Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice

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Hepatic glucose production plays a pivotal role in glucose homeostasis. After ingestion of a high-carbohydrate meal, hepatic glucose production is promptly suppressed to limit postprandial glucose excursion, whereas in response to fasting hepatic glucose production is increased, accompanied by diminished peripheral glucose utilization (25). As a major endogenous source of glucose, hepatic glucose production plays a crucial role in counterbalancing hypoglycemia and maintaining blood glucose levels in a normal range (23). Unrestrained hepatic glucose production, due to insulin deficiency or insulin insufficiency, is a contributing factor for the pathogenesis of postabsorptive hyperglycemia in type 1 and type 2 diabetes, respectively (3, 10, 18).

Hepatic glucose production is controlled by the activities of two gluconeogenic enzymes in the liver, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) (25). Both PEPCK and G-6-Pase expression are regulated at the level of transcription, inhibited by insulin, and stimulated by glucagon and glucocorticoids (16, 28, 29, 32). PEPCK and G-6-Pase promoters share a distinctive structural motif [TG(ATTTT(G)/G/T)], termed insulin response element (IRE), which is also found in the promoters of other genes that are downregulated by insulin, including insulin-like growth factor-binding protein-1 (IGFBP-1) (12, 13, 22). Using the IGFBP-1 promoter-directed expression system, the forkhead transcription factor Foxo1 (or FKHR) has been shown to play an important role in mediating insulin action and regulating target gene expression (13, 14, 19, 21, 22). In the absence of insulin, Foxo1 binds to IRE in target promoters and stimulates the promoter activity (1, 13, 31).

In response to insulin, Foxo1 undergoes phosphatidylinositol 3-kinase-dependent phosphorylation and is excluded from the nucleus, resulting in inhibition of Foxo1-dependent transcription (2, 5, 6, 13, 22, 27). There are at least three consensus protein phosphorylation sites in Foxo1, and mutations in two of these sites lead to its nuclear sequestration, resulting in constitutive transactivation of target promoters (13). Rena et al. (26) recently identified two other novel phosphorylation sites in Foxo1 that are also critical for its nuclear exclusion.

Foxo1 comprises two structural domains, the amino domain, which is responsible for DNA binding to target genes.
promoters, and the carboxyl transactivation domain, which functions to stimulate promoter activity (19, 31). The carboxyl domain of Foxo1, when fused to a heterologous Gal4 DNA-binding domain, can stimulate Gal4 promoter activity in an insulin-responsive manner (31). In contrast, the amino domain of Foxo1 (designated Foxo1-Δ256) has been shown to act as a dominant negative mutant to inhibit PEPCk and G-6-Pase expression in cultured cells (21). Like its wild-type protein, Foxo1-Δ256 is phosphorylated in response to insulin (19), but phosphorylation does not seem to interfere with its subcellular localization, presumably because its relatively small size (molecular mass ~27 kDa) makes it readily diffusible across the nuclear pore independent of the translocation mechanism required by larger molecules (molecular mass >50 kDa) (11).

In this study, we have seized this unique property of Foxo1-Δ256 to address the effect of Foxo1 inhibition on gluconeogenesis in cells as well as its impact on glucose metabolism in animals. We have used an adenovirus-mediated gene delivery system to transfer the Foxo1-Δ256 cDNA to the livers of normal and diabetic db/db mice. This approach has been shown to result in transduction of hepatocytes predominantly in the liver, with little transduction of cells in extrahepatic tissues including the spleen, pancreas, and kidney (15). We have demonstrated that hepatic Foxo1-Δ256 expression produces a profound impact on glucose metabolism in adult mice. Furthermore, we have shown that Foxo1 expression is deregulated, as reflected in the significantly increased production and localization of Foxo1 in the nucleus in the livers of diabetic db/db mice. Hepatically produced Foxo1-Δ256 interfered with Foxo1 function via competitive binding to Foxo1-targeted sites in the PEPCK and G-6-Pase promoters, resulting in inhibition of gluconeogenic gene expression in the liver and reduction of fasting hyperglycemia in diabetic db/db mice.

**EXPERIMENTAL PROCEDURES**

**Recombinant adenoviral vectors.** The recombinant adenoviral vector Ad-CMV-Foxo1-Δ256 expressing a mutant version of Foxo1 containing its amino domain (corresponding to fl in HEK-293 cells and purified by CsCl density centrifugation) has been described (21). To prepare adenoviruses, Ad-CMV-Foxo1-Δ256 was propagated in HEPK-293 cells and purified by CsCl density centrifugation as described (7). The titer of Ad-CMV-Foxo1-Δ256 was 2.5 × 10^{12} viral particles (vp/ml) and 1.6 × 10^{11} plaque-forming units (pfu/ml). The control Ad-RSV-LacZ vector, with a titer of 10^{12} viral particles (vp/ml) and 1.6 × 10^{11} pfu/ml, has been previously described (7).

**Cell culture.** Hepatoma H4IIE cells were cultured as previously described (8). To transduce H4IIE cells, 100 μl of the Ad-CMV-Foxo1-Δ256 vector at a defined multiplicity of infection (MOI) were added to cells. Control cells were transduced with 100 μl of the Ad-RSV-LacZ vector at the same MOI or 100 μl of PBS buffer. After 24 h of incubation, cells were collected and frozen at −70°C.

**Western blot analysis.** Cells were resuspended in 200 μl of M-PER (mammalian protein extraction reagent; Pierce, Rockford, IL) and lysed according to the manufacturer’s instructions. Aliquots (20 μl) of protein lysates were resolved on 12% SDS-polyacrylamide gels, and proteins were blotted onto a piece of nitrocellulose membrane, which was subsequently probed with goat anti-Foxo1 antibody (1:1,000 dilution, N-18; Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with anti-goat IgG conjugated with horseradish peroxidase (1:2,000 dilution). Protein bands were detected by ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

**Northern blot analysis.** Aliquots (10 μg) of RNA were resolved on 1.2% formaldehyde agarose gels. After electrophoresis, RNA was blotted onto a piece of Hybond N+ membrane (Amersham Pharmacia Biotech), which was subsequently hybridized with specific DNA probes that were radio-labeled with [α-32P]dCTP by use of the Amersham random priming kit. RNA bands were visualized by autoradiography and quantified by PhosphorImager (Molecular Dynamics). DNA probes were amplified from mouse liver RNA by use of primers as follows: PEPCk forward, 5′-ATGCTCTCCTC-AGCTGCTAATGGTC-3′ (corresponding to PEPCk cDNA, 1–25 nt); PEPCk reverse, 5′-CTTTGACCTGCTGCAACATC-3′ (PEPCk cDNA, 391–410 nt); G-6-Pase forward, 5′-GAAGGGAAATGACACCTTCT-3′ (G-6-Pase cDNA, 4–24 nt); G-6-Pase reverse, 5′-TGGCGGTTTGGCCAAAACAG-3′ (G-6-Pase cDNA, 317–336 nt); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5′-TGAAGGTCGGTG- GAACGGAT-3′ (GAPDH cDNA, 5–25 nt); and GAPDH reverse, 5′-CAGGGGGCTAAGCAGTGTG-3′ (GAPDH cDNA, 454–474 nt). All primers were obtained commercially (Integrated DNA Technologies, Coralville, IA). The DNA probe for Foxo1-Δ256 mRNA was made from the XbaI-EcoRI DNA fragment covering the 5′-half of the Foxo1 protein coding sequence (1–621 nt) from the plasmid pCMV-Foxo1-Δ256.

**Apoptosis assay.** The level of cellular apoptosis was determined by caspase-3 activity and DNA fragmentation assays. H4IIE cells at 90% confluence were transduced with the Foxo1-Δ256 vector at different MOIs varying from 0 to 400 vp/cell as well as with the control vector at the highest MOI. After 48 h of transduction, cells were harvested and lysed in 200 μl of 1 × DTT lysis buffer (Roche Diagnostics, Mannheim, Germany). Aliquots (100 μl) were used for measuring caspase-3 activity using a fluorometric immunosorbent enzyme assay kit (Roche Diagnostics). For DNA fragmentation assay, genomic DNA of H4IIE cells transduced by the Foxo1-Δ256 or control vectors were prepared using the nuclear DNA purification kit (Clontech Laboratory, Palo Alto, CA). Isolated genomic DNA (20 μg) was fractionated on 0.7% agarose gels and visualized under UV light after staining with ethidium bromide.

**Animal studies.** CD1 mice at 6 wk of age were purchased from Charles River Laboratory (Wilmington, MA), and db/db mice at 6 wk of age were purchased from the Jackson laboratory (Bar Harbor, ME). Animals were fed standard rodent chow diet and water ad libitum in sterile cages (5 mice per cage) in a barrier animal facility with a 12:12-h light-dark cycle. For vector administration, animals were injected through tail veins with an adenoviral vector diluted in PBS buffer as described (8). An adjustment in injection volume (μl) was made to ensure the same amount of viral particles per kilogram of body weight (vp/kg) in different animals. Before and on different days after vector administration, blood glucose levels were measured using the Glucometer Elite (Eugene Diagnostic, War掣ington, NJ). Blood was collected from tail vein into capillary tubes precoated with potassium-EDTA (Sarstedt, Numbrecht, Germany), and plasma was separated after centrifugation of blood samples at 6,000 rpm for 5 min. Plasma lactate concentrations were measured using the lactate assay kit (Sigma Diagnostics, St.
Liver histology. Liver tissue collected from euthanized animals was fixed in 10% phosphate-buffered formalin. Paraffin-embedded tissue was sectioned (5 μm thick) and stained with hematoxylin and eosin (H-E) or with X-gal, as previously described (8).

Quantification of hepatic glycogen content. Two hundred milligrams of liver tissue were homogenized in 400 μl of 0.03 N HCl. The homogenates were centrifuged at 12,000 rpm for 2 min at room temperature. One aliquot (100 μl) of the supernatant was mixed with 400 μl of 1.25 N HCl and incubated at 95°C for 1 h for determination of both glycogen content and residual glucose concentrations in the liver. In addition, another aliquot (100 μl) was mixed with 400 μl of 1.25 N HCl but incubated at room temperature for measurement of residual glucose concentrations in the liver. After incubation, 20-μl samples were mixed with 1 μl of glucose oxidase reagent (Sigma) and incubated at 37°C for 30 min, followed by the addition of 100 μl of 12 N H2SO4. The absorbance of each sample was read at 540 nm. In parallel, rabbit liver glycogen type III (Sigma) at different concentrations ranging from 30, 50, 70, and 90 to 110 μg/ml were treated identically and used as standards. The absorbance of standards was plotted against glycogen concentrations to yield a standard curve, from which the relative glycogen concentrations in unknown samples were calculated. After the residual glucose concentrations were subtracted, hepatic glycogen contents in different treatment groups of mice were compared.

Tissue RNA isolation. RNA isolation from liver tissue was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA). Twenty milligrams of liver tissue were homogenized in 300 μl of cell lysis buffer with a rotor-stator tissue homogenizer. Total RNA was purified from homogenized cells, and the RNA concentration was determined spectrophotometrically.

Real-time quantitative RT-PCR. Real-time quantitative RT-PCR was used for quantification of the relative mRNA abundance in the liver by use of the Roche LightCycler-RNA amplification kit (Roche Diagnostics). For each reaction, aliquots (250 ng in 10 μl) of liver RNA were mixed with 4 μl of the LightCycler RT-PCR reaction mix SYBR green I, 2.5 μl of 25 mM MgCl2, 2 μl of the primer mix (containing 5 μM primers for forward and reverse reactions), 1 μl of RNase-free H2O, and 0.5 μl of the LightCycler RT-PCR enzyme mix in a final volume of 20 μl in capillary reaction tubes provided by the kit. In addition, aliquots of denatured cDNA (~250 bp), corresponding to the test mRNA at a series of concentrations ranging from 0.3, 1, 3, and 10 to 30 ng, were treated identically as above and used as controls for standard curve construction. As a negative control, a separate reaction without RNA sample was included in each reaction. All reactions were carried out by RT at 55°C for 10 min, followed by PCR amplification using the program containing 30 cycles of 95°C for 30 s, 55°C for 10 s, and 72°C for 14 s for each cycle. At the end of reaction, the cycle threshold (Ct) values, i.e., the cycle numbers at which fluorescence signals exceed background, were obtained for standard and unknown samples. A standard curve was constructed by plotting the Ct values as a function of the log concentration of standard cDNA (ng). On the basis of the Ct values of unknown samples, their relative mRNA concentrations were deduced from the standard curve. The primers used for real-time quantitative RT-PCR have been described as above, except for glucokinase (GK) primers GK forward, 5'-CTGGATGACAGGAGCCAAGATG-3' (corresponding to mouse GK cDNA, 3–24 nt), and GK reverse, 5'-AGTTGTTTCTTCCAGTCTT-3' (GK cDNA, 321–250 nt). β-Actin primers were purchased from Promega (Madison, WI).

Immunoprecipitation. Immunoprecipitation of Foxo1 from liver tissue was performed using the Catch and Release immunoprecipitation system (Upstate Biotechnology, Lake Placid, NY). Twenty milligrams of liver tissue were homogenized in 500 μl of M-PER. After centrifugation at 14,000 rpm for 10 min in a Microfuge, aliquots of protein lysates (500 μg) were incubated with 4 μg of rabbit anti-Foxo1 antibody and applied to a protein A-agarose spin column. Subsequent affinity column chromatography was performed according to the manufacturer’s instruction. Likewise, hepatic nuclear factor 3β (HNF-3β) was immunoprecipitated using rabbit anti-HNF-3β antibody (Upstate Biotechnology), and GK was immunoprecipitated using rabbit anti-GK antibody (Rockland Immunocchemicals, Boyertown, PA). Immunoprecipitated proteins were studied by Western blot analysis, and the intensity of protein bands was quantified by densitometry using NIH Image software version 1.62 (National Institutes of Health, Bethesda, MD).

Fractionation of cytoplasmic and nuclear proteins. Cytoplasmic and nuclear protein extracts were fractionated by differential lysis of cells in two consecutive steps using the NE-PER extraction reagents (Pierce). Forty milligrams of liver tissue were homogenized in 400 μl of ice-cold CER-I solution (Pierce) supplied with 40 μl of protease inhibitor cocktail (Pierce). Subsequent steps for fractionation of cytoplasmic and nuclear extracts were according to the manufacturer’s instructions. Protein concentrations in the cytoplasmic and nuclear extracts were determined using the Micro BSA protein assay kit (Pierce), and aliquots (20 μg) were resolved on 12% SDS-polyacrylamide gels, followed by Western blot analysis using anti-Foxo1 antibody (1:1,000 dilution, N-18). The intensity of the protein bands was quantified by densitometry using NIH Image software version 1.62.

Statistics. Statistical analyses of data were performed by analysis of variance (ANOVA) using StatView software (Abacus Concepts). Pairwise comparisons were performed to study the significance between different treatments. Data are expressed as means ± SE. P values < 0.05 are statistically significant.

RESULTS

Effect of Foxo1-Δ256 production on gluconeogenesis in H4IIE cells. To verify the production of Foxo1-Δ256 from the Foxo1-Δ256 vector, H4IIE cells were transduced with the Foxo1-Δ256 vector using the LacZ vector as a control. After 24 h of transduction, cells were lysed, and protein lysates were subjected to Western blot analysis. As shown in Fig. 1A, transduction of H4IIE cells by the Foxo1-Δ256 vector resulted in the production of an immunoreactive protein corresponding to Foxo1-Δ256 (predicted molecular mass 27 kDa). In the same immunoblot, there was no detectable production of Foxo1-Δ256 in control cells. These results confirm that the Foxo1-Δ256 vector was capable of expressing Foxo1-Δ256.

To study the effect of Foxo1-Δ256 production on gluconeogenesis, the expression levels of endogenous PEPCK and G-6-Pase genes were studied by Northern blot analysis after adenoviral mediated production of
Fig. 1. Effects of production of the mutant of forkhead transcription factor Foxo1 (devoid of its carboxyl domain, Foxo1-Δ256), on gluconeogenic gene expression in H4IIE cells. A: adenoviral mediated production of Foxo1-Δ256 in H4IIE cells. Arrow indicates the protein band of Foxo1-Δ256 in a 12% SDS-polyacrylamide gel, as visualized by Western blot analysis. MOI, multiplicity of infection. B–D: Northern blot analysis of phospho-enol-pyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G-6-Pase), and Foxo1-Δ256 mRNA. Endogenous PEPCK (B), G-6-Pase (C), and Foxo1-Δ256 (D) gene expression in H4IIE cells were studied by Northern blot analysis and quantified by PhosphorImager using GAPDH mRNA as an internal control. Data were obtained from 5, 3, and 2 independent measurements for PEPCK, G-6-Pase, and Foxo1-Δ256 mRNAs, respectively. E: intracellular levels of caspase-3 activity. F: genomic DNA integrity of the Foxo1-Δ256 and control vector-transduced H4IIE cells. MW, molecular weight. *P < 0.001 vs. control values, by ANOVA.
Foxo1-Δ256 in H4IIE cells. As shown in Fig. 1, B–D, transduction of H4IIE cells with the Foxo1-Δ256 vector resulted in a dose-dependent inhibition on cAMP-dexa-methasone-stimulated expression of both PEPCK and G-6-Pase genes. The degree of inhibition is correlated with both MOI and the level of Foxo1-Δ256 mRNA in the cells. In contrast, the intracellular levels of GAPDH mRNA remained unchanged irrespective of Foxo1-Δ256 expression. Likewise, transduction of H4IIE cells with the control vector at the highest MOI did not result in significant alterations in the expression levels of PEPCK and G-6-Pase genes. These results demonstrate that Foxo1-Δ256 production inhibited the endogenous expression of PEPCK and G-6-Pase in H4IIE cells.

To determine whether Foxo1-Δ256 expression causes cellular apoptosis, H4IIE cells transduced with the Foxo1-Δ256 and control vectors were subjected to apoptosis assays. As shown in Fig. 1E, intracellular caspase-3 activity in the Foxo1-Δ256 and control vector-transduced cells remained at background levels. When total genomic DNA was analyzed on agarose gels, no significant differences were detected in H4IIE cells transduced with different vector doses compared with control cells (Fig. 1F).

**Impact of hepatic Foxo1-Δ256 expression on glucose metabolism in normal mice.** The effect of hepatic Foxo1-Δ256 expression on glucose metabolism was addressed as follows. CD1 mice were stratified by body weight and randomly divided into three groups to ensure a similar mean body weight (21.2 ± 1.1 g) in different groups, which were respectively treated with the Foxo1-Δ256 vector at the dose of 3 × 10^{12} vp/kg body wt, the control vector at the same dose, and PBS.

After vector administration, blood glucose levels were measured and compared between different treatment groups. As shown in Fig. 2A, administration of the Foxo1-Δ256 vector resulted in a significant reduction in blood glucose levels (106 ± 6 vs. 150 ± 7 mg/dl in PBS-treated controls). In contrast, blood glucose levels in the control vector-treated animals remained unchanged.

The effect of hepatic Foxo1-Δ256 production on glucose metabolism was further studied by subjecting animals to a 15-h fast followed by the determination of their fasting blood glucose levels. Blood glucose levels were reduced after fasting, but the amplitude of blood glucose reduction in the LacZ vector-treated animals was not significantly different from that in PBS-treated controls (Fig. 2B). However, under the same fasting condition, the Foxo1-Δ256 vector-treated animals exhibited a much greater reduction in their fasting blood glucose levels (56 ± 9 vs. 93 ± 9 mg/dl in PBS-treated controls; Fig. 2B). When serum alanine aminotransferase (ALT) levels were assayed under fasting conditions, no significant differences were detected between the Foxo1-Δ256 vector-treated (101 ± 20 U/l) and PBS- (74 ± 11 U/l) and control vector-treated animals (112 ± 30 U/l). Thus the observed reduction in blood glucose levels was not due to hepatotoxicity associated with vector treatment but attrib-

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**Fig. 2.** Effects of hepatic Foxo1-Δ256 production on blood glucose and plasma lactate metabolism in normal mice. A: blood glucose levels under nonfasting conditions. B: blood glucose levels after a 15-h fast. C: plasma lactate levels. Data were obtained on day 4 for nonfasting blood glucose and plasma lactate levels and on day 5 for fasting blood glucose levels after hepatic Foxo1-Δ256 expression. Six mice were used per group. *P < 0.05, **P < 0.01 vs. controls; NS, not significant, all by ANOVA.
utable to the effect of Foxo1-Δ256 expression on glucose metabolism in the liver.

To investigate the potential effect of Foxo1-Δ256 on gluconeogenic substrate metabolism, plasma lactate levels were determined after 4 days of hepatic Foxo1-Δ256 expression. As diagrammed in Fig. 2C, plasma lactate levels in the Foxo1-Δ256 vector-treated mice (118 ± 10 mg/dl) were not significantly different from control values in PBS (98 ± 6 mg/dl)- and LacZ vector (109 ± 15 mg/dl)-treated mice.

Effect of Foxo1-Δ256 production on hepatic gluconeogenic gene expression. To study the molecular basis of Foxo1-Δ256-mediated blood glucose reduction in mice, total liver RNA was prepared from individual animals after hepatic Foxo1-Δ256 expression and subjected to real-time quantitative RT-PCR analysis. PEPCK and G-6-Pase mRNA levels were measured using β-actin mRNA as an internal control. As shown in Fig. 3, when normalized to β-actin mRNA, the relative concentrations of both PEPCK and G-6-Pase mRNAs in the liver were significantly reduced in the Foxo1-Δ256 vector-treated mice. In contrast, the relative levels of PEPCK and G-6-Pase mRNAs remained unchanged in the control vector-treated animals (Fig. 3, A and B). These results indicate that hepatic Foxo1-Δ256 expression significantly decreased the expression level of gluconeogenic genes, but not of the β-actin gene, in the liver.

GK is a key enzyme involved in the glycolysis pathway. To determine the possible effect of hepatic Foxo1-Δ256 production on GK expression, we quantified the hepatic GK mRNA content in different treatment groups. A small reduction in the expression levels of GK mRNA was detected after hepatic Foxo1-Δ256 expression, but this reduction was not significant compared with the control vector-treated animals (Fig. 3C), indicating that Foxo1-Δ256 production does not affect GK expression in the liver.

Because G-6-Pase is responsible for the terminal step of both gluconeogenesis and glycogenolysis by catalyzing the hydrolysis of glucose 6-phosphate to glucose (25), a reduction of hepatic G-6-Pase expression might adversely affect glycogen metabolism in the liver. To examine the effect of hepatic Foxo1-Δ256 expression on glycogenolysis, animals were killed, and hepatic glycogen content was determined after 1 wk of Foxo1-Δ256 expression in the liver. Consistent with the inhibitory effect of Foxo1-Δ256 on G-6-Pase mRNA expression in the liver, we detected a ~20% increase in hepatic glycogen content in the Foxo1-Δ256 vector-treated mice (Fig. 3D), but this increase was not significant compared with the control value in the LacZ

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**Fig. 3.** Relative hepatic mRNA abundance and glycogen content. Animals were killed after 1 wk of treatment. Liver tissue was collected and used for determination of hepatic mRNA levels and glycogen content. The amount of mRNA corresponding to β-actin, PEPCK, G-6-Pase, and glucokinase (GK) out of total RNA in the liver was estimated by real-time quantitative RT-PCR. After normalization to β-actin mRNA, the relative abundances of PEPCK (A), G-6-Pase (B), and GK mRNA (C) were determined. D: Hepatic glycogen content was determined as described in EXPERIMENTAL PROCEDURES and is expressed as mg glycogen/g wet liver tissue. *P < 0.05, **P < 0.001, by ANOVA; n = 6.
vector-treated animals (4.4 ± 0.9 vs. 5.3 ± 1.2 mg/g liver in the Foxo1 vector-treated mice, \( P > 0.05 \) by ANOVA).

To study the potential effects of hepatic Foxo1-Δ256 production on the expression of its full-length protein Foxo1 and other forkhead transcription factors such as HNF-3β, total protein lysates extracted from the livers of treated mice were immunoprecipitated using the specific antibodies against Foxo1 and HNF-3β, respectively. Immunoprecipitated proteins were subjected to Western blot analysis. As shown in Fig. 4, protein bands corresponding to Foxo1 and HNF-3β were detected but with no significant differences in quantity between the Foxo1-Δ256 vector-treated vs. control animals. Immunoprecipitation with anti-Foxo1 antibody also resulted in copurification of Foxo1-Δ256 from the liver protein extracts of the Foxo1-Δ256 vector-treated mice but not of the control vector-treated animals. In addition, the expression levels of GK protein were analyzed by immunoprecipitation using specific anti-GK antibody. Consistent with the data of GK mRNA (Fig. 3C), a small, but insignificant, reduction in hepatic GK protein content was detected in the Foxo1-Δ256 vector-treated mice (Fig. 4).

To study the potential toxic effect on the liver as a result of Foxo1-Δ256 production or vector treatment, the liver tissue sampled from euthanized animals after 1 wk of hepatic Foxo1-Δ256 expression was examined in a microscope after staining with H-E and X-gal. No morphological lesions, such as inflammation and parenchymal necrosis, were observed in the livers of animals treated with either the Foxo1-Δ256 or control vector. Using the LacZ vector as a standard, we determined the transduction efficiency to be ~70% with a standard deviation of 5–10% among animals.

**Hepatic Foxo1 expression in db/db mice.** Augmented hepatic gluconeogenesis is causative for postabsorptive hyperglycemia in type 2 diabetes. Given the functional importance of Foxo1 in mediating insulin action in controlling hepatic gluconeogenesis, hepatic Foxo1 expression might be altered in diabetic db/db mice. To test this idea, we studied the level of Foxo1 expression as well as its subcellular distribution in the livers of diabetic db/db mice. Hepatic RNA prepared from diabetic db/db mice (blood glucose levels >500 mg/dl) and their heterozygous littermates (135 ± 18 mg/dl) were subjected to real-time RT-PCR analysis for determination of Foxo1, PEPCK, G-6-Pase, and β-actin mRNAs, respectively. After normalization to β-actin mRNA, the relative levels of Foxo1, PEPCK, and G-6-Pase mRNA concentrations were compared between diabetic db/db and nondiabetic control mice. As shown in Fig. 5A, hepatic Foxo1 mRNA levels were significantly increased in diabetic db/db mice compared with nondiabetic controls. Furthermore, this pattern of hepatic Foxo1 expression correlated with increased PEPCK and G-6-Pase expression in the livers of diabetic db/db mice (Fig. 5, B and C).

In addition to its regulation at the level of transcription, the transactivation activity of Foxo1 is regulated by its phosphorylation-dependent intracellular redistribution in response to insulin. Phosphorylation of Foxo1 results in its translocation from the nucleus to the cytoplasm, which in turn limits its access to target promoters and its ability to enhance promoter activity (1, 2, 14). To determine the possible alteration in subcellular distribution of Foxo1 in insulin-resistant livers, hepatic protein lysates were prepared from the livers of diabetic db/db and nondiabetic control mice, and the relative Foxo1 protein concentrations in the cytoplasmic vs. nuclear fractions were determined by Western blot analysis. In nondiabetic control mice, Foxo1 was present predominantly in the cytoplasm of hepatocytes, as a relatively larger proportion of Foxo1 was detected in the cytoplasmic fraction, accounting for >85% of total cellular Foxo1 proteins (Fig. 6A). However, in diabetic db/db mice, the relative amount of Foxo1 protein in the cytoplasm was decreased, accompanied by a corresponding increase of Foxo1 in the nucleus (Fig. 6B). Thus we detected a quantitative difference in the subcellular distribution of Foxo1 in hepatocytes in normal vs. insulin-resistant livers. Close to 50% of total intracellular Foxo1 proteins were localized in the nucleus in the livers of db/db mice, which was threefold higher than the corresponding number (12 ± 3%) in the livers of lean controls (Fig. 6C).

**Effect of hepatic Foxo1-Δ256 expression on glucose metabolism in db/db mice.** To test whether hepatic Foxo1-Δ256 expression can inhibit gluconeogenesis and reduce hyperglycemia, we delivered the Foxo1-Δ256 cDNA into the livers of diabetic db/db mice. Diabetic db/db mice at 12 wk of age (mean body wt 42.7 ± 2.4 g) were stratified by blood glucose levels and randomly assigned to three different groups to ensure similar mean blood glucose levels (520 ± 25 mg/dl) in different groups. One group of db/db mice was treated with the Foxo1-Δ256 vector at 3 × 10^{12} vp/kg per animal, the second group was treated with the control vector at the same dose, and the third group was mock-treated with PBS. In addition, one group of heterozygous lean littermates (mean body wt 22.4 ± 0.5 g)
was included as nondiabetic controls. Blood glucose levels were determined under both nonfasting and fasting conditions after hepatic Foxo1Δ256 expression. As shown in Fig. 7A, hepatic production of Foxo1Δ256 significantly reduced the degree of hyperglycemia (from >500 mg/dl to 283 ± 40 mg/dl) 3 days after vector administration. In contrast, the control vector-treated diabetic mice continued to manifest severe hyperglycemia (515 ± 42 mg/dl). To further illustrate the effect of Foxo1Δ256 on glucose metabolism, animals were fasted for determination of fasting blood glucose levels. As shown in Fig. 7B, in response to an 8-h fast, blood
glucose levels in the Foxo1-Δ256 vector-treated diabetic mice were further reduced to a normal range (80–160 mg/dl). In contrast, hyperglycemia (495–1100 mg/dl) persisted in the control vector-treated diabetic mice after fasting.

To study the effect of hepatic Foxo1-Δ256 production on gluconeogenesis, animals were killed after 5 days of hepatic Foxo1-Δ256 expression, and liver tissue was collected for preparation of total RNA, which was subsequently analyzed by real-time RT-PCR. As shown in Fig. 7, C and D, the expression levels of PEPCK and G-6-Pase mRNAs relative to β-actin mRNA were significantly decreased in response to hepatic Foxo1-Δ256 expression, which correlated with the reduction of hyperglycemia in the Foxo1-Δ256 vector-treated diabetic mice. In contrast, the relative levels of PEPCK and G-6-Pase mRNAs in the control vector-treated diabetic mice remained unchanged, which coincided with the lack of reduction in their fasting hyperglycemia (Fig. 7).

**DISCUSSION**

We showed that hepatic production of Foxo1-Δ256 inhibited both PEPCK and G-6-Pase, but not GAPDH, expression in H4IIE cells. The degree of inhibition on PEPCK and G-6-Pase expression correlated closely with the level of Foxo1-Δ256 production in the cells. Furthermore, we extended our studies from in vitro to in vivo, demonstrating in animals that hepatic expression of Foxo1-Δ256 resulted in blood glucose reduction in treated mice. To further illustrate the effect of Foxo1-Δ256 on hepatic glucose metabolism, treated animals were subjected to a prolonged fast. Given the importance of hepatic gluconeogenesis in glucose homeostasis during fasting, an inhibition on gluconeogenesis by Foxo1-Δ256 would have a greater impact on fasting blood glucose metabolism in the animals. The Foxo1-Δ256 vector-treated mice exhibited a greater reduction in fasting blood glucose levels after the prolonged fast. In contrast, the fasting blood glucose levels in control animals remained within the normal range. These data are in agreement with previous observations that adenoviral mediated expression of Foxo1-Δ256 suppressed cAMP-dexamethasone-stimulated PEPCK and G-6-Pase expression in primary culture of mouse hepatocytes and kidney epithelial cells (21). Together, these results indicate that hepatic Foxo1-Δ256...
Δ256 expression has the effect of inhibiting gluconeogenic activity in the liver. Tang et al. (30) observed that overproduction of the Foxo1-AAA, a mutant form that could not undergo PI 3-kinase-dependent phosphorylation, caused apoptosis in HEK-293 cells. Likewise, FKHRL-1, another member of the forkhead transcription factor family, was shown to induce apoptosis when present in the nucleus in nonphosphorylated forms (4). Although the underlying mechanism is not clear at present, it appears that Foxo1-mediated apoptosis is caused by enhanced transactivation of target promoters by Foxo1 in the nucleus, since the overproduction of only a constitutively active Foxo1-AAA mutant, but not the wild-type Foxo1, was associated with cellular apoptosis (30). This is consistent with our findings that elevated production of Foxo1-Δ256, lacking the transactivation domain of Foxo1, did not induce apoptosis. These data suggest that the effect of Foxo1-Δ256 on gluconeogenesis is specific to Foxo1-Δ256 expression but not due to cellular apoptosis.

A hallmark of type 2 diabetes is unrestrained hepatic gluconeogenesis, accounting for the pathogenesis of postabsorptive hyperglycemia. We showed that hepatic expression of Foxo1 was impaired in db/db mice, as evidenced by significantly elevated Foxo1 expression in the liver as well as increased localization of Foxo1 protein in the nucleus. These data imply that Foxo1, when deregulated in the liver, is a contributing factor to the pathogenesis of postabsorptive hyperglycemia in diabetes. A significant elevation in hepatic Foxo1 expression, coupled with its loss of response to insulin-dependent subcellular redistribution, is attributable to its enhanced transactivation activity in stimulating gluconeogenic gene expression and exacerbating the degree of fasting hyperglycemia in type 2 diabetes. Consistent with this interpretation is the recent observation that Foxo1 haploinsufficiency was associated with reduced hepatic gluconeogenesis, resulting in reversal of diabetic phenotype in insulin-resistant diabetic mice (20).

It is noteworthy that our measurement of the effect of Foxo1-Δ256 on hepatic gluconeogenesis was based on the alterations in PEPCK and G-6-Pase mRNA expression after Foxo1-Δ256 production in the liver. However, we observed under our experimental conditions that Foxo1-Δ256 exerted an inhibitory effect on PEPCK and G-6-Pase expression in cultured hepatocyte cells as well as in the liver, which correlated with the reduction of blood glucose levels in both normal and diabetic mice. These results, together with previous reports (19, 21), suggest that functional inhibition of Foxo1, caused by hepatic expression of its dominant negative mutant, resulted in inhibition of gluconeogenic activity in the liver and reduction of fasting hyperglycemia in diabetic db/db mice.

What is the molecular mechanism underlying the inhibitory effect of Foxo1-Δ256 on hepatic gluconeogenesis? Previous studies have characterized Foxo1 as a mediator of insulin action in controlling the expression of genes that are downregulated by insulin (17, 24). These genes share a consensus IRE sequence in their respective promoters (24). This highly conserved IRE DNA element serves as a target site of Foxo1 in mediating insulin action. In the absence of insulin, Foxo1 binds to IRE and acts as a transactivator to enhance target promoter activity, whereas in response to insulin Foxo1 is phosphorylated and translocated from the nucleus to the cytoplasm, resulting in inhibition of target gene expression (2, 19, 21, 22). HNF-3β, another forkhead transcription factor, also functions as a coactivator through the glucocorticoid’s response unit, and a truncated version of HNF-3β, lacking its carboxyl transactivation domains, is shown to inhibit glucocorticoid-stimulated PEPCK and G-6-Pase gene expression in hepatoma cells (34). HNF-3β has been shown to bind the DNA region that encompasses the IRE in target promoters, but its DNA binding activity is not subject to insulin inhibition (9, 33). One potential mechanism is that hepatically produced Foxo1-Δ256 downregulated Foxo1 or HNF-3β expression in the liver, consequently resulting in attenuation of Foxo1-and/or HNF-3β-mediated transactivation of gluconeogenic gene expression. However, this possibility was ruled out when we showed that the expression levels of both Foxo1 and HNF-3β in the liver remained unchanged in response to hepatic Foxo1-Δ256 expression. A second potential mechanism is that Foxo1-Δ256, when expressed in the liver, binds to IRE in a competitive manner, interfering with the binding and recruiting of Foxo1 and other accessory transcription factors including HNF-3β to their cognate sites in target promoters, resulting in inhibition of gluconeogenic gene expression. Consistent with this explanation is the demonstration that Foxo1-Δ256 retains the ability to bind its target IRE site in the PEPCK and G-6-Pase promoter, respectively, when analyzed by a DNA mobility shift assay (data not shown), but its binding does not result in transactivation of target promoters because of the lack of its carboxyl transactivation domain.

In summary, our data are consistent with the idea that Foxo1 is an important mediator of insulin action in modulating gluconeogenesis in the liver. In diabetic db/db mice, hepatic Foxo1 expression became deregulated, resulting in elevated production and increased nuclear localization of Foxo1, which was attributable to augmented gluconeogenesis in the liver. We showed that functional interference of Foxo1, caused by hepatic expression of its mutant Foxo1-Δ256, was associated with reduced hepatic gluconeogenic activity and improved fasting glycemia in diabetic db/db mice.

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DISCLOSURES
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REFERENCES


