TRIB3 Mediates Glucose-Induced Insulin Resistance

Via a Mechanism that Requires the Hexosamine Biosynthetic Pathway

Wei Zhang1*, Jiarong Liu2, Ling Tian1, Qinglan Liu2, Yuchang Fu1, and

W. Timothy Garvey1,3

1 Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, AL 35294-3360, USA
2 Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35223-7331, USA
3 Birmingham Veterans Affairs Medical Center, Birmingham, AL 35233, USA

Running Title: Glucose Induction of TRIB3 Requires the Hexosamine Biosynthetic Pathway

*Correspondence to: Wei Zhang, MD
Department of Nutrition Sciences
The University of Alabama at Birmingham
1825 University Boulevard
Birmingham, AL 35294-3360
Tel: 205-996-5620
Fax: 205-996-5896
Email: siweizh@uab.edu
ABSTRACT

In the current study, we studied the role of TRIB3 in glucose-induced insulin resistance, and whether the induction of TRIB3 by glucose is dependent upon the nutrient-sensing hexosamine biosynthetic pathway (HBP) known to mediate glucose toxicity in diabetes.

In diabetic rats, TRIB3 expression in skeletal muscle was increased after 10 days of hyperglycemia, both glycemia and muscle TRIB3 were restored towards normal by insulin therapy. In L6 myocytes, the induction of TRIB3 by high glucose or glucosamine was reversible upon removal of these substrates. To assess the role of HBP in the induction of TRIB3, we demonstrated that the ability of high glucose to augment TRIB3 expression was prevented by azaserine, an inhibitor of GFAT which is the rate-limiting enzyme in the HBP pathway. TRIB3 expression was also substantially stimulated by glucosamine which bypasses GFAT, accompanied by decrease in insulin-stimulated glucose transport rate, and both responses were not affected by azaserine. Further, knockdown of TRIB3 inhibited, and TRIB3 overexpression enhanced, the ability of both high glucose and glucosamine to induce insulin resistance.

These data provide the mechanistic link between the HBP flux and insulin resistance, and point to TRIB3 as a novel target for treatment of glucose-induced insulin resistance.

KEYWORDS: TRIB3; nutrient sensor; hexosamine biosynthetic pathway; insulin resistance; glucose transport system; glucose toxicity.
INTRODUCTION

Insulin resistance is a major metabolic defect that helps establish and sustain hyperglycemia in Type 2 Diabetes Mellitus (T2DM) diabetes, and involves impaired insulin-stimulated glucose uptake into skeletal muscle (1; 2). A component of insulin resistance in diabetic patients is induced by hyperglycemia itself (i.e., ‘glucose toxicity’) (3). Patients with Metabolic Syndrome and/or Prediabetes are insulin resistant; however, as glucose tolerance deteriorates into overt T2DM, the superimposition of hyperglycemia worsens overall insulin resistance. This latter component of insulin resistance is known as glucose-induced insulin resistance or glucose toxicity (3-6). Intensive therapy leading to euglycemia can reverse glucose-induced insulin resistance, and put diabetes into remission particularly in recently-diagnosed patients, whether by weight reduction (7), sulfonylureas (8; 9), or insulin therapy (3), and the increase in whole-body insulin sensitivity is paralleled by increased glucose transport rates in adipocytes (10) and skeletal muscle (7). Likewise, patients with T1DM in poor glycemic control exhibit insulin resistance which can be reversed by intensified insulin therapy (11). Rats made diabetic by streptozotocin (STZ) exhibit a reduction in insulin-stimulated glucose transport in muscle and fat, which can be reversed by euglycemia induced by exogenous insulin or by promotion of glycosuria with phlorizin (12; 13). Finally, multiple in vitro studies demonstrate direct effects of glucose to impair insulin-stimulated glucose transport in perfused target tissues (14) and cultured cell systems (15; 16). Thus, a large body of data supports the contention that glucose per se can induce desensitization of insulin's action to stimulate glucose uptake.

The mechanism by which glucose induces insulin resistance involves decreased activity of the glucose transport effector system and impaired translocation of intracellular GLUT4 glucose transporters to the cell surface in adipocytes and skeletal muscle (15; 17; 18). Furthermore, Marshall et al have shown that the ability of glucose to regulate its own uptake is dependent on its intracellular metabolism via the hexosamine biosynthetic pathway (19-22). The first and rate-limiting enzyme for
this pathway is glutamine:fructose-6-P amidotransferase (GFAT), which converts fructose-6-P to glucosamine-6-P, and the major end-product, N-acetylglucosamine-6-P, serves as the substrate for both N-linked and O-linked protein glycosylation. Studies have established that O-linked glycosylation, catalyzed by UDP–N-acetylglucosaminyl transferase (OGT), is linked to the induction of insulin resistance (23; 24), and that this action involves effects on gene transcription (22). Involvement of hexosamine pathway products in glucose-induced insulin resistance is classically supported by two key observations: (a) azaserine, a glutamine antagonist which irreversibly inhibits GFAT, specifically blocks the ability of glucose to regulate glucose transport, and (b) glucosamine, which enters the hexosamine pathway distal to GFAT, can alone impair the glucose transport system with high potency in a manner that is not affected by azaserine.

While glucose-induced insulin resistance involves augmented glucose metabolism via the hexosamine pathway, the actual identity of genes that diminish glucose transport system activity remain unknown despite more than a decade of study. We now demonstrate for the first time that glucose-induced insulin resistance is dependent upon induction of TRIB3 in a process that requires HBP metabolism. Our interest in TRIB3 was first initiated when we used high-density cDNA microarrays (25; 26) to identify TRIB3 as an upregulated gene in skeletal muscle from T2DM patients. Our subsequent work demonstrated that TRIB3 protein levels were elevated in skeletal muscle from hyperglycemic rodent models and from T2DM patients in a manner that was correlated with fasting glucose, that TRIB3 levels were regulated by media glucose concentrations in L6 cells, and that overexpression of TRIB3 blocked insulin-stimulated glucose transport rates (27). These data suggested that TRIB3 is a glucose-responsive gene that could mediate insulin resistance in muscle. The tribbles homolog 3 (TRIB3, also named as TRB3, NIPK, SIKP3) has been identified as an inhibitor of Akt activity by physically binding to its phosphorylation site. TRIB3 has also been reported to affect a number of functions, such as pancreatic beta-cell survival and insulin secretory capacity, adipose and
muscle cell differentiation, as well as endoplasmic reticulum (ER) stress (28; 29). However, in addition to the AKT signaling pathway, studies show alternative pathways through which TRIB3 can exert effects on metabolism such as the PPAR-gamma signaling cascade (30). The current study demonstrates that TRIB3 is a mechanistic link between HBP and glucose-induced insulin resistance.

MATERIALS AND METHODS

**Diabetic rat.** All experimental procedures were approved by the Animal Care Committee of University of Alabama at Birmingham, AL, USA. Hyperglycemia was induced by streptozotocin (STZ, 50mg/kg BW, IP). Five-week-old, male Sprague-Dawley rats (Charles River, USA) were placed on ad libitum normal chow diet and randomized to three groups: control, diabetic, and diabetic + insulin treated (Humalog®, 1.28U/100g ip, twice a day). Blood glucose was measured using a Sirrus chemistry auto analyzer (Stanbio Laboratory, TX); tissue samples were snap-frozen in liquid nitrogen and stored at -80°C for later use.

**Glucosamine injection.** Eight-week-old C57BL/6 mice (Jackson Laboratories) were given standard chow diet and housed in a 12-h light/dark cycle. After one week of accommodation, mice in treatment group were injected with glucosamine (70mg/kg/day, tail vein injection) for seven days, and control mice received injections with equal volumes of PBS buffer. Mice were euthanized and tissue samples were snap-frozen in liquid nitrogen for later experiment.

**Cell culture.** Rat L6 myoblasts (ATCC, VA) were cultured in DMEM (5mM glucose, Invitrogen, CA) supplemented with 10% fetal bovine serum (FBS) , 100 units/ml penicillin, and 100 mg/ml streptomycin. Upon reaching 80% confluence, the culture medium was changed to DMEM with 2% horse serum (5mM glucose, Invitrogen, CA) to induce differentiation and refreshed every 2 days. L6 myotubes were fully differentiated after 7 days and ready for experiments. 3T3-L1 cells were grown in DMEM containing 25 mM glucose and 10% calf serum. Two days after full confluence, cells
started differentiating in DMEM containing 25 mM glucose, 0.5 mM isobutylmethylxanthine, 1 µM
dexamethasone, 10 µg/ml insulin, and 10% FBS for 3 days and then in DMEM containing 25 mM
 glucose, 10 µg/ml insulin and 10% FBS for 2 days. After which, cells were maintained in DMEM with
5 mM glucose and 10% FBS until used for experiments on 10–14 days after initiation of the
differentiation. For experiment, cells were cultured in 5mM glucose, 25mM glucose, or 25mM glucose
with azaserine for 24 hours.

**Lentiviral-mediated overexpression and knockdown of TRIB3.** Wild-type TRIB3 gene was
kindly provided by Dr. Kiss-Toth (University of Sheffield, UK). Lentivirus vector was constructed and
packaged by ADV Bioscience (Birmingham, AL). The stop codon in the TRIB3 cDNA was removed
and c-Myc was attached to the C terminal. The modified cDNA was ligated into lentivector (pHR-EF-
IRE5-Bla) at the BamHI and XhoI sites. DNA sequencing was used to ensure proper insertion.
Lentiviral shRNA (Sense: 5'- GAT CCA GGA AGA AAC CGT TGG AGT TTG TCA AGA GCA
AAC TCC AAC GGT TTC TTC CTT TTT GG -3'; Antisense: 5'- AAT TCC AAA AA GGA AGA
AAC CGT TGG AGT TTG CTC TTG ACA AAC TCC AAC GGT TTC TTC CTG -3') locates at
rat TRIB3 cDNA position 212 (NM_144755). To establish stably transfected cell lines, procedures
were performed as previously described (27).

**Glucose transport assays.** Glucose transport rates were measured in L6 muscle cells as
previously described (31). Briefly, same numbers of L6 cells were plated in each well. Cells were
grown and differentiated into myotubes as described above. Insulin resistance was induced by
culturing L6 myotubes in 25mM high glucose or 5mM glucose medium with 1mM glucosamine for 18
to 24hrs. Myotubes were incubated in serum free DMEM for 3 hours, and followed by 45 minutes
incubation in the absence or presence of 100 nM insulin stimulation. Basal and the maximally insulin-
stimulated 2-D glucose transport rates were determined and normalized to total protein quantity as
described (31). Percent decrement of glucose transport of myotubes cultured in 25mM glucose or 1mM
glucosamine as compared to controls in 5mM glucose was calculated as an indicator of glucose/glucosamine-induced insulin resistance.

**RNA isolation and analysis.** RNeasy columns (Qiagen, CA) were used to isolate total RNA from L6 cells, and total RNA from tissue samples was extracted using Trizol reagent (Invitrogen). cDNA was synthesized by VILO kit (Invitrogen) following the manufacturer’s instructions. StepOnePlus™ 96-well machine (Applied Biosystems, CA) was applied for real-time quantitative PCR analysis. PCR products were detected using Sybr Green and normalized to 18S ribosomal RNA, using the following sequences: rat 18S, 5’-GGAGGATGAGGTGGAGCGAGT-3’ (5’ primer) and 5’-GCCTCTCCAGGTCTCACGC-3’ (3’ primer); rat TRIB3, 5’-AGAGTCTGGAAACGGGTATT-3’ (5’ primer), 5’-AGTTGCGTCGATTTGCTTC-3’ (3’ primer).

**Protein isolation and immunoblot.** Proteins were extracted from tissues or cells in lysis buffer. The Bicinchoninic Acid kit (Sigma, USA) was used for quantifying protein concentrations. Membranes were incubated with anti-TRIB3 antibody (Calbiochem, or SantaCruz) or anti-O-GlcNAc antibody (CTD110.6 mouse mAb, Cell Signaling) and followed by anti-rabbit or anti-mouse IgG (Santa Cruz). Images were captured by using enhanced chemiluminescence (Pierce) on a ChemiDoc™ XRS imager (BioRad), and Image Lab software (BioRad) was used for quantification.

**Statistical analysis.** Experimental results are shown as the mean ± S.D. Statistical analyses were conducted using the unpaired Student's t test assuming unequal variance unless otherwise indicated. Significance was defined as *p<0.05 or **p<0.01.

**RESULTS**

Increased TRIB3 expression parallels with up-regulated protein O-GlcNAc modification levels.
Increased protein O-GlcNAc modifications are classically observed under high glucose/hyperglycemia conditions (19-22), however, recent studies have shown up-regulated protein O-GlcNAcylation levels in cancer cells and cardiomyocytes under glucose deprivation (32-34). In the current study, we observed up-regulated protein O-GlcNAc modification levels under both glucose deprivation (0mM) and high glucose (25mM) conditions in cultured L6 muscle cells (Figure 1). Consistent with our previous finding (27), TRIB3 expression was significantly induced by both glucose deprivation and high glucose (Figure 1). This paralleled up-regulation of TRIB3 expression and protein O-GlcNAcylation levels suggests us that hexosamine biosynthetic pathway could participated in the regulation of TRIB3 by glucose.

**TRIB3 expression is induced by high glucose and glucosamine in a reversible manner.** We previously reported that TRIB3 expression was up-regulated in skeletal muscle from hyperglycemic T2DM patients and insulin-resistant rodent models, and was induced by exposure to high glucose in L6 myotubes (27). In Figure 2A, we have confirmed that TRIB3 expression is augmented in L6 myotubes treated with 25 mM glucose DMEM medium for 24 hours (~2.3 fold, P<0.05, Fig.2A) when compared with cells incubated with 5 mM glucose. We now further demonstrate that the increase in TRIB3 protein induced by exposure to 25 mM glucose is restored to baseline over the subsequent 24 hours in the presence of 5mM glucose DMEM medium (P<0.05, Fig.2A). Similarly, TRIB3 protein was induced in L6 cells treated with 1 mM glucosamine, and this effect was also reversed upon removal of glucosamine as TRIB3 levels returned towards baseline (Fig. 2B). Consistently, this induction and restoration of TRIB3 expression were associated with up-regulation and down-regulation of protein O-GlcNAcylation levels (Fig. 2). To assess whether the glucose-responsive expression of TRIB3 was reversible in vivo, we induced diabetes in rats by administering moderate dose streptozotocin, and observed a ~2-fold increase in TRIB3 protein levels in skeletal muscle (p<0.05). As shown in Figure 3, restoration of euglycemia using exogenous insulin
injections was accompanied by a reduction in muscle TRIB3 to baseline levels. These results indicate that the induction of TRIB3 in muscle by high glucose can be reversed in the presence of lower glucose concentrations both in vitro and in vivo.

**TRIB3 expression is induced by hexosamine biosynthetic pathway metabolism both in vitro and in vivo.** Based on previous reports that glucose induced-insulin resistance required glucose metabolism via the hexosamine biosynthetic pathway (HBP) (16-22), we hypothesize that induction of TRIB3 by glucose was also dependent upon the HBP. To test this idea, we cultured L6 myotubes in high glucose medium with and without azaserine, an inhibitor of GFAT, the rate limiting enzyme for glucose metabolism via the HBP. As shown in Figure 4A, azaserine prevented the induction of TRIB3 by 25 mM glucose. On the other hand, glucosamine, which enters the HBP distal to GFAT, increased TRIB3 protein levels in the muscle cells to the same extent as 25 mM glucose; however, azaserine was not able to block induction of TRIB3 by glucosamine. The increase in TRIB3 induced by both glucose and glucosamine was accompanied by reductions in both basal and insulin-stimulated glucose transport activity as shown in Fig. 5, consistent with the functional consequences that HBP metabolism exerts on the glucose transport effector system (16-22). Since we had earlier shown that glucosamine infusion causes insulin resistance in rats (17; 18), we injected mice daily with glucosamine, and observed a 2.6-fold increase in skeletal muscle TRIB3 protein as well as a 64.7% decrease in phosphorylation of Akt at Thr308 (both p<0.05) compared with control rats injected with saline (Figure 4 B). We further examined the role of the HBP and TRIB3 induction in another insulin target cell, namely 3T3-L1 adipocytes. In fully differentiated adipocytes, TRIB3 protein expression was also significantly induced by high glucose, and, consistent with the findings in L6 muscle cells, this induction of TRIB3 was also blocked by azaserine (Figure 6). These results showing effects of both glucose and glucosamine, with the ability of azaserine to block the action of glucose but not glucosamine, fulfill the classic criteria for demonstrating that the effects are dependent upon HBP metabolism.
**Induction of insulin resistance by both glucose and glucosamine is dependent upon TRIB3.**

We previously showed that TRIB3 knockdown in L6 muscle cells led to significant increase in both basal and insulin-stimulated glucose uptake rates along with enhanced phosphorylation of insulin receptor substrate-1 (IRS-1) at Tyr612 and insulin-stimulated phosphorylation of Akt on Ser473. To determine whether TRIB3 is necessary for glucose-induced insulin resistance, we treated control and TRIB3 knockdown muscle cells with high glucose and glucosamine. Figure 7 demonstrates that incubation of control cells for 24 hrs in 25 mM high glucose (Fig. 7B), or in 1mM glucosamine (Fig. 7C), led to significant decrements in both basal and insulin-stimulated glucose transport rates. Moreover, in TRIB3 knockdown cells, the ability of glucose and glucosamine to decrease basal and insulin stimulated glucose transport rates was reduced by ~50%. These results demonstrate that glucose-induced insulin resistance is at least partially dependent on TRIB3.

**DISCUSSION**

In the current study, we show that the ability of high glucose to diminish glucose transport system activity in muscle cells is dependent upon induction of TRIB3 and glucose metabolism via the HBP. When combined with our previous observations, the novel current results provide critical information to indicate that induction of TRIB3 via the HBP is relevant to the pathophysiology of glucose toxicity in diabetes. In prior studies, we have shown that TRIB3 expression is significantly increased in skeletal muscle biopsies from patients with T2DM when compared with insulin sensitive
individuals, and is positively correlated with fasting blood glucose levels (27). Also, elevated muscle TRIB3 expression was observed in multiple hyperglycemic rodent models including STZ-induced diabetic rats, Zucker diabetic fatty rats, and db/db mice (27). In vitro, incubation of cultured L6 myocytes in the presence of high glucose, under conditions that reduce basal and insulin-stimulated glucose transport rates, increased TRIB3 mRNA and protein expression (27). Finally, stable overexpression of TRIB3 in L6 muscle cells employing a lentiviral expression vector led to suppression of insulin-stimulated glucose uptake and GLUT4 translocation to the cell surface, as well as insulin-stimulated Akt phosphorylation (27). These previous studies established that TRIB3 is a glucose responsive gene in muscle, and can reduce basal and insulin-stimulated glucose transport activity together with insulin-stimulated GLUT4 translocation and Akt phosphorylation.

Our previous data notwithstanding, it was still not clear whether TRIB3 induction was responsible for glucose-induced insulin resistance. The current data address this issue by addressing three critical aspects that pertain to the pathophysiology of glucose toxicity. First, we found that the induction of TRIB3 is reversible under conditions that restore full activity of the glucose transport effector system. This was the case in vitro after removal of high glucose or glucosamine from the medium, and in vivo when streptozotocin-induced diabetic mice were made euglycemic by treatment with exogenous insulin. These results are consistent with studies in poorly-controlled patients with T2DM or T1DM demonstrating that there is a component of insulin resistance that can be reversed by several weeks of intensive insulin treatment (3-14). The second criterion was that TRIB3 induction in response to high glucose should involve increased glucose metabolism via the HBP, which has been shown to mediate glucose toxicity in cell models and in vivo systems including diabetic patients (19-22). The current data demonstrate that the induction of TRIB3 fulfills the classic criteria (16-22) for participation of the HBP, namely that (a) azaserine, an antagonist of GFAT, the rate limiting enzyme for the pathway blocked the ability of glucose to augment TRIB3 expression, and (b) glucosamine,
which enters the hexosamine pathway distal to GFAT, can alone impair the glucose transport system with high potency in a manner that is not affected by azaserine. The third aspect of the current data is that shRNA-mediated suppression of TRIB3 in L6 myocytes blocked the ability of high glucose and glucosamine to diminish basal and insulin-stimulated glucose transport rates by ~50% compared with that observed in control cells. Furthermore, TRIB3 over-expression increased the ability of high glucose and glucosamine to decrease insulin-stimulated transport without modulating effects on basal transport activity. Therefore, the current data indicate that glucose-induced insulin resistance is, at least in part, dependent upon induction of TRIB3 which in turn is dependent upon glucose metabolism via the HBP. Thus, TRIB3 is a missing link between HBP metabolism and diminished activity of the glucose transport effector system under conditions of glucose toxicity.

The pathophysiological role of TRIB3 in mediating glucose toxicity with chronic hyperglycemia should not be confused with the short-term physiological role of TRIB3, which regulates nutrient metabolism during feeding and fasting (27). We found that short-term fasting in rats enhanced whole-body insulin sensitivity concomitant with decrements in TRIB3 mRNA in muscle and increments of TRIB3 in adipose tissue, when compared to non-fasted controls. On the other hand, rats fed a western diet for 7 days became insulin resistant concomitant with increments in TRIB3 in muscle and a decrease in adipose. Furthermore, TRIB3 upregulation impaired insulin sensitivity at the cell level while TRIB3 downregulation led to an increase in basal and insulin stimulated glucose transport rates. Thus, TRIB3 influenced tissue glucose uptake oppositely in muscle and fat according to conditions of nutrient deprivation and excess (48). With nutrient excess, TRIB3 upregulation limits excessive uptake of glucose into muscle, while the decrease in TRIB3 expression in adipose leads to the increase in glucose uptake needed for glycerol/triglyceride synthesis; thereby, redirecting fuel from muscle to adipose tissue for storage. The opposite scenario accompanies nutrient deprivation. Fasting reduces muscle TRIB3 levels resulting in increased glucose transport in a setting where glycogen
stores are depleted. When the next meal is eventually consumed and insulin levels rise, these changes would facilitate the restoration of muscle glycogen stores. In adipose tissue, fasting increases TRIB3 and impairs glucose uptake under conditions when fat is geared for lipolysis as opposed to lipogenesis. These physiological roles for TRIB3 to acutely regulate fuel metabolism in fat and muscle during feeding and fasting are in contradistinction to the pathophysiological role in diabetes where chronic hyperglycemia results in persistent upregulation of muscle TRIB3, thus contributing to glucose-induced insulin resistance.

Remaining questions not fully addressed by the current study pertain to the mechanisms by which HBP metabolism leads to the increase in TRIB3 expression, and the explanation of how TRIB3 mediates desensitization of the glucose transport effector system. Regarding the former question, increased flux through the hexosamine pathway rapidly elevates UDP-N-acetylglucosamine levels (23; 24) providing substrate for both N-linked and O-linked protein glycosylation. O-GlcNAcylation has been reported as a common posttranslational modification regulating activity for many important cytosolic and nuclear proteins (35), including the transcription factors Sp1 (36), c-myc (37), CREB (38), Stat 5 (38), PDX-1 (39), and glycogen synthase (40). Studies have established that O-linked glycosylation is linked to the induction of insulin resistance (19; 23; 24), and that this action is dependent upon changes in gene transcription (22). Increased protein O-GlcNAc modifications are classically observed under high glucose/hyperglycemia conditions (19-22), however, recent studies have shown up-regulated protein O-GlcNAcylation levels under glucose deprivation in cancer cells and cardiomyocytes (32-34). In the current study, we found that protein O-GlcNAcylation levels were also paradoxically increased under no/low glucose and high glucose conditions in L6 myotubes. Additionally, the up-regulated protein O-GlcNAcylation levels was accompanied with induction of TRIB3 protein levels which strongly indicates that O-GlcNAcylation could be the mechanism via which HBP metabolism regulates TRIB3 gene expression. To date, despite its clinical importance, the
actual proteins being modified by O-glycosylation and the identity of regulated genes involved in glucose-induced insulin resistance have remained a mystery for 2 decades. The current data indicate that O-glycosylation involves proteins that produce induction of TRIB3 as a key gene mediating glucose-induced insulin resistance. In fact, the TRIB3 promoter region has three classes of binding sites that are compelling candidates for glucose regulation (41-44), including Sp1, E-Box, and USF binding sites. Sp1 was the first transcription factor shown to be regulated by O-glycosylation (45), and the regulation of TGF-β1 (46), PAI-1 (47), and acetyl-CoA carboxylase (48) are regulated by high glucose and/or glucosamine via posttranslational O-linked glycosylation of SP1. In any event, further studies are needed to address the mechanisms by which HBP metabolism results in induction of TRIB3.

The second question involves TRIB3 effects on insulin action and glucose transport. We (27; 49) and others (50) have shown that TRIB3 interacts with Akt and blocks its phosphorylation and activation in response to insulin. However, TRIB3 may exert its influence via other potential mechanisms. For example, TRIB3 acts like a scaffolding protein and interacts with multiple cytosol and nuclear proteins including several transcription factors (28), protein kinases (50) and other proteins (29) which lead to multiple biological effects including modulation of stress response, cell viability, and glucose or lipid metabolism.

In summary, our findings include: 1) The induction of TRIB3 is accompanied with up-regulation of protein O-GlcNAcylation levels under both glucose deprivation and high glucose conditions; 2) The induction of TRIB3 by high glucose and glucosamine in L6 myocytes is reversible upon removal of these substrates. 3) Strptozotocin-induced diabetes in rats is associated with increased expression of TRIB3 in skeletal muscle, and TRIB3 levels are restored to baseline by euglycemia achieved with exogenous insulin therapy. 4) TRIB3 induction under conditions of glucose toxicity involves increased HBP metabolism. 5) Knockdown of TRIB3 in L6 muscle cells significantly prevented, and TRIB3 overexpression enhanced, the development of insulin resistance induced by high
glucose or glucosamine. When considered in light of our previous data showing that muscle TRIB3 levels are increased in T2DM patients and that TRIB3 diminishes activity of the insulin-responsive glucose transport system, the current study substantiates the role of TRIB3 as a mechanistic link between the HBP metabolism and glucose-induced insulin resistance. The data identify TRIB3 as a novel potential target for treatment of glucose toxicity in diabetes.

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No potential conflicts of interest were reported relevant to this article.

Wei Zhang designed the study, performed experiments, analyzed the data and wrote, reviewed and edited the manuscript. Jiarong Liu, Ling Tian and Qinglan Liu helped with animal experiment and glucose transport experiment. Yuchang Fu and W. Timothy Garvey designed the study, interpreted the data and reviewed the manuscript. Wei Zhang is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES


FIGURE LEGENDS

Figure 1  Induction of TRIB3 is accompanied with up-regulation of protein O-Glycosylation levels

Panel A: L6 myotubes were cultured in medium contains 0mM, 2.5mM, 5mM, 10mM, 15mM and 25mM glucose for 24hrs. Representative western blot films of protein O-GlcNAc modification and TRIB3 protein expression were shown. Panel B: quantitative analysis of protein O-GlcNAc modification area. Panel C: quantitative analysis of TRIB3 protein. ** P < 0.01, results were expressed as means ± SD, n=3-6, results were calculated from three independent experiments.

Figure 2  TRIB3 is induced by high glucose and glucosamine (GlcN) in a reversible manner in culture L6 myotubes. Representative western blot films of protein O-GlcNAcylation levels and TRIB3 were shown. Panel A: L6 myotubes were cultured in 5mM glucose (control), 25mM glucose for 24hrs (high glucose) or 25mM glucose for 24hrs followed by 5mM glucose for 24hrs (remove high glucose). Panel B: L6 myotubes were cultured in 5mM glucose (control), 1mM glucosamine (GlcN) for 24hrs or 1mM glucosamine for 24hrs followed by 5mM glucose for 24hrs (remove GlcN). * P < 0.05, results were expressed as means ± SD, n=3-6, experiment was repeated at least three times.

Figure 3  Increased TRIB3 expression in hyperglycemia was reversed by insulin treatment in STZ-induced diabetic rats. Panel A: Plasma glucose levels of control, STZ-diabetic rats (STZ) and STZ-diabetic rats received insulin treatment (STZ+Insulin). Panel B: Protein expression of TRIB3 in skeletal muscle of control, STZ-diabetic rats (STZ), and STZ-diabetic rats treated with insulin (STZ+Insulin). Hyperglycemia was confirmed after 5days of STZ-injection, and insulin was
administered twice (7am and 7pm) daily successively for 7 days. * P < 0.05, results were expressed as means ± SD, n=6.

**Figure 4** Activation of hexosamine biosynthetic pathway is required for induction of TRIB3 by glucose. Panel A: In L6 myotubes, inhibition of HBP by azaserine (40uM) blocked the induction of TRIB3 expression by 25mM glucose, without affecting the induction of TRIB3 expression by 1mM glucosamine. Culture medium with 25mM high glucose or 1mM glucosamine was added with 40umol/L azaserine for 24hrs. Panel B: representative analysis of TRIB3 expression and AKT phosphorylation in skeletal muscle of mice injected with saline or glucosamine (70mg/kg/day) for 7 days. * P < 0.05, vs. control; # P < 0.05 vs. indicated group.

**Figure 5** Induction of TRIB3 by high glucose and glucosamine is accompanied with decreased glucose transport rate. Panel A&B: TRIB3 mRNA levels of L6 myotubes cultured in medium with or without presence of 25mM high glucose or 1mM glucosamine. Panel C: Basal and insulin-stimulated glucose rate of L6 myotubes cultured in 5mM or 25mM glucose medium for 24hrs; Panel D: Basal and insulin-stimulated glucose rate of L6 myotubes cultured in 5mM glucose medium with or without presence of 1mM glucosamine for 24hrs. * P < 0.05, ** P < 0.01, results were from 3 to 6 repeated experiments and expressed as means ± SD

**Figure 6** GFAT inhibitor, azaserine, blocked induction of TRIB3 protein by high glucose in 3T3-L1 adipocytes.
Fully differentiated 3T3-L1 adipocytes were incubated in 5mM or 25mM high glucose with or without azaserine (40uM) for 24hrs. Representative western blot film was shown and protein bands were quantified by Image Lab software (BioRad). * P < 0.05, n=6 and results were expressed as means ± SD.

**Figure 7** Knockdown of TRIB3 inhibited, and TRIB3 overexpression enhanced, the ability of both high glucose and glucosamine to induce insulin resistance.

Panel A: Representative western blot film showing efficacy of TRIB3 overexpress and TRIB3 knockdown in L6 myotubes. 24hr high glucose (25mM) (Panel B) or glucosamine (1mM) (Panel C) incubation significantly reduced basal and insulin-stimulated glucose transport, TRIB3 knockdown (black bar) inhibited, and TRIB3 overexpression (Gray bar) enhanced the ability of both high glucose (Panel B) and glucosamine (Panel C) to induce insulin resistance in L6 myotubes. Development of insulin resistance induced by high glucose or glucosamine was calculated as percentage of decrement of glucose transport of cells cultured in 25mM high glucose or 1mM glucosamine as compared with cells cultured in 5mM glucose medium. * P < 0.05, vs. control. N=3-6 for each group. Results were from three to six independent experiments.
Figure 1. Induction of TRIB3 is accompanied with up-regulation of protein O-Glycosylation levels.

88x150mm (300 x 300 DPI)
Figure 2. TRIB3 is induced by high glucose and glucosamine (GlcN) in a reversible manner in culture L6 myotubes.
Figure 3. Increased TRIB3 expression in hyperglycemia was reversed by insulin treatment in STZ-induced diabetic rats.
Figure 4. Activation of hexosamine biosynthetic pathway is required for induction of TRIB3 by glucose. (A. GFAT inhibitor, azaserine, blocked induction of TRIB3 by high glucose without affecting glucosamine induced TRIB3; B. In vivo glucosamine injection in mice led to impaired AKT phosphorylation and increased TRIB3 expression.)
Figure 5. Induction of TRIB3 by high glucose and glucosamine is accompanied with decreased glucose transport rate.

160x119mm (300 x 300 DPI)
Figure 6. GFAT inhibitor, azaserine, blocked induction of TRIB3 protein by high glucose in 3T3-L1 adipocytes.
Figure 7. Knockdown of TRIB3 inhibited, and TRIB3 overexpression enhanced, the ability of both high glucose and glucosamine to induce insulin resistance.