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The role of Sirt3 in the changes of skeletal muscle mitophagy induced by hypoxic training

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Abstract. We aimed to explore the role of Sirt3 in the regulation of skeletal muscle mitophagy with hypoxic training. C57BL/6J mice were randomly divided into four groups: C group (control), HT group (mice performed a hypoxic training of living in an environment with an oxygen concentration of 13.8% and treadmill exercise under normoxia for 6 weeks), T group (mice were subjected to an intraperitoneal (i.p.) injection of the Sirt3 inhibitor 3-(1H-1,2,3-triazol-4-yl) pyridine (3-TYP) 50 mg/kg three times *per* week for 6 weeks) and THT group (the hypoxic training of HT group with i.p. injection of 3-TYP in T group). The results showed that 6 weeks of hypoxic training could improve ATP synthesis in skeletal muscle. After the combined intervention of 3-TYP injection and hypoxic training, Sirt3, FOXO3a, and SOD2 protein contents were still lower than those in hypoxic training group. Hypoxic training cannot improve the negative effect of Sirt3 inhibition on muscle PINK1/Parkin signal. This study demonstrated that Sirt3 plays a key role in mediating skeletal muscle mitophagy by hypoxic training. The results of our study also provided the first evidence that mitophagy caused by hypoxic training might be transduced through the Sirt3-FOXO3a signaling pathway.

Key words: Hypoxic training — Sirt3 — Mitophagy — Skeletal muscle — Mitochondria

Introduction

Hypoxic training is considered an effective method, which can improve the oxygen transport capacity and induce a higher metabolic stress in athletes (Wang et al. 2019), promote adaptive changes, and improve athletic performance (Çolak et al. 2021). Hypoxic training, which is consist of exercise and hypoxia, has a great physiological load in skeletal muscle and profoundly promote mitochondrial remodeling to match the energy demand (Zhao et al. 2021). Mitochondrial biogenesis and mitochondrial autophagy (i.e., mitophagy) are key processes during the adaptation of skeletal muscle in exercise activity, resulting in a better mitochondrial function for ATP production (Guan et al. 2019). Relationships between mitochondrial biogenesis and various exercise models have been studied comprehensively but fewer research investigated the influence of hypoxic training on muscle mitophagy. There are two pathways of mitophagy, namely PTEN-induced putative kinase 1/Parkin (PINK1/Parkin) and BCL2/adenovirus E1B 19Kda protein-interacting protein 3/BL2/adenovirus E1B 19Kda protein-interacting protein 3-like (Bnip3/Nix). One recent report has found that hypoxic training can improve the PINK1/Parkin pathway of skeletal muscle mitophagy more effectively than normoxic training, which may be one of the possible mechanisms for hypoxic training to efficiently promote muscle energy metabolism (Zhao et al. 2021). Studies

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in mammals have found that the proteins dynamin-related protein 1 (Drp1) and mitochondrial fission factor (MFF) are the substrates of Parkin (Hood et al. 2019).

Silent information regulator 3 (Sirt3) is mainly located in the mitochondria with high metabolic environments such as in skeletal muscle and heart (Ahn et al. 2008). It can activate forkhead box class O 3a (FOXO3a), superoxide dismutase 2 (SOD2) and catalase, promote reactive oxygen species (ROS) clearance, and enhance antioxidant defense capabilities in skeletal muscle cells (Aldakkak et al. 2008; Sundaresan et al. 2009; Koltai et al. 2018). Exercise can increase the activity of Sirt3 to a certain extent, and then directly or indirectly resist oxidative stress through its downstream target molecules. Aerobic exercise can upregulate antioxidant enzymes to inhibit oxidative stress, and concomitantly increase the level of mitophagy. Vargas-Ortiz et al. (2015) confirmed that aerobic training can increase Sirt3 expression in skeletal muscle of male adolescents. Sirt3 knockout of diabetic mice showed inhibition of mitophagy, resulting in cardiac hypertrophy (Yu et al. 2017). Zhao's experiment reported that sustaining short-duration exercise can regulate mitophagy signal by increasing the expression of PINK/Parkin, inhibit the production of ROS, enhance the activity of antioxidant SOD2, and increase the level of Sirt3 in cardiomyocytes. This suggests that sustained short-duration exercises can increase Sirt3 levels, and improve mitochondrial quality control and mitochondrial morphology (Zhao et al. 2018).

Preliminary studies indicated hypoxic training can effectively improve human cardiopulmonary function and enhance muscle mitophagy, and skeletal muscle mitophagy is positively regulated by Sirt3 which is closely related to the oxidative phosphorylation of muscle mitochondria. Therefore, this study hypothesized that hypoxic training enhances the Sirt3 and mitophagy signaling pathways in skeletal muscle, and Sirt3 signaling involves and contributes to the upregulation of skeletal muscle mitophagy induced by hypoxic training.

Material and Methods

Animal models

C57BL/6 mice (male, eight weeks) underwent a 12-hour light-dark cycle, freely eating and drinking. The food included carbohydrate, water, protein, fat, crude fiber and other ingredients, and the mixing ratio is 50%, 9%, 18%, 6%, 4%, and 13%, respectively. They were randomly assigned into four groups: C group (control), HT group (mice performed a hypoxic training), T group (mice were subjected to an injection of a specific Sirt3 inhibitor 3-TYP), and THT group (3-TYP+hypoxic training); n = 6/group. All experiments were approved by Research Commission on Ethics of Shanghai University of Sport.

Mice in group C did not receive any intervention. Mice in HT group performed a hypoxic training for 6 weeks (6 days/week, oxygen concentration was 13.8%, living in a hypoxic room every day for 8 h from 9:30 am to 5:30 pm. Treadmill training was under normoxia at 6:30 pm. From the first week to the sixth week, the speeds of the treadmill were 10, 12, 14, 16, 18, 20 m/min. Training time was increased by 10 min *per* week from 30 min in the first week to 80 min in the sixth week). Mice in the T group were intraperitoneally (i.p.) injected with 50 mg/kg of 3-TYP (3-TYP (3-(1H-1,2,3-triazol-4-yl) pyridine; HY-1083, Med Chem Express, USA) dissolved in dimethyl sulfoxide; i.p. injection was given at 5:00 pm on every Monday, Wednesday, and Friday. The THT group received 3-TYP injection (50 mg/kg every Monday, Wednesday, and Friday) after living in hypoxia, and normoxic training was performed 30 min post-injection. The mice were sacrificed 24 h after the last hypoxic training, and skeletal muscle samples were collected. About 1 mm^3 gastrocnemius muscle tissue was placed in a special fixator for electron microscopy. The remaining skeletal muscle tissues were stored at –80°C for biochemical testing and protein analysis.

Sirt3 levels test

The Sirt3 levels of gastrocnemius muscle was detected using the mouse mitochondrial acetylase 3 antibody ELISA detection kit (Jianglai Biological, JL46300), and all procedures were operated in accordance with the experimental protocol in the manual. The fluorescence intensity was measured with a microplate reader at a wavelength of 450 nm.

Western blotting analysis

Gastrocnemius samples in RIPA buffer (Beyotime Biotech) were placed on ice for 20 min. After homogenization, it was centrifuged and taken the supernatant. After protein quantification with the BCA protein concentration determination kit (Beyotime Biotech), 4×loading buffer was added to adjust the protein loading volume, and it was placed at 100°C for 10 min. After obtaining the protein sample, 50 μg of protein in each well was loaded. Then we used 70 mV (30 min) and 110 mV (60 min) separated the protein by SDS-PAGE and transferred proteins to the PVDF membrane. Membranes were blocked with 5% nonfatdry milk for 2 hours and added the primary antibody after washer with TBST (a mixed liquid formed by dissolving Tris-bas, Glycine and Tween 20 in deionized water) and incubated overnight in 4°C. The second antibody was added and incubated for 1 h after washer with TBST. Primary antibody information: Sirt3 (Cell Signaling Technology, 1:1000), FOXO3a (Cell Signaling Technology,

1:1000), SOD2 (Cell Signaling Technology, 1:1000), PINK1 (Proteintech Group, 1:1000), Parkin (Proteintech Group, 1:1000), Bnip3 (Boster Biological Technology,1:1000), Nix (Proteintech Group, 1:700), Drp1 (Cell Signaling Technology, 1:1000), MFF (Cell Signaling Technology, 1:1000). Second antibody was HRP goat anti-rabbit IgG antibody (Proteintech Group, 1:1000). Membranes were observed by ultra-sensitive chemiluminescence detection kit (abs920, Absin) and proteins relative contents were calculated by ImageJ software, and standardized with α-tubulin (Proteintech Group, 1:1000).

ATP synthesis ability analysis

Quadriceps sample were added RIPA buffer to prepared the homogenate, and BCA protein determination kit was used to measure the protein concentration of the homogenate. According to the experimental protocols of manufacturers, we used the microplate reader to test the ATP content and citrate synthase activity (Nanjing Jiancheng Institute of Biological Engineering, A095-1-1, A108-1-2) at 636 nm and 412 nm.

Transmission electron microscopy (TEM) observation

Gastrocnemius muscle samples were placed in PBS containing 1% osmic and 0.1 M phosphate buffer. Afterward, the fixed samples were dehydrated in graded alcohol series. Tissues were permeabilized in a mixture of acetone and 812 embedding medium (1:1) and pure 812 embedding medium overnight at 4°C, after which they were embedded. Ultramicrosection (UC7) was used to cut muscle sections (70 nm) which were stained with uranium and leaded for 15 min. Sections were observed under a transmission electron microscope, photographed by Gatan CCD camera, and collected images for analysis.

Statistical analysis

The experimental data was processed by IBM SPSS Statistics 25 and the values were represented as Mean ± SD. Two-way ANOVA with Turkey *post hoc* test was used to evaluate the data. $p < 0.05$ was considered as statistically difference.

Results

Effects of different interventions on the alteration of body weight

After 6 weeks of intervention, we compared the weight gain of the mice. It was found that the increased body weight in HT group and THT mice was significantly lower than that in C group ($p < 0.05$). These results implied that hypoxic training can reduce the increased extent of body weight of mice, and the 6 weeks of Sirt3 inhibition has no effect on the increased process of body weight in mice (Fig. 1).

Effects of different interventions on Sirt3 levels

The change of Sirt3 levels in skeletal muscle was measured by ELISA method. Compared with C group, Sirt3 levels was increased significantly in HT group ($p < 0.01$) and was decreased significantly in T group ($p < 0.05$). Compared with HT group, Sirt3 levels in THT group was decreased ($p < 0.01$). Interestingly, Sirt3 levels in THT group was increased greatly compared with T group ($p < 0.05$) (Fig. 2A). The above results suggested that: after the combined intervention of 3-TYP injection and hypoxic training, Sirt3 levels was higher than that of 3-TPY injection alone, but the effect did not achieve the response by hypoxic training alone.

Effects of different interventions on Sirt3 and related proteins in skeletal muscle

Compared with C group, HT group elevated expression of Sirt3 ($p < 0.01$); Sirt3 levels in THT group were lower than those of HT group ($p < 0.01$) (Fig. 2B). The levels of FOXO3a in HT group were increased compared with C and THT group ($p < 0.01$) (Fig. 2C). SOD2 expressions in the HT group were higher than those in C group ($p < 0.01$) and THT group ($p < 0.01$) (Fig. 2D). The above results indicated that hypoxic training could increase the protein contents of Sirt3, FOXO3a, and SOD2. After the combined intervention of 3-TYP injection and hypoxic training, Sirt3, FOXO3a, and SOD2 protein contents were still lower than those in the hypoxic training group.

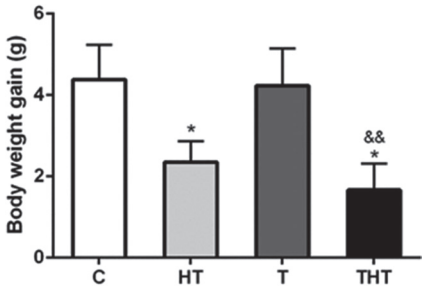

Figure 1. Changes in weight gain of mice after interventions. Body weight gain = body weight post-intervention – body weight pre-intervention. C, control group; HT, hypoxic training group; T, Sirt3 inhibitor 3-TYP group; THT, 3-TYP+hypoxic training group. $n = 6$; * $p < 0.05$ *vs.* C; && $p < 0.01$ *vs.* T. Data are mean ± SD.

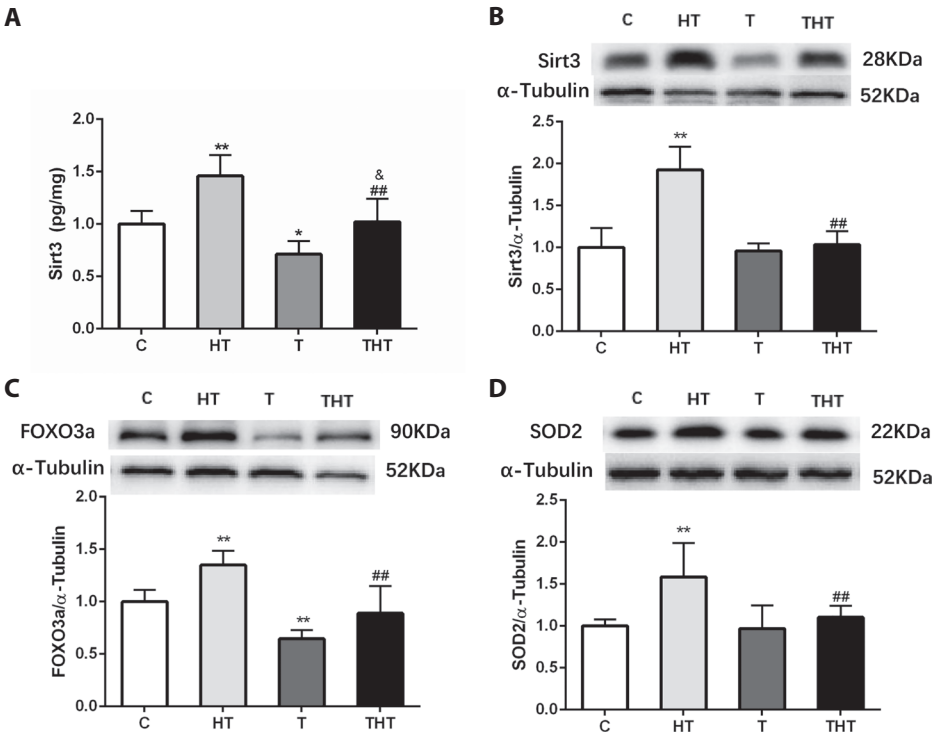

Figure 2. Alterations of Sirt3 levels and Sirt3-related proteins expressions in different groups. **A.** Sirt3 levels (pg/ml) after Sirt3 inhibition and hypoxic training interventions. Sirt3 (**B**), FOXO3a (**C**) and SOD2 (**D**) expressions after Sirt3 inhibition and hypoxic training interventions evaluated by Western blot. Standardized with α-tubulin. $n = 6$; * $p < 0.05$, ** $p < 0.01$ *vs.* C; ## $p < 0.01$ *vs.* HT; & $p < 0.05$ *vs.* T. All the results were presented as mean ± SD. For abbreviations, see Figure 1.

Effects of different interventions on mitophagy proteins in skeletal muscle

Compared with C group, PINK1 expression in HT group were significantly increased ($p < 0.01$) and PINK1 contents in THT group was lower than that in HT group ($p < 0.05$) (Fig. 3A). Parkin in THT group was still lower than that in HT group ($p < 0.05$) and was higher than that in T group ($p < 0.01$), respectively (Fig. 3B). The protein expression of Bnip3 was not changed after other interventions ($p > 0.05$) except T group (Fig. 3C). Nix expression in THT group were significantly lower than that in HT group ($p < 0.05$) (Fig. 3D). The protein expression of Drp1 did not change significantly in HT group ($p > 0.05$) and only Drp1 level of the THT group was significantly higher than that of the T group ($p < 0.05$) (Fig. 3E). MFF expression of THT group were also lower than those of HT group ($p < 0.05$) (Fig. 3F). These results indicated that Sirt3 inhibition intervention can effectively reduce the overall muscle PINK1/Parkin signal, and hypoxic training cannot improve the negative effect of Sirt3 inhibition on muscle PINK1/Parkin signal. Bnip3 may not participate in this regulatory process.

Changes of ATP synthesis capacity in skeletal muscle following interventions

Mitophagy can effectively remove damaged mitochondria, improve mitochondrial quality, and facilitate mitochondrial oxidative phosphorylation (Palikaras et al. 2015). We tested ATP content and citrate synthase (CS) activity to evaluate the alteration of ATP synthesis ability (Fig. 4). ATP content and CS activity in HT group were higher than those in C group ($p < 0.01$, $p < 0.05$), and ATP contents in THT group was still lower than that in HT group ($p < 0.05$). These results implied that hypoxic training could enhance quadriceps muscle ATP content and CS activity, but hypoxic training could not completely improve the decline in ATP content caused by Sirt3 inhibition.

TEM examination of skeletal muscle after different interventions

Images from our TEM observations showing features of gastrocnemius cell myofibrils and mitochondria morphology were presented in Figure 5. In each group, we observed that the myofibril sarcomere structure was unchanged, and the Z-line trend was straight and had no obvious pathological defects. These results indicated that hypoxic training did not change the structure of skeletal muscle myofibrils.

We also observed the ultrastructure of mitochondria in skeletal muscle: the mitochondria of gastrocnemius in C group were small and distributed on both sides of the Z line. The mitochondrial double-layer membrane structure was clear and complete, and most of the mitochondrial cristae structure was relatively full and dense; the mitochondria volume and number of the HT group significantly increased. The skeletal muscle mitochondria in the T group were unevenly distributed. Some mitochondrial matrix become

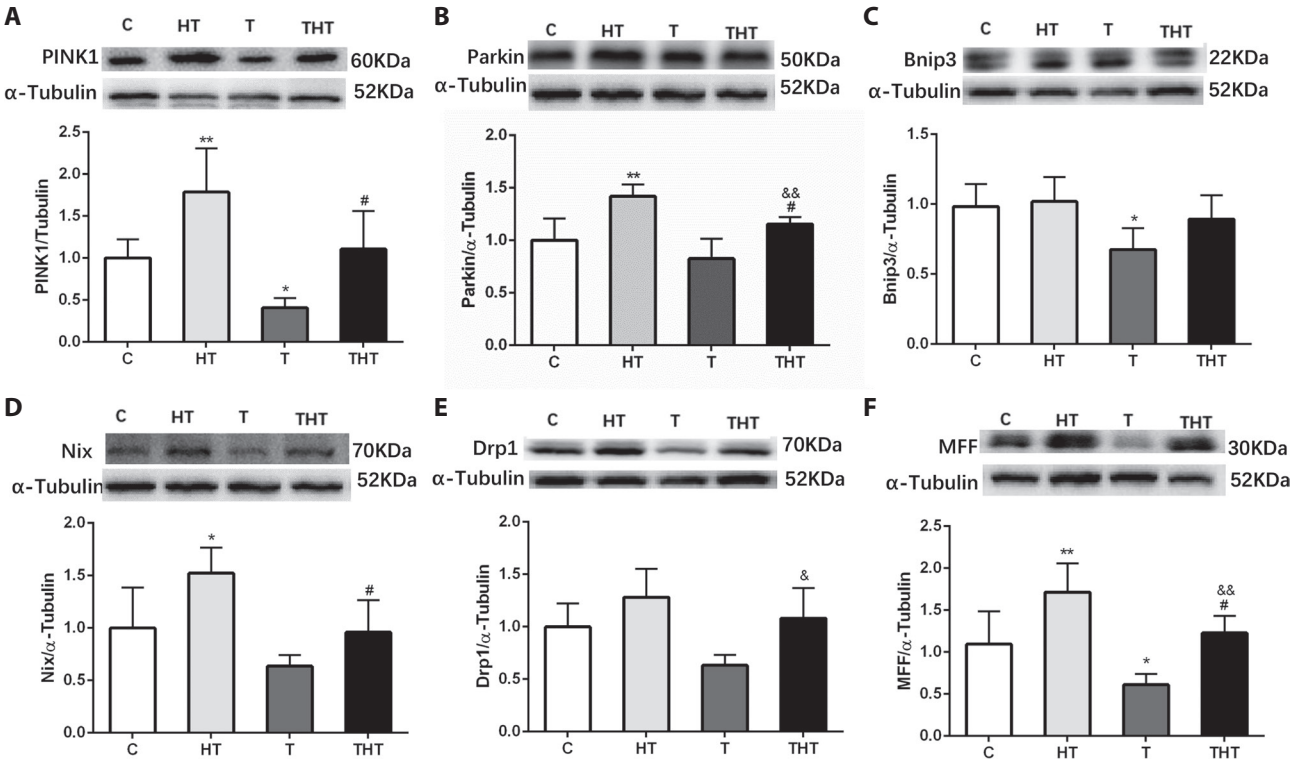

Figure 3. Alterations of mitophagy proteins expressions in different groups. PINK1 (**A**), Parkin (**B**), Bnip3 (**C**), Nix (**D**), Drp1 (**E**) and MFF (**F**) expressions after Sirt3 inhibition and hypoxic training interventions evaluated by Western blot. Standardized with α-tubulin. $n = 6$; * $p < 0.05$, ** $p < 0.01$ *vs.* C; # $p < 0.05$ *vs.* HT; & $p < 0.05$, && $p < 0.01$ *vs.* T. All the results were presented as mean ± SD. For abbreviations, see Figure 1.

shallow, swollen, vacuoles, and most of the cristae were small and the structure was unclear, showing clear mitochondrial damages. The mitochondrial cristae of the THT group were relatively visible and the degree of mitochondrial damage was relieved. The changes of mitochondrial ultrastructure were consistent with the alterations of the energy production and mitophagy signaling in the skeletal muscle.

Discussion

Present study explored the effects of hypoxic training on Sirt3-related signaling and mitophagy signaling of skeletal muscle in mice, and investigate the role of Sirt3 in the response of muscle mitophagy induced by hypoxic training. Total data suggested that 6-week of hypoxic training enhanced mitophagy signals such as PINK1/Parkin and Nix, paralleled with the upregulation of Sirt3, FOXO3a, and SOD2 levels in skeletal muscle. After pharmacological inhibition of Sirt3, some mitophagy proteins contents were reduced to varying degree in skeletal muscle. After inhibition of Sirt3, mice then undertook hypoxic training, and we failed to observe the mitophagy increased response in skeletal muscle just like the results in hypoxic training alone. Thus, these data suggest that Sirt3 is required for the enhancement of mitophagy of skeletal muscle resulting from hypoxic training.

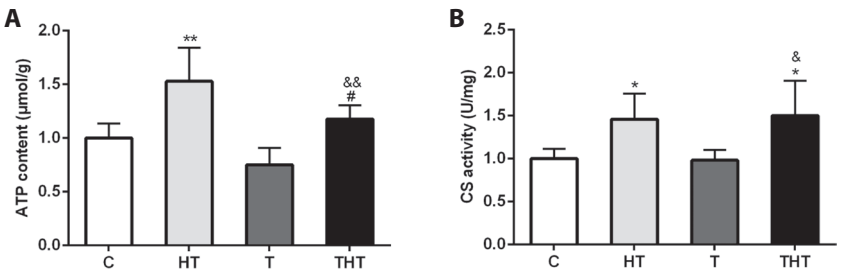

Figure 4. Changes of ATP content and citrate synthase (CS) activity in different groups. ATP content (**A**) and CS activities (**B**) after Sirt3 inhibition and hypoxic training interventions. $n = 6$; * $p < 0.05$, ** $p < 0.01$ *vs.* C; # $p < 0.01$ *vs.* HT; & $p < 0.05$, && $p < 0.01$ *vs.* T. All the results were presented as mean ± SD. For abbreviations, see Figure 1.

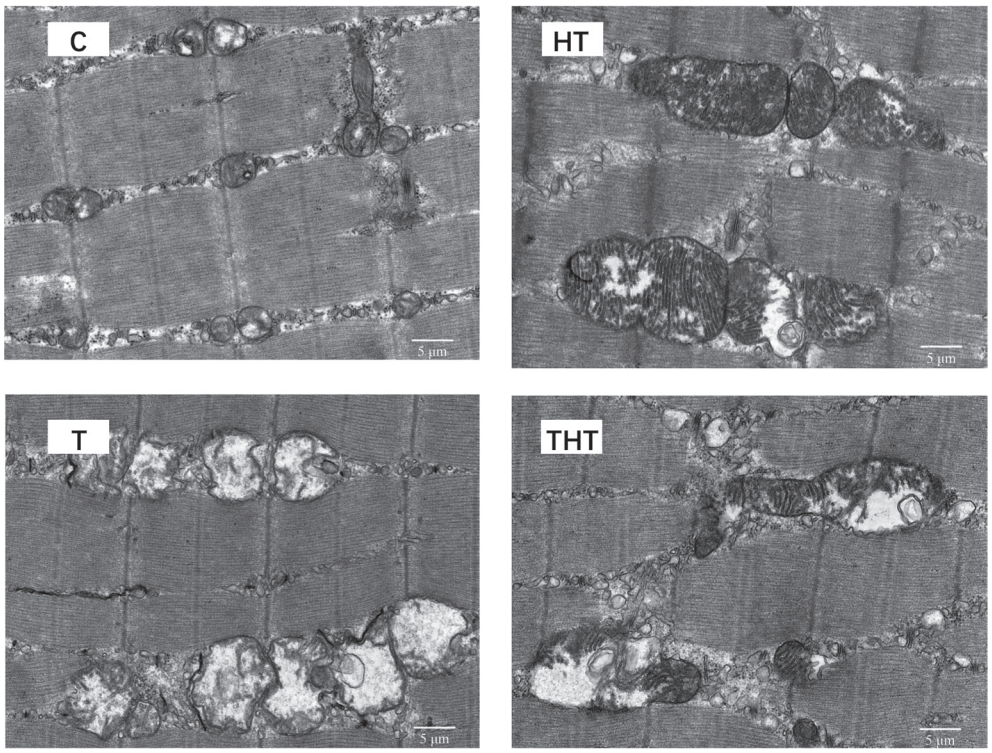

Figure 5. The ultrastructure of skeletal muscle mitochondria in different groups observed by TEM (magnification ×6000). Scale bars indicate 5 μm. For abbreviations, see Figure 1.

Dysfunctional or excessive mitochondria can be eliminated by mitophagy under hypoxia or exercise (Lemasters 2005; Li et al. 2018). The most widely studied mitophagy pathway is the PINK1/Parkin system. When some factors lead to the loss of mitochondrial membrane potential, PINK1 accumulate on the outer mitochondrial membrane. This accumulation leads to phosphorylation of PINK1 and activation of Parkin (Narendra et al. 2010). Mitophagy also includes the Bnip3/Nix pathway (Hanna et al. 2012). Study has shown that the level of Bnip3 protein in skeletal muscle increased after exercise training, and the ability of mitophagy increased (Lira et al. 2013).

Sirt3 is a mitochondrial NAD-dependent deacetylase with deacetylase activity. Sirt3 elevation can improve mitochondrial function and oxidation state, mainly through the interaction with FOXO3a to activate SOD2 and catalase. FOXO3a, a downstream target of Sirt3, can upregulate Bnip3/ Nix level (Salminen et al. 2013; Tseng et al. 2013; Yu et al. 2017). In addition, studies on rat cardiac ischemia-reperfusion have shown that FOXO3a is activated by Sirt3 activation, which then activates PINK1 (Das et al. 2014). Confocal microscopy showed that FOXO3a coexists with PINK1 and Parkin, suggesting Sirt3-FOXO3a-pink1-parkin constitutes a signal transduction cascade that jointly mediates mitophagy (Das et al. 2014). SOD2 is essential for maintaining cell oxidation balance and protecting mitochondria from oxidative attacks (Zeng et al. 2019).

This study mainly used the pharmacological inhibition of Sirt3 to analyze the influence of Sirt3 on mitophagy signaling under different intervention conditions. 3-TYP was used as an inhibitor of Sirt3, which can inhibit Sirt3 activity, but does not affect Sirt3 protein expression (Pi et al. 2015; Zhai et al. 2017). By referring to the existing literature, this experiment used i.p. injection to give 50 mg/kg of the body weight, and the injection was performed every two days (Zhai et al. 2017; Ye et al. 2019). The results of the study found that the Sirt3 protein level did not change by Western blot test, which is consistent with the previous research (Zhai et al. 2017; Ye et al. 2019).

In our experiment, 8-h hypoxic exposure combined with normoxic exercise may resulted in robust antioxidant response in skeletal muscle, thus accumulating a high level of Sirt3, FOXO3a, and SOD2. This study also found that, after i.p. injection of 3-TYP, the protein level of FOXO3a decreased, and mitochondria displayed pathological damages, indicating that the inhibition of Sirt3 function would damage the function of mitochondria. After the intervention of Sirt3 inhibitor combined with hypoxic training, the Sirt3-related proteins contents were still lower than hypoxic training group. Our results suggested that in the absence of Sirt3, hypoxic training cannot increase the protein levels of its downstream related proteins, indicating that Sirt3 plays a key role in the process of increasing FOXO3a and SOD2 expressions by hypoxic training.

In this study, hypoxic training increased the protein levels of PINK1 and Parkin. It reported that the mitophagy induced by hypoxic training is dependent on the PINK1/Parkin pathway. After the intervention of Sirt3 inhibitor injection combined with hypoxic training, the protein levels of PINK1 and Parkin were still lower than those of the hypoxic training group. It meant that Sirt3 plays an important role in the process of hypoxic training promoting Parkin-mediated mitophagy. The mechanism may be that in the absence of Sirt3, p53-Parkin binding is enhanced, and Parkin-mediated mitophagy is inhibited (Li et al. 2018). Hypoxic training can increase the Sirt3 levels, which may reduce the binding capacity of p53 and Parkin, promote the translocation of Parkin to mitochondria, and improve the ability of mitophagy. Interestingly, after the intervention of Sirt3 inhibitor combined with hypoxic training, Parkin expression was higher than that of the Sirt3 inhibitor group. It suggested that hypoxic training can partially reverse the decline of Parkin protein expression by the Sirt3 inhibition, and Sirt3 is not the only factor that hypoxic training regulates Parkin-mediated skeletal muscle mitophagy.

During this series of interventions, the protein expression of Bnip3 did not change significantly which was consistent with our previous study (Zhao et al. 2021). The pathway of mitophagy that affected by hypoxia is currently controversial. Some studies believed that hypoxic conditions mainly affect mitophagy through the Bnip3 and FUNDC1 pathways (Gustafsson and Dorn 2019; Wang et al. 2020). However, recent studies have shown that hypoxic training did not increase the protein expression level of Bnip3 in skeletal muscle, but it can significantly increase the expression of Parkin (Zhao et al. 2021). Our study did not find evidence of the interconnection between hypoxic training and Bnip3.

Mitochondrial division state is a prerequisite for mitophagy (Shirihai et al. 2015). In cardiomyocytes, Bnip3 can induce Drp1 to translocate to mitochondria, which in turn promotes Parkin to translocate to mitochondria in a Drp1-dependent manner (Lee et al. 2011). As the receptor of Drp1, MFF recruits Drp1 to mitochondria and divides severely damaged mitochondria (Jin et al. 2018). However, this view is controversial. A study have found that mitophagy can also occur in Drp1-deficient cells, and Drp1 is not necessary for mitophagy (Yamashita and Kanki 2017). In our study, Drp1 did not change significantly under various intervention conditions. Our study also found that hypoxic training can increase the MFF protein expression and upregulate the initial signal of mitochondrial fission. After the intervention of Sirt3 inhibitor injection combined with hypoxic training, the MFF expression was lower than that of the hypoxic training group. It implied that Sirt3 may play a critical role in the regulation of skeletal muscle mitochondrial fission by hypoxic training. However, the MFF expression after the intervention of Sirt3 inhibitor combined with hypoxic training was significantly higher than that of the Sirt3 inhibitor group. It indicated that, in the response of the mitochondrial fission of skeletal muscle by hypoxic training, other factors may have overlapping function just as Sirt3 in mediating muscle mitochondrial fission. Recent literatures have confirmed that AMPK also promotes mitochondrial fission of skeletal muscle by phosphorylation of MFF (Seabright et al. 2020). It was speculated that AMPK may cooperate with Sirt3 to regulate MFF to affect the mitochondrial fission and participate in the process of mitophagy mediated by PINK1/Parkin.

This study has certain limitations. Firstly, there were many training modes for hypoxic training methods. Due to the limitations of experimental conditions, this study did not investigate the effects of other hypoxic training methods such as "live low-train high", "intermittent hypoxic training" on skeletal muscle mitophagy. In addition, this study took hypoxic training as a holistic intervention method. We did not separately analyze the effects of hypoxia exposure and exercise training on mitophagy under the same experimental conditions.

In conclusion, our research indicated that 6 weeks of hypoxic training could improve mitochondrial mitophagy and ATP synthesis in skeletal muscle in mice. Sirt3 inhibitory intervention compromises the promotion of mitophagy signaling in skeletal muscle caused by hypoxic training. Sirt3 plays a key role in mediating skeletal muscle mitophagy by hypoxic training. Simultaneously, the results of our study also provided the first evidence that mitophagy caused by hypoxic training might be transduced through the Sirt3-FOXO3a signaling pathway.

Conflict of interest. The authors declare that they have no competing interests.

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