Doctor of Philosophy

Role of Trib3 in obesity and insulin resistance.

비만과 인슐린 저항성에서의 Trib3 의 역할.

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Abstract

Obesity, as a severe risk factor that threatens health, has been increasing consistently worldwide for decades, along with diabetes, a representative metabolic disorder. Insulin resistance is known to be closely related to obesity and diabetes, and by improving high insulin resistance, obesity and diabetes can be improved effectively. Although insulin resistance increases due to obesity, which in turn causes diabetes, it is unclear to this day why obesity causes diabetes.

This study examined the physiological role of Trib3 in skeletal muscle that is important in glucose metabolism by using Trib3 transgenic mice, which are mice exhibiting an overexpression of Trib3. Metabolic cage studies, glucose tolerance tests, insulin tolerance tests, and glucose uptake assays confirmed that insulin sensitivity was weakened in Trib3 transgenic mice. At the cellular level, AKT's activity and AKT2's downregulation caused by an overexpression of Trib3 were confirmed. Ubiquitination assay and ATG7 knockout cell line testing confirmed that Trib3 regulated AKT2's homeostasis autophagy dependently. The expression of Trib3 increased in the skeletal muscle tissue of obese mice induced by a High Fat Diet (HFD) and due to such, AKT's activity decreased. In particular, AKT2 was downregulated significantly compared to AKT1 due to the manifestation of Trib3.

The increase of Trib3 in the skeletal muscle tissue of obesity-induced mice downregulates AKT2, which is important to insulin signaling pathways, ultimately causing insulin resistance. Therefore, Trib3 is deemed to play an important role in obesity and insulin resistance.

Key words: Obesity, Insulin resistance, Trib3, AKT2, Autophagy

Contents

List of Figures

Introduction

Obesity is defined as the state of excessive fat accumulation that presents severe health risks worldwide, and is a major risk factor for metabolic diseases such as hyperlipidemia, hypertension, insulin resistance, and diabetes.

Diabetes, a metabolic disease, is closely related to obesity. According to the Organization for Economic Cooperation and Development (OECD) and Centers for Disease Control and Prevention (CDC), obesity rates in the world are steadily increasing.

In general, approximately 75% of adults with type 2 diabetes are overweight or obese [1, 2]. Type 2 diabetes is a form of diabetes that accounts for 95% of all diabetic patients [3]. It occurs in response to the insulin resistance or when the body stops producing enough insulin. Obese patients develop impaired insulin signaling and sensitivity [4, 5]. Therefore, these patients need to make more insulin than healthy people in order to regulate their blood glucose; however, if insulin is not controlled, then pancreas is chronically exposed to glucose. Consequently, the function of the pancreas is affected, leading to diabetes [6].

While various studies have reported that obesity is the major risk factor for diabetes mellitus, the exact mechanism of diabetes remains unclear.

Insulin synthesis and secretion

Insulin is a hormone secreted from the beta cells in pancreas, and plays a pivotal role in

regulating blood glucose level. It is created in two stages [7]. Initially, preproinsulin, a molecule with a signal peptide, is removed by hydrolysis and becomes proinsulin. Then, the C-peptide of proinsulin is removed by hydrolysis and eventually becomes insulin [8].

Synthesized insulin is stored in the cytoplasmic granules, and secreted via various processes when glucose is influxed. After glucose is released by GLUT2, it is converted by hexokinase to form glucose-6-phosphate (G6P), which is then converted by glycolysis and TCA cycle pathway into pyruvate, thus generating ATP. The generated ATP blocks the membrane-bound ATP sensitive K+ channels of pancreatic β-cells, which lead to membrane depolarization. Voltage-gated calcium channels (VGCCs) mediate Ca2+ entry into the cells in response to membrane depolarization. As a result, increase of intracellular Ca2 promotes the synthesis and secretion of insulin.

Insulin is also secreted due to hormone stimulation. Once acetylcholine or cholecystokinin is released by the parasympathetic nerve or duodenum, respectively, it binds to the Gq-coupled receptors of G protein-coupled receptor (GPCR) and stimulates insulin secretion by increasing the intracellular Ca2 concentration. Incretin (GLP1, GIP), vasoactive intestinal peptide (VIP), and Peptide YY (PYY) bind to a GPCR, which stimulates insulin secretion by increasing the cyclic adenosine monophosphate (cAMP).

Insulin signaling pathway

Insulin regulates crucial energy homeostasis including glucose and lipid metabolism. The insulin signaling pathway begins when the insulin binds to the insulin receptor (IR). Thus, the insulin receptor substrate (IRS) is phosphorylated by tyrosine, which further activates phosphoinositide 3-kinase (PI3K). This plays an essential role in insulin functioning, mainly via the activation of the AKT/PKB cascades. PI3K activates the AKT (serine/threonine protein kinase) that further triggers various substrates and regulates the insulin effect. Forkhead box O (FoxO) is suppressed by AKT, which regulates metabolism and autophagy. In contrast, AMP-activated protein kinase (AMPK) is known to directly regulate FoxO3 and activate the transcriptional activity. The insulin signaling pathway inhibits autophagy via the ULK1 kinase, which is further inhibited by AKT and mTORC1, and activated by AMPK. Insulin signaling inhibits gluconeogenesis in the liver, via disruption of CREB/CBP/mTORC2 binding. In addition, PI3K/AKT-mediated insulin signaling pathway stimulates glucose transporter 4 (GLUT4) to be transported to the plasma membrane and triggers glucose uptake in the muscle and adipocytes. Thereafter, activation of GSK3β is suppressed by AKT, which inhibits glycogen synthase in glycogen synthesis. As a result, AKT activates glycogen synthase, which improves glucose translocation, glucose utilization, and cellular metabolism. [9, 10].

AKT, also known as protein kinase B (PKB), is a serine/threonine kinase that plays an important role in multiple cellular processes such as cell proliferation, cell survival, apoptosis, and glucose metabolism [11]. AKT exists in three isoforms, namely, AKT1, AKT2, and AKT3

[12]. AKT1 is ubiquitously expressed and linked to cell survival pathways. AKT2 is mainly expressed in the insulin target tissues, such as liver, adipose tissues, and skeletal muscle tissues [13]. AKT3 is exclusively expressed in the brain tissue and is linked to brain development [14]. AKT1 knockout mice exhibited growth retardation and increased apoptosis [15]. Akt2 knockout mice, having normal AKT1, exhibited metabolic disorders such as insulin resistance and a diabetic phenotype [16]. Therefore, AKT2 is the most important isoform for insulin signaling pathway and glucose uptake [17, 18].

Insulin resistance

Insulin reduces blood glucose and provides this glucose for energy. Insulin resistance is a severe condition in which the body's cells become resistant to the effects of insulin. In this condition, insulin-induced glucose uptake is impaired in various insulin-related tissues. Eventually, insulin signaling pathway is disrupted in numerous tissues. It is known that insulin resistance leads to hyperinsulinemia. To prevent hyperglycemia by insulin resistance, the pancreatic β-cells produce sufficient insulin to regulate the blood glucose level. Type 1 diabetes indicates hyperglycemia and hypoinsulinemia; however, type 2 diabetes exhibits hyperglycemia and hyperinsulinemia. Insulin resistance is a major risk factor for development of type 2 diabetes. Several risk factors are known to be associated with induction of insulin resistance. These include genetic mutation, obesity, hyperinsulinemia, inflammation, fatty

liver, and ER stress. As a result, insulin resistance induces a decrease in the glucose inflow in various insulin-related tissues, particularly in skeletal muscle tissue.

Skeletal muscle tissues are the major sites of insulin action. The decrease in insulin in the skeletal muscle impaired the insulin-stimulated glucose uptake, which is one of the causes of the insulin resistance. Insulin resistance can be induced by lipids. Activation of PKCθ by diacylglycerol (DAG) impairs insulin signaling pathway in the skeletal muscle [19]. Elevation of ceramide in the skeletal muscle results in activation of protein phosphatase 2A (PP2A) and causes dephosphorylation (inactivation) of AKT2 [20]. Therefore, translocation of GLUT4 is impaired in the skeletal muscle. Intracellular inflammatory pathways are also linked to the development of insulin resistance. Activation of (I kappa B kinase) IKK increases ceramide synthesis and activation of JNK1, which impair the insulin signaling pathway via insulin receptor substrate 1 (IRS1) serine phosphorylation [21]. Under various conditions such as acute exercise, starvation, and high fat diet, the ER stress induces insulin resistance in the skeletal muscle [22, 23], which leads to induction of ATF6 and PGC1α mediated adaptive response.

Overview of Trib3 protein structure and function

Trib3 was initially discovered in the 2000s as a cell cycle regulator, which is essential for regulating cell divisions, specifically in drosophila development [24, 25]. It is a

serine/threonine pseudokinase that is induced by the transcription factor, its expression is upregulated in response to a variety of stress signals that include hypoxia, ER stress, and nutrient starvation.

Trib3 is one of three isoforms of the Tribbles subfamily, which include Trib1, Trib2, and Trib3 [26]. Several studies reported that Tribbles could interact with kinase dependent proteins; however, they lose the protein kinase activity [27, 28]. Tribbles domains completely lose VAIK and DFG domains, which are essential for the kinase catalytic core and interaction of bound ATP [29]. Therefore, the Tribbles subfamily is classified as pseudokinases [26, 30]. Additionally, the C-terminal region of Trib3 includes a COP1 binding domain, which is essential for association of E3 ubiquitin ligase COP1 protein. Trib3 is known to function as an adaptor molecule that promotes the degradation of target proteins. For instance, the aforementioned CDC25 is a cell cycle molecule, and Trib3 promotes CDC25 ubiquitination by associating with the ubiquitin E3 complex. Trib3 controls cell cycle progression and cell migration by regulating CDC25 [24]. It is associated with SMAD ubiquitin regulatory factor 2 (Smurf2) and facilitates its degradation via Trib3-dependent ubiquitination [31]. Moreover, Trib3 also stimulates the ubiquitination of ACC by recruiting COP1 to ACC [32], and is presumed to mediate the interaction between substrate and ubiquitin ligase and is known to trigger ubiquitination.

Trib3 on ER Stress

Considering various functions of Trib3, it has been reported that Trib3 is induced by endoplasmic reticulum (ER) stress. The ER is a cellular organelle that is the crucial for protein folding and secretion, and lipid biosynthesis. Various cellular stresses or conditions can disturb ER homeostasis, thus preventing protein folding and may cause misfolding or malfolding of proteins to accumulate in the ER, termed as ER stress [33, 34]. The cells respond to ER stress by initiating a defensive process, called the unfolded protein response (UPR), which promotes cell survival by reducing the accumulation of misfolding and malfolding proteins. The ER stress signaling can be divided into three representative pathways as follows: activating transcription factor 6 (ATF6), protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), and inositol-requiring enzyme 1 (IRE1). These three pathways play a pivotal role in the regulation and initiation of the UPR. Under normal conditions, the ER chaperone binding immunoglobulin protein (BiP) binds to the ATF6, PERK, and IRE1, keeping them inactive. On accumulation of unfolded proteins in the ER, BiP is separated from ATF6, PERK, and IRE1, and these proteins become active. ATF6 translocates to the Golgi and is cleaved by the proteases SP1 and SP2. Cleaved ATF6 is a transcription factor that regulates the expression of chaperones and enzymes that are important for ER functions. Trib3 is induced by ER stress and via activating transcription factor 4 (ATF4)-C/EBP homologous protein (CHOP) pathway [35]. The activation of PERK leads to phosphorylation of eIF2α in its inactive form.

Phosphorylated (inactivated) eIF2α inhibits protein translation, and upregulates protein translation such as ATF4, CHOP, and Trib3 [36]. For instance, ER stress triggers c-Jun N-terminal kinase (JNK) activation in the liver, which leads to IRS1 activation and subsequently insulin resistance [21]. High glucose and free fatty acids can also cause ER stress, which essentially contributes in β-cell dysfunction and death during progression to diabetes in the pancreatic β-cells [37-40]. In cardiac myocytes and tissues, increased expression of Trib3 by ER stress blocked the insulin-stimulated AKT activation [41]. Trib3 leads to insulin resistance by suppressing PI3K/AKT, which is important for insulin signaling pathway [42]. In the fasting state, activated CREB and PGC-1α protein upregulate the expression of Trib3 and induce insulin resistance by suppressing the insulin action [43]. Although the overexpression of Trib3 in mouse liver results in impaired glucose homeostasis and increased blood glucose, knockdown of Trib3 increased the activation of AKT in hepatocytes and improved glucose metabolism [42]. Moreover, The Trib3 expression was increased in the liver of a patient suffering from obesity accompanied by insulin resistance; however, to date, sufficient information is lacking on the role of Trib3 in skeletal muscle tissue [44].

Obesity is known to induce ER stress. Several studies have reported that Trib3 is associated with obesity and diabetes, wherein the expression of Trib3 is increased in the liver of diet induced obesity and ob/ob mouse [45Thus, various studies reported that Trib3 is a negative regulator of the insulin signaling pathway, in particular AKT, in several tissues, including the

liver and skeletal muscle tissue [42, 46]. Trib3 expression is induced in the liver and skeletal muscle tissue under nutrient starvation and fasting conditions, which inhibits insulin signaling pathway by binding directly to AKT and disrupts activation of this kinase. Overexpression of Trib3 in mouse liver and skeletal muscle tissue results in increased blood glucose and impaired glucose homeostasis [42]. In contrast, inhibition of Trib3 expression in mouse liver and skeletal muscle tissue by RNAi improves glucose homeostasis [46, 47]. In a previous study, Trib3 expression was increased in the obese and diabetic models in mice and humans [46, 48]. Hence, Trib3 is considered to be important for association between obesity and insulin resistance by regulating AKT.

Insulin resistance in liver, β-cells, adipocytes, brain, and specifically skeletal muscle tissue represents the crucial defect in obesity or type 2 diabetes. Among these, the skeletal muscle tissues comprise 40%–50% of the total human body mass, which is responsible for 90% of insulin-dependent glucose uptake [49], and thus, play a crucial role in glucose homeostasis

The Significance of this study

This study aimed to demonstrate whether Trib3 mediates the expression of AKT protein in skeletal muscle cells and tissue and to determine the effect of Trib3 on the insulin resistance using skeletal muscle specific Trib3 overexpressing transgenic mice. This is the first study to demonstrate that the downregulation of AKT2 protein level increased Trib3. This increase of

Trib3 in the skeletal muscle tissue downregulated the AKT2 expression and inhibited glucose uptake during impaired glucose homeostasis. Hence, overexpression of Trib3 in mice induced insulin resistance. Thus, this study suggests that Trib3 may be the therapeutic target for insulin resistance via regulation of AKT homeostasis.

Materials and Methods

Animals

The mice were housed in a cage in a temperature-controlled environment at 23 °C with a 12-h (8:00 am–8:00 pm) light–dark cycle with free access to water and a normal chow diet (Purina Rodent Chow, Seoul, Korea). To generate skeletal muscle-specific Trib3 overexpressing transgenic mice (Trib3 TG), the flag-tagged mouse Trib3 was cloned next to the 9.5 kb of skeletal muscle-specific α-actin promoter, as previously described [50]. The purified Trib3 TG construct and α-actin promoter were injected in the zygote pronucleus of F1 mice. The expression of a chimeric mice α-skeletal globin gene was examined and found to be restricted to the skeletal muscle tissues. These mice were selected and backcrossed to C57BL6/J mice. For the experiments, heterozygous Trib3 TG mouse were bred with C57BL6/J mice and their phenotypes were compared with the littermate mice.

To generate streptozotocin (STZ)-induced diabetic mouse models, STZ powder was dissolved in 0.1 M Na citrate buffer, at pH 4.5, and then filtered. Thereafter, the mouse were injected with vehicle (0.1 M Na citrate buffer, pH 4.5, and filtered) or STZ (50 mg/kg mouse in 0.1 M Na citrate buffer, pH 4.5, and filtered) in wild type and Trib3 TG mice via an intraperitoneal injection for 5 days consecutively. At 3 weeks after STZ injection of these mice, the increase in their blood glucose levels (from approximately 300–350 mg/dl) was confirmed.

The quadricep muscles separated from two groups were used for western blot.

All animal experiments conducted in this study were approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences, Asan Medical Center, Seoul, Korea.

Cell Culture

The HEK293T, C2C12, and HeLa cell lines were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Corning, Corning Life Sciences, NY, USA) and 1% penicillin/streptomycin at 37 °C. 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 Trex cells and hygromycin and blasticidin-resistant cells were selected. All cell lines were incubated in a humid environment with 5% CO2 at 37 °C

Antibodies & Reagents

Primary antibodies against the following epitope or proteins were purchased from the indicated sources: Flag (Sigma-Aldrich, St. Louis, MO, USA); AKT1, AKT2, total AKT, phospho-AKT, ACC, CREB, p62, ATG7, phospho-PKC Substrate, UCP1, SDHA, Pyruvate Dehydrogenase, AP2, Cytochrome C (Cell Signaling Technology, Danvers, MA, USA);

HSP90, HRP-conjugated HA (Santa Cruz Biotechnology. Santa Cruz, CA, USA); Trib3 (AB Frontier, Seoul, Korea). Secondary antibodies conjugated to HRP were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

The following reagents were purchased from the indicated sources: Bafilomycin A1 and doxycycline (Sigma-Aldrich, St. Louis, MO, USA); TPA, rapamycin, and MG132 (Tocris Bioscience, Bristol, UK); thapsigargin and tunicamycin (Calbiochem, San Diego, CA, USA).

Western blot

To prepare protein samples from the cultured cells, PBS-washed cells were lysed in 1% SDS lysis buffer (1% SDS, 10 mM Tris and 5 mM EDTA, pH 7.4). Using a plastic cell scraper, the cells were scraped off the culture dish and transferred to a precooled microcentrifuge tube. To prepare protein samples from the mouse tissue samples, the tissues were dissected and then transferred to storage immediately; however, the sample tube was preserved into liquid nitrogen for snap freezing of the tissue. The tissue samples were ground in liquid nitrogen and lysed with RIPA buffer (1% NP-40, 150 mM NaCl, 25 mM Tris-HCl pH 7.6, 1% sodium deoxycholate, 0.1% SDS), supplemented with a proteinase (Roche, Basel, Switzerland) and phosphatase inhibitor. The BCA protein assay is conducted for protein quantification in a sample. Each protein sample was boiled in 5X SDS sample buffer at 100 °C for 10 min. Equal amount of protein was run in SDS-PAGE, transferred to the nitrocellulose membranes, and

blocked in Tris-buffered saline with Tween 20 containing 3% BSA for 1 h at room temperature. Blots were incubated overnight at 4 °C with primary antibodies. The antigen–antibody complexes were visualized using peroxidase-conjugated antibodies and were detected by Amersham ECL Chemiluminescent detection reagent (GE Healthcare).

Co-immunoprecipitation

HEK293T cells were transfected with the HA-AKT1/2 and FL-Trib3 plasmids for co-immunoprecipitation. After transfection at 24 h, the protein samples were harvested with HEPES lysis buffer (1 mM EDTA, 1 mM EGTA, 20 mM HEPES, 200 mM NaCl, 1% Triton X-100, 5 mM Na-pyrophosphate, 20 mM β-glycerophosphate, and 50 mM NaF), supplemented with a proteinase inhibitor (Roche, Basel, Switzerland). Samples were incubated on ice for 30 min, and were then centrifuged at 13,000 rpm × 10 min at 4 °C, following which the supernatant was transferred to a fresh tube. FLAG-bead (Invitrogen) was washed thrice (centrifuged at 2000 rpm × 10 min at 4 °C) in HEPES lysis buffer. Thereafter, the supernatant and FLAG-bead were transferred to a new tube and the volume was adjusted to 1 ml with HEPES lysis buffer. After incubation at 4 °C for 5 h on a rotator, FLAG-bead was washed thrice to remove nonspecific binding. The supernatant was aspirated completely and the FLAG-bead was eluted by 2× SDS sample buffer.

Ubiquitination assay

For ubiquitination assay, the cells were transfected with the plasmids encoding 6 × His-tagged ubiquitin and indicated expression plasmids. After transfection at 24 h, the protein samples were harvested with 1% SDS lysis buffer (1% SDS, 10 mM Tris-HCl, PH 7.4) and the total ubiquitinated protein was precipitated with cobalt-coated Talon beads. Whole ubiquitinated proteins were eluted from the Talon beads and separated using SDS PAGE. Ubiquitination was analyzed using an immunoblot with the indicated antibody.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the indicated cell and tissue samples via homogenization 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 the first-stand cDNA with a random hexamer (Toyobo, Osaka, Japan). The mRNA expression of AKT1, AKT2, Trib3, and GAPDH was analyzed using RT-PCR. We used the following primer sequences presented in Table 1. The RNA was performed for reverse transcription reaction and initial denaturation, followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 15 s at 72°C.

Primer	Sequence (5'-3')
Mouse AKT1-F	GAAGCTGGAGAACCTCATGC
Mouse AKT1-R	CTTCATAGTGGCACCGTCCT
Mouse AKT2-F	TTTGCACTCGAGAGATGTGG

Mouse AKT2-R	TTTGCACAAGCCAAAGTCAG
Mouse Trib3-F	GGAACCTTCAGAGCGACTTG
Mouse Trib3-R	TCTCCCTTCGGTCAGACTGT,
Mouse GAPDH-F	ACCACAGTCCATGCCATCAC
Mouse GAPDH-R	TCCACCACCCTGTTGCTGTA

Table.1 Primer list F : Forward R : Reverse

Subcellular fractionation

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

Glucose tolerance test and insulin tolerance test

For the glucose tolerance test (GTT), overnight-fasted male mice were intraperitoneally injected with glucose (1.5~2 g/kg), and the glucose level was measured every 30 min. For the insulin tolerance test (ITT), male mice (fasting for 4–5 h) were intraperitoneally injected with insulin (Humulin, Lilly, USA, 1~1.2 unit/kg), and glucose levels were monitored using the blood collected from a tail vein with an Accu-Chek Performa glucometer (Roche, Basel, Switzerland).

Metabolic cage study

Metabolic cage studies were conducted as described previously [51]. Oxygen (O2) consumption, carbon dioxide (CO2) production, respiratory exchange ratio (RER), locomotor activity, and food intake were monitored using an indirect calorimeter (Columbus Instruments, Columbus, OH, USA or TSE systems, Bad Homburg, Germany). Metabolic cage allows accurate sensors to measure the amount of oxygen and carbon dioxide entering and exiting the chamber. The two types of measurements are the result of the respiration of a single mouse in the cage. The mice were monitored for 24–48 h and results were acquired during the nighttime (12 h) and daytime (12 h) of the light–dark cycle. Oxygen (O2) consumption and carbon dioxide (CO2) production are calculated in ml/kg/hr. Energy expenditure is calculated using O2 consumption and calorific value. Locomotor activity was evaluated during 24 h whenever

the count of infrared beam was broken in the cage. The respiratory exchange ratio (RER) is calculated as the ratio of the volume of CO2 produced to the volume of O2 used, or VCO2/VO2. In general, the range for RER is 0.7–1.0. An RER of 0.7 indicates the mixed fat use, but a 1.0 indicates the predominant use of carbohydrates. Because an RER of 1.0 requires less O2 than an RER of 0.7, the value of 1.0 represents more energy efficiency per molecule of O2. Mouse body fat and lean mass were analyzed using MRI (EchoMRI LLC, Houston, TX, USA), according to the manufacturer's instructions.

Glucose uptake assay

Mouse soleus muscles were dissected from both hind legs of Trib3 WT and KO mice. The tissues were plated in 100 μl growth media in a 96-well plate, and were washed with PBS and starved in a serum free media. Thereafter, the tissues were again washed with PBS and incubated in KRPH (Krebs-Ringer-Phosphate-HEPES buffer)/2% BSA for 40 min. Next, 10 mM 2-Deoxy-D-glucose (2-DG) was added to the tissues (control and insulin treated tissues), followed by another wash with PBS to remove 2-DG. The total lysate was extracted from the tissues by adding an extraction buffer. Furthermore, the lysate was diluted as 1/10 with an assay buffer. The output was measured at OD 412 nm on a microplate reader in a kinetic mode, until the standard value (100 pmol/well) reached 1.5~2.0 OD value.

Statistical analysis

Data are represented as mean ± SEM, and statistical significance was determined 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 as statistically significant.

Results

The overexpression of skeletal muscle tissue specific Trib3 does not affect the progress of obesity, but deteriorates glucose homeostasis.

In the preceding studies, the expression of Trib3 was known to be increased by stress signals such as metabolic stress or ER stress. To identify this, the mouse myoblast cell line, C2C12 cells, was differentiated into myotubes. C2C12 myotubes treated with thapsigargin (an inhibitor of Ca2+ store in the endoplasmic reticulum) [52] or tunicamycin (an inhibitor of N-linked glycosylation) [53] increased the expression of Trib3 (Figure 1a). Even when Palmitate was treated to cause metabolic stress, the expression of Trib3 increased (Figure 1b). Based on such results, a hypothesis was made that the Trib3 of the skeletal muscle tissue would control the insulin resistance in obese conditions. To identify this, we generated Trib3 TG mice, which feature overexpress Trib3 specifically in skeletal muscle tissue. It was confirmed that 9.5kb skeletal α-actin promoter was expressed specifically in skeletal muscle tissue (Figure 2a, b). There was no significant difference in the mRNA level of type I muscle in the soleus and type II muscle in the gastrocnemius and extensor digitorum longus [EDL] (Figure 3a). However, when they were checked at the protein level, the expression of Trib3 increased more in the gastrocnemius and EDL muscles (Figure 3d). Through this, it was revealed that the expression

of Trib3 was controlled at a posttranscriptional level.

Compared to the wild type mice, Trib3 TG mice demonstrated similar food intake day and night (Figure 4a). Moreover, similar levels of activity and energy expenditure were demonstrated (Figure 4b, 4c) and there were no differences, even in the body weights from the 4th week to 15th week after birth (Figure 4d). There were no differences in O_2 consumption, CO_2 production, energy expenditure, and physical activity between the wild type mice and Trib3 TG mice either (Figure 5). As seen in Figure 3d, tissue weight was checked through an sacrifice on wild type mice and Trib3 TG mice that generally exhibited no differences in their body weights. There were no differences in weight in all livers, epididymal white adipose tissue (EWAT), inguinal white adipose tissue (IWAT), brown adipose tissue (BAT), gastrocnemius, or quadriceps, and triceps tissues (Figure 6b). Even when the fat mass and lean body masses were checked through MRI analysis, there were no differences (Figure 6c, 6d). Through this, it was confirmed that even if the expression of skeletal muscle tissue specific Trib3 was increased, it did not affect obesity.

Although there were no differences in the body weights of wild type mice and Trib3 TG mice in general, a metabolic cage study was conducted to check their glucose homeostasis. During the daytime, the respiratory exchange ratio [RER] of two groups did not show a difference, but at nighttime, the RER of Trib3 TG mice decreased by approximately 15%

(Figure 7a). Insulin sensitivity is very important in glucose homeostasis, and to identify this, glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were conducted. It was confirmed that due to the weakened insulin sensitivity, the glucose homeostasis was weakened in Trib3 TG mice (Figure 7b and 7c). Additionally, the soleus muscle separated from two groups was used to conduct glucose uptake tests based on insulin stimulation, and it was confirmed that glucose uptake decreased in Trib3 TG mice by approximately 22% (Figure 7d). To summarize, it is believed that glucose homeostasis weakened in Trib3 TG mice probably because insulin sensitivity weakened in skeletal muscle tissues.

Trib3 in skeletal muscle tissues downregulates AKT2, ultimately suppressing the insulin signaling pathway.

As we have seen in the previous figures, Trib3 was important to the insulin signaling pathway, and we have confirmed the level of AKT, which is another protein that is important to the insulin signaling pathway. After 16hrs of fasting in the two mouse groups, the quadriceps were separated. The expression of Trib3 increased in Trib3 TG mice and phosphorylation of AKT decreased, it was confirmed that AKT activity decreased. In case of insulin was injected after 16hrs of fasting, AKT's phosphorylation increased in both wild type and Trib3 TG mice. AKT's phosphorylation in Trib3 TG mice increased more than the mice that were not injected

with insulin, but compared to the wild type mice that were injected with insulin, it decreased. Interestingly, although there were no differences in the AKT1 protein level in wild type mice and Trib3 TG mice, it was confirmed that the AKT2 protein level decreased in Trib3 TG mice (Figure 8a). AKT3 was excluded as it usually expresses in the brain. To identify it additionally, hyperglycemia was induced in both groups through streptozotocin (STZ) injection (Figure 8b). After the quadriceps were separated in the two mouse groups, it was confirmed that the expression of Trib3 increased in two groups due to STZ injection, and that the expression of AKT2 decreased (Figure 8b). For additional confirmation at the cellular level, Trib3 was overexpressed by using adenovirus, and it was confirmed that following the increase of Trib3 expression, the protein levels of AKT2 decreased in both C2C12 cells (Figure 9a) and HeLa cells (Figure 9b). To determine at which stage Trib3 controls the expression of AKT, HA-AKT1/2 and Flag-Trib3 plasmids were transfected in HEK293T cells (Figure 9c) and C2C12 cells (Figure 9d). It was confirmed that the AKT2 was downregulated by Trib3 in all cells of the two types. However, when mRNA levels were checked through RT-PCR, there were no changes in AKT2 caused by Trib3 (Figure 10). Through such data, Trib3 seems to regulate AKT2 in post-transcriptional levels.

The localization of Trib3 is important to AKT1/2 expression regulation.

Previous studies showed that Trib3 regulates AKT by directly binding with AKT, but it is unknown why AKT2 is more downregulated. We thought that Trib3 would have more affinity with AKT2 than AKT1. To identify this, a co-immunoprecipitation test was conducted, but Trib3's interaction with AKT1/2 was similar (Figure 11). For further identification, we checked AKT1/2's subcellular localization with Trib3 through a microscope. Trib3-GFP mainly expressed in the nucleus. While AKT1-RFP expressed evenly in the nucleus and cytoplasm, AKT2-RFP more expressed in the nucleus than AKT1-RFP (Figure 12a). When further identification was conducted through subcellular fractionation, abundant expression of Trib3 was confirmed in the nucleus, conforming the microscopic data. AKT2 was more expressed in the nucleus than AKT1. As for the reduction of AKT2 caused by Trib3, it reduced more significantly in the nucleus (Figure 12b). Consequently, we were able to determine that the regulation of AKT1/2 protein level was determined by Trib3's localization.

Trib3 regulates AKT protein through AKT2's ubiquitination

One of the functions of Trib3 revealed in the preceding studies was the function as a adapting molecule that links substrate with E3 ubiquitin ligase [45]. Trib3 degrades CDC25 protein in Drosophila through ubiquitination. Thus, we conducted ubiquitination assay to determine how Trib3 downregulates AKT. Previously, In case of AKT and Trib3 were co-

transfected, the expression of AKT2 decreased. Therefore, later on, inducible systems which were capable of inducing the expression of Trib3 were used. As for Trib3's induction by Doxycycline, the polyubiquitination of AKT2 was increased compared to AKT1 (Figure 13). The ubiquitin-proteasome and the autophagy-lysosome pathway are the two major protein degradation systems in eukaryotes. The two systems are responsible for protein homeostasis and protein quality control [54]. To identify how Trib3 regulates AKT2, the proteasome inhibitor, MG132 and the autophagy inhibitor, bafilomycin A1 were treated to C2C12 myotubes. Although MG132 treatment did not affect the endogenous AKT2 level, bafilomycin A1 increased AKT2 level (Figure 14a, 14b). Corresponding to such, when bafilomycin A1 was treated when AKT1/2 and Trib3 were co-transfected, Trib3-induced AKT2's downregulation has weakened (Figure 14c). Autophagy is a cellular process that maintains protein homeostasis through the clearing damaged organelles, removing abnormal proteins (aggregated or misfolded proteins). Autophagy related 7 (ATG7), E1 enzyme, plays on essential role in this pathway. Deletion of ATG7 was shown to induce the accumulation of p62 and LC3B (Figure 15a). The expression of AKT2 was higher in the ATG7 knockout cell than the control cell (Figure 15b). Moreover, AKT2's ubiquitination the by Trib3 was accumulated further by bafilomycin A1 treatment (Figure 16). Such results indicate that Trib3 regulates AKT2's homeostasis through autophagy dependent AKT2's ubiquitination.

AKT2 is downregulated even in the skeletal muscle tissue of diet-induced obese mice.

As Trib3 regulates AKT2, which is important to insulin signaling pathways, Trib3 can be considered to be the regulator that links obesity and insulin resistance. As in the previous study, the expression of Trib3 in the skeletal muscle tissue of obese mice induced by a High Fat Diet (HFD) had increased. As before, although there was no change in AKT1, we confirmed that AKT's activity and AKT2's protein level decreased (Figure 17a). Additionally, Trib3 overexpression (Adeno-Trib3) decreased insulin-stimulated-glucose uptake in C2C12 myoblasts by approximately 28% (Figure 17b). In summary, obesity causes cellular stress, and this increases the expression of Trib3. AKT2 is an important protein in insulin signaling pathway, specially downregulated by increased Trib3 via the autophagy dependent pathway. AKT2's downregulation impairs insulin signaling pathway and glucose uptake in skeletal muscle tissue. As a result, it results in insulin resistance (Figure 18).

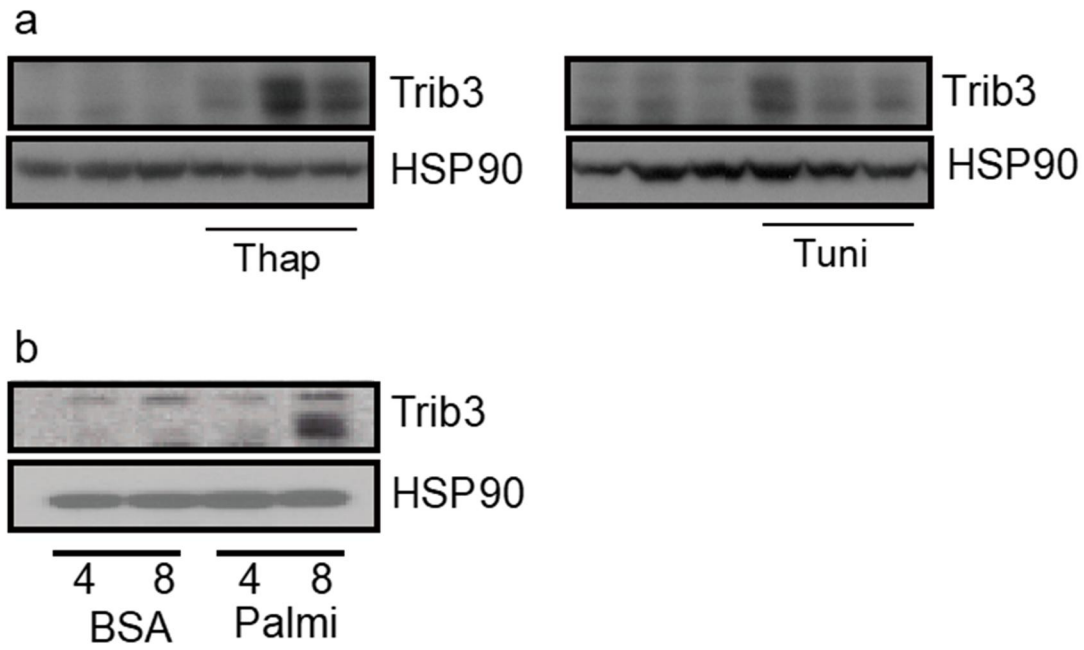

Figure 1. The ER Stress induces Trib3 expression in C2C12.

(a) To detect the increase in the expression of Trib3 under cellular stress, C2C12 myotubes were treated with two drugs, thapsigargin (0.5 μM, 12 h) or tunicamycin (1 μM, 12 h), which were known to induce ER stress. (b) The increase of Trib3 expression in the presence of BSA-conjugated palmitate (12 h) in C2C12 myotubes. HSP90 protein is used as a loading control in western blot analysis

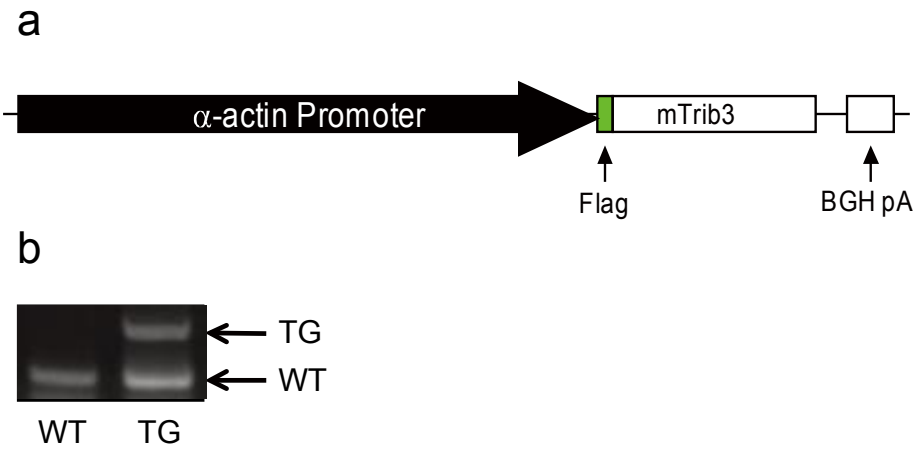

Figure 2. Schematic representation of a Trib3 TG construct.

(a) Schematic representation of a Trib3 TG construct. (b) Comparison of genotyping results of control litter mates and Trib3 TG mice by using PCR.

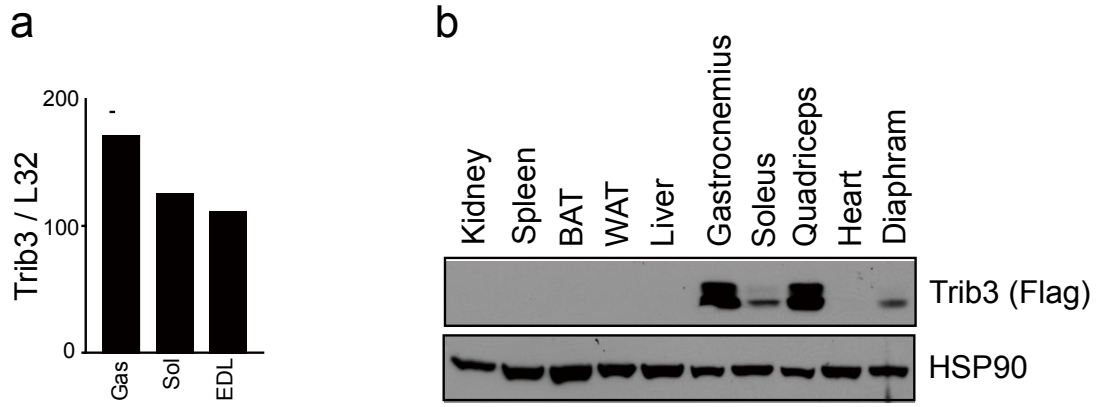

Figure 3. The expression of Trib3 is regulated at the post-transcriptional level.

(a) A bar graph represents the Trib3 mRNA expression level in gastrocnemius (Gas), soleus (Sol), and extensor digitorum longus (EDL) muscle tissues. (b) Western blots reveal the Trib3 expression levels in various tissues of Trib3 TG mice. Flag antibody detects the overexpressed Flag-tagged mouse Trib3 proteins in the skeletal muscle tissue. HSP90 protein is used as a loading control in western blot analysis.

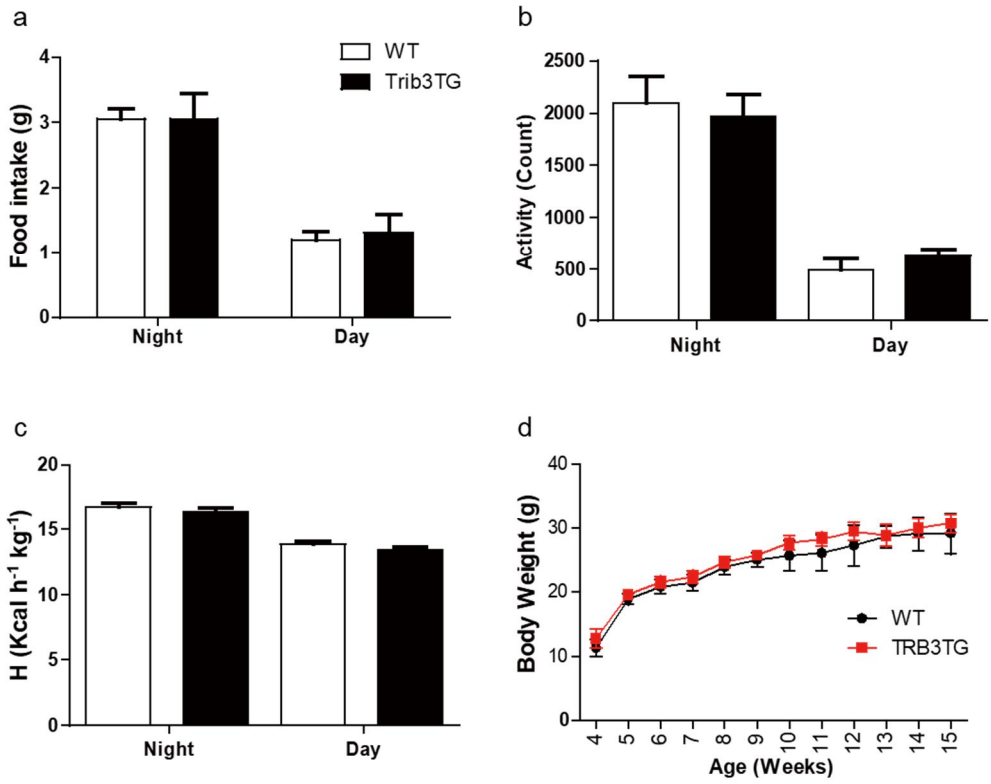

Figure 4. Overexpression of skeletal muscle-specific Trib3 does not affect obesity.

(a) Average of food intake in control and Trib3 TG mice who were fed normal chow for 24 h (n = 7–8 per group). (b) Physical activity. (c) Average of energy expenditures in control and Trib3 TG mice who were fed normal chow (n = 4 per group). The X-axis represents a period of 12 h daytime and 12 h nighttime of the light–dark cycle. (d) Average of the body weight growth curve in control and Trib3 TG mice maintained on a normal chow (n = 7–8 per group). The X-axis represents age of mice in weeks.

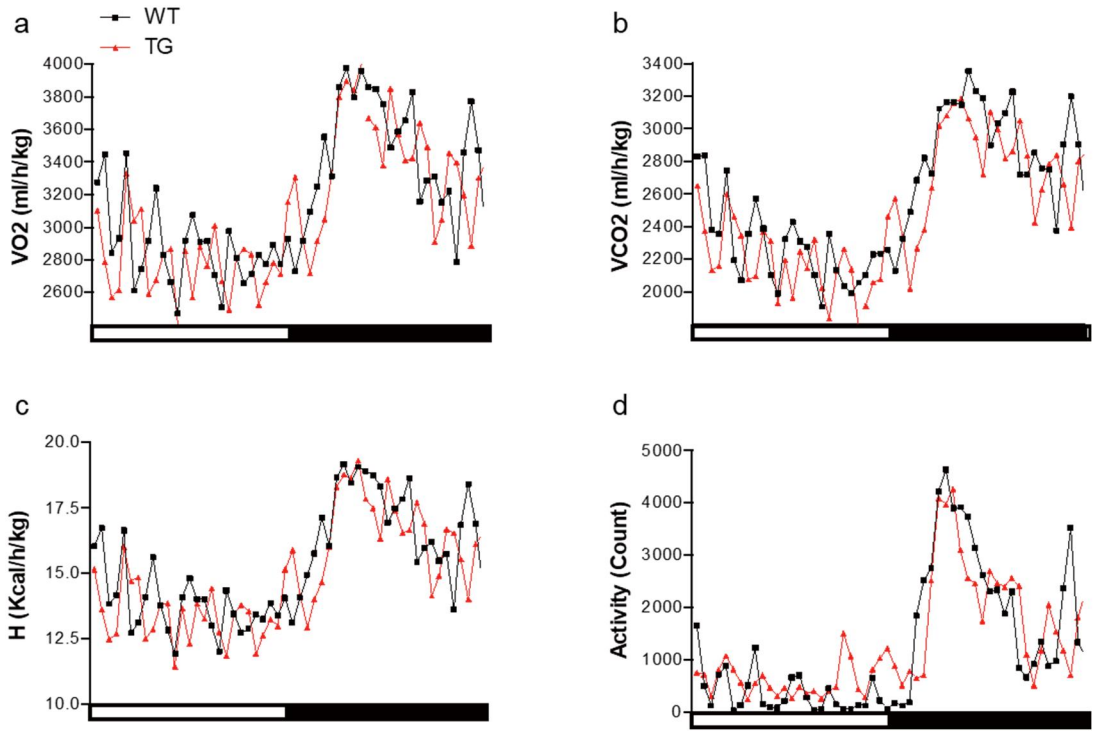

Figure 5. Overexpression of skeletal muscle-specific Trib3 does not affect the metabolic rate.

Line graphs represent the relative (a) O2 consumption, (b) CO2 production, (c) energy expenditure, and (d) physical activity in the control and Trib3 TG mice fed normal chow using indirect calorimetry. The X-axis represents a period of 12 h daytime and 12 h nighttime of the light–dark cycle.

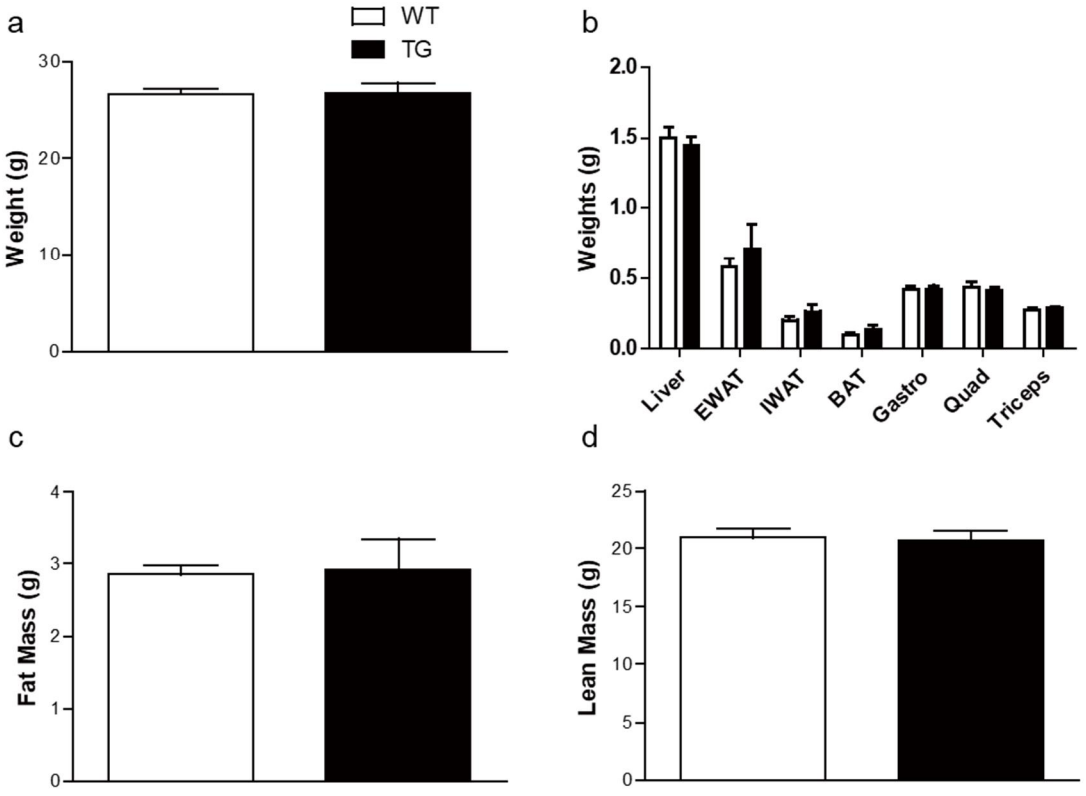

Figure 6. Overexpression of skeletal muscle-specific Trib3 does not affect the body mass.

Average of (a) body weight gain, (b) weight of various metabolic organs in control and Trib3 TG mice fed normal chow (n = 4 mice per group). A bar graph show the relative (c) fat mass and (d) lean mass in control and Trib3 TG mice through MRI analysis. The bar graphs are displayed with the white bar as the control mice and the black bar as the Trib3 TG mice. There are no significant differences between two groups.

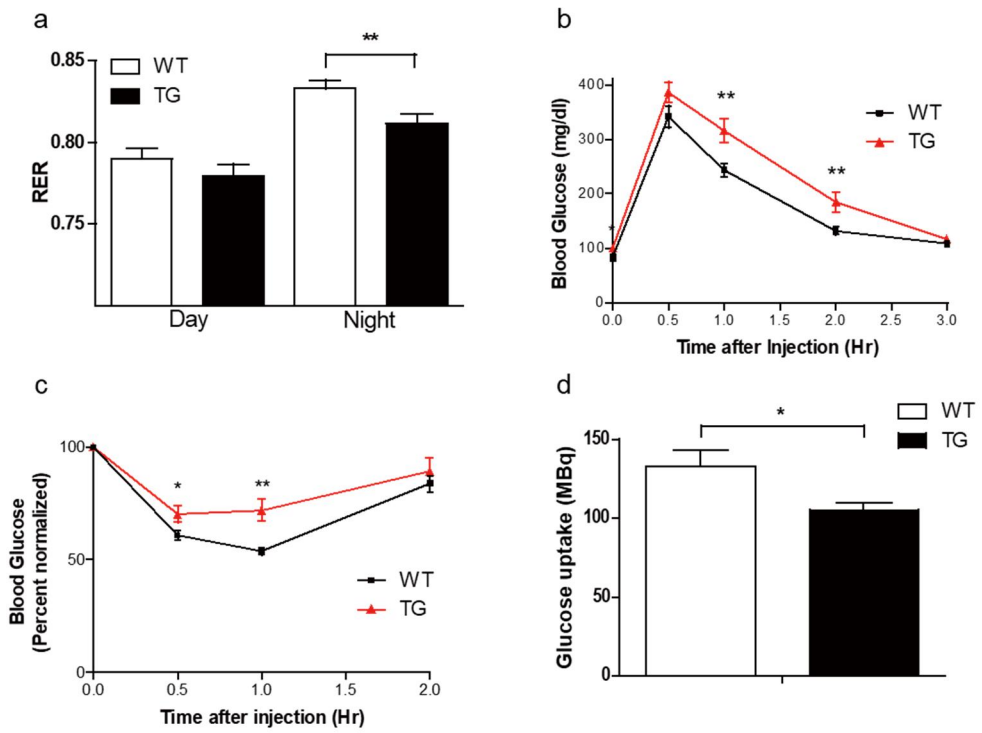

Figure 7. Impaired glucose homeostasis in skeletal muscle in Trib3 TG mice.

(a) A bar graph presents the respiratory exchange ratios (RERs) in the control and Trib3 TG mice who were fed normal chow (n = 4 per group). The X-axis represents a period of 12 h daytime and 12 h nighttime of the light–dark cycle. The bar graphs are displayed with the white bar as the control mice and the black bar as the Trib3 TG mice. (b) Glucose tolerance test (GTT) and (c) insulin tolerance test (ITT) in control and Trib3 TG mice who were fed normal chow (n = 6–7 per group). The X-axis represents the time duration after insulin injection (h). (d) A bar graph represents the average of glucose uptake in the soleus muscle of control and Trib3 TG mice (n = 3). Significant differences between the control and Trib3 TG mice ($p < 0.05$) were found.

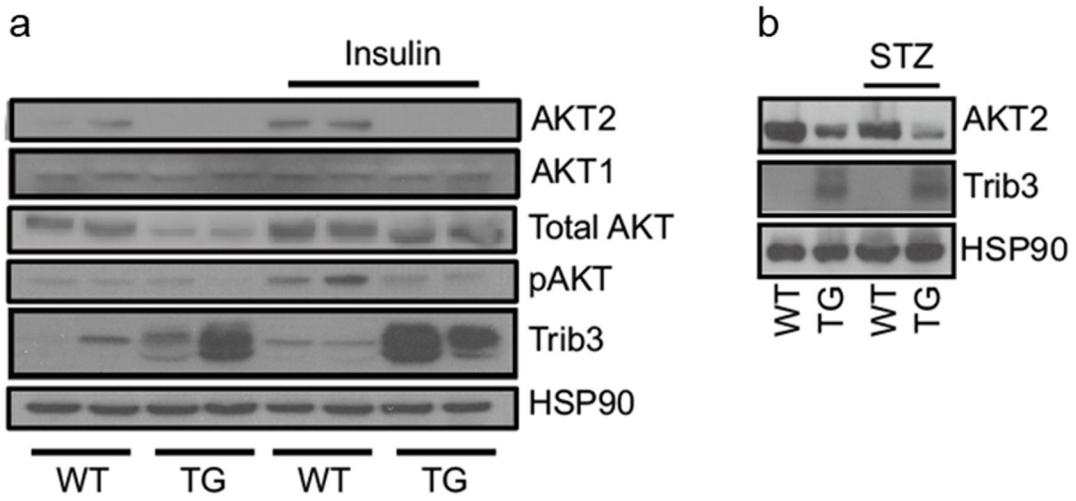

Figure 8. Trib3 specifically downregulates AKT2 protein expression in the skeletal muscle tissue of the Trib3 TG mice.

(a) To detect the changes in the expression of AKT1, AKT2, total AKT, and AKT phosphorylation, tissue samples were 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). (b) To identify the effect of hyperglycemia induced by STZ injection in quadriceps muscle tissue of control and Trib3 TG mice, the tissue samples were collected from the quadriceps of control and Trib3 TG mice. Western blots present the changes in the AKT2 and Trib3 expression levels.

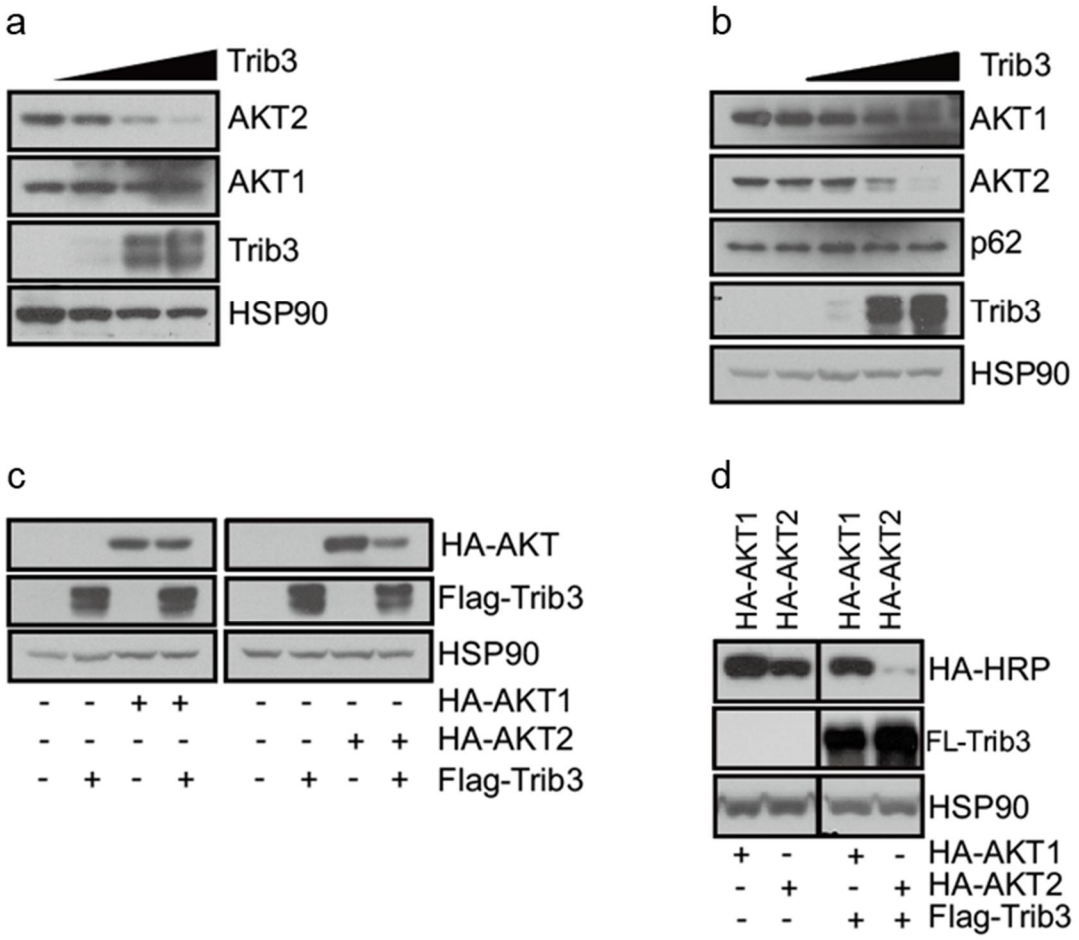

Figure 9. Trib3 specifically downregulates AKT2 protein expression at the cellular level.

Western blot reveals the changes in AKT1 and AKT2 protein levels by increasing the expression of Trib3 (Adeno-Trib3) in (a) C2C12 myoblasts and (b) HeLa cells. Western blot represent the protein levels of HA-AKT1/2 and Flag-Trib3 in (c) HEK293T cells and (d) C2C12 myoblasts at 24 h after transfection. HSP90 is used as a loading control in the western blot analysis.

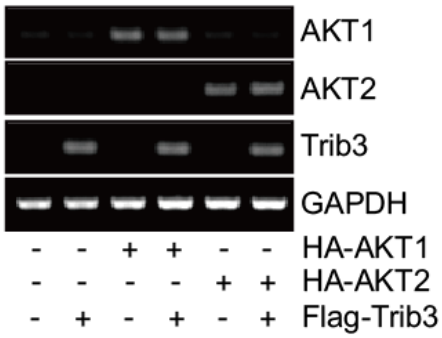

Figure 10. The mRNA level of AKT was not affected by Trib3.

RT-PCR data indicate the mRNA levels of HA-AKT1/2 and Flag-Trib3 in HEK293T cells at 24 h after transfection. GAPDH is used as a loading control in RT-PCR analysis.

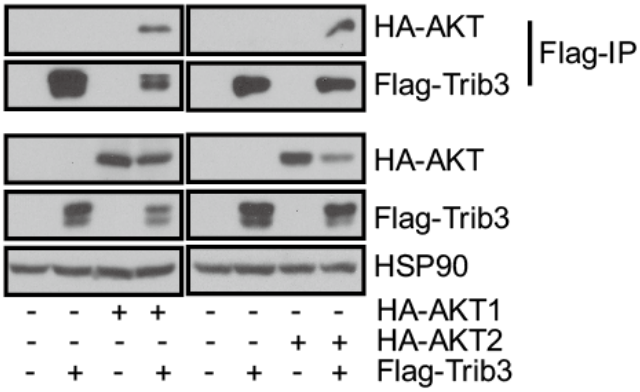

Figure 11. Trib3 interacts with AKT1/2.

(a) Western blot from co-immunoprecipitation between HA-AKT1/2 and Flag-Trib3 in HEK293T cells at 24 h after transfection. No significant differences were observed between HA-AKT1/2 and Flag-Trib3. HSP90 is used as a loading control in western blot analysis

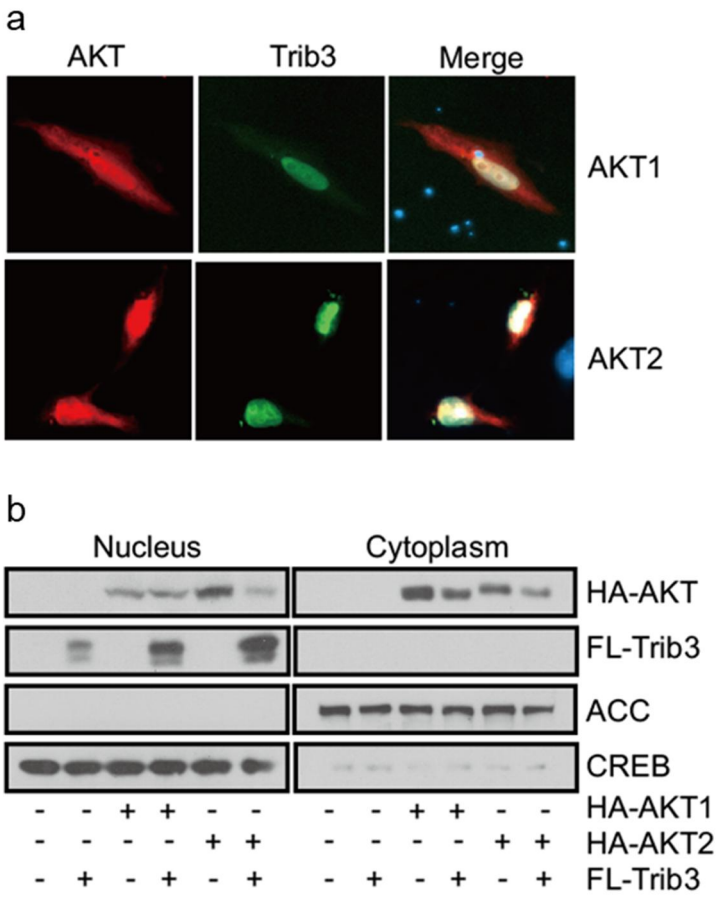

Figure 12. Association of Trib3 with AKT1/2 in the nucleus and cytoplasm.

(a) The representative images depict the subcellular localization of RFP-AKT1/2 and GFP-Trib3 in the HeLa cells. (b) Western blots present the protein levels of HA-AKT1/2 and Flag-Trib3 in HEK293T cells after the cellular fractionation assay. ACC and CREB proteins are used as the cytoplasm and nucleus markers, respectively.

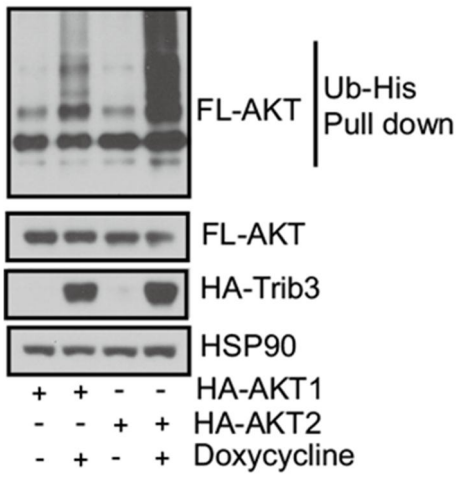

Figure 13. Trib3 induces AKT1/2 ubiquitination.

(a) Western blots reveal the ubiquitination of FL-AKT1/2 by Trib3 in Trib3 inducible cells at 24 h after transfection. Doxycycline treatment (6 h) induced expression of HA-Trib3 Trib3 inducible cells. HSP90 is used as a loading control in western blot analysis.

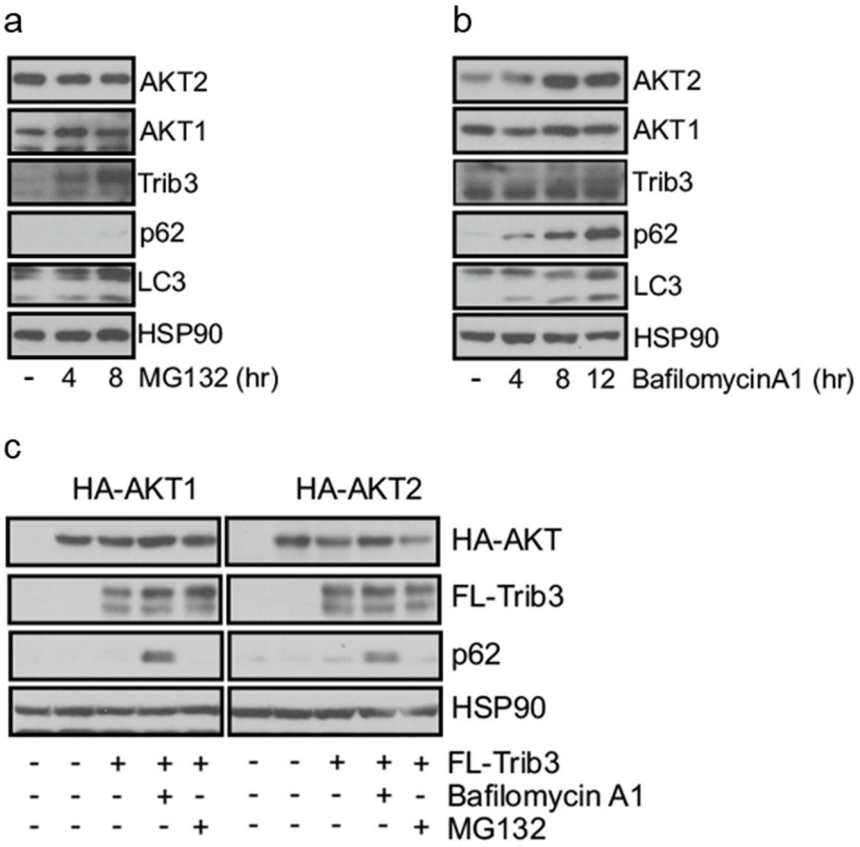

Figure 14. Trib3-induced AKT2's downregulation was weakened by an autophagy inhibitor.

Western blots present the changes in endogenous AKT1/2 and Trib3 expression levels in C2C12 myotubes in the presence of (a) MG132 or (b) bafilomycin A1. (c) To identify the effect of MG132 and bafilomycin A1 treatment, HEK293T cells were transfected with HA-AKT1/2 and Flag-Trib3. HSP90 is used as a loading control in western blot analysis..

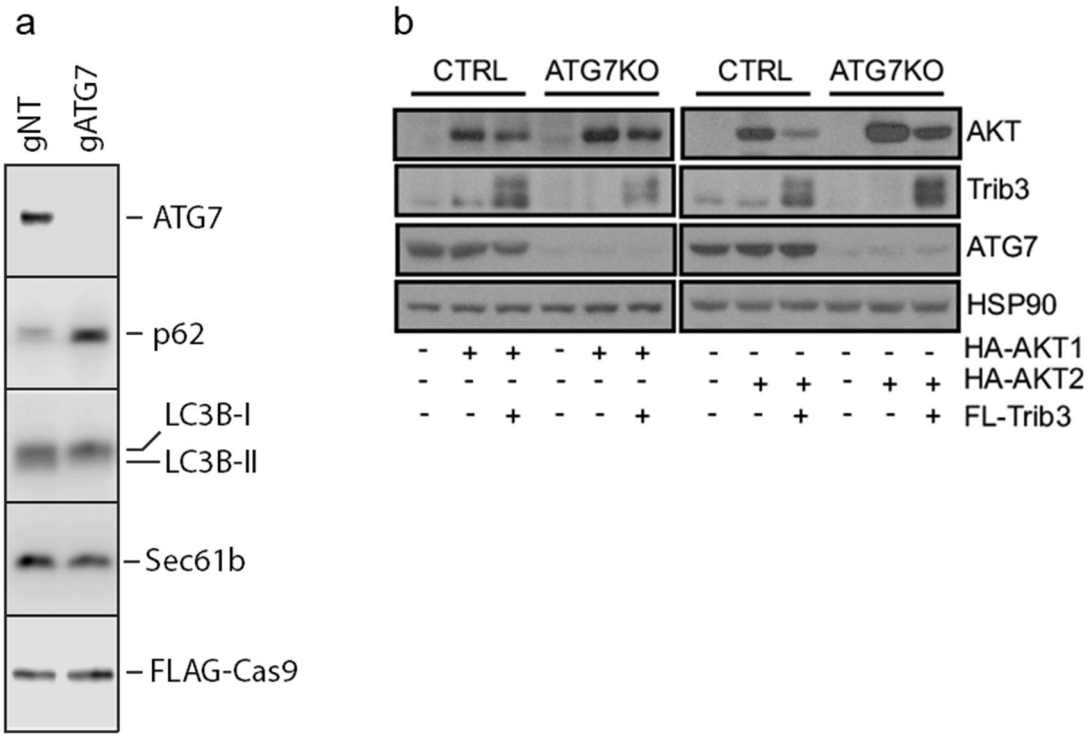

Figure 15. Ablation of autophagy induces increase of AKT2.

(a) Deletion of ATG7 in HEK293T cells using the CRISP/Cas9 system with ATG7-specific guide RNA. Western blots represent the knockout of ATG7 gene, which blocks the autophagy pathway via accumulation by p62 and LC3B. (b) Western blots present the changes in the AKT1/2 and Trib3 expression levels in control and ATG7KO cells. HSP90 is used as a loading control in western blot analysis.

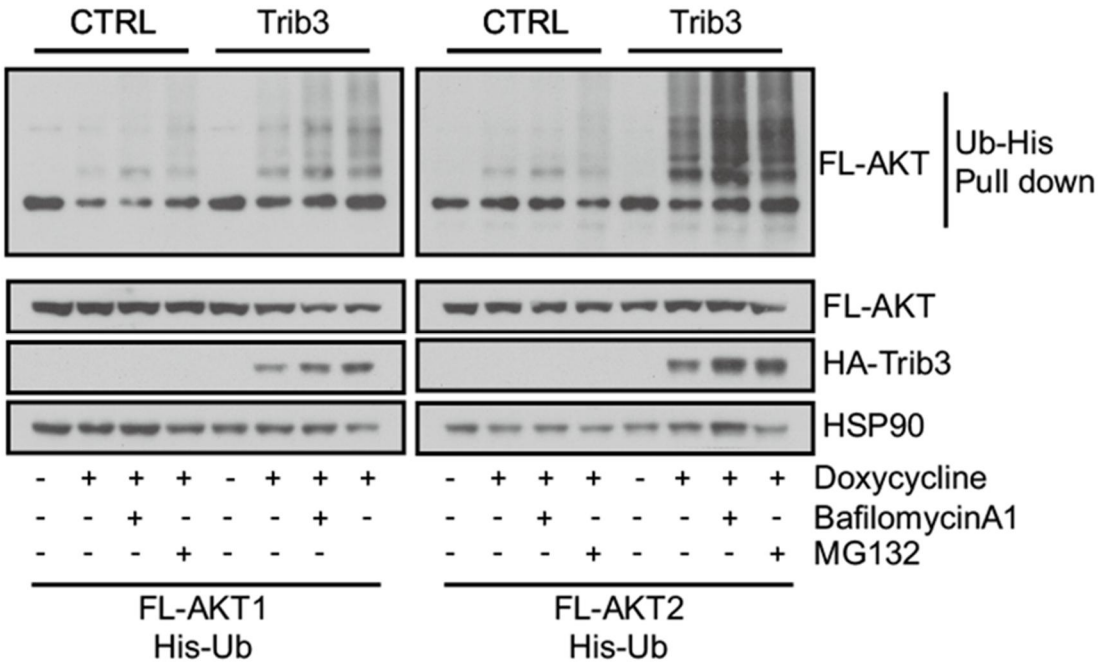

Figure 16. Trib3-induced AKT1/2 ubiquitination and AKT2 protein homeostasis via the autophagy pathway.

The effect of bafilomycin A1 and MG132 on the Trib3-induced AKT1/2 ubiquitination in control and Trib3 inducible cells. Doxycycline treatment (6 h) induced expression of HA-Trib3 Trib3 inducible cells. HSP90 is used as a loading control in western blot analysis.

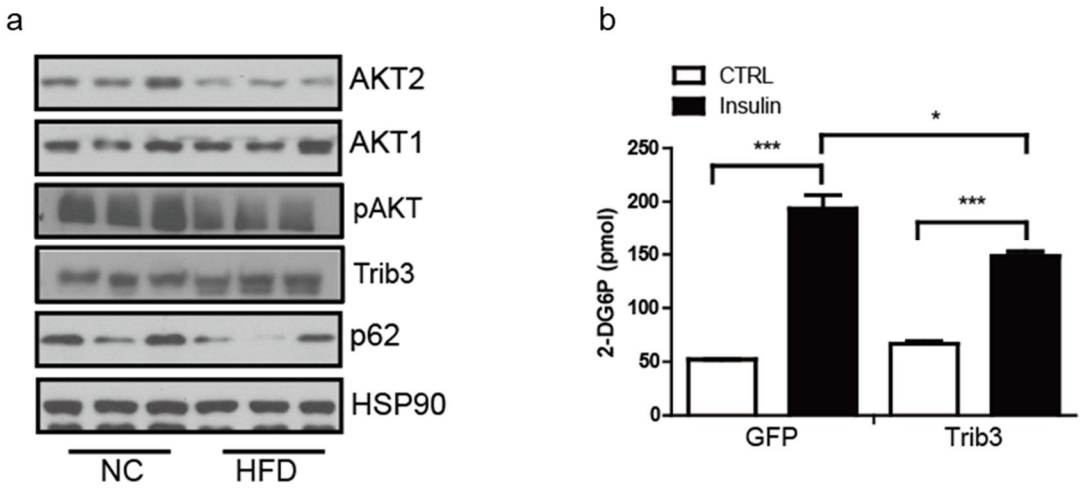

Figure 17. The increase of Trib3 in obese conditions inhibits insulin-stimulated glucose uptake by AKT2 downregulation.

(a) Western blot represent the changes in the AKT1, AKT2, total AKT, and AKT phosphorylation and Trib3 expression levels in the quadriceps of normal chow or HFD-fed mice. (b) Insulin-stimulated glucose uptake assay in Adeno-GFP (control) infected and Adeno-Trib3 infected C2C12 myoblasts. The bar graphs are displayed with the white bar as the vehicle treatment and the black bar as the insulin treatment. The X-axis represents the kind of adenovirus (control GFP or Trib3).

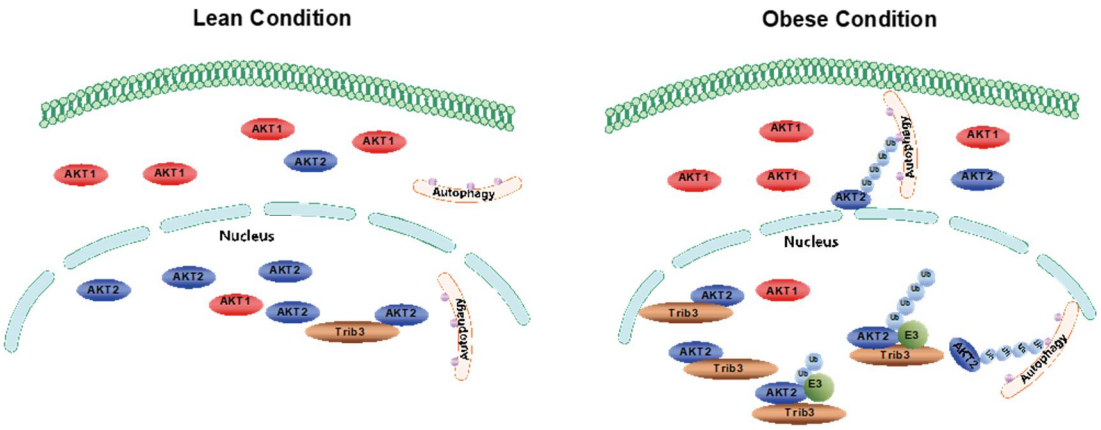

Figure 18. Schematic representation of insulin resistance in obesity.

In obese conditions, the obesity-induced cellular stress upregulates the expression of Trib3, which leads to the downregulation of AKT2 protein levels. Trib3, in turn, promotes insulin resistance.

Discussion

Although the previous studies reported that the expression of Trib3 increased in hyperglycemic and obesity conditions [44, 45], the expression of Trib3 is more relevant in insulin resistance and diabetes than in obesity. The Trib3 increased in the liver of a patient suffering from obesity accompanied by insulin resistance [44]. In the mouse model study, the increased Trib3 prevented hepatic glucose production in obese mice induced by HFD and ob/ob mice [45]. In addition, Trib3's knockdown increased AKT's activity in hepatocytes and improved glucose metabolism [42, 45, 47, 53]. The liver is an organ that produces and adjusts glucose through gluconeogenesis in the fasting state. However, skeletal muscle is also the main organ that regulates glucose uptake and glucose homeostasis. Although insulin sensitivity differs depending on the target organ, it is most influenced in skeletal muscles. When glucose uptake takes place, the required insulin amount is the biggest in skeletal muscle, and upon suppression of lipolysis, the required insulin amount is the lowest, so the majority of glucose use is determined by glycogen synthesis in the skeletal muscle. There are reports showing that in hyperinsulinemic conditions, 80~90% of glucose uptake takes place in skeletal muscle [53].

In this study, we described that the Trib3 increases in obesity conditions and that it plays an important role in glucose homeostasis. Even in previous studies, Trib3 increased in the skeletal muscle tissue of patients accompanied by insulin resistance correspondingly [56]. Meanwhile, in case of Trib3's knockdown or Trib3 whole-body knockout mice, glucose uptake increased

in the skeletal muscle tissues [46, 57]. Based on such previous studies, we conducted a test by using Trib3 transgenic mice in which Trib3 was overexpressed in the skeletal muscle tissues. The Trib3 TG mice demonstrated similar weight and tissue weight with wild type mice. Despite so, weakened insulin sensitivity was demonstrated compared to wild type mice, and this is believed to be caused by the increase of Trib3's expression in the skeletal muscle tissue. This is equivalent to the previous studies that demonstrated insulin resistance in the livers of mice [42, 47, 58]. Just like the study in which Trib3 was overexpressed in the liver [45], AKT's activity and protein levels decreased in the skeletal muscle tissue of Trib3 TG mice. Trib3 regulates the homeostasis of protein by causing degradation through proteasomes by binding with substrates or by causing ubiquitination by linking with E3 ubiquitin ligase [24, 32, 59]. This study confirmed that AKT, especially, AKT2's ubiquitination increased by Trib3 (Figure 10a).

In the majority of the previous studies, research was conducted only pertaining to the AKT's activity (phosphorylation) level and not the total AKT protein. Therefore, this study will be interesting as we can determine during which stage Trib3 regulates the AKT protein.

AKT2's downregulation by Trib3 can be explained with Trib3 and AKT1/2 protein's localization. While AKT1 is distributed in the cytoplasm of the skeletal muscle, AKT2 is abundantly expressed in the nucleus [60, 61]. The binding of Trib3 and AKT1/2 demonstrated similarities, but it was confirmed through microscopic analysis that Trib3 and AKT2 were

abundantly expressed in the nucleus. Through such, it was confirmed that Trib3 specifically downregulates AKT2 than AKT1 (Figure 9). Additionally, it was reported that AKT2 is more important than AKT1 in the insulin signaling pathway [62, 63]. Therefore, the role of Trib3 in the insulin signaling pathway is also considered to be important.

As seen in Figure 10, Trib3 regulates autophagy dependent downregulation of the AKT2. This can be seen in the increase of AKT2's accumulation by autophagy inhibitor, bafilomycin A1. As Trib3 and AKT2 are more abundant in the nucleus than AKT1, ubiquitination can take place as the Trib3-AKT2 complex moves into the cytoplasm. In another case, it can also take place in nucleus when Trib3 recruits E3 ubiquitin ligase to the nucleus. Future studies are necessary to determine the correlation between AKT2's ubiquitination caused by Trib3 and E3 ubiquitin ligase in terms of insulin resistance.

This study demonstrated that Trib3 specifically regulates AKT2 protein's homeostasis. The Trib3 increased in the skeletal muscle tissue in the obese condition and due to such, AKT's activity and AKT2's expression decreased. In Trib3 TG mice, insulin resistance was induced by the weakened insulin sensitivity and glucose homeostasis. Consequently, this study demonstrated that Trib3 will become an important therapeutic target in terms of insulin resistance through the regulation of AKT2 protein.

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국문 요약

비만은 건강을 위협하는 심각한 위험 인자로서 대표적인 대사질환인 당뇨병과 함께 수십년간 전세계적으로 꾸준히 증가하고 있다. 인슐린 저항성은 비만과 당뇨병과 밀접하게 관련 있는 것으로 알려져 있는데, 높은 인슐린 저항성을 개선함으로써 비만과 당뇨병을 효과적으로 개선할 수 있다. 비만으로 인해 인슐린 저항성이 증가하고 이는 당뇨병을 유발하게 되지만 아직까지 비만이 어떻게 당뇨병을 유발하는지 명확하지 않다.

본 연구에서는, Trib3 를 과발현 시킨 Trib3 형질전환 마우스를 이용하여 포도당대사에서 중요한 골격근조직에서 Trib3 의 생리학적 역할을 알아보았다. 대사케이지 연구, 당부하검사, 인슐린부하검사, 근육에서의 당 흡수 검사를 통해 Trib3 형질전환 마우스에서 인슐린 민감성이 약해진 것을 확인하였다. 세포 수준에서, Trib3 의 과발현으로 인한 AKT 의 활성과 AKT2 의 하향조절을 확인하였다 유비퀴틴화 분석 (Ubiquitination assay)과 ATG7 유전자를 제거한 세포주에서의 실험을 통해 Trib3 가 자가포식작용 (Autophagy) 의존적으로 AKT2 단백질의 항상성을 조절하는 것을 확인하였다. 고지방식으로 비만을 유도한 마우스의 골격근조직에서 Trib3 의 발현이 증가되었고, 이로 인해 AKT 의 활성이 감소하였다. 특히, Trib3 의 발현에 의해 AKT1 에 비해 AKT2 가 현저히 하향조절 되었다.

비만을 유도한 마우스의 골격근조직에서 Trib3 의 증가는 인슐린 신호전달 경로에 중요한 AKT2 를 하향조절 시켜 인슐린 저항성을 유발한다. 때문에 Trib3 는 비만과 인슐린 저항성에 있어 중요한 역할을 할 것이라 보여 진다.

<핵심 단어> 비만, 인슐린 저항성, Trib3, AKT2, 자가포식작용