Original Paper

Skeletal Muscle Tissue Trib3 Links Obesity with Insulin Resistance by Autophagic Degradation of AKT2

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Key Words

Tribbles • AKT2 • Obesity • Insulin resistance • Autophagy

Abstract

Background/Aims: Obesity is a serious health risk factor strongly associated with insulin resistance and type 2 diabetes; however, the underlying mechanisms associating obesity with insulin resistance remain unknown. In this study, we explored the physiological role of Trib3 in regulating glucose metabolism in skeletal muscle tissues in a Trib3 transgenic mice model. **Methods:** Glucose metabolism in transgenic mice overexpressing Trib3 specifically in the skeletal muscle was examined by glucose/insulin tolerance test, metabolic cage studies, and glucose uptake assay. The effect of Trib3 overexpression on AKT phosphorylation and AKT protein turnover were assessed by RT-PCR and immunoblot analysis. Subcellular distribution of Trib3 and AKT1/2 was determined by microscopic analysis, co-immunoprecipitation experiments, and limited-detergent extraction of subcellular organelles. Ubiquitin assay was performed and ATG7 deficient cell line was employed to address the mechanisms of Trib3-dependent AKT protein homeostasis. **Results:** We found that Trib3 expression in skeletal muscle is elevated in obese conditions, and transgenic mice that overexpressed Trib3, specifically in skeletal muscle tissues, displayed impaired glucose homeostasis by suppressing insulin-stimulated glucose uptake. Disruption of insulin signaling in skeletal muscle Trib3 transgenic mice may occur due to the specific downregulation of AKT2 but not AKT1. Autophagy regulated AKT2 protein turnover, and Trib3 overexpression stimulated autophagic degradation of AKT2 by promoting AKT2 ubiquitination. **Conclusion:** Because diet-induced obesity upregulates Trib3 and downregulates AKT2 in skeletal muscle tissues, Trib3 may play a key role in establishing an association between obesity and insulin resistance by regulating AKT2 protein homeostasis.

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Introduction

Increasing hepatic glucose production and decreasing glucose uptake in skeletal muscles impairs insulin signaling and induces hyperglycemia, and chronic elevation of circulating glucose levels damages β -cells, ultimately resulting in type 2 diabetes. Although both environmental factors and genetic susceptibility contribute to insulin sensitivity and resistance, the regulation of AKT activity may play a pivotal role in insulin-mediated signal transduction because mice lacking *Akt* develop diabetes due to their resistance to insulin signaling [1-3].

Tribbles (Trib) was first identified during *Drosophila* mutant screening to identify genes regulating cell proliferation and division [4, 5]. One unique feature of Trib is its kinase-like domain (Trib domain), which is conserved across species from *Drosophila* to humans. The Trib domain lacks a VAIK motif, which is required for phosphatase interaction of bound ATP for kinase function. Thus, Trib is considered a decoy kinase or scaffolding protein [6]. In fact, it has been shown to modulate cellular signaling pathways by directly interacting with various proteins including cdc25, C/EBP, 12-LOX, MEK-1, MKK4, MKK7, and ATF4 [4, 5, 7-10]. Three Trib isoforms, Trib1, Trib2, and Trib3, were found in mammals. Among the three isoforms, Trib3 was identified as a binding partner for AKT by yeast two-hybrid screening [11]. In liver, Trib3 is induced in a PGC1 α -dependent manner in a fasting state and through direct interaction with AKT, Trib3 blocks phosphorylation of AKT, disrupts insulin signaling, and inhibits the suppression of hepatic glucose production. Therefore, mice with hepatic Trib3 overexpression become resistant, and hepatic Trib3 knockdown become sensitive to insulin signaling [11, 12].

Skeletal muscle tissues are responsible for 90% of insulin-dependent glucose uptake [13] and thus play a crucial role in glucose homeostasis. Skeletal muscle Trib3 expression may be regulated by metabolic status, as elevated Trib3 expression has been observed in diabetic patients and its expression is reversibly regulated by altering glucose metabolism [14, 15].

Here, we specifically assessed skeletal muscle Trib3 function in regulating the insulin signaling pathway, demonstrating that skeletal muscle Trib3 expression is upregulated under metabolically stressful conditions, and through direct interaction, Trib3 specifically downregulated AKT2 expression and attenuated insulin signaling. Furthermore, skeletal muscle-specific Trib3 expression in mice becomes resistant to insulin signaling. Because upregulation of skeletal muscle Trib3 directly inhibits insulin signaling and contributes to insulin resistance, these data along with that of previous studies on Trib3 function in the liver suggest that Trib3 is a potential target for improving insulin sensitivity in patients with type 2 diabetes.

Materials and Methods

Animals

The mice were kept in a temperature-controlled germ-free environment on a 12-h light/12-h dark cycle (lights on at 8:00 AM and off at 8:00 PM) with free access to water and a normal chow diet (Purina Rodent Chow, 38057, Seoul, Korea). To generate skeletal muscle-specific Trib3-overexpressing transgenic mice (Trib3TG), Flag-tagged mouse Trib3 was cloned next to the 9.5 kb of skeletal muscle-specific α -actin promoter as previously described [16]. The Trib3TG construct was pronuclear injection, and chimeras containing SMactin-Trib3TG in germ cells were selected and backcrossed to C57BL6/J mice at least five times. For the experiments, we crossed heterozygotes of Trib3TG mice with pure C57BL6/J mice and compared their phenotypes with those of sibling mice (control). To generate diabetic mouse models, streptozotocin (STZ (50 mg/kg body weight in 10 mmol/L sodium citrate buffer, pH 4.5)) was administered to control and Trib3TG mice intraperitoneally for 5 consecutive days. Vehicle (10 mmol/L sodium citrate buffer, pH 4.5)

or STZ treated control and Trib3TG mice were sacrificed 3 weeks post STZ injection and quadriceps tissues were collected for further analysis. After terminating the experiments, we sacrificed the mice and confirmed transgenic Trib3 expression in skeletal muscle tissues and excluded the Trib3 TG mice that did not express transgenic Trib3. All animal studies were conducted according to an approved protocol by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences, Asan Medical Center, Seoul, Korea.

Glucose tolerance test and insulin tolerance test

For the glucose tolerance test (GTT), overnight-fasted male mice were intraperitoneally injected with glucose (2 g glucose/kg of mouse), and the glucose level was measured every 30 min. For the insulin tolerance test (ITT), insulin (Humulin; Lilly, Indianapolis, IN, USA) was intraperitoneally administered to male mice (fasting for 4–5 h) at 1 unit/kg of mouse, and glucose levels were monitored using blood collected from a tail vein with an Accu-Chek Performa glucometer (Roche, Basel, Switzerland).

Indirect calorimetry and MRI

The mice were individually housed for acclimation for 2 days prior to conducting the experiments. O₂ consumption, CO₂ production, locomotor activity, and food intake were monitored using an indirect calorimeter (Columbus Instruments, Columbus, OH, USA or TSE systems, Bad Homburg, Germany). Mouse body fat and lean mass were analyzed on MRI (EchoMRI LLC, Houston, TX, USA) according to the manufacturer's instructions.

Reagents

A final concentration of 0.4 μM bafilomycin A1 (Tocris Bioscience, Bristol, UK), 0.2 μM rapamycin, 10 μM MG132 (Tocris Bioscience), 0.5 μM thapsigargin, 1 μM tunicamycin, 10 μM MG132 (Calbiochem, San Diego, CA, USA), and 1 μg/ml doxycycline (Sigma-Aldrich, St. Louis, MO, USA) was utilized as indicated in the figures.

Cell culture experiments

The HEK293T, HeLa, and C2C12 cell lines were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Corning, Corning Life Sciences, NY, USA) and 1% P/S at 37°C in a humid environment with 5% CO₂. For the ectopic expression of AKT1/2 or Trib3, plasmids containing HA-, Flag-, GFP-, or RFP-tagged AKT1/2 or Trib3 were transfected in HEK293T or HeLa cell lines using polyethylenimine (PEI) (Sigma-Aldrich) or Lipofectamine (Invitrogen, San Diego, CA, USA) as indicated in the figures. Gene delivery in the C2C12 cell line was conducted either by transfection using Lipofectamine supplemented with a Plus Reagent (Invitrogen), or recombinant adenovirus encoding Trib3 as indicated in the figures. The ATG7 knockout cell line was generated using the CRISPR/Cas9 system. To generate inducible Trib3-expressing cell lines, HA-tagged Trib3 cloned into the pcDNA5-FRT/TO vector (Invitrogen) were transfected to Flp-In T-REx cells and hygromycin-resistant cells were selected.

Reverse transcription-polymerase chain reaction

Total RNA was extracted using the RNA Mini Kit (Favorgen, Ping-Tung, Taiwan) according to the manufacturer's instructions. A 500 ng sample of total RNA was used to synthesize first-strand cDNA with a random hexamer (Toyobo, Osaka, Japan), and mRNA expression of AKT1, AKT2, Trib3 and endogenous level of GAPDH was analyzed using reverse transcription-polymerase chain reaction (RT-PCR). We used the following primers (forward and reverse, respectively) for amplification of Trib3; GGAACCTTCAGAGCGACTTG and TCTCCCTTCGGTCAGACTGT; AKT1; GAAGCTGGAGAACCTCATGC and CTTCATAGTGGACCGTCCT; AKT2; TTTGCACTCGAGAGATGTGG and TTTGCACAAGCCAAAGTCAG, and GAPDH; ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA.

Antibodies and Western blot

To prepare protein samples from the cultured cells, PBS-washed cells were lysed in 1% SDS in 10 mM Tris and 5 mM EDTA at pH 7.4. Mouse tissue samples were snap-frozen and either maintained at –80°C or immediately ground in liquid nitrogen and lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with a proteinase inhibitor (Roche, Basel, Switzerland) and phosphatase inhibitor. Antibodies for Atg7, p62, AKT1, AKT2, total AKT, phospho-AKT (Cell Signaling Technology, Danvers, MA,

USA), Flag (Sigma-Aldrich), and HRP-conjugated HA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used for immunoblotting. HSP90 (Santa Cruz Biotechnology) was used as a loading control to verify equal loading for each immunoblot.

Analysis of subcellular localization of Trib3 and AKT

HeLa cells were plated in a confocal dish (SPL Life Sciences, Pocheon, Korea) at approximately 30%–40% confluence, and RFP-tagged AKT1/2 and/or GFP-tagged Trib3 were introduced using PEI reagents. Subcellular localization of AKT1/2 and Trib3 was monitored using a confocal microscope (LSM 710, Carl Zeiss, Oberkochen, Germany). Flag-tagged AKT1/2 and HA-tagged Trib3 were transfected into HeLa or HEK293T cells for nuclear and cytoplasmic fractionation. Protein samples were prepared with an extraction buffer (250 mM sucrose, 20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, and 1 mM EGTA) to separate nuclear and cytosolic fractions by centrifugation according to the manufacturer's instructions (Thermo Fisher, Waltham, MA, USA).

Ubiquitination assay

Ubiquitination assay was performed as previously described with few modifications [17]. Briefly, plasmids encoding 6XHis-tagged ubiquitin and FL-AKT1 or FL-AKT2 were transfected into Flp-In T-REx cells incorporated with HA-Trib3 as indicated in the figures. Trib3 expression was induced by doxycycline treatment at 24 h after transfection. Protein samples were prepared in 1% SDS lysis buffer (1% SDS in 100 mM Tris-Cl (pH 7.4), and total ubiquitinated protein was precipitated with cobalt-coated Talon beads. Whole ubiquitinated proteins were eluted from the Talon beads and separated using SDS PAGE, and AKT1 or AKT2 ubiquitination was analyzed using an immunoblot with Flag antibody. To determine Trib3-dependent ubiquitination of AKT1/2, basic Flp-In T-REx cells, which do not contain HA-Trib3, were used as the control.

Glucose uptake assay

The glucose uptake assay was performed according to the manufacturer's instructions (Abcam, UK). Briefly, soleus muscles dissected from control and Trib3TG mice were rinsed using PBS and incubated twice for 30 min in Krebs-Ringer Phosphate-HEPES (KRPH) buffer containing 2% BSA under continuous gas flow (95% O₂, 5% CO₂). After the 1-h pre-incubation period, soleus strips were transferred to 1 ml of fresh KRPH buffer containing 2% BSA and 0.1 mM 2-deoxy-D-glucose (2-DG) with or without insulin. At the end of the experiment, individual soleus strips were washed twice with 2% BSA, and KRPH and lysates were prepared with extraction buffer. 2-DG uptake at an optical density of 412 nm was measured using a microplate reader (Biotek, Winooski, VT, USA). C2C12 cells were infected with an adenovirus containing either control GFP or Trib3. Lysates were prepared with an extraction buffer with or without insulin treatment, and the 2-DG uptake amount was measured as described above.

Statistical analysis

Data are presented as mean ± SEM, and statistical analyses were performed by an unpaired Student *t* test using the Graphpad Prism Program. In this study, *P* < 0.05, *P* < 0.01, and *P* < 0.001 are represented as *, **, and ***, respectively, and were considered statistically significant.

Results

Trib3 overexpression in skeletal muscle tissues displays impaired glucose homeostasis without affecting obesity

Trib3 expression is elevated by stress signals such as oxidative and endoplasmic reticulum (ER) stress [10, 18, 19]. To examine the regulation of Trib3 expression in muscle cells, we differentiated C2C12 cells into myotubes. C2C12 myotubes treated with the Ca²⁺ release of thapsigargin or the glycosylation blocker tunicamycin increased Trib3 expression (Supplementary Fig. S1a). For all supplemental material see www.karger.com/doi/10.1159/000492264. Mimicking nutritional overload with free fatty acid also increased Trib3 expression in C2C12 myotubes (Supplementary Fig. S1b).

As Trib3 expression was upregulated due to stress signaling in skeletal muscle tissues, we

hypothesized that skeletal muscle Trib3 would mediate insulin resistance and development of type 2 diabetes in the obese condition. To test our hypothesis, we generated transgenic mice that overexpressed Trib3 specifically in skeletal muscle tissues (hereafter referred to as Trib3TG mice). Supplementary Fig. S2a, b, and d show that a 9.5-kb skeletal α -actin promoter can express Trib3 specifically in mouse skeletal muscle tissues. Although type I (soleus) and type II (gastrocnemius and extensor digitorum longus [EDL]) muscles of Trib3TG mice (Supplementary Fig. S2c) had comparable Trib3 mRNA expression, transgenic Trib3 protein expression was significantly greater in type II compared to type I fibers in enriched muscle tissues (Supplementary Fig. S2d), implying that Trib3 expression is also tightly regulated at the post-transcriptional level.

Trib3TG mice consumed a comparable amount of food (Fig. 1a), with equivalent activity (Fig. 1b), O_2 consumption, CO_2 production, and overall energy expenditure to those of wild-type control mice (Fig. 1c and Supplementary Fig. S3), hence their overall weights were similar (Fig. 1d). The weights of the liver, epididymal white adipose tissue (WAT), inguinal WAT, brown adipose tissue (BAT), and skeletal muscle tissue of Trib3TG mice as well as their total fat and lean body masses were also comparable with those of control sibling mice (Supplementary Fig. S4).

Despite having comparable body weights, nighttime respiratory quotients (respiratory exchange ratio [RER]) of Trib3TG mice were significantly lower compared to those of their control litter mates (Fig. 2a). Regarding their lower RER, Trib3TG mice exhibited impaired glucose homeostasis on the glucose and

Fig. 1. Skeletal muscle-specific Trib3 overexpression does not affect obesity. (a) The 24-h food intake in control (WT) and Trib3TG mice maintained on a normal chow diet ($n = 7-8$ per group). (b) Physical activity. (c) Energy expenditures in normal chow-fed controls and Trib3TG mice aged 6 months old ($n = 4$ per group). (d) Body weight growth curve of control and Trib3TG mice maintained on a normal chow diet ($n = 7-8$ per group).

Fig. 2. Glucose homeostasis is impaired in Trib3TG mice. (a) Respiratory exchange ratios (RERs) in control and Trib3TG mice maintained on a normal chow diet ($n = 4$ per group). (b) Glucose tolerance test (GTT) and (c) insulin tolerance test (ITT) of normal chow-fed control and Trib3TG mice ($n = 6-7$ per group). (d) Glucose uptake into soleus muscle explants of control and Trib3TG mice ($n = 3$).

Fig. 3. Trib3 downregulates AKT2 specifically at the post-transcriptional level. (a) Immunoblots were used to examine the level of AKT phosphorylation, total AKT, AKT1, and AKT2 collected from the quadriceps of control and Trib3TG mice after 16 h of fasting or 16 h of fasting followed by insulin administration (1 unit/kg insulin intraperitoneally). The HSP90 level is shown as a loading control. (b) Immunoblots showing the endogenous protein level of AKT1 and AKT2 by increasing the amount of Trib3 (Adeno-Trib3) in C2C12

myoblasts. (c) Protein and (d) mRNA levels of AKT1 and AKT2 by Trib3 in HEK293T cells at 48 h after transfection of HA-AKT1/2 and Flag-Trib3 were examined using immunoblot and RT-PCR, respectively.

insulin tolerance test (Fig. 2b and c). Further, the muscles of Trib3TG mice exhibited lower insulin-stimulated glucose uptake compared to those of control mice (Fig. 2d). Overall, these results suggest that impaired glucose homeostasis in Trib3TG mice occurred due to altered insulin sensitivity in muscle tissues.

Skeletal muscle Trib3 inhibits insulin signaling by downregulating AKT2

Because Trib3 appears to affect insulin signaling, we examined the AKT expression and activity in skeletal muscle tissues of Trib3TG and control sibling mice. Overall, 16 h of fasting in both the control and mutant mice downregulated AKT activity (AKT phosphorylation) in skeletal muscle tissues, and 16 h of fasting followed by an intraperitoneal injection of insulin induced AKT phosphorylation in the skeletal muscle tissues of control mice, which was less induced in the skeletal muscles of Trib3TG mice. Notably, when we assessed AKT expression in skeletal muscle tissues, we found that the protein level of AKT2, but not that of AKT1, was specifically downregulated in mutant mice (Fig. 3a). Interestingly compared with vehicle treatment, streptozotocin (STZ) induced hyperglycemia further downregulated the protein level of AKT2 in skeletal muscle tissue of Trib3TG mice (Supplementary Fig.

Fig. 4. Co-localization of Trib3 and AKT2 in the nucleus. (a) Interaction between AKT1/2 and Trib3 was examined using co-immunoprecipitation. (b) Subcellular localization of RFP-tagged AKT1/2 and GFP-tagged Trib3 in HeLa cells. (c) Immunoblots showing a cellular fraction assay in HEK293T cells that were transfected with HA-AKT1/2 and Flag-Trib3. CREB and ACC were used as the nucleus and cytoplasmic markers, respectively.

S5a). Considering the decreased AKT2 protein level in skeletal muscle tissues of Trib3TG mice, the ectopic expression of Trib3 in C2C12 myoblasts and HeLa cells also specifically decreased the level of endogenous proteins in AKT2 (Fig. 3b and Supplementary Fig. S5b). To further investigate how and at which stages Trib3 regulates AKT expression, we transfected plasmids encoding HA-tagged AKT and Flag-tagged Trib3, in which expression was derived from CMV promoter in HEK293T and C2C12 myoblasts. Both AKT1 and AKT2 protein levels were downregulated by co-expression of Trib3; however, AKT2 downregulation was significantly more dramatic than that of AKT1 (Fig. 3c and Supplementary Fig. S5c). Decreased AKT protein levels were not accompanied by an alteration in mRNA levels, suggesting that Trib3 induced the downregulation of the AKT2 protein level at post-transcriptional levels (Fig. 3d).

Subcellular localization of Trib3 determines the specificity for AKT2 versus AKT1

Multiple studies have demonstrated that Trib3 regulates insulin signaling in the liver by directly interacting with AKT. Based on our observation that Trib3 preferentially downregulated AKT2 versus AKT1, we considered that Trib3 may bind to AKT2 with a higher affinity than to AKT1 and may regulate the stability of AKT2; however, co-immunoprecipitation experiments showed comparable Trib3 binding between AKT2 and AKT1 (Fig. 4a). Because the co-immunoprecipitation experiment could not distinguish whether protein-protein interactions were formed in the cells physiologically or *in vitro* during incubation with an antibody, we attempted to examine the subcellular localization of Trib3 and AKT1/2. GFP-tagged Trib3 was primarily expressed in the nucleus, and whereas RFP-tagged AKT1 was evenly distributed in the cytoplasm and nucleus of cells, AKT2 was more enriched in the nucleus (Fig. 4b). In support of the microscopic observations, cellular fractionation also confirmed that AKT2 was more abundant in the nucleus than was AKT1, and although Trib3 co-expression decreased the protein amounts in both AKT1 and AKT2, nuclear AKT2 expressions appeared to be more dramatically downregulated by Trib3 (Fig. 4c).

Fig. 5. Trib3 stimulates AKT2 ubiquitination and regulates AKT2 protein stability via the autophagy pathway. (a) Trib3 induction by doxycycline treatment stimulates AKT2 ubiquitination. Immunoblots showing the endogenous levels of AKT1/2 and Trib3 in C2C12 myotubes by (b) MG132 and (c) bafilomycin A1 treatments. (d) Immunoblots were used to examine the effect of bafilomycin A1 treatment on Trib3-mediated AKT1/2 protein levels in HEK293T cells transfected with HA-AKT1/2 and Flag-Trib3. (e) The effect of bafilomycin A1 and MG132 on the accumulation of ubiquitinated AKT1/2 was analyzed in control- and Trib3-inducible cells following doxycycline treatment. (f) Immunoblots showing the protein levels of AKT1/2 and Trib3 in control and ATG7KO cells.

Fig. 6. Elevation of skeletal muscle Trib3 in obesity inhibits glucose uptake by stimulating AKT2 degradation. (a) Immunoblots showing the relative amounts of Trib3 and AKT protein in the quadriceps of normal chow- or 60% HFD-fed mice. (b) Glucose uptake assay in control GFP-infected and Trib3-infected C2C12 myoblasts. (c) Schematic diagram proposing the role of skeletal muscle Trib3 in promoting insulin resistance in obesity.

Trib3 regulates AKT protein homeostasis by stimulating the polyubiquitination of AKT2

Previous studies have demonstrated that Trib3 functions as an adapting molecule linking substrate proteins to E3 ubiquitin ligase and regulates substrate protein homeostasis [20]. We conducted an ubiquitination assay to assess whether this applies to the case of Trib3 and AKT2. Because Trib3 co-transfection significantly downregulated the AKT2 protein level, we utilized the inducible system, in which Trib3 expression is induced by doxycycline treatments, and investigated the early activity of Trib3 on AKT2 ubiquitination. Compared with control, Trib3 induction increased polyubiquitination for AKT1 and to a greater extent for AKT2 (Fig. 5a). Because AKT2 ubiquitination appeared to be stimulated by Trib3, we examined whether the protein stability of AKT2 is regulated in a proteome-dependent manner. MG132 treatment on C2C12-differentiated myotubes did not upregulate the level of AKT2 protein; instead, we found that bafilomycin A1 treatment elevated the level of AKT2 protein (Fig. 5b and c). Accordingly, bafilomycin A1, but not MG132, treatment attenuated the downregulation of Trib3-mediated AKT2 (Fig. 5d), and bafilomycin A1 further accumulated Trib3-mediated polyubiquitination of AKT2 (Fig. 5e). Moreover, the protein level of AKT2 was higher in ATG7 knockout cells than in control cells (Supplementary Fig. S6 and Fig. 5f). These results suggest that Trib3 regulates AKT2 turnover by stimulating AKT2 ubiquitination, and this process is regulated primarily in an autophagy-dependent manner.

Downregulation of AKT2 in skeletal muscle tissues of diet-induced obese mice

Because Trib3 downregulated AKT2 expression, we suspected that Trib3 is a mediator linking obesity and insulin resistance. In agreement with a previous observation, Trib3 expression was elevated in skeletal muscle tissues of obese mice induced by a 60% high-fat diet (HFD), in which we observed a decline in AKT2 expression (Fig. 6a). Finally, Trib3 overexpression attenuated insulin-stimulated glucose uptake in C2C12 cells (Fig. 6b). Overall, these results suggest that obesity-induced cellular stress signals upregulate Trib3, and that Trib3 disrupts the insulin signaling pathway by downregulating the AKT2 protein level, leading to insulin resistance (Fig. 6c).

Discussion

Elevated Trib3 is observed in hyperglycemic and obese conditions [19, 21], and although obesity and type 2 diabetes are strongly associated and difficult to separate, Trib3 expression appears to be correlated more with insulin resistance and/or diabetes than with obesity.

For instance, Oberkofler et al. examined Trib3 expression in the liver of obese patients and reviewed their insulin sensitivity. They showed that Trib3 expression was significantly elevated in obese subjects with insulin resistance, suggesting that Trib3 is a key player in obesity-induced insulin resistance [22]. As in human studies, the hepatic overexpression of Trib3 blocked the insulin-mediated suppression of hepatic glucose production and impaired glucose homeostasis, and the knockdown of Trib3 improved glucose metabolism [11, 12, 21]. While the liver is important for regulating glucose production during the fasting state, skeletal muscle is the primary tissue responsible for insulin-dependent glucose uptake and maintaining glucose homeostasis [23-26].

In the current study, we demonstrated that skeletal muscle Trib3 expression is induced by metabolic stress conditions and plays a crucial role in glucose homeostasis. This corresponds with a previous observation that skeletal muscle Trib3 expression is highly correlated with insulin resistance in human subjects [14]. Regarding the skeletal muscle Trib3 function, a recent couple of studies have presented evidences that suggested the involvement of skeletal muscle Trib3 in glucose metabolism. For example, skeletal muscle-specific LKB1 knockout mice, in which Trib3 expression was downregulated, showed an improved glucose tolerance because of an increased glucose uptake in the muscle [27]. The systemic knockdown of Trib3 with antisense oligonucleotide or Trib3 whole-body knockout mice exhibited increased glucose uptake in skeletal muscle tissues with lower circulating NEFA levels, leading to improved whole-body glucose homeostasis [28, 29]. While these studies agree that Trib3 might negatively regulate glucose metabolism in skeletal muscles, few mechanistic data address whether improved glucose uptake in skeletal muscles of antisense Trib3 knockdown or knockout mice is due to a decrease in Trib3 in the skeletal muscle tissues themselves or whether it is an indirect consequence of improved systemic insulin sensitivity due to the decreased Trib3 expression in other tissues such as the liver or adipose tissues [30]. We investigated this hypothesis using a skeletal muscle-specific Trib3-overexpressing transgenic mice model. Trib3TG mice, despite having a comparable weight to that of control mice, displayed impaired glucose homeostasis, which appeared to be a result of decreased glucose uptake in skeletal muscle tissues due to Trib3 expression.

Mechanistically, consistent with previous findings in hepatocytes [11, 12, 31], insulin-resistant phenotypes observed in Trib3TG mice appear to be caused by an inhibition of AKT signaling by Trib3. Similar to that in hepatic Trib3 overexpression, we also observed reduced AKT phosphorylation in skeletal muscle tissues of Trib3TG mice; however, this primarily contributed from the decreased total AKT protein level, particularly AKT2, rather than blocking the phosphorylation of AKT. Trib3 regulates protein activity or stability by enhancing ubiquitination and proteasome-dependent degradation of its binding substrates [4, 20, 32]. Similarly, we revealed that Trib3, without altering AKT2 mRNA level, dose-dependently induced AKT2 ubiquitination. In regulating AKT activity by the ubiquitination process, Suizu et al. showed that TTC3 bound and preferentially ubiquitinated phosphorylated, or activated AKT [33]. We speculate that TTC3 is involved as a feedback regulatory mechanism to block prolonged AKT activation and that skeletal muscle Trib3, when induced under an obese or hyperglycemic condition, specifically regulates glucose metabolism by targeting AKT2. Past investigations of skeletal muscle tissue have substantially advanced our understanding of Trib3 as a negative regulator of glucose metabolism [27, 34-36]. However, while focusing primarily on the suppressive effect of Trib3 on insulin-stimulated AKT activation, the proteolytic activity of Trib3 might be overlooked. In fact, most of the previous studies analyzed the level of AKT phosphorylation but not that of total AKT protein. It will be interesting to determine which of the known Trib3-interacting proteins is subjected to degradation and how and what conditions allow Trib3 to make this decision.

Contrary to our observations, An et al. reported that transgenic overexpression of Trib3 in skeletal muscle tissue of mice did not significantly alter whole-body glucose metabolism or insulin-stimulated AKT phosphorylation [35]. On the other hands, Zhang et al. showed that skeletal muscle-specific conditional Trib3 transgenic mice exhibited insulin resistance with blunted insulin-stimulated AKT phosphorylation compared with the control group,

and skeletal muscle-specific conditional Trib3 knockout mice remained sensitive to insulin signaling in hyperglycemic conditions [36]. We do not fully understand why there are discrepancies among these studies. However, since inhibition of insulin signaling by skeletal muscle Trib3 appears to be strengthened by metabolic stress [36], it is possible that there are additional factors that boost the function of Trib3 synergistically and promote insulin resistance in hyperglycemia or obesity. Considering that muscle Trib3 expression is dynamically regulated in response to subtle changes in glucose level [14, 37], it is tempting to revisit these mouse models and examine glucose metabolism in a variety of conditions.

The preferential downregulation of AKT2 versus AKT1 by Trib3 might be explained by the co-localization of Trib3 with AKT2 versus AKT1. Although the expression and subcellular distribution of AKT isoforms appear to be regulated in a cell type-dependent manner [38], AKT1 is predominantly found in the cytoplasm, and AKT2 is highly enriched in the nucleus in skeletal muscles [39, 40]. Accordingly, we found Trib3 mainly in the nuclear fraction co-localized with AKT2 [10]. Thus, although Trib3 has a similar binding affinity to AKT1 and AKT2, subcellular localization of Trib3 might endow a specificity to AKT2. Trib3TG mice phenotypes appear to be consistent with those reported in previous studies; namely, that AKT2—rather than AKT1—is primarily associated with the regulation of glucose homeostasis because insulin-stimulated glucose uptake is primarily controlled by AKT2 and not by AKT1 [41, 42]. AKT1-null mice exhibited a growth defect with normal glucose metabolism [43, 44], while AKT2-null mice displayed impaired insulin-mediated suppression of hepatic glucose production and skeletal muscle glucose uptake [1, 2].

Another interesting finding was the autophagy-dependent regulation of AKT2 protein stability. We demonstrated that AKT2 turnover was strongly inhibited due to a block in the autophagy pathway, and consistent with our findings, some reports have suggested an association between AKT and autophagosome [45, 46]. By using immunoprecipitation coupled with mass spectrometry, we observed the association of Trib3 with the autophagy receptors optineurin and p62 (data not shown), thus Trib3 may link AKT2 to an autophagosome. However, because Trib3-dependent AKT2 ubiquitination was further accumulated by a bafilomycin A1 treatment, our data rather suggest that Trib3 triggers the association of ubiquitinated AKT2 to an autophagy receptor and degradation by stimulating AKT2 ubiquitination. The topologies of ubiquitin may be associated with sorting polyubiquitinated proteins by proteasome or autophagy. For example, while polyubiquitination via lysine 48 residues of ubiquitin targets to proteasome, lysine 63-linked polyubiquitination appears to stimulate the formation of inclusion and clearance via the autophagy pathway [47-49]. Although both Trib3 and AKT2 primarily reside in the nucleus, there is a chance that Trib3-AKT2 complex will migrate to cytoplasm, and cytoplasmic E3 ligase stimulates ubiquitination and autophagic degradation of AKT2. Alternatively, Trib3 might recruit E3 ligase to AKT2 in the nucleus, and nuclear autophagy could process ubiquitinated AKT2 [50, 51]. Identifying E3 ligase, which promotes AKT2 ubiquitination together with Trib3, and the mechanism of how these processes target AKT2 to autophagosome, perhaps examining the topologies of Trib3-dependent AKT2 polyubiquitination and whether that E3 ligase is associated with insulin resistance is worth considering for future studies.

Collectively, we showed that Trib3 regulates ubiquitination and autophagy-dependent downregulation of the AKT2 protein, and this current study appears to be the first to report on the specific regulation of AKT2 versus AKT1 activity. Because skeletal muscle Trib3 expression is increased in obesity and skeletal muscle-specific Trib3 transgenic mice presented insulin resistance with impaired glucose homeostasis, our study, together with previous studies, suggests that Trib3 is a key player in mediating obesity-induced type 2 diabetes, and developing drugs that specifically disrupt Trib3 binding to AKT2 might be a beneficial strategy for treating insulin resistance and type 2 diabetes.

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Disclosure Statement

The authors declare that there is no conflict of interests.

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