Original Article

Fibroblast Growth Factor-21 Improves Pancreatic β-Cell Function and Survival by Activation of Extracellular Signal–Regulated Kinase 1/2 and Akt Signaling Pathways

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Fibroblast growth factor-21 (FGF-21) is a recently discovered metabolic regulator. Here, we investigated the effects of FGF-21 in the pancreatic β-cell. In rat islets and INS-1E cells, FGF-21 activated extracellular signal–regulated kinase 1/2 and Akt signaling pathways. In islets isolated from healthy rats, FGF-21 increased insulin mRNA and protein levels but did not potentiate glucose-induced insulin secretion. Islets and INS-1E cells treated with FGF-21 were partially protected from glucolipotoxicity and cytokine-induced apoptosis. In islets isolated from diabetic rodents, FGF-21 treatment increased islet insulin content and glucose-induced insulin secretion. Short-term treatment of normal or *db/db* mice with FGF-21 lowered plasma levels of insulin and improved glucose clearance compared with vehicle after oral glucose tolerance testing. Constant infusion of FGF-21 for 8 weeks in *db/db* mice nearly normalized fed blood glucose levels and increased plasma insulin levels. Immunohistochemistry of pancreata from *db/db* mice showed a substantial increase in the intensity of insulin staining in islets from FGF-21–treated animals as well as a higher number of islets per pancreas section and of insulin-positive cells per islet compared with control. No effect of FGF-21 was observed on islet cell proliferation. In conclusion, preservation of β-cell function and survival by FGF-21 may contribute to the beneficial effects of this protein on glucose homeostasis observed in diabetic animals. *Diabetes* 55:2470–2478, 2006

Pancreatic β-cell dysfunction is a central component of the pathogenesis of all forms of diabetes. Type 1 diabetes manifests from the autoimmune destruction of β-cells, whereas type 2 diabetes is characterized by reduced β-cell mass and marked functional defects, including impaired first-phase insulin secretion, increased proinsulin-to-insulin ratio, and elevated rate of β-cell apoptosis (1–3). The glucose-sensing and insulin-signaling pathways have been shown to play important roles in insulin secretion as well as β-cell growth and survival. For example, mice lacking insulin receptors, insulin receptor substrate-2, or Akt (protein kinase B) display marked defects in glucose sensing, insulin secretion, and β-cell mass (4–6). The amount of secreted insulin is determined by the secretory activity of the β-cell and the total number of β-cells in the pancreas. Glucose plays an essential role in the control of secretory activity of β-cells. Metabolism of glucose leads to an increase in the ATP-to-ADP ratio, membrane depolarization, Ca^{2+} influx, and stimulation of insulin secretion (7). β-Cell mass is governed by the balance between β-cell growth and β-cell death (apoptosis). Type 2 diabetic patients display a progressive loss of β-cells caused by an increased rate of β-cell apoptosis (8). However, the cause and mechanism(s) responsible for the increased apoptosis rate in type 2 diabetes are not well understood (9). Preventing β-cell death and increasing survival of the β-cell can be a valuable therapeutic approach to treat and cure type 2 diabetes.

Members of the fibroblast growth factor (FGF) family play important roles in defining and regulating the development and function of endocrine tissues as well as modulating various metabolic processes (10–15). FGFs modulate cellular activity via five distinct high-affinity FGF receptors (16).

FGF-21 is a recently described member of the FGF family (17). We have previously demonstrated that FGF-21 is a potent regulator of glucose homeostasis (18). FGF-21 enhances glucose uptake in adipocytes, protects from diet-induced obesity when overexpressed in transgenic animals, and lowers blood glucose and triglyceride levels when administrated to diabetic animals (18). We have also found that plasma glucagon levels were lower in FGF-21–overexpressing transgenic mice and in FGF-21–administered diabetic mice, an effect resulting from inhibition of glucagon secretion in the pancreatic islets (18). In the current study, we have investigated the effects of FGF-21 on β-cell function and survival. We demonstrate that although FGF-21 does not acutely stimulate insulin secretion in isolated islets from normal rats, it does increase insulin secretion and insulin content from diabetic islets and protects β-cells from apoptosis via activation of extracellular signal–regulated kinase (ERK)1/2 and Akt signaling pathways.

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BAD, Bcl-XL/Bcl-2–associated death promoter; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal–regulated kinase; FGF, fibroblast growth factor; FRS, FGF receptor substrate; GLP, glucagon-like peptide; p90RSK, 90-kDa ribosomal S6 kinase; PCNA, proliferating cellular nuclear antigen.

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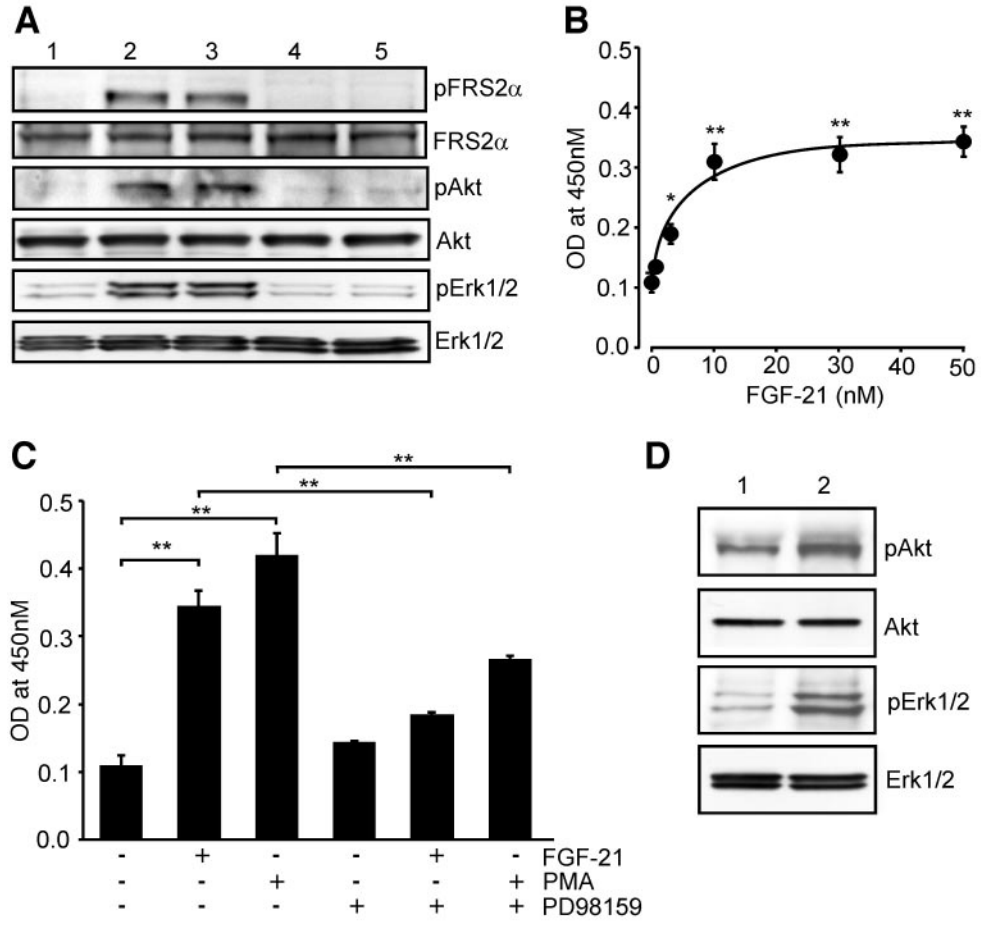

FIG. 1. FGF-21 induces phosphorylation of FRS2α, ERK1/2, and Akt. *A*: INS-1E cells were treated for 5 min with either vehicle (*lane 1*), 50 nmol/l FGF-21 (*lane 2*), 5 nmol/l FGF-1 (*lane 3*), 50 nmol/l FGF-21 with 20 μmol/l SU5402 (*lane 4*), or 20 μmol/l SU5402 (*lane 5*). Phospho-specific antibodies were used to determine phosphorylation of FRS2α, ERK1/2, and Akt. *B*: Dose-response curve for stimulation of ERK1/2 activity by FGF-21 in INS-1E cells as measured by ELISA. *C*: Histogram showing ERK1/2 activity after stimulation of INS-1E cells with 50 nmol/l FGF-21 and 200 nmol/l 4β-phorbol 12-myristate 13-acetate (PMA) and the effects of 20 μmol/l PD98059 in INS-1E cells under basal conditions and after FGF-21 (50 nmol/l) stimulation for 10 min. *D*: Rat islets were treated with either vehicle (*lane 1*) or 50 nmol/l FGF-21 (*lane 2*) for 10 min. Phospho-specific antibodies were used to determine phosphorylation of ERK1/2 and Akt. Results are the means ± SE of 3–5 separate experiments. OD, optical density; p, phospho-.

RESEARCH DESIGN AND METHODS

Intact pancreatic islets were isolated from male Wistar and GK rats (250–300 g; Charles River Laboratories, Sulzfeld, Germany) as well as *db/db* mice (C57BL/KsOlaHsd-Leprdb; Harlan, Borchen, Germany), as previously described (19,20). Islets were cultured overnight in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) heat-inactivated FCS (Sigma-Aldrich), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). INS-1E cells (passages 55–70) were cultured in RPMI-1640 medium with L-glutamine, 1 mmol/l sodium pyruvate, 10 mmol/l HEPES, 5% heat-inactivated FCS, 50 μmol/l 2-mercaptoethanol (Invitrogen), 10,000 IU/ml penicillin, and 10,000 μg/ml streptomycin, as previously described (21).

Insulin secretion assay. Insulin secretion in pancreatic islets was performed as previously described (20), using three islets in 300 μl of Earle's balanced salt solution (Invitrogen) supplemented with glucose and test substances as indicated. For determination of insulin content, islets were subjected to a lysis buffer containing 75% ethanol, 23.5% water, and 1.5% concentrated HCl. Insulin concentrations in the collected supernatant and islet lysates were measured with enzyme-linked immunosorbent assay (ELISA).

Real-time quantitative PCR. Real-time PCR was performed with islet total RNA samples produced with an RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. An ABI Prism 7900 HS sequence detection system (Applied Biosystems) was used. PCR for each mRNA was performed in quadruplicate on cDNA samples. A master mixture for each PCR was set up in a total reaction volume of 30 μl, comprised of 5 μl cDNA (in a 1:5 dilution), 15 μl 2× TaqMan Universal Master Mix, No AmpErase UNG (Applied Biosystems, Foster City, CA), 5 μl of diethyl pyrocarbonate–treated water (Ambion, Huntingdon, U.K.), and 1.5 μl 20× primer/probe set (Assay-on-Demand assay mix; Applied Biosystems). From this mixture an aliquot of 5 μl was placed in each of the four wells. Assay-on-demand identification for Ins2 was Rn01774648_g1. The thermal cycling conditions were: 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min.

Immunoblotting. INS-1E cells were lysed in radioimmunoprecipitation buffer (50 mmol/l Tris-HCl, pH 8.0, 150 mmol/l NaCl, 1% NP-40, 0.5% Na-deoxycholate, 5 mmol/l EDTA, and 0.1% SDS) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Protein samples were separated by SDS-PAGE, transferred to polyvinylidine fluoride membrane, and probed with antibodies against the following proteins: Akt, phospho-Akt (Ser473), 90-kDa ribosomal S6 kinase (p90RSK), phospho-p90RSK (Ser380), phospho-ERK1/2 (Thr202/204), S6 ribosomal protein, and phospho-S6 ribosomal protein (Ser235/236; Cell Signaling Technology, Beverly, MA); ERK1/2 and phospho-BAD (Ser136; Upstate, Charlottesville, VA); and anti-BAD (BD Bioscience, San Diego, CA). For the quantification of the Western blots, a VersaDoc imaging system (Bio-Rad, Munich, Germany) and Quantity One 1-D analysis software (version 4.4; Bio-Rad) were used.

Caspase activity and DNA fragmentation assays. INS-1E cells were plated in a 96-well plate (20,000 cells per well). Apoptosis was induced by incubation of cells with 20 ng/ml interleukin-1β (Sigma-Aldrich) and 40 ng/ml tumor necrosis factor-α (Sigma-Aldrich) for 48 h. FGF-21, glucagon-like peptide (GLP)-1, and kinase inhibitors were added 24 h before cytokine challenge. Caspase 3/7 activity was measured with Apo-ONE caspase assay according to the manufacturer's instructions (Promega, Madison, WI). DNA fragmentation was measured with a Cell Death ELISA Plus assay according to the manufacturer's instructions (Roche Applied Science). To measure caspase 3/7 activation in isolated islets, groups of 100 islets were incubated with or without FGF-21 for 72 h. Islet apoptosis was then induced either by addition of a cytokine mixture analogous to the mixture used in INS-1E cells or by changing

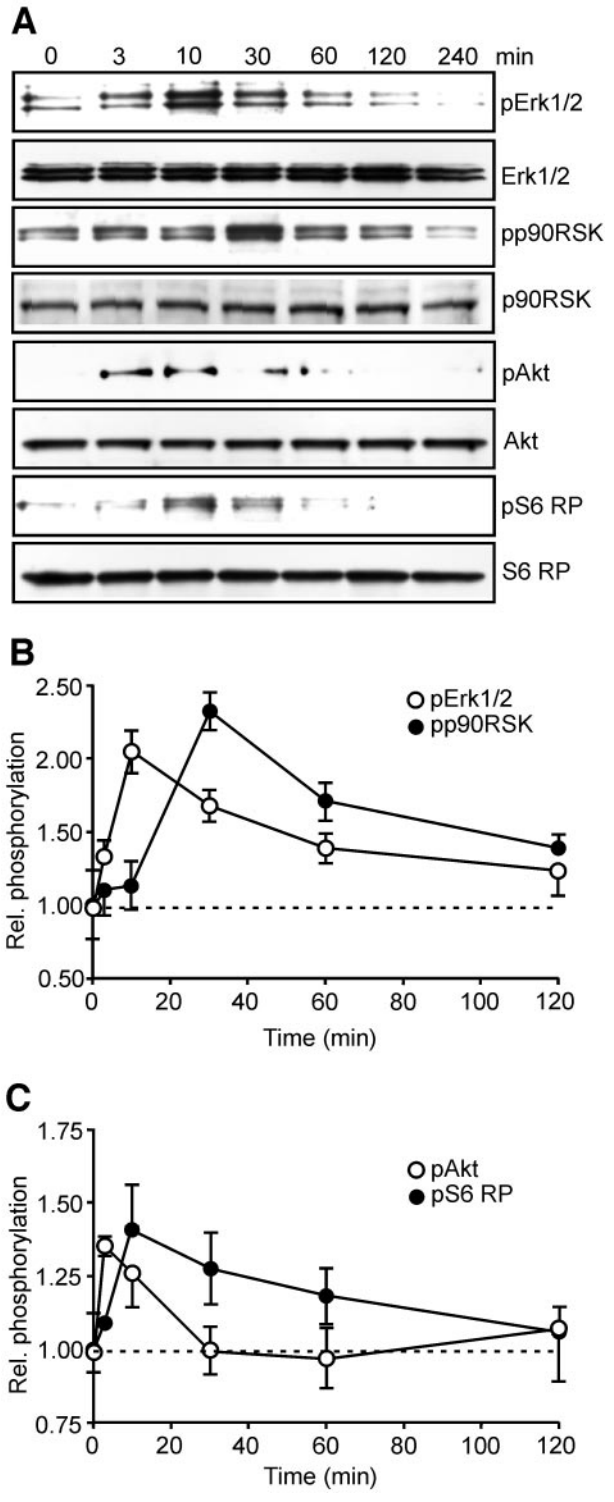

FIG. 2. FGF-21 activates ERK1/2 and Akt signaling pathways. *A*: INS-1E cells were stimulated for 0, 3, 10, 30, 60, 120, and 240 min with 50 nmol/l FGF-21. Phospho-specific antibodies were used to determine phosphorylation of ERK1/2, p90RSK, Akt, and S6 ribosomal protein (S6 RP). *B*: Time-dependent changes in relative phosphorylation of ERK1/2 and p90RSK in INS-1E cells stimulated with 50 nmol/l FGF-21. *C*: Same as for panel *B*, except that relative phosphorylation for Akt and S6 ribosomal protein is depicted. p, phospho-; Rel., relative.

the regular culture medium to a medium containing 1% fetal bovine serum, 27 mmol/l glucose, and 0.4 mmol/l free fatty acid. Islets were cultured for another 24 h, washed, and transferred into a 96-well plate (10 islet per well) for caspase activity determination.

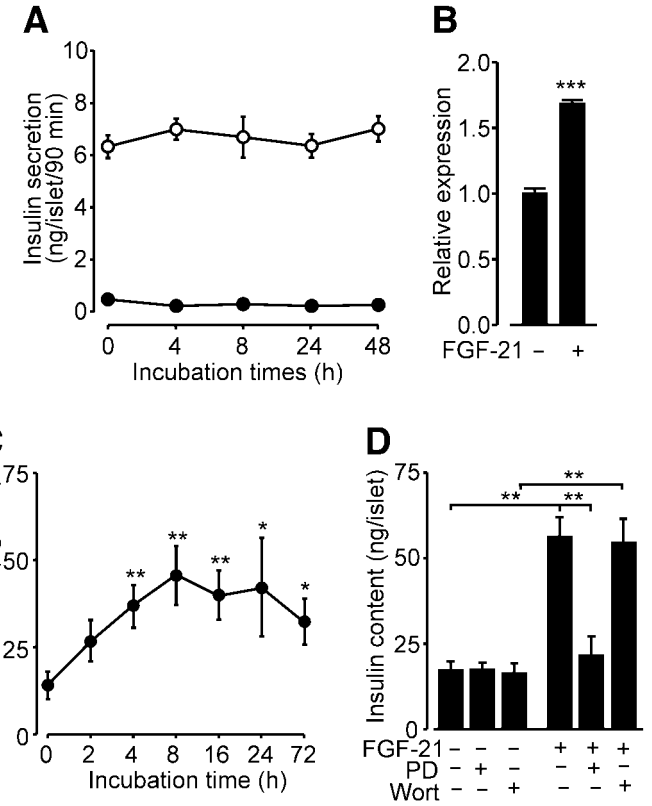

FIG. 3. FGF-21 increases insulin mRNA and insulin content but does not modulate insulin secretion in islets isolated from healthy rats. *A*: Rat islets were treated for 0, 4, 8, 24, and 48 h with 50 nmol/l FGF-21 before exposure to 3 mmol/l glucose (●) or 15 mmol/l glucose (○) for 90 min. Results are the means ± SE of six separate experiments. *B*: Relative insulin gene mRNA expression in rat islets under basal conditions and after exposure to FGF-21 (50 nmol/l) for 24 h. Results are the means ± SE of four separate experiments. *C*: Insulin content in rat islets exposed for 0, 2, 4, 8, 16, 24, and 48 h to 50 nmol/l FGF-21. Results are the means ± SE of six separate experiments. *D*: Insulin content in rat islets under basal conditions and after stimulation with 50 nmol/l FGF-21 for 16 h. Where indicated, islets were also treated with 20 μmol/l PD98059 (PD) or 100 nmol/l wortmannin (Wort). Results are the means ± SE of six separate experiments.

Phospho-ERK1/2 ELISA. INS-1E cells were plated in a 24-well plate (250,000 cells per well). After 72 h of culture, the cells were starved for 1 h in Earle's balanced salt solution with 3 mmol/l glucose before treatment with agonists for 10 min. After the treatment the cells were frozen on dry ice, lysed with cell extraction buffer (Biosource, Camarillo, CA), boiled, and subjected to a PathScan phospho-p44/42 mitogen-activated protein kinase (T202/Y204) sandwich ELISA kit (Cell Signaling Technology).

In vivo protocols. The protocols used in these studies were approved by the Eli Lilly Research Laboratories institutional animal care and use committee. Mice were maintained in a controlled environment (20 ± 2°C, 50–60% humidity, 12-h light/dark cycle, lights on at 6 A.M.). FGF-21 treatment of C57BL/6 mice was as follows: 8-week-old C57BL/6NTac mice were grouped according to body weight and then dosed daily for 7 days with 0.45 mg/kg s.c. FGF-21. An oral glucose tolerance test was performed as described below. FGF-21 treatment of *db/db* mice was as follows: 8-week-old male *db/db* mice (C57BL/KsOlaHsd-Leprdb; Harlan, Indianapolis, IN) were dosed twice daily for 4 days with 2.5 mg/kg s.c. (1.25 mg/kg per dose) FGF-21. For the oral glucose tolerance test, the mice were fasted overnight (16 h) and bled to obtain a specimen for fasting glucose and insulin measurements before they were given a dose (2.5 g/kg) of 50% dextrose solution by oral gavage. Blood glucose levels were determined by a Precision-G blood glucose testing system (Abbott Diagnostics, Abbott Park, IL), and insulin levels were determined by radioimmunoassay (Linco Diagnostics, St. Charles, MO). Chronic treatment of *db/db* mice with FGF-21 was as follows: 8-week-old male *db/db* mice (C57BL/KsOlaHsd-Leprdb; Harlan, Indianapolis, IN) were implanted with osmotic minipumps (model 1002; Durect, Cupertino, CA) for subcutaneous administration of FGF-21 in 150 mmol/l NaCl, resulting in ~10 ng/ml plasma levels. Every 2 weeks the pumps were replaced, for a total of 8 weeks.

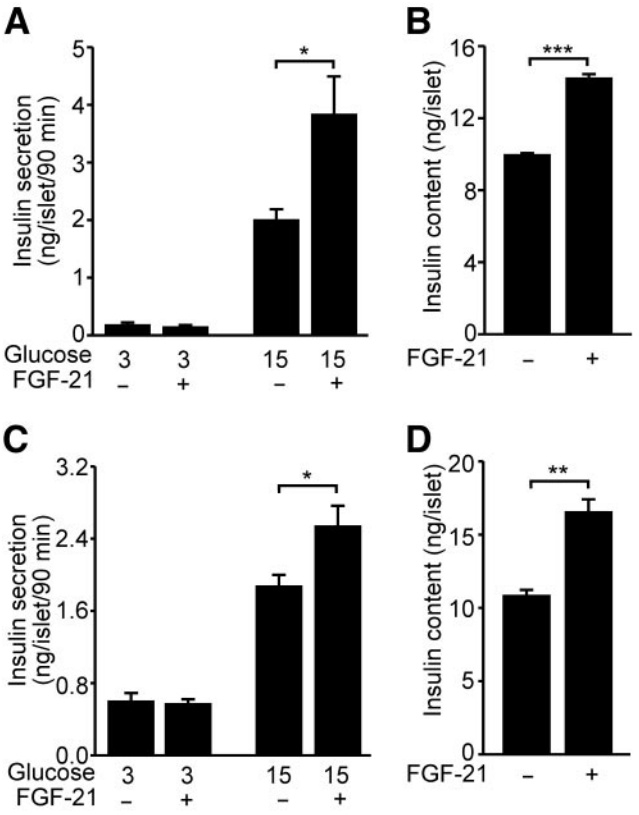

FIG. 4. FGF-21 increases insulin secretion and insulin content in islets isolated from diabetic rodents. Islets were isolated from GK rats (*A* and *B*) or *db/db* mice (*C* and *D*) and treated for 48 h with either vehicle or 50 nmol/l FGF-21. *A* and *C*: Histogram depicting insulin secretion at 3 and 15 mmol/l glucose in vehicle- and FGF-21–treated islets. *B* and *D*: Insulin content in islets treated with vehicle or FGF-21. Results are the means ± SE of six separate experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Immunostaining. Pancreata were fixed overnight in 3% paraformaldehyde and transferred into 70% ethanol before processing through paraffin. Next, 5-μm sections were cut and placed on positively charged slides. The slides were baked overnight at 60°C in an oven and then deparaffinized in xylene and rehydrated through graded alcohols to water. Antigen retrieval was performed by immersing the slides in Target Retrieval Solution (Dako, Glostrup, Denmark) for 20 min at 90°C in a water bath. Immunostaining steps were performed on a Dako autoimmunostainer. Incubations were performed at room temperature, and Tris-buffered saline plus 0.05% Tween 20, pH 7.4 (Dako), was used for all washes and diluents. Slides were blocked with protein-blocking solution for 25 min, followed by washing. Both insulin and glucagon primary antibodies (Dako) were added to the slides and incubated for 10 min. An indirect antibody linked to either Alexa 488 or Alexa 568 dye (20 μg/ml for 1 h) was used to detect the primary antibody.

Proliferating cellular nuclear antigen image analysis. Images were taken at 20×, using a digital camera (SPOT RT Color; Diagnostic Instruments) mounted on a Leica DMRXE upright brightfield microscope (Leica Microsystems, Bannockburn, IL). An area of interest was hand drawn around each islet. Brown cells and total cells were then counted within each islet. The analysis was automated, using ImagePro Plus software (Media Cybernetics, Silver Spring, MD).

Statistical analysis. Results are the means ± SE for the indicated number of experiments. Statistical significances were evaluated using Student's *t* test for pairs of data, Dunnett's test for multiple comparisons to a control, and Tukey's test when multiple comparisons between groups were required.

RESULTS

FGF-21 activates FGF receptor downstream signaling cascades in β-cells. In previous studies, we showed in 3T3-L1 adipocytes that FGF-21 caused phosphorylation of FGF receptor substrate (FRS)2, a docking protein linking FGF receptors to the ERK1/2 signaling pathway (18). Here, we extend this observation to demonstrate that the FRS2α protein is expressed in INS-1E cells and that FRS2α undergoes tyrosine phosphorylation after stimulation of INS-1E cells for 5 min with 50 nmol/l FGF-21 or 5 nmol/l FGF-1 (Fig. 1*A*). SU5402 (20 μmol/l), an inhibitor of FGF receptor tyrosine kinase activity (22), blocked FGF-21–induced FRS2α phosphorylation (Fig. 1*A*) as well as FGF-1–mediated FRS2α phosphorylation (data not shown). FGF-21 and -1 also induced phosphorylation of FGF receptor downstream targets. Similarly, the phosphorylation of ERK1/2 and Akt kinases was also inhibited by SU5402 (Fig. 1*A*).

The effect of FGF-21 on ERK1/2 phosphorylation was further examined in INS-1E cells with ERK1/2-specific ELISA. The stimulatory effect of FGF-21 on ERK1/2 phosphorylation was concentration dependent (Fig. 1*B*). Approximating the dose-response phosphorylation data points to the Hill equation yielded a half-maximal effective concentration (FGF-21 concentration producing 50% of the maximum response) of 4 ± 1 nmol/l, a cooperativity factor of 1.9 ± 0.8, and a maximal response of 220 ± 23%.

Figure 1*C* shows that a maximally stimulatory concentration of FGF-21 produced phosphorylation of ERK1/2 comparable to that of the phorbol ester 4β-phorbol 12-myristate 13-acetate (200 nmol/l). Furthermore, the ERK1/2 phosphorylation was blocked with PD98059 (20 μmol/l), an inhibitor of mitogen-activated protein/ERK kinases, the direct upstream kinases of ERK1/2. These data suggest that FGF-21 produces ERK1/2 activation in INS-1E cells via stimulation of a classical receptor tyrosine kinase signaling pathway. We extend this finding in INS-1E cells to rat islets, where we observed strong ERK1/2 and Akt phosphorylation after stimulation with 50 nmol/l FGF-21 (Fig. 1*D*).

Figure 2*A* shows a time course of FGF-21–induced phosphorylation of ERK1/2 and its downstream target p90RSK as well as Akt and its downstream target S6 ribosomal protein in INS-1E cells. FGF-21 (50 nmol/l) treatment caused a rapid phosphorylation of ERK1/2 (maximum ≤10 min) and Akt (≤3) followed by a gradual return of activity toward basal over the next 2 h (Fig. 2*B* and *C*). A similar but delayed phosphorylation of p90RSK (maximum ≤30 min) and S6 ribosomal protein (≤10) was also observed (Fig. 2*B* and *C*).

FGF-21 increases insulin content but not insulin secretion in islets isolated from healthy rats. Acute addition of 50 nmol/l FGF-21 to rat islets or incubation of islets for 4–48 h with FGF-21 did not affect subsequent insulin secretion induced by 3 or 15 mmol/l glucose stimulation (Fig. 3*A*). However, incubation of rat islets for 24 h with 50 nmol/l FGF-21 elevated insulin mRNA levels (Fig. 3*B*) and produced a time-dependent increase in insulin content (Fig. 3*C*). After 8 h incubation with FGF-21, the increase in insulin content reached its maximum (230%) and remained relatively constant for up to 72 h of exposure to FGF-21 (Fig. 3*C*). The stimulatory effect of FGF-21 on insulin content in rat islets was mediated by activation of the ERK1/2 pathway and was abolished with PD98059 (20 μmol/l) (Fig. 3*D*). On the contrary, inhibition of the Akt signaling pathway with the phosphatidylinositol 3-phosphate kinase inhibitor wortmannin (100 nmol/l) did not affect the ability of FGF-21 to stimulate an increase in islet insulin content (Fig. 3*D*).

FGF-21 enhances insulin secretion and content in diabetic rat and mouse islets. Next, we investigated the effects of FGF-21 treatment on insulin secretion and

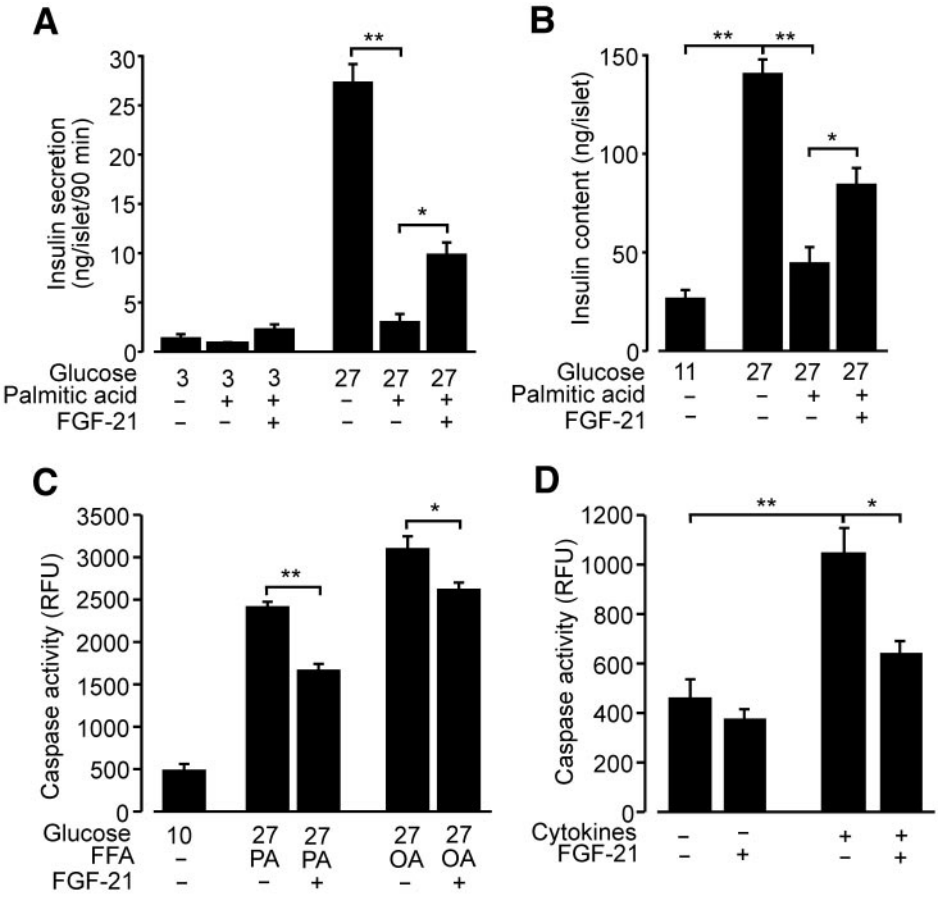

FIG. 5. FGF-21 partially restores insulin secretion and content as well as reduces caspase 3/7 activity in dysfunctional rat islets. *A*: Insulin secretion in rat islets exposed for 48 h to 27 mmol/l glucose alone or in combination with 0.4 mmol/l palmitic acid, in the absence and presence of 50 nmol/l FGF-21. *B*: Insulin content in rat islets exposed for 48 h to either 11 or 27 mmol/l glucose alone or in combination with 0.4 mmol/l palmitic acid, in the absence and presence of 50 nmol/l FGF-21. *C*: Caspase 3/7 activity determined in rat islets exposed for 72 h to a combination of 27 mmol/l glucose and 0.4 mmol/l palmitic acid or 0.4 mmol/l oleic acid, in the absence and presence of 50 nmol/l FGF-21. *D*: Same as in panel *C*, except that rat islets were exposed to a combination of interleukin-1β (20 ng/ml) and tumor necrosis factor-α (40 ng/ml) for 24 h. Results are the means ± SE of six to eight separate experiments. *$P < 0.05$; **$P < 0.01$. FFA, free fatty acid; OA, oleic acid; PA, palmitic acid; RFU, relative fluorescence units.

content in islets isolated from diabetic GK rats. Insulin content in islets isolated from GK rats was somewhat lower than insulin content in islets from healthy rats (Fig. 4*A*). Treatment of GK rat islets with 50 nmol/l FGF-21 for 48 h produced a 45% increase in insulin content (Fig. 4*A*). In addition, FGF-21 treatment strongly potentiated glucose-induced insulin secretion in GK rat islets, whereas basal insulin secretion was not affected by FGF-21 treatment (Fig. 4*B*). Potentiation of glucose-induced insulin secretion with FGF-21 was not specific for GK rat islets and was also observed in islets isolated from diabetic *db/db* mice. We found that 48-h in vitro FGF-21 treatment (50 nmol/l) of islets isolated from *db/db* mice enhanced glucose (15 mmol/l)-induced insulin secretion and insulin content compared with control islets (Fig. 4*C* and *D*).

FGF-21 protects rat islets and INS-1E cells from glucolipotoxicity and cytokine-induced apoptosis. The above data suggest that the stimulatory action of FGF-21 on insulin content is translated into enhanced glucose-evoked insulin secretion in islets from diabetic but not from healthy animals. Therefore, we investigated the effects of FGF-21 in rat islets under conditions of islet insulin depletion with 27 mmol/l glucose and 0.4 mmol/l palmitic acid for 48 h (23). Culture of rat islets in 27 mmol/l glucose strongly increased the insulin secretory response (Fig. 5*A*) and intracellular insulin content (Fig. 5*B*) compared with that observed in islets incubated in normal culture medium. Exposure of islets to a combination of high glucose (27 mmol/l) and palmitic acid (0.4 mmol/l) resulted in a pronounced decrease in glucose-induced insulin secretion (Fig. 5*A*) and insulin stores (Fig. 5*B*). Under the latter experimental conditions, FGF-21 (50 nmol/l) treatment partially restored the secretory capacity of islets (Fig. 5*A*) and islet insulin content (Fig. 5*B*). These data suggest that FGF-21 can reduce β-cell dysfunction under conditions of islet glucolipotoxicity.

We next investigated whether FGF-21 can promote β-cell survival under conditions of induced glucolipotoxicity. The caspase family of proteases plays a key role in the execution of apoptotic β-cell death in response to high glucose and lipid load (24). Rat islets were exposed for 24 h to a combination of 27 mmol/l glucose and 0.4 mmol/l palmitic acid. The induction of glucolipotoxicity in the islets increased caspase 3/7 activity compared with control islets (Fig. 5*C*). Importantly, simultaneous exposure of the islets to 50 nmol/l FGF-21 produced a reduction in caspase 3/7 activity induced by a combination of high glucose and lipids (Fig. 5*C*). We also determined caspase 3/7 activity in rat islets exposed to 20 ng/ml interleukin-1β and 40 ng/ml tumor necrosis factor-α for 24 h. Figure 5*D* shows that

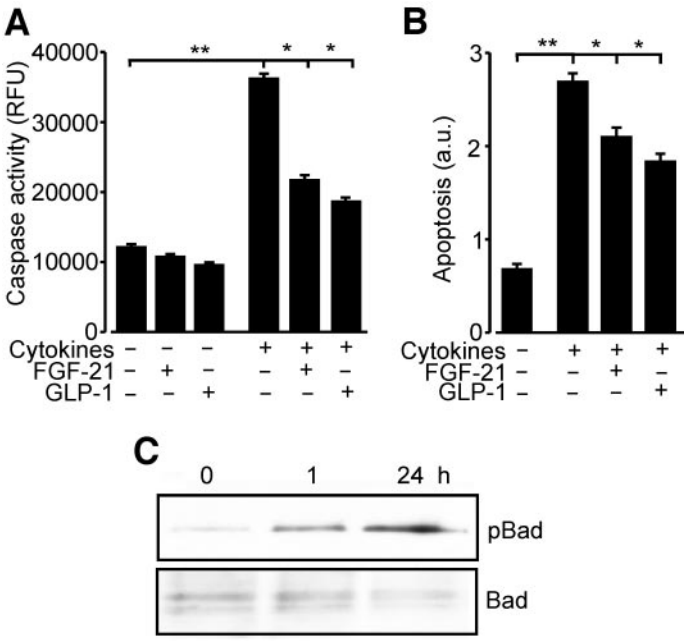

FIG. 6. FGF-21 reduces caspase 3/7 activity in INS-1E cells exposed to cytokines. *A*: Caspase 3/7 activity was determined after exposure to a combination of interleukin-1β (20 ng/ml) and tumor necrosis factor-α (40 ng/ml) for 48 h, in the absence and presence of either 50 nmol/l FGF-21 or 50 nmol/l GLP-1. *B*: DNA fragmentation in INS-1E cells after treatment with cytokines, FGF-21, and GLP-1 as described in panel *A*. Results are the means ± SE of three separate experiments. *C*: INS-1E cells stimulated for 0, 1, or 24 h with 50 nmol/l FGF-21. Specific antibodies for BAD and phosphorylated BAD (Ser136) were used to determine expression and phosphorylation of BAD. *$P < 0.05$; **$P < 0.01$. RFU, relative fluorescence units.

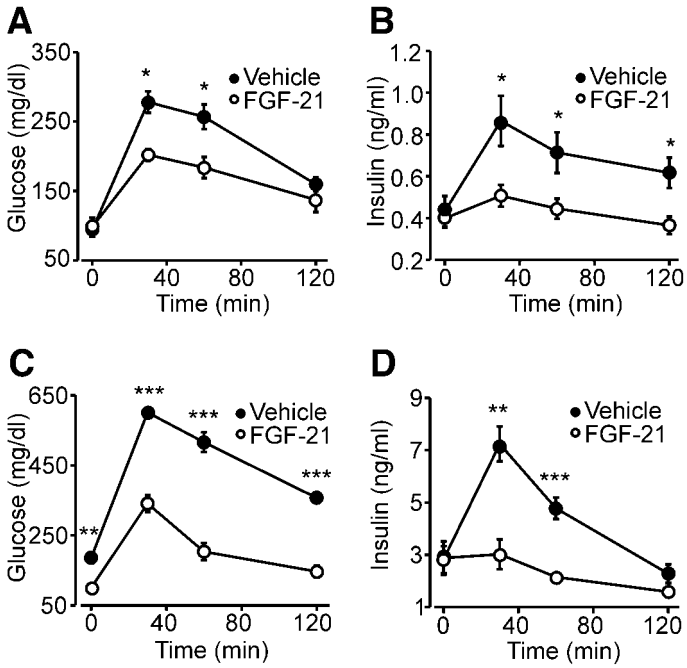

FIG. 7. FGF-21 improves glycemic control in mice. *A* and *B*: Plasma glucose (*A*) and plasma insulin (*B*) levels in oral glucose tolerance tests performed in healthy C57BL/6 mice treated for 7 days with vehicle or FGF-21 (0.45 mg · kg^{-1} · day^{-1}). *C* and *D*: Plasma glucose (*C*) and plasma insulin (*D*) levels in oral glucose tolerance tests performed in diabetic *db/db* mice treated for 4 days with vehicle or FGF-21 (2.5 mg · kg^{-1} · day^{-1}). Results are the means ± SE of six animals. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

caspase 3/7 activity doubled after cytokine treatment and that simultaneous exposure of the islets to 50 nmol/l FGF-21 reduced cytokine-induced caspase 3/7 activity by 38%.

In a separate series of experiments, we examined the effects of FGF-21 treatment on caspase 3/7 activity in INS-1E cells exposed to the cytokine combination for 48 h. Cytokine treatment induced a threefold increase in caspase 3/7 activity (Fig. 6*A*). Cytokine-induced caspase 3/7 activity was reduced by 39% in the presence of FGF-21 (50 nmol/l). A comparable 49% reduction in caspase 3/7 activity was observed in the presence of GLP-1 (Fig. 6*A*). In addition to blocking caspase activation, FGF-21 also inhibited the late step of apoptosis/DNA fragmentation (Fig. 6*B*). Cytokine treatment of INS-1E cells resulted in a threefold increase in DNA fragmentation, an effect that was partially reduced by 50 nmol/l FGF-21 or 50 nmol/l GLP-1.

It is well established that Akt-induced phosphorylation of Bcl-XL/Bcl-2–associated death promoter (BAD) suppresses apoptosis and promotes cell survival (25). Figure 6*C* shows a time-dependent increase in phosphorylation of BAD at Ser-136 after exposure of INS-1E cells for up to 24 h with 50 nmol/l FGF-21. This observation suggests that phosphorylation of BAD is likely to underlie the protective effect of FGF-21 on apoptosis in β-cells.

FGF-21 improves glucose tolerance in normal and *db/db* mice. In our previous study, we demonstrated that FGF-21 treatment decreased plasma glucose levels in diabetic animal models (18). Here, we confirmed the glucose-lowering effects of FGF-21 in diabetic *db/db* mice: lower glucose and insulin levels after an oral glucose challenge were observed in *db/db* mice treated with FGF-21 (Fig. 7*C* and *D*). Moreover, subcutaneous administration of FGF-21 for 7 days in normal C57BL/6 mice decreased both plasma glucose and plasma insulin levels measured after an oral glucose challenge, whereas the compound treatment did not alter fasted glucose and insulin levels in healthy mice (Fig. 7*A* and *B*).

Long-term treatment of *db/db* mice with FGF-21 preserves β-cell function and mass. We evaluated the potential of FGF-21 to preserve β-cell function and mass in vivo by constant subcutaneous infusion for 8 weeks of FGF-21 (11 μg · kg^{-1} · h^{-1}) in *db/db* mice. Figure 8*A* shows that fed plasma glucose levels in FGF-21–treated animals were 42% lower than in the control group. Interestingly, despite the lower fed plasma glucose level, the corresponding plasma insulin levels were elevated by 132% in the FGF-21–treated group compared with controls (Fig. 8*B*). No significant difference in plasma glucagon levels between vehicle and FGF-21–treated mice was observed (data not shown). Interestingly, FGF-21 treatment decreased plasma glucose but increased plasma insulin levels measured after an oral glucose challenge (Fig. 8*C* and *D*). The higher plasma insulin level in the FGF-21–treated group was associated with increased insulin content in the islets, as revealed by intense insulin staining (green) compared with islets obtained from the control group (Fig. 8*E*). Histological examination of pancreas sections from FGF-21– and vehicle-treated animals revealed a 340% increase in the number of islets per section (Fig. 8*F*), 280% more β-cells per section (Fig. 8*G*), and 130% more β-cells per islet (Fig. 8*H*). Finally, we observed a small but not significant reduction in staining of proliferating cellular nuclear antigen (PCNA), a cell prolifera-

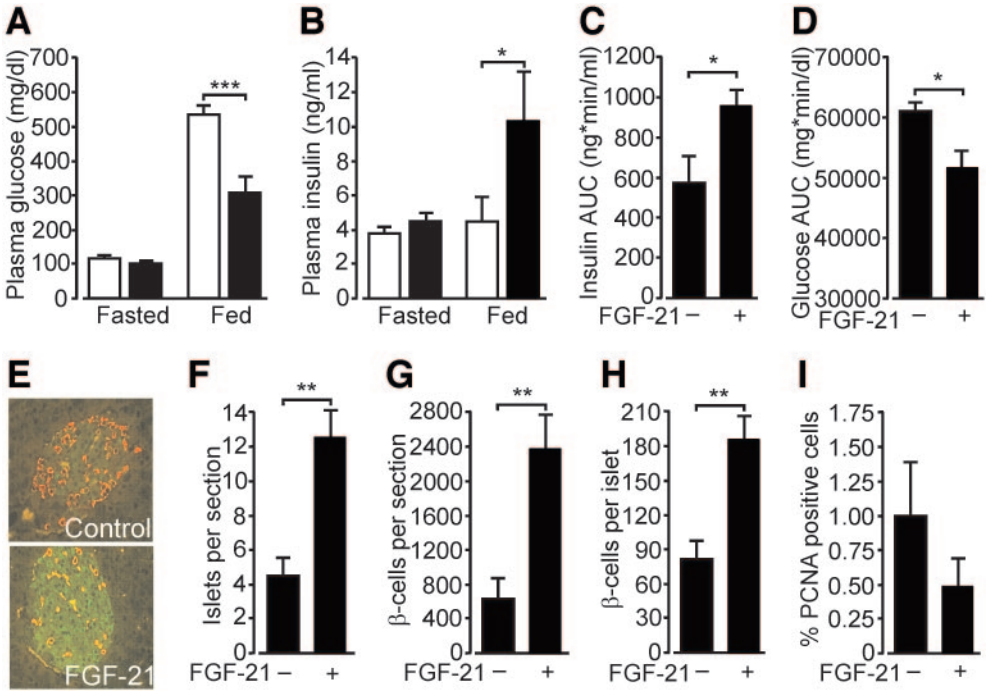

FIG. 8. FGF-21 preserves islet and β-cell number and improves glycemic control in *db/db* mice. *A* and *B*: Fasted and fed plasma glucose (*A*) and insulin (*B*) levels in *db/db* mice treated for 8 weeks with either vehicle (□) or FGF-21 (■; 11 μg · kg^{-1} · h^{-1}). *C* and *D*: Plasma insulin (*C*) and glucose (*D*) area under the curve (AUC) after oral glucose tolerance tests in vehicle- and FGF-21–treated *db/db* mice. *E*: Insulin (green) and glucagon (red) staining of pancreatic islet sections from vehicle- and FGF-21–treated *db/db* mice. Histograms depicted average number of islets per section (*F*) and the number of β-cells per section (*G*) and per islet (*H*), as well as the percent PCNA-positive islet cells (*I*) from vehicle- and FGF-21–treated *db/db* mice. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

tion marker, in cells within islets from the FGF-21–treated animals (Fig. 8*I*).

DISCUSSION

We recently discovered that FGF-21 stimulates glucose uptake in adipocytes and reduces plasma glucose and triglyceride levels to near-normal levels in animal models of type 2 diabetes (18). In this study, we provide novel information on the activity of FGF-21 in the pancreatic β-cell. Specifically, we demonstrate that FGF-21 preserves β-cell function, insulin content, and β-cell mass both in vitro and in vivo in diabetic mice.

FGF-21 is a typical FGF molecule with respect to its ability to stimulate FRS2α phosphorylation and activation of ERK1/2 and Akt signaling pathways. The effect of FGF-21 on ERK1/2 and Akt activity in INS-1E cells is transient and returned to basal within 2 h. It is well established that glucose-induced activation and nuclear translocation of ERK1/2 stimulates insulin gene transcription in β-cells (26–28). However, activation of ERK1/2 is not required for stimulation of insulin secretion (26,29). Our data show that FGF-21 stimulates insulin biosynthesis in both normal rat and diabetic mouse islets. The effect of FGF-21 on insulin content was mediated via ERK1/2 activation and was abolished by PD98059. However, ERK1/2 activation and increased insulin content did not translate into higher glucose-induced insulin secretion in islets from healthy animals. This clearly contrasts with a stimulation of glucose-evoked insulin secretion in islets isolated from diabetic rodents and treated in vitro with FGF-21. These data support the view that insulin biosynthesis is not rate limiting for glucose-induced insulin secretion under normal conditions, when only a small fraction of intracellular insulin is released after glucose challenge (30,31). However, insulin biosynthesis becomes an important factor for glucose-induced insulin response under diabetic conditions, characterized by a lack of adaptation of insulin biosynthesis rates to increased insulin demand (30,31). This conclusion is reinforced by our finding that FGF-21 partially rescued glucose-induced insulin secretion in rat islets exposed for a prolonged period of time to elevated glucose and free fatty acid levels. Several lines of evidence indicate that the loss of insulin stores participates in the deterioration of the insulin response to glucose and, consequently, the deterioration of glucose homeostasis as a whole. Thus, pancreatic insulin content is strongly decreased in rats with chronic hyperglycemia (32) and in *Psammomys obesus* after high-energy diet feeding (33), as well as in animals oversecreting insulin (31).

Administration of FGF-21 to diabetic *db/db* mice led to a significant lowering of circulating glucose levels, and, interestingly, in the long-term treated animals, it was associated with increased plasma insulin levels. Immunohistological examination of the pancreata revealed stronger insulin staining in the islets from the long-term FGF-21–treated animals. Furthermore, the number of islets per pancreas section as well as the number of β-cells per islet were significantly increased in the FGF-21–treated animals compared with the control group. The preservation of islets and β-cells in the FGF-21–treated animals is likely to result from a combination of at least two mechanisms. First, the ability of FGF-21 to reduce plasma glucose and circulating triglyceride levels (19) will in turn decrease β-cell glucolipotoxicity and consequently the rate of β-cell apoptosis. Second, FGF-21 directly reduces the rate of apoptosis in β-cells after cytokine treatment and glucose-lipid overload. The latter pathway is likely to result from

FGF-21 activation of the Akt signaling pathway. Activation of Akt has been convincingly linked to β-cell survival (5,34–36) and is involved in the mechanism of compensatory β-cell growth in insulin-resistant rats (37).

We previously demonstrated that FGF-21 is mitogenically inactive in vitro when tested in several otherwise FGF-sensitive cell lines and primary cells (19). This observation is reinforced in the current study because FGF-21 treatment of *db*/*db* mice for 8 weeks was not associated with an increased number of PCNA-positive islet cells. Furthermore, the rate of proliferation was not affected by incubation of INS-1E cells with 50 nmol/l FGF-21 for 48 h at either 3 or 15 mmol/l glucose (data not shown). The exact reason for the nonmitogenic nature of FGF-21 is currently unknown, but it is tempting to speculate that the transient activation of Akt is insufficient to trigger a mitotic response.

FGF-21 is preferentially expressed in liver (17), but the regulation of FGF-21's expression and secretion is unknown. Interestingly, we have been able to detect expression of FGF-21 in human, rat, and mouse islets as well as purified rat β-cells and INS-1E cells (S.S., unpublished observations). This observation suggests that FGF-21 production and secretion might be regulated and dependent on the metabolic status of the β-cell. Because FGF-21 has a short elimination half-life (18), local release of FGF-21 during high metabolic demand could represent a physiologically important mechanism not only for maintaining and improving β-cell performance but also for whole-body metabolic control via inhibition of glucagon release from pancreatic α-cells and stimulation of glucose uptake in adipocytes (18).

In summary, this study demonstrates that FGF-21 stimulates insulin gene transcription and insulin biosynthesis via activation of ERK1/2 signaling in the pancreatic β-cell. FGF-21 also promotes β-cell survival without inducing mitogenicity. Inhibition of β-cell apoptosis with FGF-21 is likely mediated via activation of the Akt signaling pathway. The ability of FGF-21 to increase insulin biosynthesis and promote β-cell survival resulted in a strong reduction in circulating glucose levels and an increased number of islets and β-cells in *db*/*db* mice after long-term administration of FGF-21. Thus, our data supports FGF-21 as a novel metabolic factor that exhibits the therapeutic characteristics essential for effective treatment of type 2 diabetes.

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