

TWEAK promotes exercise intolerance by decreasing skeletal muscle oxidative phosphorylation capacity

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TWEAK promotes exercise intolerance by decreasing skeletal muscle oxidative phosphorylation capacity

Shuichi Sato¹, Yuji Ogura¹, Vivek Mishra^{1,3}, Jonghyun Shin¹, Shephali Bhatnagar¹, Bradford G Hill² and Ashok Kumar^{1*}

Abstract

Background: Proinflammatory cytokine tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and its receptor Fn14 are the major regulators of skeletal muscle mass in many catabolic conditions. However, their role in muscle metabolism remains largely unknown. In the present study, we investigated the role of TWEAK on exercise capacity and skeletal muscle mitochondrial content and oxidative metabolism.

Methods: We employed wild-type and TWEAK-knockout (KO) mice and primary myotube cultures and performed biochemical, bioenergetics, and morphometric assays to evaluate the effects of TWEAK on exercise tolerance and muscle mitochondrial function and angiogenesis.

Results: TWEAK-KO mice showed improved exercise tolerance compared to wild-type mice. Electron microscopy analysis showed that the abundance of subsarcolemmal and intermyofibrillar mitochondria is significantly increased in skeletal muscle of TWEAK-KO mice compared to wild-type mice. Furthermore, age-related loss in skeletal muscle oxidative capacity was rescued in TWEAK-KO mice. Expression of a key transcriptional regulator peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) and several other molecules involved in oxidative metabolism were significantly higher in skeletal muscle of TWEAK-KO mice. Moreover, treatment of primary myotubes with soluble TWEAK inhibited the expression of PGC-1 α and mitochondrial genes and decreased mitochondrial respiratory capacity. Deletion of TWEAK also improved angiogenesis and transcript levels of vascular endothelial growth factor in skeletal muscle of mice.

Conclusions: These results demonstrate that TWEAK decreases mitochondrial content and oxidative phosphorylation and inhibits angiogenesis in skeletal muscle. Neutralization of TWEAK is a potential approach for improving exercise capacity and oxidative metabolism in skeletal muscle.

Keywords: Skeletal muscle, Exercise tolerance, TWEAK, Fn14, PGC-1 α , PPAR δ

Background

Skeletal muscle is the largest tissue of the human body which ensures basic functions such as locomotion, metabolism, and respiration. Skeletal muscle exhibits a high level of plasticity in response to physiological stressors. For example, in response to exercise training, the proportion of slow-type fibers and mitochondrial content within fibers is noticeably increased [1]. The biogenesis

of new mitochondria and clearance of defunct mitochondria are essential to meet cellular energy demand especially during endurance exercise and to protect from many chronic conditions such as diabetes, heart failure, obesity, and aging [2,3]. Peroxisome proliferator-activated receptor (PPAR)- γ coactivator 1 α (PGC-1 α) is a key player in regulating skeletal muscle fiber composition, mitochondrial content, and oxidative metabolism, and maintenance of glucose, lipid, and energy homeostasis in response to physiological demands [4-6]. Transgenic mice overexpressing physiological levels of PGC-1 α in skeletal muscle have increased mitochondrial biogenesis and oxidative capacity,

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are more resistant to fatigue and have improved aerobic performance [7-10]. Forced expression of PGC-1 α also preserves skeletal muscle mass in various catabolic states including denervation [11,12]. PGC-1 α improves mitochondrial biogenesis by coactivating nuclear respiratory factor (NRF)-1 [13], NRF-2 [14] and estrogen-related receptor α [15-17] which, in turn, augments the expression of hundreds of nuclear-coded mitochondrial genes [18]. Recent evidence also suggests a role for mitochondria in preventing muscle protein degradation in catabolic conditions [19,20]. Thus, preserving mitochondria content and function is essential to countering muscle wasting and to improving muscle homeostasis.

Inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) are some of the important mediators of loss of skeletal muscle mass and function in many catabolic conditions [21-24]. TNF- α has been reported to augment muscle protein degradation both *in vitro* and *in vivo* [25-27]. Moreover, TNF receptor 1 knockout (KO) mice are protected from diet-induced obesity due to increased thermogenesis [28]. Importantly, TNF- α inhibits the expression of PGC-1 α levels in myotubes [29]. In animal models of cancer cachexia, mitochondrial content and biogenesis are reduced with increased levels of IL-6 during the progression of the condition whereas inhibition of IL-6 activity attenuates tumor-induced loss of skeletal muscle [30,31]. It is also notable that while moderate exercise attenuates inflammation [32-34], acute exercise more robustly increases the levels of inflammatory cytokines in obese compared with lean subjects suggesting that rigorous exercise can further aggravate the condition of overweight individuals [35]. Despite these observations, cause-and-effect relationships between different inflammatory mediators, exercise tolerance, and mitochondrial function in skeletal muscle have not been clearly established using genetic mouse models.

TNF-like weak inducer of apoptosis (TWEAK) is a major proinflammatory cytokine of the TNF super family [36] that functions by binding to Fn14 receptors on target cells [37-39]. TWEAK-Fn14 signaling mediates unique and context-dependent pleiotropic effects [40]. TWEAK has been recently identified to be a key mediator that causes skeletal muscle loss that occurs in response to denervation, immobilization, and starvation [21,41,42]. Moreover, TWEAK-transgenic mice overexpressing physiological levels of TWEAK in skeletal muscle showed a higher proportion of fast-type fibers (glycolytic) in soleus and extensor digitorum longus (EDL) muscle [41] indicating that elevated levels of TWEAK cause a slow-to-fast type fiber switch. However, the role and mechanisms by which TWEAK affects exercise capacity and bioenergetic function have not been studied.

In the present study, we have used TWEAK-knockout (KO) mice to investigate the role of TWEAK in exercise

tolerance and skeletal muscle bioenergetic function. Our results show that, compared with wild-type mice, TWEAK-KO mice have increased exercise tolerance, which is associated with higher levels of skeletal muscle subsarcolemmal and intermyofibrillar mitochondria and enhanced oxidative phosphorylation capacity. TWEAK represses the expression of PGC-1 α and several other molecules involved in mitochondrial biogenesis and oxidative metabolism *in vivo* and in cultured myotubes. Furthermore, vascularization and expression of vascular endothelial growth factor (VEGF) are increased in skeletal muscle of TWEAK-KO mice compared with wild-type mice. These results support our hypothesis that TWEAK causes exercise intolerance by suppressing mitochondrial oxidative metabolism and angiogenesis.

Methods

Cell culture

Primary myoblasts were isolated from hind limb muscle of mice and cultured following the same protocol as previously described [43]. Briefly, hind limb skeletal muscles from mice were aseptically isolated, minced into a coarse slurry, and enzymatically digested at 37°C for one hour by adding 200 IU/ml collagenase I (cat # LS004196; Worthington, Lakewood, NJ, USA) and 0.1% pronase (EMD Chemicals, Billerica, MA, USA). The digested slurry was filtered through a 70 μ m filter and spun, and isolated cells were resuspended and cultured initially in F-10 medium (containing 20% fetal bovine serum (FBS) and supplemented with 10 ng/ml basic fibroblast growth factor) and then in F-10 plus (Dulbecco's) Modified Eagle's Medium ((D)MEM) (1:1 ratio) based culture medium supplemented with 15% FBS on culture dishes coated with 10% matrigel (BD Biosciences, San Jose, CA, USA). Differentiation in primary myoblast cultures was induced by replacing the growth medium with differentiation medium (2% horse serum in (D)MEM).

Animals

TWEAK-KO mice were provided by Dr. Avi Ashkenazi (Genentech South, San Francisco, CA, USA) and have been previously described [40]. All the mice were in the C57BL/6 background, and their genotype was determined by PCR from tail DNA. All animal procedures were approved (protocol # 10129) by the Institutional Animal Care and Use Committee and conformed to the American Physiological Society's Guiding Principles in the Care and Use of Animals.

Treadmill running protocol

Mice were subjected to treadmill running following the same previously described protocol [44]. In brief, 4.5-month-old wild-type and TWEAK-KO mice were matched for body weight and randomly assigned to

either a sedentary or exercise group. Mice were subjected to an acute bout of treadmill (Eco3/6 treadmill; Columbus Instruments, Columbus, OH, USA) running at 15 m/minute for 90 minutes. All mice in the exercise group finished the 90-minute trial and were visibly exhausted. Mice were sacrificed within 30 minutes after exercise to study mitochondrial function.

Exercise tolerance test

The exercise tolerance test on mice was performed following a method as previously described [45]. Briefly, all animals were run on a treadmill (Columbus Instruments) at 10 m/minutes for five minutes at 0% degree incline for acclimation for three days. On the exercise testing day, animals ran on the treadmill with a fixed slope of 10%. Mice first ran at 10 m/minute for five minutes and the speed was increased by 2 m/minute every two minutes until they were exhausted or a maximal speed of 46 m/minute was achieved. The criterion of exhaustion was defined as the inability of the animal to run on the treadmill for 10 seconds despite mechanical prodding. Running time and maximum speed achieved was measured whereas running distance, work and power were calculated.

Transmission electron microscopy

Soleus muscles from wild-type and TWEAK-KO mice were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer overnight followed by fixing in 1% cacodylate-buffered osmium tetroxide. The tissue was dehydrated through a series of graded alcohols, and embedded in LX-112 plastic (Ladd Research Industries, Williston, VT, USA). Longitudinal sections (80 nm) were cut using an ultramicrotome (LKB, Rockville, MD, USA) and stained with uranium acetate and lead citrate. Samples were analyzed using a transmission electron microscope (Philips CM 12; HZB, Berlin, Germany) operating at 60 kV. The pictures were captured at 8,800x magnification using a 3.2 megapixel digital camera (Sia-7C; Kodak, Rochester, NY, USA) at room temperature. No imaging medium was used to visualize the pictures, and images were stored as JPEG files. Image levels were equally adjusted using Photoshop CS2 software.

Succinate dehydrogenase (SDH) staining

SDH staining was performed as previously described [46]. Briefly, transverse sections (8 μ m) were cut from the mid-belly of the TA muscles on a cryostat at -20°C and stored at -80°C until SDH staining was performed. The sections were dried at room temperature for 30 minutes before incubation in a solution made up of 0.2 M phosphate buffer (pH 7.4), 0.1 M MgCl_2 , 0.2 M succinic acid (Sigma Chemical Company, St. Louis, MO, USA)

and 2.4 mM nitroblue tetrazolium (NBT, Sigma) at 37°C in a humidity chamber for 45 minutes. The sections were then washed in deionized water for three minutes, dehydrated in 50% ethanol for two minutes, and mounted for viewing with DPX mount medium (Electron Microscopy Sciences, Hatfield, PA, USA). Digital photographs were taken from each section at 10X magnification under a Nikon Eclipse TE 2000-U microscope (Nikon, Melville, NY, USA) with a Nikon digital camera (Digital Sight DS-Fi1), and fibers were quantified with imaging software (Image J, NIH). At least 700 fibers were counted to determine SDH-positive fibers in each section in a blinded fashion. The percentage of SDH stained fibers was then determined based on a criteria using integrated optical density.

Measurement of mitochondrial bioenergetics

Mitochondrial oxidative capacity was measured in isolated mitochondria and in cultured myotubes using a Seahorse Bioscience XF24 extracellular flux analyzer (Billerica, MA, USA). For measurements in isolated mitochondria, tissue from the limb muscle (approximately 50 mg) was isolated within 30 minutes of exercise and homogenized in 1 ml of isolation buffer (220 mM mannitol, 70 mM sucrose, 5 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 1 mM ethylene glycol tetraacetic acid (EGTA), 0.3% fatty acid-free BSA, pH 7.2) using a Potter Elvehjem tube and a Teflon pestle. The homogenate was centrifuged at $500\times g$ for five minutes at 4°C . The supernatant containing mitochondria was centrifuged at $10,000\times g$ for five minutes. After two wash-centrifugation steps in BSA-free isolation buffer, the mitochondria were suspended in respiration buffer (120 mM KCl, 25 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM MgCl_2 , 5 mM KH_2PO_4 , pH to 7.2). Protein in the mitochondrial suspension was estimated using the Lowry DC assay (Biorad, Hercules, CA, USA) and 5 to 12.5 μ g of mitochondrial protein was sedimented in XF culture plates as described previously [47]. Complex I-mediated, state 3 respiratory activity was determined by measuring the oxygen consumption rate (OCR) after injection of pyruvate (5 mM), malate (2.5 mM) and ADP (1 mM). The OCR of mitochondria after exposure to oligomycin (1 μ g/ml) was used to estimate state 4 activity; exposure to carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 2 μ M) was used to examine the uncoupled rate of respiration. Finally, succinate (10 mM) and rotenone (1 μ M) were injected to assess maximal Complex II-mediated respiratory capacity. Data are expressed as pmol O_2 /min/ μ g protein. For measurements in cultured cells, differentiated myoblasts were exposed to soluble TWEAK (R&D Systems, Minneapolis, MN, USA) for 72 hours followed by the mitochondrial function assay outlined previously [48-51].

RNA isolation and quantitative real time-PCR

RNA isolation and quantitative real time-PCR (qRT-PCR) were performed as previously described [41]. Briefly, total RNA was isolated from homogenized mouse tissues using the TRIzol reagent (Invitrogen, Grand Island, NY, USA) and an RNeasy Mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturers' protocols. First strand cDNA for PCR analyses were made using a reverse transcription system with 1 µg of purified RNA using oligo (dT) primer (Applied Biosystems, Grand Island, NY, USA) and the Omniscript reverse transcription kit (QIAGEN). The quantification of mRNA expression was performed using the SYBR green dye method on a sequence detection system (Applied Biosystems, model 7300). Primers were designed using Vector NTI software (Invitrogen). Primer sequences are available on request. The thermal conditions consisted of an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and extension at 60°C for one minute, and, for a final step, a melting curve of 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds. All reactions were performed in duplicate to reduce variation. Data normalization was accomplished using the endogenous control (β -actin), and the normalized values were subjected to a $2^{-\Delta\Delta C_t}$ formula to calculate the fold change between control and experimental groups.

Immunostaining for CD31

After cutting 8 µm thickness frozen sections of TA muscles, the sections were fixed by cold acetone for 10 minutes and dried in air for 30 minutes. The tissues were rinsed with phosphate buffered saline (PBS) twice, blocked in 2% BSA solution for one hour at room temperature followed by incubation with primary antibodies in 2% BSA solution (laminin, anti-rabbit 1:300, Sigma and CD31, anti-mouse 1:30, BD Biosciences, San Jose, CA, USA) at 4°C in a humidity chamber overnight. The next day, the sections were rinsed with PBS for five minutes three times and incubated with secondary antibody (Alexa Fluor-conjugated 488 anti-rabbit, 1:2500 and 546 anti-mouse, 1:2500, Invitrogen) in 2% BSA solution for one hour at room temperature. After washing twice with PBS and once with deionized water, the sections were mounted with DPX mounting medium. Digital photographs were taken from each section under a Nikon Eclipse TE 2000-U microscope (Nikon) with a Nikon digital camera (Digital Sight DS-Fi1). Number of CD31-positive vessels per myofiber was quantified using NIS Elements BR 3.00 software (Nikon).

Statistical analysis

The results are presented as means \pm standard deviation (SD). Student's *t*-test was used to compare the difference

between control and treatment groups. A value $P < 0.05$ was considered statistically significant.

Results

Improvement in exercise tolerance in TWEAK-KO mice

We first studied the role of TWEAK in exercise tolerance in mice. Male 4.5-month old wild-type and TWEAK-KO mice were acclimated for three days for treadmill running prior to the exercise tolerance test. After acclimation, the mice were run on a 10% slope until the mice were unable to run on the treadmill for 10 seconds despite mechanical prodding. TWEAK-KO mice ran longer compared to wild-type mice (1,483 seconds for TWEAK-KOs versus 1,170 seconds for the controls, Figure 1A). Since TWEAK-KO mice could keep running on higher speed (30.6 m/minute for TWEAK-KOs versus 25.5 m/minute for the controls, Figure 1B), the difference in running distance became greater than 47% (463 meter for TWEAK-KOs versus 314 meter for the controls, Figure 1C). Work and power generated by TWEAK-KO mice were also higher compared to wild-type mice. TWEAK-KO mice generated 22.2 J of work while control mice generated 15.2 J of work (a difference of 46%) during the exercise test (Figure 1D). In turn, TWEAK-KO mice exerted 14% more power than the controls (14.9 mW for TWEAK-KO versus 13.0 mW for the wild-type, Figure 1E) on the treadmill. These data established that TWEAK-KO mice have significantly improved exercise tolerance.

Ablation of TWEAK improves mitochondrial content in skeletal muscle of mice

We have previously shown that deletion of TWEAK increases the proportion of type I fibers in soleus and EDL muscles of mice [41]. In this study, we investigated whether TWEAK affects mitochondrial content in skeletal muscle of mice. The soleus muscle of 4.5-month old TWEAK-KO and wild-type mice were isolated and used to measure mitochondrial content by performing transmission electron microscopy. As shown in Figure 2A, the abundance and size of mitochondria was found to be increased in TWEAK-KO compared to wild-type mice. Quantitative analysis of mitochondria in electron micrographs showed that the levels of subsarcolemmal and intermyofibrillar mitochondria were increased by 42% and 32%, respectively, in TWEAK-KO mice compared to wild-type mice (Figure 2B and Figure 2C). Because gastrocnemius (GA) muscle contains a mixture of slow and fast twitch fibers and greatly influences running capacity on the treadmill, we measured mRNA levels of fiber type-specific myosin heavy chain (MyHC) isoforms in GA muscle of wild-type and TWEAK-KO mice. As shown in Figure 2D, mRNA levels of MyHC I and MyHC IIA were increased by 2- and 2.7-fold, respectively in TWEAK-KO mice compared to wild-type mice. These results further

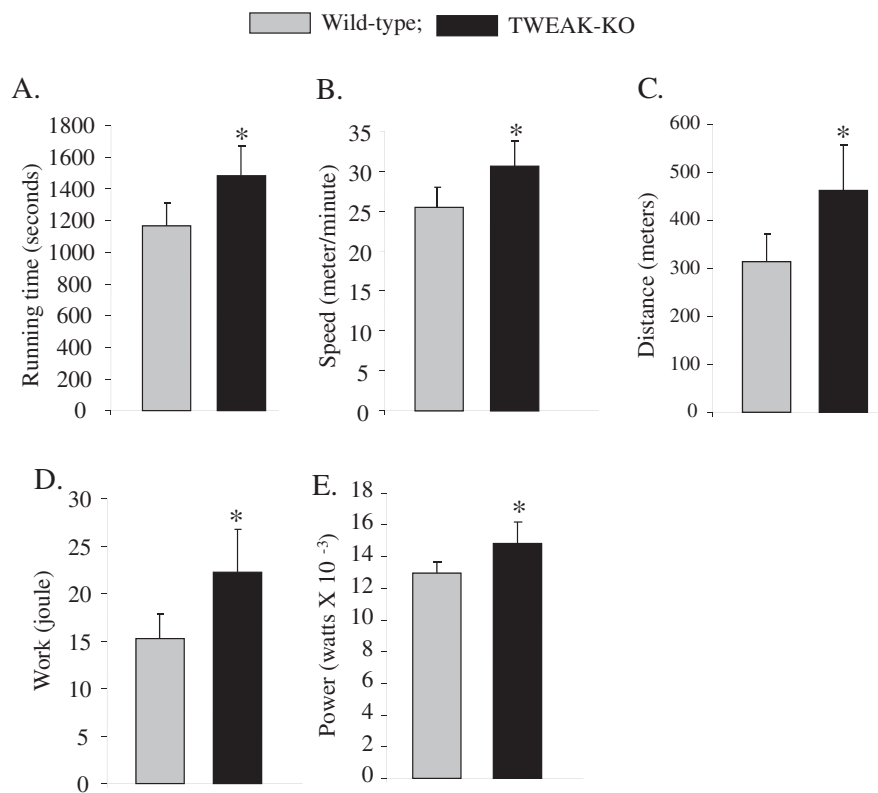


Figure 1 Treadmill exercise tolerance test. After acclimatization, mice were run on a treadmill with a 10% slope and increasing speed to exhaustion. Maximum speed and running time were monitored, and distance, work, and power were calculated based on the individual performance. (A) Running time; (B) Speed; (C) Distance; (D) Work; and (E) Power. Data are represented as mean ± SD. N = 6 in each group. *P < 0.05; values vary significantly between wild-type and TWEAK-KO mice. KO, knockout.

suggest that genetic ablation of TWEAK increases the slow-type fiber phenotype in skeletal muscle of mice.

SDH, also known as complex II in the mitochondrial respiratory chain, is a marker of oxidative capacity of skeletal muscle at the fiber level. Therefore, we performed SDH staining on TA muscle of 4.5-month- and 12-month-old wild-type mice and TWEAK-KO mice and quantified the percentage of SDH-positive fibers. Compared to wild-type mice, fibers in TWEAK-KO mice stained more darkly for SDH (Figure 3A). Furthermore, there was a significant increase in the number of SDH-positive fibers in TWEAK-KO mice compared to wild-type mice at the age of 4.5 months (Figure 3B). Likewise, 12-month-old TWEAK-KO had more SDH-positive fibers compared to age-matched wild-type mice (Figure 3A and Figure 3C). Taken together, these results suggest that ablation of TWEAK in mice is sufficient to improve mitochondrial content and oxidative capacity of skeletal muscle.

TWEAK-KO mice demonstrate increased expression of PGC-1 α and metabolic genes in skeletal muscle

To understand the mechanisms by which TWEAK affects exercise capacity and mitochondrial content in skeletal

muscle, we investigated whether TWEAK modulates the expression of PGC-1 α , a critical regulator of the mitochondrial biogenetic program in skeletal muscle [9,10]. Interestingly, the mRNA levels of PGC-1 α were found to be significantly higher in 4.5-month-old TWEAK-KO mice compared to age-matched wild-type mice (Figure 4). Similarly, mRNA levels of PPAR δ were significantly higher in skeletal muscle of TWEAK-KO mice compared to corresponding wild-type mice (Figure 4). In contrast, mRNA levels of glycolytic enzymes such as hexokinase-2 (HK II) and phosphoglycerate mutase 2 (PGAM2) were reduced in skeletal muscle of TWEAK-KO mice compared to wild-type mice. No significant change was observed in the expression of pyruvate dehydrogenase kinase-4 (PDK4). Furthermore, mRNA levels of mitochondrial carnitine palmitoyltransferase I (mCPT1), which is required for the transport of long-chain fatty acyl-CoAs from the cytoplasm into the mitochondrion, were significantly elevated in TWEAK-KO mice compared to wild-type mice (Figure 4). These results suggest that a deficiency of TWEAK reduces expression of glycolytic genes and augments the expression of genes involved in oxidative metabolism in skeletal muscle.

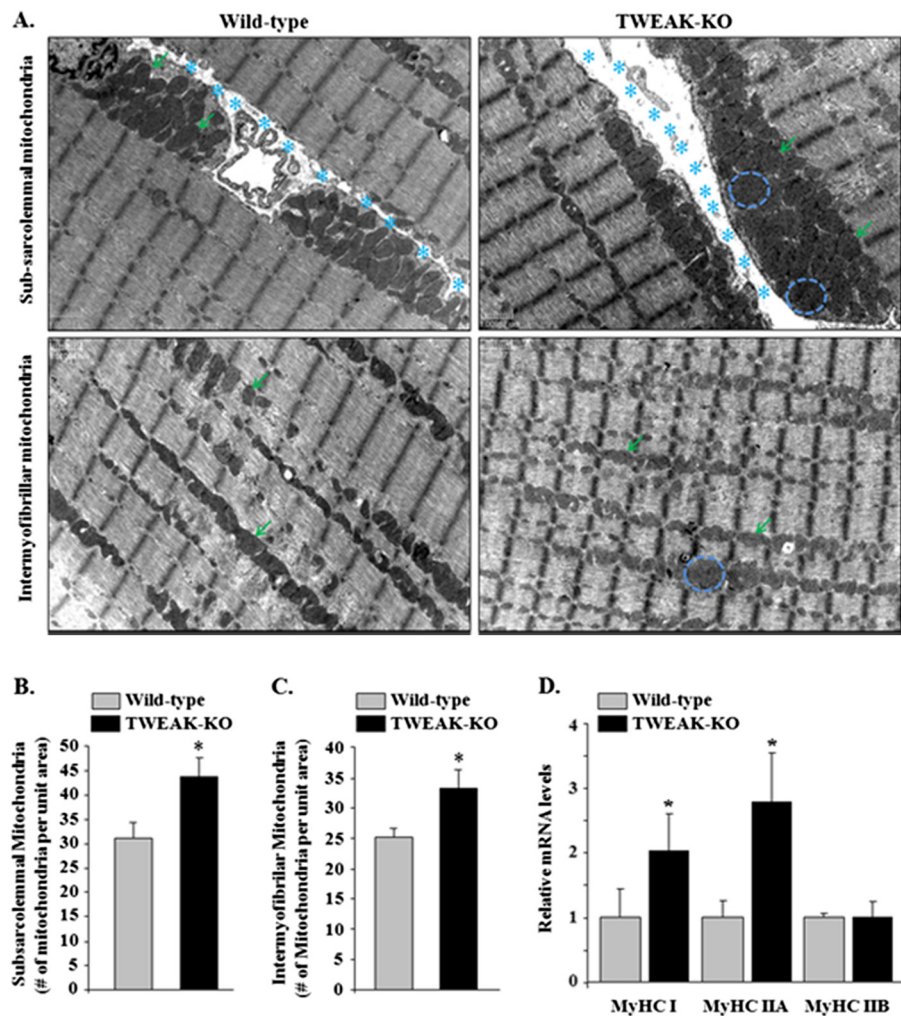


Figure 2 Transmission electron microscopy (TEM) analysis for mitochondria morphology and content and expression of muscle fiber type-specific genes in TWEAK-KO mice. Soleus muscle of six-month-old wild-type and TWEAK-KO mice were isolated and longitudinal sections were processed for TEM analysis. (A) Representative images of longitudinal soleus muscle are shown. The abundance and the size of mitochondria were increased in TWEAK-KO mice compared to wild-type mice. Arrows point to mitochondria in muscle sections. Circles are used to show representative enlarged mitochondria. Stars (*) point to subsarcolemmal space in longitudinal sections. Scale bar: 1 μ m. (B) Quantification of the number of subsarcolemmal mitochondria in soleus muscle of wild-type and TWEAK-KO mice. (C) Quantification of the number of intermyofibrillar mitochondria in soleus muscle sections of wild-type and TWEAK-KO mice. (D) Relative mRNA levels of MyHC type I, IIA and IIB in gastrocnemius muscle of 4.5-month-old wild-type and TWEAK-KO mice. Data are represented as mean \pm SD. N = 3 in each group. * P < 0.05; values vary significantly from wild-type mice. KO, knockout; TWEAK, TNF-like weak inducer of apoptosis.

Ablation of TWEAK increases state 3 respiration in skeletal muscle mitochondria

We next sought to determine whether TWEAK regulates mitochondrial function in skeletal muscle. After an acute bout of treadmill running for 90 minutes, mitochondria were isolated from hind limb muscles of wild type and TWEAK-KO mice and mitochondrial function was examined by extracellular flux (XF) analysis. The sequential addition of respiratory substrates and inhibitors of oxidative phosphorylation were used to determine changes in electron transport chain activity: state 3 respiration was

induced by addition of pyruvate, malate and ADP; state 4 respiration was induced by addition of oligomycin; FCCP was added to determine maximal complex I-mediated respiratory activity; and, lastly, succinate and rotenone were added to determine maximal complex II-mediated respiratory activity. As shown in Figure 5A and 5B, mitochondria from TWEAK-KO mice showed significantly increased state 3 respiratory activities compared with wild type mice. Mitochondrial coupling (as determined by calculating respiratory control ratios) and other indices of mitochondrial function were not significantly different

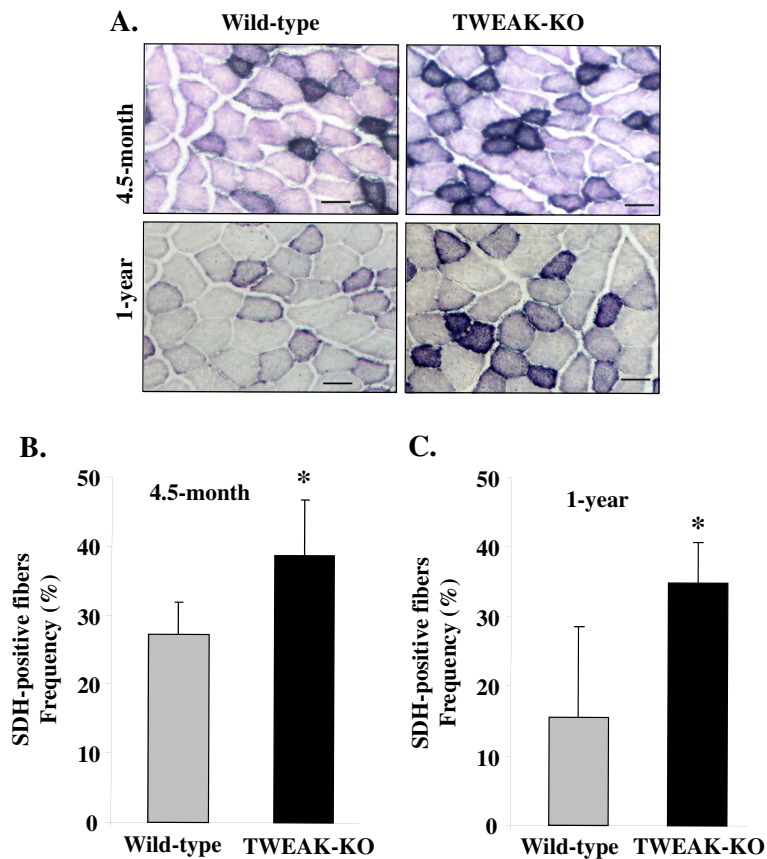


Figure 3 Succinate dehydrogenase (SDH) staining analysis for oxidative capacity in TA muscle of TWEAK-KO mice. Frozen transverse TA muscle sections from 4.5- or 12-month old wild-type and TWEAK-KO mice were used to stain for SDH. **(A)** Representative SDH-stained images are presented here. TA muscle of TWEAK-KO showed relatively dark staining for SDH compared to wild-type mice. Scale bars: 50 μ m. Quantification of SDH-positive fibers in TA muscle of **(B)** 4.5-month, and **(C)** 12-month old wild-type and TWEAK-KO mice. Data are presented as mean \pm SD. * P < 0.05; values vary significantly from wild-type mice. KO, knockout; TA, tibial anterior; TWEAK, TNF-like weak inducer of apoptosis.

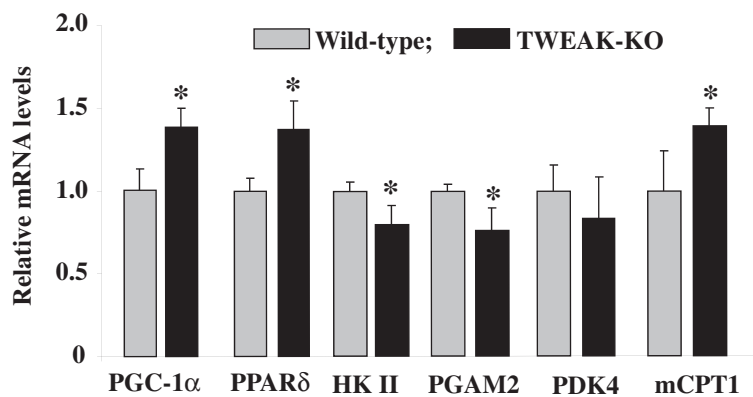
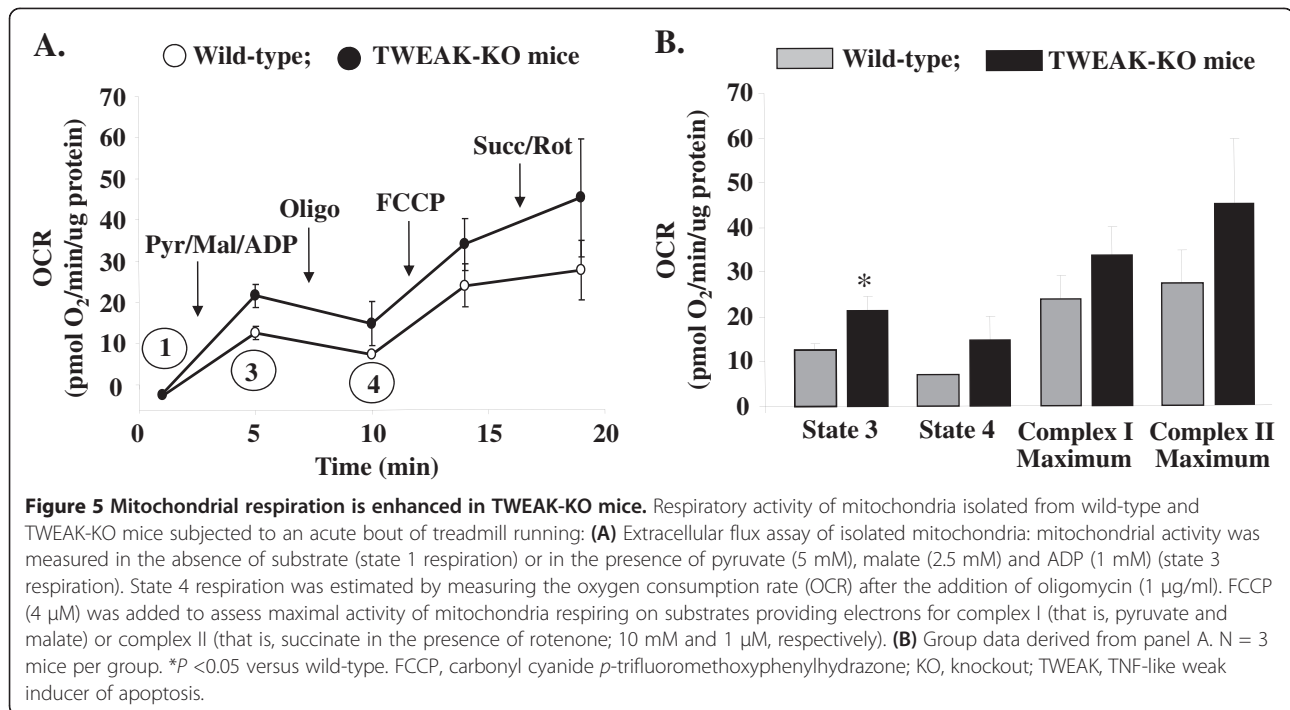


Figure 4 qRT-PCR analysis of transcript levels of metabolic genes in skeletal muscle of wild-type and TWEAK-KO mice. TA muscle from 4.5-month-old wild-type and TWEAK-KO mice were isolated and processed to measure mRNA levels of PGC-1 α , PPAR δ , HK II, PGAM2, PDK4, and mCPT1. Data are represented as mean \pm SD. N = 4 in each group. * P < 0.05. values vary significantly from wild-type mice. HK II, hexokinase-2; KO, knockout; mCPT1, mitochondrial carnitine palmitoyltransferase 1; PDK4, pyruvate dehydrogenase kinase-4; PGAM2, phosphoglycerate mutase 2; PGC-1 α , PPAR coactivator 1 α ; PPAR, peroxisome proliferator-activated receptor; TA, tibial anterior; TWEAK, TNF-like weak inducer of apoptosis.



between wild type and KO mice. These data suggest that absence of TWEAK augments mitochondrial oxidative capacity in skeletal muscle in exercised mice.

TWEAK represses the expression of PGC-1α and genes regulating oxidative metabolism in cultured primary myotubes

We next studied whether TWEAK affects the expression of PGC-1α and other genes in cultured primary myotubes. Primary myoblasts prepared from wild-type mice were differentiated into myotubes by incubation in differentiation medium for 96 hours followed by treatment with TWEAK for 72 hours. As shown in Figure 6, addition of soluble TWEAK dramatically reduced the expression of PGC-1α in myotubes. Treatment with TWEAK also significantly reduced the mRNA levels of several mitochondrial genes encoding proteins related to oxidative metabolism, such as ATP synthase subunit beta (ATP5b), cytochrome c oxidase subunit I (Cox I), Cox subunit IV (COX IV), COX - 7b, cytochrome c (Cyt c), medium-chain acyl-coenzyme A dehydrogenase (MCAD), PPARδ, and mCPT1 (Figure 6). These results further suggest that TWEAK inhibits the mitochondrial biogenetic program in skeletal muscle.

TWEAK decreases maximal respiratory capacity and increases glycolysis in cultured primary myotubes

Our studies in isolated mitochondria derived from skeletal muscle after exercise suggest that the absence of TWEAK increases mitochondrial oxidative capacity. To

assess the effects of TWEAK on mitochondrial function directly, we exposed differentiated myotubes to TWEAK and then assessed mitochondrial function as described previously [48-51]. As shown in Figure 7A and 7B, exposure of myotubes to TWEAK for 72 hours did not affect the basal mitochondrial OCR, nor did it affect ATP-linked OCR or proton leak. However, the FCCP-stimulated OCR, indicative of maximal respiratory capacity, was remarkably diminished by TWEAK treatment. In addition, a modest, yet significant, increase in the non-mitochondrial OCR occurred in myotubes exposed to 100 ng/ml TWEAK, which may be due to increased production of reactive oxygen species. The diminishment in mitochondrial respiratory capacity caused by TWEAK was associated with an increase in the extracellular acidification rate (ECAR), a surrogate measure of glycolysis (Figure 7C). Collectively, these results suggest that TWEAK directly modulates the bioenergetic capacity of skeletal muscle.

Ablation of TWEAK improves angiogenesis in skeletal muscle of mice

There is a causal relationship between PGC-1α and angiogenesis. Skeletal muscle-specific overexpression of PGC-1α in mice results in increased angiogenesis, which may contribute to enhanced exercise performance [52]. Since TWEAK suppresses PGC-α in skeletal muscle, we next sought to determine whether TWEAK also regulates angiogenesis in skeletal muscle of mice. TA muscle of 4.5-month-old wild-type and TWEAK-KO mice

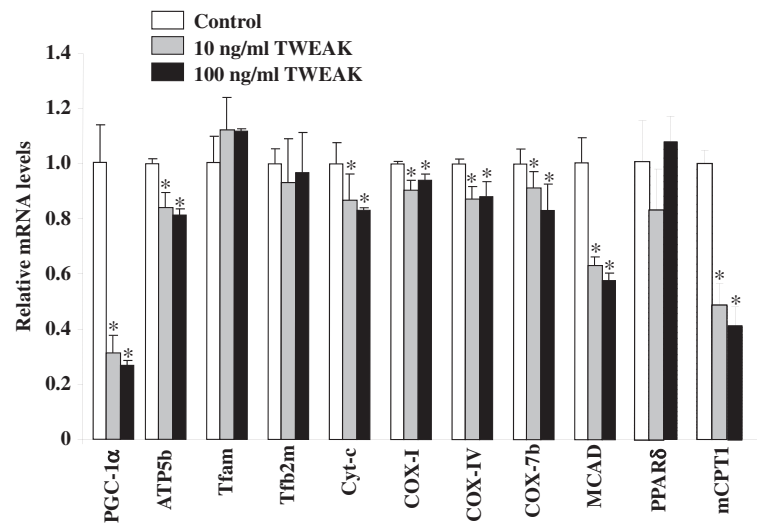


Figure 6 Effect of TWEAK on expression of PGC-1α and mitochondria oxidative metabolism genes in cultured primary myotubes.

Primary myotubes prepared from wild-type mice were treated with soluble TWEAK protein (10 ng/ml or 100 ng/ml) for 72 hours followed by RNA isolation and performing qRT-PCR to study transcript levels of PGC-1α, Tfam, Tfam2m, Cyt-c, Cox I, Cox IV, Cox 7b, and MCAD. Data are presented as mean ± SD. * $P < 0.05$; values vary significantly from untreated myotubes. Cox, cytochroms c oxidase; Cyt c, cytochrome c; MCAD, medium-chain acyl-coenzyme A dehydrogenase; PGC-1α, PPAR coactivator 1α; PPAR, peroxisome proliferator-activated receptor; Tfam, transcription factor A mitochondrial; TWEAK, TNF-like weak inducer of apoptosis.

was isolated and immunostained with antibodies against CD31 (also known as platelet endothelial cell adhesion molecule-1), an endothelial-specific marker for capillaries. Laminin staining was used to mark the periphery of fibers. As shown in Figure 8A, the number of CD31-positive capillaries was considerably increased in TWEAK-KO mice compared with wild-type mice. The capillary-to-fiber ratio was increased by 29% in TWEAK-KO mice (Figure 8B). Since VEGF positively regulates angiogenesis, we also measured mRNA levels of VEGF in TA muscle of wild-type and TWEAK-KO mice. As shown in Figure 8C, mRNA levels of VEGF were significantly higher in TA muscle of TWEAK-KO mice compared to wild-type mice. Furthermore, treatment with TWEAK also reduced the mRNA levels of VEGF in primary myotubes (Figure 8D). These results suggest that ablation of TWEAK stimulates angiogenesis in skeletal muscle of mice potentially through increasing the expression of VEGF.

Discussion

Exercise capacity in mammals is determined by multiple factors including skeletal muscle oxidative metabolism and vascularization. Our previous microarray study suggested that TWEAK can modulate the expression of several genes whose products are involved in mitochondrial dysfunction and fatty acid metabolism [53]. Our present study demonstrates that compared to wild-type mice, TWEAK-KO mice run longer and with higher speed during an exercise tolerance test (Figure 1). TWEAK-KO mice show augmented levels of subsarcolemmal and

intermyofibrillar mitochondria, increased SDH-positive myofibers, and elevated expression of metabolic genes such as PGC-1α, PPARδ, and mCPT-1 compared to wild-type mice (Figures 2, 3, and 4). Oxidative phosphorylation is also increased in exercised TWEAK-KO mice compared with wild-type mice (Figure 5), and treatment of myotubes with TWEAK directly decreases mitochondrial biogenetic capacity and maximal respiratory activity (Figures 6 and 7). Moreover, vascularization and expression of VEGF in skeletal muscle are enhanced in TWEAK-KO mice compared to their controls (Figure 8). These data suggest that TWEAK directly regulates the mitochondrial biogenetic program and that loss of TWEAK improves mitochondrial respiratory capacity and increases vascularization of skeletal muscle tissue, which collectively lead to improved exercise performance.

One of the potential mechanisms by which TWEAK might be attenuating exercise capacity is through diminishing the levels of PGC-1α in skeletal muscle. PGC-1α augments mitochondrial biogenesis, oxidative metabolism, and promotes fast-to-slow type fiber transition [9,13,17,54-56]. Overexpression of PGC-1α in rat primary culture cells leads to increased abundance of slow oxidative-associated MyHC isoform [57]. Skeletal muscle-specific PGC-1α KO mice demonstrate reduced endurance capacity with a shift from oxidative fiber type to glycolytic fibers and increased levels of TNF-α in skeletal muscle [45,58]. By contrast, muscle-specific overexpression of PGC-1α enhances exercise performance with increased fatty acid oxidation and decreased

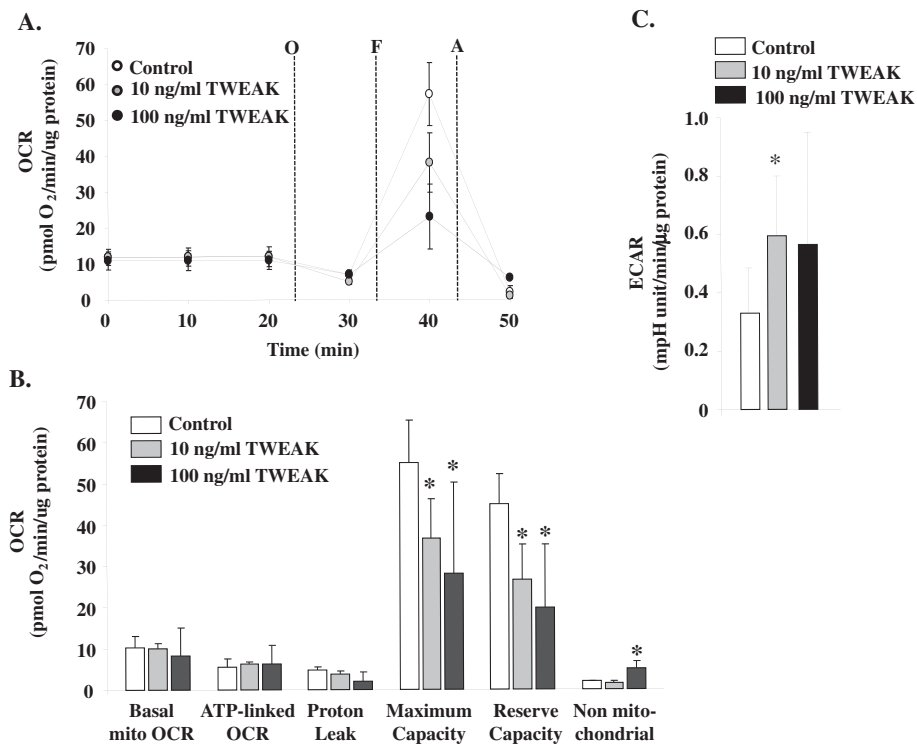
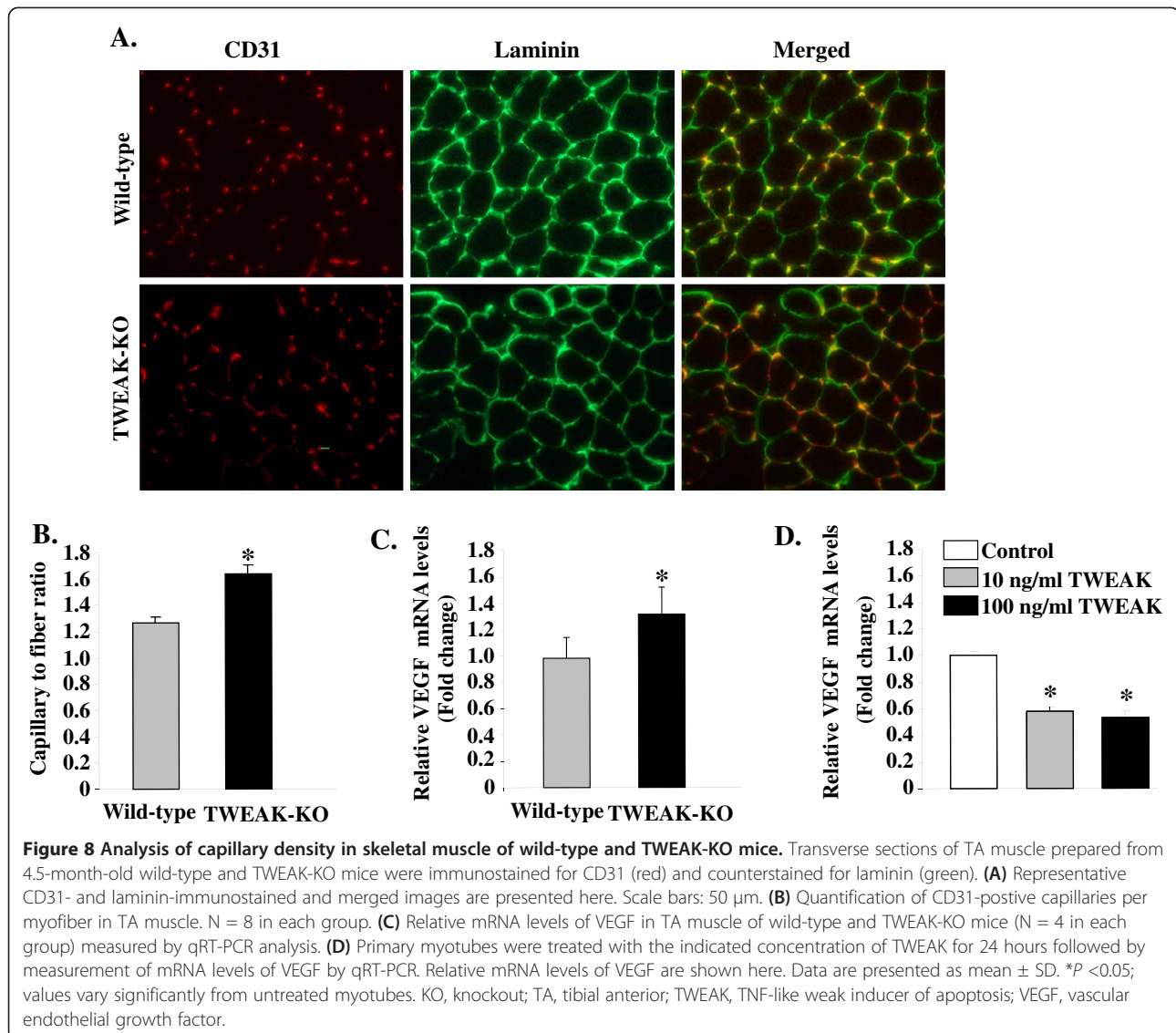


Figure 7 TWEAK regulates mitochondrial oxidative capacity and glycolytic flux. Extracellular flux analysis of primary differentiated myotubes treated with 0, 10, or 100 ng/ml TWEAK for 72 hours. **(A)** Mitochondrial function assay: after three baseline measurements, inhibitors or activators of electron transport were added sequentially to intact myotubes. Oxygen consumption rate (OCR) measurements were recorded after each exposure. O, oligomycin; F, FCCP; and A, antimycin A/rotenone. The OCR was normalized to total protein in each well. **(B)** Indices of mitochondrial function calculated from assay results in panel A. **(C)** Extracellular acidification rates (ECAR) of cells treated without or with TWEAK; ECAR is a surrogate measure of lactate and therefore is used as a measure of glycolytic flux. N = 3 per group; *P <0.05 versus control. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TWEAK, TNF-like weak inducer of apoptosis.

glycogen usage during exercise [8,52]. These findings suggest that PGC-1 α is not only essential but sufficient to determining skeletal muscle fiber composition. Our experiments demonstrate that mRNA levels of PGC-1 α are increased in skeletal muscle of TWEAK-KO mice with a concomitant increase in mitochondrial content and expression of genes whose products are involved in oxidative capacity (Figures 2 and 4). Furthermore, treatment of primary myotubes with TWEAK drastically reduced levels of PGC-1 α and other mitochondrial genes (Figure 6) further suggesting that TWEAK represses the expression of PGC-1 α leading to reduced mitochondrial content. These findings are consistent with functional analyses of mitochondrial function: ablation of TWEAK resulted in increased oxidative phosphorylation capacity in isolated mitochondria (Figure 5) and treatment of myotubes with TWEAK decreased maximal respiratory capacity (Figure 7). Although not tested in this study, it is possible that TWEAK also reduces the levels of PGC-1 α in other organs which ultimately results in reduced exercise capacity. Indeed, Shi *et al.* recently reported that TWEAK represses the expression of PGC-1 α and

other molecules involved in mitochondrial oxidative phosphorylation in cardiomyocytes and forced expression of PGC-1 α attenuates TWEAK-induced cardiac dysfunction in mice [59].

The PPARs are ligand-modulated transcription factors in which three subtypes have been identified: α , β/δ , and γ [60]. Previous studies have shown that their endogenous ligands are composed of fatty acids and lipid metabolites and, therefore, certain PPARs mediate the expression of genes whose products are involved in the regulation of fatty acid metabolism in response to changes in systemic fuel availability [60-62]. In skeletal muscle, levels of PPAR δ are relatively higher compared to PPAR α or PPAR γ [63]. Treatment with synthetic PPAR δ agonist or overexpression of PPAR δ by retroviral infection induces the levels of molecules which are involved in lipid metabolism and fatty acid oxidation, whereas overexpression of a dominant-negative PPAR δ mutant exerts opposite effects in C2C12 myotubes [64]. Furthermore, it has been shown that muscle-specific overexpression of PPAR δ in mice increases exercise tolerance with a switch to increased number of type I



muscle fibers and up-regulation of molecules related to fatty acid metabolism. In contrast, PPAR δ -null mice show decreased exercise performance compared to wild-type mice [65,66]. Although muscle relies mainly on fat and carbohydrate as energy resources, enhanced fatty acid utilization during exercise with glycogen sparing results in improved exercise endurance capacity [67-69]. Haramizu *et al.* demonstrated that the expression levels of gene products related to lipid metabolism in the muscle is correlated with the levels of fatty acid β -oxidation activity as well as exercise strength [70]. Our results demonstrate that transcript levels of PPAR δ and mCPT1 are significantly increased while the expression of molecules that are associated with glycolysis such as HK II and PGAM2 is suppressed in skeletal muscle of TWEAK-KO mice compared to wild-type mice (Figure 6). These results suggest that enhanced fatty acid oxidation

might be responsible for improvement in exercise capacity in TWEAK-KO mice. Data from cell culture experiments further support this possibility, as treatment of myotubes with TWEAK resulted in diminished maximal respiratory capacity and increased glycolytic flux (Figure 7).

Although the mechanisms by which TWEAK reduces levels of PGC-1 α in skeletal muscle remain unknown, a recent study has shown that TWEAK increases membrane translocation of adaptor protein TNF receptor associated factor 2 (TRAF2), in an Fn14 dependent manner, in cardiomyocytes [59]. Furthermore, TWEAK treatment increases the activation of canonical nuclear factor-kappaB (NF- κ B) signaling in both skeletal muscle [41] and cardiac myocytes [59]. Knockdown of TRAF2 using small hairpin RNA (shRNA) or selective blockade of I κ B kinase- β (IKK β , an upstream activator of canonical NF- κ B signaling) prevented the TWEAK-mediated suppression of

PGC-1 α in cardiomyocytes suggesting that TWEAK repression of PGC-1 α requires Fn14-TRAF2-IKK β -NF- κ B signaling cascade [59]. Previously, the effects of TNF- α on the mRNA expression of PPAR δ and its target genes have been investigated. Treatment with TNF- α reduces PPAR δ -target genes, such as mCPT1 and PGC-1 α , whereas addition of PPAR δ agonist rescues this reduction in adipocytes [71,72]. Moreover, TNF- α impairs mitochondrial biogenesis and function in skeletal muscle [73]. While TWEAK can induce the expression of molecules associated with the autophagy-lysosomal system indicating it causes mitochondria dysfunction [74], muscle-specific ablation of TRAF6, which is involved in TWEAK signaling, suppresses the activation of autophagy in response to denervation and cancer cachexia [75]. These results suggest that TWEAK is involved in reducing PPAR δ and PGC-1 α levels and their target genes leading to mitochondrial dysfunction and content. Since no change of PPAR δ expression was observed in skeletal muscle of PGC-1 α KO mice or the muscle-specific PGC-1 α overexpressing transgenic mice [66], PGC-1 α is not an upstream regulator for PPAR δ . Indeed, PGC-1 α has a synergistic effect with PPAR δ agonist to induce the expression of oxidative metabolic genes such as mCPT1 and PDK4 [76]. Furthermore, it has been shown that PGC-1 α directly coactivates the mCPT1 and PDK4 promoter via PPAR δ in a ligand-dependent manner [76,77]. Therefore, it is likely that PGC-1 α is a coactivator of PPAR δ in terms of up-regulating these metabolic genes.

Another interesting observation of the present study is that depletion of TWEAK improves angiogenesis in skeletal muscle of mice (Figure 8). Previous studies have shown that transgenic mice overexpressing PGC-1 α show increased vascularization in skeletal muscle [56]. PGC-1 α is also a positive regulator for the expression of VEGF1 which is known to promote angiogenesis [56]. Moreover, human endothelial cells treated with the PPAR δ agonist GW501516 demonstrated increased production of VEGF and expression of VEGF receptor [78,79]. Therefore, increased angiogenesis in skeletal muscle of TWEAK-KO mice appears to be a result of increased amounts of PGC-1 α and PPAR δ (Figure 4). The enhancement of vascularization in TWEAK-KO mice might further augment the capacity of skeletal muscle mitochondria to respire by increasing blood flow and delivery of O₂ and nutrients, especially during exercise.

Conclusion

In summary, our study provides initial evidence that inhibition of TWEAK increases mitochondrial biogenesis, oxidative metabolism and angiogenesis in skeletal muscle which potentially contribute to improved exercise capacity. These findings also have high clinical significance as

inhibition of TWEAK activity using neutralizing antibodies or pharmacological compounds could improve muscle function and exercise capacity in patients with metabolic disorders.

Abbreviations

ATP5b: ATP synthase subunit beta; BSA: Bovine serum albumin; Cox: Cytochrome c oxidase; FCCP: Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GA: Gastrocnemius; HK II: Hexokinase II; KO: Knockout; MCAD: Medium-chain acyl-coenzyme A dehydrogenase; mCPT1: Mitochondrial carnitine palmitoyltransferase I; MyHC: Myosin heavy chain; NF- κ B: Nuclear factor-kappaB; OCR: Oxygen consumption rate; PCR: Polymerase chain reaction; PDK4: Pyruvate dehydrogenase kinase 4; PGAM2: Phosphoglycerate mutase 2; PGC-1 α : PPAR coactivator 1 α ; PPAR: Peroxisome proliferator-activated receptor; qRT-PCR: Quantitative real-time PCR; SDH: Succinate dehydrogenase; shRNA: Small hairpin RNA; TA: Tibial anterior; TNF: Tumor necrosis factor; TRAF: TNF receptor-associated factor; TWEAK: TNF-like weak inducer of apoptosis; VEGF: Vascular endothelial growth factor.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

AK and BGH conceived and designed the study. SS, YO, VM, JS, BGH and SB performed experiments and analyzed the data. SS, BGH and AK wrote the manuscript. All authors read and approved the final manuscript.

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