Lack of TXNIP protects against mitochondria-mediated apoptosis, but not against fatty acid-induced, ER-stress-mediated beta cell death

Running title: Lipotoxicity and TXNIP

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Objective: We have previously shown that lack of thioredoxin-interacting protein (TXNIP) protects against diabetes and glucotoxicity-induced beta-cell apoptosis. Since the role of TXNIP in lipotoxicity is unknown, the goal of the present study was to determine whether TXNIP expression is regulated by fatty acids and whether TXNIP-deficiency also protects beta-cells against lipoapoptosis.

Research design and methods: To determine the effects of fatty acids on beta-cell TXNIP expression, INS-1 beta-cell and isolated islets were incubated with/without palmitate and rats underwent cyclic infusions of glucose and/or intralipid prior to islet isolation and analysis by quantitative real-time RT-PCR and immunoblotting. Using primary wild-type and TXNIP-deficient islets, we then assessed the effects of palmitate on apoptosis (TUNEL), mitochondrial death pathway (cytochrome C release) and endoplasmic reticulum (ER) stress (BiP, CHOP). Effects of TXNIP-deficiency were also tested in the context of staurosporine (mitochondrial damage) or thapsigargin (ER-stress).

Results: Glucose elicited a dramatic increase in islet TXNIP expression both in vitro and in vivo, whereas fatty acids had no such effect and, when combined with glucose, even abolished the glucose effect. We also found that TXNIP-deficiency does not effectively protect against palmitate or thapsigargin-induced beta-cell apoptosis, but specifically prevents staurosporine or glucose-induced toxicity.

Conclusions: Our results demonstrate that unlike glucose, fatty acids do not induce beta-cell expression of pro-apoptotic TXNIP. They further reveal that TXNIP deficiency specifically inhibits the mitochondrial death pathway underlying beta-cell glucotoxicity, whereas it has very little protective effects against ER-stress-mediated lipoapoptosis.
Pancreatic beta cell loss by apoptosis is a major factor in the pathogenesis of type 1 and type 2 diabetes (1-5). Two highly interconnected intrinsic signaling pathways, i.e. the mitochondrial death pathway and endoplasmic reticulum (ER) stress can lead to beta cell apoptosis (6). In addition, while multiple processes can activate either one or both pathways and thereby contribute to the phenomenon of beta cell loss, glucotoxicity and lipotoxicity are key stimuli especially in type 2 diabetes (7; 8). However, the detailed molecular mechanisms involved have just begun to be unraveled.

Recently, we discovered that thioredoxin-interacting protein (TXNIP) acts as a critical link between glucotoxicity and pancreatic beta cell apoptosis (9) and that TXNIP deficiency protects against streptozotocin (STZ) as well as against obesity-induced diabetes (10). TXNIP (also called vitamin D3-upregulated gene 1 (VDUP1), or thioredoxin-binding protein 2 (TBP-2)) is a ubiquitously expressed 50kD protein (11; 12). As suggested by its name, TXNIP binds and inhibits thioredoxin, a thiol-oxidoreductase and major cellular reducing system member, and thereby promotes oxidative stress and regulates the cellular redox state (13-17).

Originally, we identified TXNIP as the most dramatically upregulated gene in response to glucose in a human islet oligonucleotide microarray study (18), found that its expression was increased in islets of diabetic mice (9) and further demonstrated that it induces pancreatic beta cell death (19). More recently, we found that TXNIP is essential for glucotoxic beta cell death (9) and discovered that the functional beta cell mass was significantly increased in TXNIP-deficient HcB-19 mice harboring a nonsense mutation in their TXNIP gene as well as in beta cell-specific TXNIP knock out mice (bTKO) (10). Of note, this was the case despite the fact that HcB-19 mice are hyperlipidemic (10; 20; 21). Moreover, generation of a double-mutant congenic BTBR\textit{lep}\textsubscript{ob/ob}\textit{txnip}\textsubscript{hcb/hcb} mouse lacking leptin as well as TXNIP revealed that TXNIP deficiency was able to reduce beta cell apoptosis >50-fold, to increase pancreatic beta cell mass and to prevent diabetes in this very severe model of type 2 diabetes associated with marked obesity, insulin resistance and hyperlipidemia (10). Together these findings raised the possibility that TXNIP deficiency may not only play a role in glucotoxicity, but also be protective against lipotoxicity. The aim of the present study was therefore to assess whether fatty acids regulate beta cell TXNIP expression \textit{in vitro} and \textit{in vivo} and to ascertain whether lack of TXNIP protects beta cells against lipoapoptosis.

**RESEARCH DESIGN AND METHODS**

**Animal studies.** All animal studies were approved by the respective Institutional Animal Care and Use Committees and the NIH principles of laboratory animal care were followed. 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 (12; 21; 22). Beta cell-specific TXNIP knock out mice (bTKO) and lox/lox control littermates were generated by the Cre-LoxP system and are described in detail elsewhere (10).

Male Wistar rats (Charles River, St.-Constant, QC, Canada) were housed under standard conditions. \textit{In vivo
infusion studies were performed as described previously (23). In brief, indwelling catheters were placed in the left carotid artery and the right jugular vein under general anesthesia and animals were allowed to recover for 5 days. They were then randomized into 4 groups receiving either 0.9% saline, 50% glucose, 20% Intralipid (with heparin 20 units/ml) or glucose plus Intralipid through Harvard infusion pumps. These were administered in alternating 4h cycles of glucose or saline followed by intralipid or saline for 4h and the infusion profile was repeated for a total of 72h until sacrifice. All animals received the same volume of fluid and had free access to food and water during the infusions.

**Islet isolation.** Mouse pancreatic islets were isolated by collagenase digestion as described previously (24; 25). 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 incubated at low or high glucose with or without palmitate.

Rat pancreatic islets were isolated by collagenase digestion and gradient centrifugation as described previously (23).

**Tissue culture:** Mouse islets (C3H, HcB-19, lox/lox and bTKO), were incubated in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 1% BSA and 1% penicillin-streptomycin and 2.8mM or 16.7 mM glucose for 24h at 37°C in the presence or absence of 1 mM palmitate. Rat islets were cultured for 24h in the presence of 2.8 or 16.7 mM glucose with or without 0.5 mM palmitate as described previously (26).

INS-1 cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 11.1 mM glucose and supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1mM sodium pyruvate, 2mM L-glutamine, 10mM HEPES, and 0.05mM 2-mercaptoethanol.

Culture media containing palmitate was prepared as described previously (27) with minor modifications. The stock solution was prepared by dissolving sodium palmitate (Sigma) in ethanol:water (1:1, vol:voll) at 65°C for 15 minutes at a final concentration of 150 mmol/l. Aliquots of stock solution were complexed with fatty-acid–free BSA (10% in water, Sigma) by incubation for 1h at 37°C and then diluted in culture media. The final molar ratio of fatty acid:BSA was 5:1 for all experiments. The final ethanol concentration was ≤0.33% (vol:voll). The control condition included a solution of vehicle (ethanol:water) mixed with fatty-acid–free BSA at the same concentration as the palmitate solution. Staurosporine
and thapsigargin were from Invitrogen and were dissolved in DMSO.

**TUNEL:** For TUNEL ~100 isolated mouse islets were mixed with 15µl 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 5µm sections.

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, Westgrove, PA). The Vectashield with DAPI mounting solution (VECTOR, Burlingame, CA) was used for visualization of nuclei.

**Quantitative real-time RT-PCR:** RNA was extracted using the RNeasy Mini kit (Qiagen), converted to cDNA with the SuperScript III First-Strand Synthesis Super Mix (Invitrogen) and analyzed on a Prism 7000 Sequence Detection System (Applied Biosystems). TXNIP was measured using primers recognizing rat TXNIP, forward: 5’-CGAGTCAAAAGGCTCGGAT-3’, reverse: 5’-TTCATAGCGCAAGTAGTCCAAGGT-3’. BiP was amplified using the forward primer 5’-ACGTCCAACCCGGAGAAACA-3’ and the reverse primer 5’-TTCAGGTGCGTCCGATGA-3’ and ChOP with 5’-TGGCACAAGCTTGCTGAAGAG-3’ and 5’-TCAGGCCTCGATTCCC-3’, respectively. All samples were corrected for the 18S ribosomal subunit (Applied Biosystems) run as an internal standard.

**Immunoblotting:** Protein extraction and immunoblotting were performed as described previously (9) using the following antibodies: TXNIP (JY2, MBL International Co., Woburn, MA) (1:400), monoclonal cleaved caspase-3 (Cell Signaling, Boston, MA) (1:200), beta-actin (Abcam, Cambridge, MA) (1:200), anti-mouse IgG (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-rabbit IgG (Biorad, Hercules, CA).

**Cytochrome C release:** INS-1 cells were incubated at 5 mM or 25 mM glucose with or without 1mM palmitate for 24h prior to cell fractionation.

Cytosolic and mitochondrial cell fractions were obtained and analyzed for cytochrome C by immunoblotting as described previously (9) using a rabbit cytochrome C antibody (Cell Signaling) and anti-rabbit IgG (Santa Cruz).

**Statistical analysis:** To calculate the significance of a difference between two means, we used two-sided Student’s t-tests and a p-value of <0.05 was considered statistically significant. To compare data sets of more than two groups, one way ANOVA was used followed by Holm-Sidak tests for all pairwise multiple comparisons.

**RESULTS**

**Fatty acids do not induce TXNIP expression in vitro or in vivo.** We previously showed that glucose acts as a potent stimulus of beta cell TXNIP expression (9; 18; 19). However, the effects of fatty acids on TXNIP expression have not been studied. To first examine whether fatty acids modulate TXNIP expression in vitro, isolated rat or mouse islets were incubated in the presence or
absence of palmitate at low or high glucose concentrations and TXNIP expression was assessed by quantitative real-time RT-PCR and immunoblotting (Fig. 1A-C). Fatty acids did not alter TXNIP expression in these experiments and, interestingly, the concomitant presence of palmitate completely abolished the stimulatory effect of glucose on TXNIP expression. This was also confirmed in vivo in islets isolated from rats after cyclic 20% intralipid infusions with and without additional glucose infusions as described in Research Design and Methods. While cyclic glucose infusions led to a significant increase in TXNIP expression as compared to saline, Intralipid infusions alone had no effect and, when combined with glucose, suppressed the glucose-stimulated TXNIP induction (Fig. 1D). This suggests that unlike glucose, fatty acids do not increase TXNIP expression in islets and, further, block the effect of glucose on TXNIP expression.

**TXNIP deficiency does not protect against beta cell lipoapoptosis.** TXNIP deficiency effectively protects pancreatic beta cells against glucotoxic cell death (9). To test whether it may also protect against lipotoxic beta cell apoptosis, we incubated isolated islets of TXNIP-deficient HcB-19 and control C3H mice in the presence or absence of 1 mM palmitate. Palmitate led to a significant 5-fold increase in TUNEL-positive beta cells in islets from both C3H and TXNIP-deficient HcB-19 islets (Fig. 2). We also performed a palmitate dose-response curve and assessed beta cell apoptosis by cleaved caspase-3 measurements as well as by TUNEL. With both methods a very similar, dose-dependent increase in apoptosis was seen (supplemental material Fig. S1 available in the online appendix at http://diabetes.diabetesjournals.org), confirming that our TUNEL experiments were providing adequate quantification of beta cell apoptosis. In addition, we analyzed the effects of palmitate at 24h (Fig. 2A-D) as well as at 48h (Fig. 2E-G). In both C3H and HcB-19 islets, we observed that the percent of apoptotic beta cells almost doubled at 48h demonstrating that we were still in the dynamic range with our 24h our findings and suggesting that lack of TXNIP is not able to protect against lipoapoptosis.

To further substantiate this observation, we performed analogous experiments using isolated islets from beta cell-specific TXNIP knock out mice (bTKO) and control lox/lox mice. As observed using Hcb-19 islets, beta cell specific deletion of TXNIP in bTKO islets was not able to prevent the >5-fold increase in beta cell apoptosis induced by palmitate (Fig. 3A), while it completely prevented glucotoxicity-induced beta cell apoptosis (Fig. 3B) consistent with our earlier findings (9). These results suggest that the protective effect of TXNIP deficiency might be pathway-specific and that glucose and fatty acids might affect different features of beta cell apoptosis. The two major intrinsic pathways implicated in pancreatic beta cell death are mitochondrial damage and endoplasmic reticulum (ER) stress (6). Since we previously showed that TXNIP is involved in mitochondria-mediated apoptosis (9), we first compared the effects of glucotoxicity and lipotoxicity on this pathway. Indeed we found that while incubation at high glucose induced the mitochondrial death pathway as shown by the pronounced release of cytochrome C from the mitochondria into the cytosol, palmitate had no such effect and when added to high glucose even normalized the cytochrome C distribution (Fig. 3C),
suggesting that mitochondria-mediated apoptosis does not play a major role in lipoapoptosis. In contrast, we found that palmitate increased the expression of the ER stress markers BiP and ChOP in INS-1 cells and rat islets (supplemental material Fig. S2 in the online appendix). While these findings are consistent with previous studies and suggest that beta cell lipoapoptosis is primarily associated with ER-stress (28-36) there is also a significant body of work implicating mitochondria in lipotoxicity (37-41), a discrepancy that is most likely due to the intricate crosstalk between mitochondria and the ER (42; 43).

Lack of TXNIP protects from staurosporine-induced but not thapsigargin-induced beta cell apoptosis. Combined with the finding that TXNIP deficiency only protected against glucotoxic but not lipotoxic cell death, our results suggest that lack of TXNIP may specifically inhibit mitochondria-, but not ER-stress-mediated beta cell apoptosis. To directly test this hypothesis, we treated isolated islet of bTKO and lox/lox mice with staurosporine, a well known stimulus of the mitochondrial death pathway. While staurosporine led to a significant >10-fold increase in beta cell apoptosis in lox/lox islets as compared to islets incubated with DMSO vehicle only, TXNIP-deficient bTKO islets were completely protected against staurosporine-induced apoptosis (Fig. 4A-B). (Of note, staurosporine did not increase TXNIP expression (1.0 vs 1.0-fold ±0.02) demonstrating that the protection conferred by TXNIP deficiency is not limited to stimuli that increase TXNIP expression, but rather to those that induce mitochondrial apoptosis.)

In marked contrast, incubation with the ER-stress inducer thapsigargin led to a ∼10-fold increase in beta cell apoptosis in both lox/lox and bTKO islets and while the percentage of apoptotic beta cells was slightly lower in the bTKO islets, TXNIP deficiency had no significant protective effect (p=0.119) (Fig. 4C-D), indicating that the protective effects of TXNIP deficiency are largely restricted to mitochondria-mediated apoptosis. In order to compare the effects of staurosporine and thapsigargin, we had to achieve similar levels of beta cell apoptosis and therefore used this higher dose of 25 μM of thapsigargin. However, dose-response experiments at significantly lower thapsigargin concentrations confirmed that lack of TXNIP was not effective in protecting against thapsigargin-induced beta cell death even at apoptosis rates of as low as 2% (Fig. 5).

DISCUSSION

The results of this study uniquely identify TXNIP as a specific mediator of the mitochondrial death pathway in beta cells under glucotoxic conditions, while revealing that TXNIP does not play a significant role in ER-stress mediated lipoapoptosis.

Recently, we discovered that TXNIP represents a critical link between glucotoxicity and beta cell apoptosis (9) and that lack of TXNIP protects against STZ- and obesity-induced diabetes, raising the possibility that TXNIP deficiency might also be protective against lipotoxicity. However, using islets of TXNIP-deficient HcB-19 and bTKO mice, the results of the present study indicate that lack of TXNIP is unable to prevent or inhibit beta cell apoptosis induced by fatty acids, while it effectively protects against glucose-induced beta cell death (Fig. 2 and 3).

Even though both, glucotoxicity and lipotoxicity play important roles in the
Lipotoxicity and TXNIP

Pathogenesis of diabetes and diabetic beta cell loss (8) and culminate in beta cell apoptosis, different signaling pathways are involved and the relative contribution from mitochondrial and ER stress remains under discussion (28-33; 37-41; 44). This controversy is most likely due to the intricate crosstalk between these two organelles and the apoptosis pathways that ultimately lead to beta cell death (42; 43) (Fig. 6). In fact, mitochondria represent the major source of ATP and reactive oxygen species (ROS), which in turn can stimulate ER stress and activate apoptosis signal-regulating kinase 1 (ASK1) (45). On the other hand, the ER supplies the mitochondria with calcium, a process that is now believed to require physical interaction between the organelles mediated by mitofusin 2 (46). In addition, ChOP has been reported to promote ROS formation, whereas ChOP deletion reduced oxidative stress and enhanced beta cell survival (47). Moreover, ER stress also activates ASK1 through formation of an IRE1 (serine-threonine protein kinase) – TRAF2 (tumor necrosis factor receptor-associated factor 2) – ASK1 complex and ASK1 leads to mitochondria-dependent caspase activation and apoptosis (48). Interestingly, thioredoxin (Trx) directly binds to and inactivates ASK1 and TXNIP deficiency increases the availability of Trx for this interaction (49), suggesting that it may thereby decrease ASK1 activity and apoptosis. Finally, Trx in conjunction with glutaredoxin and NADPH has also been shown to control exocytosis, insulin secretion and beta cell signaling (50).

Given this extensive signaling network, a clear separation between the pathways involved is in general impossible. However, our results demonstrate that the protective effects of TXNIP deficiency are predominantly limited to the prevention of mitochondria-mediated beta cell apoptosis, while ER-stress induced apoptosis remained largely unaffected (Fig. 4-5). Consistent with this observation TXNIP deficiency was ineffective in preventing beta cell lipotoxicity, while it had significant beneficial effects in the context of beta cell glucotoxicity, which is in alignment with our previous findings (9). Nevertheless, and despite the fact that at lower thapsigargin concentrations TXNIP deficiency had absolutely no protective effects (Fig. 5), there was a trend to slightly lower levels of apoptosis in bTKO islets at the higher thapsigargin dose (Fig. 4D) suggesting that lack of TXNIP might be able to partially reduce cell death associated with severe ER stress potentially by inhibiting mitochondrial pathways activated by ER stress and/or those involved in the ER-mitochondrial crosstalk (Fig. 6).

While we have shown that incubation of beta cells and primary islets at high glucose induces TXNIP expression and that diabetic mice have elevated islet TXNIP levels (9; 18; 19), this is the first demonstration that repeated infusions of glucose can lead to a significant increase in beta cell TXNIP expression (Fig. 1D). These in vivo infusion experiments were not designed to induce or assess beta cell apoptosis (26) and previous work has suggested that glucose infusion does not cause beta cell apoptosis (51). However, it is tempting to speculate that postprandial glucose excursions in diabetic or pre-diabetic patients may result in a similar increase in beta cell TXNIP expression and given the strong pro-apoptotic properties of TXNIP this may, over time, contribute to the gradual beta cell loss observed (52) as well as to the overall
Lipotoxicity and TXNIP

detrimental effects of postprandial hyperglycemia appreciated clinically (53).

Surprisingly, fatty acids not only failed to induce TXNIP expression, but essentially blocked glucose-induced TXNIP expression both in vitro and in vivo (Fig. 1). This is consistent with a recent observation in INS-1E cells, where, fatty acid-induced insulin secretion also led to inhibition of TXNIP expression (54). In addition, palmitate has been shown to lead to exclusion of ChREBP out of the nucleus and thereby to cause a fatty acid “sparing” effect on glucose-induced transcription (55). Since we have shown that glucose-induced beta cell TXNIP expression is mediated by ChREBP (56), these processes may also contribute to the observed reduction in glucose-induced TXNIP expression in response to palmitate. The inhibition of glucose-induced TXNIP expression and cytochrome C release by the concomitant presence of fatty acids also suggests that when both fuels are elevated under glucolipotoxic conditions, the TXNIP-independent, fatty acid-mediated ER-stress apoptotic pathway may take precedence over TXNIP-mediated mitochondrial cell death.

In summary, we have found that lack of TXNIP primarily protects against beta cell apoptosis mediated by the mitochondrial death pathway under glucotoxic conditions, whereas lipoapoptosis mediated by ER-stress could not be prevented by TXNIP deficiency. Nevertheless, it is likely that TXNIP deficiency also affects components of the ER-mitochondrial crosstalk. Moreover, our results indicate that unlike glucose, fatty acids do not induce TXNIP expression revealing the specificity of both, the upstream control of TXNIP expression as well as the downstream TXNIP effects on apoptotic signaling. Together, these findings provide new insight into the specific regulation and function of TXNIP that further helps in establishing the role of TXNIP as a target for diabetes therapy. In addition, they enhance our understanding of the molecular pathways controlling the life and death of the pancreatic beta cell.

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FIGURE LEGENDS

Figure 1
In vitro and in vivo effects of fatty acids on islet TXNIP expression.
(A) Isolated rat islets were incubated at 2.8 mM glucose (LG) or 2.8 mM glucose and 0.5 mM palmitate (LG+FA), 16.7 mM glucose (HG) or 16.7 mM and 0.5 mM palmitate (HG+FA) for 24h and TXNIP expression was analyzed by real-time RT-PCR, n=4. (B-C) Isolated wild-type mouse islets were incubated at LG or HG with or without palmitate (1 mM) for 24h and analyzed for changes in TXNIP protein levels by immunoblotting. Bars represent mean fold change ±SEM in TXNIP protein corrected for beta actin; three independent experiments were performed, one representative immunoblot is shown. (D) Male Wistar rats received cyclic infusions of saline 0.9%, glucose 50%, intralipid 20% or glucose and intralipid for a total of 72h as described in the methods section prior to sacrifice. Their islets were isolated and analyzed for TXNIP mRNA expression using quantitative real-time RT-PCR. Bars represent mean fold change ±SEM compared to saline. Four independent experiments were performed.

Figure 2
Beta cell apoptosis in TXNIP-deficient HcB-19 and control C3H mice in response to palmitate. Isolated islets were incubated at 11.1 mM glucose in the absence or presence of 1mM palmitate (FA) and the effects on beta cell apoptosis were assessed by TUNEL. (A) Representative islet images, white arrows point at TUNEL-positive apoptotic nuclei, red=insulin, blue=nuclei. Quantification of palmitate-induced beta cell apoptosis after 24h (B-D) and after 48h (E-G), bars represent means ±SEM. Over 12 islets and over 700 beta cell nuclei were analyzed per group.

Figure 3
Different effects of lipotoxicity and glucotoxicity on TXNIP knock out (bTKO) beta cells and mitochondria-mediated apoptosis. Isolated islets of beta cell-specific TXNIP knock out (bTKO) and control (lox/lox) mice were incubated at (A) 11.1 mM glucose +/- palmitate (1 mM) or (B) at 5 versus 25 mM glucose for 24h and analyzed by TUNEL. At least 12 islets and over 700 beta cell nuclei were analyzed per group. Bars represent mean fold change ±SEM in beta cell apoptosis. (C) Cytochrome C release from the mitochondria (M) into the cytosol (C) was measured by subcellular fractionation and immunoblotting in INS-1 cells incubated for 24h at low glucose (LG) (5mM) or high glucose (HG) (25mM) with or without palmitate (FA). Bars represent means ±SEM of three independent experiments and a representative immunoblot is shown. * p<0.01 (M) HG vs LG; ** p<0.01 (C) HG vs LG

Figure 4
Different response of bTKO beta cells to mitochondria (staurosporine) or ER-stress (thapsigargin) mediated apoptosis. Islets isolated from lox/lox and bTKO mice were incubated for 24h in the presence or absence of staurosporine (0.5 µM) (A-B) or thapsigargin (25 µM) (C-D) and beta cell apoptosis was assessed by TUNEL. Over 12 islets and over 700 beta cell nuclei were analyzed per group. Representative images are shown; insulin=red, nuclei=blue, TUNEL-positive nuclei=green (white arrows). Bars represent means ±SEM.
**Figure 5**
Comparison of beta cell apoptosis in bTKO and control lox/lox islets exposed to different doses of thapsigargin.
Islets isolated from lox/lox and bTKO mice were incubated for 24h in the presence or absence of thapsigargin at the designated concentrations and beta cell apoptosis was assessed by TUNEL. Over 12 islets and over 700 beta cell nuclei were analyzed per group. Bars represent means ±SEM.

**Figure 6**
Schematic representation of TXNIP deficiency-mediated protection against beta cell apoptosis. Excess glucose or fatty acids ultimately lead to beta cell apoptosis by mitochondrial (staurosporine-) or ER-stress (thapsigargine-induced) pathways. Despite extensive crosstalk, TXNIP deficiency primarily blocks glucose-induced mitochondria-mediated beta cell apoptosis.

**Figure 1**
Figure 3

A. FA-induced apoptosis (fold change)

B. Glucose-induced apoptosis (fold change)

C. Cytochrome C localization (%)

Cytochrome C
β-actin
Figure 4

(A) Δ control staurosporine

lox/lox

bTKO

(C) control thapsigargin

lox/lox

bTKO

Figure 5

N.S.

N.S.

N.S.

0 uM 5 uM 10 uM

thapsigargin

*p<0.01 thapsigargin vs control

Lipotoxicity and TXNIP
Figure 6

[Diagram showing the relationship between TXNIP deficiency, gluc, staurosporine, fatty acid, thapsigargin, and various cellular processes including mitochondrial stress, cytochrome C release, and ER stress.]