

# Posttranslational regulation of thioredoxin-interacting protein

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## Abstract

Thioredoxin-interacting protein (Txnip) is a metabolic regulator, which modulates insulin sensitivity and likely plays a role in type 2 diabetes. We studied the regulation of Txnip in 3T3-L1 adipocytes. Cells were incubated under different conditions and Txnip was measured by immunoblotting. We confirmed that high glucose markedly increases Txnip expression by promoting transcription. Insulin decreases Txnip protein levels. Rapamycin under most conditions decreased Txnip, suggesting that mTOR complex-1 is involved. The acute effects of insulin are mainly posttranscriptional; insulin (100 nM) accelerates Txnip degradation more than tenfold. This effect is cell type specific. It works in adipocytes, preadipocytes and in L6 myotubes but not in HepG2 or in HEK 293 cells or in a pancreatic  $\beta$ -cell line.

The ubiquitin/proteasome pathway is involved. Degradation of Txnip occurred within 15 min in the presence of 3 nM insulin and overnight with 0.6 nM insulin. Proteasomal Txnip degradation is not mediated by a cysteine protease or an anti-calpain enzyme. Okadaic acid (OKA), an inhibitor of phosphoprotein phosphatases (pp), markedly reduced Txnip protein and stimulated its further decrease by insulin. The latter occurred after incubation with 1 or 1000 nM OKA, suggesting that insulin enhances the phosphorylation of a pp2A substrate. Incubation with 0.1  $\mu$ M Wortmannin, a PI3 kinase inhibitor, increased Txnip protein twofold and significantly inhibited its insulin-induced decrease. Thus, while OKA mimics the effect of insulin, Wortmannin opposes it. In summary, insulin stimulates Txnip degradation by a PI3 kinase-dependent mechanism, which activates the ubiquitin/proteasome pathway and likely serves to mitigate insulin resistance.

## Key Words

- ▶ Txnip
- ▶ Adipocytes
- ▶ Glucose-induced insulin resistance
- ▶ Insulin action

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## Introduction

Thioredoxin-interacting protein (Txnip) is a 50 kDa protein, named for its capacity to bind to thioredoxin and thereby may mediate effects of the cellular redox state (Nishiyama *et al.* 1999, Patwari *et al.* 2006). Originally, it was referred to as vitamin D-3 upregulated protein 1, because it was induced by treatment with 1,2, 5-dihydroxyvitamin D-3 in HLA-60 cells (Chen & DeLuca 1994, Nishiyama *et al.* 1999). In a strain of mice with spontaneous deletion of Txnip, Bodnar identified its activity similar to that observed in familial combined hyperlipidemia in man

(Bodnar *et al.* 2002). Recent studies have indicated a possible role of Txnip in diabetes. High glucose induces Txnip in pancreatic  $\beta$  cells, where it promotes apoptosis (Minn *et al.* 2005). Txnip regulates the oxidation state of PTEN, which is active in the reduced form. PTEN is a lipid phosphatase that regulates phosphatidylinositol (3,4,5)-triphosphate availability, and in turn, Akt activity (Hui *et al.* 2008). Txnip deficiency disrupts the fasting–feeding metabolic transition (Sheth *et al.* 2005). Txnip deficiency increases insulin signaling (Yoshihara *et al.* 2010). High

glucose tends to increase Txnip expression and insulin decreases it (Parikh *et al.* 2007). Based on this knowledge, we examined the regulation of Txnip in 3T3-L1 adipocytes, which develop insulin-resistant glucose transport after chronic (18 h) exposure to high glucose in the presence of low-dose insulin (Nelson *et al.* 2000). We confirmed the observation (Parikh *et al.* 2007) that high glucose induces Txnip expression, primarily via promoting transcription (Minn *et al.* 2005). We further showed that rapamycin under most conditions decreased Txnip levels, suggesting that mTOR complex-1 is involved in its regulation. Interestingly, the rapid effects of insulin in decreasing Txnip reflect a marked acceleration of Txnip protein degradation in these cells. This does not involve cysteine or calpain proteases, but appears to involve phosphorylation of PI3 kinase, leading to activation of the ubiquitin/proteasome pathway.

## Materials and methods

3T3-L1 fibroblasts and adipocytes were cultured and differentiated in high (25 mM) glucose and preincubated in low (5 mM) and high (25 mM) glucose before experiments as previously described (Nelson *et al.* 2000). At the time of the studies more than 95% of the cells demonstrated a phenotype characteristic of adipocytes. L6 myoblasts (the kind gift of Dr Amira Klip, University of Toronto, Toronto, Canada) were cultured and differentiated into myotubes as previously described (Mitsumoto & Klip 1992). INS-1 cells (the kind gift of Dr Christopher Newgard, Duke University, Durham, NC, USA) were cultured as described (Hohmeier *et al.* 2000). HEK 293 cells (QBioGene, MP Biomedicals, Santa Ana, CA, USA) and HepG2 cells (ATCC, Manassas, VA, USA) were cultured according to the supplier's protocols.

Western blots were carried out as previously described (Dignam *et al.* 1983, Nelson *et al.* 2002a,b). In ubiquitin immunoblotting experiments membranes were autoclaved in water for 30 min prior to blocking. Mouse MAB specific for Txnip was obtained from MBL (Woburn, MA, USA) and used at a 1:1000 dilution. Ubiquitin,  $\beta$ -tubulin, lamin A/C, phospho-Akt (Ser473) and Akt antibodies were purchased from Cell Signaling (EMD Millipore, Billerica, MA, USA) and used at a 1:1000 dilution. HRP-labeled secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA, USA) and used at a 1:10 000 dilution. ECL detection was performed and quantified using Pierce (Rockford, IL, USA) West Pico and West Dura ECL reagents and the Kodak Image Station 4000MM Pro with Carestream Molecular Imaging Software (Rochester, NY, USA). Nuclear and cytosolic extracts were prepared as previously described (Dignam *et al.* 1983).

RNA was isolated using the RNeasy kit (Qiagen) and cDNA was prepared using the iScript kit (Bio-Rad) and Bio-Rad iCycler. RT-PCR was performed using mouse Txnip primers (forward, 5'-CGAGTCAAAGCCGTCAG-GAT-3' and reverse, 5'-TTCATAGCGCAAGTAGTCAAAGT-3' or forward, 5'-TCCAGAGTTTCTCCAGTTGC-3' and reverse, 5'-CGTAAAGTCAGGGCTTGAAA-3') and GAPDH primers (forward, 5'-CAGCAAGGACACTGAG-CAAG-3' and reverse, 5'-GGTCTGGGATGGAAATTGATG-3') or  $\beta$ -actin (forward, 5'-AAGAGCTATGAGCTGCCTGA-3' and reverse, 5'-TACGGATGTCAACGTCACAC-3'), iQ SYBR green supermix (Bio-Rad) and the Bio-Rad iCycler iQ.

Calpain and cathepsin L activities were assessed using the calpain or cathepsin L Activity Assay kits (BioVision, Milpitas, CA, USA). Cells were preexposed or not to inhibitors at indicated concentrations for 1 h and then processed according to the manufacturer's directions.

Statistical analysis was performed by ANOVA or two-tailed Student's *t*-test as appropriate. Graphs illustrate means  $\pm$  S.E.M.;  $P < 0.05$  was considered significant.

## Results

### The effects of high glucose, chronic low-dose insulin and acute high-dose insulin on Txnip in 3T3-L1 adipocytes

Cells were incubated for 18 h in the presence of 1% fetal bovine serum (FBS) with either 5 or 25 mM glucose with or without the addition of 0.6 nM insulin. They were then exposed (or not) to a maximally stimulating dose of insulin (100 nM) for 15 min, lysed and analyzed for Txnip protein (Fig. 1). Exposure to chronic low-dose insulin significantly decreased Txnip. Incubation in 25 mM glucose, as compared with 5 mM glucose, increased Txnip, and this effect was particularly pronounced after incubation in low-dose insulin ( $P < 0.01$ ). Incubation with high-dose insulin for only 15 min decreased Txnip by ~80% in the presence of high or low glucose.

The cellular specificity of this insulin effect was investigated. It occurred in preadipocytes, i.e. 3T3-L1 fibroblasts that did not receive any treatment leading to differentiation (Fig. 1B) and in L6 myotubes (Fig. 1C). However, insulin had no effect on Txnip expression in liver-derived HepG2 cells or in HEK 293 cells (embryonic kidney cells), or in a pancreatic  $\beta$ -cell line (INS-1 cells) (data not shown). However, both the HepG2 cells and the INS-1 cells showed markedly increased Txnip expression in response to high glucose, irrespective of their treatment with insulin.

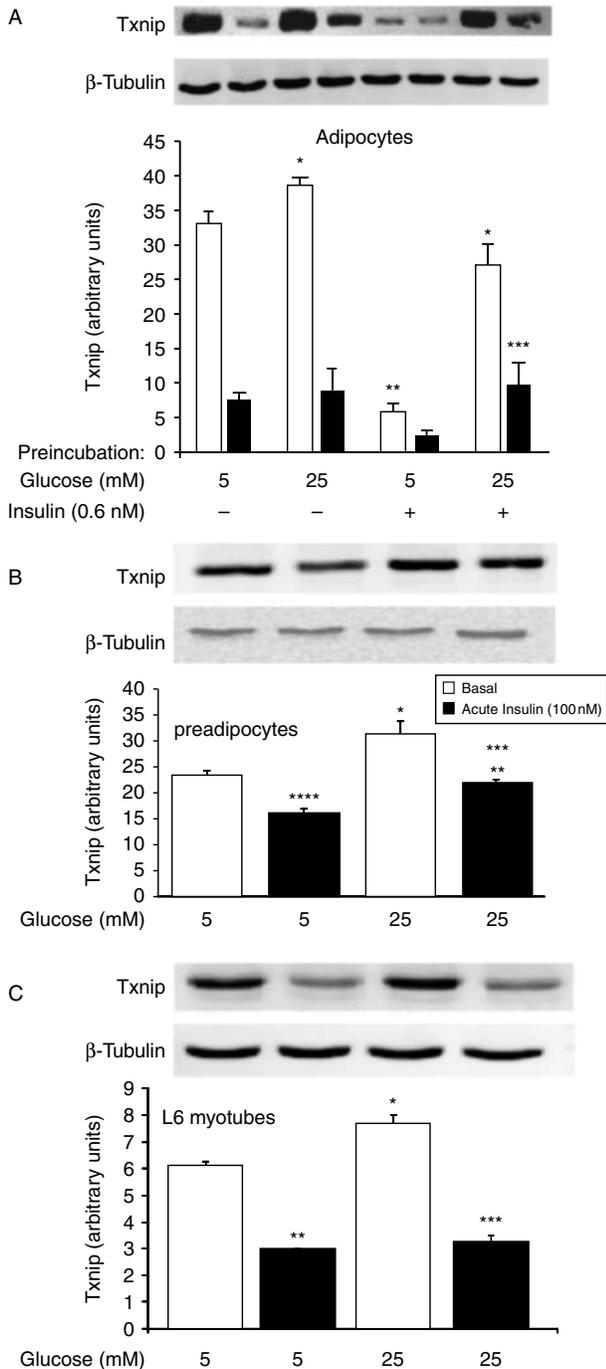
### Rapamycin, a specific inhibitor of mTOR complex-1, decreases Txnip

The design of these experiments was identical to that of Fig. 1, with the exception of incubating cells in the presence of 100 nM rapamycin (Sigma) for 1 h in place of exposure to high-dose insulin. Rapamycin (Fig. 2A) significantly inhibited Txnip by ~20% in cells exposed to

low or high glucose. There was no effect in cells that had been exposed to low glucose and chronic low-dose insulin, where Txnip was already suppressed. Rapamycin was also ineffective after acute stimulation with high insulin (data not shown). The significant inhibition by rapamycin ( $P < 0.005-0.04$ ,  $n = 7$ ) suggests that mTOR complex-1 participates in the regulation of Txnip abundance. In Fig. 2B cells were incubated with rapamycin for 1 h and then exposed to a high dose of insulin (or not) for 15 min. Acute insulin had no effect in rapamycin-treated cells, suggesting that mTOR activity may be required for the insulin effect.

### The effects of high glucose and acute insulin on the cellular distribution of Txnip

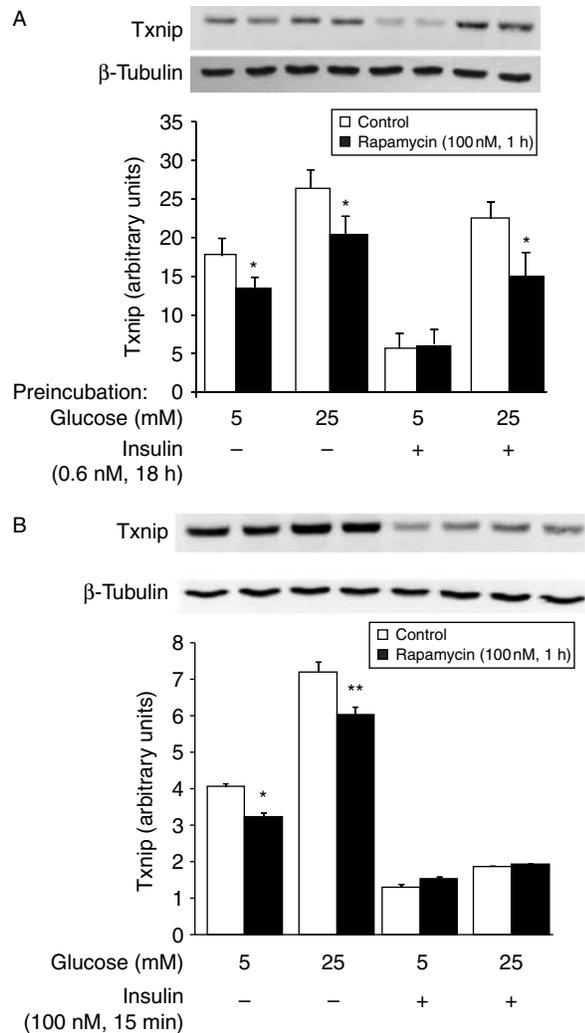
Txnip is distributed in the cytoplasm and the nucleus (Nishinaka *et al.* 2004; Fig. 3). Exposure to high glucose increased Txnip in both compartments, with a greater increase in the nucleus. Acute high-dose insulin decreased Txnip in both compartments. However, acute insulin seems to favor the localization of Txnip in the nucleus, which resulted in doubling of the ratio of concentration of Txnip in the nucleus vs the cytoplasm. Since the effect of acute insulin seems to be primarily that of accelerating Txnip degradation (discussed below), this effect may reflect the distribution of enzymes, e.g. those that accelerate Txnip degradation may be primarily cytosolic. The use of lamin and tubulin for localization of nuclear



**Figure 1**

The effects of high glucose, chronic low-dose insulin and acute high-dose insulin on Txnip. (A) 3T3-L1 adipocytes were incubated for 18 h in DMEM containing 1% FBS, with either 5 or 25 mM glucose, with or without low-dose (0.6 nM) insulin. They were then stimulated or not (basal) with 100 nM insulin for 15 min, lysed and analyzed for Txnip abundance by immunoblotting. Representative blots are shown on top for Txnip and  $\beta$ -tubulin, while the bar graph illustrates the quantitative analysis of seven replicates from four separate experiments. \*Indicates the significant effect of high glucose,  $P < 0.05-0.01$ . High glucose stimulates, chronic insulin inhibits significantly ( $**P < 0.01$ ), particularly in the presence of low glucose, but acute (15 min) high-dose insulin inhibits significantly ( $***P < 0.01$ ), even in the presence of high glucose. Clearly, high glucose and insulin exert opposing effects on Txnip. (B) 3T3-L1 preadipocytes were treated as in (A) without chronic insulin treatment. Representative blots are shown for Txnip and  $\beta$ -tubulin. The bar graph is representative of six replicates from three separate experiments. High glucose stimulates ( $*P < 0.04$ ,  $***P < 0.01$ ) and acute insulin inhibits significantly ( $***P < 0.001$  and  $**P < 0.03$ ). (C) L6 myotubes were treated as in (A) without chronic insulin treatment. Representative blots are shown for Txnip and  $\beta$ -tubulin. The bar graph is representative of six replicates from three separate experiments. High glucose stimulates in basal ( $*P < 0.005$ ) and insulin inhibits significantly ( $**P < 0.001$  and  $***P < 0.001$ ).

and cytosolic fractions is explored in Fig. 3D, where both components were stained with the same antibody. We estimate that the nuclear staining of lamin was enriched ~30-fold vs the cytosol, while the cytosolic staining of tubulin was enriched ~75-fold vs the nucleus.



**Figure 2**

Rapamycin, a specific inhibitor of mTOR complex-1 activity, decreases Txnip. (A) The experimental design was identical to Fig. 1 except that instead of acute insulin stimulation the cells were exposed for 1 h to 100 nM rapamycin before immunoblotting. A representative blot is shown on top, while the bar graph illustrates the quantitative analysis of seven replicates from four separate experiments. Significant rapamycin effects ( $P < 0.005$ – $0.04$  by paired analysis) are indicated by \*. Rapamycin was ineffective when Txnip level was low, e.g. after preincubation in 5 mM glucose with 0.6 nM insulin. (B) Cells exposed to 5 or 25 mM glucose in 1% FBS for 18 h were incubated 1 h with 100 nM rapamycin before stimulating or not with 100 nM insulin for 15 min followed by immunoblotting. A representative blot is shown on top, while the bar graph illustrates the quantitative analysis of six replicates from three separate experiments. The effect of rapamycin in decreasing Txnip was only detected in the samples that did not receive insulin ( $*P < 0.001$  and  $**P < 0.05$ ).

### Acute insulin markedly accelerates the degradation of Txnip protein

Since the effect of insulin in decreasing Txnip is very rapid, it is unlikely to be purely a decrease in protein synthesis. To test the effect of insulin on Txnip degradation, adipocytes were incubated for 18 h in 5 mM glucose. During the last 30 min of incubation, the dishes were supplemented with 1  $\mu$ g/ml cycloheximide (Sigma), a protein synthesis inhibitor. Cells were then treated with or without 100 nM insulin for 15 min. Cells were lysed at intervals and Txnip was quantified by immunoblotting. The half-life of Txnip was ~42.7 min in control cells, and this accelerated to ~2.5 min after the addition of insulin (Fig. 4A).

Since 100 nM insulin is a supraphysiological concentration, we tested whether lower concentrations would also promote Txnip proteolysis. Cells were treated with cycloheximide as described above, and were further treated with or without insulin at various concentrations between 1 and 100 nM. The lowest insulin concentration, which accelerated Txnip proteolysis within 15 min, was 3 nM (Fig. 4B). It is of note that after overnight exposure to insulin, 0.6 nM insulin appeared to be effective (Fig. 1).

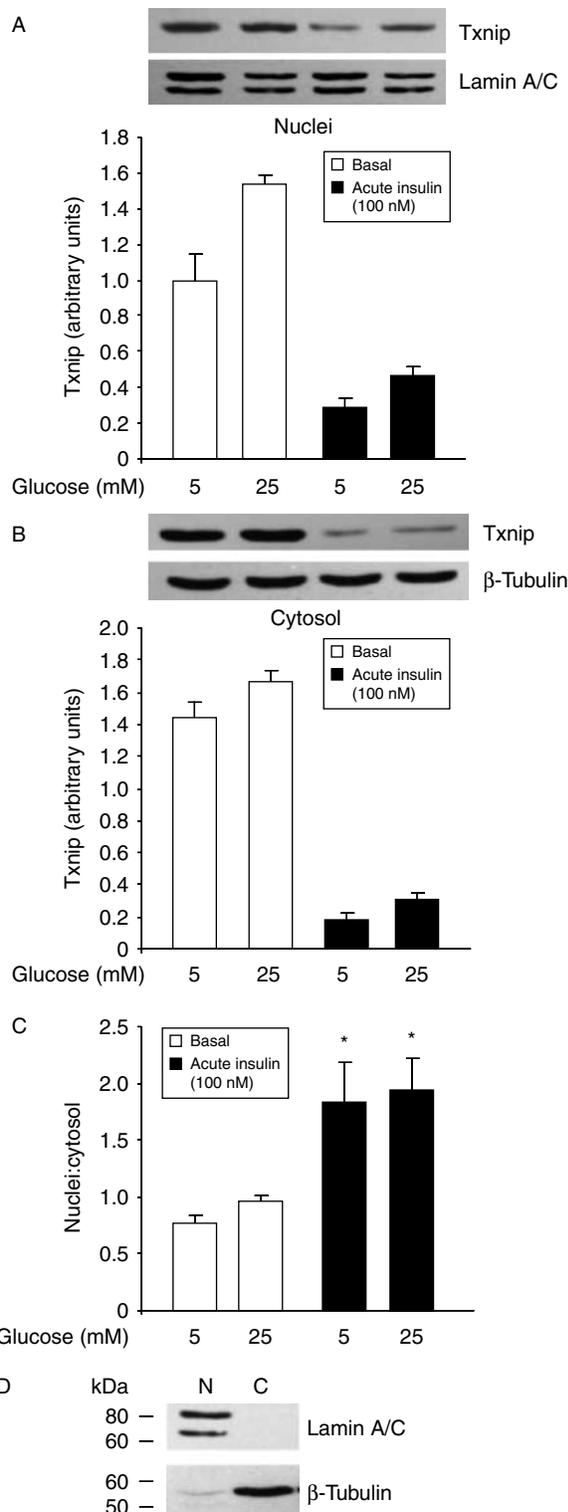
To document that the effect of insulin on Txnip concentration was at the level of proteolysis, Txnip mRNA was measured by RT-PCR in cycloheximide-treated cells that had been exposed to 100 nM insulin or to vehicle for 15 min. (Fig. 4C and D) No difference was detected. It is noteworthy that overnight exposure to high glucose increased Txnip mRNA (Fig. 4D) and this effect was independent of insulin treatment.

### Role of the ubiquitin/proteasome pathway

Based on the above data, it is apparent that the major acute effect of insulin on Txnip abundance is mediated by acceleration of protein degradation. Therefore, we further investigated whether this effect was mediated by the ubiquitin/proteasome pathway. In a first series of experiments (Fig. 5A), we incubated cells in 5 mM glucose with or without a proteasome inhibitor, 50  $\mu$ M Z-Leu-Leu-Leu-al (Sigma), for 1 h, with a subsequent stimulation with 100 nM insulin or vehicle control for 15 min. Using this experimental design, modified from Pagano *et al.* (1995), we observed that the insulin-induced decrease in Txnip was abolished in cells treated with the proteasome inhibitor ( $P < 0.01$ ; note that in Fig. 6 several potential inhibitors were tested and none of them reproduced the effect of the proteasome inhibitor).

In a second series of experiments (Fig. 5B) we immunoprecipitated the cell extracts with anti-Txnip antibody followed by western blotting with anti-ubiquitin antibody, in an effort to determine whether short-term

insulin stimulation increased the ubiquitination of Txnip. These experiments showed that while Txnip decreased markedly in response to insulin stimulation, the ubiquitin associated with Txnip did not, resulting in increased ubiquitin binding per unit of Txnip (Fig. 5B and C). The ratio of ubiquitin/Txnip (Fig. 5B) increased significantly between 2 and 4 min after exposure to insulin in cells that had been incubated overnight with 5 mM glucose ( $P < 0.02$ ) or with 25 mM glucose ( $P < 0.005$ ). The greater ubiquitination noted in Fig. 5C as compared with Fig. 5B is due in part to a change in technique in that the proteasome inhibitor used in Fig. 5A was added to the cells at the beginning of preparation, thereby preventing the degradation of ubiquitinated products. The ubiquitin/Txnip ratios 4 min after exposure to insulin compared with basal increased by 60% ( $P < 0.005$ ). Taken together, the data indicate that the ubiquitin/proteasome pathway plays an important role in Txnip degradation, supporting the observations by Zhang *et al.* (2010).

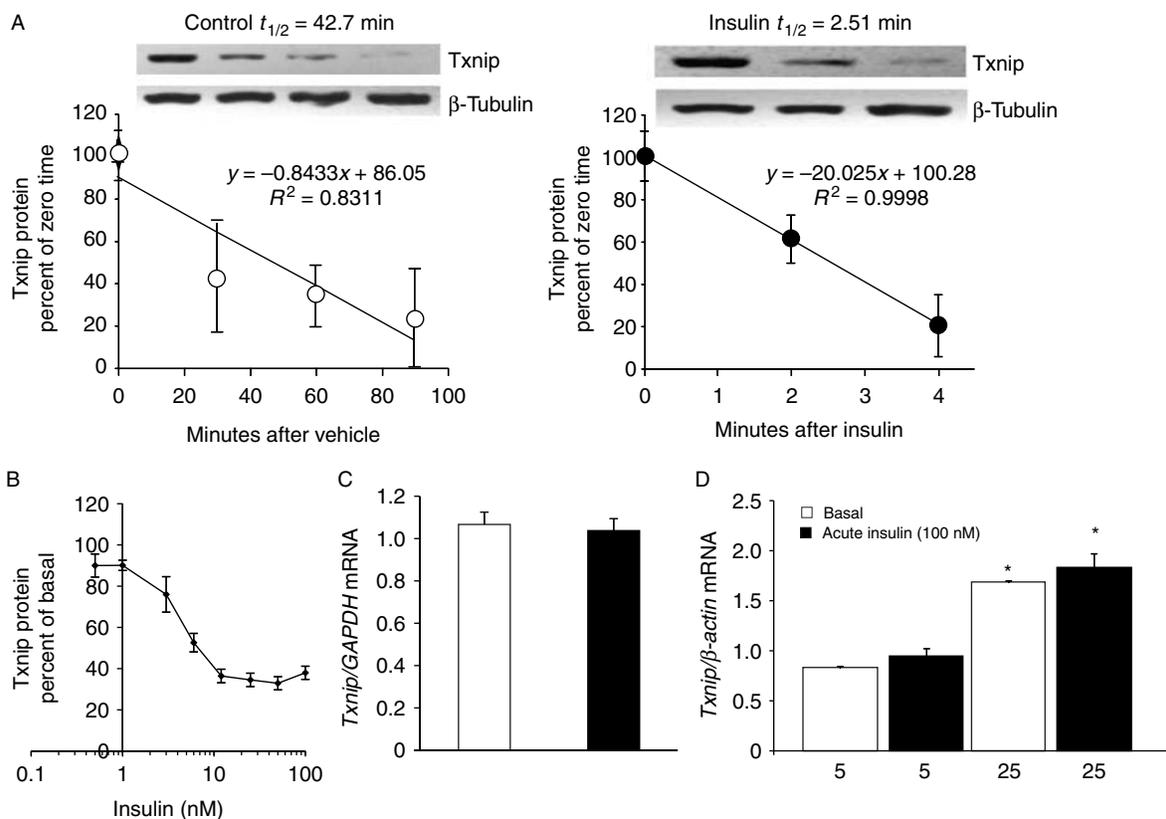


### Lack of effect of cysteine protease or calpain inhibitors

We were also interested in whether the rapid insulin-stimulated degradation of Txnip was mediated by a cysteine protease or an enzyme directed against calpain. Cells were incubated with E64, a cysteine protease inhibitor (Sigma), a calpain inhibitor I (Leu-leu-norleu-al; Sigma), a calpain inhibitor III (MDL 28170; Sigma). The concentrations used ranged from 2 to 400  $\mu$ M, and the duration of treatment from 1 to 4 h to overnight. Calpain inhibitor III was also tested in a medium with or without  $Ca^{++}$  (Fig. 6). Finally, we tested calpeptin (benzyloxycarbonylleucyl-norleucinal), an inhibitor of calpain I and II (Calbiochem, EMD Millipore, Billerica, CA), at a concentration of 1 or 5  $\mu$ M for 1 h (data not shown). We did not observe an increase in Txnip with or without insulin stimulation in response to these

### Figure 3

The effects of high glucose and acute insulin on the distribution of Txnip between nuclei and cytosol. (A, B and C) Cells were incubated for 18 h in media containing low (5 mM) or high (25 mM) glucose, 1% FBS and stimulated or not with 100 nM insulin for 15 min. Before analysis of Txnip by immunoblotting, cytosolic and nuclear extracts were prepared (Dignam *et al.* 1983). High glucose increases Txnip more in nuclei than in the cytosol. Acute insulin markedly decreases Txnip in nuclei and in the cytosol, but also promotes the transfer of Txnip into nuclei. Ratio of nuclei:cytosol increased approximately twofold after 15 min with insulin ( $*P < 0.05$ ). (D) Representative western blots of nuclear and cytosolic preps for the proteins lamin A/C (nuclear marker) and  $\beta$ -tubulin (cytosolic marker) showing appropriate fraction enrichment.

**Figure 4**

Acute insulin markedly increases the degradation of Txnip protein. (A) Adipocytes were incubated for 18 h in media containing 5 mM glucose, 1% FBS, and then protein synthesis was blocked for 1 h with 1  $\mu$ g/ml cycloheximide. Then cells were stimulated with 100 nM insulin or vehicle (controls) and lysed at the times indicated for assessment of Txnip by immunoblotting. Txnip was expressed as the percentage of the value present at the time insulin or vehicle was added. The half-life of Txnip in controls was 42.7 min, which accelerated to 2.5 min after the addition of insulin ( $n=6-11$ ). (B) The experiment was repeated, except that the cells

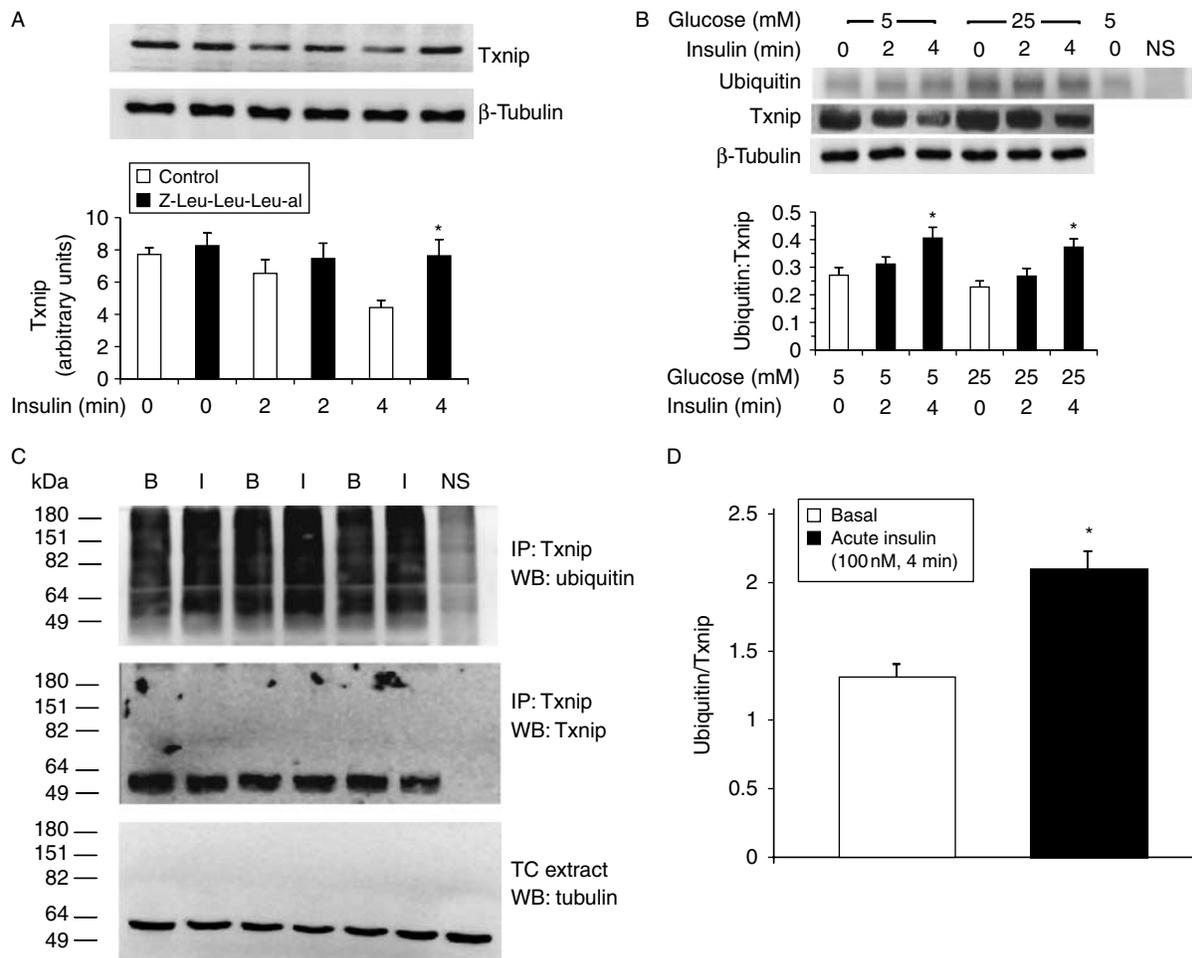
were incubated for 15 min with varying concentrations of insulin indicated in the abscissa ( $n=4$ ). (C) Under identical conditions the effect of exposing cells for 15 min to 100 nM insulin on the expression of Txnip mRNA was tested as described in Materials and methods. Values are expressed as the ratio of Txnip, GAPDH ( $n=6$ ). (D) Cells were first incubated for 18 h in media containing 5 or 25 mM glucose, 1% FBS, then treated as in (A) with 1  $\mu$ g/ml cycloheximide, and mRNA was measured as described in Materials and methods. Values are expressed as the ratio of Txnip: $\beta$ -actin ( $n=6$ ,  $*P<0.001$ ).

inhibitors. Therefore, we conclude that the insulin effect is not mediated by a cysteine protease or by a calpain protease.

#### Treatment with okadaic acid markedly decreases Txnip

Okadaic acid (OKA, Axxora Farmingdale, NY, USA) is a potent inhibitor of phosphoprotein phosphatase (pp). At very low concentrations, e.g.  $\sim 1$  nM, it is specific for pp2A, while at higher concentrations, e.g.  $\sim 1000$  nM, it non-specifically inhibits other pp-s, such as pp1 (Takai & Mieskes 1991). When we incubated adipocytes for 15 or 30 min with OKA (1 or 1000 nM) it had no effect on Txnip in the basal or in the insulin-stimulated condition (data not shown). This likely reflects the relatively slow penetration of OKA

across the cell membrane, particularly in the presence of very low concentrations of the inhibitor. However, after 60 min exposure to 1000 nM OKA, a 64% decrease in Txnip was observed. This effect was not seen with 1 nM OKA in the basal state (Fig. 7). Exposure to 100 nM insulin for 15 min decreased Txnip by 73% without OKA. Addition of 1 nM OKA increased the decline in Txnip in the presence of insulin significantly (by 60%,  $P<0.05$ ), suggesting that insulin promotes the degradation of Txnip by enhancing the phosphorylation of a protein substrate of pp2A. Increasing OKA concentration to 1000 nM further accelerated protein degradation in the presence of insulin, although the difference between the effects of 1 and 1000 nM OKA under these conditions was not statistically significant (Fig. 7A).

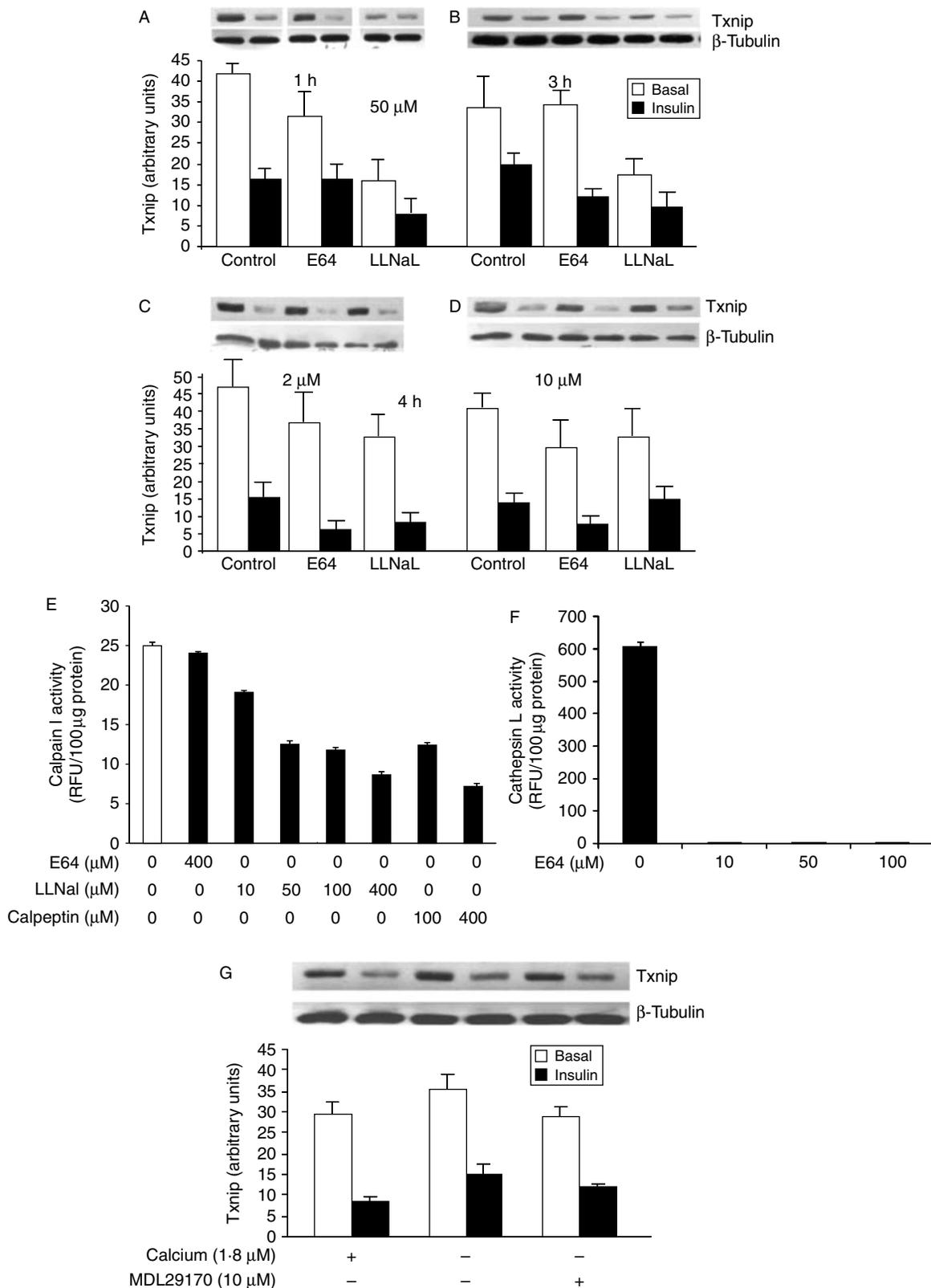
**Figure 5**

Involvement of the ubiquitin/proteasome pathway in Txnip degradation. (A) 3T3-L1 adipocytes were incubated in media containing 5 mM glucose, 1% FBS for 18 h and then incubated for 1 h with or without the proteasome inhibitor Z-Leu-Leu-Leu-al (50  $\mu$ M; Sigma) and then stimulated for 2 or 4 min with 100 nM insulin or with vehicle. While Txnip as assessed by immunoblotting decreased significantly after 4 min exposure to insulin, this was prevented in cells exposed to Z-Leu-Leu-Leu-al (\* $P$ <0.01,  $n$ =7). (B) Cells were preincubated for 18 h in 5 or 25 mM glucose, 1% FBS, treated with 1  $\mu$ g/ml cycloheximide for 1 h and then stimulated (or not) with 100 nM insulin for 2 or 4 min. Cells were then extracted and immunoprecipitated with anti-Txnip antibody and immunoblots were performed with anti-ubiquitin antibody. As a comparison we show the cellular extracts, immunoblotted with anti-Txnip antibody. NS (non-specific binding) represents a nonimmune control. The bar graph in (B) quantifies the ratios of ubiquitin/Txnip expression at

0 time and 2 and 4 min after exposing the cells to insulin;  $n$ =7 from three experiments. \*Indicates a significant increase between 2 and 4 min postinsulin,  $P$ <0.02 and  $P$ <0.005 after incubation in 5 or 25 mM glucose respectively. (C) Cells were preincubated for 18 h in 5 mM glucose, 1% FBS and treated with 1  $\mu$ g/ml cycloheximide and 50  $\mu$ M Z-Leu-Leu-Leu-al for 1 h then stimulated (or not) with 100 nM insulin for 4 min. Cells were then extracted in the presence of Z-Leu-Leu-Leu-al and immunoprecipitated with anti-Txnip antibody and immunoblots were performed with anti-ubiquitin antibody. Western blots of Txnip immunoprecipitates from basal (B) or insulin (I) treated samples and nonimmune serum control (NS) were performed using ubiquitin and Txnip antibodies. Postnuclear extracts were immunoblotted for  $\beta$ -tubulin to confirm equal protein loading of the immunoprecipitates. Representative western blots are shown. The bar graph in (D) quantifies the ratio of ubiquitin/Txnip in basal and insulin after 4 min (\* $P$ <0.005).

As expected, exposure to insulin markedly activated Akt, increasing Akt phosphorylation approximately five-fold, without affecting the expression of total Akt (Fig. 7B). However, Akt activation was not affected by OKA, suggesting that in 3T3-L1 adipocytes Akt activation is

not controlled by the phosphatase inhibited by OKA (Valverde *et al.* 2000), although this is speculative at this point. Alternatively, the insulin-induced activation of Akt may be so powerful that the effects of a phosphatase could not be detected.



### Inhibition of PI3 kinase increases Txnip

Cells were preincubated with or without cycloheximide for 90 min, and during the last 60 min with or without 0.1  $\mu\text{M}$  Wortmannin (Axxora), an inhibitor of PI3 kinase (Fig. 8A). In cells not exposed to cycloheximide, Wortmannin doubled Txnip abundance in the basal state, while treatment with 0.1  $\mu\text{M}$  insulin (for 3 or 15 min) still decreased it, although proportionally less than in the controls. In cycloheximide-treated cells, Txnip was much lower, however treatment with Wortmannin increased Txnip and also significantly ( $P < 0.03$ ) inhibited the insulin stimulation of Txnip degradation (the latter is based on comparing the percent decrease in Txnip between 3 and 15 min postinsulin stimulation). The fact that insulin still promoted Txnip degradation after treatment with 0.1  $\mu\text{M}$  Wortmannin may indicate that PI3 kinase was only partially inhibited by this concentration. To test this, we repeated the experiment, but used 1  $\mu\text{M}$  Wortmannin to block PI3 kinase. Under these conditions insulin no longer decreased Txnip in the cells (Fig. 8B).

The activation of Akt was exquisitely controlled by PI3 kinase activity. In the absence of Wortmannin, Akt was markedly activated by insulin. The presence of 0.1  $\mu\text{M}$  Wortmannin, which only partially inhibited the effect of insulin on Txnip degradation, showed near complete inhibition of Akt activation. This effect was further increased by incubation with 1  $\mu\text{M}$  Wortmannin (Fig. 8C).

To answer the question whether or not Akt was involved in the effect of insulin on Txnip degradation we tested the effect of the Akt inhibitor VIII (10  $\mu\text{M}$ ; EMD Millipore, Billerica, MA, USA) on this process (Fig. 8D). The reagent inhibited the phosphorylation of Akt on Ser 473 in response to insulin nearly completely; however, it had no effect on the insulin-stimulated decrease in Txnip abundance. The data suggest that Akt is not involved in the stimulation of Txnip degradation by insulin.

Clearly, OKA and Wortmannin exert opposing effects on Txnip abundance. While OKA mimics the effect of insulin, Wortmannin opposes it. The data suggest

that insulin promotes Txnip degradation by a PI3 kinase-dependent mechanism.

### Discussion

Txnip is a member of the  $\alpha$ -arrestin family. Of the six known mammalian members of the family, Txnip is the only one that binds to thioredoxin, yet other members of the family have been shown to alter glucose metabolism (Patwari *et al.* 2009). This strengthens the concept that Txnip belongs to a new class of metabolic regulators that can operate independently of thioredoxin (Patwari *et al.* 2009). One of the outstanding properties of Txnip is its ability to modulate insulin sensitivity. Txnip-deficient mice fed a high-fat diet gain more weight and accumulate larger fat deposits than the wild-type controls, yet their insulin sensitivity and response is markedly increased, in the whole animal, in muscle and in adipocytes (Chutkow *et al.* 2010). This is in part due to Txnip inhibiting the expression and the activity of peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ; Chutkow *et al.* 2010) and in part to other effects. *In vitro* studies indicate that Txnip also regulates PPAR $\alpha$  expression and activity (Oka *et al.* 2009) as well as Foxo4 activity (Dansen *et al.* 2009).

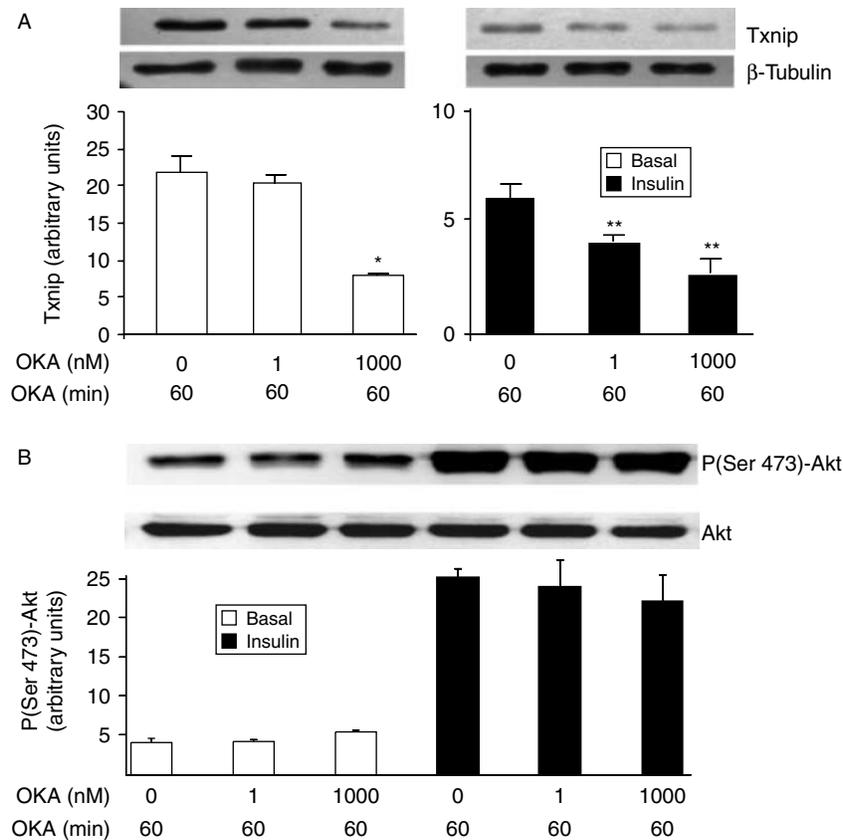
The expression of Txnip is highly regulated by the glucose concentration in several cell culture models and in animals and man *in vivo* (Minn *et al.* 2005, Parikh *et al.* 2007, Chen *et al.* 2008b, Chutkow *et al.* 2010). Our data in 3T3-L1 adipocytes confirm these observations. The glucose effect is clearly transcriptional, at least in part as evidenced by the fact that it is regulated by a classical carbohydrate response element situated in the promoter region of Txnip DNA (Minn *et al.* 2005).

The opposing effect of insulin to glucose on Txnip has also been observed by others (Parikh *et al.* 2007). Since exposure of several hours to insulin was used, the extremely rapid response to insulin, which suggests a posttranslational effect, was not observed in early experiments. We found that a major effect of insulin on Txnip

### Figure 6

No evidence for involvement of a cysteine or calpain protease in the insulin-stimulated degradation of Txnip. Cells were incubated in 5 mM glucose, 1% FBS containing media for 18 h and then exposed to cysteine protease inhibitor E-64, a calpain inhibitor I (LLNaL in (A, B, C and D)) or a calpain inhibitor III (MDL 29170 in (E)) (all from Sigma) for various times and concentrations and then stimulated or not with 100 nM insulin for 15 min. (A and B) Inhibitor concentration was 50  $\mu\text{M}$  and duration of pretreatment was 1 and 3 h respectively. (C and D) Pretreatment was 4 h, (C) with inhibitor concentrations of 2  $\mu\text{M}$  and (D) 10  $\mu\text{M}$ .

(E and F) Incubations were performed as described in (A, B, C and D) and cells were processed using the Calpain 1 or Cathepsin L Activity Kits (EMD Millipore) to confirm the potency of the inhibitors ( $n = 6$ ). (F) E64 was maximally inhibitory at each concentration tested. (G) Cells were exposed to 10  $\mu\text{M}$  MDL 29170 for 4 h in the presence or absence of 1.8 mM calcium from the media. While some of the inhibitors tended to decrease basal Txnip protein levels, none of them diminished the inhibitory effect of insulin on Txnip as assessed by immunoblotting ( $n = 3$  in (A, B, C and D) and  $n = 4$  in (G)).

**Figure 7**

OKA mimics the effect of insulin. Cells were incubated in media containing 5 mM glucose, 1% FBS for 18 h and exposed to OKA, 1 or 1000 nM for 1 h following which they were stimulated or not (basal) with 100 nM insulin for 15 min (A). Txnip was measured by immunoblotting. OKA had no effect in 15 or 30 min (data not shown). However, after 60 min exposure, under

basal conditions 1000 but not 1 nM OKA significantly decreased Txnip ( $*P < 0.01$  in (A)). After stimulation with insulin, both 1 and 1000 nM OKA further decreased Txnip ( $**P < 0.05$ ,  $n = 4$  in (A)). Under these conditions, OKA had no effect on P(Ser 473)-Akt phosphorylation or amount of Akt protein ( $n = 4$  in (B)).

abundance is mediated by acceleration of Txnip degradation in 3T3-L1 adipocytes (Robinson & Buse 2010). Because Txnip is thought to bind to thioredoxin via its CXXC motif (Patwari *et al.* 2006) we investigated whether the insulin-accelerated degradation of Txnip was mediated by a cysteine or calpain protease, but found no evidence to support this hypothesis.

In more recent work, the effect of insulin in accelerating Txnip degradation has been recognized, and mechanisms to explain this have been proposed. Chutkow & Lee (2011) made the interesting observation that thioredoxin exerts its effects on adipogenesis by interacting with Txnip, which results in enhancement of Txnip protein stability. They extended this observation to the effect of insulin, which appeared to increase Txnip protein degradation by weakening the interaction between thioredoxin and Txnip in 3T3-L1 adipocytes. However, the

mechanism by which insulin inhibits the binding of Txnip to thioredoxin has not been determined.

The above experiments were carried out in 3T3-L1 adipocytes, but there was no indication that they did not reflect what happens in cells in general. We were therefore astonished when we tested a number of cell types and found that the ability of insulin to promote Txnip degradation varied between cells. Figure 1 shows that the greatest effect (Txnip decrease in response to insulin/basal concentration) was observed in 3T3-L1 adipocytes, which was followed by L6 myocytes, with the least, albeit significant, effect observed in preadipocytes. There was no effect of insulin at all on Txnip expression in cells derived from liver, embryonic kidney or insulin-producing pancreatic  $\beta$  cells (data not shown). Clearly, the cause for this cell specificity needs to be determined. What comes to mind is that the cells that respond to insulin with

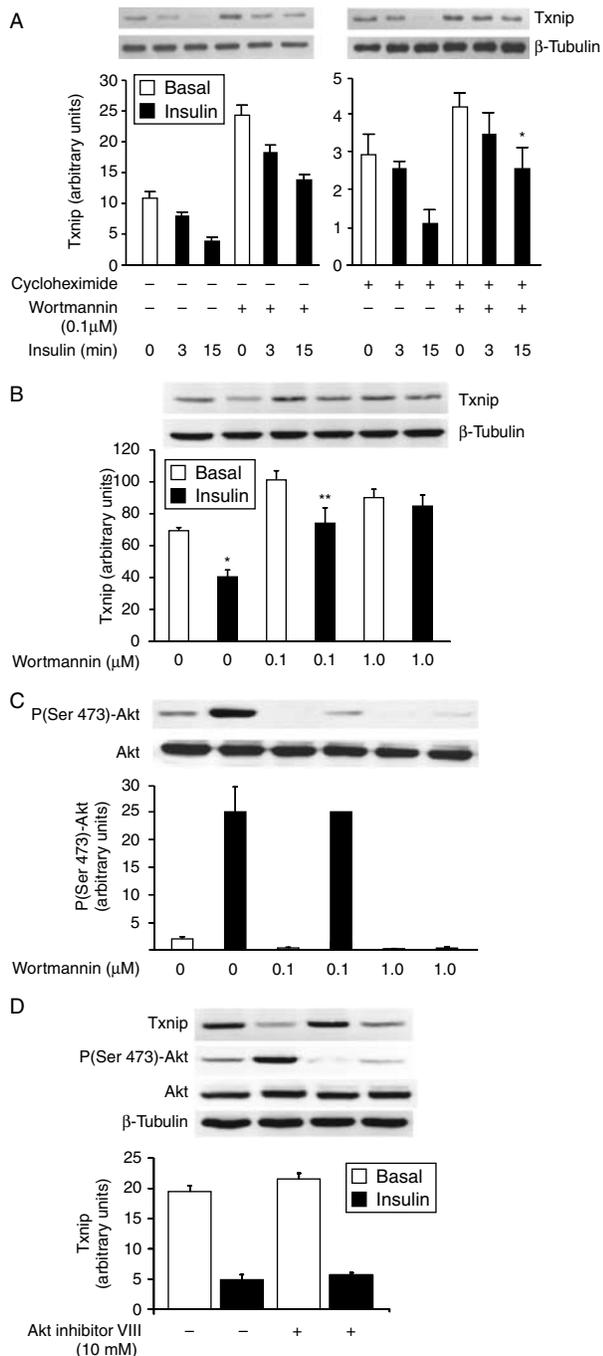
accelerated degradation of Txnip are those that can express Glut4 and respond to insulin with translocation of Glut4 to the cell membrane.

A pp inhibitor (OKA) and an inhibitor of PI3 kinase (Wortmannin) exerted opposing effects on Txnip. While OKA mimicked the effect of insulin, Wortmannin inhibited it. Taken together, these findings suggest that a Ser/Thr phosphorylation event may play a role in the

insulin-induced activation of Txnip proteolysis, and that the effect of insulin, like many of its other metabolic effects, may be mediated by activation of PI3 kinase.

We also found that rapamycin decreased Txnip in 3T3-L1 adipocytes, although to a lesser degree than acute insulin. While we did not study the mechanism of the rapamycin effect, the fact that it was not additive to the effects of insulin suggests that it may involve the same posttranslational mechanism. Our data suggest that mTOR activity may be required for the insulin effect on Txnip degradation. Rapamycin decreased Txnip abundance, indicating that mTOR stimulation would enhance it. Since mTOR is activated by insulin, and in general by metabolic success, mTOR's effect on Txnip would act as a negative regulator of mTOR activity.

In general, the effect of Txnip is anti-insulin. Increased Txnip expression leads to insulin-resistant glucose transport in muscle and in adipocytes (Chutkow *et al.* 2010, Yoshihara *et al.* 2010), and in pancreatic  $\beta$  cells chronic increases in Txnip expression cause apoptosis (Minn *et al.* 2005, Chen *et al.* 2008a,b, Corbett 2008). Hyperglycemia-mediated increase in Txnip induces accelerated transcription of interleukin 1 $\beta$  (IL1 $\beta$ ), in human and in mouse adipocytes, and the increased production of IL1 $\beta$  likely contributes to the insulin resistance of diabetes (Koenen *et al.* 2011). Since the most potent stimulus for Txnip expression is increased glucose concentration, one has to assume that its purpose may be to protect the organism from the deleterious effects of hyperglycemia, e.g. that caused by oxygen radicals. The rapid downregulation by



**Figure 8**

Treatment with a PI3 kinase inhibitor increases Txnip. Cells were incubated in media containing 5 mM glucose, 1% FBS for 18 h and then for 90 min with or without 1  $\mu$ g/ml cycloheximide, during the last 60 min with or without 0.1  $\mu$ M Wortmannin. (A) They were then stimulated or not (basal) with 100 nM insulin for 3 or 15 min and Txnip was measured by immunoblotting. Wortmannin increased Txnip abundance in the basal state, in cells treated or not with cycloheximide. In cells treated with cycloheximide it also inhibited the insulin-induced acceleration of Txnip degradation ( $*P < 0.03$ ,  $n = 6$ ). Since Wortmannin did not abolish the insulin effect (A), the experiment was repeated, in the presence of cycloheximide, except that the effects of 0.1 and 1  $\mu$ M Wortmannin were compared (B). Wortmannin 0.1  $\mu$ M inhibited but did not abolish the insulin effect on protein degradation, but that was abolished by 1  $\mu$ M Wortmannin ( $*P < 0.001$  and  $**P < 0.04$ ,  $n = 6$ ). (C) At both concentrations Wortmannin significantly reduced both basal and insulin-stimulated P(Ser 473)-Akt with no effect on Akt protein levels. (D) Cells preincubated in 5 mM glucose, 1% FBS for 18 h were treated with 1  $\mu$ g/ml cycloheximide with or without 10  $\mu$ M Akt inhibitor VIII (EMD Millipore) for 1 h then stimulated or not with 100 nM insulin for 15 min. No effect was detected on the insulin-induced decrease in Txnip, while Akt phosphorylation was nearly completely inhibited by the Akt inhibitor.

insulin of the glucose-induced increase in Txnip expression introduces an interesting regulatory mechanism, which would depend not only on the magnitude of the insulin response to glucose, but also render the regulation to be cell and tissue specific, since it would reflect the cell type, the number of cellular insulin receptors as well as the access of a given tissue to insulin. The hyperglycemia-induced marked increase in Txnip expression likely plays a role in insulin resistance, as observed in uncontrolled type 1 diabetes (Yki-Jarvinen *et al.* 1987), while the insulin-induced rapid degradation of Txnip likely contributes to the restoration of insulin sensitivity upon insulin treatment (Yki-Jarvinen *et al.* 1987).

The mechanism by which insulin promotes Txnip degradation is also of interest. It appears that insulin promotes the phosphorylation of a protein, or of Txnip itself, leading to activation of the ubiquitin/proteasome pathway, which then rapidly catalyzes Txnip degradation. To our knowledge this is a novel effect of insulin. It is reminiscent of the effect of insulin on IRS-1 and -2 degradation, which after prolonged exposure to insulin activates the ubiquitin/proteasome pathway, which leads to the degradation of IRS proteins and presumably to insulin resistance (reviewed in references, Gual *et al.* (2005) and Petroski (2008)). In preliminary experiments, we found no evidence of Txnip phosphorylation (data not shown).

The proteasomal degradation of Txnip has been documented, and Itch has been identified as the E3 ubiquitin ligase for Txnip (Zhang *et al.* 2010, Chutkow & Lee 2011). Our results support this concept. The rapid response to insulin suggests that it may be the most effective agent to combat insulin resistance in response to hyperglycemia as well as other stresses, which induce hyperexpression of Txnip (reviewed in Zhang *et al.* (2010)). Txnip also acts as a tumor suppressor and many human tumors are low in Txnip (Zhang *et al.* 2010). Therefore, vigorous treatment with insulin may favor tumorigenesis and spreading of metastases (Zhang *et al.* 2010). The complex mechanisms that determine the concentration of Txnip are of particular interest because Txnip is an important regulator of intermediary metabolism (Chutkow *et al.* 2010) and likely plays a role in the development of insulin resistance and type 2 diabetes (Parikh *et al.* 2007, Chen *et al.* 2008b). Although the mechanisms by which increased cellular abundance of Txnip causes insulin resistance, e.g. the impaired translocation of GLUT4 to the cell membrane in muscle and in adipocytes, have not been identified, recent work has clarified the role of Txnip in causing apoptosis in pancreatic  $\beta$  cells. Txnip is a critical node in the

endoplasmic reticulum (ER) intracellular signaling pathway called the unfolded protein response, which when activated leads to apoptosis. Thus, Txnip is a potential therapeutic target for diabetes and ER stress-related human diseases, e.g. Wolfram syndrome (Lerner *et al.* 2012, Osowski *et al.* 2012).

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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#### Author contribution statement

K A R and J W B carried out the experiments, and M G B planned the study, interpreted results and wrote the paper.

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