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BH3-Only Molecule Bim Mediates β -Cell Death in IRS2 Deficiency



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***Irs2*-deficient mice develop type 2-like diabetes due to a reduction in β -cell mass and a failure of pancreatic islets to undergo compensatory hyperplasia in response to insulin resistance. In order to define the molecular mechanisms, we knocked down *Irs2* gene expression in mouse MIN6 insulinoma cells. Insulin receptor substrate 2 (IRS2) suppression induced apoptotic cell death, which was associated with an increase in expression of the BH3-only molecule Bim. Knockdown (KD) of Bim reduced apoptotic β -cell death induced by IRS2 suppression. In *Irs2*-deficient mice, *Bim* ablation restored β -cell mass, decreased the number of TUNEL-positive cells, and restored normal glucose tolerance after glucose challenge. FoxO1 mediates Bim upregulation induced by IRS2 suppression, and FoxO1 KD partially inhibits β -cell death induced by IRS2 suppression. These results suggest that Bim plays an important role in mediating the increase in β -cell apoptosis and the reduction in β -cell mass that occurs in IRS2-deficient diabetes.**

The insulin receptor substrate (IRS) proteins are key molecules in insulin-, growth hormone-, and IGF-1-induced signaling pathways. After binding to the receptor, IRS1 and IRS2 are phosphorylated and activate the phosphatidylinositol 3-kinase/AKT and mitogen-activated protein kinase pathways, and mediate the effects of insulin, growth hormone, and IGF-1 on cell growth, survival, development, glucose homeostasis, and metabolism (1).

Irs1- and *Irs2*-deficient mice demonstrate growth retardation and type 2 diabetes, respectively (2,3). *Irs1* and *Irs2* knockout (KO) mice show similar levels of insulin resistance. β -Cell mass undergoes a compensatory increase

in *Irs1* KO mice to approximately double the levels seen in wild-type (WT) mice, whereas the β -cell mass in *Irs2* KO mice is only 40% of that of WT (2,3). The mechanisms responsible for the reduction of β -cell mass in *Irs2* KO mice have not been clarified (4).

A variety of physiological death signals, as well as pathological cellular stress, can trigger the genetically programmed pathway of apoptosis (5). BCL-2 family members, including BH3-only molecules Bid, Bim, and Puma and multiple-BH-domain Bax and Bak, play a pivotal role in “mitochondrial” apoptotic cell death. BH3-only molecules such as Bim, Puma, Bad, and Bid are involved in regulating β -cell death. For example, PUMA activation contributes to pancreatic β -cell apoptosis in type 1 diabetes (6). Bid is essential for death receptor-induced apoptosis of pancreatic β -cells (7). Hyperglycemia/glucotoxic stress increases Bad protein expression in human and mouse pancreatic islets and causes β -cell death (8).

Bim was initially identified as a Bcl2-interacting protein and is expressed in hematopoietic, epithelial, neuronal, and germ cells (9). There are at least three main isoforms, Bim_{EL}, Bim_L, and Bim_S, which are the most potent inducers of apoptosis (10). Bim is constitutively expressed in many cell types but is maintained in an inactive form through binding to the microtubule-associated dynein motor complex (11). Bim_{EL} and Bim_L have a binding site for dynein light chain 1, which decreases their proapoptotic activity via sequestration to the cytoskeleton (11), whereas Bim_S is free to exert its potent proapoptotic activity (12). Bim is critical for apoptosis and homeostasis in the lymphoid and myeloid compartments (13). With age, *Bim* KO mice develop splenomegaly, lymphadenopathy, and hypergammaglobulinemia (14). Bim mediates β -cell apoptosis

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induced by chronic exposure to high glucose and the FasL system (15).

Using real-time quantitative reverse transcription PCR (QRT-PCR) in IRS2 knockdown (KD) MIN6 insulinoma cells, expression of the BH3-only molecule Bim was significantly increased, suggesting that it may play a role in β -cell apoptosis in IRS2 deficiency. The current study was undertaken to define the role of Bim in mediating β -cell apoptosis induced by IRS2 suppression.

RESEARCH DESIGN AND METHODS

MIN6 Cell Culture, Quantification of mRNA Levels, Lentivirus-Mediated Short Hairpin RNA Expression, and Western Blot

MIN6 cell culture, RNA isolation and first-strand cDNA synthesis, and preparation of pLKO.1-Pdx1 short hairpin RNA (shRNA) lentivirus all were performed as previously described (16). TaqMan assay numbers (Invitrogen) were as follows: mouse actin B, 4352933; IRS2, Mm003038438_m1; Bim, Mm00437796_m1; and Puma, Mm00519268_m1. The pLKO-Bim shRNA (TRCN0000009692), IRS2 shRNA (TRCN00000055110), and FoxO1 (TRCN0000054880) lentiviral vectors were purchased from Thermo Scientific. Lentivirus was added to the medium on day 1. The blots were probed with antibodies against IRS2 (3089; Cell Signaling), Puma (7467; Cell Signaling), cleaved caspase-3 (9661; Cell Signaling), FoxO1 (2880; Cell Signaling), p-AKT and AKT (9916; Cell Signaling), Bcl-xL (2762; Cell Signaling), Bcl-2 (554218; Pharmingen), BAD (sc-943; Santa Cruz Biotechnology), Mcl-1 (sc-819; Santa Cruz Biotechnology), Bim (202000; Calbiochem), and β -actin (A-2066; Sigma-Aldrich).

Quantitation of Cell Death

Cell death was quantified by propidium iodide (PI) staining followed by flow cytometric analyses (FACS) using a FACS Caliber (BD Bioscience) and FlowJo software (17). PI intercalates into double-stranded nucleic acids. PI is excluded by viable cells but can penetrate membranes of dying or dead cells. Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone; 20 μ mol/L) was added to the medium 2 h prior to treatment of MIN6 cells by IRS2 shRNA lentivirus. Z-VAD was added to the cells on days 1 and 3.

Cell Viability

Cell viability was assessed by methylene blue staining (18). In brief, MIN6 cells were washed twice with PBS and stained with 2% methylene blue (weight for volume) in 50% ethanol for 15 min with shaking at room temperature. Cells were then washed twice with PBS and air dried before being photographed. Viable cells take up methylene blue.

BrdU Staining

Cell proliferation was assessed by BrdU staining. For in vitro BrdU labeling studies, MIN6 cells were incubated with 10 μ mol/L BrdU for 2 h and stained with BrdU-FITC (BD Biosciences) according to the manufacturer's instructions. In brief, cells were washed with PBS, fixed in 70%

ethanol at 4°C, resuspended in 2N HCl/0.5% Triton X-100, incubated for 30 min at room temperature, neutralized with 0.1 mol/L sodium tetraborate (pH 8.5), rinsed in PBS containing 1% BSA and 0.5% Tween 20, stained with the above-mentioned anti-BrdU antibody, and then analyzed by FACS.

Retrovirus Infection

Human Bim, Bcl-2, and Bcl-xL were cloned into the retroviral expression vector MSCV-IRES-GFP (pMIG) (Addgene). The production of amphotropic retroviruses using the 293GPG packing cell line was performed as described previously (19). MIN6 cells were infected with these retroviral vectors at multiplicity of infection of 10.

Isolation Primary Mouse Pancreatic Islets

Mouse islets were isolated by using collagenase and filtration as previously described (20).

In Vivo Characterization of Mice

The *Irs2*^{+/-} mice were obtained from The Jackson Laboratory. *Bim*^{+/-} mice (21) were provided by Dr. Emily Cheng (Memorial Sloan Kettering Cancer Center, New York, NY). Male mice were fed a regular diet (Teklad irradiated 5% fat diet) and provided with water ad libitum. Intraperitoneal glucose tolerance tests were performed after a 16-h fast (2 g/kg dextrose) at 12 weeks of age. Insulin levels were measured after 16-h fasting and 10 min after glucose challenge. Insulin tolerance tests were performed after a 4-h fast by administering human recombinant insulin (0.75 units/kg). We quantified β -cell area from anti-insulin-stained pancreas sections counterstained with hematoxylin using the intensity thresholding function of the integrated morphometry package in ImageJ. The TUNEL labeling used the Dead End Fluorometric TUNEL System (Promega). All of the experiments in this study using animal protocols were approved by The University of Chicago Animal Studies Committee.

Imaging Studies of Pancreatic Islets

Formalin-fixed pancreas sections underwent antigen retrieval in boiling citrate buffer (pH 6.0) for 10 min before labeling with antibodies against insulin (A0564; DAKO), glucagon (G2654; Sigma-Aldrich), Ki-67 (642501; BioLegend), and DAPI (P-36931; Invitrogen). Images were obtained on an Evos microscope (Advanced Microscopy Group).

Statistical Analysis

The two-tailed unpaired Student *t* test was used to assess the statistical significance of differences between two sets of data. Differences were considered significant when $P < 0.05$. In all experiments, the number of asterisks is used to designate the following levels of statistical significance: *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ compared with the control group or WT group; ### $P < 0.001$, ## $P < 0.01$, and # $P < 0.05$ compared with the IRS2 KD or *Irs2*^{-/-} group. Results are presented as mean \pm SEM.

RESULTS

Reduced IRS2 Expression Leads to Apoptosis in MIN6 Insulinoma Cells

To investigate whether reduced IRS2 expression increases β -cell death, we used IRS2 shRNA lentivirus to knock down expression of IRS2 in MIN6 cells. IRS2 mRNA levels were significantly reduced to $26 \pm 2\%$ of control cells on day 5 after infection with lentiviral IRS2 shRNA (Fig. 1A), and IRS2 protein levels were decreased to $21 \pm 2\%$ of control (Fig. 1B). Methylene blue staining demonstrated the decreased viability of cells in which IRS2 expression had been knocked down (Fig. 1C). The effects of IRS2 KD on β -cell viability was further explored using PI staining followed by FACS to quantify β -cell death. The proportion

of PI staining-positive cells increased from $34 \pm 3\%$ on day 3 after infection with IRS2 shRNA lentivirus to $50 \pm 3\%$ on day 5 and $74 \pm 4\%$ on day 7 (all $P < 0.001$), respectively (Fig. 1D). Z-VAD, a pan-caspase inhibitor, significantly inhibited the increase in PI staining-positive cells (Fig. 1E). The PI staining-positive cells decreased from $35 \pm 2\%$ in IRS2 KD cells to $11 \pm 1\%$ in Z-VAD/IRS2 KD cells ($P < 0.001$) (Fig. 1E). These changes induced by IRS2 suppression were associated with increased caspase-3 activation and cleaved caspase-3 protein levels (Fig. 1F). Immunoblot analysis showed that Z-VAD significantly inhibited the increase in cleaved caspase-3 protein levels induced by IRS2 KD from 4.2- to 1.8-fold in Z-VAD/IRS2 KD cells ($P < 0.001$) (Fig. 1F). To further define the

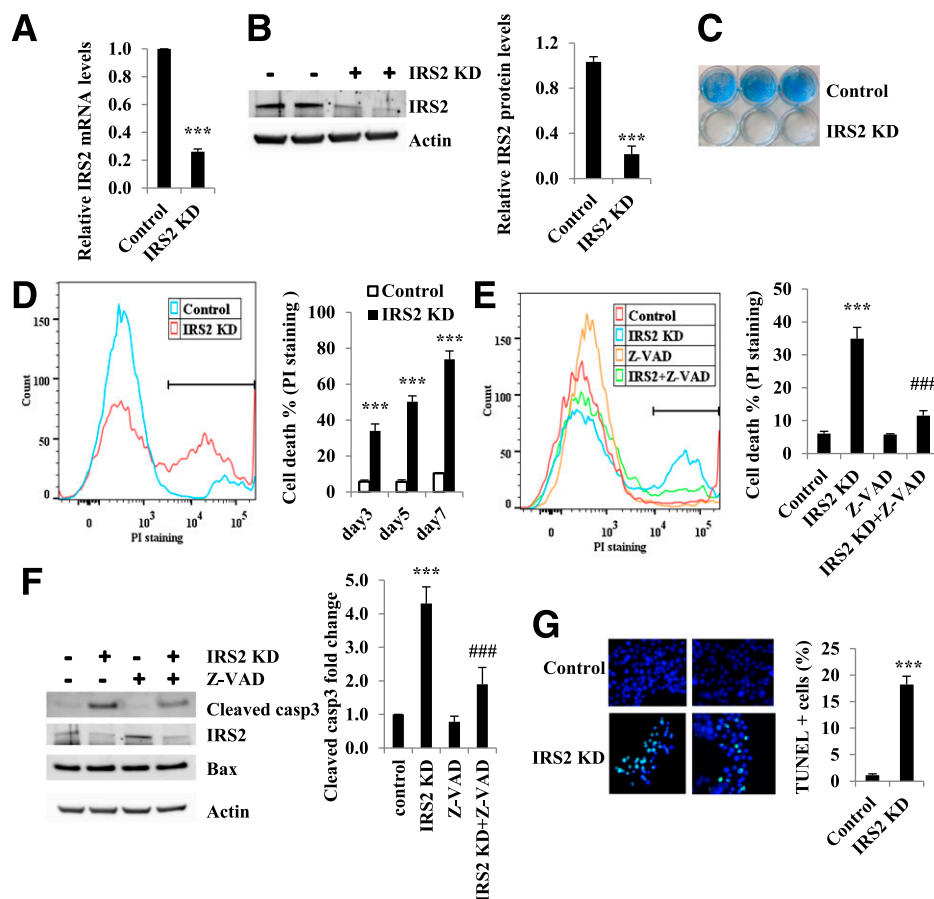


Figure 1—Reduced IRS2 expression leads to apoptosis in MIN6 cells. **A:** mRNA levels in control and IRS2 KD cells. MIN6 cells were infected with a lentivirus vector that drives expression of a shRNA that targets the IRS2 transcript (IRS2 KD) or a control lentivirus vector without a specific target. After 5 days infection, IRS2 mRNA levels were measured by real-time reverse transcription PCR ($n = 3$). **B:** IRS2 and caspase-3 protein levels in IRS2 KD cells. Three days after IRS2 shRNA lentivirus infection, IRS2 protein and cleaved caspase-3 protein were assayed by Western blot (three independent experiments). **C:** Cell viability in IRS2 KD cells was assessed by methylene blue staining. After 5 days, cells exposed to the IRS2 shRNA lentivirus showed a marked decrease in methylene blue uptake. **D:** Cell death was determined by PI staining in MIN6 cells. Three days after IRS2 shRNA lentivirus infection, the percentage of cell death is shown by representative histograms (left). The results are shown (right). **E:** MIN6 cells were treated with caspase inhibitor ($20 \mu\text{mol/L}$ Z-VAD) for 2 h prior to infection with IRS2 shRNA lentivirus, and cell death was determined 3 days later by PI staining ($n = 3$). **F:** Cleaved caspase-3 protein levels were assayed by Western blot. The bar graph depicts the relative changes in the levels of the indicated proteins using densitometry analysis of the Western blots in **F** ($n = 3$). *** $P < 0.001$ compared with control group; ### $P < 0.001$ compared with IRS2 KD group. **G:** TUNEL labeling of IRS2 KD MIN6 cells. Three days after IRS2 shRNA lentivirus infection, apoptotic cells were assayed by TUNEL staining. Quantitative TUNEL data are shown. *** $P < 0.001$ compared with control group. Values are mean \pm SEM.

effects of IRS2 suppression on β -cell survival, TUNEL assay was used to measure cell apoptosis. The results showed that IRS2 suppression significantly increased the number of TUNEL-positive cells from 1.1% in control cells to 18.2% in IRS2 KD cells ($P < 0.001$) (Fig. 1G). Collectively, these findings showed that reduced IRS2 expression leads to apoptotic β -cell death.

Bim Is Upregulated in MIN6 Cells and Islets After IRS2 Suppression

Because BCL-2 family members are widely involved in apoptotic cell death, we used real-time quantitative PCR to determine whether the expression of proapoptotic Bcl2

family members is regulated by IRS2 KD. IRS2 KD resulted in a onefold increase in Bim mRNA and no increase in Puma mRNA in MIN6 cells (Fig. 2A). Western blotting showed a corresponding onefold increase in Bim protein ($P < 0.01$) and 2.8-fold increase in cleaved caspase-3 protein ($P < 0.01$), respectively, whereas IRS2 KD was not associated with an increase in Puma protein levels (Fig. 2B).

To determine if similar changes occur in vivo, pancreatic islets isolated from *Irs2*^{-/-} mice at 5–6 weeks showed an increase by 40% in Bim mRNA levels (Fig. 2C). These results demonstrate that IRS2 downregulation is associated with upregulation of Bim in both MIN6 cells and islets.

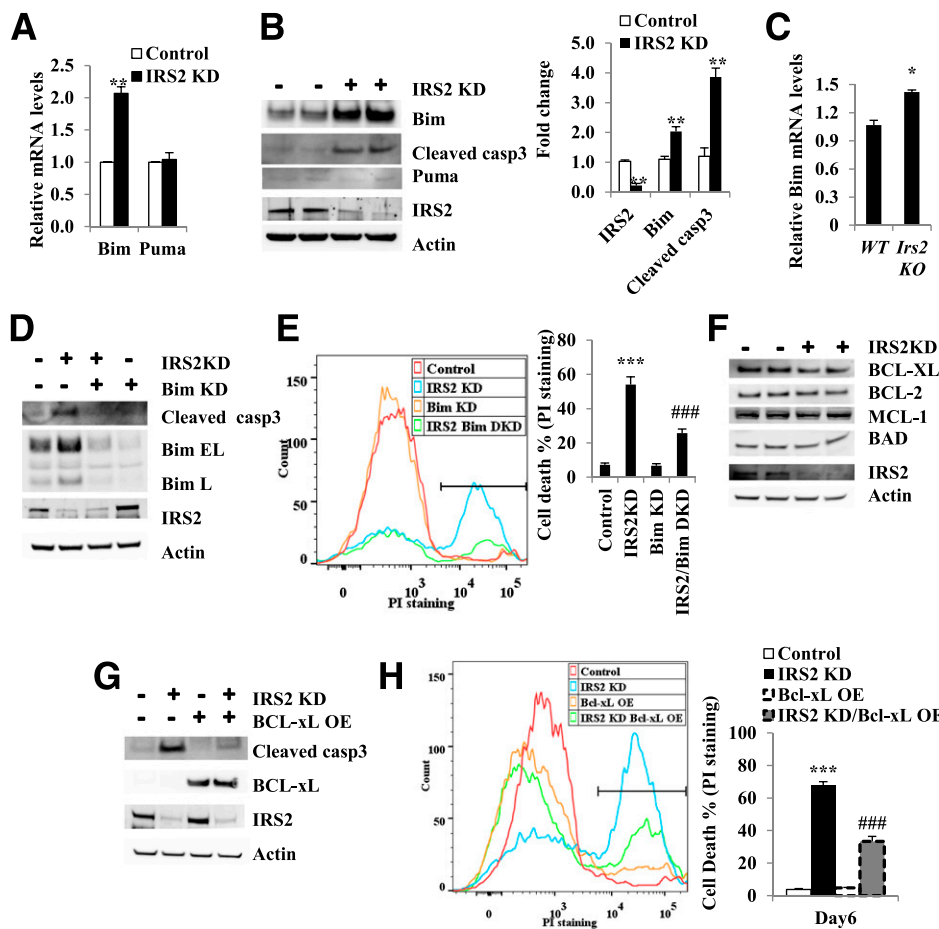


Figure 2—Bim is upregulated in MIN6 cells after IRS2 suppression. **A:** Bim and Puma mRNA levels in control and IRS2 KD cells. Five days after IRS2 KD in MIN6 cells, Bim and Puma mRNA levels were measured by QRT-PCR in MIN6 cells ($n = 3$). $^{**}P < 0.01$ compared with control group. **B:** Western blot of IRS2 KD cells. Three days after IRS2 KD in MIN6 cells, immunoblot analysis was performed to determine IRS2, Bim, Puma, and cleaved caspase-3 protein levels in IRS2 KD MIN6 cells. The bar graph depicts the relative changes in the levels of the indicated proteins using densitometry analysis of the Western blots in **B** ($n = 3$). Values are mean \pm SEM. **C:** Bim mRNA levels were measured by QRT-PCR in islets from 5–6-week-old male *Irs2*^{-/-} mice on normal chow ($n = 3$ –4). **D:** Western blot of IRS2/Bim DKD cells. Three days after IRS2/Bim DKD in MIN6 cells, immunoblot of IRS2, Bim, and cleaved caspase-3 in cells. **E:** Measurement of cell death. Five days after Bim/IRS2 DKD in MIN6 cells, cell death was determined by PI staining ($n = 3$). $^{***}P < 0.001$ compared with control group; $^{###}P < 0.001$ compared with IRS2 KD group. **F:** Western blot of IRS2 KD MIN6 cells. Three days after IRS2 KD in MIN6 cells, immunoblot of BCL-2, BCL-XL, and MCL-1. **G:** Western blot of IRS2 KD/Bcl-xL–overexpressing cells. Three days after IRS2 KD and/or Bcl-xL overexpression (OE) in MIN6 cells, immunoblot of cleaved caspase-3, BCL-xL, and IRS2 proteins in MIN6 cells. **H:** Cell death was determined by PI staining in IRS2 KD/Bcl-xL OE cells. The percentage of cell death is shown by representative histograms (*left*). The results are shown (*right*). $^{***}P < 0.001$ compared with control group; $^{###}P < 0.001$ compared with IRS2 KD group. Values are mean \pm SEM.

Bim Contributes to β -Cell Apoptosis Induced by IRS2 Suppression

To determine whether Bim upregulation plays a role in the increase in β -cell death observed after IRS2 suppression, lentiviral shRNA was used to suppress Bim expression in MIN6 cells. Consistent with the results described above, IRS2 suppression increased Bim protein (Fig. 2D). This effect was significantly inhibited by Bim KD (Fig. 2D). Caspase-3 activation was also inhibited in Bim/IRS2 double KD (DKD) cells compared with IRS2 KD cells alone (Fig. 2D). After IRS2 KD, $50.2 \pm 3.9\%$ of the MIN6 cells took up the PI stain. In the Bim/IRS2 DKD group, only $25.7 \pm 2.4\%$ ($P < 0.001$ compared with IRS2 alone) took up the PI stain, indicative of a 25% increase in cell viability (Fig. 2E). The consequences of increased Bim expression on MIN6 cells with normal IRS2 expression were next determined. Overexpression of Bim achieved by using a retroviral vector at multiplicity of infection of 10 resulted in an increase in MIN6 cell death (Supplementary Fig. 1).

The Bcl-2 family has both proapoptotic members, including Bid, Bim, Puma, Bax, and Bak, and antiapoptotic members, such as Bcl-2, Bcl-xL, and Mcl-1. The presence of an antiapoptotic molecule such as Bcl-2 or Bcl-xL can inhibit the role of proapoptotic molecules after a death signal. To determine whether IRS2 KD induced changes in the expression levels of antiapoptotic molecules, we measured the expression levels of Bcl-2, Bcl-xL, and Mcl-1. The results showed that IRS2 KD induced a decrease in protein levels of Bcl-xL. The protein levels of Bcl-2 and Mcl-1 were unchanged in IRS2 KD cells compared with control cells (Fig. 2F). To determine whether increasing Bcl-xL expression can inhibit cell death induced by upregulation of Bim, we forced expression of Bcl-xL in MIN6 cells. The results showed that Bcl-xL prevented caspase-3 activation and inhibited an increase in cleaved caspase-3 protein induced by IRS2 suppression (Fig. 2G). Bcl-xL overexpression also decreased IRS2 KD-induced cell death. After Bcl-xL overexpression, the PI stain-positive cells decreased from $67.9 \pm 3.8\%$ in IRS2 KD cells to $44.6 \pm 1.9\%$ ($P < 0.001$) in IRS2 KD/Bcl-xL cells on day 6 (Fig. 2H). Bcl-2 overexpression also inhibited IRS2 KD-induced cell death at an earlier time point (Supplementary Fig. 2).

Taken together, these findings demonstrate that Bim contributes to the increase in apoptotic β -cell death induced by IRS2 suppression and Bcl-xL inhibits Bim upregulation-induced MIN6 cell death.

Effect of Bim Ablation in Adult $Irs2^{-/-}$ Mice

To define the role of Bim in β -cell death associated with IRS2 deficiency in vivo, the ability of a loss-of-function Bim mutation to rescue β -cell failure in $Irs2^{-/-}$ mice was tested. Pancreatic islets from $Irs2^{-/-}$ mice were small and contained reduced numbers of insulin-containing β -cells (Fig. 3A), and the β -cell area was $\sim 63\%$ smaller than that in age-matched WT mice ($P < 0.001$) (Fig. 3B). Pancreatic islets from $Bim^{-/-}$ mice appeared normal (Fig. 3A).

β -Cells in $Irs2^{-/-}$ islets also exhibited increased TUNEL labeling (Fig. 3C). These parameters did not differ in $Bim^{-/-}$ and control mice. In contrast, in $Irs2^{-/-}Bim^{-/-}$ mice, β -cell area was restored to $\sim 62\%$ of the value in WT mice and β -cell mass approximately doubled compared with $Irs2^{-/-}$ mice ($P < 0.01$) (Fig. 3B). The number of TUNEL-positive cells was significantly reduced in islets from $Irs2^{-/-}Bim^{-/-}$ mice compared with $Irs2^{-/-}$ mice ($P < 0.01$) (Fig. 3C). To determine whether the effects of Bim ablation were solely the result of inhibition of β -cell death, or whether there was also an effect on β -cell proliferation, $Irs2^{-/-}Bim^{-/-}$ islets were stained for the proliferative marker Ki-67. Proliferation of β -cells was decreased in $Irs2^{-/-}$ islets compared with WT and was significantly improved by Bim ablation in $Irs2^{-/-}Bim^{-/-}$ islets. Surprisingly, Bim ablation decreased the number of Ki-67-positive cells in $Bim^{-/-}$ islets compared with WT. These results indicate that the effects of Bim were manifest on both β -cell proliferation as well as death. Thus, Bim ablation in $Irs2^{-/-}$ mice inhibits β -cell apoptosis and β -cell proliferation and enhances β -cell mass.

Bim Gene Ablation Prevents Diabetes in $Irs2^{-/-}$ Mice

To determine whether enhanced β -cell survival due to Bim ablation prevents the diabetic phenotype in $Irs2^{-/-}$ mice, we performed glucose tolerance tests and insulin tolerance tests on $Irs2^{-/-}$ and $Irs2^{-/-}Bim^{-/-}$ mice. Both male and female $Irs2^{-/-}$ mice develop increased fasting blood glucose and impaired glucose clearance (Fig. 4A–C and E). $Irs2^{-/-}Bim^{-/-}$ mice exhibited significantly lower fasting blood glucose and improved glucose tolerance (Fig. 4A–C and E). The area under the blood glucose curve decreased $38\% \pm 2.3\%$ (male) and $40\% \pm 3.3\%$ (female) in $Irs2^{-/-}Bim^{-/-}$ mice compared with $Irs2^{-/-}$ mice, respectively ($P < 0.001$) (Fig. 4D and F). $Irs2^{-/-}Bim^{-/-}$ female mice exhibited a normal response to glucose challenge compared with WT mice. $Irs2^{-/-}Bim^{-/-}$ male mice showed significantly improved but not normal glucose tolerance (Fig. 4F). $Irs2^{-/-}$ mice were insulin resistant compared with WT mice (Fig. 4G). The hypoglycemic response to exogenous insulin was significantly increased in $Irs2^{-/-}Bim^{-/-}$ mice, and the reduction in blood glucose after insulin administration was similar to WT mice in these animals (Fig. 4G). At 12 weeks, fasting insulin levels in $Irs2^{-/-}$ mice were 50% of the values in WT animals (Fig. 4H), and these levels were increased in the $Irs2^{-/-}Bim^{-/-}$ mice under basal conditions and after glucose challenge in mice compared with WT mice (Fig. 4H). Together with the above results, we conclude that Bim^{-/-} ablation prevents the diabetic phenotype in the $Irs2^{-/-}$ mouse by reducing apoptotic β -cell death, preserving glucose tolerance, and increasing response to exogenous insulin.

Role of FoxO1 in Bim Upregulation Induced by IRS2 Suppression

Loss-of-function mutations of the forkhead transcription factor FoxO1 rescue β -cell failure in $Irs2^{-/-}$ mice (22), and FoxO1 has been shown to contribute to apoptosis by

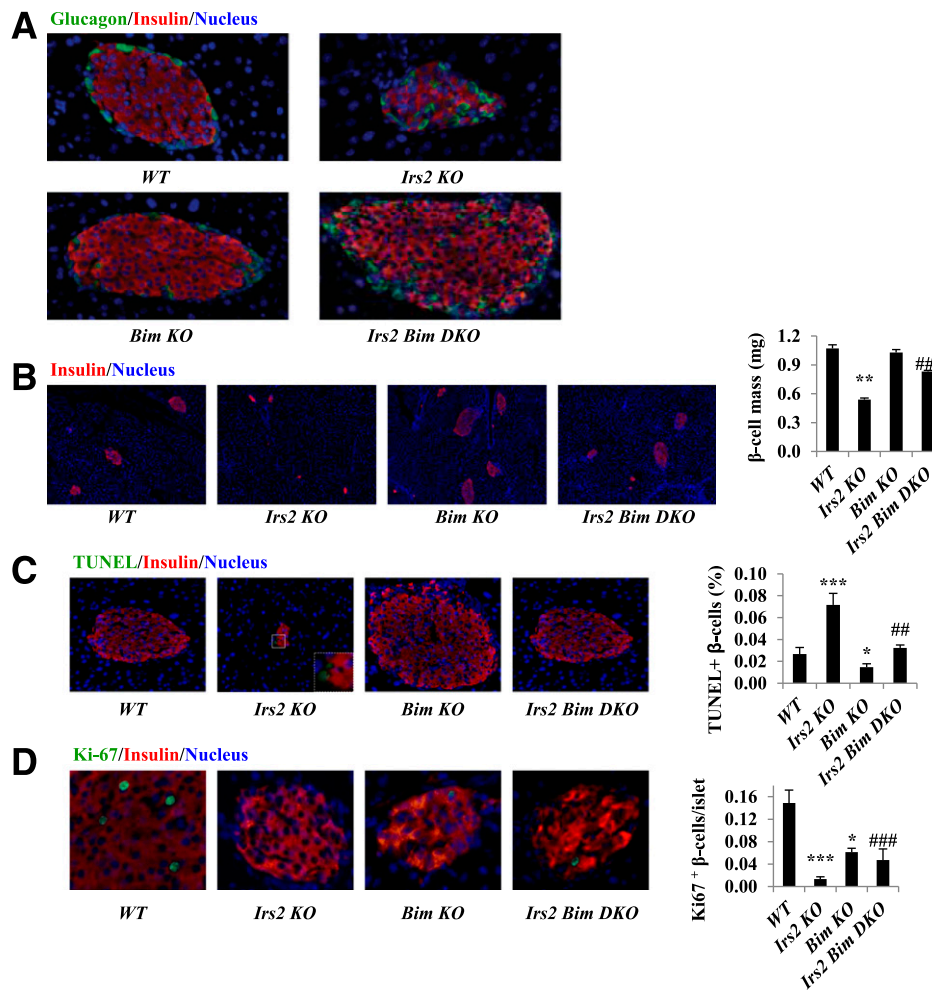


Figure 3—*Bim* ablation protects β -cells in adult *Irs2*^{-/-} mice. **A:** Morphology of adult mouse islets after 18 weeks on a normal diet; anti-insulin and antiglucagon antibodies were used to stain β -cells (red) and α -cells (green), respectively. **B:** Histological analysis of pancreatic islets and quantitative analysis of β -cell mass are shown ($n = 3$ –5 per group). ** $P < 0.01$ compared with the WT mice; ### $P < 0.01$ compared with *Irs2*^{-/-} mice. **C:** TUNEL labeling of adult pancreatic β -cells. Quantitative TUNEL data are shown. * $P < 0.05$ and *** $P < 0.001$ compared with the WT mice; ## $P < 0.01$ compared with *Irs2*^{-/-} mice. Original magnification $\times 200$. **D:** Ki-67 staining of β -cells; group Ki-67 data are on the right. * $P < 0.05$ and *** $P < 0.001$ compared with the WT mice; ### $P < 0.001$ compared with *Irs2*^{-/-} mice. Original magnification $\times 200$. All group data are mean \pm SEM of $n = 3$. DKO, double KO.

increasing transcription of *Bim* (23). These associations between FoxO1 and β -cell apoptosis prompted us to undertake experiments to determine if FoxO1 plays a role in promoting the upregulation of *Bim* induced by IRS2 suppression.

In order to determine whether FoxO1 participates in *Bim*-mediated β -cell death induced by IRS2 suppression, we studied the ability of FoxO1 suppression to prevent β -cell apoptosis induced by IRS2 KD. FoxO1 KD significantly decreased *Bim* expression by $43 \pm 7.8\%$ compared with control cells ($P < 0.05$) (Fig. 5D). Consistent with this result, IRS2 KD significantly increased *Bim* protein levels by $80 \pm 13\%$ ($P < 0.01$), in contrast with a $20 \pm 10\%$ increase in *Bim* protein in FoxO1/IRS2 DKD cells (Fig. 5E). Therefore, FoxO1 KD significantly inhibited the increase in *Bim* protein in FoxO1/IRS2 DKD cells compared with IRS2 KD cells ($P < 0.05$) (Fig. 5E). The

PI staining-positive cells significantly increased in a time-dependent manner in IRS2 KD Min6 cells ($P < 0.001$) (Fig. 5F). On day 3, $18.0 \pm 2.8\%$ of IRS2/FoxO1 DKD cells stained positive with PI compared with $33.4 \pm 2.8\%$ of IRS2 KD cells ($P < 0.05$). By day 5, there were no significant differences in the number of PI-positive cells between IRS2 KD and IRS2/FoxO1 DKD cells (Fig. 5F).

To determine if there were differences in cell proliferation, BrdU incorporation was measured in IRS2 KD and IRS2/FoxO1 DKD cells. In contrast to apoptosis, BrdU incorporation was not significantly different in the two groups (Fig. 5G). Taken together, FoxO1 does contribute to *Bim* upregulation induced by IRS2 KD and FoxO1 suppression and temporarily reduces, but does not eliminate, apoptosis in MIN6 in which IRS2 has been suppressed.

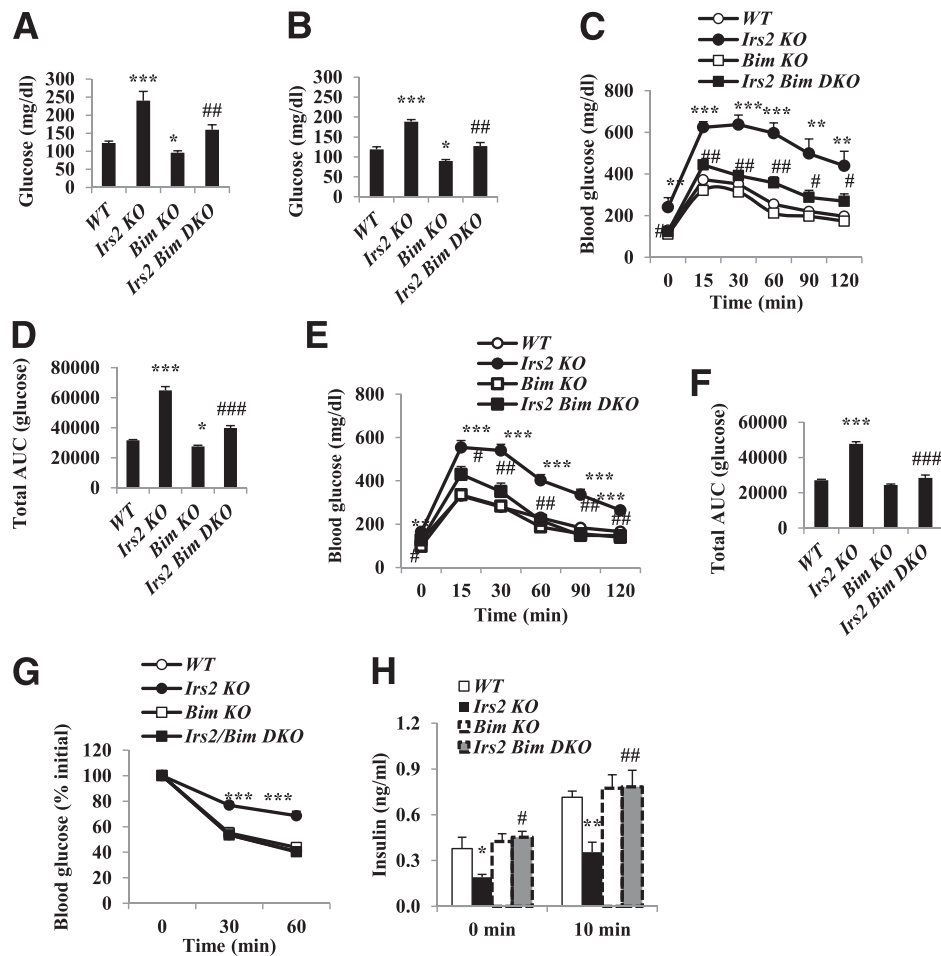


Figure 4—Bim gene ablation reduces hyperglycemia in *Irs2*^{-/-} mice. Fasting glucose levels were measured in male (A) and female (B) mice at 16 weeks ($n = 8-12$). * $P < 0.05$ and *** $P < 0.001$ compared with the WT mice; ## $P < 0.01$ compared with *Irs2*^{-/-} mice. Blood glucose levels after intraperitoneal injection of dextrose (2 g/kg) in male (C) and female (E) mice at 12 weeks. ** $P < 0.01$ and *** $P < 0.001$ compared with the WT mice; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with *Irs2*^{-/-} mice. Area under the blood glucose curves (AUC) using the data from panel C (D) or panel E (F) ($n = 8-12$) in the four mouse groups designated. ** $P < 0.01$ and *** $P < 0.001$ compared with the WT mice; ## $P < 0.01$ compared with *Irs2*^{-/-} mice. G: Glucose levels in response to insulin (0.75 units/kg body weight) in the four mouse groups designated at 13 weeks ($n = 8-10$). H: Insulin levels measured fasting and 10 min after intraperitoneal dextrose at 12 weeks ($n = 8-10$). ** $P < 0.01$ compared with the WT mice; # $P < 0.05$ compared with *Irs2*^{-/-} mice. Values are mean \pm SEM. DKO, double KO.

The phosphorylation and degradation of FoxO1 in Min6 cells was also measured. Consistent with previous reports (24), insulin treatment for 5 or 15 min stimulated FoxO1 phosphorylation in control cells (Fig. 5B), but not in IRS2 KD cells (Fig. 5B). FoxO1 protein levels were dramatically reduced in control cells during insulin stimulation; however, insulin failed to reduce FoxO1 protein levels in IRS2 KD cells (Fig. 5B). The phosphorylation of FoxO1 also promotes its exclusion from the nucleus (25). Nuclear and cytoplasmic proteins were extracted from control and IRS2 KD MIN6 cells under serum-free conditions or after incubation with 100 nmol/L insulin for 10 min. Under basal conditions, more FoxO1 protein was detected in nuclear fractions in IRS2 KD cells than in control cells (Fig. 5C). Whereas insulin treatment barely changed nuclear FoxO1 in control cells (Fig. 5C), the nuclear FoxO1 was dramatically increased after insulin

treatment of IRS2 KD cells (Fig. 5C). These data demonstrate that IRS2 KD increases nuclear FoxO1 and decreases its degradation during insulin stimulation of MIN6 cells.

DISCUSSION

Previous studies have demonstrated that *Irs2* KO mice develop hyperglycemia associated with a lack of a compensatory increase in β -cell mass and insulin secretion in response to insulin resistance (2). Reduced β -cell mass has actually been demonstrated in these mice (3,26). The present studies were undertaken to define the molecular mechanisms responsible for a lack of compensatory response and a reduction in pancreatic β -cell mass induced by IRS2 deficiency. The results demonstrate that IRS2 deficiency induces β -cell apoptotic cell death mediated by caspase-3 activation and detected by increased

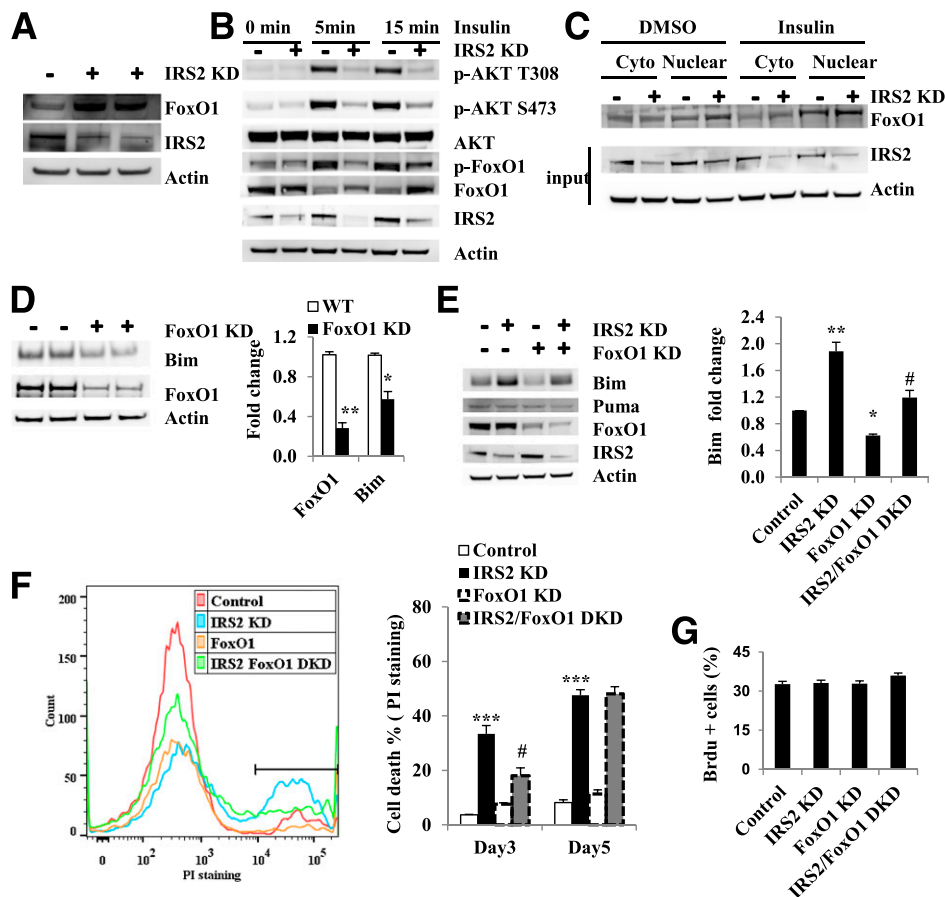


Figure 5—FoxO1 regulates Bim upregulation, and FoxO1 suppression partially rescues MIN6 cells from apoptosis induced by IRS2 suppression. **A:** FoxO1 protein levels were increased in IRS2 KD MIN6 cells. Three days after IRS2 shRNA lentivirus infection, FoxO1 protein was analyzed by Western blot. **B:** Insulin effects on phosphorylation of AKT and FoxO1 in IRS2 KD MIN6 cells. Two days after IRS2 shRNA lentivirus infection, serum-starved (overnight) cells were stimulated with 100 nmol/L insulin for 5 and 15 min. IRS2, AKT, and FoxO1 proteins were assayed by Western blot ($n = 3$). **C:** FoxO1 nuclear/cytoplasmic translocation in IRS2 KD MIN6 cells. Two days after IRS2 shRNA lentivirus infection, serum-starved (overnight) cells were stimulated with 100 nmol/L insulin for 10 min, and cytosolic and nuclear proteins were analyzed by Western blot. **D:** Bim protein levels in FoxO1 KD cells. Three days after FoxO1 shRNA lentivirus infection, Bim protein was analyzed by Western blot. The bar graph depicts the relative changes in the levels of the indicated proteins using densitometry analysis of the Western blots in **D** ($n = 3$). * $P < 0.05$, ** $P < 0.01$, compared with WT group. **E:** Bim protein in IRS2/FoxO1 DKD cells. Three days after IRS2/FoxO1 shRNA lentivirus infection, Bim protein was analyzed by Western blot. The bar graph depicts the relative changes in the levels of the indicated proteins using densitometry analysis of the Western blots in **E** ($n = 3$). * $P < 0.05$, ** $P < 0.01$, compared with control cells. **F:** Cell death was determined by PI staining in MIN6 cells. MIN6 cells were infected with IRS2/FoxO1 shRNA lentivirus, and cell death was determined 3 and 5 days later by PI staining ($n = 3$). The percentage of cell death on day 3 is shown by representative histograms (*left*). *** $P < 0.001$ compared with control group; # $P < 0.05$ compared with IRS2 KD group. **G:** BrdU incorporation in IRS2/FoxO1 DKD cells. BrdU incorporation into cells was measured 4 days after IRS2 KD. Values are mean \pm SEM.

TUNEL labeling in both MIN6 cells and mouse islets. Bim plays an important role in regulating β -cell apoptosis induced by IRS2 suppression. Bim is a BH3-only molecule that is essential for mitochondrial-dependent apoptosis and for inducing cell death in different cell types, including neurons, T and B lymphocytes, and macrophages (13,27–30). Recent studies have indicated that Bim is upregulated by ER stress in β -cells and that Bim, Puma, and Bax are required for β -cell apoptosis induced by glucose and ribose toxicity (31). The present experiments demonstrated that in IRS2 deficiency, KD of Bim significantly reduced β -cell apoptosis. The current studies also demonstrate that *Irs2*^{-/-} mice develop type 2 diabetes

with reduced pancreatic β -cell mass and insulin resistance. Reduced Bim expression improves β -cell mass in *Irs2*^{-/-} mice, indicating that Bim is an important mediator of β -cell apoptosis induced by IRS2 suppression.

In mouse pancreas, Bim is expressed in the acinar cells and endocrine cells of the islets of Langerhans and the columnar epithelial cells lining the pancreatic ducts. Bim is also expressed in β -cells of human islets (32). Regulation of Bim in β -cells contributes to type 1 and 2 diabetes (33,34). In mice, IRS2 mediates peripheral insulin action and pancreatic β -cell growth and function. In *Irs2*^{-/-} mice, effects on β -cell apoptosis but also insulin action pathways in hepatocytes contribute to *Irs2* deficiency-induced

glucose intolerance and insulin resistance (2,26). In this study, *Bim* KO rescues the *Irs2* KO phenotype and restores normal glucose tolerance. The improvement in glucose levels is multifactorial. The reduction in β -cell apoptosis results in increased insulin secretion, and this is undoubtedly a major, if not the major, factor in lowering glucose. In addition, insulin sensitivity is also improved in the *Irs2/Bim* double KO mice. We do not fully understand the mechanisms behind this unanticipated finding. It is well known that reducing hyperglycemia leads to improved insulin sensitivity. However, the finding also raises the interesting possibility that Bim could have effects on insulin action pathways outside the pancreatic β -cells. Important roles for Bim have been documented in a number of cell types, including hepatocytes, neurons, adipocytes, and muscle cells. For example, transcriptional regulation of Bim by FoxO3a mediates hepatocyte lipoapoptosis (35). Bim mediates motor neuron loss in amyotrophic lateral sclerosis, and Bim is required for β -amyloid-induced neuronal apoptosis in Alzheimer disease (30). Glucose oxidase stimulation induces apoptosis by increasing Bim expression in 3T3-L1 adipocytes (36). In cultured muscle cells, oxidative stress induces cell apoptosis by regulating Bim expression (37). Furthermore, Bim deficiency in hematopoietic cells renders mice resistant to autoimmune encephalomyelitis and diabetes (13). Possible effects on pancreatic α -cells should also be considered. Although we were unable to detect Bim in α -cells, this could be related to the sensitivity of our antibody.

Cell survival or death is decided by the balance between proapoptotic and antiapoptotic factors. In IRS2 KD MIN6 cells, antiapoptotic molecule Bcl-xL was dramatically decreased and Mcl-1 and Bcl-2 are unchanged. Forced overexpression of the Bcl-xL- and Bcl-2-inhibited Bim induced β -cell apoptosis. Thus, both decreasing expression of Bcl-xL and increasing expression of Bim were involved in β -cell apoptosis induced by KD of IRS2.

β -Cell death and β -cell proliferation are the two major mechanisms to regulate pancreatic β -cell mass. In this study, Bim was involved in regulating both β -cell death and β -cell proliferation. Although β -cell death is significantly decreased in *Bim* KO mice, proliferation of β -cells is lower in these mice compared with WT mice. When β -cell mass is considered, the effects of Bim on β -cell death and proliferation balance each other and, as a result, β -cell mass in *Bim* KO mice is similar to that in WT mice.

We further characterize the role of FoxO1 in regulating Bim expression. The phosphorylation of FoxO1 by insulin receptor signaling inhibits FoxO1 activity and increases FoxO1 degradation. In MIN6 cells in which IRS2 had been suppressed, the phosphorylation of Akt was almost fully blocked compared with control cells. Since Akt-dependent phosphorylation of FoxO1 dramatically decreased, FoxO1 protein increased in IRS2 KD cells. FoxO1 regulates genes of both the extrinsic and intrinsic apoptotic pathways (38). FoxO1 KD inhibited Bim upregulation in IRS2 KD

cells. Surprisingly, FoxO1 KD only inhibited β -cell death induced by IRS2 suppression transiently. The effect was observed only at early time points. One explanation is that IRS2 KD can induce Bim and/or FoxO1-independent apoptotic cell death. Another interpretation of this result is that IRS2 suppression may induce other forms of β -cell death in addition to apoptosis. Our recent studies have shown that autophagy and necrosis are also implicated in β -cell death induced by *Pdx1* deficiency (16,39). IRS2 has been implicated in activation of autophagy in neuronal cells (40).

In conclusion, we have shown that BH3-only molecule Bim plays a role in mediating β -cell apoptosis caused by IRS2 deficiency. Genetic ablation of *Bim* protects β -cells from apoptosis and preserves β -cell mass and decreases insulin resistance in *Irs2*^{-/-} mice. FoxO1 was involved in Bim upregulation in IRS2-suppressed cells. These results suggest Bim may be a novel target for therapeutic interventions in diabetes associated with reductions in β -cell mass.

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