^{*}P < 0.05$. *D*: Islet insulin content in β-cell$^{Pdx1-/-}$ islets versus β-cell$^{Pdx1+/+}$ islets; $n = 24$ and 33 for β-cell$^{Pdx1+/+}$ and β-cell$^{Pdx1-/-}$ islets, respectively. $^{****}P < 0.0003$.

determinant necessary for GLP-1–mediated inhibition of α-cell secretory activity. Although insulin remains a prime candidate for the β-cell–derived product inhibiting glucagon secretion, the complex relationship between islet β-cells and α-cells leaves open the possibility that other β-cell factors, such as GABA or zinc, may also contribute to inhibition of glucagon secretion (29).

Our experiments examining the importance of Pdx1 for the β-cell actions of GLP-1R agonists were prompted by studies demonstrating activation of *Pdx1* gene expression following administration of GLP-1R agonists. GLP-1R agonists increase *Pdx1* gene expression and levels of nuclear Pdx1 protein (30), enhance binding of Pdx1 to the insulin gene promoter (31), and stimulate differentiation of exocrine cell lines toward a β-cell–like phenotype in a Pdx1-dependent manner (24). Although the precise signals linking GLP-1 receptor signaling to *Pdx1* activation remain unknown, Ex-4 increases the binding of the transcription factor Foxa2 to specific elements in the *Pdx1* gene promoter (24). Our data demonstrate that Pdx1 is essential for an appropriate β-cell response linking GLP-1R activation to stimulation of insulin gene expression, as well as insulin biosynthesis and secretion.

Pdx1 expression in β-cells appears essential for normal cell proliferation as revealed in studies of transgenic mice expressing a mutant Pdx1 protein (32), and mice with loss of a single Pdx1 allele (Pdx1+/−) exhibit a relative reduction of β-cell mass and islet number with increasing age (6). Pdx1+/− mice continue to maintain normal levels of glucose-stimulated insulin secretion but exhibit increased caspase activation and β-cell apoptosis in islets cultured in normal glucose concentrations (6). GLP-1R knockout islets exhibit a similar phenotype, demonstrating normal glucose competence (33) despite a reduction in islet number (34) and increased susceptibility to β-cell apoptosis after streptozotocin administration (19). Although GLP-1R agonists activate pathways coupled to both stimulation of cell proliferation and inhibition of apoptosis (20), the downstream mediators of these GLP-1R–dependent responses remain poorly understood. Recent studies using islet cell lines have demonstrated that the kinase Akt is essential for the proliferative and cell

survival effects of GLP-1 (35), consistent with our finding that Akt1 gene expression was reduced in β-cell$^{Pdx1-/-}$ mice. Our studies therefore identify Pdx1 as an essential mediator for the β-cell proliferative and cytoprotective actions of GLP-1R agonists.

Studies of Pdx1+/− mice demonstrated either relatively preserved (6) or modestly reduced glucose-stimulated insulin secretion (12) and a paradoxically increased insulin secretory response to 10 nmol/l GLP-1 (12). In contrast, glucose-stimulated insulin secretion was clearly abnormal in β-cell$^{Pdx1-/-}$ mice, and isolated islets from β-cell$^{Pdx1-/-}$ mice exhibited defects in both glucose-stimulated insulin secretion and islet insulin content. Furthermore, in contrast to the enhanced secretory response to GLP-1 in Pdx1+/− mice, Ex-4 or forskolin failed to stimulate insulin secretion in β-cell$^{Pdx1-/-}$ mice. Nevertheless, β-cell$^{Pdx1-/-}$ islets retain responsiveness to IBMX, tolbutamide, and KCl, indicating that the β-cell$^{Pdx1-/-}$ islets exhibit highly selective rather than generalized defects in insulin secretion.

Extinction of β-cell Pdx1 expression was associated with reduced expression of several mRNA transcripts encoding proteins important for differentiated islet function, including transcription factors, secreted hormones, ion channels, and signaling molecules (Table 1). Furthermore, the GLP-1R–dependent induction of insulin and amylin gene expression was significantly reduced in islets from β-cell$^{Pdx1-/-}$ mice. Intriguingly, the levels of mRNA transcripts for Kir6.2 and sulfonylurea receptor (SUR) were reduced in β-cell$^{Pdx1-/-}$ islets, and SUR knockout mice exhibit a defective insulin secretory response to GLP-1 administration (36). In contrast, basal levels of mRNA transcripts for key proteins required for differentiated islet function, such as glucokinase, IRS2, CREB, and the transcription factors Nkx2.2, Foxa2, and Isl1, were normal in β-cell$^{Pdx1-/-}$ islets. The reduced expression of selective RNA transcripts encoding proteins important for differentiated β-cell function likely contributes to the acquired defects in glucose- and Ex-4–stimulated insulin secretion observed in β-cell$^{Pdx1-/-}$ islets.

Studies of rat islets following adenoviral transduction with a dominant-negative Pdx1 gene demonstrated reduced glucose-stimulated insulin secretion, decreased islet ATP content, and reduced expression of a number of mitochondrial genes important for ATP generation (37). Similarly, Pdx1+/− islets exhibit reduced NAD(P)H generation in response to glucose stimulation (12). These observations, taken together with experiments demonstrating that GLP-1 stimulates mitochondrial ATP generation in MIN6 islet cells (38), implicate an emerging role for mitochondrial energy homeostasis as an essential component of the downstream pathways linking GLP-1 receptor activation to enhanced β-cell function.

In summary, the data shown here demonstrate that GLP-1R activation produces multiple pleiotropic effects in the β-cell that require functional Pdx1-dependent pathway(s). Furthermore, the inhibitory action of GLP-1R agonists on the islet α-cell also requires intact β-cell Pdx1 expression. As GLP-1 exerts many effects on the islet β-cell via generation of cyclic AMP (7,39), it is possible that GLP-1R agonists activate Pdx1 in part through a cyclic AMP-dependent pathway that may be partly dysfunctional in β-cell$^{Pdx1-/-}$ islets. Given the importance of Pdx1 for the glucoregulatory actions of GLP-1, our data predict that diabetes characterized by defective Pdx1 action, as exemplified by human subjects with maturity-onset diabetes of the young (MODY4), may exhibit subnormal responses to treatment with GLP-1R agonists.

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