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Fructose-1,6-Bisphosphatase Overexpression in Pancreatic β-Cells Results in Reduced Insulin Secretion

A New Mechanism for Fat-Induced Impairment of β-Cell Function

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OBJECTIVE—Fructose-1,6-bisphosphatase (FBPase) is a gluconeogenic enzyme that is upregulated in islets or pancreatic β-cell lines exposed to high fat. However, whether specific β-cell upregulation of FBPase can impair insulin secretory function is not known. The objective of this study therefore is to determine whether a specific increase in islet β-cell FBPase can result in reduced glucose-mediated insulin secretion.

RESEARCH DESIGN AND METHODS—To test this hypothesis, we have generated three transgenic mouse lines overexpressing the human FBPase (huFBPase) gene specifically in pancreatic islet β-cells. In addition, to investigate the biochemical mechanism by which elevated FBPase affects insulin secretion, we made two pancreatic β-cell lines (MIN6) stably overexpressing huFBPase.

RESULTS—FBPase transgenic mice showed reduced insulin secretion in response to an intravenous glucose bolus. Compared with the untransfected parental MIN6, FBPase-overexpressing cells showed a decreased cell proliferation rate and significantly depressed glucose-induced insulin secretion. These defects were associated with a decrease in the rate of glucose utilization, resulting in reduced cellular ATP levels.

CONCLUSIONS—Taken together, these results suggest that upregulation of FBPase in pancreatic islet β-cells, as occurs in states of lipid oversupply and type 2 diabetes, contributes to insulin secretory dysfunction. ***Diabetes*** **57:1887–1895, 2008**

Type 2 diabetes is characterized by a chronic elevation of plasma glucose concentration, causing complications such as retinopathy, neuropathy, and nephropathy and increasing the risk of cardiovascular disease and stroke. Although insulin resistance may be the initiating defect, hyperglycemia in type 2 diabetes results from a relative deficiency of circulating insulin (1). Progressive deterioration in β-cell function is likely to result from exposure to the diabetic milieu (i.e., hyperglycemia and hyperlipidemia), thus setting up a positive feedback loop in which hyperglycemia and/or hyperlipidemia impairs β-cell function, leading to further hyperglycemia (2–7).

Chronic high fatty acid exposure results in increased basal and blunted glucose-mediated insulin secretion and reduced β-cell mass (2,5,8,9). This is associated with the pancreatic β-cells undergoing adaptive changes such that genes that are highly expressed under normal conditions, for example, insulin, PDX-1, and GLUT2, are underexpressed and genes that are poorly expressed in the pancreatic β-cells, such as hexokinase I, glucose-6-phosphatase, c-Myc, and acetate dehydrogenase, are shown to be upregulated (10,11).

One of the genes that is upregulated in β-cell lines under conditions of high fatty acid exposure is fructose-1,6-bisphosphatase (FBPase) (10–12), a regulated enzyme in the gluconeogenic pathway that catalyzes the dephosphorylation of fructose-1,6-bisphosphate to fructose-6-phosphate. FBPase is abundant in the liver and the kidneys but is poorly expressed in the pancreatic β-cells under normal conditions. In addition, FBPase was upregulated fivefold in islets from the diabetes-susceptible obese BTBR mouse compared with the diabetes-resistant C57BL/6 mouse (13). We have previously shown that FBPase is upregulated in the liver of mice or rats fed a high-fat diet (14,15) and in the New Zealand Obese (NZO) mouse, an obese model of type 2 diabetes (14,16). We have recently demonstrated that transgenic mice with specific overexpression of FBPase in the liver displayed increased glycerol gluconeogenesis (17).

From the abovementioned studies, it is evident that upregulation of FBPase is induced by fatty acids. However, whether an increase in FBPase alone can be detrimental to cellular function and in particular to β-cell insulin secretory rates has not been investigated.

To determine whether an increase in FBPase impairs insulin secretion, we generated both transgenic mice and stably transfected pancreatic β-cell lines (MIN6) overexpressing the human FBPase (huFBPase) gene. We demonstrated that overexpression of FBPase in β-cells results in impaired glucose-stimulated insulin secretion, which is associated with decreased glucose metabolism, resulting in reduced cellular ATP levels and cell proliferation.

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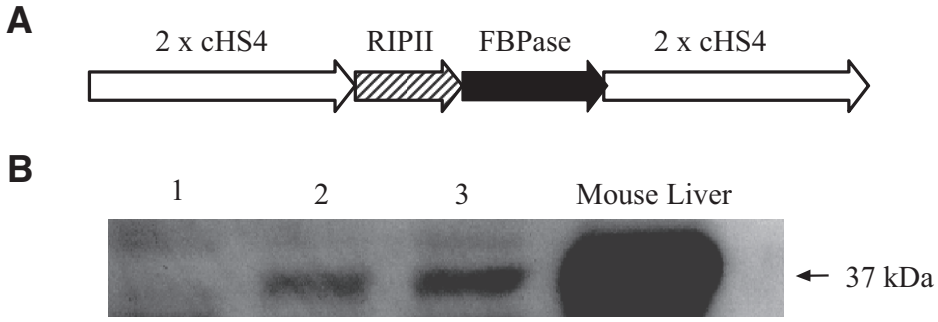

FIG. 1. Generation and testing of the transgenic construct. *A*: The complete 7,645-bp linear-insulated RIP-huFBPase construct, which consists of 695 bp of RIPII, the first intron of the insulin gene, a cDNA encoding huFBPase, and a downstream polyadenylation site (Poly A) flanked by two sets of the cHS4 insulator sequences. *B*: Transient transfection of FBPase in βTC-3 cells with the construct depicted in *A*, showing its capacity to produce the expected 37-kDa protein immunoreactive with the FBPase antibody in tissue culture. *Lane 1*, untransfected βTC-3 cells; *lanes 2* and *3*, transiently transfected βTC-3 cells, compared with a mouse liver sample.

RESEARCH DESIGN AND METHODS

MIN6 cells were obtained from Prof. Jun-ichi Miyazaki (Osaka University, Osaka, Japan). The RIP7 expression vector was provided by Prof. Thomas Kay (St. Vincent's Institute of Medical Research, Victoria, Australia). The FBPase primary antibody was a gift from Dr. Hideo Mizunuma (Akita University, Akita, Japan). Oligonucleotide primers were synthesized by Gene Works (Hindmarsh, South Australia, Australia). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, glutamine, and penicillin/streptomycin were from Invitrogen Australia (Mount Waverley, Victoria, Australia).

Human islet preparation. Human islets were prepared as previously described (18). Briefly, pancreata were removed from heart-beating deceased donors and disaggregated by infusing the ducts with cold Liberase enzyme (Liberase Human Islet; Roche Applied Science, Indianapolis, IN). Dissociated islets and acinar tissue was separated on a continuous Biocoll (Biochrom, Berlin) density gradient (polysucrose 400 and amidotrizolic acid).

Generation of transgenic construct. Standard molecular biology techniques were used to generate the transgenic construct (19) (Fig. 1*A*). Pancreatic β-cell specificity of transgene expression was directed by a segment of the rat insulin II gene promoter (RIP), generated by PCR amplification of the RIP7 expression vector (20). The amplified fragment included a 659-bp promoter/enhancer region, the first exon and intron of the rat insulin II gene, a *Cla*I cloning site in the second exon, and the class II E_α poly A terminator sequence (20). This fragment was then ligated into a pUC-based expression vector, designated the insulated transgenic vector, in which the promoter fragment was flanked on each side by two tandem copies of the chicken Hyper-Sensitive-4 (HS4) insulator sequence (21). The huFBPase fragment was generated by PCR amplification of the human liver cDNA as previously described (17) and ligated into the *Cla*I cloning site. The complete DNA sequence of the insulated RIP-huFBPase plasmid was determined, and expression of huFBPase was confirmed by transient transfection in a βTC3 cell line (22) and Western blot analysis. The insulated RIP-huFBPase expression fragment (Fig. 1*A*) was separated from the pUC-19 backbone by *Avr*II and *Spe*I restriction enzyme digestion and gel purification.

Transgenic mice. Transgenic mice were generated at the Central Microinjection Service of the Walter and Eliza Hall Institute of Medical Research (Parkville, Victoria, Australia) by injecting the insulated RIP-huFBPase DNA fragment (Fig. 1*A*) into the pronuclei of C57BL/6 fertilized oocytes, following established procedures (23). Offspring were genotyped for the presence of the transgene by PCR amplification of tail DNA using a forward primer from the RIP promoter (5′-CCTAAGTGACCAGCTACAGTC-3′) and a reverse primer from the coding region of huFBPase (5′-GCTGGGTCAACTCGCCCGTG-3′). Primers amplifying a glyceraldehyde-3-phosphate dehydrogenase fragment of 650 bp were included as an internal control for the PCR analysis. PCR reagents were obtained from New England Biolabs (Beverly, MA). The amplified DNA products were resolved on a 2% agarose gel.

Physiological studies were carried out on third- and fourth-generation heterozygous mice. Control mice for all experiments were the transgene-negative littermates. Mice were fed ad libitum with a standard rodent laboratory diet and kept under a 12-h light/12-h dark cycle (light on at 0700 h).

RNA and cDNA preparation. Total RNA was extracted from tissues and cultured cells using the Chomczynski and Sacchi procedure (24). RNA was treated with DNaseI (RNase-free; Ambion) before cDNA synthesis using 2 μg RNA and random primers with the Promega Reverse Transcription kit.

Real-time PCR analysis. Primers were designed for SYBR-green real-time PCR using the Primer-Express software (Applied Biosystems, Foster City, CA). The primers for β-actin (GenBank accession no. NM_007393) were forward, 5′-CGTGAAAAGATGACCCAGATCA-3′, and reverse, 5′-CACAGCCT GGATGGCTACGT-3′. The primers for total FBPase were homologous to both mouse (GenBank accession no. NM_019395) and human (GenBank accession no. NM_000507) liver FBPase sequences. The sequences were forward, 5′-AGC CTT CTG AGA AGG ATG CTC-3′, and reverse, 5′-GTC CAG CAT GAA GCA GTT GAC-3′. The transgene-specific primers for RIP-huFBPase mRNA but not the endogenous mouse FBPase sequence were forward, 5′-GTG TTG ACG TCC GTG TCG AA-3′, and reverse, 5′-ACC AGC TAC AGT CGG AAA CCA-3′. All primer sets were designed to amplify a product spanning an exon-intron boundary of the genomic DNA.

Each sample was run in triplicate for each set of primers (i.e., FBPase and β-actin). Gene expression was determined using ABI PRISM 7900 HT system (Applied Biosystems). The relative quantification comparative C_t, also known as the $\Delta\Delta C_t$ method using the ABI sequence detection software (SDS), was used.

Western blot analysis. FBPase protein levels were determined by immunoblotting as previously described (25). Protein lysates were resolved by SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride membranes (PolyScreen PVDF membrane; Perkins Elmer), and probed with the appropriate secondary antibody by standard protocols. Immunoreactive proteins were visualized with ECL Western blotting detection reagents (Amersham Bioscience).

Intravenous glucose tolerance test. The intravenous glucose tolerance test (IVGTT) was performed as previously described (26). A bolus of glucose (1 g/kg) was injected via a carotid artery catheter and blood was sampled at 0, 2, 5, 10, 15, and 30 min after the injection. Plasma was stored at −20°C for future glucose and insulin analysis.

Intraperitoneal glucose tolerance test. The intraperitoneal glucose tolerance test was performed as previously described (26). A bolus of glucose (2 g/kg) was injected intraperitoneally and blood was sampled at 0, 15, 30, 60, and 120 min after the injection. Plasma was stored at −20°C for future glucose and insulin analysis.

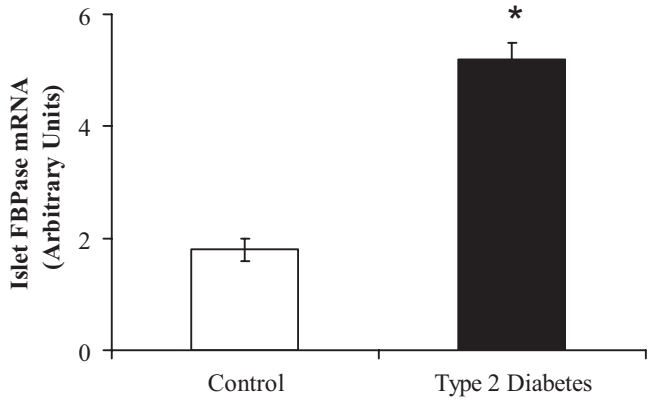

FIG. 2. Islet FBPase expression in patients with type 2 diabetes. FBPase mRNA levels in islets isolated from patients with type 2 diabetes ($n = 3$) and in age- and weight-matched control subjects ($n = 6$) was determined using real-time PCR. Results are presented as means ± SE. *$P < 0.05$.

Islet isolation and culture. Islets were isolated from the pancreas by collagenase digestion as previously described (27,28). After islets were freed from exocrine tissue, they were hand-picked under a stereomicroscope (Olympus, Tokyo, Japan) and transferred for overnight culture in RPMI 1640 with 10% (vol/vol) heat-inactivated fetal calf serum (FCS), in a 37°C humidified atmosphere of 95% air:5% CO_2.

After overnight culture, batches of islets were preincubated for 90 min in Krebs-Ringer bicarbonate buffer (KRBB) with 2.8 mmol/l glucose. Triplicate batches of five islets were then transferred to tubes containing 1 ml KRBB supplemented with 2.8 mmol/l in the absence or presence of 10 mmol/l L-arginine or 20 mmol/l glucose. After a 60-min incubation of the islets at 37°C, tubes were centrifuged at 2,000 rpm for 5 min, and 0.5-ml supernatant was removed for insulin analysis. The remaining 0.5 ml containing the islets was treated with 0.18 mol/l HCl/95% ethanol followed by sonication to determine insulin content.

TABLE 1
FBPase upregulation in mouse models of obesity and diabetes

Model	*n*	FBPase levels (fold increase of corresponding control)
NZO	3	1.9 ± 0.2*
High-fat–fed C57BL/6	3	2.1 ± 0.3*
C57BL/KsJ-*db/db*	5	3.5 ± 1.0†

Data are means ± SE for the number of mice indicated. Pancreatic FBPase was measured by immunoblotting in NZO and high-fat–fed (60% [wt/wt] for 16 weeks) C57BL/6 mice, and islet FBPase mRNA levels were determined in C57BL/KsJ-*db/db* mice using real-time PCR. *$P < 0.05$, †$P < 0.01$.

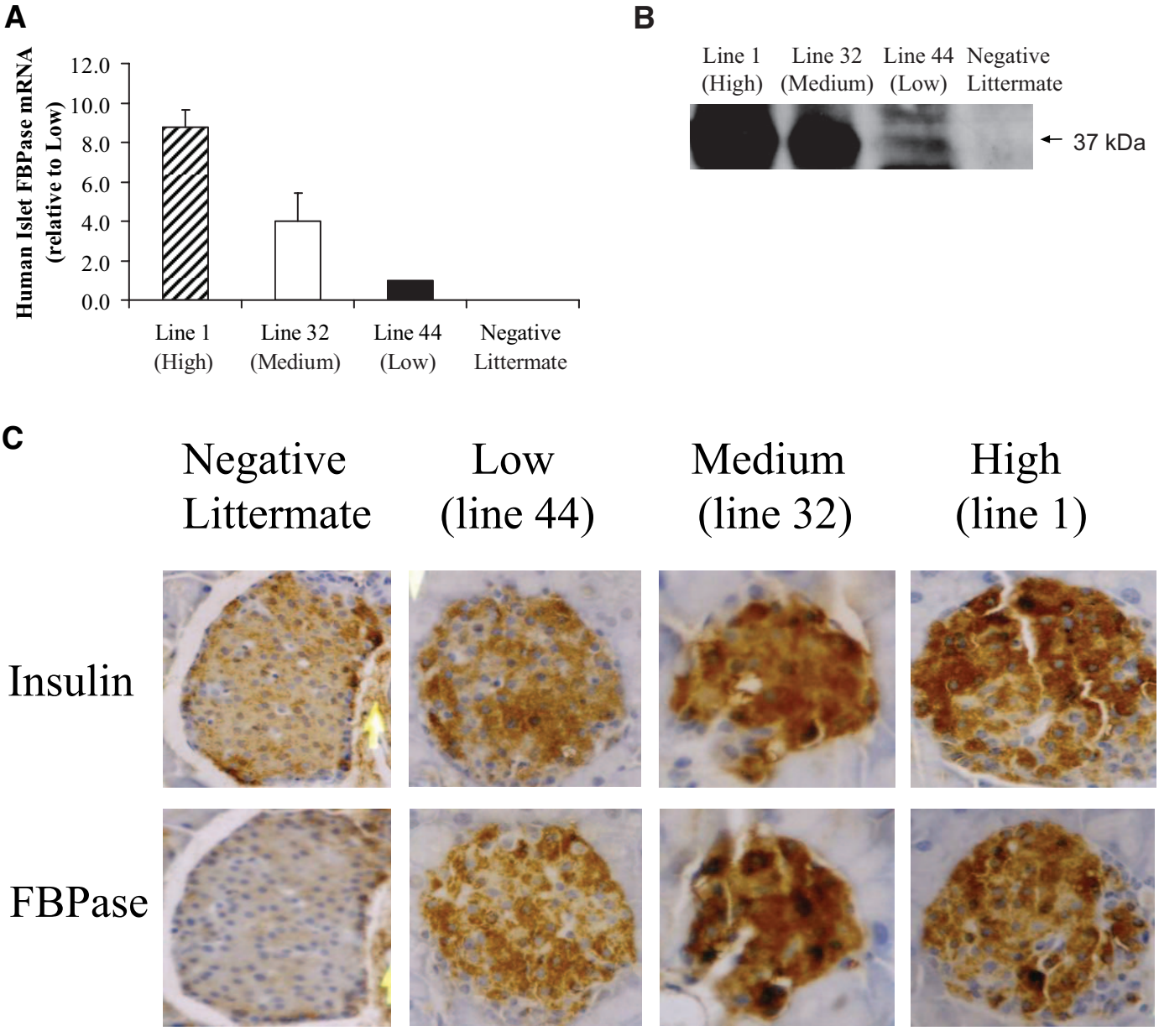

FIG. 3. Expression of the FBPase gene in transgenic mice. *A*: huFBPase mRNA levels in three transgenic mouse lines (1, 32, and 44), showing high, medium, and low expression levels of the transgene, relative to the lowest expressing transgenic line 44. As expected, the transgene was not detected (ND) in negative littermate mice. *B*: Immunoblotting of pancreatic samples from transgenic lines 1, 32, and 44, showing high, medium, and low levels of the 37-kDa FBPase protein, in accordance with the mRNA data shown in *A* above. *C*: Immunohistochemistry of pancreata from the three FBPase transgenic mouse lines (1, 32, and 44) and a negative littermate, showing insulin immunostaining in all sections, whereas FBPase immunostaining was only seen in the transgenic pancreata, localized to the insulin staining.

TABLE 2
Body weight, fasting plasma glucose, and plasma insulin concentrations; pancreatic insulin content levels; and glucose tolerance in 10-week-old β-cell FBPase transgenic mice

	Line 1 (high)	Line 32 (medium)	Line 44 (low)	Negative littermate
Male body weight (g)	21.4 ± 1.0	22.9 ± 0.4	24.4 ± 0.5	22.5 ± 0.8
Female body weight (g)	17.2 ± 0.51	16.7 ± 0.35	17.6 ± 0.10	17.7 ± 0.30
Plasma glucose (mmol/l)	7.0 ± 0.4	7.5 ± 0.3	10.3 ± 0.5	8.0 ± 0.6
Plasma insulin (ng/ml)	0.3 ± 0.02*	0.5 ± 0.10*	0.3 ± 0.10*	0.8 ± 0.16
Pancreatic insulin content (ng/μg protein)	883 ± 197	739 ± 95	694 ± 61	661 ± 72
Glucose area under the curve (mmol/l ×120 min)	2,500 ± 101*	1,773 ± 103	1,460 ± 125	1,863 ± 113

Data are means ± SE ($n = 10$). *$P < 0.05$ compared with the negative littermate.

Insulin levels were determined with a double antibody radioimmunoassay using a rat-specific insulin antibody and rat insulin as a standard (Linco Research, St. Charles, MO).

Cell culture. Pancreatic cell lines were routinely cultured in DMEM supplemented with 25 mmol/l glucose, 15% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μmol/l β-mercaptoethanol at 37°C with 5% CO_2.

Transient transfection. Cells were seeded into six-well plates at 2×10^6 cells/well 2 days before transfection. Lipofectamine 2000 (Invitrogen) was used to cotransfect cells with 1–2 μg supercoiled insulated RIP-huFBPase plasmid (Fig. 1*A*) and 0.1–0.2 μg pEGFP-C1 (Clontech Laboratories) reporter plasmid per well according to the manufacturer's protocol. Cells were harvested 48 h posttransfection, and cell pellets were stored at −80°C for subsequent analysis.

Stable transfection. Cells were transfected with the linear-insulated RIP-huFBPase fragment (Fig. 1*A*) using Lipofectamine 2000 as above, but the cotransfecting plasmid was pPGK-puro to enable selection with the antibiotic puromycin. Forty-eight hours after transfection, the cell medium was supplemented with 6 μg/ml puromycin, and resistant cells started to form colonies after ~7 days. Colonies were picked, amplified, and screened for FBPase expression by RNA and protein analysis.

Cell proliferation assay. Cell proliferation was assessed over a 7-day period using trypan blue dye exclusion. At day 0, cells were seeded at 1×10^5 cells/well in 0.5 ml DMEM in 24-well plates. Cells were then counted manually at days 1, 3, 5, and 7.

FBPase enzyme activity assay. Proteins were extracted by homogenizing 1×10^6 cells in 0.5 ml homogenization buffer (50 mmol/l triethanolamine, pH 7.2, 1 mmol/l dithiothreitol, and 10 mmol/l EDTA) followed by centrifugation at 40,000*g* for 40 min at 4°C. FBPase enzyme activity was determined using a spectrophotometric-coupled enzyme assay as previously described (16,25).

Insulin secretion and content in MIN6 cells. Cells were seeded at 3×10^5 cells/well in 0.5 ml DMEM in 24-well plates. After 24 h, cultured cells were washed in modified KRBB containing 2.8 mmol/l glucose and preincubated for 30 min in 0.5 ml of the same medium at 37°C. This medium was then replaced with 0.5 ml prewarmed KRBB containing 2.8 mmol/l glucose, 2.8 mmol/l glucose plus 10 mmol/l methyl-pyruvate, 16.8 mmol/l glucose, or 16.8 mmol/l glucose plus a nonglucose secretagogue cocktail. The secretagogue cocktail contained 0.1 mmol/l 3-isobutyl-1-methylxanthine (IBMX), 10 mmol/l arginine, and 5 μmol/l carbamylcholine chloride (carbachol) (29,30). The cells were incubated for 60 min at 37°C, and an aliquot of medium was removed for analysis of insulin. To measure the total insulin content, the cell monolayers were washed twice in PBS and harvested in 0.5 ml 0.18 mol/l HCl/95% ethanol per well followed by sonification for 20 min in a Branson 2200 bath sonicator (Branson Ultrasonics, Danbury, CT). The cell lysates were centrifuged at 13,000 rpm for 10 min, and supernatants were retained and stored at −20°C until analysis. Insulin levels were determined with a double-antibody radioimmunoassay using rat-specific insulin antibody and rat insulin as a standard (Linco Research).

Measurement of glucose utilization and glucose oxidation. Glucose utilization of cells was measured using the production of tritiated water from the metabolism of D-[5-^{3}H]glucose as previously described (31,32). Tritiated H_2O was then measured by liquid scintillation counting. Glucose oxidation was estimated by assessing the formation of $^{14}CO_2$ from the metabolism of [U-^{14}C]glucose (33). The amount of $^{14}CO_2$ was determined by liquid scintillation counting.

Measurement of glycolytic intermediates. Fructose 1,6-bisphosphate and fructose 6-phosphate assays were performed on the supernatant as described before (34,35).

Measurement of cellular ATP content. The amount of ATP was measured fluorimetrically using the ATP bioluminescent assay kit from Sigma (St. Louis, MO) as previously described (30).

Immunohistochemistry. For immunohistochemical detection of FBPase and insulin, pancreata from control and transgenic mice were fixed in freshly made 4% paraformaldehyde, processed for paraffin, embedded, and sectioned at 5 μm. Slides were incubated with either a guinea-pig anti-porcine insulin antibody (DAKO, Carpentaria, CA) diluted 1:100 or rabbit-anti-rat FBPase antibody diluted 1:100. The sections were then washed with Tris hydrochloride (10 mmol/l, pH 7.4) for 10 min and then incubated with the DAB (3,3′-diaminobenzidine) chromagen for 1 min. Sections were counterstained with hematoxylin and mounted with distyrene plasticizer and xylene mounting media and observed under a microscope.

Ethics approval. All of the animal studies were approved by the Austin Health Animal Ethics committee. Consent for collection of the human tissue samples was obtained by the Red Cross Transplantation Service, and St. Vincent's Sydney Hospital Human Research Ethics Committee gave approval for the human studies.

Statistical analysis. Area under the curve was calculated using the trapezoidal rule. Results are presented as means ± SE. Statistical significance was assessed using the Student's t test. Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

To confirm the literature reports that FBPase is upregulated by lipid oversupply, we first assessed the pancreatic FBPase levels in a naturally occurring obese animal model, the NZO mouse; in a diet-induced obesity animal model, the C57BL/6 mouse fed a 60% fat diet for 16 weeks; and in the diabetic C57BL/KsJ-*db/db* mouse. We found that both the NZO and high-fat–fed C57BL/6 mice had a twofold increase in pancreatic FBPase protein levels, whereas there was a 3.5-fold increase in FBPase mRNA levels in *db/db* mice compared with their respective controls (Table 1).

To determine whether patients with type 2 diabetes have elevated FBPase, pancreatic islets were isolated from three diabetic and six age-matched (50.6 ± 3.4 vs. 51 ± 4.4 years) and BMI-matched (26.5 ± 0.5 vs. 27.8 ± 4.0 kg/m^2) control subjects as previously described (18). FBPase mRNA levels were measured by real-time PCR and were corrected for expression using the housekeeping gene TATA-box binding protein. FBPase levels were increased by twofold in the human diabetic islets ($P < 0.05$; Fig. 2). Taken together, these data support previous experimental evidence that FBPase is upregulated by lipid oversupply/diabetes in animal models and suggests for the first time that islet FBPase may also be increased in patients with type 2 diabetes.

To determine whether a specific upregulation of FBPase in islet β-cells can result in defective insulin secretion, transgenic mice were generated. A construct was generated with the human liver FBPase cDNA, driven by the minimal (654 bp) RIP and flanked by the chicken HS4 (cHS4) insulator sequences (Fig. 1*A*). To test whether this construct expressed protein, it was transiently transfected into the immortalized pancreatic β-TC3 cell line. Immunoblotting of transiently transfected cells showed a band immunoreactive with the anti-FBPase antibody at the expected size of 37 kDa (Fig. 1*B*), confirming that the construct was capable of producing FBPase. The RIPII-

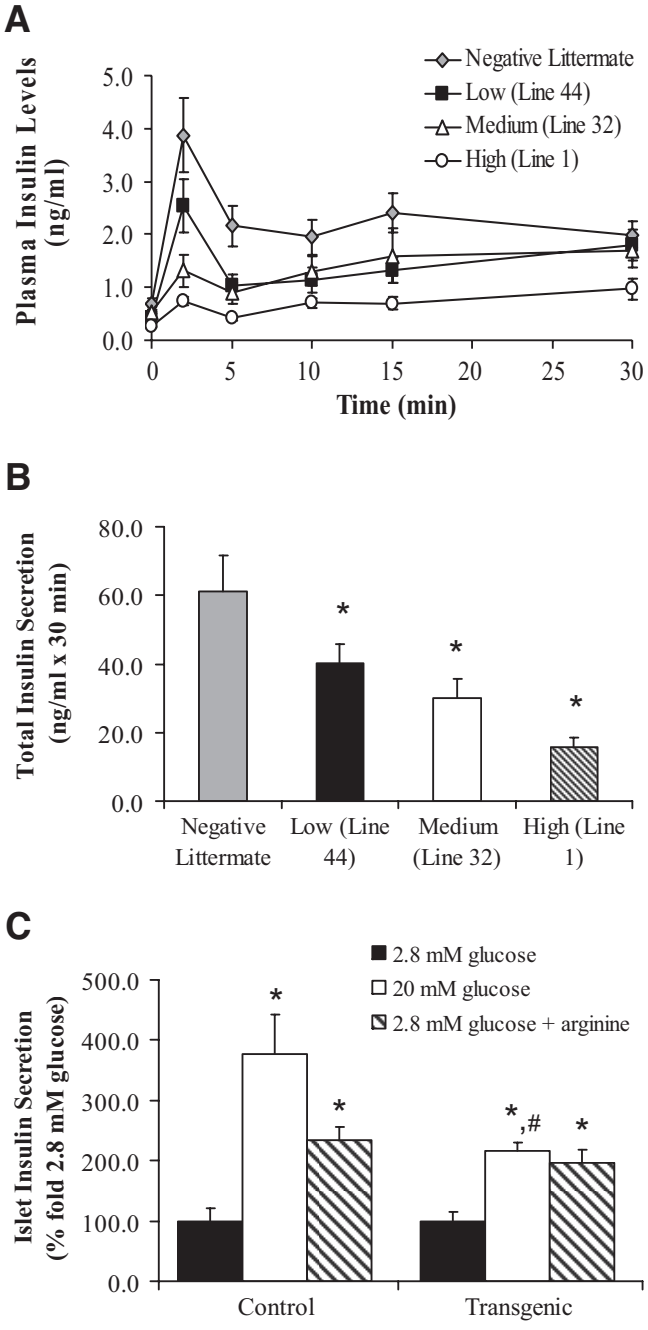

FIG. 4. Insulin secretory responses in FBPase transgenic and negative littermate mice. *A*: Plasma insulin levels in FBPase transgenic and negative littermate mice during the IVGTT. *B*: Total insulin secretion, calculated as the area under the insulin curve over the 30 min of the IVGTT in the three transgenic FBPase and negative littermate mice. Results are presented as means ± SE (n = 6–14). *$P \leq 0.05$; **$P < 0.01$. *C*: Insulin secretion measured from islets isolated from line 32 transgenic and negative littermate control mice. Results are presented as means ± SE (n = 3). *$P < 0.05$ compared with 2.8 mmol/l glucose; #$P < 0.05$ compared with control islets.

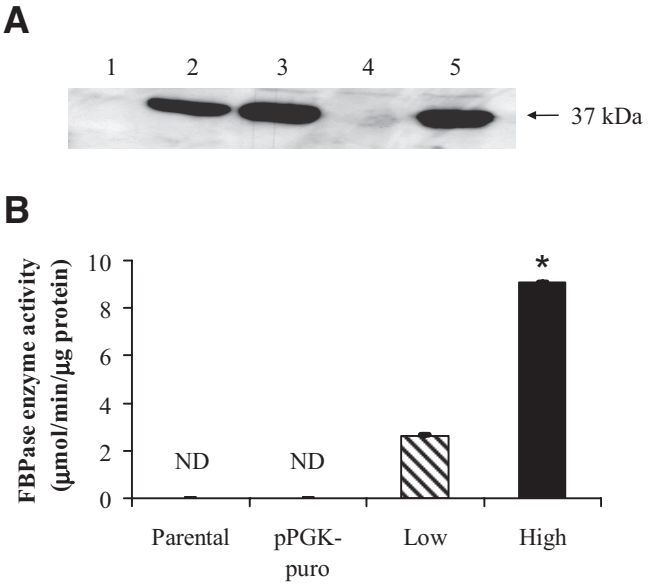

FIG. 5. Generation of MIN6 cells overexpressing huFBPase. *A*: MIN6 cells were cotransfected with 1–2 µg transgene construct shown in Fig. 1*A* and 0.1–0.2 µg pPGK-puro selection plasmid. Eighteen puromycin-resistant colonies were isolated, of which three colonies were selected to be extensively studied with no (pPGK-puro), low, and high levels of the FBPase protein, respectively, as assessed by immunoblotting. *Lane 1*, parental MIN6 cells; *lane 2*, low FBPase; *lane 3*, high FBPase; *lane 4*, pGK-puro (no FBPase); *lane 5*, mouse liver control. *B*: FBPase enzyme activity in the cell lines depicted in *A*, showing increased activity in the cells with the highest FBPase protein levels, whereas the parental MIN6 and pPGK-puro cells had no detectable (ND) enzyme activity. Results are presented as means ± SE (n = 3). *$P < 0.05$ compared with low-expressing FBPase transgenic cells.

huFBPase chimeric gene was microinjected into mouse embryos, and four founder mice (on the C57BL/6 genetic background) were obtained. One of the lines did not express the transgene and was therefore excluded from further analysis. The studies presented here were performed using three transgenic mouse lines denoted 1, 32, and 44. The three lines expressed the transgene at different levels. The highest expresser (line 1) exhibited an ~9 times higher transgene mRNA level than the lowest expresser (line 44), as estimated by real-time PCR (Fig. 3*A*). No expression of the transgene was detected in the liver, muscle, and brain of the transgenic mice (data not shown). Immunoblotting confirmed line 1 as the highest expresser of FBPase, followed by lines 32 and 44, whereas very low levels of FBPase protein were detected in the pancreata of negative littermate mice (Fig. 3*B*). Although it is not quantitative, immunohistochemistry of FBPase transgenic and negative littermate pancreata showed islet FBPase staining in the same region as insulin immunostaining, suggesting β-cell–specific expression of the transgene in the transgenic mice, whereas the negative littermate mice showed no islet FBPase staining (Fig. 3*C*).

Body weight of the three FBPase transgenic lines was not different to the negative littermates (Table 2). Fasting plasma glucose levels were not different, except for a slight increase in line 44 compared with the negative littermate mice (Table 2). However, fasting plasma insulin concentrations were significantly lower in all three FBPase transgenic lines compared with negative littermates (Table 2). Pancreatic insulin content was comparable between transgenic mice and that of the negative littermate mice (Table 2).

When subjected to an IVGTT, all three lines showed a significant impairment in insulin secretion (Fig. 4*A*). Consequently, the area under the insulin curve was reduced in all three transgenic lines compared with the negative littermates (Fig. 4*B*). Glucose tolerance was significantly impaired only in line 1 (Table 2), which had the highest expression of FBPase and most significant defect in insulin secretion compared with the negative littermates.

Insulin secretion was also determined in vitro in isolated islets from line 32 and the data presented in Fig. 4*C*. In

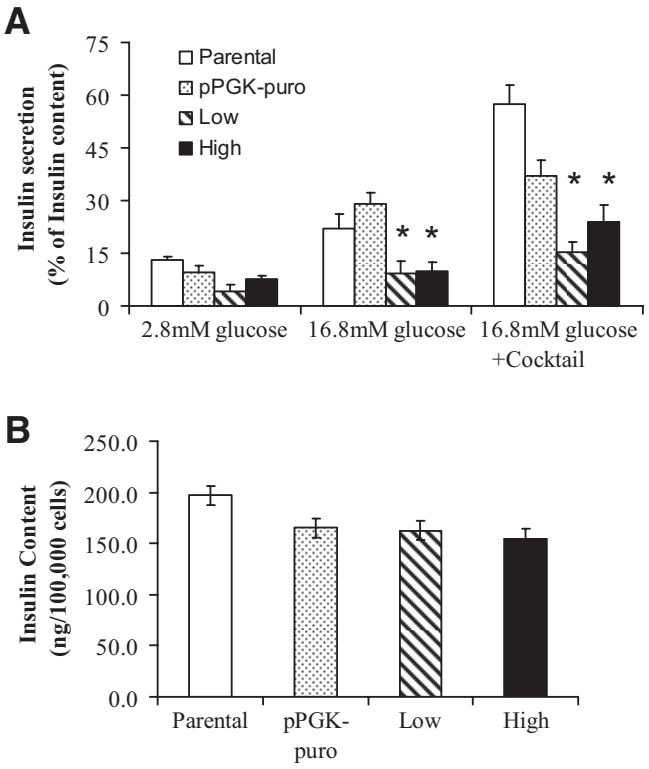

FIG. 6. Glucose- and non–glucose-induced insulin secretion in FBPase transgenic MIN6 cells. *A*: The cells were incubated in KRBB containing 2.8 mmol/l glucose, 16.8 mmol/l glucose, or 16.8 mmol/l glucose plus a cocktail of secretagogues, including 10 mmol/l arginine, 0.1 mmol/l IBMX, and 5 μmol/l carbachol for 1 h. Medium was taken to determine levels of insulin secretion. *B*: Total cellular insulin content was determined by lysing the cells with 0.18 mol/l HCl/95% ethanol, followed by sonification. Insulin secretion was then expressed as relative to total cellular insulin content of cells. Results are presented as means ± SE of three independent experiments. *$P < 0.05$ compared with parental MIN6 cells.

response to high glucose, line 32 transgenic islets secreted significantly less insulin compared with negative littermate islets. Furthermore, in response to arginine, which is proposed to act distal to the ATP-sensitive K^+ channel by directly depolarizing the plasma membrane, there was no difference in insulin release between transgenic and negative littermate islets.

To investigate the biochemical mechanism by which FBPase upregulation resulted in significantly reduced glucose-mediated insulin secretion, transgenic cell lines (MIN6) overexpressing the huFBPase gene were generated and characterized as follows. The FBPase and pPGK-puro constructs were stably transfected into MIN6 cells. Eighteen colonies, which grew in puromycin, were selected and checked for FBPase expression and protein levels. From this screen, three colonies were chosen to be more extensively studied: a colony that had no FBPase expression but grew in puromycin as a result of the pPGK-puro transgene, a colony with relatively low expression, and a colony with relatively high expression of FBPase (Fig. 5*A*). FBPase enzyme activity was also assayed and shown to be elevated in the high-expressing cells compared with the low-expressing cells (Fig. 5*B*). Furthermore, the parental and pPGK-puro cells had undetectable activity, concurring with the immunoblotting data (Fig. 5*A*).

To determine whether the overexpression of FBPase affected insulin secretory function, the parental MIN6, pPGK-puro, and the two overexpressing cell lines were incubated with basal glucose (2.8 mmol/l), high glucose (16.8 mmol/l), or high glucose plus a cocktail of secretagogues (arginine, IBMX, and carbachol), and insulin release was determined. All cell lines released similar levels of insulin under basal conditions, although there was a trend for insulin secretion to be lower in the FBPase-transfected cells (Fig. 6*A*), reflecting the lower basal plasma insulin levels in the transgenic mice (Table 2). Both the parental MIN6 and pPGK-puro cells showed increases in insulin secretion in response to high glucose and high glucose plus the secretagogue cocktail, respectively (Fig. 6*A*). In contrast, FBPase-overexpressing cells did not increase insulin release in response to 16.8 mmol/l glucose and showed a much-blunted response to glucose plus the cocktail secretagogue compared with the parental cell line (Fig. 6*A*). Insulin content was determined and found to be not significantly different in the transfected cells compared with the parental MIN6 (Fig. 6*B*). Therefore, overexpression of FBPase significantly reduced glucose-mediated insulin secretion, reproducing and confirming what was found in vivo in the transgenic mice overexpressing FBPase in β-cells.

A mechanism by which increased FBPase may reduce insulin secretion is by decreasing glucose utilization and energy production. Because islets from patients with type 2 diabetes have markedly decreased expression of phosphofructokinase-1 (PFK-1) (18), increased FBPase would be expected to further impair glycolysis. Therefore, glucose utilization and glycolytic intermediates were measured. All cell lines had comparable glucose utilization when cultured at basal (2.8 mmol/l) glucose concentrations (Fig. 7*A*, *left*). However, in response to high (16.8 mmol/l) glucose, glucose utilization (assessed using 5-^{3}H-glucose) was significantly lower in cells overexpressing FBPase compared with the parental and pPGK-puro controls (Fig. 7*A*, *right*). Furthermore glucose oxidation (measured using [U-^{14}C]glucose) was reduced in the high-overexpressing FBPase compared with the parental MIN6 cells when incubated with both 2.8 and 20 mmol/l glucose (Fig. 7*B*). FBPase catalyzes the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate and is opposed by PFK-1 in the glycolytic pathway. Overactivity of FBPase would be expected to result in overabundance of the product and a reduction of the substrate. Figure 7*C* shows that the substrate (fructose-1,6-bisphosphate) was lower and the product (fructose-6-phosphate) was higher in the FBPase-overexpressing cell lines compared with the parental MIN6 cells.

To determine whether the decrease in glucose utilization caused a reduction in energy levels, cellular ATP levels were measured and found to be comparable among the four cell lines at basal (2.8 mmol/l) glucose (Fig. 7*D*). However, with high (16.8 mmol/l) glucose culture, the FBPase-overexpressing cell lines showed decreased ATP levels compared with the parental and control cell lines (Fig. 7*D*). Together, these data suggest that decreased glucose utilization and energy production may be responsible for the reduced insulin secretion, in β-cells overexpressing the human liver FBPase gene.

A reduction in energy availability may affect cell growth. To study this, the proliferation rate of the four cell lines was determined over 7 days. There was an increase in cell number in all cell lines over the 7-day test period (Fig. 8*A*). However, the cell lines overexpressing FBPase showed a slower rate of cell proliferation compared with the paren-

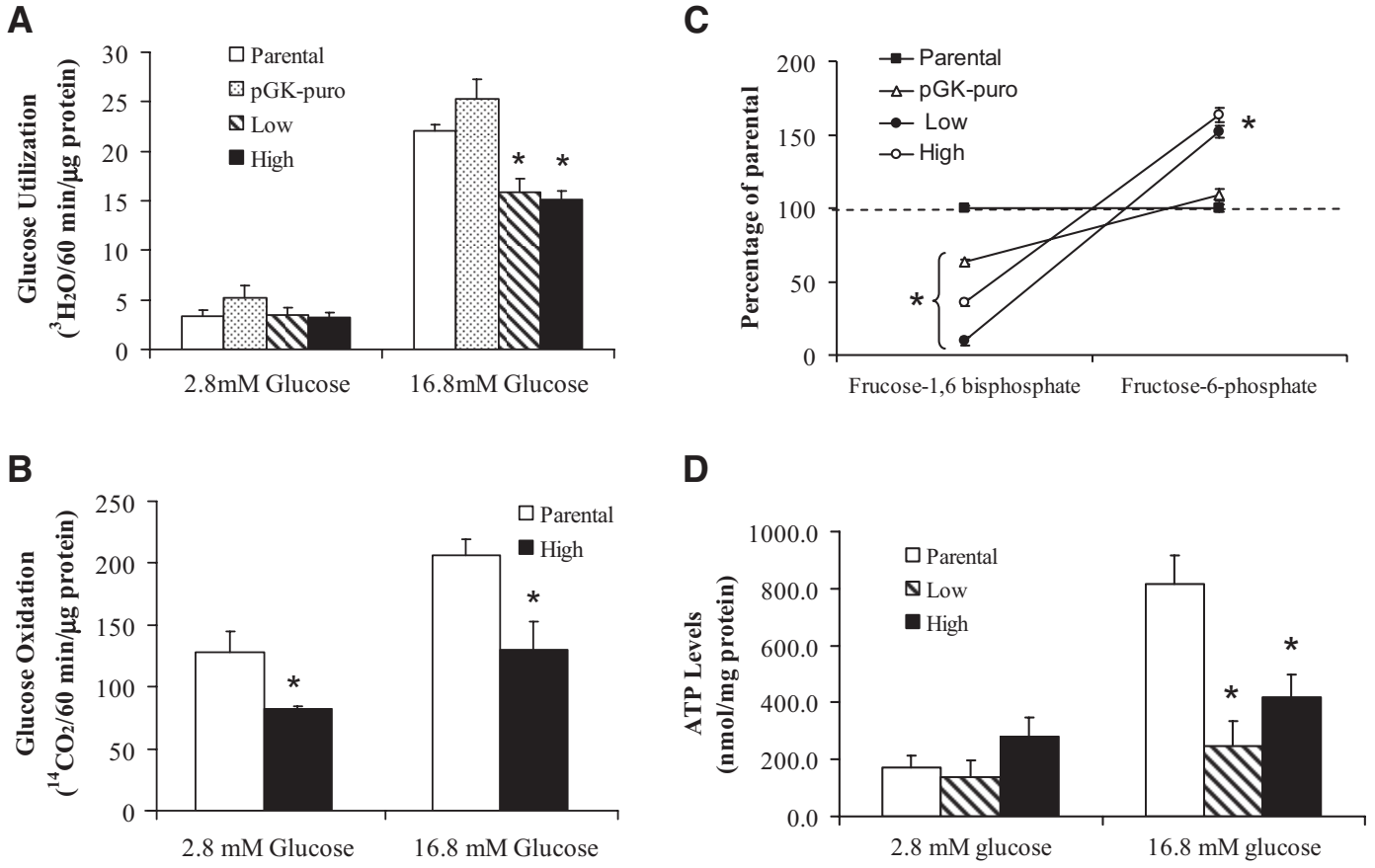

FIG. 7. Glucose utilization, glucose oxidation, glycolytic intermediate, and cellular ATP levels in MIN6 cells overexpressing FBPase. *A*: Glucose utilization was measured by the conversion of [5-^{3}H]glucose into ^3H_2O as described in RESEARCH DESIGN AND METHODS. Results are expressed as means ± SE of three independent experiments. **P* < 0.05 compared with parental MIN6 cells. *B*: Glucose oxidation was measured by the conversion of [U-^{14}C]glucose into $^{14}CO_2$ as described in RESEARCH DESIGN AND METHODS. Results are expressed as means ± SE of five independent experiments. **P* < 0.05 compared with parental MIN6 cells. *C*: Fructose-1,6-bisphosphate and fructose-6-phosphate levels in parental, pGK-puro (no FBPase), low-FBPase, and high-FBPase MIN6 cells. Results are expressed as means ± SE of three independent experiments. **P* < 0.05 compared with control MIN6 cells. *D*: Cellular ATP content was measured in parental, low-FBPase, and high-FBPase MIN6 cells. Results are expressed as means ± SE of three independent experiments. **P* < 0.05 compared with parental cells.

tal and the pPGK-puro control cell lines, respectively (Fig. 8*A*). This difference in cell proliferation was particularly pronounced at days 5 and 7.

To confirm that the reduction in growth rate was due to a deficiency in energy availability secondary to reduced rate of glycolysis, cells were supplemented with sodium pyruvate to a final concentration of 2.5 mmol/l. As can be seen in Fig. 8*B*, pyruvate supplementation normalized growth rates at 5 and 7 days in the low-expressing and at 5 days in the high-expressing FBPase-MIN6 cells. These data further support the assertion that increased expression of FBPase results in impaired cellular function by reducing energy availability.

To investigate whether pyruvate stimulation improves the insulin secretory function of FBPase-overexpressing MIN6 cells, the parental and low-expressing cells were pretreated with pyruvate (to a final concentration of 2.5 mmol/l), and insulin secretory function was determined at basal glucose (2.8 mmol/l) concentrations. We chose the low-expressing cell line because we have shown in Fig. 8*B* that in this cell line, 2.5 mmol/l pyruvate supplementation rescued the cell proliferation defects in FBPase-overexpressing cells. In concordance with these data, pyruvate stimulation also resulted in significantly higher insulin secretory rates in the FBPase-transfected cells, compared with control cells transfected with the puromycin gene only (insulin content 68.3 ± 2.6 vs. 16.4 ± 1.5%, $n = 4$, $P < 0.05$).

DISCUSSION

Islet β-cell dysfunction is a key characteristic of patients with type 2 diabetes that results in hyperglycemia. Hyperglycemia, in conjunction with the dyslipidemia, which is often present in patients with type 2 diabetes, can further exacerbate the defects in insulin secretion. A number of studies have shown that FBPase is upregulated in response to high glucose or fatty acid conditions (10–12,36). Importantly, direct evidence for the stimulatory effect of fat on FBPase expression has been provided in vitro using pancreatic β-cell lines incubated with palmitate (10,11). Although the mechanism for this induction is not known, we speculate that fatty acids increase the transcription of the FBPase gene. We have extended these results and have shown here that in obese (NZO) or diabetic (C57BL/KsJ-*db-db*) mice and in a high-fat–fed mouse model, there is a two- to threefold increase in pancreatic/islet FBPase levels. Furthermore, we show for the first time that increased FBPase expression has direct relevance to human disease because its expression was increased twofold in islets from patients with type 2 diabetes. These studies, however, do not provide any information on whether this increase in FBPase has functional consequences in islet β-cells. To determine whether overexpression of FBPase impairs insulin secretion, we generated three lines of transgenic mice with different levels of islet FBPase over-

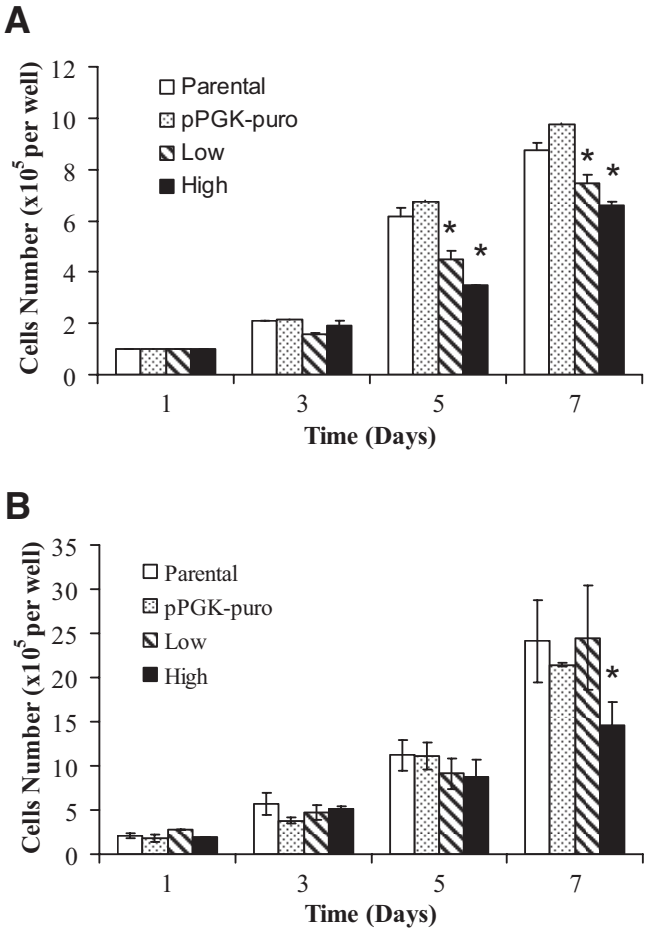

FIG. 8. Cell proliferation rates in MIN6 cells overexpressing FBPase. *A*: Cells were counted on days 1, 3, 5, and 7 after seeding. Results are expressed as means ± SE of three independent experiments. **P* < 0.05 compared with parental cells. *B*: The cell culture medium was supplemented so that the final concentration of pyruvate was 2.5 mmol/l, and cells were counted on days 1, 3, 5, and 7 after seeding. Results are expressed as means ± SE of three independent experiments. **P* < 0.05 compared with parental cells.

expression. We demonstrated that insulin secretion in response to glucose was reduced in a dose-dependent manner with greater overexpression, causing more severe impairment of secretion. Interestingly, despite a reduction in insulin secretion in all three transgenic lines tested, glucose tolerance was impaired only in line 1 with the highest FBPase overexpression and the greatest reduction in secretion. This probably reflects the robust nature of the mouse and that a severe reduction in insulin secretion is required before it affects glucose tolerance. All of this suggests that upregulation of islet FBPase by high glucose/lipid levels can result in reduced glucose-mediated insulin secretion in vivo. Using MIN6 cells, an in vitro β-cell model, we went on to show that the most likely mechanism by which FBPase overexpression impairs insulin secretion is a reduction in glycolytic flux resulting in reduced energy production. Arginine-induced secretion was not impaired in the transgenic islets. Because arginine acts by directly depolarizing the plasma membrane, this result suggests that the secretory machinery is functioning appropriately and supports the notion of decreased glycolytic flux as the cause of reduced insulin secretion in the transgenic mice.

Many mechanisms for β-cell dysfunction and death have been proposed, including high glucose–induced oxidative stress (8), fatty acid–induced defects in secretion and activation of apoptotic pathways (30,37), islet amyloid–mediated β-cell death (38,39), and others (40). Some of these may be inherent defects that are exposed when the islet is stressed with obesity and excess nutrients. It is clear from our own data and that of other researchers that the upregulation of FBPase is a consequence of the diabetic milieu. The question then arises: What role does this particular abnormality play in the pathogenesis of type 2 diabetes? We show in this study that FBPase is not normally expressed at high levels in the islet. Furthermore, we and others have shown that this enzyme is upregulated by fat. Noting this, together with the data in this report that a specific upregulation of FBPase causes impaired insulin secretion, we suggest that the obese/diabetic milieu of increasing lipidemia stimulates the expression of islet FBPase, which contributes to the deteriorating function of the β-cell, resulting in further diminution of insulin release.

We show in this report that FBPase expression is increased in patients with type 2 diabetes and that a specific increase in this gene can lead to impaired insulin secretory function. However, this is not the only protein whose expression is altered in diabetes. It has been shown that there are >200 genes upregulated in islets from patients with type 2 diabetes compared with control subjects (18), and Busch et al. (10) have shown that >100 genes are overexpressed >1.9-fold when MIN6 cells are cultured in high-fat conditions (including FBPase). One approach to determine the functional consequences would be to systematically inhibit each of the overexpressed genes in diabetes and to determine whether cellular function is improved. Although for most genes, this would have to be done in vitro using, for example, siRNA technology, for FBPase, this can now be done in vivo because specific inhibitors of this enzyme have been developed as therapeutic targets to treat type 2 diabetes (41,42). MB06322 (CS-917) is one such compound that efficiently inhibits the enzyme and reduces gluconeogenesis in hepatocytes and in vivo in ZDF rats, resulting in a substantial lowering of plasma glucose levels in these diabetic animals (41,42). It would be of interest to determine whether this or other compounds that inhibit islet FBPase can improve insulin secretory function and the diabetic phenotype of animal models, such as the ZDF rat or C57BL/KsJ-*db/db* mouse, or even in patients with type 2 diabetes.

It is of interest that reduction in glycolytic rate and the ensuing reduction in ATP levels resulted in a slowing of β-cell growth in vitro. It is important to note that the insulin secretory experiments were performed after 24 h of seeding the same amount of cells, a time that there was no difference in cell number regardless of whether FBPase was expressed (Fig. 8). We therefore do not believe that differences in cell number can explain the blunting in glucose-mediated insulin release with FBPase overexpression. As stated above, we provide evidence that the decreased insulin release is due to reduced glycolytic flux.

Taken together, we have demonstrated that obese and hyperglycemic mice and patients with type 2 diabetes display an increase in the expression of pancreatic islet FBPase. We consequently generated transgenic mice and cell lines with a specific increase in islet β-cell FBPase, and these were shown to have reduced insulin secretion associated with impaired glucose metabolism and ATP generation. This study identifies islet FBPase as a possible therapeutic target for the improvement of insulin secretory function in type 2 diabetes.

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