

# Endoplasmic Reticulum Stress–Induced Apoptosis Is Partly Mediated by Reduced Insulin Signaling Through Phosphatidylinositol 3-Kinase/Akt and Increased Glycogen Synthase Kinase-3 $\beta$ in Mouse Insulinoma Cells

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**An imbalance between the rate of protein synthesis and folding capacity of the endoplasmic reticulum (ER) results in stress that has been increasingly implicated in pancreatic islet  $\beta$ -cell apoptosis and diabetes. Because insulin/IGF/Akt signaling has been implicated in  $\beta$ -cell survival, we sought to determine whether this pathway is involved in ER stress–induced apoptosis. Mouse insulinoma cells treated with pharmacological agents commonly used to induce ER stress exhibited apoptosis within 48 h. ER stress–induced apoptosis was inhibited by cotreatment of the cells with IGF-1. Stable cell lines were created by small-interfering RNA (siRNA) with graded reduction of insulin receptor expression, and these cells had enhanced susceptibility to ER stress–induced apoptosis and reduced levels of phospho–glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ). In control cells, ER stress–induced apoptosis was associated with a reduction in phospho-Akt and phospho-GSK3 $\beta$ . To further assess the role of GSK3 $\beta$  in ER stress–induced apoptosis, stable cell lines were created by siRNA with up to 80% reduction in GSK3 $\beta$  expression. These cells were found to resist ER stress–induced apoptosis. These results illustrate that ER stress–induced apoptosis is mediated at least in part by signaling through the phosphatidylinositol 3-kinase/Akt/GSK3 $\beta$  pathway and that GSK3 $\beta$  represents a novel target for agents to promote  $\beta$ -cell survival. *Diabetes* 54:968–975, 2005**

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eIF2 $\alpha$ , eukaryotic initiation factor 2 $\alpha$ ; ER, endoplasmic reticulum; GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; JNK, c-Jun NH<sub>2</sub>-terminal kinase; IKRD, insulin receptor knock down; PARP, poly-ADP-ribose polymerase; PERK, PKR-like ER kinase/pancreatic eIF2 $\alpha$  kinase; PI3, phosphatidylinositol 3; Q-VD-OPh, N-(2-quinolyl)valyl-aspartyl-(2,6-difluorophenoxy)methyl ketone; siRNA, small-interfering RNA.

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**M**olecular mechanisms involved in various forms of pancreatic islet  $\beta$ -cell failure are being discovered, and most recently the endoplasmic reticulum (ER) has been shown to mediate signals that may contribute to this process (1,2). All cells regulate the capacity of the ER to fold and process proteins and thereby control the balance between protein demand and folding capacity. An imbalance in this process triggers an aberrant process referred to as ER stress, which if unabated can lead to apoptosis. Pancreatic  $\beta$ -cells have highly developed ER, and they also abundantly express ER stress transducer proteins including Ire1 $\alpha$ , PERK (pancreatic ER kinase or PKR-like ER kinase), and BiP (3). Recent studies have shown that these cells may be particularly vulnerable to ER stress. A targeted disruption of Chop, a C/EBP homologous protein strongly implicated in ER stress–induced apoptosis, resulted in resistance to nitric oxide–induced apoptosis in  $\beta$ -cells as well as amelioration of  $\beta$ -cell failure caused by a mutated insulin gene (Akita mouse) (4,5). PERK is activated by ER stress, and it in turn phosphorylates eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), which leads to attenuation in protein synthesis. Loss of PERK (3,6) or a mutant eIF2 $\alpha$  incapable of undergoing phosphorylation by PERK (eIF2 $\alpha$ <sup>S51A</sup>) in mice (7) leads to diabetes due to destruction of pancreatic  $\beta$ -cells. Mutations in the human EIF2AK3 (PERK) gene are the cause of a rare recessive disorder, the Wolcott-Rallison syndrome, which is characterized by early-onset diabetes (8). These studies highlight that ER stress is a likely contributor to the  $\beta$ -cell dysfunction in diabetes.

Recent evidence has indicated the importance of ER stress and reduced insulin signaling in the fat-feeding model of diabetes (9). In these experiments, it was shown that fat feeding was associated with markers of ER stress, C-Jun NH<sub>2</sub>-terminal kinase (JNK) activation, and insulin resistance in the liver (9). Genetic mouse models deficient in insulin or IGF-1 receptors, or in insulin receptor substrate-1 or -2, exhibit various impairments in  $\beta$ -cell mass and/or function (10–19). Insulin/IGF signaling through phosphatidylinositol 3 (PI3)-kinase and Akt are well-established activators of survival in numerous cell types, and overexpression of Akt specifically in pancreatic islet  $\beta$ -cells resulted in marked expansion of cell number and

size (20,21). These mice have been shown to resist streptozotocin-induced  $\beta$ -cell apoptosis and diabetes.

Glycogen synthase kinase  $\beta$  (GSK3 $\beta$ ) was the first substrate shown to be phosphorylated by Akt (22). GSK3 $\beta$  is a serine/threonine protein kinase whose major control is a negative one by Akt-mediated phosphorylation. Overexpression of a constitutively active GSK3 $\beta$  in a PC12 cell line was associated with cell death (23), while apoptosis initiated by PI3-kinase inhibition, or serum or growth factor starvation, was reduced in the presence of GSK3 $\beta$  inhibition (24). Recently, it was established that GSK3 $\beta$  is an obligatory factor in ER stress-induced apoptosis of human neuroblastoma cells (25). Expression of GSK3 $\beta$  in pancreatic islets, as well as its possible role in growth factor-mediated growth and survival, has been little studied. The relationship between IGF-1, GSK3 $\beta$ , and survival of insulinoma cells in culture was suggested by the rapid and sustained phosphorylation of GSK3 $\beta$  following IGF-1 treatment (26). The results of these studies together suggest that inhibition of GSK3 $\beta$  by growth factor-mediated PI3-kinase/Akt signaling may be an important mechanism to promote  $\beta$ -cell survival.

In the current study the hypothesis tested was that the ER stress-induced apoptosis is mediated at least in part by decreased insulin signaling through the PI3-kinase/Akt pathway in pancreatic islet  $\beta$ -cells. Pharmacological agents known to result in ER stress (25) were shown to result in apoptosis in glucose-sensitive mouse insulinoma cells (MIN6) that was associated with reduced Akt and GSK3 $\beta$  phosphorylation. Cotreatment with IGF-1 partially reversed these effects. A stable cell line with reduced insulin signaling by silencing the insulin receptor was shown to have reduced GSK3 $\beta$  phosphorylation and enhanced susceptibility to ER stress-induced apoptosis. Additionally, reduced expression of GSK3 $\beta$ , utilizing small interfering RNA (siRNA), resulted in significant protection from ER stress-induced apoptosis. These studies show that ER stress-induced apoptosis is mediated at least in part by growth factor signaling through the PI3-kinase/Akt/GSK3 $\beta$  pathway. Modulation of this pathway is shown to protect islet  $\beta$ -cells against ER stress-induced apoptosis, and it may represent an important novel area for therapeutic intervention in clinical diabetes.

## RESEARCH DESIGN AND METHODS

**Cell culture, transfection of insulinoma cells, and selection of stably transfected clones.** MIN6-cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/l glucose, with 15% fetal bovine serum, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 100  $\mu$ g/ml L-glutamine, and 5  $\mu$ l/l  $\beta$ -mercaptoethanol in humidified 5% CO<sub>2</sub>/95% air at 37°C (27). Parental MIN6 cells used for plasmid transfection were between passages 24 and 26. An siRNA-expressing plasmid system (pSUPER vector) (28) was used to reduce the insulin receptor or GSK3 $\beta$  expression. Target sequence against mouse insulin receptor was 5'-ACTGCATGGTTGCCCATGA-3', 5'-CATAGTCGACTGCGGTAT-3' for GSK3 $\beta$ KD $\Delta$ 50 cells, and 5'-CACCAGTGAAGCTTGTGC-3' for GSK3 $\beta$ KD $\Delta$ 80 cells was used to silence GSK3 $\beta$ . A total of 10  $\mu$ g pSUPER vector along with 1  $\mu$ g pCDNA3.1 plasmid containing a neomycin selection cassette was transfected using 40  $\mu$ l of *TransIT-LT1* transfection reagent (Mirus, Madison, WI) for each 10-cm plate. The transfected cells were first selected with culture medium containing 500  $\mu$ g/ml G418 (Mediatech, Herndon, VA) for 4 weeks, and then isolated colonies of the surviving cells (defined as passage 4) were maintained in culture medium with 200  $\mu$ g/ml G418. Protein levels and mRNA expression were tested at passage 7–8, and clones were further maintained by weekly passaging. MIN6 cells transfected with empty pSUPER vector were designated as MIN6-Con, and those with reduced insulin receptor or GSK3 $\beta$  expression were designated as IRKD (for

"insulin receptor knock down") or GSK3 $\beta$ KD (for "GSK3 $\beta$  knock down"), respectively. Transformed cells were used for experiments herein between passages 9 and 18, which corresponded to passages 33 and 42 of parental MIN6 cells.

**Detection of apoptosis.** After treatment with reagents (thapsigargin, tunicamycin, Brefeldin-A, IGF-1 [Sigma, St. Louis, MO] or caspase inhibitor Q-VD-OPh [*N*-(2-quinolyl)valyl-aspartyl-(2,6-difluorophenoxy)methyl ketone] [Calbiochem, San Diego, CA]), cells were harvested and apoptosis rates were measured by Annexin-V assay (BioVision, Mountain View, CA) using flow cytometry as previously described (29).

**Western blot analysis.** Protein was extracted with a lysis buffer (200 mmol/l Tris, pH 7.5, 1.5 mol/l NaCl, 0.1 mol/l EDTA, 0.5 mol/l EGTA, Triton X-100, 0.25 mol/l sodium pyrophosphate, 0.25 mol/l glycerophosphate, 200 mmol/l sodium orthovanadate, okadaic acid, distilled water, and protease cocktail tablet). Protein samples (20–40  $\mu$ g) were separated by electrophoresis through 10% polyacrylamide/0.1% SDS gels, transferred to polyvinylidene fluoride (PVDF) membranes, and then immunoblotted. Immunodetection was performed with Western Lightning (PerkinElmer Life Sciences, Boston, MA). Antibodies used in this study are as follows: anti-actin (Sigma), anti-phospho-Ser473 Akt, anti-Akt, anti-phospho-GSK3, anti-phospho-Ser9 GSK3 $\beta$ , anti-GSK3 $\beta$ , anti-poly-ADP-ribose polymerase (PARP), anti-JNK, anti-phospho-JNK (Cell Signaling, Boston, MA), and anti-CHOP10 (Santa Cruz Biotechnology, Santa Cruz, CA).

**Quantitative RT-PCR.** One microgram total RNA was used to prepare cDNA, primed with random hexamers, and reverse-transcribed with Superscript II (Invitrogen Carlsbad, CA). Quantitative RT-PCR was performed with SYBR Green dye as described (30) using the ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA). Values were normalized to the amounts of 18S ribosomal RNA. All PCR reactions were performed as at least replicates of four. Sequences of primers for detecting GSK3 $\beta$  used in this study are: forward 5'-ACCAATATTCTGGGGACA-3' and reverse 5'-GTGCCTTGATTGAGGGAAT-3'.

## RESULTS

**ER stress provoked by different agents induced apoptosis in MIN6 cells.** Thapsigargin has been previously shown to reduce cell viability in a dose- and time-dependent manner in mouse insulinoma cells (31). Thapsigargin inhibits the ER calcium ATPase and blocks the sequestration of calcium by the ER, resulting in increased intracellular calcium, accumulation of misfolded proteins, and activation of apoptosis (32). Thapsigargin-induced apoptosis in MIN6 cells in proportion to the dose and duration of treatment for up to 48 h (Fig. 1A). Brefeldin-A, which specifically blocks protein transport from the ER to the Golgi apparatus, and tunicamycin, which inhibits NH<sub>2</sub>-linked glycosylation and protein folding in the ER, also induced apoptosis in MIN6 cells after 48 h of treatment (Fig. 1B). At the highest dose of thapsigargin tested (1  $\mu$ mol/l), Q-VD-OPh (10  $\mu$ mol/l) resulted in a 44% reduction of apoptosis ( $n = 6$ ,  $P < 0.001$ , data not shown). These experiments showed that ER stress induced by several different pharmacological reagents resulted in apoptosis in insulinoma cells, thus serving as a model to study this process.

**Insulin/IGF signaling pathways alter ER stress-induced apoptosis in MIN6 cells.** IGF-1 has previously been shown to protect islet  $\beta$ -cells from growth factor depletion and cytokine-mediated apoptosis (33–35). This protection is mediated via PI3-kinase/Akt signaling and phosphorylation of its downstream targets. In the current experiments, we tested the effects of this growth factor on ER stress-induced apoptosis. Treatment of the cells with IGF-1 (100 nmol/l) significantly reduced the apoptosis after 48 h of exposure to the highest dose of thapsigargin (1  $\mu$ mol/l) (control 7.5  $\pm$  0.6%, thapsigargin 69.1  $\pm$  6.3%, thapsigargin + IGF-1 36.8  $\pm$  2.3%;  $P < 0.01$  vs. thapsigargin alone) (Fig. 1C).

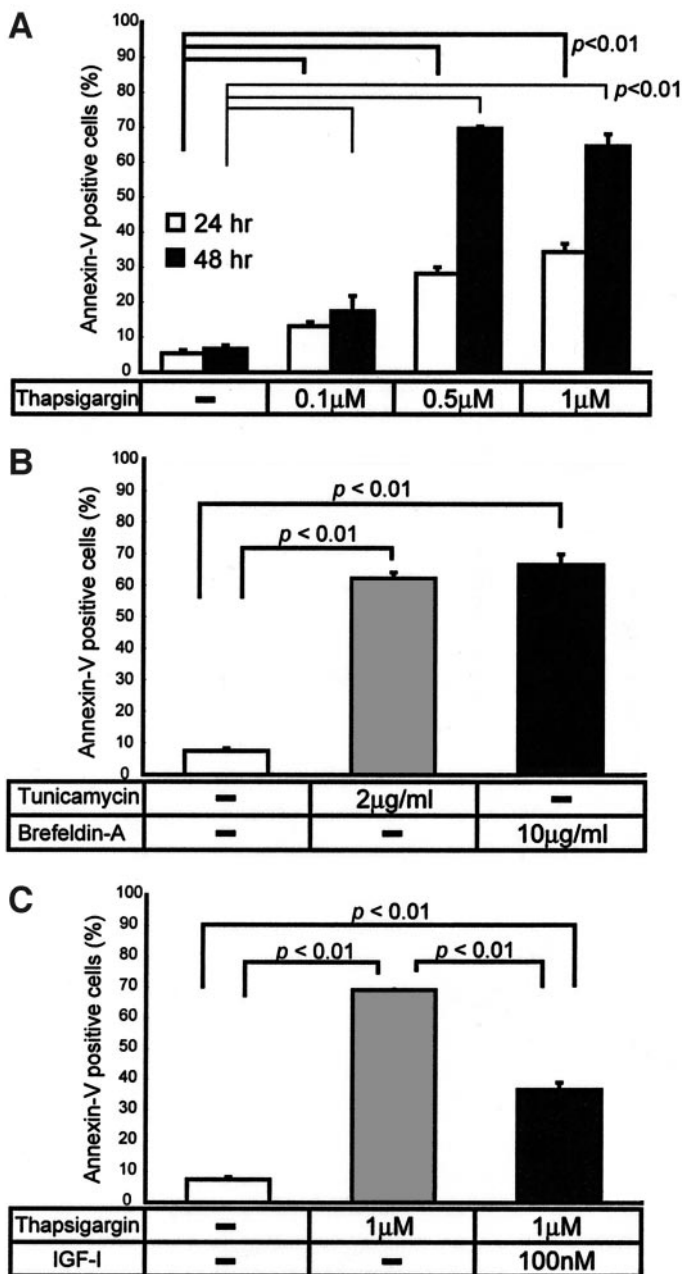


FIG. 1. ER stress-induced apoptosis in MIN6 insulinoma cells. **A:** Thapsigargin-induced apoptosis as measured by an Annexin-V assay that quantitates cell surface phosphatidyl-serine. **B:** Effects of tunicamycin (2  $\mu$ g/ml) or Brefeldin-A (10  $\mu$ g/ml) on apoptosis in MIN6 cells after 48-h treatment. **C:** IGF-1 inhibits thapsigargin-induced apoptosis. MIN6 cells were cultured in the presence and absence of thapsigargin (1  $\mu$ mol/l) with or without IGF-1 (100 nmol/l) for 48 h then rates of apoptosis were measured by Annexin-V assay. Data are shown with SEM ( $n = 6-8$ ).

The observation that IGF-1 treatment reduced ER stress-induced apoptosis suggested that chronic inhibition of insulin signaling might be associated with enhanced ER stress-induced apoptosis. As recently described (36), a vector-based siRNA was used to create MIN6 cells with reduced insulin receptor expression (IRKD cells). These cells had stably reduced levels of the insulin receptor mRNA as well as protein by 50 and 80% (IRKD $\Delta$ 50 and IRKD $\Delta$ 80, respectively) compared with those of control cells stably transformed with an empty vector. Function-

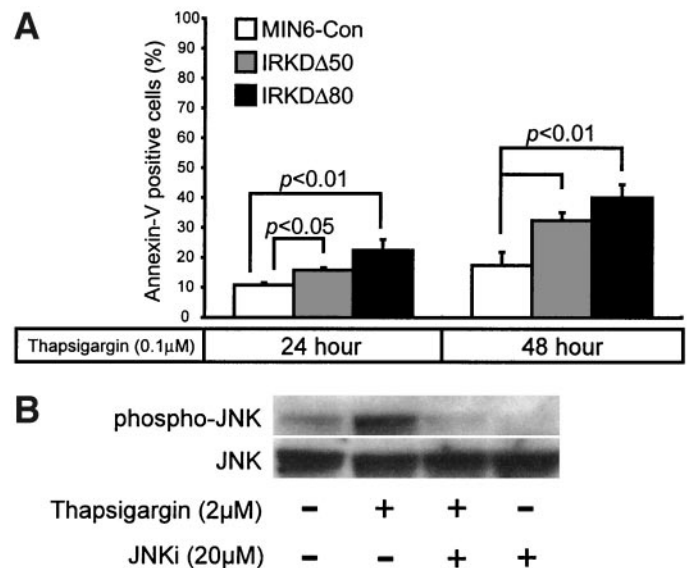
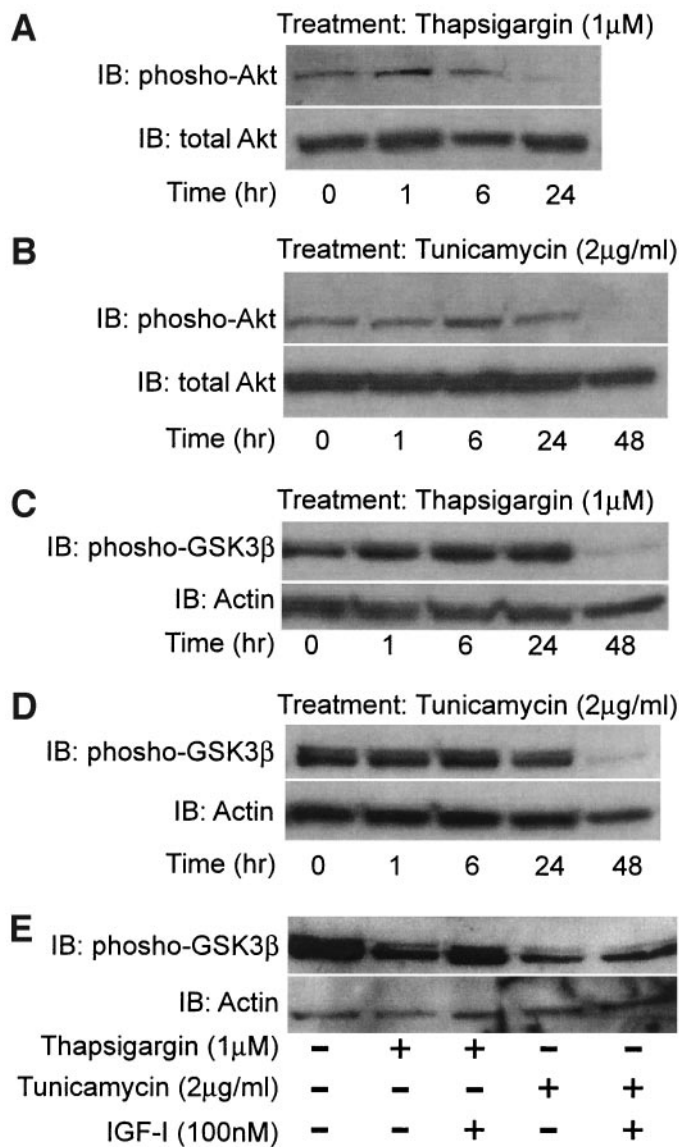


FIG. 2. Insulin/IGF signaling pathways modulate ER stress-induced apoptosis in MIN6 cells. **A:** Apoptosis rates were measured following 24- to 48-h thapsigargin (0.1  $\mu$ mol/l) treatment in IRKD $\Delta$ 50 and IRKD $\Delta$ 80 cells, which had reduced IR expression by 50 or 80%, respectively. Data are shown with SEM ( $n = 6-9$ ). **B:** Activity of JNK was measured by phospho-JNK after 6 h of 2  $\mu$ mol/l thapsigargin treatment in MIN6 cells. JNK inhibitor SP600125 (20  $\mu$ mol/l) was used to show that the level of JNK phosphorylation correlates with its activity.

ally perturbed insulin receptor signaling was confirmed with the absence of insulin-stimulated insulin receptor substrate-1 tyrosine phosphorylation. Additionally, Akt phosphorylation was reduced and responded poorly to glucose stimulation. In the current studies, when IRKD cells were treated with 0.1  $\mu$ mol/l thapsigargin, the rates of apoptosis were significantly increased in both IRKD $\Delta$ 50 and IRKD $\Delta$ 80 cells compared with control cells with both 24- and 48-h treatments (Fig. 2A). The sum of these results suggested that insulin/IGF signaling, perhaps through PI3-kinase/Akt, could modulate ER stress-mediated apoptosis. **Agents producing ER stress are associated with activation of JNK.** Recent study has shown that JNK activation is associated with fat feeding-induced ER stress, and JNK activation results in decreased insulin signaling in liver and adipose tissue (9). To test whether JNK is activated by ER stress in pancreatic  $\beta$ -cells, we assessed its activity by measuring phospho-JNK in MIN6 cells following thapsigargin treatment. As seen in Fig. 2B, phospho-JNK was increased after thapsigargin treatment, and a specific JNK inhibitor significantly reduced its phosphorylation. These data demonstrated that JNK was activated by ER stress in  $\beta$ -cells and suggested that the ER stress-induced apoptosis might be associated with reduced insulin signaling.

**Agents producing ER stress resulted in inhibition of phosphorylation of Akt and GSK3 $\beta$ .** To examine whether agents producing ER stress in  $\beta$ -cells results in altered insulin/IGF signaling, we next assessed whether treatment with these agents were associated with reduction of Akt activity, since Akt is a well-known downstream target of insulin/IGF/PI3-kinase signaling (37). Akt activity was measured with an antibody specific for phospho-Ser473 Akt (38). As seen in Fig. 3A, treatment of MIN6 cells with thapsigargin (1  $\mu$ mol/l) resulted in marked reduction

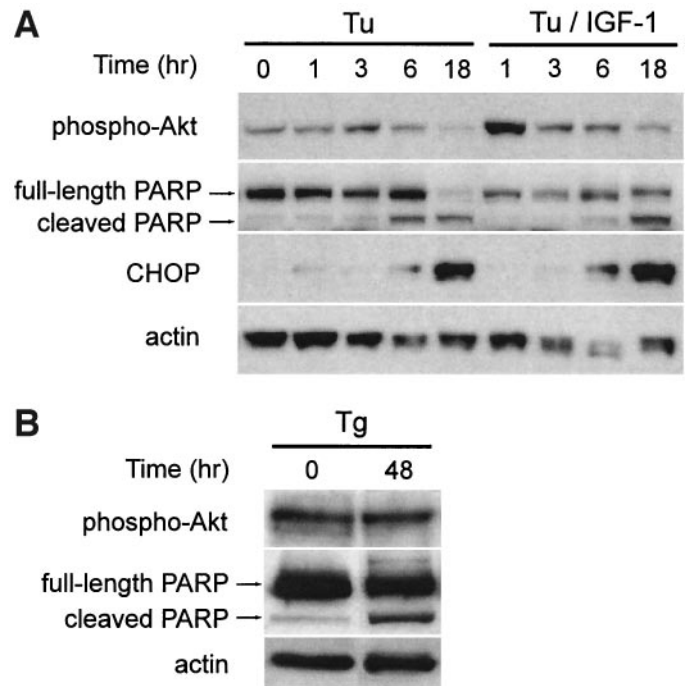




**FIG. 3.** ER stress-inducing agents and Akt or GSK3 $\beta$  phosphorylation. MIN6 cells were treated with 1  $\mu$ mol/l thapsigargin (A) or 2  $\mu$ g/ml tunicamycin (B), and phospho-Ser473 Akt was detected using Western blot analysis. MIN6 cells were treated with 1  $\mu$ mol/l thapsigargin (C) or 2  $\mu$ g/ml tunicamycin (D), and phospho-Ser9 GSK3 $\beta$  was detected using Western blot analysis. E: MIN6 cells were treated with 1  $\mu$ mol/l thapsigargin or 2  $\mu$ g/ml tunicamycin in the absence or presence of 100 nmol/l IGF-1 for 48 h. Using Western blot analysis phospho-Ser9 GSK3 $\beta$  was detected. These results are representative of three independent experiments.

in phospho-Akt by 24 h, with no apparent alteration in total Akt protein. Similar reduction in phospho-Akt was also observed following 24-h treatment with tunicamycin, with more marked reduction at 48 h, and with no obvious change in total Akt protein over this time period (Fig. 3B).

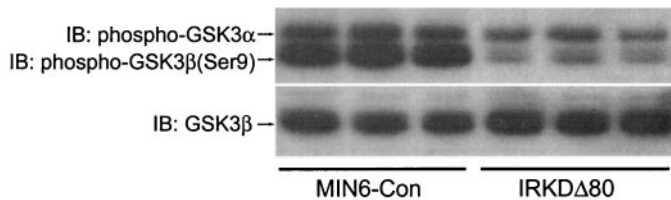
Akt is a serine/threonine kinase that regulates a number of downstream effectors that contribute to cell survival (37). One proapoptotic substrate whose activity is inhibited by Akt phosphorylation is GSK3 $\beta$ . Recently ER stress-induced apoptosis in neuronal cells was shown to be associated with decreased Akt and enhanced GSK3 $\beta$  activity (25). Blocking GSK3 $\beta$  activity markedly reduced ER stress-induced apoptosis in this neuronal cell model system. To study the potential role of GSK3 $\beta$  in ER stress



**FIG. 4.** IGF-1 protects MIN6 cells from ER stress-induced apoptosis without altering the magnitude of ER stress. A: MIN6 cells were treated with 2  $\mu$ g/ml tunicamycin with or without 100 nmol/l IGF-1 and phospho-Ser473 Akt; PARP cleavage and CHOP induction were assessed by Western blot analysis. B: Isolated human islets were treated with 2  $\mu$ mol/l thapsigargin for 48 h. Akt phosphorylation and PARP cleavage were assessed by Western blot analysis.

responses in MIN6 insulinoma cells, we determined the activity of GSK3 $\beta$  by using a phospho-Ser-9-specific antibody. Thapsigargin-treated cells exhibited a marked reduction in the Ser9-phospho-GSK3 $\beta$ , the inactive form of the enzyme, at 48 h with no apparent change in total protein levels (Fig. 3C). Similar results were observed with tunicamycin (2  $\mu$ g/ml) (Fig. 3D). Cotreatment with IGF-1 (100 nmol/l) appeared to ameliorate the ER stress-induced reduction in phospho-GSK3 $\beta$  following either thapsigargin (1  $\mu$ mol/l) or tunicamycin (2  $\mu$ g/ml) treatments (Fig. 3E).

To more carefully examine the relationships between ER stress-induced decrease in phospho-AKT and activation of apoptosis, MIN6 cells were treated with tunicamycin (2  $\mu$ g/ml) for up to 18 h, cellular proteins were assayed for phospho-AKT level, CHOP-10, an ER stress marker, and cleaved PARP, a measure of caspase activation. Phospho-AKT briefly increased, but after treatment for 18 h it was decreased, while CHOP-10 expression was gradually increased during this period, indicating the accumulation of ER stress. Cleavage of PARP also progressively increased over this time period (Fig. 4A). These data demonstrated that decreased phospho-AKT was tightly associated with accumulation of ER stress and activation of caspases. As the previous experiment had shown that IGF-1 treatment ameliorated ER stress-induced apoptosis, to examine if this is associated with reduced ER stress, we examined the level of ER stress in the IGF-1-cotreated MIN6 cell samples. As seen in Fig. 4A, treatment with IGF-1 (100 nmol/l) was associated with enhanced phospho-AKT level and decreased caspase activation but with equal induction of CHOP-10 compared with untreated cells. Thus, IGF protects MIN6 cells from ER stress-



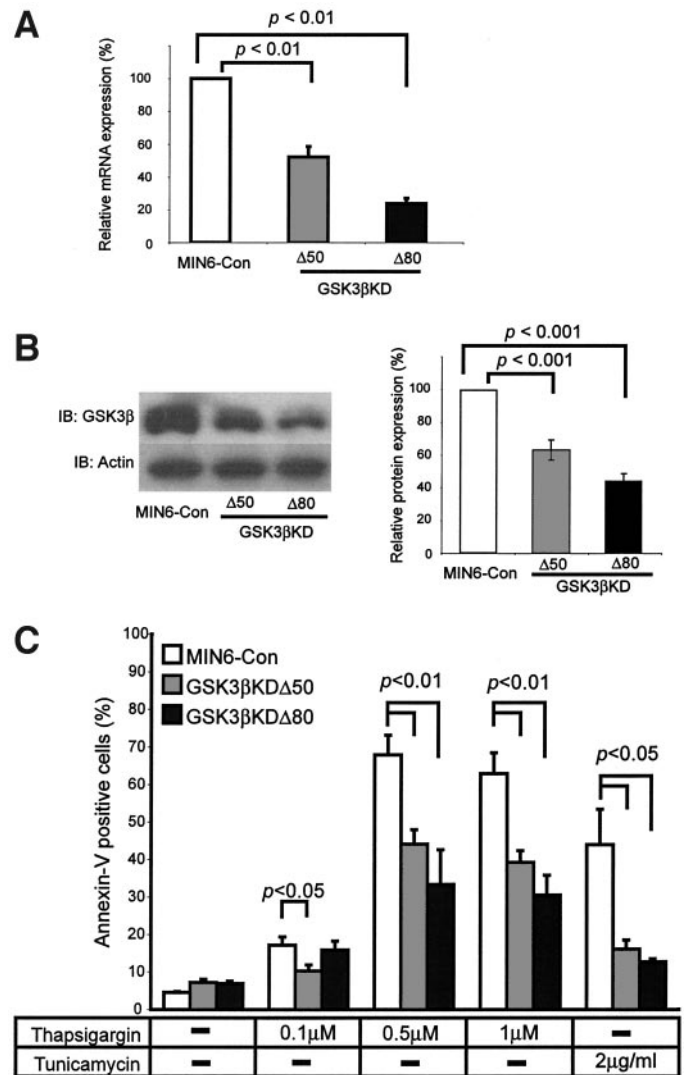
**FIG. 5.** GSK3 $\beta$  phosphorylation in IRKD cell. Western blot analysis of phospho-GSK3 $\beta$  in MIN6-Con and IRKD $\Delta$ 80 cells was performed with whole-cell lysates. GSK3 $\beta$  phosphorylation was assessed by using antibody for phospho-GSK3 (detecting both phospho-GSK3 $\alpha$  and -GSK3 $\beta$ ), and then GSK3 $\beta$  was detected as loading control. The results are representative of two identical blots.

induced apoptosis without apparent alteration of the magnitude of ER stress.

To determine whether ER stress-induced reduction of insulin receptor signaling is also occurring in primary cultures, we treated human islets with thapsigargin and examined the level of AKT phosphorylation and apoptosis. As seen in Fig. 4B, phospho-AKT was decreased (the ratios of phospho-Akt/actin at 0 and 48 h were 96 and 72%, respectively) and caspase activation was increased, seen as cleavage of PARP, in human islets after 48 h of thapsigargin treatment.

**Decreased expression of GSK3 $\beta$  by siRNA reduced ER stress-induced apoptosis.** Having demonstrated that ER stress-activated apoptosis was associated with reduction in AKT/GSK3 $\beta$  phosphorylation, we predicted that the previously observed enhanced apoptosis of the IRKD cell lines (Fig. 2A) would be associated with reduced GSK3 $\beta$  phosphorylation. As shown in Fig. 5, IRKD $\Delta$ 80 cells had marked reduction in phospho-GSK3 $\beta$ , with no apparent change in total GSK3 $\beta$  protein. This observation confirmed that sensitivity of MIN6 cells to ER stress-induced apoptosis is associated with altered insulin signaling through modulation of GSK3 $\beta$  activity.

The observed reduction in AKT/GSK3 $\beta$  phosphorylation occurring with ER stress may not necessarily be a primary event in the apoptosis but rather a consequence of cells undergoing apoptosis. If GSK3 $\beta$  is affecting ER stress-induced apoptosis, reduced GSK3 $\beta$  expression/activity would predictably result in resistance to this process. To accomplish this, we again employed siRNA to reduce expression of GSK3 $\beta$ . Several cell lines with reduced expression were established, and the reduced expression was examined at both mRNA and protein levels. Two of the derived cell lines showing 50 and 80% reduction (designated as GSK3 $\beta$ KD $\Delta$ 50 and GSK3 $\beta$ KD $\Delta$ 80, respectively) in both mRNA (Fig. 6A) and protein expression (Fig. 6B) were chosen for the subsequent experiments. When these cells were treated with the highest concentrations of thapsigargin, the rate of apoptosis was reduced by 51%. With tunicamycin treatment, the rates of apoptosis were significantly decreased by as much as 71% compared with cells transfected with vector alone (Fig. 6C). Thus, it was concluded that GSK3 $\beta$  is a contributing factor in ER stress-induced apoptosis in MIN6 cells. To determine whether the magnitude of ER stress was altered in GSK3 $\beta$  knockdown cells, we examined the level of CHOP-10 induction. As seen in Fig. 7, activation of CHOP-10 expression in GSK3 $\beta$ KD cells was largely comparable to that in control cells. Similarly, CHOP-10 activation was not signif-



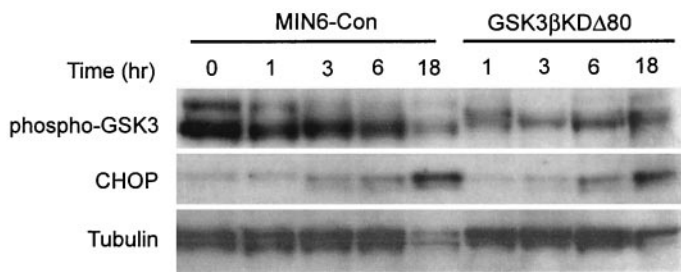
**FIG. 6.** siRNA-mediated reduction of GSK3 $\beta$  led to resistance to ER stress-induced apoptosis. **A:** GSK3 $\beta$  mRNA levels were detected by quantitative RT-PCR analysis as described in RESEARCH DESIGN AND METHODS. Expressions of GSK3 $\beta$  mRNA in GSK3 $\beta$ KD $\Delta$ 50 and - $\Delta$ 80 cells relative to MIN6-Con cells were shown with SEM. This is combined data from three individual samples. **B:** GSK3 $\beta$  protein expressions were detected by Western blot analysis (left). Actin was also probed to show equal loading of proteins. This is a representative of three identical blots. Quantifications of the levels of GSK3 $\beta$  protein were shown with SEM (right). **C:** MIN6-Con, GSK3 $\beta$ KD $\Delta$ 50, and GSK3 $\beta$ KD $\Delta$ 80 cells were treated with thapsigargin as indicated or tunicamycin (2  $\mu$ g/ml) for 48 h, and then apoptosis rates were measured by Annexin-V assay. Data are shown with SEM ( $n = 6$ ).

icantly changed in IRKD cells (data not shown), and these findings are consistent with our observation that insulin receptor signaling protects MIN6 cells from ER stress-induced apoptosis without altering the level of ER stress.

## DISCUSSION

This study reports three novel and potentially important observations relevant to insulin/IGF signaling and ER stress-induced apoptosis in pancreatic islet  $\beta$ -cells. First, pretreatment of insulinoma cells with IGF-1 significantly reduced ER stress-induced apoptosis, and cells with reduced expression of the insulin receptor had enhanced susceptibility to this process. Second, ER stress-induced apoptosis was associated with activation of JNK kinase





**FIG. 7. ER stress-induced apoptosis in GSK3βKDΔ80 cells.** MIN6-Con and GSK3βKDΔ80 cells were treated with 2 μmol/l thapsigargin as indicated. Phosphorylation of GSK3β protein and Chop induction were detected by Western blot analysis. Tubulin was also probed to show equal loading of proteins.

and reduced insulin signaling, evidenced by reduced phosphorylation of Akt and GSK3β, and these reductions were partially reversed by cotreatment with IGF-1. Third, and most important, siRNA-mediated reduction of GSK3β expression resulted in resistance to ER stress-induced apoptosis. These results thus highlight a new mechanism whereby signaling through the insulin/IGF pathway in pancreatic islet β-cells mediates ER stress-induced apoptosis and may provide a means to enhance survival. Because of the likely involvement of ER stress in common forms of diabetes (39), these observations have potential clinical implications.

Insulin/IGF signaling has been implicated in pancreatic β-cell growth, function, and survival (rev. in 40). The observation that cell lines with reduced expression of the insulin receptor have enhanced susceptibility to ER stress-induced apoptosis (Fig. 2A) suggested a possible mechanism for the reduced islet β-cell mass observed in the mouse models of diabetes with insulin receptor (βIRKO) or the insulin receptor substrate-2 deficiencies. Additionally, the current results suggest that the decrease in islet β-cell function that accompanies diabetes may be related to impaired insulin signaling in β-cells and enhanced susceptibility to ER stress.

GSK3β is a well-characterized downstream target of growth factor-activated PI3-kinase/Akt signaling (41,42). We showed that ER stress is associated with apparent reduced insulin signaling evidenced by attenuated Akt phosphorylation and resultant dephosphorylation of GSK3β (Fig. 3). IGF-1 treatment reduced ER stress-induced apoptosis and was also associated with reductions in the dephosphorylation of Akt and GSK3β. The conclusion that GSK3β modulates susceptibility to ER stress-induced apoptosis, rather than its activity being altered by apoptosis or impaired insulin secretion, was shown by reduction of GSK3β expression. These cells had partial but highly significant resistance to ER stress-induced apoptosis (Fig. 6C). Another downstream target of PI3-kinase/Akt is the forkhead or Foxo transcription factors. These proapoptotic proteins are also silenced by insulin/IGF-activated phosphorylation through Akt activation (37) and could contribute to the enhanced susceptibility of the IRKD cells to ER stress. This possibility has yet to be evaluated. Taken together, this study provides evidence supporting an important role for GSK3β as at least one of the components connecting insulin/IGF signaling with resistance to ER stress-induced apoptosis in islet β-cells.

The mechanisms by which GSK3β facilitates apoptosis

have yet to be identified in this model system. Several transcription factors are potential targets whereby this kinase could promote apoptosis (rev. in 43). For example, activation of heat shock factor-1 induces the expression of heat shock proteins and attenuates stress-induced cell death (44,45). GSK3β phosphorylates and inhibits heat shock factor-1 activation and, thus, increases cellular susceptibility to stress-induced apoptosis (46). Similarly, CREB upregulates the expression of the anti-apoptotic protein bcl-2, and the inhibition of CREB activity by GSK3β may contribute to the proapoptotic effects of GSK3β (47,48). However, the precise proapoptotic targets of GSK3β during ER stress in β-cells remain to be identified.

The pathways mediating ER stress-activated apoptosis are complex and only partially defined, as recently reviewed (49). ER stress induced by thapsigargin or tunicamycin led to a gradual reduction of phosphorylation of Akt and GSK3β in MIN6 cells. At 24 h, 30% of the cells treated with thapsigargin were Annexin-V positive, while there was no apparent decrease in GSK3β phosphorylation (Figs. 1 and 3). These results indicated that reduced phosphorylated Akt and GSK3β can only partially account for the apoptosis induced by ER stress. Furthermore, IGF-1 cotreatment significantly but only partially protected the cells from apoptosis. Finally, while up to 80% reduction in expression of GSK3β resulted in a highly significant reduction in ER stress-induced apoptosis, it did not completely eliminate apoptosis (Fig. 6C). These results leave little doubt that there are other mechanisms involved in ER stress-activated apoptosis that are independent of the growth factor/PI3-kinase/Akt/GSK3β pathway.

Akt and GSK3β may be possible targets for pharmacological intervention to promote β-cell survival. Pharmacologic GSK3β inhibition has been investigated as a potential treatment for type 2 diabetes, since increased GSK3β activity was linked to insulin resistance (50,51). Studies have shown that inhibition of GSK3β leads to improved insulin sensitivity or insulin mimetic action in vitro, and Ring et al. (52) reported that administration of a selective GSK3β inhibitor acutely improved hyperglycemia in murine diabetic models. The current study, furthermore, identified GSK3β inhibition as a potential therapeutic target to possibly preserve β-cell mass. In conclusion, we showed direct evidence that GSK3β is involved in ER stress-induced apoptosis in a pancreatic β-cell model. Further studies will likely clarify the signaling pathways from ER stress to Akt/GSK3β and will identify GSK3β downstream targets responsible for apoptosis. These results may ultimately provide additional therapeutic targets for protecting pancreatic β-cells.

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