Exendin-4 Uses Irs2 Signaling to Mediate Pancreatic β Cell Growth and Function*

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The insulin receptor substrate 2 (Irs2) branch of the insulin/insulin-like growth factor-signaling cascade prevents diabetes in mice because it promotes β cell replication, function, and survival, especially during metabolic stress. Because exendin-4 (Ex4), a long-acting glucagon-like peptide 1 receptor agonist, has similar effects upon β cells in rodents and humans, we investigated whether Irs2 signaling was required for Ex4 action in isolated β cells and in Irs2−/− mice. Ex4 increased cAMP levels in human islets and MIN6 cells, which promoted Irs2 expression and stimulated Akt phosphorylation. In wild type mice Ex4 administered continuously for 28 days increased β cell mass 2-fold. By contrast, Ex4 failed to arrest the progressive β cell loss in Irs2−/− mice, which culminated in fatal diabetes; however, Ex4 delayed the progression of diabetes by 3 weeks by promoting insulin secretion from the remaining islets. We conclude that some short term therapeutic effects of glucagon-like peptide 1 receptor agonists can be independent of Irs2, but its long term effects upon β cell growth and survival are mediated by the Irs2 branch of the insulin/insulin-like growth factor signaling cascade.

Diabetes mellitus is a complex disorder that arises from various causes, including dysregulated glucose sensing and impaired insulin secretion (maturity-onset diabetes of youth, MODY), autoimmune-mediated β cell destruction (type 1), or insufficient compensation for peripheral insulin resistance (type 2) (1). Type 2 diabetes is the most prevalent form. It usually occurs at middle age and afflicts more than 30 million people over the age of 65 but is appearing with greater frequency in children and adolescents (2). Dysregulated insulin signaling exacerbated by chronic hyperglycemia promotes a cohort of systemic disorders, including dyslipidemia, hypertension, cardiovascular disease, and female infertility (3, 4). The search for strategies to promote β cell function and regeneration has lead to the discovery that glucagon-like peptide-1 (GLP1) receptor agonists increase insulin biosynthesis and secretion from β cells, inhibit glucagon secretion from α-cells, and promote peripheral insulin sensitivity and satiety in type 2 diabetics (5–9). During a meal, GLP1 is secreted into the circulation from L cells located in the intestine (10); however, GLP1 is quickly inactivated by circulating dipeptidyl-peptidase IV, which diminishing its usefulness as an injectable therapeutic. Compounds that inhibit dipeptidyl-peptidase IV or GLP1 homologs like exendin-4 (Ex4) that are not degraded by dipeptidyl-peptidase IV display improved therapeutic efficacy (11–16). Administration of Ex4 to rodents or humans with type 2 diabetes increases first-phase insulin secretion and increases β cell mass, which can compensate for peripheral insulin resistance (8, 9, 17, 18). Recently, a synthetic Ex4 called Exenatide (Byetta, Amylin/Lilly) has gained Food and Drug Administration approval as an injectable treatment for type 2 diabetes (15). Because Exenatide is the first in a new class of drugs for the treatment of type 2 diabetes, it is important to understand its molecular mechanism of action.

The GLP1 receptor (GLP1R) is coupled to adenylyl cyclase through Gs, which increases cAMP levels that activate protein kinase A (10). Protein kinase A has immediate effects upon the KATP and Ca2+ channel activity to mobilize secretory vesicles into readily releasable pools required for rapid insulin secretion (8, 19, 20). Moreover, protein kinase A activates the transcription factor cAMP response element-binding protein (CREB) in β cells, which stimulates the expression of various genes that play a direct role in glucose sensing (GLUT2 and glucokinase) and insulin secretion (PDX1 and insulin itself) (21, 22). However, the long term effects of stable GLP1R agonists upon β cell growth, function, and survival are more difficult to understand. Recent reports suggest that exendin-4 activates various protein kinases that promote cell growth and survival, including Akt, Iak, protein kinase C, C5r, or the epidermal growth factor receptor (7, 21, 23). Studies on the role of the insulin and insulin-like growth factor receptors in β cells reveal an essential role for the phosphatidylinositol (PI) 3-kinase → Akt cascade to promote nuclear exclusion of Foxo1 (24); however, the mechanism coupling GLP1R to the Akt → Foxo1 cascade is unclear (7).

Irs2 is a substrate of the insulin and IGF1 receptor tyrosine kinases that is essential for compensatory β cell growth, function, and survival throughout life (25). Irs2 expression is strongly induced in β cells by Ex4 and other cAMP agonists through the activation of CREB (26, 27). Irs2 binds to the SH2 domains of common signaling effectors, including enzymes (phosphoinositide 3-kinase, the phosphatase SHP2, or the tyrosine kinase Fyn) or adapters (GRB2, NCK, CRK, SHB, and others). The production of PI-3,4,5-trisphosphate by PI 3-kinase recruits the Ser/Thr kinases Pdk1 and Akt to the plasma membrane where Akt is activated by Pdk1-mediated phosphorylation (28). Akt phosphorylates many proteins that play important physiological roles (28): glycogen synthase kinase 3β, the BAD-BCL2 heterodimer (apoptosis inhibition), TSC1-TSC2 (protein synthesis and nutrient sensing), and FOXO (transcriptional regulation). Moreover, Irs2 signaling can play a unique role in this cascade because of its ability to coordinate the degradation of...
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Foxy1. Consequently, the IRS2 branch of the insulin/insulin-like growth factor signaling pathway strongly promotes β cell growth and survival, which is essential for compensatory β cell function during physiological or metabolic stress (29–31).

In this report we show that the long term effects of exendin-4 upon β cell survival and replication do not develop in Irs2−/− mice. We conclude that these long term effects are mediated at least in part through increased expression of Irs2.

MATERIALS AND METHODS

Antibodies—Primary antibodies used were as follows: rabbit anti-glucagon and guinea pig anti-insulin (Zymed Laboratories Inc., San Francisco, CA); rabbit polyclonal antibodies against Akt and phosphorylated Akt (pAktSer-473), Erk1 and Erk2 and phosphorylated Erk (pErk) was purchased from Cell Signaling Technology, Inc., Beverly, MA; rabbit anti-Irs2 and rabbit pan anti-p85 (Upstate USA, Chicago, IL); mouse anti-BrdUrd (Roche Applied Science); goat anti-PDX-1 (Santa Cruz Biotechnology, Santa Cruz, CA). The rabbit anti-Irs-1 was prepared in our laboratory against a glutathione S-transferase fusion protein of rat Irs-1 amino acids 735–900 as previously described (32). Secondary antibodies were used as follows: rhodamine-conjugated donkey anti-rabbit IgG and fluorescein isothiocyanate-conjugated goat anti-guinea pig IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA); horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology).

Mice—Wild type (WT) and Irs2−/− mice on a C57BL/6 background were genotyped by PCR as previously described (31). Mice were fed ad libitum with a standard diet (Purina diet 5058, with 9% fat) and kept under a 12-h light/dark cycle. Blood samples were collected via tail bleeds, and circulating glucose levels were determined using a glucometer (Glucometer Elite from Bayer, Pittsburgh, PA). Serum insulin levels were determined from tail bleed samples using an insulin enzyme-linked immunosorbent assay kit (Crystal Chemical Inc., Downers Grove, IL). Random-fed glucose and insulin measurements were performed in the morning as previously described (31). Intraportal injections of tests (IPGTT) were conducted with male Irs2−/− mice at 6–7 weeks of age and male WT mice at 8–10 weeks of age. For the IPGTT, all mice were fasted overnight for 15–16 h before an intraperitoneal injection of 2 g of glucose/kg of body weight as previously described (33). For insulin tolerance tests (ITT), male WT mice 8–10 weeks of age and male Irs2−/− mice at 6–10 weeks of age were used. All mice were fasted for 6 h before an intraperitoneal injection of 1 unit of human insulin per kg of body weight as previously described (27). For both the IPGTT and ITT, glucose was measured in tail blood samples at time intervals indicated in the figure legends.

Drug Treatment—All Ex4 (Sigma) treatments were initiated in mice at 4 weeks of age when WT and Irs2−/− animals displayed equivalent normal glucose homeostasis and sustained functional pancreatic β cell mass. Litter-matched WT and Irs2−/− mice were randomly divided into control (saline-treated) or experimental (Ex4-treated) groups. Ex4 was administered with an osmotic pump (300 pmol/kg of body weight/day) until the mice died or the experiment was terminated when the animals were 12 weeks of age. Osmotic pumps were changed as required to maintain the desired drug dosage. Some experiments were conducted with Ex4 administration via intraperitoneal injection (150 pmol/kg of body weight) every 12 h to confirm the results generated using the osmotic pump.

Immunohistochemistry of the Pancreas and Quantitation of β Cell Mass—The pancreas samples were prepared and analyzed as previously described (27). The pancreas was dissected and fixed in Bouin’s solution overnight. Serial 5-μm paraffin-embedded tissue sections were mounted on slides. After rehydration, sections were co-immunostained overnight at 4°C for the presence of glucagon-positive α-cells (rabbit anti-glucagon, 1:100 in phosphate-buffered saline and 1% Triton-X (PBST)) and insulin-positive β cells (guinea pig anti-insulin, 1:100 in PBST). Detection was performed using rhodamine- and fluorescein isothiocyanate-labeled secondary antibodies (1:100 in PBST) for 1 h at room temperature. The β cell area was measured by acquiring images from two sets of 8–10 distal, random non-overlapping images (10× magnification) of insulin- and glucagon-stained pancreatic sections. Seven male mice were analyzed from each genotype and treatment group. Results of the β cell quantification are expressed as the percent-age of the total surveyed area containing insulin-positive cells. Ratios of β to α cells were calculated from the mean total insulin-positive (β cell) area divided by the mean total glucagon-positive (α cell) area.

Islet proliferation and β cell size were examined by intraperitoneally injecting male WT and Irs2−/− mice 5–6 weeks of age with 5-bromo-2-deoxyuridine (BrdUrd; 100 μg/g of body weight in saline; Roche Applied Science) and then co-immunostaining for insulin and BrdUrd. Six hours post-BrdUrd injection, the pancreas perfused with 4% parafomaldehyde (pH 7.2) solution in saline. The pancreas was removed, fixed in 4% paraformaldehyde (pH 7.2) saline solution overnight at room temperature, and embedded in paraffin blocks, and 5-μm longitudinal sections were subsequently immunostained to determine the level of BrdUrd incorporation as previously described (33). BrdUrd-positive β cell ratios were calculated as BrdUrd-positive β cells over total β cell nuclei per section, two sections per animal, seven animals per genotype.

Hyperglycemic Clamp—Mice were anesthetized with intraperitoneal injections of ketamine and xylazine (100 mg- and 10 mg/kg of body weight). A cannula was surgically implanted into the left jugular vein, externalized in the interscapulums, and sealed as previously described (34). After a recovery period of 5–6 days, the hyperglycemic clamp procedure was performed with conscious and overnight fasted mice as previously described (34). The fasted blood glucose level was determined as described above before the beginning of the clamp procedure. Bolus glucose (375 mg of glucose/kg of body weight) was infused through the cannula for 5 min at the beginning of the clamp procedure, and 20% glucose was administered through the cannula to maintain blood glucose levels at 5.5 mM above the determined fasted blood glucose level. Glucose and insulin levels were determined in blood samples collected via tail bleeds at 0-, 5-, 10-, 40-, 50-, and 60-min post-glucose bolus glucose infusion.

Pancreas Perfusion—Mice treated with saline or Ex4 for at least 4–6 weeks were fasted for 15 h and anesthetized with 35 mg/kg of body weight sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL) administered via intraperitoneal injection. The abdominal aorta and portal vein were cannulated with PE-10 and PE-50 tubing, respectively, for perfusion and sample collection. The perfusate was composed of Krebs-Ringer bicarbonate buffer containing 3% dextran (Amersham Biosciences), 1% free fatty acid-free bovine serum albumin (Sigma) and equilibrated to a pH of 7.4 with 9.5% O2, 5% CO2. Five mM glucose in perfusate buffer was infused for 2 min to stabilize the pancreas in the condition of in situ perfusion. The pancreas was sequentially perfused at 1 ml/min with buffer containing 5 mM glucose (10 min), 16.7 mM glucose (20 min), 5 mM glucose (15 min), 30 mM glucose (20 min), 5 mM glucose (15 min), and 30 mM glucose with 20 mM arginine (20 min).

S. Guo and M. F. White, unpublished results.
The perfusion eluate was collected every minute, and the insulin content in each fraction was measured using a radioimmunoassay technique performed by the Joslin Diabetes Center Assay Core. At the end of the experiment the pancreas was excised and homogenized, and the insulin was extracted overnight in acid-ethanol at −20 °C. The following day the samples were centrifuged at 10,000 rpm for 10 min at 4 °C, and supernatant was stored at −20 °C. The pellet was sonicated, and insulin was extracted a second time overnight in acid-ethanol at −20 °C. After centrifugation the following day, the extracts were pooled, and the insulin concentration was measured using a radioimmunoassay performed by the Joslin Diabetes Center Assay Core.

**Pancreatic Islet Isolation**—Pancreatic islets were isolated by collagenase digestion of whole pancreas excised from male WT and Irs2−/− mice 5–6 weeks of age as previously described (27). Digested pancreatic tissue from 6 mice per genotype was pooled for analysis. Human islet isolations were performed in the Islet Core Laboratory of the Juvenile Diabetes Foundation Center for Islet Transplantation at Harvard Medical School using the method of Ricordi and co-workers (35). The human islets were incubated for 48 h in culture medium to allow recovery and to eliminate acinar cells. Following the experimental manipulations indicated in the figure legends, islets were harvested and lysed for analysis in ice-cold radioimmunoprecipitation assay lysis buffer composed of PBS, 20 mM Tris (pH 7.4), 2 mM EDTA, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 12 mM α-glycerol phosphate, and 10 μg/ml aprotinin. After 30 min on ice, lysates were centrifuged at 12,000 rpm for 10 min at 4 °C. The protein content was determined using the dye-binding Bradford-based assay (Bio-Rad).

**Immunoprecipitation and Immunoblotting**—Cell or tissue lysates with equal amounts of protein were directly resolved by SDS-PAGE or immunoprecipitated with specific antibodies as indicated before SDS-PAGE. Samples were then transferred to nitrocellulose for immunoblotting. The indicated proteins were detected by immunoblotting with specific antibodies against Irs1 or Irs2, Akt or pAktSer-473, Erk1, Erk2 or pErk, or Pdx-1, as previously described (27). In several cases the primary phosphospecific antibody was washed away for 1 h in 60 mM Tris-base (pH 9.0) containing 1% SDS and 8 ml/liter β-mercaptoethanol followed by a 1-h wash in PBS containing 0.1% Tween 20. The stripped gel was immunoblotted with a nonphospho-specific antibody for normalization.

**Quantitative Reverse Transcription-PCR**—Total RNA was isolated from saline- or Ex4-treated human islets with Trizol (Invitrogen). The cDNA synthesis was performed with the RETROscript kit (Ambion Inc., Austin, Texas) using random decamers. The quantitative reverse transcription-PCR was carried out using the QuantiTect SYBR green PCR kit (Qiagen Inc., Valencia, California) on an iCycler PCR instrument (Bio-Rad). The PCR reactions were performed following the kit instructions. The following primers were used to detect human IRS2, cyclophilin, PDX-1 glucokinase, and GLUT2:

- IRS2 forward, 5′-AGAGTGGACCGGTACCTATGGA-3′;
- IRS2 reverse, 5′-CTTACCTCTCTGGACCTGTGTC-3′;
- cyclophilin forward, 5′-GGCTCCTCCCTTGGACGTTGGA-3′;
- cyclophilin reverse, 5′-CACCCCTGACCATATAACCCTGGAGA-3′;
- GLUT2 forward, 5′-CTCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
tyrosine phosphorylation was unchanged (Fig. 1, b and c). Akt phosphorylation (pAKTSer-473) increased about 2-fold, whereas its expression was unchanged. These results suggest that Ex4 promotes the IRS2 → AKT cascade in human islets (Fig. 1, b and c).

GLP1R agonists can stimulate the phosphorylation of Erk1 and Erk2 in /H9252 cells through an insulin-insensitive mechanism that depends upon /Ca2+ regulation of MEK1 (36). To confirm this effect of Ex4, we incubated human islets with Ex4 or Bt2cAMP for 30 min and measured the phosphorylation of ERK1 or ERK2 with phosphospecific antibodies. Although the expression of ERK1 or ERK2 was not changed during this experiment, the phosphorylation of both isoforms increased about 2-fold by a low or high concentration of Ex4 and by 1 mM Bt2cAMP (Fig. 1d). Thus, before IRS2 expression increases (data not shown), GLP1R agonists stimulate ERK1 and ERK2 phosphorylation in human islets.

**FIGURE 1.** Expression of IRS2 in human islets. a, the mRNA expression levels of IRS2, PDX-1, glucokinase, and GLUT2 in human islets incubated for 4 or 8 h with control vehicle (Me2SO), 2.5 nM Ex4, or 1 mM Bt2cAMP. The levels of mRNA for IRS2, PDX-1, glucokinase (GK), and GLUT2 were determined by real-time reverse transcription-PCR. The relative quantification analysis was performed by calculating the ratio of the PCR product level of the gene of interest divided by the amount of internal control cyclophilin. The relative quantified values of each gene for 2.5 nM Ex4 and 1 mM Bt2cAMP treatment were then divided by values obtained with Me2SO control treatment. The values represent the fold increase due to Ex4 and Bt2cAMP treatment over Me2SO control data. The data are expressed as the means ± S.E. calculated from three separate sets of human islets. b, expression and phosphorylation (p) of IRS1, IRS2, and AKT in human islets. After 48 h of incubation to allow recovery and to eliminate acinar cells, washed human islets (2000 –3000 islet equivalents) were incubated with Me2SO, 0.5 or 2.5 nM Ex4 in serum-free media for 8 h. At the end of the incubation the islets were exposed to 10 nM insulin-like growth factor 1 for 10 min. Islet lysates were prepared as described under “Materials and Methods.” Tissue lysates with equivalent amounts of protein were immunoprecipitated with specific antibodies against IRS1, IRS2, or AKT, resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phosphotyrosine (PY20) antibody (IRS1 and IRS2) or phospho-specific antibody directed toward phospho-pAktSer-473. The blots were stripped and reblotted with antibodies against IRS1, IRS2, or AKT as indicated. Blots shown are representative of three sets of experiments. The adjacent graph (right of panel b) was generated by quantifying the autoradiograph in ImageQuant TM using a 16-bit TIFF image produced with an Epson 4990 scanner. The -fold change results shown are representative of three separate experiments. c, expression and phosphorylation of Erk1 and Erk2 in human islets stimulated for 30 min with Ex4 or Bt2cAMP (1 mM). Islet lysates were prepared as described under “Materials and Methods” and for panel b. Phosphorylated Erk1 or Erk2 (pErk1 and pErk2) and total Erk1 or Erk2 was quantified by scanning the autoradiograph obtained from two separate experiments. The ratios pErk1/Erk1 or pErk2/Erk2 ± average deviation calculated from two separate experiments are plotted in the figure. Cntr, control.

Irs2 Mediates Ex4-stimulated Akt Phosphorylation and Pdx1 Expression—Several studies suggest that the PI 3-kinase → Akt cascade mediates the trophic effects of GLP1R agonists that promote compensatory β cell function (7, 37, 38). We used Min6 cells to determine whether Irs2 mediates the effects of Ex4 upon Akt phosphorylation in a β cell line. Ex4 (2.5 nM) increased 4-fold the concentration of cAMP in Min6 cells, which verified that they responded to GLP1R agonists (Fig. 2a). To control the expression of Irs2, the Min6 cells were transfected with siRNA against Irs2 (siRNAIrs2) or a scrambled siRNA (siRNAscram) as the control (Fig. 2b). Ex4 doubled the expression of Irs2 in Min6 cells transfected with siRNAIrs2 without increasing the expression of Irs1 or Akt (Fig. 2, b and d). By contrast, the siRNAIrs2 reduced the expression of Irs2 by 2-fold and blocked the effect of Ex4 to increase Irs2 (Fig. 2, b and c); however, it had no effect upon Irs1 or Akt expression (Fig. 2, b and d).
Ex4 increased Akt phosphorylation (pAktSer-473) in the siRNA scram Min6 cells, consistent with increased signaling from Irs2 (Fig. 2, b and e). However, Ex4 failed to stimulate Akt phosphorylation in Min6 cells transfected with siRNA Irs2 (Fig. 2, b and e). These results support the hypothesis that increased expression of Irs2 mediates the effect of Ex4 upon Akt phosphorylation.

Next, we measured Pdx1 expression in Min6 cells transfected with siRNA scram or siRNA Irs2 (7, 39). Pdx1 is a homeobox-containing transcription factor required for endocrine pancreatic development and β cell function in adults (40, 41). Its expression can be increased in adult murine islets by activation of the Akt→Foxo1 signaling pathway by Irs2 (24, 27). Transgenic expression of Pdx1 promotes β cell function and prevents diabetes in Irs2−/− mice, suggesting that Pdx1 mediates some effects also controlled by the Irs2→Akt cascade (33). Consistent with this hypothesis, Ex4 doubled the expression of Pdx1 in siRNA scram Min6 cells but failed to increase Pdx1 in Min6 cells transfected with siRNA Irs2 (Fig. 2f). These results suggest that increased expression of Pdx1 by Ex4 requires Irs2 signaling (7, 39).

Tyrosine-phosphorylated Irs proteins can bind Grb2, which activates the mSOS→Raf→Mek1→Erk1/2 cascade (42). To determine whether Irs2 plays a role in Ex4-stimulated Erk1/2 phosphorylation, we transfected Min6 cells with siRNA scram or siRNA Irs2 24 h before stimulation with Bt2cAMP (1 mM) or Ex4 (2.5 nM). Erk1 and Erk2 phosphorylation was activated by each agonist whether or not Irs2 expression was inhibited by siRNA Irs2 (Fig. 2c). Thus, Irs2 was not involved in the rapid effects of Ex4→GLP1R signaling to activate Erk1/2 in the Min6 β cell line.

Ex4 Delays the Progression of Diabetes in Irs2−/− Mice—Rodent and human studies suggest that GLP1R agonists improve glucose homeostasis by enhancing glucose-stimulated insulin secretion and promoting compensatory expansion of β cell mass (9, 38). To establish whether Irs2 signaling was required for the long term therapeutic effects of GLP1R agonists, we treated male WT or Irs2−/− mice with Ex4 via osmotic pump delivery from 4 weeks of age until the Irs2−/− mice died at 12 weeks. Body weight was not altered by Ex4 treatment in WT mice up to 12 weeks of age, and Ex4 did not prevent the usual weight loss that commenced at 9 weeks of age in Irs2−/− mice (Fig. 3a). Ex4 treatment had no effect upon random-fed blood glucose or insulin levels of WT mice during the experimental period (Fig. 3, b and c). By contrast, the Irs2−/− mice displayed moderate compensatory hyperinsulinemia and slightly elevated glucose when the Ex4 treatment was initiated (Fig. 3, b...
and c). At 8 weeks of age, Ex4 improved glucose tolerance in Irs2−/− mice to a level comparable with untreated wild type mice; however, Ex4 also improved glucose tolerance in wild type mice, leaving a significant difference between Ex4-treated wild type and Irs2−/− mice (Fig. 3d). Although Ex4 increased the circulating insulin in Irs2−/− mice, it had no detectable effect upon circulating insulin levels in wild type mice (Fig. 3c). Ex4 also slightly increased the ability of exogenous insulin injections to suppress circulating glucose in wild type and Irs2−/− mice (Fig. 3, e and f). However, the effect of Ex4 upon glucose tolerance was largely due to temporarily increased circulating insulin, as Ex4-treated or untreated Irs2−/− mice died when their circulating insulin levels fell below 1 ng/ml and glucose rose above 600 mg/dl (Fig. 3b). Ex4 extended the lifespan of Irs2−/− mice about 18 days, coincident with the maintenance of circulating insulin (Fig. 3c).

FIGURE 3. Metabolic analyses of WT and Irs2−/− mice after Ex4 treatment. a, body weight was measured every week as indicated. b, blood glucose concentrations were measured from tail bleeds of random-fed male mice. Results are presented as the mean ± S.E. (n = 9–11) obtained from WT or Irs2−/− mice treated from 4 weeks of age with either control (cont) saline or Ex4. For Irs2−/− mice the Ex4-treated group was significantly different from the control group (*, p < 0.05; **, p < 0.01; ***, p < 0.001). c, serum insulin concentrations were measured from tail bleeds of random fed male mice by enzyme-linked immunosorbent assays. Results are presented as the mean ± S.E. (n = 9–11). For Irs2−/− mice the Ex4-treated group was significantly different from the control group (*, p < 0.05; **, p < 0.01; ***, p < 0.001). d, intraperitoneal glucose tolerance tests were performed on overnight-fasted male WT mice 8–10 weeks of age and male Irs2−/− mice 6–7 weeks of age after intraperitoneal injections of D-glucose (2 g/kg of body weight). Glucose was measured in tail blood samples at the indicated time intervals. Results are presented as the mean ± S.E. (n = 10). For Irs2−/− mice the Ex4-treated group was significantly different from the control group (*, p < 0.05; **, p < 0.01; ***, p < 0.001). e, insulin tolerance tests were performed on male WT mice at 8–10 weeks of age treated for 4–6 weeks with control saline or Ex4 (*, p < 0.05; **, p < 0.01). f, ITTs were performed on male Irs2−/− mice 6–10 weeks of age when their 6-h fasted blood glucose levels were similar after 2–6 weeks of control or Ex4 treatment (*, p < 0.05; **, p < 0.01; ***, p < 0.001 Ex4 versus control-treated groups). For e and f, mice were fasted for 6 h before ITT. Glucose was measured in tail blood samples at the indicated time post-insulin injection. Results are expressed as the mean ± S.E.
The Role of Irs2 during Ex4-induced Insulin Secretion—GLP1 promotes insulin secretion at least in part by rendering B cells more sensitive to glucose and increasing insulin secretory capacity (10, 12, 43). To determine whether Irs2 mediates the effects of Ex4 upon insulin secretion, wild type or Irs2−/− mice were treated continuously without or with Ex4 beginning at 4 weeks of age. After 3–4 weeks, circulating insulin was determined at regular intervals during a hyperglycemic clamp maintained at 5.5 mM above the base-line fasting blood glucose level. Fasting blood glucose levels were determined before beginning the hyperglycemic clamp procedure. Bolus glucose (375 mg of glucose/kg of body weight) was administered via venous infusion for 5 min to begin the clamp, and continuous venous infusion of 20% glucose was administered to maintain blood glucose levels at 5.5 mM above the determined fasting glucose levels. Significant differences were noted between the saline and Ex4-treated WT mice (*, p < 0.05; ***, p < 0.001); as well as between the saline- and Ex4-treated Irs2−/− mice (*, p < 0.05). Ex4-treated groups were significantly different from the control groups in both WT and Irs2−/− mice. By contrast, circulating insulin was barely increased at 5 min in saline- and Ex4-treated wild type mice and Irs2−/− mice. Groups of 4-week-old mice were treated daily with control (cont) saline or 300 pm Ex4/kg of body weight until 7–8 weeks of age. Glucose-stimulated insulin secretion and pancreatic perfusion in Ex4-treated WT and Irs2−/− mice. a, serum insulin concentrations during hyperglycemic clamp in conscious and overnight-fasted WT and Irs2−/− mice. Groups of 4-week-old mice were treated daily with control saline or 300 pm Ex4/kg of body weight until 7–8 weeks of age. Fasting blood glucose levels were determined before beginning the hyperglycemic clamp procedure. Bolus glucose (375 mg of glucose/kg of body weight) was administered via venous infusion for 5 min to begin the clamp, and continuous venous infusion of 20% glucose was administered to maintain blood glucose levels at 5.5 mM above the determined fasting glucose levels. Significant differences were noted between the saline and Ex4-treated WT mice (*, p < 0.05; ***, p < 0.001); as well as between the saline- and Ex4-treated Irs2−/− mice (*, p < 0.05). b, average glucose infusion rates for maintenance of blood glucose levels at 5.5 mM above fasting glucose levels between 40–60 min during the hyperglycemic clamp procedure. Ex4-treated groups were significantly different from the control groups in both WT and Irs2−/− mice (*, p < 0.05). c and d, insulin secretion from perfused pancreas of 4 male wild type mice and 4 male Irs2−/− mice treated with saline control or Ex4 for 4–6 weeks beginning at 4 weeks of age. The pancreas was sequentially perfused with Krebs-Ringer bicarbonate buffer (1 ml/min) containing 3% dextran supplemented with 5 mM glucose (for 10 min), 16.7 mM glucose (for 20 min), 5 mM glucose (for 15 min), and 30 mM glucose (for 20 min). Insulin was determined by radioimmunoassay in each pancreatic perfusate eluate fraction collected at 1-min intervals. The total insulin released during the 80-min pancreatic perfusion was calculated by numeric integration of the secretion curves using Origin (OriginLab Corp.). e, the integrated insulin secretion during the 80-min experiments is plotted for male and female experimental mice under the conditions described in panels c and d. There was a statistically significant difference between Ex4-treated and saline-treated groups within each mouse genotype and gender group (*, p < 0.05; **, p < 0.01). f, the pancreatic insulin content was determined in acid-ethanol extractions of total pancreas from 6–7-week-old WT or Irs2−/− mice before and after the 80-min perfusion experiments depicted, as described for panels c and d. Significant differences were noted between the saline- and Ex4-treated wild type mice (*, p < 0.05) as well as between the saline- and Ex4-treated Irs2−/− mice (**, p < 0.01).
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(45, 46). Moreover, at 5–6 weeks of age (1 week after beginning Ex4 treatment) reduced about 50% under all experimental conditions, but the insulin production with the increased basal secretion (Fig. 4a). Consequently, Ex4 treatment doubled the total capacity for insulin secretion from wild type islets during the 80-min perfusion experiment (Fig. 4c). By contrast, the response of the male Irs2−/− pancreas to low and high glucose stimulation and to Arg stimulation was sluggish and significantly reduced (Fig. 4c and d).

Female Irs2−/− mice develop diabetes more slowly than male mice, which permits the investigation of healthier mice between 4–8 weeks of age. Glucose-stimulated insulin secretion was also reduced in 7–8-week-old female Irs2−/− pancreas that as yet did not display diabetic symptoms; however, the magnitude of the reduction was less severe. As found with the male mice, Ex4 increased the basal level of insulin secretion established during 5 mM glucose perfusion in the female Irs2−/− pancreas, but it did not normalize the response to glucose or Arg stimulations (Fig. 4e and data not shown). Thus, Ex4 doubled the amount of insulin secreted during the 80-min protocol, but the overall capacity was significantly reduced in Irs2−/− mice regardless of gender or the extent of diabetic disease development (Fig. 4e). By 25–30 weeks of age female Irs2−/− mice developed severe diabetes whether or not Ex4 treatment was conducted (44).

Finally, we measured the insulin content of the male WT and Irs2−/− pancreas by acid-ethanol extraction before or after an 80-min perfusion protocol. Ex4 increased the insulin content about 50% in WT mouse pancreas (Fig. 4e). By contrast, the insulin content of Irs2−/− pancreas was decreased by Ex4, which was consistent with the inability to balance the insulin production with the increased basal secretion (Fig. 4f). After the 80-min perfusion protocol, the pancreas insulin content was reduced about 50% under all experimental conditions, but the insulin content was nearly exhausted in Ex4-treated Irs2−/− pancreas. These data support the hypothesis that Irs2 signaling mediates the long term effects of GLP1R agonists upon β cell function.

Irs2 Mediates β Cell Growth during Ex4 Treatment—Ex4 increases the rate of β cell proliferation in islets by stimulating β cell proliferation with the increased basal secretion and growth of β cells or β cell precursors in human and rodent tissues (7, 13). Because Irs2 plays a critical role in β cell growth, we investigated whether it was required for Ex4-stimulated β cell growth. Ex4 treatment between 4 and 8 weeks of age increased β cell area about 2-fold in WT mice (Fig. 5a). By contrast, β cell area decreased 10% in control Irs2−/− islets during this time interval, and Ex4 treatment had no impact on this β cell loss (Fig. 5a) (30, 31). Moreover, at 5–6 weeks of age (1 week after beginning Ex4 treatment), fewer glucagon-positive α-cells infiltrated into the center of the islet sections (Fig. 5b). By 10 weeks of age, glucagon-positive α-cells were significantly reduced in both Irs2−/− and WT mice, increasing the ratio of β/α cells in both Irs2−/− and WT mice (Fig. 5, a and b). This is consistent with previously reports that pancreatic and glucagon cells can be reduced 50% in rats and humans by Ex4 treatment (45, 46).

To determine whether Ex4 stimulates β cell mitogenesis in the Irs2−/− mice treated with Ex4 for 1 week, 5–6-week-old mice were injected with the thymidine analog BrdUrd, and its incorporation into DNA was determined 6 h later. Ex4 increased BrdUrd incorporation into the nuclei of WT β cells 2-fold (Fig. 5a). By contrast, BrdUrd incorporation was significantly reduced in control saline-treated Irs2−/− islets and was not increased by Ex4 treatment (Fig. 5a). Consistent with these results, Ex4 treatment increased the levels of both Irs2 and Pdx1 in wild type islets, whereas Ex4 had no effect upon Pdx1 expression in islets from Irs2−/− mice (Fig. 5c). Because transgenic Irs2 or Pdx1 can restore β cell function in Irs2−/− mice, our results suggest that Irs2 signaling is required for the effects of Ex4 upon compensatory β cell function and growth.

**FIGURE 5. The impact of Ex4 treatment on pancreatic islet morphology.** a, α and β cell quantitation was determined by point counting morphometry from tissue sections immunostained for glucagon and insulin, respectively. All mice used were male at 8–10 weeks of age 4 weeks after control saline or Ex4 treatment. β cell proliferation is depicted as BrdUrd (BrdU-positive) cells. BrdUrd incorporation was determined on double-labeled (BrdUrd and insulin) pancreas sections from male mice 5–6 weeks of age 1–2 days after saline or Ex4 treatment and expressed as a percentage of total nuclear. Results are expressed as the mean ± S.E. of 7 mice per group (*, p < 0.05; **, p < 0.01 when compared with saline-treated mice of same genotype). b, representative islets from n = 7 mice per treatment group in (a) (original magnification). c, representative islets from n = 7 mice per treatment group in (a) (100 × original magnification). Green indicates insulin-staining β cells, and red indicates glucagon-staining α-cells. a, c expression of Irs2 and Pdx1 in pancreatic islets isolated from WT and Irs2−/− mice after in vivo Ex4 treatment. Isolated islets from WT and Irs2−/− mice were incubated with vehicle or 2.5 mM Ex4 for 8 h in Dulbecco’s modified Eagle’s medium with high glucose. Pdx1 and Irs2 protein levels were determined by immunoblot analysis. Cell lysates with equivalent protein concentrations were resolved by SDS-PAGE and transferred to nitrocellulose, and the resultant blot was probed with anti-Irs2 or Pdx1 antibodies (upper and middle panels). Protein loading was controlled and visualized with a pan anti-p85 immunoblot (lower panel).
DISCUSSION

Most if not all effects of GLP1R agonists upon β cell function are mediated through the activation of adenyl cyclase and the production of cAMP (7, 10). However, various heterologous signaling proteins (PI 3-kinase, Akt, protein kinase Cζ, and Erk1/2) are also activated by GLP1 (23, 39, 47). Several mechanisms are proposed to link GLP1R to these signaling proteins, including the activation of the epidermal growth factor receptor or p60Src or increased synthesis of IRS2 (26, 38, 48, 49).

Compounds that increase cAMP in β cells, including GLP1R agonists such as Ex4, activate the CREB, which strongly stimulates Irs2 gene expression (26). The trophic effects of GLP1R agonists upon murine β cell growth and survival are completely lost in Irs2−/− mice even though Erk1 and Erk2 are still activated. We conclude that Irs2 signaling is essential for the long term effects of GLP1R agonists upon β cell growth, function, and survival.

Activation of the PI 3-kinase → Akt cascade plays an essential role for the long term effects of GLP1/Ex4 upon β cell growth and survival (37, 50). Recent work with transgenic mice suggests that Irs2 provides one of the most potent regulatory signals for the PI 3-kinase cascade in β cells (27, 51). Pancreatic β cells are almost entirely lost in systemic Irs2−/− mice unless proteins in the cascade are modified (including down-regulation of protein tyrosine phosphatase Ptp1b, the phospholipid phosphatase Pten, or the transcription factor Foxo1 or up-regulation of Akt or Pdx1) to promote compensatory β cell growth, function, and survival (24, 27, 33, 52, 53). Up-regulation of Irs2 itself in pancreatic β cells rescues diabetes in Irs2−/− mice, obese mice, and streptozotocin-induced diabetic mice (27). Whereas GLP1 fails to prevent diabetes in Irs2−/− mice, it succeeds in other diabetic models where the potential to increase Irs2 exists; that is, obese or aged rodents and partial pancreatic-tectomy and streptozotocin-treated rats (7).

PDX-1 is required for the development of the pancreas in mice and humans, but it also promotes β cell function in adults (40, 54–56). Genetic disruption of the Pdx1 gene in β cells promotes apoptosis that progresses to diabetes (57). By contrast, GLP1R agonists increase PDX-1 expression in rodent and human β cells, which promotes β cell function and improves glucose tolerance in diabetic rodents and patients (7). The loss of Pdx1 expression in Irs2−/− mice is thought to play an important role in β cell failure because transgenic Pdx1 restores β cell mass and function that prevents diabetes in Irs2−/− mice (33).

Moreover, transgenic Irs2 or genetic suppression of Foxo1 also restores Pdx1 expression and prevents diabetes in Irs2−/− mice (27, 41, 58). Thus, the inability of Ex4 to increase Pdx1 in Irs2−/− mice (or in Min6 cells transfected with siRNAiRNA) supports the hypothesis that Irs2 signaling is essential for this important Ex4 response.

GLP1R agonists like Ex4 promote β cell function and growth (10, 58). The effects on glucose-stimulated insulin secretion appear to be independent of Irs2 signaling, which is consistent with the activation of cAMP-dependent mechanisms that are coupled directly to secretion (10). Thus, Ex4 treatment of Irs2−/− mice retards the progression toward diabetes by enhancing glucose-stimulated insulin secretion. Ex4 also reduces the number of α-cells in the wild type and Irs2−/− islets, which might improve glucose tolerance. Eventually, without Irs2, Ex4 fails to promote sufficient β cell growth needed for compensatory insulin secretion, which is essential for its long term beneficial effects upon glucose tolerance and the prevention of diabetes.

Activation of GLP1R by Ex4 promotes β cell regeneration in various rodent models, including regeneration after a partial pancreatectomy (59). The progressive loss of β cell mass in Irs2−/− mice is accompanied by increased apoptosis and reduced mitogenesis (30); transgenic Irs2 expressed in Irs2−/− β cells restores mitogenesis and inhibits apoptosis (27). By comparison, Ex4 increases BrdUrd incorporation into wild type β cells and increases β cell mass 2-fold; however, Ex4 fails to do so in Irs2−/− mice. Because β cell mass decreases similarly in Irs2−/− islets whether or not the mice were treated with Ex4, the inhibition of apoptosis by Ex4 largely fails in Irs2−/− mice. These results are consistent with an important role for Irs2 signaling during Ex4-stimulated β cell growth.

The role of IRS2 signaling in human β cells is difficult to validate directly. Our experiments with small aliquots of freshly isolated human islets show that Ex4 increases IRS2 expression at least as well as it increases the expression of other important β cell genes: GLUT2, glucokinase, or PDX-1. Because Ex4 has similar effects upon human and murine islets and β cells, it is plausible that Irs2 plays an important role in human β cell function. This indirect conclusion deserves more investigation.

GLP1 secretion can be reduced in type 2 diabetic patients, which might impair the expansion of β cell mass required to compensate for peripheral insulin resistance (9, 60). Because GLP1R is also expressed by other peripheral tissues and in the central nervous system, reduced GLP1 levels might contribute to systemic insulin resistance because of reduced IRS2 expression (61, 62). Treatment of diabetic patients with Ex4 improves insulin secretion and action (17, 61, 63, 64). The systemic and central effects of GLP1 or Ex4 are consistent with a role for increased IRS2 expression that might occur in responsive tissues.

These effects of GLP1 or Ex4 on glucose homeostasis fuels the efforts to treat diabetic patients with stable GLP1R agonists or dipeptidyl-peptidase IV inhibitors (65). Its effect on IRS2 expression suggests a rational mechanism that causes these long-term effects. Because Irs2 tyrosine phosphorylation is a strong activator of the PI 3-kinase → Akt → Foxo1 cascade in mice, regulation of IRS2 is nearly certain to promote the growth and survival of human β cells and promote central nutrient homeostasis (24, 29). The identification of other compounds that increase IRS2 expression in β cells or other tissues could be important treatments for diabetes and related metabolic disorders.

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