Glucose, dexamethasone, and the unfolded protein response regulate TRB3 mRNA expression in 3T3-L1 adipocytes and L6 myotubes

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Submitted 13 March 2006; accepted in final form 2 July 2006

Yacoub Wasef, Sherif Z., Katherine A. Robinson, Mary N. Berkaw, and Maria G. Buse. Glucose, dexamethasone, and the unfolded protein response regulate TRB3 mRNA expression in 3T3-L1 adipocytes and L6 myotubes. Am J Physiol Endocrinol Metab 291: E1274–E1280, 2006. First published July 11, 2006; doi:10.1152/ajpendo.00117.2006.—Tribbles 3 (TRB3) is a recently recognized atypical inactive kinase that negatively regulates Akt activity in hepatocytes, resulting in insulin resistance. Recent reports link TRB3 to nutrient sensing and regulation of cell survival under stressful conditions. We studied the regulation of TRB3 by glucose, insulin, dexamethasone (Dex), and the unfolded protein response (UPR) in 3T3-L1 adipocytes and in L6 myotubes. In 3T3-L1 adipocytes, incubation in high glucose with insulin did not increase TRB3 mRNA expression. Rather, TRB3 mRNA increased fourfold with glucose deprivation and two- to threefold after incubation with tunicamycin (an inducer of the UPR). Incubation of cells in no glucose or in tunicamycin stimulated the expression of CCAAT/enhancer-binding protein homologous protein. In L6 myotubes, absent or low glucose induced TRB3 mRNA expression by six- and twofold, respectively. The addition of Dex to 5 mM glucose increased TRB3 mRNA expression twofold in 3T3-L1 adipocytes but decreased it 16% in L6 cells. In conclusion, TRB3 is not the mediator of high glucose-induced insulin resistance in 3T3-L1 adipocytes, but TRB3 mRNA was elevated in 3T3-L1 adipocytes exposed to low or no glucose and in L6 myotubes exposed to low or no glucose. In 3T3-L1 adipocytes treated with dexamethasone (Dex) (4), we sought to investigate a potential role for TRB3 in the pathogenesis of both models of insulin resistance. We found no increase of TRB3 mRNA in the glucose toxicity model of insulin resistance in 3T3-L1 adipocytes, but TRB3 mRNA was elevated in 3T3-L1 adipocytes and in L6 myotubes exposed to low or no glucose and in 3T3-L1 adipocytes exposed to Dex. The low glucose- and not the Dex-induced increase in TRB3 mRNA expression appeared to be mediated, at least in part, by the unfolded protein response (UPR).

In view of previous work linking defective Akt phosphorylation to high glucose-induced insulin resistance in 3T3-L1 adipocytes (18, 19), and to altered insulin signaling in primary adipocytes treated with dexamethasone (Dex) (4), we sought to investigate a potential role for TRB3 in the pathogenesis of both models of insulin resistance. We found no increase of TRB3 mRNA in the glucose toxicity model of insulin resistance in 3T3-L1 adipocytes, but TRB3 mRNA was elevated in 3T3-L1 adipocytes and in L6 myotubes exposed to low or no glucose and in 3T3-L1 adipocytes exposed to Dex. The low glucose- and not the Dex-induced increase in TRB3 mRNA expression appeared to be mediated, at least in part, by the unfolded protein response (UPR).

MATERIALS AND METHODS

Materials. Unless otherwise noted, materials were purchased from Sigma Chemical (St. Louis, MO) and were of the highest quality available. α-Minimal essential growth medium (MEM) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from GIBCO Invitrogen (Grand Island, NY). Human recombinant insulin was a gift from Lilly Research Laboratories (Indianapolis, IN). A polyclonal antibody specific for Akt1/2, a β-tubulin antibody, and an anti-CHOP antibody were purchased from Cell Signaling (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Jackson Immunoresearch Laboratories (West Grove, PA), and enhanced chemiluminescence reagents were purchased from Pierce (Rockford, IL). TRizol reagent was purchased from Invitrogen (Carlsbad, CA). Primers for the real-time PCR (RT-PCR) assay were designed using the primer express software (Applied Biosystems, Foster City, CA), and were...
synthesized by Invitrogen, except for adiponectin primers, which were a kind gift from Dr. Richard Klein, and the 18S primers, which were as previously described (14). Gene Amp RNA PCR core kit and SYBR Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA).

**Cell culture.** 3T3-L1 fibroblasts were grown and differentiated into adipocytes in 3.5-cm culture dishes, as previously described (18, 8). Cells were grown to confluence in DMEM containing 25 mM glucose and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂. Two days after confluence, cells were placed into DMEM containing 25 mM glucose, 0.5 mM isobutylmethylxanthine, 1 μM Dex, 10 μg/ml insulin, and 10% FBS for 3 days and then for 2 days into DMEM containing 25 mM glucose, 10 μg/ml insulin, and 10% FBS. Thereafter, cells were maintained in and refed every 2 days with DMEM, 25 mM glucose, and 10% FBS until used in experiments 10–14 days after the start of treatment, at which time between 90 and 95% of the cells exhibited the adipocyte phenotype (18).

Cryopreserved L6 rat skeletal muscle cells (a kind gift of Dr. Amira Klip) were passaged as described previously (17, 31) in MEM growth medium containing 100 μg/ml penicillin, 100 μg/ml streptomycin, 250 μg of amphotericin, and 10% FBS. To obtain differentiated myotube cultures, cells were seeded at 20,000 cells/ml into MEM medium containing 2% FBS and maintained in culture for ≥1 wk postconfluence before being used in experiments.

Before experiments, 3T3-L1 adipocytes and L6 muscle cells were preincubated for 18 h at 37°C with DMEM containing 1% FBS and 0, 2.5, 5, 10, or 25 mM glucose or 5 mM glucose with 100 nM Dex. 3T3-L1 adipocytes were also preincubated in 5 or 25 mM glucose in the presence of 0.6 mM insulin. In addition, time course studies of 5 mM glucose with 1 μg/ml actinomycin D, 5 mM glucose with or without 2.5–5 μg/ml tunicamycin, and timed incubations without glucose were conducted. Cells in all experimental conditions were studied in parallel.

**RT-PCR measurement of RNA expression.** RNA was isolated from the cells with TRIzol according to the manufacturer’s protocol and was reverse transcribed to cDNA using random hexamers and the Gene Amp RNA PCR core kit according to the manufacturer’s protocol. Primer sequences are as follows, from the 5’ to the 3’ end: TRB3 in 3T3-L1 cells (mouse): forward TCTCCCTCCGGCAAGGAACT, reverse TCTCAACCGGGAATGCAAGAG; adiponectin: forward AGGAGATCGAAGTAGCTGG, reverse CCAACACTGAAATGTGTA; GAA, reverse AGGTGAAAGGCAGGGACTCA. RT-PCR was performed on an Applied Biosystems ABI Prism 7000 Sequence Detection System, using SYBR Green PCR Master Mix. Twenty-five microliters of reaction volume were used per well, and all samples were run in duplicate. The expression of target genes was normalized to the 3’ end with 18S RNA measured simultaneously.

**Glucose transport.** 3T3-L1 adipocytes, with or without pretreatment with 100 nM Dex for 18 h, as described above, were deprived of FBS for 2 h and then incubated in glucose-free Krebs-Ringer bicarbonate-HEPES buffer at 37°C without or with 100 nM insulin for 15 min. Glucose transport was initiated by the addition of [14C]glucose (50 μM, 0.05 μCi/ml) and [3H]glucose (50 μM, 0.05 μCi/ml) was added as an extracellular space marker. After 3 min at 37°C, 2-DG transport was terminated by the addition of phloretin (48 μM). The cells were placed on ice, washed 3 times with ice-cold PBS, and solubilized with 1% Triton X-100. 3H and 14C concentrations in the cell extracts were determined by liquid scintillation spectrometry. The intracellular concentration of 2-DG was calculated by correcting for the label present in the extracellular space and was normalized to the protein concentration in the extracts, which was measured spectrophotometrically against BSA standards using Coomassie protein assay reagent (18).

**Western blot for Akt, phospho-Akt, and CHOP.** In experiments where insulin-induced Akt phosphorylation was measured, cells grown on 100-mm dishes, preincubated under different conditions, were deprived of insulin (if present) and FBS for 2 h and then acutely stimulated with 100 nM insulin for 15 min. After being washed twice with HEPES-EDTA-sucrose (HES) buffer (20 mM HEPES (pH 7.4), 1 mM EDTA, 255 mM sucrose, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 μM microcystin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml aproptin), cells were lysed by shearing them 10 times through a 22-gauge needle. We chose to measure insulin-stimulated Akt activation in crude plasma membrane (PM) preparation, because membranes are enriched in activated Akt (19). Cell lysates were centrifuged at 19,000 g for 20 min at 4°C. The pellet was layered onto a 1.12-M sucrose cushion that was centrifuged at 100,000 g for 60 min. The PM layer was collected and centrifuged at 40,000 g for 20 min. The pellet was resuspended in HES buffer containing 1% Triton X-100 and solubilized for 1 h at 4°C. It was then centrifuged at 100,000 g for 10 min, and the supernatant was analyzed by SDS-PAGE, loading 20 μg of protein/lane (19). For quantitation of CHOP protein, cells from 35-mm dishes were lysed as described above in 50 mM HEPES (pH 7.4), 1 mM EDTA, and 150 mM NaCl containing the...
protease inhibitors described above. To prepare total lysates, adipocytes were centrifuged for 5 min at 1,500 \( g \). The infranatant was saved and adjusted to 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS, solubilized for 1 h at 4°C, and centrifuged for 5 min at 10,000 \( g \). The supernatant was analyzed by SDS-PAGE, loading 20 \( \mu \)g of protein/ lane. Proteins were then transferred to polyvinylidene difluoride membranes in 25 mM Tris, 192 mM glycine, and 10% methanol. After transfer, membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline and 0.1% Tween-20 and probed with either a polyclonal antibody specific for Akt1/2 (Santa Cruz Biotechnology, Santa Cruz, CA), a phosphospecific polyclonal antibody used to detect the phosphorylation status of Akt on Ser473, a \(-tubulin\) rabbit antibody, or anti-CHOP antibody. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies and enhanced chemiluminescence substrate kit were used for protein detection (19).

**Glucose measurement.** Glucose concentrations in the medium were measured using the Beckman glucose analyzer.

**Statistical analysis.** Correlation between two parameters and the significance of the correlation was determined using Pearson correlation analysis. The significance of difference between means was evaluated by two-tailed unpaired Student’s \( t \)-test or by one-way ANOVA. Multiple comparison techniques with Dunnett’s test for all pairwise comparisons vs. a control or Tukey’s test for all pairwise comparisons were used to further examine the differences given an overall significant ANOVA \( F \)-test. \( P < 0.05 \) was considered significant.

**RESULTS**

**TRB3 does not mediate glucose toxicity in 3T3-L1 adipocytes but increases with glucose deprivation.** TRB3 mRNA expression in 3T3-L1 adipocytes incubated for 18 h in 5 mM glucose (G5), 5 mM glucose with 0.6 nM insulin (G5I), 25 mM glucose (G25), and 25 mM glucose with 0.6 nM insulin (G25I) was assessed. TRB3 mRNA level was significantly increased twofold in G5I but was not increased in G25I (Fig. 1A). Since insulin-resistant glucose transport develops only after incubation in G25I, but not under the other conditions (18, 19), we concluded that increased TRB3 expression does not mediate insulin resistance in this model. Incubation of cells for 18 h in glucose concentrations of 0, 2.5, 5, 10, and 25 mM (G0, G2.5, G5, G10, and G25, respectively), or in G5I and G25I, revealed an increase in TRB3 mRNA expression in G2.5 by 47% and a 4-fold increase in G0. Furthermore, there was a significant negative correlation between TRB3 mRNA level and the final glucose concentration in the medium at the end of the 18-h incubation (Fig. 1B).

Higher levels of TRB3 mRNA at low glucose concentrations are due to increased mRNA synthesis and occur later than 6 h postincubation. One microgram per milliliter actinomycin D, an inhibitor of mRNA transcription (5), was added to cells incubated in either 0 or 25 mM glucose (G0 and G25, respectively). TRB3 mRNA expression was assessed at baseline, 1, 2, and 6 h after incubation and plotted against time (Fig. 2A). TRB3 mRNA levels decreased to \( \approx 50\% \), 2 h postincubation, in both G0 and G25. Furthermore, the trend line for log TRB3 mRNA expression was similar for G0 and G25, denoting an equal rate of decay.

To evaluate the time required for TRB3 and CHOP mRNA to increase in 3T3-L1 cells incubated in no glucose, we
conducted a time course experiment where RNA was isolated at times 0, 2, 4, 6, 8, and 12 h after incubation in medium devoid of glucose (Fig. 2B). Compared with baseline, CHOP mRNA was significantly increased as early as 4 h after incubation, whereas TRB3 mRNA was significantly increased 12 h after incubation, but not at the earlier time points.

The glucose deprivation-induced increase in TRB3 mRNA may be mediated by the UPR. 3T3-L1 cells incubated in G0 for 18 h, but not in G25 or in Dex, had a 93% increase in GADD153/CHOP protein (growth arrest and DNA damage-inducible gene, CHOP) compared with G5 (Fig. 3A), a result consistent with previous reports of glucose deprivation causing the UPR (25). To further investigate the relationship between UPR and TRB3, we tested the effect of tunicamycin, an inducer of the UPR by prevention of protein glycosylation (2) on CHOP protein and on TRB3 mRNA expression. Tunicamycin caused an increase in CHOP protein (Fig. 3B) and in TRB3 mRNA levels as early as 4 h after treatment, with the peak increase being between 6 and 8 h postincubation (Fig. 3C).

Glucose depletion increases TRB3 mRNA level in L6 myotubes. L6 myotubes are widely used as a model for skeletal muscle, since they express GLUT4 and accelerate glucose transport in response to insulin. Consistent with our results in fat cells, L6 cells incubated without glucose or with 2.5 mM glucose had a 6- and 2-fold increase in TRB3 mRNA expression, respectively, compared with those incubated in 5 mM glucose (Fig. 4).

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**A**

CHOP10 26 kDa  

b-tubulin 50-55 kDa

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**B**

CHOP10 26 kDa  

b-tubulin 50-55 kDa

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**C**

![Graph showing the effect of tunicamycin on TRB3 expression](https://via.placeholder.com/150)

Fig. 3. Relationship between TRB3 and the unfolded protein response. A: Western blot for CHOP protein in 3T3-L1 adipocytes incubated overnight with 0, 5, 25, or 5 mM glucose with 100 nM dexamethasone (Dex). CHOP protein increased by 93% in cells incubated in no glucose (*P < 0.01, n = 16), but not in the other conditions. B: addition of 2.5 μg/ml tunicamycin for 4 h to 3T3-L1 adipocytes incubated in 5 mM glucose (G5) caused a 3-fold increase in CHOP protein (*P < 0.01, n = 4). C: real-time PCR analysis of TRB3/18S mRNA in 3T3-L1 adipocytes exposed to 2.5 or 5 μg/ml tunicamycin for variable lengths of time, showing a significant increase in TRB3 expression (*P < 0.05, **P < 0.01, n = 4–8).
Trb3 increases Trb3 mRNA expression in 3T3-L1 adipocytes but not in L6 myotubes. In 3T3-L1 adipocytes, exposure to 100 nM Dex for 18 h increased Trb3 mRNA expression 2-fold while decreasing adiponectin mRNA expression by one-half (Fig. 5A and B). In L6 myotubes, exposure to Dex at the same dose and for the same duration of time decreased Trb3 mRNA expression by 16% (Fig. 5C). In 3T3-L1 cells, basal and acutely insulin-stimulated glucose transport were significantly enhanced after preincubation with 100 nM Dex (Fig. 5D). PM-associated Akt phosphorylation decreased by 18% after incubation in 100 nM Dex, by 33% after incubation in 5 mM glucose with 0.6 nM insulin, and by 50% after incubation in 25 mM glucose with 0.6 nM insulin, whereas no change was seen in cells incubated in no glucose, 2.5 mM, 10 mM, or 25 mM glucose (Fig. 5E). We previously reported that total Akt expression was unchanged after preincubation with the various glucose concentrations, with or without 0.6 nM insulin (19). Preincubation with 100 nM Dex had no effect on the expression of total Akt, as determined by Western blot of the PM preparation (99.5 ± 14.1% of that observed in controls, n = 10; data not shown).

**DISCUSSION**

We (19) have previously shown that preincubation of 3T3-L1 adipocytes for 18 h in medium containing 0.6 nM insulin in the presence of 5 or 25 mM glucose resulted in decreased Akt translocation to the PM in response to acute insulin stimulation, as well as a decrease in serine (and threonine) phosphorylation of PM-associated Akt. The inhibition of Akt activation was greater in cells preincubated in G2.5 than in G5, and only the former developed insulin resistance, as shown by a decrease in the acute maximal insulin-stimulated glucose transport (18, 19). Given the recently identified role of Trb3 as a negative modulator of Akt activity in the liver of mice (6), we sought to investigate a possible role for Trb3 in mediation of glucose toxicity in fat cells by interfering with Akt in cells incubated with high glucose in the presence of insulin. Contrary to the anticipated results, Trb3 mRNA expression did not increase in G2.5 but did increase in G5. We hypothesized that such an increase is not a direct insulin effect but is the result of decreased glucose concentrations in the medium due to incubation with insulin for 18 h. Indeed, G0 and G2.5 had a significantly higher Trb3 level than G5, both in 3T3-L1 and in L6 cells (Figs. 1B and 4). Trb3 mRNA expression negatively correlated with glucose concentrations in medium at the end of incubation, whether the paucity of glucose was the result of preincubation at low glucose concentrations (G0 and G2.5) or of glucose consumption by cells due to the presence of insulin (G5; Fig. 1B). While this article was in preparation, the induction of Trb3 by glucose or amino acid deprivation in PC3 cells was reported, which is consistent with our findings (29). Trb3 seems to be induced by some cellular stressors, such as ER stress (3, 20–22) and nutrient starvation (29), but not by other stressors, such as osmotic stress or serum deprivation (29).

Next, we found the rate of decline of Trb3 mRNA expression in cells incubated in 25 mM or no glucose, in the presence of actinomycin D, to be similar, suggesting that the increased Trb3 mRNA expression in the absence of glucose is mediated by an increased rate of transcription rather than increased mRNA stability (Fig. 2A). The second observation was that the Trb3 mRNA half-life was less than 2 h. Since the increase in Trb3 mRNA expression with glucose deprivation took 12 h to occur (Fig. 2B), low glucose may not be exerting its effects directly on Trb3 gene expression but may be acting through an intermediary event, such as the UPR.

UPR is a complex pathway initiated in response to accumulation of unfolded proteins in the ER lumen, causing an enhanced transcription of genes involved in ER protein folding and a generalized slowing of protein synthesis at the level of translation, presumably to alleviate the ER load (2, 15, 24, 27). Glucose deprivation (15, 25) and tunicamycin (2) are known inducers of the UPR. The mammalian transcription factor ATF4 (9) and the proapoptotic transcription factor CHOP (34) are downstream UPR mediators. ATF4 mediates thapsigargin and arsinite-induced activation of Trb3 promoter in HepG2 cells (21). Furthermore, ATF4 and CHOP were shown to cooperate in mediation of tunicamycin-dependent Trb3 promoter induction (20). In our hands, glucose deprivation significantly increased Chop mRNA as early as 4 h after incubation (Fig. 2B). Both lack of glucose and tunicamycin exposure increased Chop protein in 3T3-L1 adipocytes (Fig. 3A and B). Tunicamycin increased Trb3 mRNA expression (Fig. 3C). These findings are consistent with glucose deprivation inducing the UPR, and upregulating Trb3 mRNA, possibly through the ATF4/CHOP pathway. However, we observed no effect of increased Trb3 expression on insulin-induced Akt activation (compare G0 with G5; Fig. 5E). Although Akt activation was decreased in cells preincubated in G5 (with increased Trb3 induction), it was even more suppressed in cells preincubated in G25I, where Trb3 was unchanged. Thus the glucose/insulin-induced downregulation of Akt activation (19) appears to be independent of Trb3 expression.

Glucocorticoids cause insulin resistance in fat and muscle in part by decreasing insulin-stimulated Akt phosphorylation (4, 28). Since Trb3 negatively regulates Akt (6), we investigated the potential role of Trb3 in glucocorticoid-induced insulin resistance. In agreement with previous studies (4, 28, 7), exposure of 3T3-L1 cells to Dex mildly decreased PM-associated Akt phosphorylation, basal, and acutely insulin-stimulated glucose transport and adiponectin mRNA expression. Trb3 mRNA levels increased twofold in 3T3-L1 adipocytes, but no increase was found in L6 myotubes (Fig. 5A and C). A similar discrepancy was reported, with Dex causing a two- to threefold
induction of TRB3 mRNA in Fao hepatocytes (6) but no change in primary rat hepatocytes (11). Tissue and/or species specificity may explain the variability in glucocorticoid effects on TRB3 mRNA expression in different cells. Although Dex induced TRB3 mRNA in 3T3-L1 adipocytes, this effect may not account for the glucocorticoid-induced insulin resistance, since Akt phosphorylation was not impaired with glucose deprivation despite an even greater TRB3 mRNA induction. It should be noted, however, that mRNA expression does not necessarily correlate with protein expression. We were unable to quantify TRB3 protein because the available antibody (6) was not sensitive enough to detect endogenous TRB3 in our hands.

In conclusion, TRB3 does not mediate glucotoxicity in 3T3-L1 adipocytes and is probably not the mediator of glucocorticoid-induced insulin resistance. It is induced by glucose deprivation in 3T3-L1 adipocytes and in L6 myotubes, likely as a part of the UPR. Glucocorticoids induce TRB3 mRNA in 3T3-L1 and decrease its expression in L6 cells. By its interactions with ATF4/CHOP, TRB3 may be involved in regulation...
of cell survival in response to stressful conditions, such as nutrient starvation. Although TRB3 can interact with Akt in vitro and has been shown to blunt Akt activation when markedly overexpressed in liver cells (6), the moderate changes in TRB3 expression observed in 3T3-L1 adipocytes (2- to 4-fold) did not blunt insulin-induced Akt activation. One needs to keep in mind that TRB3 also appears to regulate the activation of several MAPKs, likely at the MAPKK step, and that the effects are cell type specific and can both synergize and antagonize (12). In view of recent reports linking ER stress to peripheral insulin resistance and to the development of atherosclerosis (23, 32), further studies are needed to elucidate the role of TRB3 in apoptosis and in diabetes.

ACKNOWLEDGMENTS

We thank Dr. Richard Klein for the gift of adiponectin primers, Dr. Gian Re for helpful discussions, and Dr. Amira Klip for the gift of L6 myoblasts. This work was presented in part at the 65th Annual Meeting of the American Diabetes Association, June 2003 (San Diego, CA), and was published as an abstract (Diabetes 54, Suppl 1: A322, 2005).

GRANTS

This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-02001 to M. G. Buse.

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