THIOREDOXIN-INTERACTING PROTEIN: A CRITICAL LINK BETWEEN GLUCOSE TOXICITY AND BETA CELL APOPTOSIS

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Running title: TXNIP links glucotoxicity and beta cell death

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ABSTRACT

Objective: In diabetes, glucose-toxicity affects different organ systems including pancreatic islets where it leads to beta cell apoptosis, but the mechanisms are not fully understood. Recently, we identified thioredoxin-interacting protein (TXNIP) as a pro-apoptotic beta cell factor that is induced by glucose raising the possibility that TXNIP may play a role in beta cell glucose-toxicity.

Research Design And Methods: To assess the effects of glucose on TXNIP expression and apoptosis and define the role of TXNIP, we used INS-1 beta cells, primary mouse islets, obese, diabetic BTBR.ob mice and a unique mouse model of TXNIP-deficiency (HcB-19) harboring a natural nonsense mutation in the TXNIP gene.

Results: Incubation of INS-1 cells at 25mM glucose for 24h led to a 18-fold increase in TXNIP protein as assessed by immunoblotting. This was accompanied by increased apoptosis as demonstrated by a 12-fold induction of cleaved caspase-3. Overexpression of TXNIP revealed that TXNIP induces the intrinsic mitochondrial pathway of apoptosis. Islets of diabetic BTBR.ob mice also demonstrated increased TXNIP and apoptosis as did isolated wild-type islets incubated at high glucose. In contrast, TXNIP-deficient HcB-19 islets were protected against glucose-induced apoptosis as measured by TUNEL and caspase-3, indicating that TXNIP is a required causal link between glucose-toxicity and beta cell death.

Conclusions: These findings shed new light onto the molecular mechanisms of beta cell glucose-toxicity and apoptosis, demonstrate that TXNIP induction plays a critical role in this vicious cycle and suggest that inhibition of TXNIP may represent a novel approach to reduce glucotoxic beta cell loss.
Type 2 diabetes (T2DM) is a growing public health issue characterized by peripheral insulin resistance and decompensation of the pancreatic beta cells that can no longer keep up with the increased insulin requirements, resulting in hyperglycemia. These elevated glucose levels have detrimental effects on various tissues including the pancreatic beta cell. Beta cell glucose toxicity leads to progressive beta cell dysfunction, impaired insulin gene transcription (1; 2) and irreversible beta cell loss by apoptosis (3-14) resulting in a vicious cycle with worsening of the hyperglycemia. However, the exact molecular mechanisms by which glucose toxicity leads to apoptotic beta cell loss are still not fully understood.

Recently, we identified thioredoxin-interacting protein (TXNIP) as a highly glucose-regulated pro-apoptotic factor in beta cells (15) suggesting that it may represent a potential mediator of beta cell glucose toxicity. TXNIP binds to and inhibits thioredoxin and thereby can modulate the cellular redox state and promote oxidative stress (16-20). In addition, TXNIP has been shown to exert anti-proliferative effects by inducing cell-cycle arrest at the G0/G1 phase (16; 21; 22) and TXNIP overexpression rendered fibroblasts and cardiomyocytes more susceptible to apoptosis (16; 23). Similarly, we found that TXNIP overexpression also induces apoptosis in pancreatic beta cells (15). Moreover, we demonstrated that glucose stimulates TXNIP transcription through a carbohydrate response element in the TXNIP promoter resulting in elevated TXNIP mRNA expression (15). Together, these findings raised the possibility that TXNIP might play a role in the glucotoxic beta cell death of diabetes. The present study was therefore aimed at determining whether glucose and/or diabetes upregulate TXNIP protein levels in vivo and if so how this affects beta cell apoptosis. To further investigate the causal relationship and address the question whether TXNIP is critical for glucose toxicity-induced beta cell apoptosis, we used TXNIP-deficient primary HcB-19 islets.

RESEARCH DESIGN AND METHODS

Animal studies. All mouse studies were approved by the UW Animal Care and Use Committee and the NIH principles of laboratory animal care were followed. Obese, insulin resistant and diabetic BTBR.ob mice (Jackson Laboratories, Bar Harbor, Maine) homozygous for the leptin<sup>ob</sup> mutation were used as a model of T2DM (24-26). Diabetes was confirmed by blood glucose measurements using a OneTouch Ultra glucometer. Lean and normoglycemic BTBR.lean mice were used as a control. The C3H congenic TXNIP-deficient HcB-19 (HcB) mice harboring a naturally occurring nonsense mutation in the TXNIP gene and the control C3H/DiSnA (C3H) strain have been described previously (27-29). C57BL/6 mice were purchased from Jackson.

Islet isolation. Mouse pancreatic islets were isolated by a collagenase digestion procedure as described previously (25; 26). In brief, immediately after sacrifice pancreata were inflated with 5ml collagenase solution (0.40mg/ml type XI collagenase (Sigma, St. Louis, MO) in Hanks’ balanced salt solution (HBSS) (Invitrogen) with 0.02% RIA grade BSA (Sigma)) and placed in 25ml of the same solution, gassed with 95%O₂/5%CO₂ for 5 minutes and vigorously shaken at 37°C for 14 minutes. After a quick spin the
tissue pellet was washed twice with 10ml cold HBSS, passed through a 925-micron Spectra mesh filter (Fisher, St. Louis, MO) to remove large debris and resuspended in 5ml of 25% Ficoll (Type 400-DL, Sigma) prepared with HBSS in a 50ml conical tube. 2.5ml of 23%, 20.5% and 11% Ficoll were layered carefully on top of each other and the gradient was centrifuged for 15 minutes at 800g. Layers above the 25% Ficoll containing the isolated islets were collected, washed with HBSS and the islets pelleted by a 5-minute centrifugation at 800g. To further exclude contamination by exocrine tissue, islets were handpicked under stereomicroscopic observation and used immediately for protein extraction or incubation at low or high glucose.

Human pancreatic islets were isolated from brain dead organ donors under a protocol approved by the UW Institutional Review Board and were a generous gift of Drs. Matthew Hanson and Luis A. Fernandez.

**Tissue culture:** INS-1 cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1mM sodium pyruvate, 2mM L-glutamine, 10mM HEPES, and 0.05mM 2-mercaptoethanol.

INS-1 cells with constitutive TXNIP overexpression (INS-TXNIP) and control cells overexpressing LacZ (INS-LacZ) were generated and selected as described previously (15).

INS-1 cells and isolated HcB-19, C3H, C57BL/6 or human islets were incubated in RPMI supplemented with 10% FBS and 1% penicillin-streptomycin and containing 5mM or 25mM glucose for 24h before protein extracts were prepared or cells were fixed for immunohistochemistry.

**Western blotting.** Protein extracts were prepared using a lysis buffer containing HEPES (50mM), Nonidet P-40 (10%), sodium fluoride (100mM), sodium pyrophosphate (10mM), EDTA (4mM), PMSF (1mM), leupeptin (2 M), activated sodium orthovanadate (2mM), and okadaic acid (100nM). INS-1 cells were grown in T75 flasks and scraped into 0.2ml of lysis buffer. Isolated primary mouse islets of two animals were pooled and suspended in lysis buffer (20 L per 200 islets). Proteins were separated by 4-20% SDS-PAGE, blotted onto PVDF membranes and detected using the following primary antibodies: TXNIP (1:400) (JY2, MBL International Co., Woburn, MA), monoclonal cleaved caspase-3 (1:200) (Cell Signaling, Boston, MA), beta-actin (1:200) (Abcam, Cambridge, MA) and the secondary antibodies: anti-mouse IgG (1:5000) (Amersham, Piscataway, NJ) and anti-rabbit IgG (Biorad, Hercules, CA). Bands were visualized by Lumigen PS-3 detection reagent (Amersham) and quantified by ImageQuant Version 5.1 (GE Healthcare Lifesciences).

**Immunohistochemistry and TUNEL:** For immunohistochemistry and TUNEL ~100 isolated mouse islets were mixed with 15 l of Affi-Gel Blue Gel (Biorad), fixed in 4% formaldehyde, washed in phosphate buffered saline and the pellet resuspended in 0.5ml of warm 2% Difco-Agar in an Eppendorf tube and centrifuged for 10 seconds at 10,000rpm. After solidification the agar containing the islet pellet was removed from the tube, trimmed, refixed and processed in an automated Shandon Citadel 100 machine before paraffin embedding and preparation of 5 m sections.

For TUNEL the DeadEnd Fluorometric TUNEL System Kit (Promega, Madison, WI) was used according to the
manufacturer’s instructions, but including a permeabilization step (5 min in a 1% Triton X-100 PBS solution). Beta cells were visualized by insulin staining using guinea pig anti-insulin antibody (ZYMED, San Francisco, CA) and Cy3-conjugated anti-guinea pig IgG (1:500, Jackson ImmunoResearch, Westgove, PA). The Vectashield with DAPI mounting solution (VECTOR, Burlingame, CA) was used for visualization of nuclei. Beta cell proliferation was assessed by staining 10 m pancreatic sections for Ki67 (1:200, Abcam, Cambridge, MA).

**Whole pancreas insulin content.** 16-week old male HcB-19 (n=5) and C3H control mice (n=4) were sacrificed after a 4h fast and their whole pancreas insulin content ( g/pancreas) was assessed by acid-ethanol extraction and insulin ELISA as a measure of beta cell mass.

**Insulin secretion.** Insulin secretion and glucose-induced insulin secretion (GSIS) of isolated C3H and HcB-19 islets were assessed after an overnight incubation at 5 mM glucose as described previously (30; 31). All experiments were performed in triplicates using 3 islets per tube.

**Cytochrome C release.** To obtain cytosolic and mitochondrial cell fractions, cells from two T-75 flasks per sample were trypsinized and collected by centrifugation at 1000xg for 2 min. Cells were then washed with PBS and the cell pellet resuspended in 200μl of buffer A (20mM Hpes-KOH, pH 7.5, 10mM KCl, 1.5mM MgCl₂, 1mM EGTA, 1mM dithiothreitol (DTT), 250mM sucrose, 100mM phenylmethylsulfonyl fluoride, 1μg/ml pepstatin A, 2μg/ml leupeptin, and 2μg/ml aprotinin). The homogenate was then centrifuged at 1000xg for 10 min at 4°C and the supernatant containing the cytosolic and mitochondrial fractions was centrifuged again for 15 min at 10,000xg at 4°C. The resulting supernatant contained the cytosolic fraction and the pellet contained the mitochondrial fractions. The cytosolic fractions were boiled in SDS sample buffer and frozen at -80°C until further analysis. Pellets containing mitochondria were treated with buffer B (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 250mM sucrose, 20mM Tris HCl, pH 7.4, 1mM DTT, and protease inhibitors) and were incubated on ice for 20 min. The lysate was centrifuged at 10,000xg for 30 min at 4°C. The pellet was solubilized in buffer C (300mM sucrose, 1mM EGTA, 20mM MOPS (pH 7.4), BSA 0.5mg/ml and protease inhibitors) and the resulting mitochondria enriched fractions were boiled in SDS sample buffer and stored at −80°C until further use.

Cytochrome C protein levels in the mitochondrial and cytosolic fractions were determined by Western blotting using a mouse monoclonal cytochrome C antibody (1:500, Santa Cruz biotechnology, CA, USA) and anti-mouse IgG (1:2000, Amersham, Piscataway, NJ).

**Quantitative real-time RT-PCR.** RNA was extracted using Trizol, converted to cDNA with the First Strand cDNA synthesis kit (Roche) and analyzed on a Prism 7000 Sequence Detection System (Applied Biosystems). BiP was amplified using the forward primer 5’-ACGTCCAACCCGGAGAACA-3’ and the reverse primer 5’-TTCCAAGTGCGTCCGATGA-3’ and ChOP with 5’-TGGCACAGCTTGCTGAAGAG-3’ and 5’-TCAAGGCGCTCGATTTCCT-3’, respectively. Total TXNIP was measured using primers recognizing both human and rat TXNIP, forward: 5’-ACAGAAAAGGATTCTGTGAAGGTGAT-3’, reverse: 5’-GCCATTGGCAAGGTAAGTGTAAT-3’. All
samples were corrected for the 18S ribosomal subunit (Applied Biosystems) run as an internal standard.

**Statistical analysis.** To calculate the significance of a difference between two means, we used two-sided Student’s t-tests. A p-value of <0.05 was considered statistically significant.

**RESULTS**

**TXNIP protein levels and cleaved caspase-3 expression are elevated in response to glucose in INS-1 beta cells.** We previously found that glucose induces TXNIP transcription. To now determine whether the observed glucose effects are also translated into differential expression of TXNIP at the protein level, we performed Western blot experiments using INS-1 beta cells incubated at low (5mM) or high (25mM) glucose for 24h. We observed that incubation at high versus low glucose induced TXNIP protein expression 18-fold (Fig. 1). Interestingly, we found that these elevated TXNIP protein levels were accompanied by a 12-fold increase in cleaved (activated) caspase-3 (Fig. 1C) suggesting increased apoptosis.

These findings are consistent with our previous demonstration of glucose increasing beta cell TXNIP mRNA levels (15; 32) and the observation that TXNIP overexpression promotes beta cell apoptosis (15; 30) as well as with the pro-apoptotic properties of TXNIP described in extrapancreatic tissues (16; 23). They also suggest that TXNIP-mediated beta cell apoptosis does not require constitutive overexpression of TXNIP, but rather that the glucose-induced increase in endogenous TXNIP is sufficient for this effect. This raised the possibility that TXNIP might be involved in the pathophysiology of T2DM and the associated beta cell loss.

**Diabetic mice demonstrate increased levels of TXNIP and cleaved caspase-3 in their pancreatic islets.** To test whether diabetes alters in vivo TXNIP expression in pancreatic islets, we analyzed islets isolated from obese, insulin resistant and diabetic BTBR.ob mice harboring the leptinob mutation, as a model of T2DM (24-26). These analyses revealed that TXNIP protein levels were elevated 8-fold in islets of obese diabetic BTBR.ob mice as compared to lean normoglycemic controls (BTBR.lean) and this increase in TXNIP was again accompanied by an equal rise in cleaved caspase-3 levels (Fig.2). These data are in agreement with the elevated TXNIP mRNA expression observed in islets of non-obese, insulin resistant C57BL/6.azip mice as another model of diabetes (15) and further support an in vivo role of TXNIP in diabetes and beta cell death.

However, we also wanted to assess the causal relationship between glucose-induced TXNIP expression and beta cell apoptosis and determine whether TXNIP is critical for this effect. To this end we studied TXNIP-deficient HcB-19 mice. HcB-19 mice are an inbred congenic C3H mouse strain (33; 34) that harbors a spontaneous inactivating nonsense mutation in exon 2 at codon 97, resulting in dramatically reduced TXNIP mRNA and protein levels (29).

**Glucose induces TXNIP expression and apoptosis in primary islets.** To determine the role of TXNIP in glucose toxicity-mediated beta cell apoptosis, we first analyzed wild-type islets with normal TXNIP expression isolated from control C3H mice. In analogy to the INS-1 cell experiments, we incubated isolated islets at low or high glucose for 24h and then assessed them for the expression of TXNIP, cleaved caspase-3 and by terminal uridine deoxynucleotide nick end
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labeling (TUNEL). Again, we observed that incubation at high glucose resulted in elevated TXNIP protein levels, and increased apoptosis as measured by the 7-fold increase in cleaved-caspase 3 (Fig. 3) and the 9-fold higher percentage of TUNEL positive beta cells (Fig. 4) compared to C3H islets incubated at low glucose. We also obtained very similar findings in islets isolated from C57BL/6 mice as well as in human islets (Fig. 5). In addition, incubation of human islets at 25mM glucose for 24h also led to a ~2-fold increase in TXNIP protein levels and a >10-fold increase in cleaved caspase-3.

**TXNIP deficiency protects against glucose toxicity-induced beta cell apoptosis.** In contrast, parallel experiments using isolated islets of TXNIP-deficient HcB-19 mice revealed no increase in TXNIP expression (as expected), but interestingly also failed to demonstrate any increase in cleaved caspase-3 (Fig. 3) or TUNEL positive beta cells in response to glucose (Fig. 4). These findings strongly suggest that TXNIP expression is critical for the increase in beta cell apoptosis observed in response to high glucose exposure and provide direct evidence for the protective role of TXNIP deficiency against beta cell glucose toxicity.

To address the issue of whether other factors in the mutant HcB-19 islets may have contributed to the observed protection, we repeated the experiments using isolated islets of mice with beta cell-specific deletion of TXNIP created by the Cre-loxP system and of control lox/lox littermates. While incubation at high glucose led again to a significant increase in TUNEL positive beta cells in the control islets (p=0.001) similar to C3H, C57BL/6 and human islets, this effect was completely blunted in islets from beta cell-specific TXNIP knockout mice. These findings confirm the results obtained with the HcB-19 islets and demonstrate that it is specifically the lack of TXNIP that protects beta cells against glucose toxicity-induced apoptosis.

To further investigate the contribution of TXNIP to beta cell loss in vivo, we compared the beta cell mass in TXNIP-deficient HcB-19 and control C3H mice. Interestingly, we found that functional beta cell mass was more than 2-fold higher in the TXNIP-deficient mice as indicated by a whole pancreas insulin content of 71.2 ±1.8 g/pancreas in HcB-19 mice compared to 35.0 ±3.9 g/pancreas in control C3H mice (p=0.001). At the same time, TXNIP-deficiency did not seem to affect beta cell proliferation, as no difference in the number of Ki67 positive beta cells was observed between HcB-19 and C3H pancreata in these initial experiments (data not shown). Together with our findings of TXNIP-induced beta cell apoptosis, this suggests that the net increase in beta cell mass observed in response to TXNIP deficiency, was primarily due to a decrease in beta cell apoptosis.

**TXNIP deficiency does not alter islet insulin secretion.** To assess whether TXNIP deficiency also affects beta cell function, we performed insulin secretion studies using isolated islets from HcB-19 and C3H mice. The results of these experiments demonstrated that basal and glucose-stimulated insulin secretion remained unchanged in TXNIP-deficient islets suggesting that TXNIP does not interfere with normal islet function (Table 1).

**TXNIP induces the intrinsic mitochondrial pathway of apoptosis.** We demonstrated that TXNIP deficiency protects beta cells against glucose-induced cell death and that induction of
TXNIP promotes beta cell apoptosis, but the mechanisms involved have remained unknown. Major mechanisms leading to apoptosis consist of the extrinsic pathway activated by extracellular signals and mediated by death receptors and the intrinsic pathways mediated by mitochondrial damage and/or endoplasmic reticulum (ER) stress. As the extrinsic pathway is unlikely to be involved in TXNIP-mediated apoptosis, we focused on the mitochondrial and the ER stress pathways, both of which have been implicated in pancreatic beta cell death (35).

The ER stress-mediated pathway involves depletion of ER calcium stores, accumulation of unfolded proteins and a stress response including upregulation of ER chaperones such as BiP. Severe or persistent ER stress further activates the apoptosis cascade, which includes upregulation of transcription factors such as CHOP and activation of caspase-12.

In the case of mitochondria-mediated apoptosis, the apoptotic stimulus leads to disequilibrium of pro-apoptotic Bax and anti-apoptotic Bcl-2 and disruption of the mitochondrial membrane potential resulting in release of cytochrome C into the cytosol. This represents a hallmark of this pathway and activates caspase-9.

Both pathways converge at the level of caspase-3 activation, which we consistently observed in our TXNIP studies. To determine which of these pathways is mediating the TXNIP effects, we now used stably transfected INS-1 beta cells overexpressing TXNIP (INS-TXNIP) or LacZ as a control (INS-LacZ) and measured markers of mitochondrial or ER stress-mediated apoptosis.

In control cells, cytochrome C was practically exclusively localized to the mitochondria as expected, whereas INS-TXNIP cells showed a dramatic release of cytochrome C into the cytosolic fractions (Fig. 6 A and B). In contrast, measurements of the ER stress markers BiP and CHOP failed to demonstrate any increase in the INS-TXNIP cells (Fig. 6 C and D) despite proven TXNIP overexpression in the same samples (Fig. 6 E). Together, these results suggest that TXNIP activates primarily the mitochondrial pathway of apoptosis. This notion is further supported by our previous observation of increased Bax/Bcl-2 ratio and activated caspase-9 in response to TXNIP (15).

DISCUSSION
The results of the present study reveal for the first time that a protein (TXNIP) is essential for glucose toxicity-induced beta cell apoptosis. While the effects of glucose toxicity on beta cell dysfunction and death have been described extensively (1-14) the molecular mechanisms involved have remained largely elusive. In regard to beta cell dysfunction, small heterodimer partner (SHP) has recently been identified as a factor inhibiting insulin gene transcription in response to glucose toxicity in INS-1 cells (36), but was not reported to have any effects on beta cell death.

In the present study we have identified TXNIP as a critical factor mediating glucose toxicity-induced beta cell death and have elucidated the pathways by which TXNIP induces beta cell apoptosis. We found that TXNIP overexpression leads to activation of the intrinsic mitochondrial pathway of apoptosis, while ER stress-mediated processes of cell death remained unaffected by TXNIP (Fig. 6). These findings not only shed new light on the role of TXNIP in beta cell biology and the mechanisms involved, but also identify TXNIP as a potential target to interfere with the deleterious effects of
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glucose toxicity. Such modification could help prevent beta cell apoptosis and the gradual beta cell loss that characterizes T2DM and ultimately makes T2DM patients require daily insulin injections. In fact, we found that TXNIP reduction mediates the anti-apoptotic effects of exenatide, an anti-diabetic drug recently approved for T2DM (37). The idea of TXNIP deficiency potentially enhancing beta cell mass is further supported by the phenotype of the TXNIP-deficient HcB-19 mice that, aside from elevated plasma triglycerides and ketone bodies, includes hyperinsulinemia and hypoglycemia (27; 28). Recent work suggests that lack of TXNIP may cause impaired hepatic glucose production, which could lead to hypoglycemia (38). However, since we have found that TXNIP induces beta cell apoptosis, it is also possible that lack of TXNIP may slow the natural process of beta cell death and turnover, lead to accumulation of beta cells and result in increased basal insulin production, which would contribute to the observed hypoglycemia. Indeed, our initial results now demonstrate significantly elevated whole pancreas insulin content in TXNIP-deficient HcB-19 mice compared to C3H controls, indicating increased pancreatic beta cell mass. These findings strongly support the importance of TXNIP not only as a factor conferring glucose toxicity-induced beta cell apoptosis, but also as a novel protein controlling overall pancreatic beta cell loss in vivo.

In the context of diabetes treatment, lowering TXNIP expression may also have beneficial effects in tissues other than pancreatic beta cells, where it again may protect against glucose toxicity. TXNIP expression was found to be increased in human aortic smooth muscle cells in response to incubation at high glucose and diabetic rats had elevated vascular TXNIP levels (39). Moreover, overexpression of TXNIP induced apoptosis in cardiomyocytes (23) and proliferation of smooth muscle cells (21), suggesting that controlling TXNIP expression might help reduce cardiovascular risk and morbidity in patients with diabetes. In addition, a recent human study demonstrated that TXNIP regulates glucose uptake in adipocytes and skeletal muscle and suggested that TXNIP might play a role in the pathogenesis of T2DM by altering glucose homeostasis (40). Decreased TXNIP expression might therefore also enhance glucose uptake and clearance in peripheral tissues and thereby further improve glucose control.

In *in vitro* experiments transfection and constitutive TXNIP overexpression has been shown to reduce thioredoxin activity (23) raising the possibility that the opposite might also be true and that the effects of TXNIP deficiency might be mediated by activation of thioredoxin. However, more recent studies using TXNIP-deficient HcB-19 mice revealed no difference in thioredoxin protein levels and no increase in thioredoxin activity compared to control mice (28). This suggests that other, thioredoxin-independent pathways might be involved in the protective effects of TXNIP deficiency observed in our experiments using HcB-19 islets.

Taken together, the results of the present study have revealed TXNIP as a critical mediator of glucose toxicity-induced beta cell apoptosis. Moreover, they have demonstrated that beta cell TXNIP protein levels are dramatically induced in response to glucose and that TXNIP expression is elevated in islets of different mouse models of diabetes. This suggests that TXNIP may play an important role in diabetes progression...
and the associated decline in beta cell mass. Controlling TXNIP expression may therefore represent a novel approach to protect pancreatic beta cells from progressive destruction and to preserve a sufficient amount of insulin-producing beta cell mass in patients with T2DM.

ACKNOWLEDGMENTS

We would like to thank Anna Szabo for technical assistance and Dr. Susan Bonner-Weir for helpful suggestions in regard to the islet embedding technique. This work was supported by ADA grants 7-03-JF-37 and 7-07-CD-22 and JDRF grant 1-2007-790 to Anath Shalev.
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REFERENCES

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TABLE 1. Insulin secretion from C3H and HcB-19 islets

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<td></td>
<td>C3H</td>
<td>HcB-19</td>
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<td>Low glucose (1.7 mM)*</td>
<td>1.99 (0.18)</td>
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<td>High glucose (16.7 mM)*</td>
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<td>GSIS (fold-change)</td>
<td>2.96 (0.34)</td>
<td>2.30 (0.30)</td>
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Numbers represent means of 5 independent experiments performed in triplicates. *(ng/ml/3islets). GSIS: glucose-stimulated insulin secretion
FIGURE LEGENDS

Figure 1. Glucose effects on TXNIP protein levels and apoptosis in INS-1 beta cells. INS-1 cells were incubated at low (5mM) or high (25mM) glucose for 24h and analyzed by immunoblotting for the expression of TXNIP and cleaved caspase-3. Bars represent mean fold change in protein levels corrected for -actin ±SEM (n=3 independent experiments).

Figure 2. Expression of TXNIP and cleaved caspase-3 in islets of diabetic mice. Body weight (A) and blood glucose (B) in diabetic BTBR.ob and normoglycemic BTBR.lean mice are shown for comparison. Islets of 8-week old BTBR.ob and control BTBR.lean mice were isolated and analyzed by immunoblotting for TXNIP protein levels (C) and cleaved caspase-3 (D). Three independent experiments were performed and bars represent mean fold change ±SEM in protein levels corrected for -actin.

Figure 3. Effects of high glucose exposure on caspase-3 activation in TXNIP-deficient HcB-19 and C3H control islets. Isolated primary islets of control C3H and TXNIP-deficient HcB-19 mice were incubated at low (5mM) or high (25mM) glucose for 24h and assessed for TXNIP expression and apoptosis. (A) Representative immunoblot. (B) Quantification of TXNIP protein levels in C3H and HcB-19 islets. (As expected no TXNIP protein was detected in HcB-19 islets). (C) Quantification of cleaved caspase-3 in C3H and HcB-19 islets. Bars represent mean fold change ±SEM in protein levels corrected for -actin (n=3 independent experiments). *P-value: high vs low glucose.

Figure 4. Protection against glucose toxicity-induced beta cell apoptosis in TXNIP-deficient HcB-19 islets. Isolated primary islets of control C3H (A, B) and TXNIP-deficient HcB-19 mice (C, D) were again incubated at low (5mM) or high (25mM) glucose for 24h and then analyzed by TUNEL. Representative pictures (40X) are shown, white arrows point at bright appearing TUNEL positive nuclei. (E) For quantification >500 nuclei and at least 10 different islets were analyzed per group and condition and the percentage of TUNEL positive beta cells per islet was calculated. Bars represent means ±SEM. *P-value: high vs low glucose.

Figure 5. Glucose toxicity in C57BL/6 and human islets. Islets of wild-type C57BL/6 mice (A) or isolated human islets (B) were incubated at low (5mM) or high (25mM) glucose for 24h and then analyzed by TUNEL. At least 7 different islets were analyzed per group and condition and >1000 mouse or human beta cell nuclei were evaluated; bars represent means ±SEM.

Figure 6. TXNIP effects on mitochondrial damage and ER stress. (A) Immunoblot of cytochrome C in mitochondrial (mito) and cytosolic (cyto) cell fractions obtained from INS-1 beta cells overexpressing TXNIP (INS-TXNIP) and control cells (INS-LacZ). 25 g of protein were loaded per lane and -actin is shown as a loading control. One representative of 7 independent experiments is shown. (B) Quantification of cytochrome
C in the different cell fractions. Bars represent mean fold change ± SEM of 7 independent experiments corrected for -actin.
Expression of BiP (C), CHOP (D) and TXNIP (E) as measured by quantitative real-time RT-PCR in INS-TXNIP and INS-LacZ cells. Bars represent means ± SEM of 3 independent experiments corrected for 18S.
FIGURE 1
FIGURE 2

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A

**p<0.001**

Weight (g)

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B

**p<0.001**

Blood glucose (mg/dl)

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C

**p<0.01**

TXNIP protein (fold change)

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D

**p<0.05**

Cleaved caspase-3 (fold change)

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FIGURE 3

TXNIP links glucotoxicity and beta cell death

A

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<th>Glucose (mM)</th>
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B

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<tr>
<td>C3H</td>
</tr>
<tr>
<td>HcB</td>
</tr>
</tbody>
</table>

- 5 mM gluc
- 25 mM gluc

* p<0.005

C

<table>
<thead>
<tr>
<th>Cleaved caspase-3 (fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H</td>
</tr>
<tr>
<td>HcB</td>
</tr>
</tbody>
</table>

- 5 mM gluc
- 25 mM gluc

* p<0.05
FIGURE 4

TXNIP links glucotoxicity and beta cell death

5 mM gluc

25 mM gluc

C3H

HcB

TUNEL - insulin - DAPI

E

TUNEL positive β-cells (%)

* 4

* 3

* 2

* 1

* 0

5 mM gluc

25 mM gluc

* p<0.001

C3H

HcB
FIGURE 5

TXNIP links glucotoxicity and beta cell death

A

C57BL/6 islets

TUNEL positive β-cells (%)

* p < 0.001

5 mM 25 mM

B

Human islets

TUNEL positive β-cells (%)

* p = 0.004

5 mM 25 mM
FIGURE 6

TXNIP links glucotoxicity and beta cell death