

Glucose Regulates Foxo1 Through Insulin Receptor Signaling in the Pancreatic Islet β -cell

Sara C. Martinez, Corentin Cras-Méneur, Ernesto Bernal-Mizrachi, and M. Alan Permutt

Glucose controls islet β -cell mass and function at least in part through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway downstream of insulin signaling. The Foxo proteins, transcription factors known in other tissues to be negatively regulated by Akt activation, affect proliferation and metabolism. In this study, we tested the hypothesis that glucose regulates Foxo1 activity in the β -cell via an autocrine/paracrine effect of released insulin on its receptor. Mouse insulinoma cells (MIN6) were starved overnight for glucose (5 mmol/l) then refed with glucose (25 mmol/l), resulting in rapid Foxo1 phosphorylation (30 min, $P < 0.05$ vs. untreated). This glucose response was demonstrated to be time (0.5–2 h) and dose (5–30 mmol/l) dependent. The use of inhibitors demonstrated that glucose-induced Foxo1 phosphorylation was dependent upon depolarization, calcium influx, and PI3K signaling. Additionally, increases in glucose concentration over a physiological range (2.5–20 mmol/l) resulted in nuclear to cytoplasmic translocation of Foxo1. Phosphorylation and translocation of Foxo1 following glucose refeeding were eliminated in an insulin receptor knockdown cell line, indicating that the glucose effects are mediated primarily through the insulin receptor. Activity of Foxo1 was observed to increase with decreased glucose concentrations, assessed by an IGF binding protein-1 promoter luciferase assay. Starvation of MIN6 cells identified a putative Foxo1 target, Chop, and a Chop-promoter luciferase assay in the presence of cotransfected Foxo1 supported this hypothesis. The importance of these observations was that nutritional alterations in the β -cell are associated with changes in Foxo1 transcriptional activity and that these changes are predominantly mediated through glucose-stimulated insulin secretion acting through its own receptor. *Diabetes* 55:1581–1591, 2006

Nutrients have major effects on pancreatic β -cell function and growth. The β -cells adapt to high concentrations of extracellular glucose and growth factors with concomitant enhanced secretion of insulin and an increase in mass (1). Recent evidence suggests the importance of insulin secretion and insulin receptor signaling in β -cell growth and metabolism. The insulin receptor is a member of a family of receptor tyrosine kinases, along with the IGF receptor, in which members share proximate components, including insulin receptor substrates (IRSs) and activation of phosphoinositol 3-kinase (PI3K) (2). The β -cell-specific overexpression of protein kinase B (PKB)/Akt, a downstream target of PI3K, has been shown to greatly expand the mass and augment insulin secretion of islets (3,4). Conversely, decreases in insulin receptor signaling within the β -cell result in reduced islet mass and impaired β -cell function, as demonstrated with targeted knockouts of the insulin receptor and *Irs2* in β -cells (5,6). Previous studies of glucose regulation of early gene transcription in mouse insulinoma cells (MIN6) revealed a predominant effect mediated through the insulin receptor (7,8). The downstream targets of glucose-mediated insulin signaling in pancreatic islet β -cells mediating these effects are currently unknown.

The Foxo family members of transcription factors are evolutionarily conserved downstream targets of PKB/Akt, which may be involved in nutrient regulation of β -cell mass and function. In a number of cells including fibroblasts and liver cells, phosphorylation by Akt at conserved phosphorylation sites has been shown to inhibit activity of Foxo transcription factors by promoting nuclear to cytoplasmic translocation (9,10). Mammalian members include Foxo1, Foxo3, Foxo4, and the dominantly brain-specific Foxo6 (rev. in 11). A tissue survey of isoform expression revealed that Foxo1 was the most abundant family member in β -cells (10). It was demonstrated that haploinsufficiency of *Foxo1* restored insulin sensitivity to *Insr*^{+/-} and *Irs2*^{-/-} mice (10,12). The possibility that unregulated Foxo1 activity could have harmful effects on β -cells was suggested when transgenic mice were made glucose intolerant by overexpressing Foxo1 in the liver and β -cells, although the contribution of Foxo1 to β -cell dysfunction could not be fully resolved in this model (12).

In pancreatic β -cells, the regulation and function of Foxo proteins remains largely uncharacterized. The role of Foxo family members in β -cell dysfunction could be important because of the known reduction of cell mass in diabetes and the demonstrated control of proliferation and cellular survival by Foxo in other tissues (13). Glucose has

From the Division of Endocrinology, Metabolism, and Lipid Research, Washington University School of Medicine, St. Louis, Missouri.

Address correspondence and reprint requests to M. Alan Permutt, MD, Division of Endocrinology, Metabolism, and Lipid Research, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8127, St. Louis, MO 63110. E-mail: apermutt@im.wustl.edu.

Received for publication 26 May 2005 and accepted in revised form 20 February 2006.

Additional information for this article can be found in an online appendix at <http://diabetes.diabetesjournals.org>.

DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GFP, green fluorescence protein; IGF1BP, IGF binding protein; IRKD Δ 80, insulin receptor knockdown (\sim 80%); IRS, insulin receptor substrate; K_{ATP} channel, ATP-sensitive K^+ channel; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B.

DOI: 10.2337/db05-0678

© 2006 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

been shown to activate Akt in MIN6 and INS-1 cells, for which Foxo1 is a known substrate (7,14). Glucose and IGF-1 treatment of insulinoma cells protected against free fatty-induced apoptosis and was associated with increased phosphorylation of both Akt and Foxo1 (15). These observations were extended in the current report by demonstrating that glucose regulation of Foxo1 phosphorylation is mediated through depolarization, is Ca^{2+} dependent, and requires an intact insulin receptor. Further, we demonstrate nutrient regulation of Foxo1 cellular localization and activity in β -cells and have identified a previously known nutrient regulated gene (*Gadd153* or *Chop*) as a potential transcriptional target of Foxo1.

RESEARCH DESIGN AND METHODS

Cell culture of mouse insulinoma cells. MIN6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/l glucose, with 15% fetal bovine serum (FBS), 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 $\mu\text{g}/\text{ml}$ L-glutamine, and 5 μM β -mercaptoethanol in humidified 5% CO_2 , 95% air at 37°C (16). MIN6 cells used were between passages 21–29. An siRNA-expressing plasmid system (pSUPER vector) (17) was used to reduce the insulin receptor in the insulin receptor knockdown (~80%) (IRKD80) cell line previously described (7). The target sequence against the mouse insulin receptor was 5'-ACTGCATGGTTGCC ATGA-3'. The IRKD80 cell line was maintained in culture medium with 200 $\mu\text{g}/\text{ml}$ G418. Normal media for both cell lines contains 25 mmol/l glucose and 15% FBS, while starved media for both cell lines contains 5 mmol/l glucose and 2% FBS, with G418 added for the IRKD80 cell line. The purpose of preincubating the cells under glucose and serum starvation was to optimize the opportunity to observe the effect of glucose readdition on subsequent signaling pathways. In each of the MIN6 experiments measuring activation of Foxo1, after serum and glucose deprivation, only glucose was added back. We used 2% FBS because we have found that complete serum withdrawal leads to cell death.

Islet isolation. All procedures were performed in accordance with Washington University's Animal Studies Committee. Animals were killed in a carbon dioxide chamber. Islets of 8-week-old wild-type C57BL/6 mice were isolated by collagenase distension/digestion of the pancreas (3), followed by filtering and washing through a 70- μm Nylon mesh. Isolated islets were then kept in a low-glucose (2 mmol/l) or high-glucose (11 mmol/l) media (RPMI with 10% FBS) overnight, or a HEPES-balanced Krebs-Ringer bicarbonate buffer (119 mmol/l NaCl, 4.74 mmol/l KCl, 2.54 mmol/l CaCl_2 , 1.19 mmol/l MgCl_2 , 1.19 mmol/l KH_2PO_4 , 25 mmol/l NaHCO_3 , and 10 mmol/l HEPES, pH 7.4) containing 0.5% BSA at 37°C for a period of 1 h and then either kept in the Krebs-Ringer bicarbonate/BSA solution or exposed to 25 mmol/l glucose for an additional 30 min.

Western blot analysis. Protein was extracted with a cell lysis buffer (diluted from 10 \times cell lysis buffer [Cell Signaling Technology, Boston, MA] and an additional protease inhibitor cocktail tablet [Roche] at 1 tablet/10 ml final buffer volume). Protein samples (30 μg) were separated by electrophoresis through 8% polyacrylamide, 0.1% SDS gels, and transferred to polyvinylidene fluoride or nitrocellulose membranes, followed by immunoblotting according to the protocol outlined by Cell Signaling Technology. Immunodetection was developed with ECL Advance (Amersham Biosciences, Buckinghamshire, U.K.) and imaged with a charge-coupled device camera (Alpha Innotech, San Leandro, CA). Antibodies used in this study are anti- α -tubulin (monoclonal; Sigma, St. Louis, MO) anti-phospho-Ser²⁵⁶-Foxo1 (Cell Signaling), and anti-Gadd153/Chop (mouse monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA). At least three blots were performed for each experiment with each blot reprobed for α -tubulin as a loading control.

Statistical analysis. Western blot quantitation consisted of acquiring a per-lane ratio of the chemiluminescent signal intensities from the phospho-Ser²⁵⁶-Foxo1 or Gadd153 to its respective tubulin using NIH Image J (<http://rsb.info.nih.gov/ij/>), then normalizing each signal to the first condition of the starved state in each respective blot. For the luciferase assays, ratios of luciferase to renilla were generated with SDs and SEs. Error was propagated for fold calculations between different conditions. Student's two-tailed *t* test for independent samples was used in significance calculations. The *P* value for significance is in the figure.

Plasmid constructs. Hemagglutinin-tagged Foxo1 from the pCMV5 vector (from D. Accili, Columbia University, New York, NY) was cloned into the multiple cloning site of pEGFP1 (Clontech, Mountain View, CA) to create a fusion protein with green fluorescence protein (GFP) at the NH_2 -terminus of Foxo1. The hemagglutinin-tagged $\Delta 256$ -Foxo1 and T24A-Foxo1 in the pCMV5 vector were also from D. Accili. The IGF1P-1 promoter/luciferase gene construct (p925GL3) was a generous gift from the laboratory of M. Rechler

(NIDDK, NIH, Bethesda, MD). The CHOP-promoter/luciferase gene construct (pGL2/3) was a gift from the lab of David Ron (Skirball Institute, New York University, New York, NY). The pRL-TK control vector contains the thymidine kinase promoter of the herpes simplex virus upstream of *Renilla* luciferase (Promega, Madison, WI).

GFP-Foxo1 plasmid transfection and fluorescence visualization. Two days before transfection, MIN6 or IRKD80 cells were seeded in standard media onto coverslips in six-well plates. Each well was transfected overnight with 2 μg plasmid, the pEGFP1-Foxo1, and 5 μl Lipofectamine 2000 in 250 μl OptiMem (Invitrogen, Carlsbad, CA). The next morning, the cells were given fresh media, and 24 h later, the cells were given either standard or starved media for an additional 24 h. The next day, different subsets of cells in triplicate in the starved media were either untreated or treated with glucose (25 mmol/l), glucose (25 mmol/l) and wortmannin (100 nmol/l; Sigma), IGF-1 (100 ng/ml; Sigma), or IGF-1 (100 ng/ml) and wortmannin (100 nmol/l) for a period of 1 h. Cells were then washed within the six-well plates twice in PBS and fixed in 1.5 ml 4% paraformaldehyde in PBS for 15 min at 4°C. After three washes with PBS, each coverslip was mounted onto a standard microscope slide using VectaShield with DAPI (Vector Laboratories, Burlingame, CA). The slides were viewed under a fluorescence microscope Leica DM4000 B (Leica Microsystems, Bannockburn, IL), and cells were blindly scored. At least 100 GFP-positive cells per slide were scored for either cytoplasmic or nuclear localization. Three slides and >300 GFP-positive cells were scored for each condition in both the MIN6 and IRKD80 cell lines.

Luciferase assay. MIN6 cells were plated in 12-well plates 2 days before transfection. At ~60–70% confluence, each well of cells was transfected with 100 ng IGF binding protein (IGFBP)-1/luciferase or CHOP/luciferase plasmid, 20 ng pRL-TK control vector, and either 200 ng pCMV5-Foxo1, pCMV5- $\Delta 256$ -Foxo1, pCMV5-T24A-Foxo1, or an empty pCMV5 vector unless otherwise indicated in 2 μl Lipofectamine 2000 in 100 μl OptiMem. For cell lysis, 200 μl passive lysis buffer (Promega) was used. The firefly and *Renilla* luciferase activities were measured after 24 h of incubation in the indicated media in a Monolight 3010 luminometer (BD Biosciences, San Jose, CA) using the dual-luciferase reporter assay system (Promega).

RT-PCR. Total RNA was harvested from MIN6 cells and islets grown in triplicate in either normal or starved media for a period of 24 h using DNase treatment (Gentra Systems, Minneapolis, MN). Total RNA (1 μg) from each sample was used to prepare cDNA with random hexamers as primers and reverse-transcribed with Superscript II (Invitrogen) according to the manufacturer's protocol. Primers used for the MIN6 GADD45 are as follows: GADD45 5'-TAA CTG TCG GCG TGT ACG AGG-3' and 5'-CAC CCA CTG ATC CAT GTA GCG-3' (18). Primers for cyclophilin A were the following: 5'-CAG GTC CTG GCA TCT TGT CC-3' and 5'-TTG CTG GTC TTG CCA TTC CT-3' (19). Quantitative RT-PCR of mouse islets was performed by monitoring in real-time the fluorescence of SYBR-green dye using the ABI 7000 sequence detection system. The primers used were those as generated from Primer Bank (20) and were the following: GADD45 5'-CCG AAA GGA TGG ACA CGG TG-3' and 5'-TTA TCG GGG TCT ACG TTG AGC-3; cyclophilin A 5'-GAG CTG TTT GCA GAC AAA GTT C-3' and 5'-CCC TGG CAC ATG AAT CCT GG-3'.

Microarray experiments. The microarray experiments were conducted similarly to a previously published protocol (8). At the end of the culture, cells were harvested and total RNA was extracted from cells using TRIzol reagent or RNeasy columns according to the manufacturer's protocol (Invitrogen and Qiagen, respectively). Briefly, labeled RNA were hybridized on cDNA-based microarray developed as part of the Endocrine Pancreas Consortium effort (21). Samples were labeled with either Cy3 or Cy5 and hybridized on the same array. To limit hybridization artifacts, the same samples were also labeled with the other dye and hybridized as well. A total of three hybridizations were performed and the average ratio between the two conditions taken in consideration after background subtraction and normalization. A two-step normalization was performed as previously described (8), and after evaluation of the mean variance and false-positive rate, it was estimated that genes displaying a fold change >1.5 and with a 95% CI not encompassing one could be considered statistically significant. A total of 2,744 genes presented expression levels twofold above background on all three arrays that could be taken in consideration for further analysis. The complete list of these genes is provided in the online appendix (available at <http://diabetes.diabetesjournals.org>).

RESULTS

Glucose induces rapid phosphorylation of Foxo1 in MIN6 cells. Knowing that downstream targets of glucose-regulated insulin signaling are predominant mediators of early gene transcription (13), in this study, we evaluated the regulation of Foxo1 by glucose in β -cells. To address this issue, glucose-responsive β -cell-derived MIN6 cells

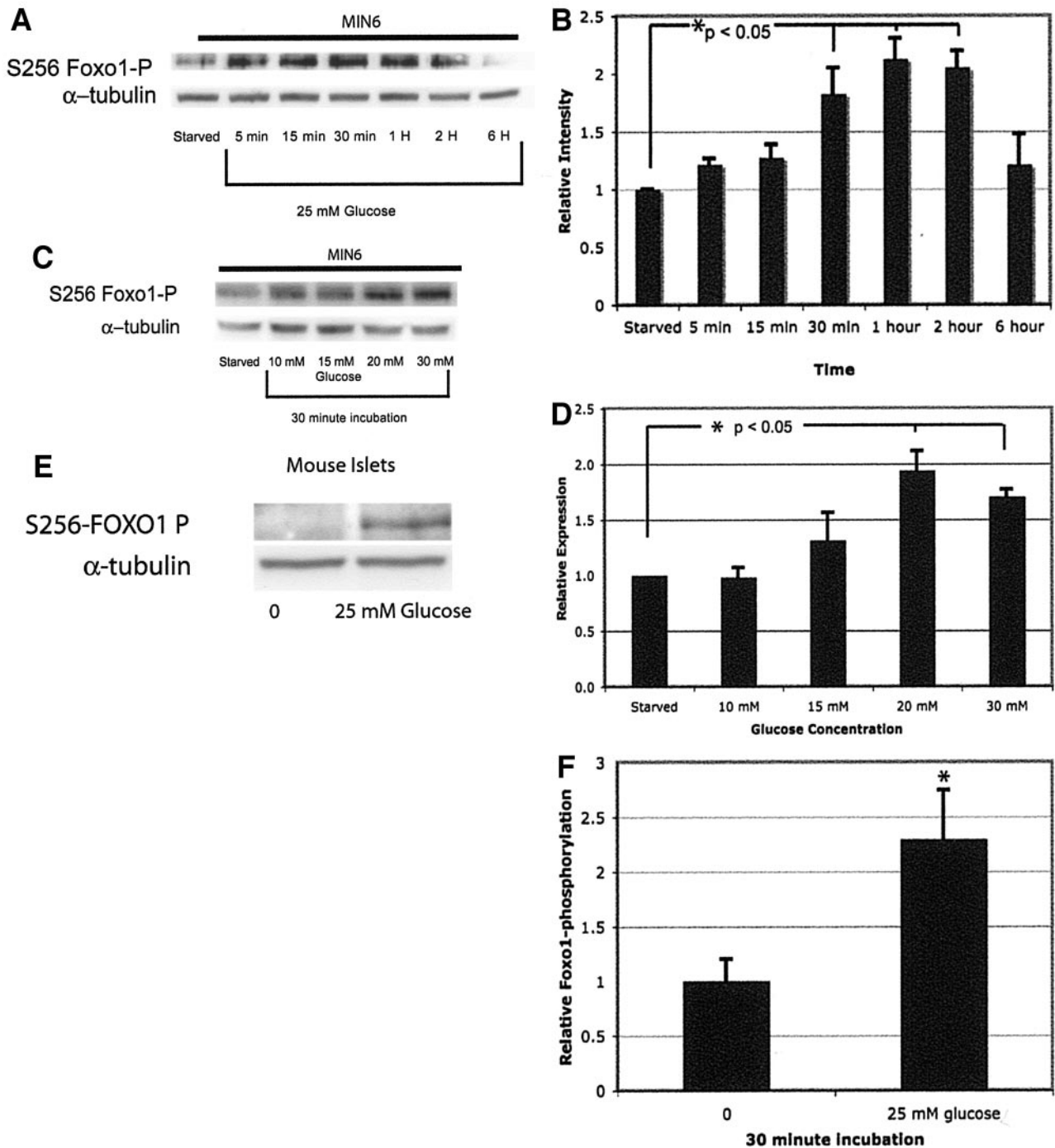


FIG. 1. Glucose induces phosphorylation of Foxo1 in MIN6 insulinoma cells. **A:** MIN6 cells were grown in starved media (5 mmol/l glucose and 2% FBS in DMEM) for 24 h and then harvested at the indicated times after glucose (25 mmol/l) exposure. Phosphorylation of Foxo1 was determined by Western blot analysis with anti-phospho-Ser²⁵⁶-Foxo1. **B:** Quantitation of Foxo1 phosphorylation in a glucose time course ($n = 4$) is shown with SEM. P value according to Student's t test. **C:** Dose response of Foxo1 phosphorylation in MIN6 cells grown in starved media and exposed to varying concentrations of glucose at 30 min. **D:** Quantitation of triplicate blots of Foxo1 phosphorylation at 30 min in MIN6 shown with SEM. **E:** Western blot of phosphorylated Foxo1 from isolated mouse islets preincubated (1 h) in Krebs-Ringer bicarbonate without glucose followed by a 30-min incubation with (25 mmol/l) or without glucose. This is a representative blot of three identical blots evaluating a total of six mice. **F:** Quantitation of Foxo1 phosphorylation from the experiment in **E**.

were initially utilized (16). Following 24 h of starvation (5 mmol/l glucose/2% FBS), there was a relatively low level of Foxo1 phosphorylation (Fig. 1). With the addition of glucose (25 mmol/l) there was a rapid phosphorylation of Foxo1, which reached a peak in intensity between 30 min and 2 h and then declined within 6 h to a baseline level (Fig. 1A and B). To determine whether Foxo1 phosphorylation responds within the range of glucose that triggers

insulin secretion, cells were treated with varying glucose concentrations from 5 to 30 mmol/l, and the effects on Foxo1 phosphorylation were observed. As shown in Figs. 1C and D, there was a gradual increase in Foxo1 phosphorylation with increasing concentrations of glucose, examined at a 30-min time point. To validate the result of the experiment on Foxo1 phosphorylation in conditions closer to the in vivo, primary cultures of mouse islets were

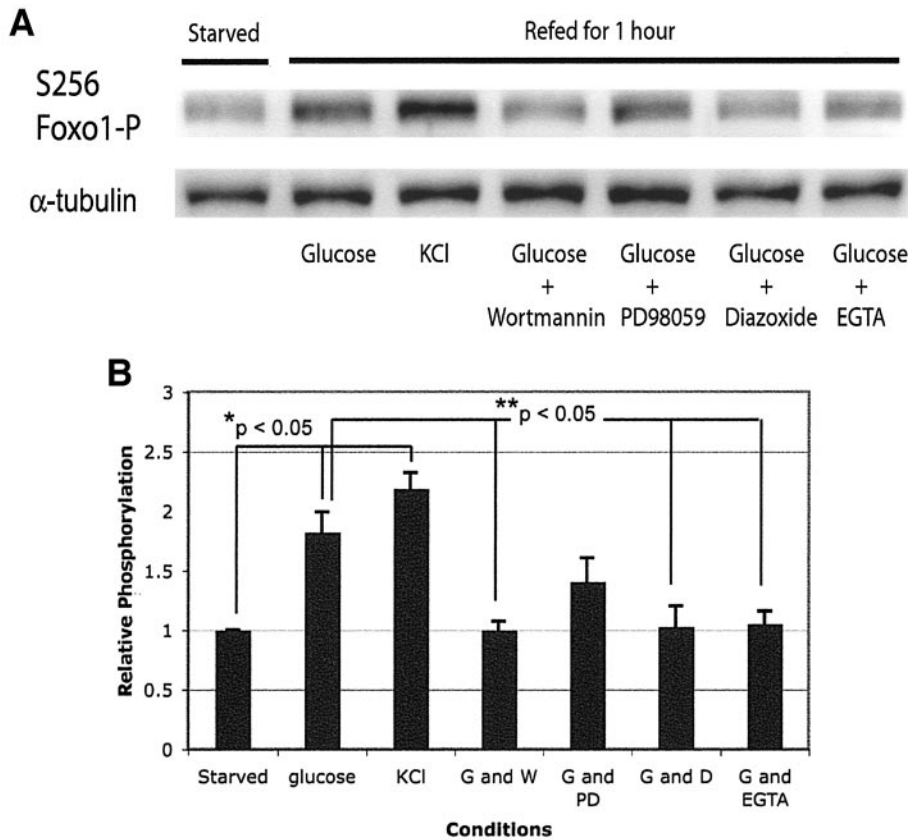


FIG. 2. Glucose-induced Foxo1 phosphorylation in MIN6 cells is voltage, calcium, and PI3K dependent. **A:** Western blot of Foxo1 phosphorylation. MIN6 were grown for 24 h in starved media (5 mmol/l glucose and 2% FBS in DMEM) and then exposed for 1 h to glucose (25 mmol/l), KCl (30 mmol/l), glucose (25 mmol/l) plus wortmannin (100 nmol/l), glucose (25 mmol/l) plus PD98059 (50 μ mol/l), glucose (25 mmol/l) plus diazoxide (500 μ mol/l), and glucose (25 mmol/l) plus EGTA (2.5 mmol/l). This blot is representative of three identical blots. **B:** Quantitation of the effects from the inhibitors on Foxo1-phosphorylation, shown with SEM. D, diazoxide; G, glucose; PD, PD98059; W, wortmannin. *Difference from starved; **difference from glucose treatment.

also evaluated. As shown in Fig. 1E, there was a significant phosphorylation of Foxo1 observed after 30 min of 25 mmol/l glucose refeeding ($P < 0.05$). These experiments established that following a period of nutrient deprivation, glucose refeeding resulted in the phosphorylation of endogenous Foxo1 in insulin-secreting β -cells.

Glucose phosphorylation of Foxo1 is dependent on depolarization and calcium influx and is mediated by intact PI3K signaling. We next evaluated the mechanism by which glucose leads to the phosphorylation of Foxo1. Treatment of pancreatic β -cells with glucose is known to result in cell depolarization, mediated through inhibition of ATP-sensitive K^+ channels (K_{ATP} channels) (22). Depolarization leads to the activation of voltage-gated calcium channels, with subsequent Ca^{2+} influx and the release of insulin (23). To determine which of these steps are required for glucose-induced Foxo1 phosphorylation, several experimental conditions were evaluated. MIN6 cells were incubated for 24 h in starvation media, after which cells were exposed for 1 h to glucose, potassium, or glucose plus one of several inhibitors. As illustrated in Fig. 2A, depolarization of the MIN6 cells by exposure to potassium (30 mmol/l) also resulted in Foxo1 phosphorylation, suggesting that depolarization could be a major component of the mechanism of glucose stimulation. To further document that depolarization is a vital component of glucose-induced phosphorylation of Foxo1, diazoxide, a K_{ATP} channel activator, obliterated Foxo1 phosphorylation by glucose. This result indicated that glucose-induced depolarization was required and that the glucose effect was not due to an osmotic change. Treatment with EGTA, a calcium-chelator, blocked the glucose response, indicating that an extracellular calcium influx was required. Two of the major signaling pathways downstream of depolar-

ization-induced calcium influx are the Ras-Mek-mitogen-activated protein kinase (24) and the PI3K pathways (14). To evaluate the possible role of these pathways, inhibitors of either Mek or PI3K signaling were used. Wortmannin (100 nmol/l), a PI3K inhibitor, significantly blocked the glucose-induced Foxo1 phosphorylation. The Mek inhibitor PD98059 (50 μ mol/l) led to inactivation of extracellular signal-related kinase (data not shown), and while there appeared to be some inhibition of glucose-induced phosphorylation, this was not significant (Fig. 2B). These results illustrated that endogenous Foxo1 phosphorylation in β -cells can be induced by depolarization alone and that the ability for glucose to enhance Foxo1 phosphorylation is dependent upon K_{ATP} channel closure, calcium influx, and the PI3K signaling pathway.

Insulin also results in a rapid and dose-dependent phosphorylation of Foxo1. Glucose-induced depolarization of β -cells is known to result in insulin secretion, and the addition of exogenous insulin in other mammalian cells has been shown to induce Foxo1 phosphorylation (25,26). In previous experiments, we demonstrated that MIN6 cells are responsive to insulin, leading to phosphorylation of IRSs and Akt (7). To determine whether exogenous insulin would also result in Foxo1 phosphorylation in MIN6 cells, insulin was added to cells after an overnight starvation. Similar to the response to glucose, the addition of insulin (100 nmol/l) resulted in the rapid phosphorylation of Foxo1 (Fig. 3A and B). This response of insulin on Foxo1 phosphorylation was also shown to be time- and dose-dependent (Fig. 3C and D).

Glucose-induced phosphorylation of Foxo1 is mediated by the insulin receptor. The results of the previous experiments are consistent with the hypothesis that glucose treatment promotes the phosphorylation of Foxo1

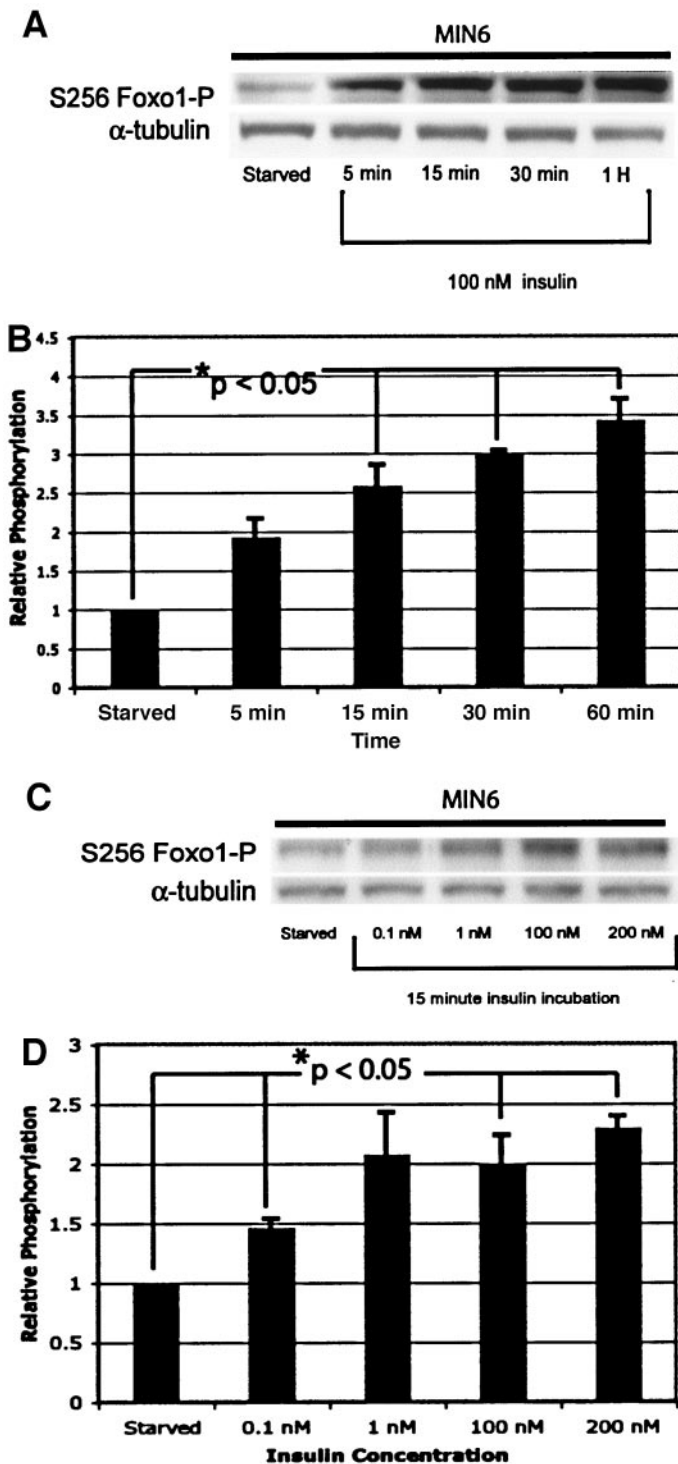


FIG. 3. Insulin induces phosphorylation of Foxo1 in MIN6 insulinoma cells. **A:** MIN6 cells were grown in the starved media for 24 h and harvested at the indicated times after exogenous administration of 100 nmol/l insulin to the media. This blot of Foxo1 phosphorylation is representative of at least three identical blots. **B:** Quantitation of the Foxo1 phosphorylation in the insulin time course is shown with SEM and *P* value. **C:** Representative of three identical blots for Foxo1 phosphorylation of MIN6 cells grown in the starved media that were exposed to varying concentrations of added insulin at 15 min. **D:** Quantitation of the Foxo1 phosphorylation as responsive to insulin concentration is shown with SEM and *P* value.

through an autocrine/paracrine effect of released insulin on its receptor. To further test this hypothesis, we repeated the same glucose time course in MIN6 cells stably

expressing small interfering RNA toward the insulin receptor, the IRKDΔ80 cell line. These cells exhibit 80% reduction in the insulin receptor, decreased IRS-1 tyrosine phosphorylation, and reduced Akt phosphorylation by glucose treatment, as compared with MIN6 cells or MIN6 stably transfected cells with an empty vector or expressing scrambled siRNA (7). In contrast to the results on Foxo1 phosphorylation on MIN6 cells and islets, similar treatment with glucose in IRKDΔ80 cells showed no change in Foxo1 phosphorylation (Fig. 4). Since the IRKDΔ80 cells have been shown to maintain a robust insulin secretory response to glucose (7), the block of glucose-induced Foxo1 phosphorylation is likely due to decreased insulin receptor function. Thus, it appears that the major effect of glucose leading to phosphorylation of Foxo1 is mediated via secreted insulin acting on its own receptor.

Glucose induces a PI3K-dependent nuclear to cytoplasmic Foxo1 translocation in β-cells. In cells other than pancreatic β-cells, phosphorylation of Foxo1 by Akt is associated with inactivation of the transcriptional activity and nuclear to cytoplasmic translocation (9,10). To determine whether the glucose-induced phosphorylation was associated with intracellular translocation within β-cells, we evaluated the intracellular localization of a GFP-tagged full-length Foxo1 in MIN6 cells following various stimuli. Cells were transiently transfected with a plasmid expressing a full-length Foxo1 protein fused with GFP at the NH₂-terminus. After a 24-h recovery period from transfection, the cells were incubated for an additional 24 h in normal or starved media. In normal media, the majority (70%) of GFP-tagged Foxo1 was in the cytoplasm of MIN6 cells (Fig. 5A and B). In contrast, incubation in starved media resulted in ~80% of the GFP-tagged Foxo1 in the nucleus. After 1 h of glucose (25 mmol/l) refeeding, there was a shift in localization from the nucleus to the cytoplasm. This shift was blocked by the simultaneous addition of the PI3K inhibitor wortmannin (100 nmol/l). It had been shown in rat insulinoma cells (INS1) that IGF-1 treatment leads to the activation of Akt in a PI3K-dependent manner and rapid phosphorylation of Foxo1 (27). Following starvation, treatment with IGF-1 (100 ng/ml) for 1 h resulted in nuclear exclusion of Foxo1, and this effect was also blocked by wortmannin (100 nmol/l).

To determine whether the nuclear to cytoplasmic translocation that was observed with refeeding required insulin receptor signaling, the experiments were repeated with the IRKDΔ80 cells. In contrast to the cytoplasmic localization of Foxo1 in MIN6 cells, IRKDΔ80 cells grown in normal media maintained most (80%) of the GFP-tagged Foxo1 in the nucleus (Fig. 5A and C). Additionally, following glucose refeeding, the majority of Foxo1 was retained in the nucleus, and wortmannin, as anticipated, did not alter this response. The only condition promoting a nuclear-to-cytoplasmic translocation of Foxo1 in the IRKDΔ80 cells was exposure to IGF-1 (100 ng/ml) for 1 h following starvation (Fig. 5A and C). These results indicated that while the insulin receptor signaling is reduced in the IRKDΔ80 cells, the PI3K signaling pathway is intact. Since the IRKDΔ80 cells have been shown to maintain glucose-stimulated insulin secretion, the block of glucose-induced Foxo1 phosphorylation is likely due to decreased insulin receptor function (28,29).

To establish intracellular localization of Foxo1 within physiologic glucose concentrations of fasting and feeding, MIN6 cells were transfected with the GFP-tagged Foxo1

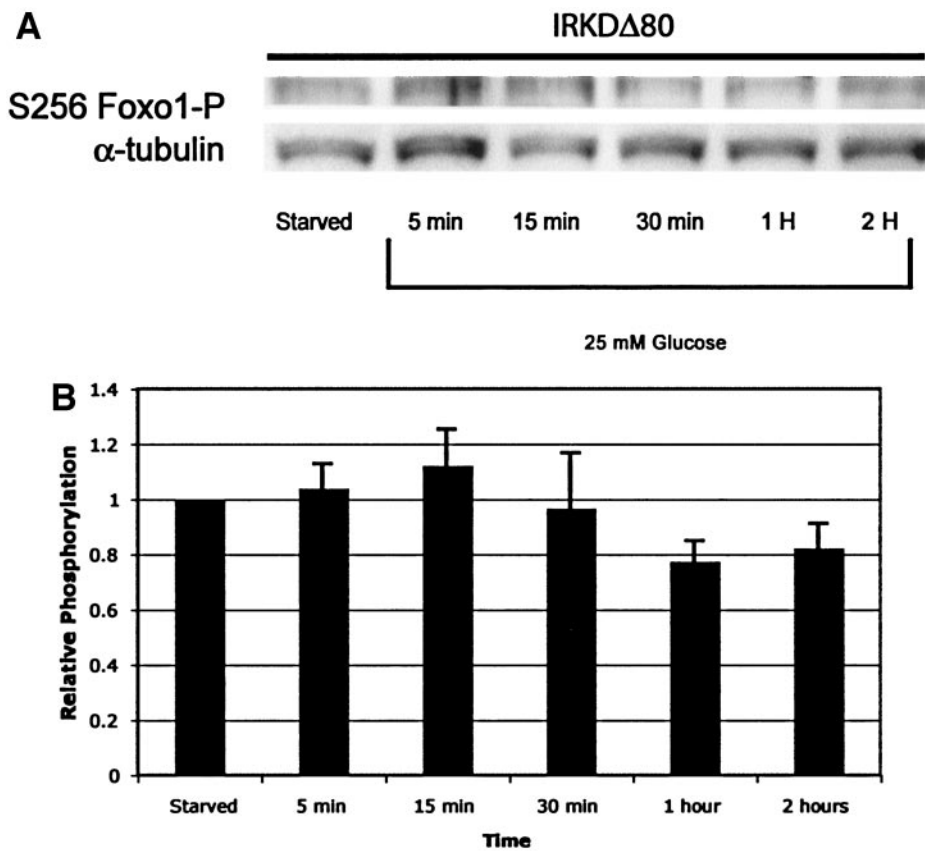


FIG. 4. Glucose-induced phosphorylation of Foxo1 is impaired in insulin receptor-deficient IRKD Δ 80 cells. **A:** IRKD Δ 80 cells were grown in the starved media for 24 h and harvested at the indicated times of glucose (25 mmol/l) exposure. Foxo1 phosphorylation was determined by Western blot analysis. This is a representative blot from four identical experiments. **B:** Quantitation of the Foxo1 phosphorylation by glucose in IRKD Δ 80 cells. None of the time points were significant for phosphorylation from the starved state.

and maintained in varying glucose concentrations overnight ranging from 2.5 to 20 mmol/l (Fig. 5D). With each increase in glucose concentration, there was an associative decrease in the nuclear localization of Foxo1 in MIN6 cells.

Foxo1 transcriptional activity is regulated by glucose. To assess the relative activity of Foxo1 in 5 mmol/l glucose compared with the normal media concentration of 25 mmol/l glucose in MIN6 cells, we used a Foxo1-dependent promoter assay. Our studies of Foxo1 activity used a promoter-luciferase construct containing an insulin response element and conserved Foxo1 binding site in the IGFBP-1 promoter (30). In the liver, IGFBP-1 transcription has been shown to be dependent on Foxo1 activity (31). We first observed that in starved cells at 5 mmol/l glucose, endogenous Foxo1 activity was increased by twofold ($P < 0.01$) compared with that in fed cells at 25 mmol/l glucose (Fig. 6A). The effect of starvation was more readily observed with cotransfection of the full-length Foxo1 plasmid.

To further characterize the dynamics of endogenous Foxo1 activity and extracellular glucose concentrations in β -cells, additional experiments were conducted comparing Foxo1 activity at 20, 10, 5, and 2.5 mmol/l glucose concentrations for a 24-h incubation (Fig. 6B). Foxo1 activity demonstrated a progressive increase in activity as the glucose concentration decreased.

In another set of experiments, we compared the effects of starvation on Foxo1 activity in the presence of a dominant-negative Foxo1, a truncated allele of Foxo1 (Foxo1 Δ 256) that retains the DNA-binding domain (amino acid residues 1–256) but lacks the transactivation domain (26). Following starvation, there was no increase in the Foxo1 transcriptional activity of cells cotransfected with Foxo1 Δ 256 ($P < 0.001$) (Fig. 6C), which strongly suggested

that the increased activity in starvation is predominantly due to endogenous Foxo1. Additionally, a mutant Foxo1 allele (Foxo1 Δ 24A) previously shown to be nuclear in hepatoma cells (26) was observed to be nuclear in MIN6 cells (data not shown). This was associated with increased Foxo1 activity in the fed state ($P < 0.01$) when compared with cotransfection with the wild-type allele of Foxo1. The results of these promoter assays demonstrated that starvation-induced nuclear localization was associated with increased Foxo1 transcriptional activity.

Glucose starvation regulates an endogenous Foxo target gene in MIN6 cells and in pancreatic islets. To determine whether the reduced phosphorylation and nuclear localization of Foxo1 could be correlated with an increase in transcription of a direct Foxo target, we evaluated the mRNA of *Gadd45* from MIN6 cells incubated for 24 h in either normal or starved media. The promoter of this gene contains consensus-binding motifs of Foxo family members and has been shown to be enhanced in activity in various tissues by direct binding of Foxo3 and Foxo4 (28,29). *Gadd45* is a gene that is upregulated in conditions of stress such as exposure to reactive oxygen species or nutrient withdrawal, leading to a G2/M cell-cycle arrest (28). Starvation of MIN6 cells (Fig. 7A) or mouse islets (Fig. 7B) for 24 h revealed an increase in the expression of *Gadd45*, demonstrated by RT-PCR and real-time RT-PCR analysis, suggesting that nutrient regulation of Foxo activity has a physiologically relevant biological function.

Glucose starvation regulates a potential Foxo target gene (*Chop*) in MIN6 cells and islet β -cells. To further substantiate the possible biological consequences of nutrient regulation of Foxo activity in β -cells, RNA of starved MIN6 cells was analyzed by microarray for additional

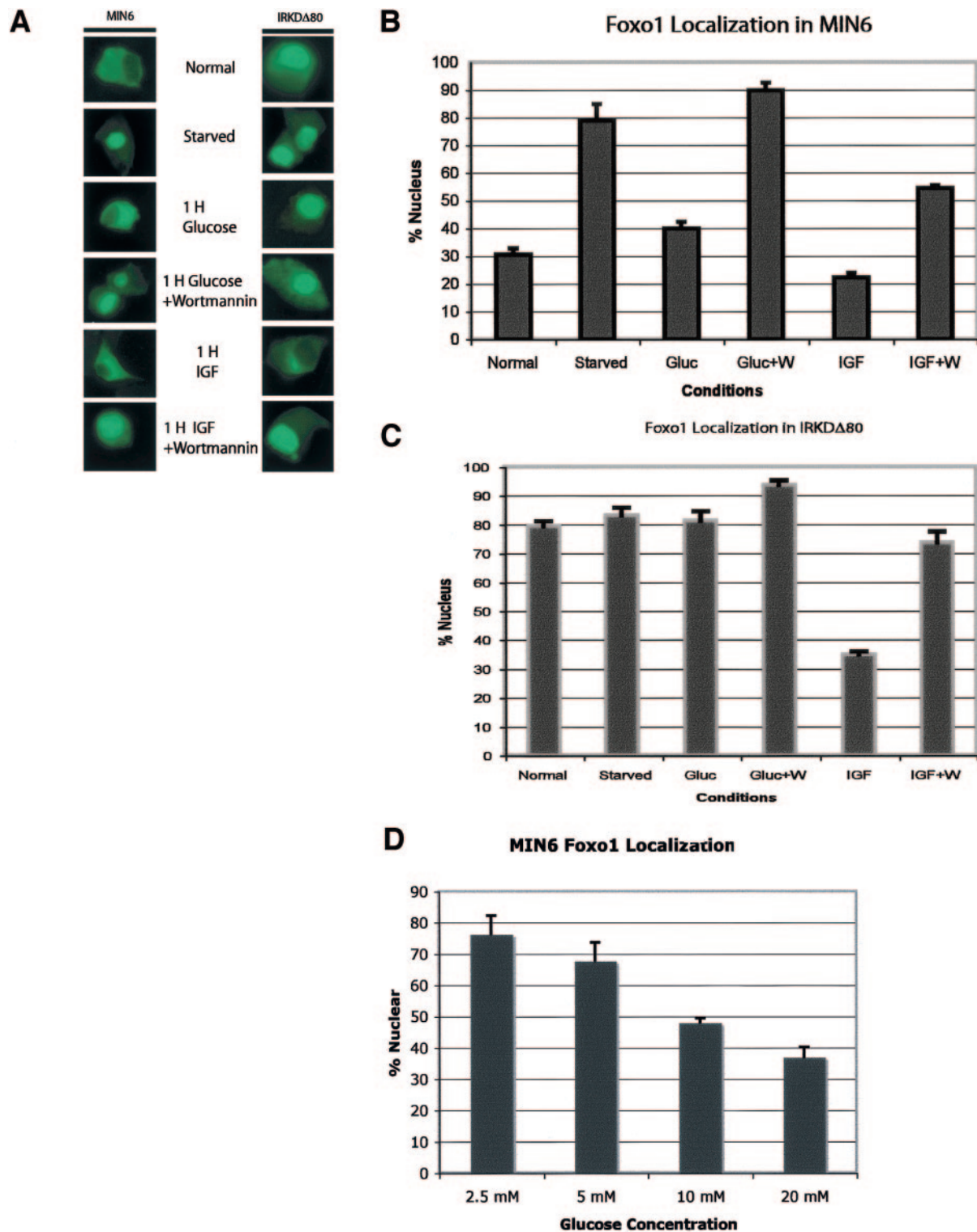


FIG. 5. Glucose promotes nuclear to cytoplasmic translocation of Foxo1 with intact insulin receptor and PI3K signaling. **A:** GFP-tagged Foxo1 localized to either the nucleus or cytoplasm of MIN6 and IRKD Δ 80 subjected to identical nutrient conditions ($n > 100$ /condition) of fed and starved media, glucose (25 mmol/l) and IGF (100 ng/ml) alone or in the presence of wortmannin (W) (100 nmol/l). Quantization of the MIN6 or IRKD80 cells in the nutrient conditions is represented in **B** and **C**, respectively. **D:** Nuclear localization of GFP-tagged Foxo1 in MIN6 cells incubated at varying glucose concentrations for 24 h.

Foxo1 targets. Of the 34 significantly upregulated genes in glucose-starved cells, the gene that was most upregulated was DNA damage-inducible transcript 3 (*Ddit3*, *Chop*, or *Gadd153*; 3.72-fold [95% CI 3.31–4.46]) (online appendix Table 1). The observation of enhanced expression of *Chop*/*Gadd153* by nutrient deprivation has been previously

described (32–34). Conversely, glucose treatment of starved MIN6 cells resulted in the downregulation of *Chop* (8). Conserved, consensus Foxo1 binding sites (35) were identified in the proximal *Chop* promoter (Fig. 7C). To confirm that the glucose-regulated changes in *Chop* mRNA were reflected at the protein level, protein from MIN6 cells

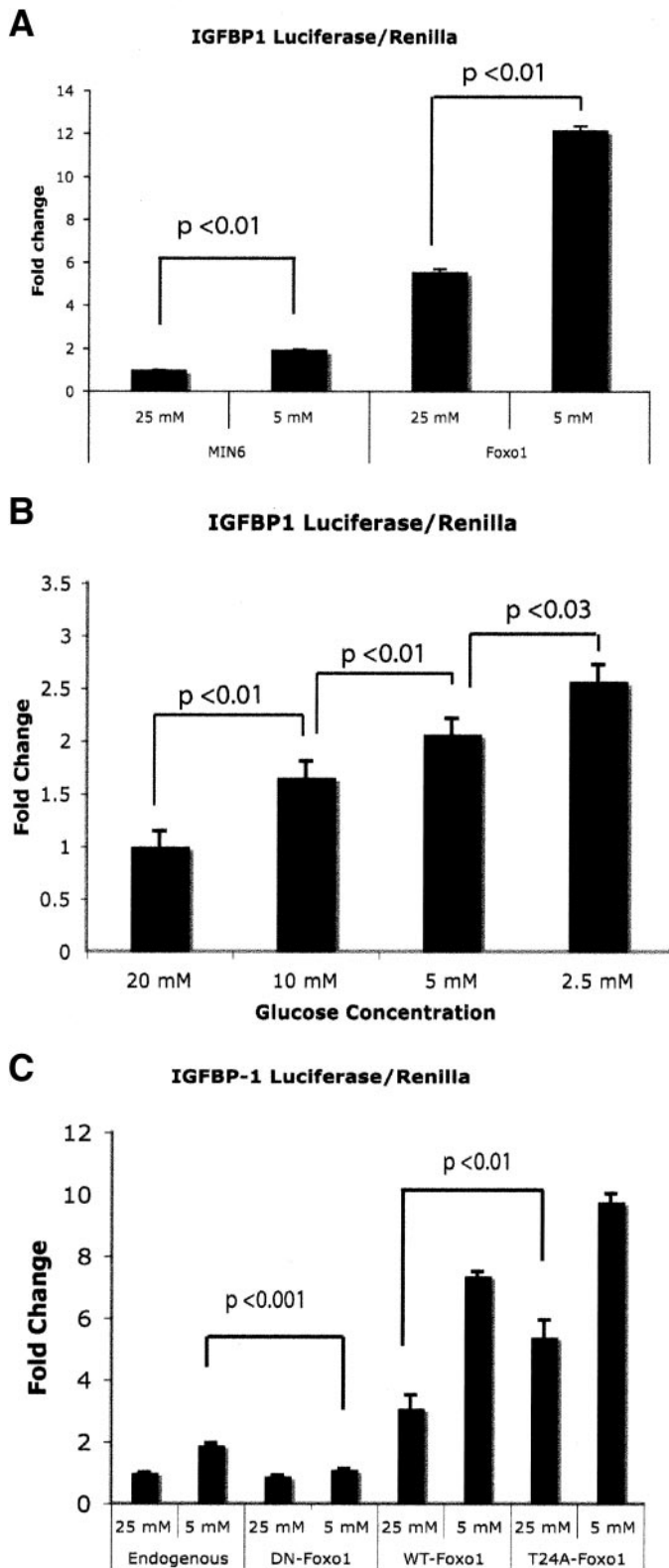


FIG. 6. Transcriptional activity of Foxo in the fed and starved state. **A:** Endogenous or transfected Foxo1 activity is increased around twofold ($P < 0.01$) in the starved state using an IGFBP1-promoter luciferase assay. **B:** Foxo1 activity is increased as glucose concentrations decrease in this 24-h incubation. **C:** Truncated allele of Foxo1 (DN-Foxo1) functions as a dominant-negative preventing endogenous Foxo1 activity in the starved state while a constitutively nuclear allele T24A-Foxo1 increases Foxo1 activity compared with a wild-type allele (WT-Foxo1).

and islets maintained in starved media overnight was examined by a Western blot that revealed elevated Chop expression (Fig. 7D and E).

The above results demonstrated an association between nutrient deprivation, Foxo1 activity, and Chop expression. To determine whether Chop expression was mediated by enhanced Foxo1 activity, MIN6 cells were transfected with a luciferase construct containing the Chop promoter. Cotransfection of the full-length Foxo1 plasmid (pCMV5-Foxo1) enhanced Chop-promoter activity in the presence of normal media (Fig. 8A). This result indicated that exogenous Foxo1 could activate the Chop reporter. Chop promoter activity was increased with nutrient deprivation, and this increase was significantly reduced with the cotransfection of the dominant-negative Foxo1 (Foxo1^{Δ256}) (Fig. 8B). While these results suggest that under starved conditions Foxo1 is regulating the promoter activity of Chop, this conclusion needs to be confirmed by chromatin immunoprecipitation, demonstrating a direct physical binding of Foxo1 to the Chop promoter.

DISCUSSION

Glucose contributes to regulation of β -cell growth and survival. The experiments presented here were designed to test the hypothesis that glucose modulates Foxo1, a transcription factor that affects both cell metabolism and growth/apoptosis, activity. We examined whether Foxo1 could be regulated by glucose in pancreatic islet β -cells. Our findings showed that starvation for glucose of both MIN6 and mouse islets resulted in reduced Foxo1 phosphorylation and movement from a predominantly cytoplasmic to nuclear localization. Glucose treatment of previously starved MIN6 cells resulted in rapid phosphorylation of Foxo1 and nuclear exclusion. What evidence is there that these results have relevance for β -cell physiology? After an overnight fast, plasma glucose concentrations are routinely <5 mmol/l. Using fluorescently tagged Foxo1 in which the cellular localization could be monitored, we showed that incubation in increasing concentrations of glucose resulted in decreases in nuclear localization. Foxo1 activity, measured with Foxo1-target promoter fused to a luciferase reporter, correlated with these observations with increased activity as glucose concentrations decreased from 10 to 2.5 mmol/l. While these results are not directly applicable to the in vivo situation, they do provide suggestive evidence that at physiological glucose concentrations found after an overnight fast, Foxo1 is transcriptionally active and is suppressed by glucose concentrations typically found upon refeeding.

These observations provided a model to evaluate the signaling mechanisms involved. Glucose effects on Foxo1 phosphorylation in the response to refeeding were shown to be dependent on depolarization, PI3K activity, and insulin secretion leading to activation of its own receptor. While modulation of Foxo1 activity by insulin has been amply demonstrated in a number of different insulin-target tissues (36–38), a novel observation in the current experiments was that in the case of the islet β -cell, a nutrient activates hormone secretion that in turn regulates Foxo1 activity through a cell-autonomous mechanism.

Evidence that glucose regulation of Foxo1 activity is mediated through insulin signaling includes the observation that KCl-induced depolarization, known to result in insulin secretion, phosphorylated Foxo1 in the absence of

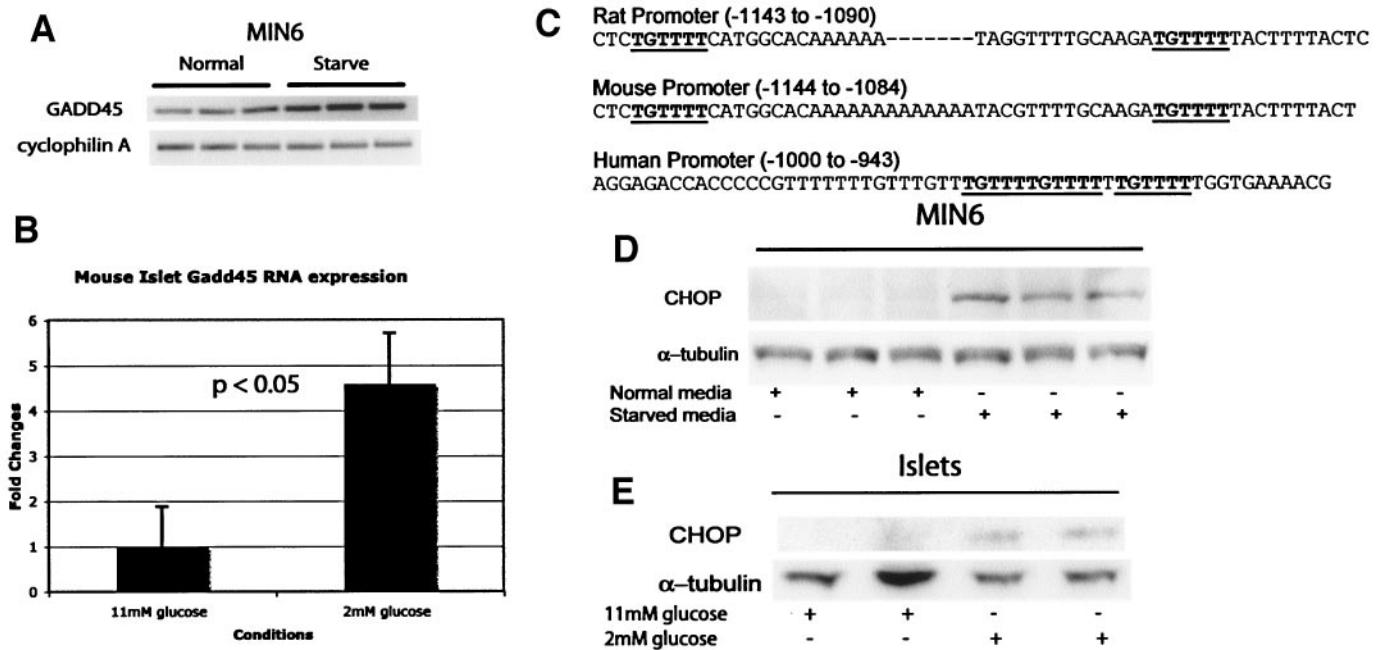


FIG. 7. Known and novel targets of Foxo1 exhibit expression in the starved state. *A*: RT-PCR of total RNA for *Gadd45*, a Foxo-dependent target, upregulated in MIN6 cells incubated in 5 mmol/l glucose/2% FBS vs. 25 mmol/l glucose/15% FBS media. *B*: *Gadd45* RNA expression changes in islets incubated in 2 or 11 mmol/l glucose for 24 h. Total RNA was extracted from islets and subjected to real-time RT-PCR. *C*: Chop promoter from rat, mouse, and human sequences contains conserved consensus binding sites for Foxo1. *D*: Chop protein is upregulated with starved media in MIN6 cells, triplicate experiment shown. *E*: Chop protein is upregulated in islets incubated in 2 compared with 11 mmol/l glucose for 24 h; experiment in duplicate is shown.

glucose. Further, diazoxide, a K_{ATP} channel opener and a known inhibitor of glucose-induced insulin secretion (39), effectively blocked glucose-induced Foxo1 phosphorylation. Calcium influx is essential for glucose-stimulated insulin secretion, and the addition of a calcium chelator inhibited glucose-induced Foxo1 phosphorylation. While we also observed that exogenous insulin resulted in Foxo1 phosphorylation, this does not exclude the possibility that this was a pharmacologic effect of the concentration added or that insulin was acting through a homologous receptor,

such as the IGF-1 receptor. However, when glucose was added to MIN6 cells with reduced insulin receptor expression (IRKD80), the glucose-stimulated insulin that was released had no effect on Foxo1 phosphorylation or localization. This suggests that the glucose effect on Foxo1 activity is mediated primarily through an autocrine/paracrine effect of insulin acting upon its own receptor.

The results of the current studies demonstrated enhanced transcriptional activity of Foxo1 in the starved state by the promoter-luciferase assay. In MIN6 cells and

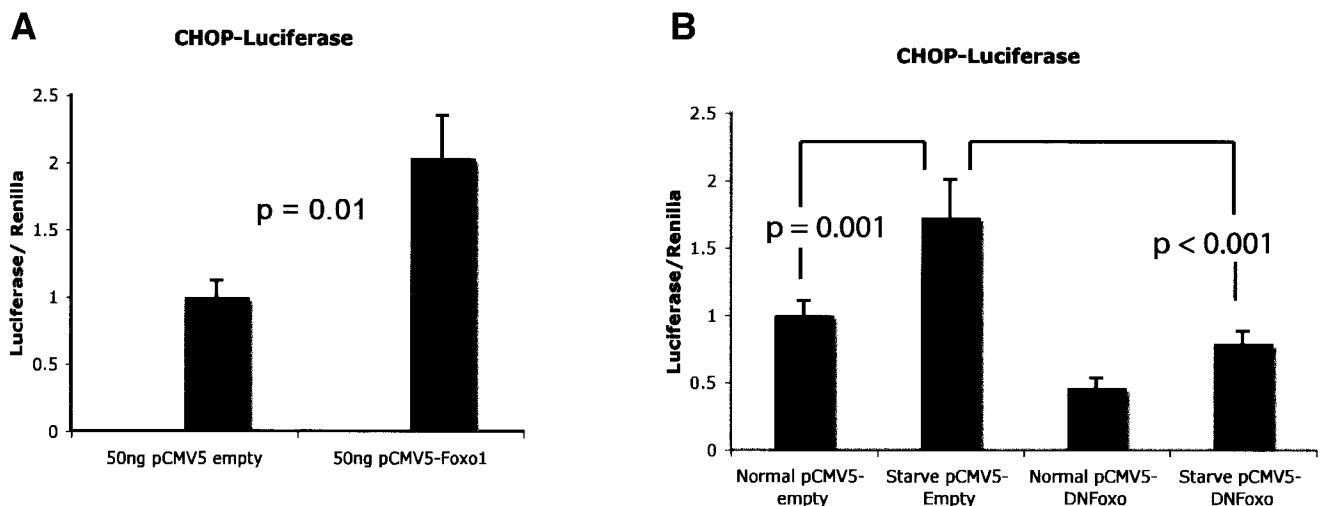


FIG. 8. Chop promoter activity is upregulated by Foxo1 and starvation and ablated with DN-Foxo1. *A*: Cotransfection of full-length Foxo1 increases *Chop*-promoter luciferase activity in MIN6 cells incubated in 25 mmol/l glucose/15% FBS. *B*: *Chop*-promoter activity is increased in MIN6 cells incubated in 5 mmol/l starved media, cotransfected with empty pCMV5 vector. Transfection of pCMV5-DNFoxo1 (dominant-negative) ablated the increase in *Chop*-promoter luciferase activity in the starved media condition.

islets, after 24 h of starvation, we observed increased *Gadd45* mRNA, previously shown to be Foxo-dependent and upregulated upon nutrient withdrawal in other tissues (18,28). It appears that a similar response in Foxo1 activity to nutrient deprivation is present in islets as well, although the targets and their physiological relevance are just beginning to be uncovered.

Consistent with previous literature, we have observed *Chop* mRNA and protein to be increased in MIN6 cells and primary islets in nutrient deprivation (32). We have extended these studies by demonstrating enhanced *Chop* promoter activation in starved media. We identified potential Foxo1 binding sites, demonstrated upregulation of *Chop* promoter activity by the addition of Foxo1, and significantly reduced endogenous *Chop* activation in starvation with the addition of a dominant-negative Foxo1. These experiments suggest a novel mechanism and link between nutrient deprivation and reduced insulin signaling contributing to *Chop* expression in β -cells via increased Foxo1 activity, although these conclusions need to be supported by direct evidence.

While it is well known that increased Foxo1 activity in insulin-responsive tissues such as the liver and muscle contributes to insulin resistance (38,40), the specific role of this transcription factor with respect to islet dysfunction present in type 2 diabetes has yet to be uncovered. Increased Foxo1 activity has been shown to promote neuronal cell death through enhanced transcription of genes involved in apoptosis (41). Pancreatic β -cells are especially susceptible to apoptosis induced by endoplasmic reticulum stress because they maintain a highly developed and active endoplasmic reticulum to secrete insulin (42). Increased *Chop* expression has been associated with endoplasmic reticulum stress and apoptosis in β -cells, concomitant with decreased insulin signaling (43). In the current experiments, we observed increased *Chop* expression in islets and MIN6 cells maintained in low-glucose media and that this nutrient-regulated expression is mediated by Foxo1 activity. Cultured mouse islets incubated under conditions of 2 compared with 15 mmol/l glucose and MIN6 cells incubated in 5 compared with 25 mmol/l glucose exhibited a threefold increase in apoptosis, and this apoptosis was blocked in MIN6 cells by the addition of constitutively active Akt, an inhibitor of Foxo1 (44).

In addition to decreasing apoptosis, glucose has been shown to be an important enhancer of proliferation in β -cells as a result of increased PI3K and Akt activity (1). Additionally, the expansion of β -cell mass through β -cell-specific overexpression of PKB/Akt could be mediated, in part, through strong inhibition of Foxo1 (3,4). Foxo family members have been shown to promote expression of the cell-cycle inhibitor *p27* and decrease expression of cyclin D (45), which induces growth arrest in diverse mammalian cell types (46–48). Pancreatic β -cells expand and proliferate as a normal response to hyperglycemia, resulting in increased insulin secretion and signaling (49). The current observations that glucose treatment of MIN6 cells results in inactivation of Foxo1 activity, provides a potential mechanism and hypothesis by which glucose suppression of Foxo1 activity may contribute to this increase in β -cell proliferation. Because of the limited capacity of adult β -cells to proliferate (50) and the demonstrated regulation of Foxo1 by glucose and intact insulin receptor signaling, the potential involvement of Foxo1 in the processes of cell-cycle arrest and apoptosis within β -cells merits further investigation. Whether aberrant regulation of Foxo1

activity contributes to the β -cell dysfunction of type 2 diabetes remains to be characterized.

ACKNOWLEDGMENTS

This work was supported in part by a National Institutes of Health grant (R37 DK16746, to M.A.P.), a Junior Faculty Award from the American Diabetes Association (to E.B.-M.), a Mentor-Based Fellowship Award from the American Diabetes Association (to C.C.-M.), and a Ruth L. Kirschstein Pre-Doctoral National Research Service Award (F32 DK06537, to S.C.M.).

The authors thank H. Harding and D. Ron (New York University) for reagents, D. Accili (Columbia) for insightful comments and reagents, N. Abumrad and K. Polonsky for helpful comments on the manuscript, Cris M. Welling for lab assistance, other members of the Permutt lab for helpful discussions, and Susan Vance for preparation of the manuscript.

REFERENCES

- Hughl SR, White MF, Rhodes CJ: Insulin-like growth factor I (IGF-I)-stimulated pancreatic β -cell growth is glucose-dependent: synergistic activation of insulin receptor substrate-mediated signal transduction pathways by glucose and IGF-I in INS-1 cells. *J Biol Chem* 273:17771–17779, 1998
- Kaburagi Y, Yamauchi T, Yamamoto-Honda R, Ueki K, Tobe K, Akanuma Y, Yazaki Y, Kadowaki T: The mechanism of insulin-induced signal transduction mediated by the insulin receptor substrate family. *Endocr J* 46 (Suppl.):S25–S34, 1999
- Bernal-Mizrachi E, Wen W, Stahlhut S, Welling CM, Permutt MA: Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. *J Clin Invest* 108:1631–1638, 2001
- Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furth EE, Polonsky KS, Naji A, Birnbaum MJ: Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. *Nat Med* 7:1133–1137, 2001
- Kubota N, Terauchi Y, Tobe K, Yano W, Suzuki R, Ueki K, Takamoto I, Satoh H, Maki T, Kubota T, Moroi M, Okada-Iwabu M, Ezaki O, Nagai R, Ueta Y, Kadowaki T, Noda T: Insulin receptor substrate 2 plays a crucial role in beta cells and the hypothalamus. *J Clin Invest* 114:917–927, 2004
- Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR: Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* 96:329–339, 1999
- Ohsugi M, Cras-Meneur C, Zhou Y, Bernal-Mizrachi E, Johnson JD, Luciani DS, Polonsky KS, Permutt MA: Reduced expression of the insulin receptor in mouse insulinoma (MIN6) cells reveals multiple roles of insulin signaling in gene expression, proliferation, insulin content, and secretion. *J Biol Chem* 280:4992–5003, 2005
- Ohsugi M, Cras-Meneur C, Zhou Y, Warren W, Bernal-Mizrachi E, Permutt MA: Glucose and insulin treatment of insulinoma cells results in transcriptional regulation of a common set of genes. *Diabetes* 53:1496–1508, 2004
- Biggs WH 3rd, Meisenhelder J, Hunter T, Cavenee WK, Arden KC: Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A* 96:7421–7426, 1999
- Kitamura T, Nakae J, Kitamura Y, Kido Y, Biggs WH 3rd, Wright CV, White MF, Arden KC, Accili D: The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *J Clin Invest* 110:1839–1847, 2002
- Accili D, Arden KC: FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell* 117:421–426, 2004
- Nakae J, Biggs WH 3rd, Kitamura T, Cavenee WK, Wright CV, Arden KC, Accili D: Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nat Genet* 32:245–253, 2002
- Tran H, Brunet A, Griffith EC, Greenberg ME: The many forks in FOXO's road. *Sci STKE* 172:1–11, 2003
- Dickson LM, Lingohr MK, McCuaig J, Hughl SR, Snow L, Kahn BB, Myers MG Jr, Rhodes CJ: Differential activation of protein kinase B and p70(S6)K by glucose and insulin-like growth factor 1 in pancreatic beta-cells (INS-1). *J Biol Chem* 276:21110–21120, 2001

15. Wrede CE, Dickson LM, Lingohr MK, Briaud I, Rhodes CJ: Protein kinase B/Akt prevents fatty acid-induced apoptosis in pancreatic beta-cells (INS-1). *J Biol Chem* 277:49676–49684, 2002
16. Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yamamura K: Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* 127:126–132, 1990
17. Brummelkamp TR, Bernards R, Agami R: A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296:550–553, 2002
18. Nemoto S, Fergusson MM, Finkel T: Nutrient availability regulates SIRT1 through a forkhead-dependent pathway. *Science* 306:2105–2108, 2004
19. Cras-Meneur C, Scharfmann R: FGFR1-IIIb is a putative marker of pancreatic progenitor cells. *Mech Dev* 116:205–208, 2002
20. Wang X, Seed B: A PCR primer bank for quantitative gene expression analysis. *Nucleic Acid Res* 31:e154, 2003
21. Kaestner KH, Lee CS, Scearce LM, Brestelli JE, Arsenlis A, Le PP, Lantz KA, Crabtree J, Pizarro A, Mazzarelli J, Pinney D, Fischer S, Manduchi E, Stoeckert CJ Jr, Gradwohl G, Clifton SW, Brown JR, Inoue H, Cras-Meneur C, Permutt MA: Transcriptional program of the endocrine pancreas in mice and humans. *Diabetes* 52:1604–1610, 2003
22. Miki T, Nagashima K, Seino S: The structure and function of the ATP-sensitive K⁺ channel in insulin-secreting pancreatic beta-cells. *J Mol Endocrinol* 22:113–123, 1999
23. Shibasaki T, Sunaga Y, Fujimoto K, Kashima Y, Seino S: Interaction of ATP sensor, cAMP sensor, Ca²⁺ sensor, and voltage-dependent Ca²⁺ channel in insulin granule exocytosis. *J Biol Chem* 279:7956–7961, 2004
24. Ishihara H, Asano T, Tsukuda K, Katagiri H, Inukai K, Anai M, Kikuchi M, Yazaki Y, Miyazaki JI, Oka Y: Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia* 36:1139–1145, 1993
25. Cunningham MA, Zhu Q, Unterman TG, Hammond JM: Follicle-stimulating hormone promotes nuclear exclusion of the forkhead transcription factor FoxO1a via phosphatidylinositol 3-kinase in porcine granulosa cells. *Endocrinology* 144:5585–5594, 2003
26. Nakae J, Barr V, Accili D: Differential regulation of gene expression by insulin and IGF-1 receptors correlates with phosphorylation of a single amino acid residue in the forkhead transcription factor FKHR. *Embo J* 19:989–996, 2000
27. Liu W, Chin-Chance C, Lee EJ, Lowe WL Jr: Activation of phosphatidylinositol 3-kinase contributes to insulin-like growth factor I-mediated inhibition of pancreatic beta-cell death. *Endocrinology* 143:3802–3812, 2002
28. Tran H, Brunet A, Grenier JM, Datta SR, Fornace AJ Jr, DiStefano PS, Chiang LW, Greenberg ME: DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science* 296:530–534, 2002
29. Furukawa-Hibi Y, Yoshida-Araki K, Ohta T, Ikeda K, Motoyama N: FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress. *J Biol Chem* 277:26729–26732, 2002
30. Ooi GT, Tseng LY, Tran MQ, Rechler MM: Insulin rapidly decreases insulin-like growth factor-binding protein-1 gene transcription in streptozotocin-diabetic rats. *Mol Endocrinol* 6:2219–2228, 1992
31. Kitamura T, Kitamura Y, Nakae J, Giordano A, Cinti S, Kahn CR, Efstratiadis A, Accili D: Mosaic analysis of insulin receptor function. *J Clin Invest* 113:209–219, 2004
32. Webb GC, Akbar MS, Zhao C, Steiner DF: Expression profiling of pancreatic beta cells: glucose regulation of secretory and metabolic pathway genes. *Proc Natl Acad Sci U S A* 97:5773–5778, 2000
33. Carlson SG, Fawcett TW, Bartlett JD, Bernier M, Holbrook NJ: Regulation of the C/EBP-related gene gadd153 by glucose deprivation. *Mol Cell Biol* 13:4736–4744, 1993
34. Huang Q, Lau SS, Monks TJ: Induction of gadd153 mRNA by nutrient deprivation is overcome by glutamine. *Biochem J* 341:225–231, 1999
35. O'Brien RM, Streeper RS, Ayala JE, Stadelmaier BT, Hornbuckle LA: Insulin-regulated gene expression. *Biochem Soc Trans* 29:552–558, 2001
36. Nakae J, Kitamura T, Kitamura Y, Biggs WH 3rd, Arden KC, Accili D: The forkhead transcription factor Foxo1 regulates adipocyte differentiation. *Dev Cell* 4:119–129, 2003
37. Nakae J, Kitamura T, Silver DL, Accili D: The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity to glucose-6-phosphatase expression. *J Clin Invest* 108:1359–1367, 2001
38. Bastie CC, Nahle Z, McLoughlin T, Esser K, Zhang W, Unterman T, Abumrad NA: FoxO1 stimulates fatty acid uptake and oxidation in muscle cells through CD36-dependent and -independent mechanisms. *J Biol Chem* 280:14222–14229, 2005
39. MacDonald PE, Sewing S, Wang J, Joseph JW, Smukler SR, Sakellaropoulos G, Wang J, Saleh MC, Chan CB, Tsushima RG, Salapatek AM, Wheeler MB: Inhibition of Kv2.1 voltage-dependent K⁺ channels in pancreatic beta-cells enhances glucose-dependent insulin secretion. *J Biol Chem* 277:44938–44945, 2002
40. Altomonte J, Richter A, Harbaran S, Suriawinata J, Nakae J, Thung SN, Meseck M, Accili D, Dong H: Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice. *Am J Physiol Endocrinol Metab* 285:E718–E728, 2003
41. Gilley J, Coffey PJ, Ham J: FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *J Cell Biol* 162:613–622, 2003
42. Zhang P, McGrath B, Li S, Frank A, Zambito F, Reinert J, Gannon M, Ma K, McNaughton K, Cavener DR: The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. *Mol Cell Biol* 22:3864–3874, 2002
43. Srinivasan S, Ohsugi M, Liu Z, Fatrai S, Bernal-Mizrachi E, Permutt MA: Endoplasmic reticulum stress-induced apoptosis is partly mediated by reduced insulin signaling through phosphatidylinositol 3-kinase/Akt and increased glycogen synthase kinase-3 α in mouse insulinoma cells. *Diabetes* 54:968–975, 2005
44. Srinivasan S, Bernal-Mizrachi E, Ohsugi M, Permutt MA: Glucose promotes pancreatic islet beta-cell survival through a PI 3-kinase/Akt-signaling pathway. *Am J Physiol Endocrinol Metab* 283:E784–E793, 2002
45. Schmidt M, Fernandez de Mattos S, van der Horst A, Klompaker R, Kops GJ, Lam EW, Burgering BM, Medema RH: Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. *Mol Cell Biol* 22:7842–7852, 2002
46. Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L, Coffey PJ: FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *J Cell Biol* 156:531–542, 2002
47. Dijkers PF, Medema RH, Pals C, Banerji L, Thomas NS, Lam EW, Burgering BM, Raaijmakers JA, Lammers JW, Koenderman L, Coffey PJ: Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). *Mol Cell Biol* 20:9138–9148, 2000
48. Stahl M, Dijkers PF, Kops GJ, Lens SM, Coffey PJ, Burgering BM, Medema RH: The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. *J Immunol* 168:5024–5031, 2002
49. Steil GM, Trivedi N, Jonas JC, Hasenkamp WM, Sharma A, Bonner-Weir S, Weir GC: Adaptation of beta-cell mass to substrate oversupply: enhanced function with normal gene expression. *Am J Physiol Endocrinol Metab* 280:E788–E796, 2001
50. Dor Y, Brown J, Martinez OI, Melton DA: Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 429:41–46, 2004