BH3-only molecule Bim mediates β-cell death in IRS2 deficiency

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ABSTRACT

Irs2 deficient mice develop type2- 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. IRS2 suppression induced apoptotic cell death which was associated with an increase in expression of the BH3-only molecule Bim. Knockdown 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 up-regulation induced by IRS2 suppression and FoxO1 knockdown 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.
INTRODUCTION

The insulin receptor substrate (IRS) proteins are key molecules in insulin, growth hormone (GH) and insulin-like growth factor (IGF-1) - induced signaling pathways. After binding to the receptor, IRS1 and IRS2 are phosphorylated and activate the phosphatidylinositol 3-kinase (PI-3K)/AKT and mitogen-activated protein kinase (MAPK) pathways, and mediate the effects of insulin, GH 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 mice, whereas the β-cell mass in IRS2 KO mice is only 40% of that of wild type (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, 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 cause 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: BimEL, BimL, and BimS 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). BimEL and BimL have a binding site for the dynein light chain 1 which decreases their pro-
apoptotic activity via sequestration to the cytoskeleton (11), while Bim<sub>5</sub> is free to exert its potent pro-apoptotic activity (12). Bim is critical for apoptosis and homeostasis in the lymphoid and myeloid compartments (13). With age, Bim-knockout mice develop splenomegaly, lymphadenopathy, and hypergammaglobulinemia (14). Bim mediates β-cell apoptosis induced by chronic exposure to high glucose and the Fas-FasL system (15).

Using real time quantitative reverse transcription 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 present 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 shRNA expression and western blot. MIN6 cell culture, RNA isolation and first strand cDNA synthesis, and preparation of pLKO.1-Pdx1 shRNA lentivirus all were performed as previously described (16). TaqMan assay numbers (Invitrogen) were: 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 days 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), Mcl-1(sc-819, Santa Cruz), Bim (202000; Calbiochem), beta-actin (A-2066; Sigma).

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). Propidium iodide intercalates into double-stranded nucleic acids. PI is excluded by viable cells but can penetrate membranes of dying or dead cells. 20 µM Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[Omethyl]-fluoromethylketone) was added to the medium 2 hours prior to treatment of MIN6 cells by IRS2 shRNA lentivirus. Z-VAD was added to the cells on day 1 and day 3.

Cell viability. Cell viability was assessed by methylene blue staining (18). Briefly, MIN6 cells were washed twice with PBS and stained with 2% methylene blue (w/v) 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 5-bromo-2′-deoxyuridine (BrdU) labeling studies, MIN6 cells were incubated with 10 µM 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 M 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 (MOI) 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 kindly provided by Dr. Emily Cheng (Memorial Sloan-Kettering Cancer Center, New York). 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-hour fast (2 g/kg dextrose) at age of 12 weeks. Insulin levels were measured after 16-hour fasting and 10 min after glucose challenge. Insulin tolerance tests were performed after a 4-hour fast by administering human recombinant insulin (0.75 U/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 terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (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 minutes before labeling with antibodies against insulin (A0564; DAKO), glucagon (G2654; Sigma), Ki-67 (642501; biolegend), and DAPI (P-36931; Invitrogen). Images were obtained on Evos microscope (Advanced microscope group).

**Statistical analysis.** The 2-tailed unpaired Student’s $t$ test was used to assess the statistical significance of differences between 2 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: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared with control group or WT group. #### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$ compared with IRS2 KD or Irs2 $^{−/−}$ group. Results are presented as mean ± 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 ± 2% of control cells on day 5 after infection with lentiviral IRS2 shRNA (Fig1. A) and IRS2 protein levels were decreased to 21 ± 2% of control (Fig1. B). Methylene blue staining demonstrated the decreased viability of cells in which IRS2 expression had been knocked down (KD) (Fig1. C). 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 ± 3% on day 3 after infection with IRS2 shRNA lentivirus to 50 ± 3% on day 5 and 74% ± 4% on day 7 (all \( P<0.001 \)), respectively (Fig1. D). Z-VAD, a pan-caspase inhibitor, significantly inhibited the increase in PI staining positive cells (Fig1. E). The PI staining positive cells decreased from 35 ± 2% in IRS2 KD cells to 11 ± 1% in Z-VAD/IRS2 KD cells (\( P<0.001 \)) (Fig1. E). These changes induced by IRS2 suppression were associated with increased caspase3 activation and cleaved caspase3 protein levels (Fig1. F). Immunoblot analysis showed that Z-VAD significantly inhibited the increase in cleaved caspase3 protein levels induced by IRS2 KD from 4.2-fold to 1.8-fold in Z-VAD/ IRS2 KD cells (\( P<0.001 \)) (Fig1. F). To further define the effects of IRS2 suppression on β-cell survival, terminal dUTP nick end labeling (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 (\( P<0.001 \)) (Fig1. G). Collectively, these findings showed that reduced IRS2 expression leads to apoptotic β-cell death.

Bim is up-regulated in MIN6 cells and islets following 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 pro-apoptotic Bcl2 family members is regulated by IRS2 KD. IRS2 KD
resulted in 1-fold increase in Bim mRNA and no increase in Puma mRNA in MIN6 cells (Fig 2A).
Western blotting showed a corresponding 1-fold increase in Bim protein ($P<0.01$) and 2.8-fold increase in cleaved caspase3 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 were isolated from $Irs2^{-/-}$ mice at 5-6 wks showed an increase by 40% in Bim mRNA levels (Fig 2C). These results demonstrate that IRS2 down-regulation is associated with up-regulation of Bim in both MIN6 cells and islets.

**Bim contributes to β-cell apoptosis induced by IRS2 suppression.** To determine whether Bim up-regulation 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 knockdown (Fig 2D). Caspase3 activation was also inhibited in Bim/IRS2 double knockdown (DKD) cells compared to IRS2 KD cells alone (Fig 2D). Following IRS2 knockdown, 50.2 ± 3.9% of the MIN6 cells took up the PI stain. In the Bim/IRS2 DKD group, only 25.7 ± 2.4% ($P<0.001$ compared to 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. Over-expression 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 pro-apoptotic members including Bid, Bim, Puma, Bax and Bak and anti-apoptotic members such as Bcl-2, Bcl-xL and Mcl-1. The presence of an anti-apoptotic molecule such as Bcl-2 or Bcl-xL can inhibit the role of pro-apoptotic molecules following a death signal. To determine whether IRS2 KD induced changes in the expression levels of anti-apoptotic 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 to control cells (Fig2. F). 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 caspase3 activation and inhibited an increase in cleaved caspase3 protein induced by IRS2 suppression (Fig2. G). Bcl-xL over-expression also decreased IRS2 KD -induced cell death. Following Bcl-xL over-expression, the PI stain positive cells decreased from 67.9 ± 3.8% in IRS2 KD cells to 44.6 ± 1.9% (P<0.001) in IRS2 KD/Bcl-xL cells on day 6 (Fig2. H). Bcl-2 over-expression also inhibited IRS2 KD-induced cell death at 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 up-regulation-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 (Fig3. A) and the β-cell area was about 63% smaller than that in age matched wild-type mice. (P<0.001) (Fig3. B). Pancreatic islets from *Bim−/−* mice appeared normal (Fig3. A). β-cells in *Irs2−/−* islets also exhibited increased TUNEL labeling (Fig3. C). These parameters did not differ in *Bim−/−* and control mice. In contrast, in *Irs2−/−/Bim−/−* mice, β-cell area was restored to about 62% of the value in wild-type mice and β-cell mass approximately doubled compared to *Irs2−/−* mice (P<0.01) (Fig3. B). The number of TUNEL positive cells was significantly reduced in islets from *Irs2−/−/Bim−/−* mice compared to *Irs2−/−* mice (P<0.01) (Fig3. C). 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 to WT. These results indicate that the effect 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 (GTT) and insulin tolerance tests (ITT) on Irs2+/+, Irs2−/− Bim−/− mice. Both male and female Irs2−/− mice develop increased fasting blood glucose and impaired glucose clearance (Fig4. A, B, C and E). Irs2−/− Bim−/− mice exhibited significantly lower fasting blood glucose and improved glucose tolerance (Fig4. A, B, C and E). The area under the blood glucose curve (AUC) decreased 38% ± 2.3% (male) and 40% ± 3.3% (female) in Irs2−/− Bim−/− mice compared to Irs2−/− mice, respectively (P<0.001) (Fig4. D and F). Irs2−/− Bim−/− female mice exhibited a normal response to glucose challenge compared to wild type mice. Irs2−/− Bim−/− male mice showed significantly improved but not normal glucose tolerance (Fig4.F). Irs2−/− mice were insulin resistance compared with wild-type mice (Fig4. G). 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 wild-type mice in these animals (Fig4. G). At 12 weeks, fasting insulin levels in Irs2−/− mice were 50% of the values in wild-type animals (Fig4. H) and these levels were increased in the Irs2−/− Bim−/− mice under basal conditions and following glucose challenge in mice compared to wild type mice (Fig4. H). 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 up-regulation 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 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 up-regulation 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 ± 7.8% compared to control cells (P<0.05) (Fig5. D). Consistent with this result, IRS2 KD significantly increased Bim protein levels by 80± 13% (P<0.01), in contrast with a 20 ± 10% increase in Bim protein in FoxO1/IRS2 DKD cells (Fig5. E). Therefore, FoxO1 KD significantly inhibited the increase in Bim protein in FoxO1/IRS2 DKD cells compared to IRS2 KD cells (P<0.05) (Fig5. E). The PI staining positive cells significantly increased in a time-dependent manner in IRS2 KD Min6 cells (P<0.001) (Fig5. F). On day 3 18.0 ± 2.8% of IRS2/FoxO1 DKD cells stained positive with PI compared to 33.4 ± 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 (Fig5. F).

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 (Fig5. G). Taken together, FoxO1 does contribute to Bim up-regulation induced by IRS2 KD and FoxO1 suppression and temporarily reduces but does not eliminate apoptosis in MIN6 in which IRS2 has been suppressed.

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 (Fig5. B), but not in IRS2 KD cells (Fig5. B). FoxO1 protein levels were dramatically reduced in control cells during insulin stimulation; however, insulin failed to reduce FoxO1 protein levels in IRS2 KD cells (Fig5. B). 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 nM insulin for 10 min. Under basal conditions, more FoxO1 protein was detected in nuclear fractions in IRS2 KD cells than in control cells (Fig 5. C). While insulin treatment barely changed nuclear FoxO1 in control cells (Fig 5. C), the nuclear FoxO1 was dramatically increased following insulin treatment of IRS2 KD cells (Fig 5. C). 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 knockout mice develop hyperglycemia associated with a lack of a compensatory increase in beta cell mass and insulin secretion in response to insulin resistance (2). Reduced β-cell 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 caspase3 activation and detected by increased TUNEL labeling in both MIN6 cells and mouse islets. Bim plays an important role in regulating beta 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 (27-31). 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 (32). The present experiments demonstrated that in IRS2 deficiency, knockdown 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 (33). Regulation of Bim in β-cells contributes to type 1and II diabetes (34, 35). In mice, IRS2 mediates peripheral insulin action and pancreatic beta 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 knockout rescues the IRS2 knockout 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 knockout 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 (36). Bim mediates motor neuron loss in amyotrophic lateral sclerosis and Bim is required for beta-amyloid-induced neuronal apoptosis in Alzheimer's disease (37). Glucose oxidase stimulation induces apoptosis by increasing Bim expression in 3T3-L1 adipocytes (38). In cultured muscle cells, oxidative stress induces cell apoptosis by regulating Bim expression (39). 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 pro-apoptotic and anti-apoptotic factors. In IRS2 KD MIN6 cells, anti-apoptotic molecule Bcl-xL was dramatically decreased and Mcl-1 and Bcl-2 is unchanged. Forced over-expression 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 knockdown 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 beta-cell death is significantly decreased in Bim KO mice, proliferation of beta cells is lower in these mice compared to wild type 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 to control cells. Since Akt-dependent phosphorylation of FoxO1 dramatically decreased, FoxO1 protein increased in IRS 2KD cells. FoxO1 regulates genes of both the extrinsic and intrinsic apoptotic pathways (40). FoxO1 KD inhibited Bim up-regulation in IRS2 KD cells. Surprisingly, FoxO1 KD only inhibited β-cell death induced by IRS2 suppression transiently. The effect was seen 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 form of β-cells 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, 41). IRS2 has been implicated in activation of autophagy in neuronal cells (42).

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 up-regulation 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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No potential conflicts of interest relevant to this article were reported.

Dr. Kenneth S. Polonsky and Dr. Decheng Ren are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
FIGURE LEGENDS

FIG.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 an 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 caspase3 protein levels in IRS2 KD cells. 3 days after IRS2 shRNA lentivirus infection, IRS2 protein and cleaved caspase3 protein were assayed by Western blot (3 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 methylene blue uptake. (D) Cell death was determined by PI-staining in MIN6 cells. 3 days after IRS2 shRNA lentivirus infection, the percentage of cell death is shown by representative histograms (left). The results are shown (right). (E+F) MIN6 cells were treated with caspase inhibitor, 20 µM Z-VAD, for 2 hours prior to infection with IRS2 shRNA lentivirus and cell death was determined 3 days later by PI-staining (n=3) (E). Cleaved caspase3 protein levels were assayed by western blot (F). The bar graph depicts the relative changes in the levels of the indicated proteins using densitometry analysis of the Western blots in Figure 1. F (n=3). ***P<0.001 compared to control group. ###P<0.001 compared to IRS2KD group. (G) TUNEL labeling of IRS2 KD MIN6 cells. 3 days after IRS2 shRNA lentivirus infection, Apoptotic cells were assayed by TUNEL staining. Quantitative TUNEL data are shown. ***P<0.001 compared to control group. Values are mean ± SEM.

FIG.2. Bim is up-regulated in MIN6 cells following IRS2 suppression. (A) Bim and Puma mRNA levels in control and IRS2 KD cells. 5 days after IRS2 KD in MIN6 cells, Bim and Puma mRNA levels were measured by real time quantitative reverse transcription-PCR (QRT-PCR) in MIN6 cells (n=3). **P<0.01 compared to control group. (B) Western blot of IRS2 KD cells. 3 days after IRS2 KD in MIN6 cells, immunoblot analysis was performed to determine IRS2, Bim, Puma and cleaved caspase3 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 Figure 2. B (n=3). Values are mean ± SEM. (C) Bim mRNA levels were measured by QRT-PCR in islets from 5-6 week old male Irs2<sup>−/−</sup> mice on normal chow (n=3-4). (D) Western blot of IRS2/Bim DKD cells. 3 days after IRS2/Bim DKD in MIN6 cells, immunoblot of IRS2, Bim and cleaved caspase3 in cells. (E) Measurement of Cell death. 5 days after Bim/IRS2 DKD in MIN6 cells, cell death was determined by PI-staining (n=3). ***P<0.001 compared to control group. ###P<0.001 compared to IRS2 KD group. (F) Western blot of IRS2 KD MIN6 cells. 3 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. 3 days after IRS2 KD and/or Bcl-xL overexpression (OE) in MIN6 cells, immunoblot of cleaved caspase3, 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 to control group. ###P<0.001 compared to IRS2 KD group. Values are mean ± SEM.
FIG. 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 anti-glucagon 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<0.05, **P<0.01, ***P<0.001 compared to the WT mice. ##P<0.01 compared to Irs2−/− mice. (C) TUNEL labeling of adult pancreatic β-cells. Quantitative TUNEL data are shown. *P<0.05, ***P<0.001 compared to the WT mice. ##P<0.01 compared to Irs2−/− mice. Original magnification, X200. (D) Ki-67 staining of β-cells; group Ki-67 data are on the right. *P<0.05, ***P<0.001 compared to the WT mice. ###P<0.001 compared to Irs2−/− mice. Original magnification, X200. All group data are mean ± SEM of n = 3.

FIG. 4. Bim gene ablation reduces hyperglycemia in Irs2−/− mice. (A+B) Fasting glucose levels were measured in male (A) and female (B) mice at 16 weeks (n=8-12). *P<0.05, ***P<0.001 compared to the WT mice. ##P<0.01 compared to Irs2−/− mice. (C+E) Blood glucose levels after intraperitoneal injection of dextrose (2g/kg) in male (C) and female (E) mice at 12 weeks. **P<0.01, ***P<0.001 compared to the WT mice. #P<0.05, ##P<0.01, ###P<0.001 compared to Irs2−/− mice. (D+F) Area under the blood glucose curves (AUC) using the data from C (panel D) or E (panel F) (n=8-12) in the 4 mouse groups designated. *P<0.01, ***P<0.001 compared to the WT mice. #P<0.05, ##P<0.01 compared to Irs2−/− mice. (G) Glucose levels in response to insulin (0.75 U/kg body weight) in the 4 mouse groups designated at 13 weeks (n=8-10). (H) Insulin levels measured fasting and 10 minutes after intraperitoneal dextrose at 12 weeks (n=8-10). **P<0.01 compared to the WT mice. #P<0.05 compared to Irs2−/− mice. Values are mean ± SEM.

FIG. 5. FoxO1 regulates Bim up-regulation and FoxO1 suppression partially rescues MIN6 cells from apoptosis induced by IRS2 suppression. (A) FoxO1 protein levels were increased in IRS2 KD MIN6 cells. 3 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. 2 days after IRS2 shRNA lentivirus infection, serum-starved (overnight) cells were stimulated with 100nM insulin for 5 and 15 min. IRS2, AKT and FoxO1 proteins were assayed by Western blot (n=3). (C) FoxO1 nuclear/cytosolic translocation in IRS2 KD MIN6 cells. 2 days after IRS2 shRNA lentivirus infection, serum-starved (overnight) cells were stimulated with 100nM insulin for 10 min, cytosolic and nuclear proteins were analyzed by Western blot. (D) Bim protein levels in FoxO1 KD cells. 3 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 Figure 5. (D) (n=3). (E) Bim protein in IRS2/FoxO1 DKD cells. 3 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 Figure 5. (E) (n=3). (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 to control group. #P<0.05 compared to IRS2KD group. (G) BrdU incorporation in IRS2/FoxO1 DKD cells. BrdU incorporation into cells was measured 4 days after IRS2 KD. Values are mean ± SEM.
FIG. 3

A Glucagon/Insulin/Nucleus

WT

Irs2 KO

Bim KO

Irs2 Bim DKO

B Insulin/Nucleus

WT

Irs2 KO

Bim KO

Irs2 Bim DKO

C TUNEL/Insulin/Nucleus

WT

Irs2 KO

Bim KO

Irs2 Bim DKO

D Ki-67/Insulin/Nucleus

WT

Irs2 KO

Bim KO

Irs2 Bim DKO
SUPPLEMENTARY DATA

Supplementary Figure 1. Over-expression of Bim induces β-cell apoptosis. A: Over-expression of Bim in MIN6 cells. 2 days after infection with Bim retrovirus in MIN6 cells, the protein levels of BIM were assayed by western blot. ***P < 0.001 vs. control cells. B: Bim over-expression induced cell death. 2 days after infection with Bim retrovirus, cell death was determined by PI-staining in MIN6 cells. ***P < 0.001 vs. control cells. Values are mean ± SEM.
**Supplementary Figure 2.** Bcl-2 over-expression inhibits IRS2KD-induced cell death. 

**A:** The expression levels of Bcl-2 in MIN6 cells. 2 days after MIN6 cells were infected with Bcl-2 retrovirus, the protein levels of BCL-2 were assayed by western blot. 

**B:** Cell death was determined by PI-staining in IRS2 KD/Bcl-xL OE cells. ***P<0.001 compared to control group. #P<0.05 compared to IRS2 KD group. Values are mean ± SEM.
Supplementary Figure 3. Bim expression was undetectable in α cells in mouse islets. Morphology of adult mouse islets after 18 weeks on a normal diet; anti-Bim and anti-glucagon antibodies were used to stain α cells.