Effects and Mechanisms of Trib3 Gene in Blood Flow Restriction Training-Induced Skeletal Muscle Hypertrophy in Rats

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Introduction. Maintaining skeletal muscle mass is considered a critical component in the pursuit of a better quality of life. Although researchers have endeavoured to prevent or delay skeletal muscle atrophy, they have yet to fully unravel the molecular mechanisms involved in the progression of the disease.BFRT is a training modality that has emerged in recent years, and a large number of studies have demonstrated that only low-intensity resistance training or low-intensity sustained training is required to achieve improved muscle strength in BFRT.The Trib3 gene is the main differentially expressed gene in the BFRT-induced skeletal muscle hypertrophy model in the rat as identified in our previous study. The Trib3 gene is the main differentially expressed gene in the BFRT-induced rat skeletal muscle hypertrophy model, and is also a negative regulator of the insulin signalling pathway, which is able to regulate Akt phosphorylation. Therefore, based on the results of the existing studies, this study will deeply investigate the effect of Trib3 gene in BFRT-induced skeletal muscle hypertrophy in rats, and its impact on the pathways related to protein synthesis and degradation in skeletal muscle.

Methods**.** Thirty 8-week-old Sprague-Dawley male rats were selected and randomly divided into three groups of 10 rats each, which were given different disposition regimens: the control group (CON), which was given saline injection only; the model group (MOD), which was given cisplatin injection (1 mg/kg); and the BFRT group (BFR), which was given cisplatin (1 mg/kg) +BFRT. in the 24 hours after the last blood flow restriction of BFR, the body weight

of rats in each group was recorded and executed, and the muscle tissues of gastrocnemius (GAS) and soleus (SOL) muscles were collected by sufficiently separating the right leg of rats, weighed and recorded their weights. The qRT-PCR method was used to detect Trib3 gene expression, IGF-1/PI3K/Akt, TOR/S6K1/4EBP1, and FOXO/atatatropine in the skeletal muscles of rats in each group. and FOXO/atrogin-1/MuRF-1 mRNA expression levels.

Results**.** The body weight of rats in the BFR group was significantly increased after treatment compared with the MOD group; the weights of GAS and SOL were further compared between the groups after treatment. Compared with the CON group, the expression of Trib3 in GAS and SOL was significantly higher in both the MOD and BFR groups, and the expression of Trib3 in GAS and SOL was significantly lower in the BFR group than in the MOD group.The expression levels of IGF-1, PI3K and Akt mRNA in GAS and SOL in the BFR group were significantly higher than those in the CON and MOD groups and the expression levels of IGF-1, PI3K and Akt mRNA in GAS and SOL in the MOD group were significantly higher than those in the CON and MOD groups. The expression levels of IGF-1, PI3K and Akt mRNA in GAS and SOL of rats in the BFR group were significantly higher than those in the CON and MOD groups, and the expression levels of mTOR, S6K1 and 4EBP1 in GAS and SOL of rats in the MOD group were significantly lower than those in the CON group. The expression levels of FOXO, atrogin-1 and MuRF-1 mRNA in the GAS and SOL of rats in the BFR group were significantly lower than those in the CON and MOD groups, and the expression levels of mTOR, S6K1 and 4EBP1 mRNA in the GAS and SOL of rats in the MOD group were significantly higher than those in the CON group.

Conclusion**.** BFRT-induced skeletal muscle hypertrophy in rats inhibits the expression of Trib3 gene, and the inhibition of Trib3 is able to activate the GF-1R / PI3K / AKT pathway and further up-regulate the expression of mTOR, S6K1 and 4EBP1, and down-regulate the expression of FOXO, atrogin-1 and MuRF-1 mRNA, which can play a role in promoting the synthesis of proteins and inducing the the role of skeletal muscle illegitimacy.

Keywords. Trib3；BFRT；Skeletal muscle hypertrophy；IGF-1/PI3K/Akt；mTOR/S6K1/4EBP1；FOXO/atrogin-1/MuRF-1

INTRODUCTION

Skeletal muscle accounts for more than 40% of body mass and is responsible for daily movement and energy metabolism [1]. It is a ductile tissue that adapts its structure and function to internal and external environmental stimuli [2]. Skeletal muscle, as a highly malleable tissue, responds to internal and external environmental stimuli and it is able to change the size, function and metabolism of muscle fibres depending on whether the stimulus is anabolic or catabolic [3]. In addition, skeletal muscle supports daily movement, force production and energy storage, which are essential for daily life [4]. However, pathological stimuli can disrupt the regulation of skeletal muscle integrity, leading to loss of muscle mass and mechanometabolic function [5]. Loss of skeletal muscle mass, also known as muscle atrophy, is a common phenomenon caused by lack of physical activity, and some pathophysiological conditions such as cancer, diabetes, and heart failure can also lead to muscle atrophy [6]. Maintaining skeletal muscle mass is considered a key component in the quest for a better quality of life.

Rehabilitation training requires a minimum resistance load of 60% to 70% 1RM to improve muscle strength and at least 70% to 85% 1RM to increase muscle size, which cannot be accomplished in the elderly sarcopenia, postoperative, bedridden, and chronically ill populations [7-9]. Such high-intensity training is applicable to a narrower group of people and may also cause adverse events such as muscle fibre tears and rhabdomyolysis, and more than 90% of weight training-related injuries are caused by muscle overload, and the incidence of sports injuries is significantly and positively correlated with the age-related loss of muscle strength [10,11]. How to carry out efficient rehabilitation training for muscle atrophy, foreign researchers have proposed a new rehabilitation training method called "blood flow restriction training (BFRT)" [12], which uses a compression device to externally compress the proximal limb to restrict venous return. BFRT can effectively improve muscle fitness, increase aerobic endurance and prevent muscular atrophy, and it has the advantages of small load stimulus, low risk of injury and easy acceptance compared with traditional

high-intensity strength training, thus providing a new way for people with exercise intervention dysfunction to improve muscle fitness, improve aerobic endurance and prevent muscular atrophy, and it has the advantages of small load stimulus, low risk of injury and easy acceptance compared with traditional high-intensity strength training, thus providing a new way for people with exercise intervention dysfunction to improve muscle fitness and prevent muscular atrophy [12]. It has the advantages of low load stimulation and easy acceptance compared with traditional high-intensity strength training, thus providing a new therapeutic pathway for people with dysfunctional exercise interventions [15,16]. In recent years, it has been used in clinical rehabilitation of chronic diseases such as cardiovascular disease, postoperative orthopaedics, spinal cord injury, and musculoskeletal pain, which suggests that BFRT has a broad research prospect and application value, but the mechanism of its treatment for muscular dystrophy is still unclear [17-9].

In our previous study, in order to explore the underlying mechanism, based on the successful establishment of an animal model, we further used Genechip Rat Genome of Affymetrix to complete the resolution of the gene expression quantitative change curves of this blood-blocking-induced skeletal muscle hypertrophy model, and screened out a total of 1,649 genes with more than 2-fold changes after blood-blocking, of which the most significant change was in the Trib3 is also a negative regulator of the insulin signalling pathway and can regulate Akt phosphorylation [21,22]. Referring to the results of previous studies, the mechanism of BFRT-induced skeletal muscle hypertrophy is closely related to skeletal muscle protein synthesis and degradation. Based on the results of previous studies, the present study will deeply investigate the effect of Trib3 gene in BFRT-induced skeletal muscle hypertrophy in rats and its influence on the pathways related to skeletal muscle protein synthesis and degradation.

1 MATERIALS AND METHODS

1.1 Animals

All animal treatments were approved by the Animal Centre of Zhejiang University of Traditional Chinese Medicine. Thirty 8-week-old Sprague-Dawley male rats, each about 310 g, were selected and housed at a temperature of 23°C, a humidity of 40%-60%, and a light-dark cycle of 12:12 h. The above animals were ad libitum fed a standard diet and water, and after one week of acclimatisation, the rats were randomly divided into three groups of 10 rats each and were given different disposal regimens: the control group (CON), which was only given a saline injection; model group (MOD), given cisplatin injection (1 mg/kg); BFRT group (BFR), given cisplatin (1 mg/kg) + BFRT. animals were given cisplatin (1 mg/kg) once a day for 3 days. after 3 days of cisplatin administration to the rats in the BFR group, modelling was performed using the right hind limb blood flow restriction method according to the methodology of our previous study in the femoral joints near the upper part of the knee using a rubber band to block venous outflow for 30 minutes, twice a week for 2 weeks. Twenty-four hours after the last blood flow restriction of BFR, the body weight of rats in each group was recorded and anaesthetised by administering an intraperitoneal injection of sodium pentobarbital (1.5 ml-kg-1 body weight) and then executed, and the right leg of the rats was fully separated to collect the muscle tissues of the gastrocnemius muscle (GAS) and the soleus muscle (SOL), and the moisture attached to the muscle was quickly sucked up with clean filter paper, and then a precision The tissues were weighed on a precision balance and their weights were recorded. The tissues were frozen in liquid nitrogen and stored at -80°C until analysis.

1.2 RNA extraction

Total RNA was extracted by adding 1 ml of Trizol (Invitrogen, Carlsbad, CA) to 100 mg of GAS and SOL, respectively. 200 ml of chloroform was mixed with the GAS and SOL samples, respectively, after complete mixing. After vortexing, the EDL and SOL samples were incubated at 4°C for 5 minutes and then centrifuged at 12,000 rpm for 10 minutes. The upper aqueous phase was then transferred to another fresh tube and precipitated by adding an equal volume of isopropanol at - 20°C for 2 h. The

RNA was centrifuged (12,000 rpm) for 10 min at 4°C, then washed with 75% ethanol and dried in air. The RNA was then dissolved in DEPC-treated water and stored at -80°C for subsequent experiments.

1.3 qRT-PCR

The quantity and quality of total RNA samples were determined by UV spectrophotometer (756, China) and electrophoresed before reverse transcription. qRT-PCR was performed using an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, USA). 3 μg of total RNA was reverse transcribed to cDNA using oligonucleotide (dT) tailed primers and RevertAid reverse transcriptase (Thermofisher) according to the steps in the instructions. qPCR conditions consisted of an initial denaturation at 93°C for 3 min, followed by 40 cycles of 93°C for 10 s, 55°C for 10 s, and 72°C for 30 s. Under identical conditions, the Gapdh was also amplified as an internal reference gene. qPCR forward and reverse primers are shown in Table 1. Gene expression was counted using 2-ΔCT.

1.4 Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism version 9.1.0. Post hoc analyses were performed using one-way ANOVA and Tukey 's honestly significant difference test. p-value < 0.05 was considered statistically significant.

2 RESULTS

2.1 Comparison of body weight and skeletal muscle weight of rats in each group

By comparing the weights of rats in each group, we found that the weights of rats in the MOD and BFR groups decreased significantly compared with those in the CON group after modelling, which suggests that cisplatin injection has led to the successful establishment of the rat skeletal muscle atrophy model (Fig. 1-A); the weights of rats in the BFR group increased significantly compared with those of the MOD group after treatment (Fig. 1-B); the weights of the GAS and the SOL of the rats in the BFR group were compared with the CON group, and it was found that the weights of GAS and SOL decreased significantly in both the MOD and BFR groups compared with the

CON group, but those of the BFR group decreased significantly. Further, the weights of GAS and SOL in each group were compared, and it was found that the weights of GAS and SOL in both MOD and BFR groups decreased significantly compared with CON group, but the weights of GAS and SOL in BFR group were significantly higher than those in MOD group (Figure 1-C), which indicated that the treatment with BFRT method could effectively improve the condition of skeletal muscle atrophy in rats.

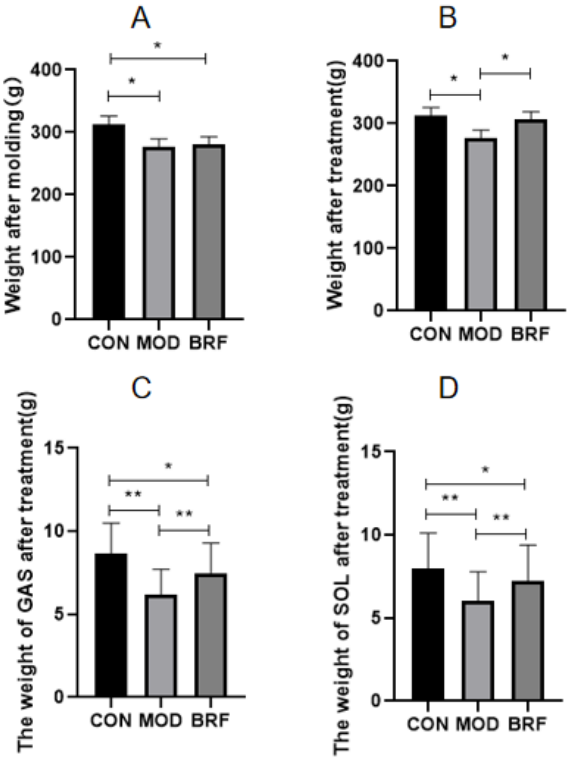

Figure 1. Comparison of weight and skeletal muscle of rats in each group.（A）Figure 1 Comparison of weight and skeletal muscle of rats in each group.(B)Comparison of body weight of rats in each group after treatment.（C）Comparison of GAS weight in each group after treatment .(D) Comparison of SOL weight in each group after treatment.*$p<0.05$,**$p<0.001$.

2.2 Mediation of skeletal muscle atrophy and hypertrophy by Trib3 gene

To determine the role of the Trib3 gene, obtained in our previous study, in the improvement of skeletal muscle atrophy in rats by BFRT, we determined the expression of Trib3 in the skeletal muscle of rats in each group after treatment. It was found that the expression of Trib3 in GAS and SOL was significantly higher in both the MOD and BFR groups compared with the CON group, however, the expression of

Trib3 in GAS and SOL was significantly lower in the BFR group than in the MOD group (Figure 2). With these data, it is suggested that skeletal muscle atrophy increases the expression of Trib3 in the skeletal muscle of mice, while BFRT can inhibit the increase of Trib3 in the skeletal muscle. These results suggest that Trib3 is a key gene mediating skeletal muscle atrophy and hypertrophy.

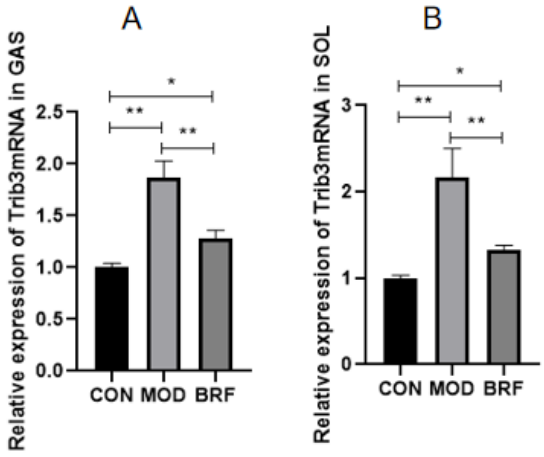

Figure 2. Expression of Trib3 gene in skeletal muscle cells of each group after treatment.(A) Relative expression of Trib3mRNA in GAS.(B) Relative expression of Trib3mRNA in SOL. *p<0.05,**p<0.001.

2.3 Role of IGF-1/PI3K/Akt pathway after Trib3 inhibition by BFRT

The IGF-1/PI3K/Akt pathway is a major mechanism for maintaining skeletal muscle protein homeostasis. In order to explore the role of Trib3 in the treatment of skeletal muscle atrophy in rats by BFRT, we compared the expression of IGF-1, PI3K, and Akt mRNA in the GAS and SOL of rats in each group, PI3K and Akt mRNA expression levels in GAS and SOL of rats in the BFR group were significantly higher than those in the CON and MOD groups, and IGF-1, PI3K and Akt mRNA expression levels in GAS and SOL of rats in the MOD group were significantly lower than those in the CON group (Figure 3). The above data suggest that BFRT inhibition of Trib3 can activate the IGF-1/PI3K/Akt pathway, which promotes the synthesis of skeletal muscle proteins and induces the hypertrophy of skeletal muscle, thus treating skeletal muscle atrophy.

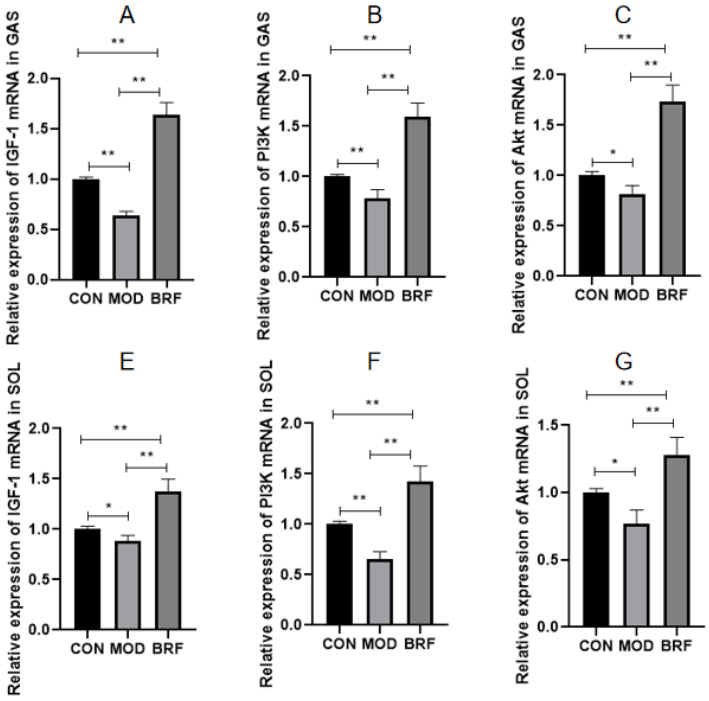

Figure 3. Effects of BFRT on IGF-1/PI3K/Akt pathway after Trib3 inhibition. (A) Relative expression of IGF-1 mRNA in GAS. (B) Relative expression of PI3K mRNA in GAS. (C) Relative expression of Akt mRNA in GAS. (D) Relative expression of IGF-1 mRNA in SOL. (E) Relative expression of PI3K mRNA in SOL. (F) Relative expression of Akt mRNA in SOL. *p<0.05,**p<0.001.

2.4 Effects of BFRT on mTOR, S6K1 and 4EBP1 after Trib3 inhibition

Phosphorylation and activation of Akt promotes protein synthesis through activation of the mechanistic target of rapamycin (mTOR), and phosphorylation of mTOR mediates phosphorylation of ribosomal S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4-E-binding protein 1 (4EBP1), which induces protein synthesis. Therefore, we further compared the expression of mTOR, S6K1 and 4EBP1 in the GAS and SOL of rats in each group, and found that the mRNA expression levels of mTOR, S6K1 and 4EBP1 in the GAS and SOL of rats in the BFR group were significantly higher than that of rats in the CON group and the MOD group, and the mRNA expression levels of mTOR, S6K1 and 4EBP1 mRNA expression levels were all significantly lower than those in the CON group (Figure 4). The above data suggest that BFRT inhibition of Trib3 can up-regulate the expression of mTOR, S6K1 and 4EBP1 by activating the IGF-1/PI3K/Akt pathway, which may be a key mechanism to inhibit Trib3 to promote skeletal muscle hypertrophy.

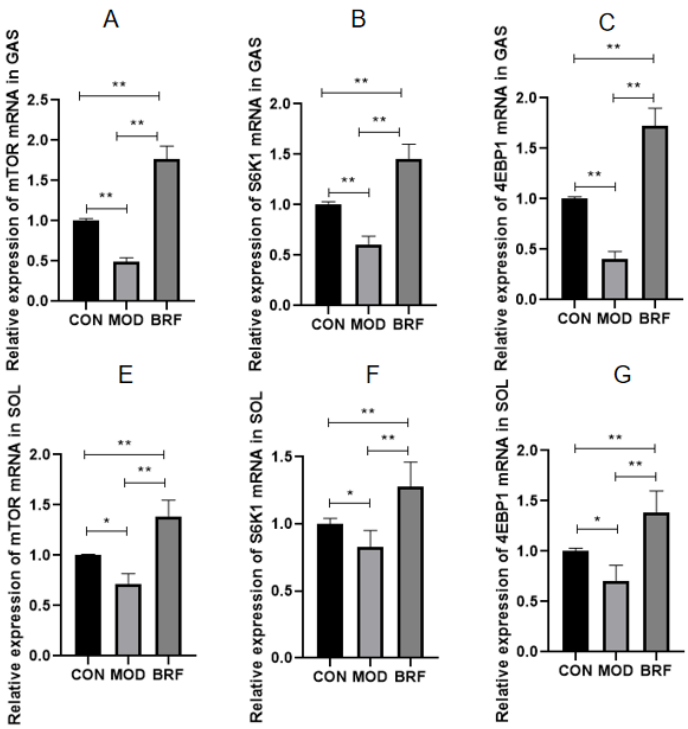

Figure 4. Effects of BFRT on mTOR, S6K1 and 4EBP1 after inhibiting Trib3. (A) Relative expression of mTOR mRNA in GAS. (B) Relative expression of S6K1 mRNA in GAS. (C) Relative expression of 4EBP1 mRNA in GAS. (D) Relative expression of mTOR mRNA in SOL. (E) Relative expression of S6K1 mRNA in SOL. (F) Relative expression of 4EBP1 mRNA in SOL. *p<0.05,**p<0.001.

2.5 Effects on FOXO, atrogin-1 and MuRF-1 after Trib3 inhibition by BFRT

Activation of Akt inhibits proteolysis through phosphorylation of forkhead box O families (FOXOs), another downstream target of Akt and a transcription factor that induces transcription of genes involved in protein degradation, including muscle muscle atrophy F-box (atrogin-1) and muscle RING finger-1 (MuRF-1). We also compared the expression of FOXO, atrogin-1 and MuRF-1 in the GAS and SOL of rats in each group, and found that the mRNA expression levels of FOXO, atrogin-1 and MuRF-1 in the GAS and SOL of rats in the BFR group were significantly lower than those in the CON and MOD groups, and that mTOR in the GAS and SOL of rats in the MOD group, S6K1 and 4EBP1 mRNA expression levels in GAS and SOL of rats in the MOD group were significantly higher than those in the CON group (Figure 5). The above data suggest that BFRT inhibition of Trib3 can down-regulate the expression of FOXO, atrogin-1 and MuRF-1 by activating the IGF-1/PI3K/Akt pathway, which may be an important mechanism that also inhibits Trib3 to promote

skeletal muscle hypertrophy.

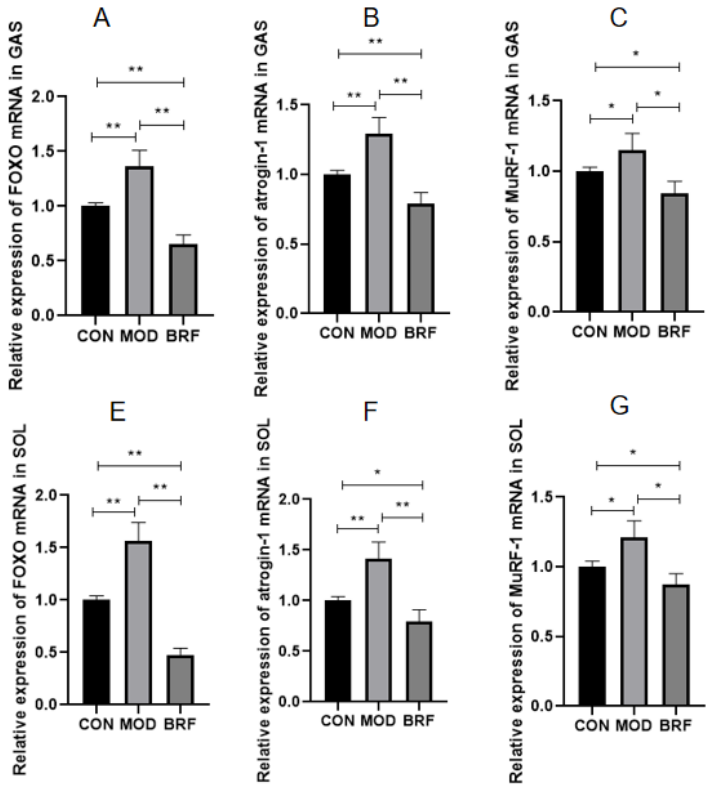

Figure 5. Effects of BFRT on FOXO, atrogin-1 and MuRF-1 after inhibiting Trib3. (A) Relative expression of FOXO mRNA in GAS. (B) Relative expression of atrogin-1 mRNA in GAS. (C) Relative expression of MuRF-1 mRNA in GAS. (D) Relative expression of FOXO mRNA in SOL. (E) Relative expression of atrogin-1 mRNA in SOL. (F) Relative expression of MuRF-1 mRNA in SOL. *p<0.05,**p<0.001.

3 DISCUSSION

Skeletal muscle, as the largest organ of movement in the body, the loss of muscle mass and integrity is associated with an increased prevalence of chronic diseases such as hypertension, type 2 diabetes, obesity and cancer, so maintaining skeletal muscle mass and function is considered a powerful strategy for preventing and overcoming pathological conditions [24,25].BFRT is a training modality that has emerged in recent years, and a large number of studies have demonstrated that only low-intensity resistance training or low-intensity continuous training is necessary in BFRT to achieve the effect of improving muscle strength and improving the function of the

cardiovascular system. A large number of studies have proved that in BFRT, only low-intensity resistance training or low-intensity continuous training can achieve the effect of improving muscle strength and cardiovascular system function, and that the elderly and BFRT can achieve the effect of effectively improving muscle mass and preventing muscle atrophy by adopting small-loaded resistance training, and that BFRT can cause less muscle damage than traditional resistance training, and that subjective fatigue and pain are lower than traditional high-intensity resistance training, which is a powerful exercise strategy for the elderly, bedridden and chronically ill people [24,25]. This is a less risky and more acceptable training method for the elderly, bedridden and chronically ill [26-28]. There are few studies on the key targets and their mechanisms of BFRT for the treatment of skeletal muscle atrophy.

Trib3 is considered to be a negative regulator of the insulin signalling pathway, mainly regulating Akt phosphorylation [21,22]. Its expression is involved in the pathophysiology of several diseases, including diabetes and obesity, leading to insulin resistance [29]. Recently, it was found that overexpression of Trib3 in mouse skeletal muscle decreases protein synthesis and increases protein degradation by disrupting the ground state of Akt and its downstream molecules [30]. Therefore, in the present study, we investigated the regulatory role of Trib3 in the treatment of skeletal muscle atrophy with BFRT and its possible mechanisms. In this study, we used cisplatin injection for 3 days to create a rat model of skeletal muscle atrophy, and the expression of Trib3 mRNA in rat skeletal muscle was significantly elevated after modelling, whereas the expression of Trib3 mRNA in rat skeletal muscle was significantly decreased after BFRT intervention. Our study reports for the first time that BFRT intervention inhibits Trib3 expression in rat skeletal muscle, suggesting that Trib3 is a key gene in BFRT-induced skeletal muscle hypertrophy.

Previous studies have shown that exercise prevents the loss of muscle mass and strength by stimulating the release of IGF-1 from skeletal muscle and other secretory-like organs, which promotes skeletal muscle protein anabolism [31, 32]. At the molecular level, IGF-1 activates the autophagy/lysosomal system in skeletal muscle cells, regulates mitochondrial function, and alleviates excessive oxidative

stress and inflammatory responses [33]. Increasing skeletal muscle IGF-1 levels can reduce oxidative stress and protein ubiquitination degradation and alleviate skeletal muscle loss [34]. Recent studies have found that exercise-induced skeletal muscle hypertrophy in rats is closely associated with activation of the IGF-1R / PI3K /AKT pathway [35]. The present study confirmed that inhibition of Trib3 by BFRT significantly activated the IGF-1/PI3K /AKT pathway, suggesting that we can achieve activation of this pathway by inhibiting Trib3 gene expression in skeletal muscle in future studies. One of the major factors regulating skeletal muscle protein synthesis is Akt, which in vivo induces the activation of skeletal muscle hypertrophy. It has been reported that two weeks after activation of Akt in adult animals, the expression of signalling proteins involved in protein synthesis, such as mammalian mTOR and p70S6K, is increased, skeletal muscle cell size is tripled and the number of myofibres is increased. mTOR promotes the phosphorylation of 4EBP and S6K1 and initiates protein synthesis. kt activates phosphorylated FOXO, which leads to its translocation from the nucleus to the cytoplasm. In the cytoplasm, FOXO inactivates and inhibits the activation of the ubiquitin ligases atrogin-1 and MuRF-1. In this study, in order to further elucidate the mechanism of BFRT inhibition of Trib3-induced skeletal muscle hypertrophy in rats, the expression of the pro-protein synthesis factors mTOR, S6K1, and 4EBP1, and the pro-protein degradation factors FOXO, atrogin-1, and MuRF-1 mRNA was simultaneously detected in each group. The results showed that the mRNA expression levels of mTOR, S6K1 and 4EBP1 were significantly increased after Trib3 inhibition by BFRT, while the expression of FOXO, atrogin-1 and MuRF-1 mRNA was significantly decreased.

Taken together, the above findings suggest that BFRT-induced skeletal muscle hypertrophy in rats inhibits Trib3 gene expression, and the inhibition of Trib3 is able to activate the GF-1R / PI3K / AKT pathway and further up-regulate the expression of mTOR, S6K1, and 4EBP1 and down-regulate the expression of FOXO, atrogin-1, and MuRF-1 mRNA, which can play a role in promoting protein synthesis and induce skeletal muscle illegitimacy.

CONCLUSION

The highlight of this study is that a simple modelling protocol published in a previous study by our team was used to create a model of BFRT-induced skeletal muscle hypertrophy, and for the first time, it was found that BFRT induction inhibits Trib3 expression in rat skeletal muscle. The drawback is that we were unable to obtain Trib3 knockdown rats to further confirm the effect of Trib3 inhibition on the GF-1R / PI3K /AKT pathway, as well as downstream signalling pathways, by means of reverse validation. We will actively pursue the development of this model in our future studies in order to obtain more comprehensive research data.

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