Resistance Training Alleviates Skeletal Muscle Atrophy in Rats Exposed to Hypoxia by inhibiting Autophagy Mediated by Acetyl-FoxO1

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Abstract

Background: Skeletal muscle atrophy induced by hypoxia could affect the physical fitness and training effect of the athletes in the rapid altitude, and also affect the production and life of the general public. Resistance training in a hypoxic environment could effectively alleviate the occurrence of muscular atrophy. Whether autophagy lysosomal pathway, as an important proteolysis pathway, is involved in this process, and whether FoxO1, the key gene of atrophy, plays a role by regulating autophagy is unclear.

Methods: Male Sprague-Dawley (SD) rats were randomly divided into normoxic control group (group C), normoxic resistance-training group (group R), hypoxic control group (group H), and hypoxic resistance-training group (group HR). The H and HR groups were exposed to 12.4% oxygen for four weeks. The R and HR groups underwent incremental loaded training by climbing a ladder every other day for four weeks.

Results: Compared to parameters in group H, resistance training increased lean body mass (LBM) and wet weight and decreased the expression of atrogin1 of the extensor digitorum longus (EDL) after four weeks ($P < 0.05$). Resistance training decreased the levels of FoxO1 and Ac-FoxO1 and the extent of their localization in the nucleus and cytoplasm, respectively ($P < 0.05$), as well as the LC3II/LC3I ratio, the integrated optical density (IOD) of LC3 and the levels of autophagy-related gene 7 (Atg7), and elevated the levels of sequestosome 1 (SQSTM1/p62) ($P < 0.05$). Most differentially expressed autophagy-related genes (ATGs) interacted with FoxO1, and the functions of these ATGs were mainly enriched in the early autophagy phase.

Conclusions: Our findings demonstrate that resistance training lowers the levels of both nuclear FoxO1 and cytoplasmic Ac-FoxO1, as well as reduced autophagic flux in the EDL of rats exposed to hypoxia.

Background

Plateau/hypoxic training, plateau tourism, and hypoxic weight loss promote individuals to experience hypoxic environments, which induce a series of adverse effects, including skeletal muscle atrophy. Such reduction in the size and strength of muscles affects not only the general public health, but also the competitive levels of athletes [1, 2]. Although appetite, food intake, and gastrointestinal function are attenuated during hypoxia, these changes occur only at the early stages of hypoxic stimulation and are not sufficient to induce skeletal muscle atrophy. Rather, this atrophy is attributed primarily to an alteration in protein metabolism, with the rate of protein degradation becoming approximately 3 times faster than synthesis [3, 4]. Resistance training is the most effective training method for promoting muscle hypertrophy and is therefore effective non-pharmaceutical treatment for various pathological or age-related muscle atrophies. However, it is unclear whether resistance training may also alleviate hypoxia-induced muscle atrophy. It is a fascinating possibility that resistance training might also attenuate hypoxia-induced muscle atrophy, thereby avoiding the use of sometimes illicit drugs.

The autophagy-lysosome pathway (ALP) is an important pathway for protein degradation [5, 6]. The balance of autophagic flow may be disrupted by multiple factors—such as diet, environment, physical activity, systemic disease, and/or genetics—to induce muscle atrophy. Although insufficient autophagy can cause muscle atrophy, this effect results in a chronic loss and is more common in aging. However, excessive autophagy can cause a rapid loss in muscle mass due to the continued clearance of necessary cellular components. Whether and how autophagy is involved in hypoxia-induced muscle atrophy remains unclear. Exercise has been found to induce B-cell lymphoma-2 (Bcl-2) phosphorylation, providing a molecular regulatory link to autophagy.

In studies of exercise-regulated autophagy, endurance exercise represents the most commonly investigated method. Earlier findings suggested that resistance training may not influence autophagy, but more recent observations indicating that such training attenuates age-related muscle atrophy have challenged this view. However, the role of resistance training in relieving autophagy in hypoxia-induced muscle atrophy remains an unanswered question.

Forkhead box protein O1 (FoxO1), a member of the FoxOs family of transcription factor, is an important regulator of muscle mass [7]. FoxO1 transgenic mice exhibit decreased muscle mass and impaired muscular function [8]. Autophagic levels are directly regulated by FoxO1 to induce hypotrophy-induced muscle atrophy but are not changed in FoxO1 skeletal-muscle-specific knockout mice, confirming that the induction of autophagy requires FoxO1 [9]. Previous studies have suggested that FoxO1 nuclear localization is a prerequisite for its function, but more recent observations indicate that FoxO1 in the cytoplasm can also regulate autophagy, for which acetylation modification is an important regulation mode. Indeed, reduction of acetylase activity inhibits the atrophy induced by FoxO in C2C12 myotubes deprived of nutrients, as well as in disused muscle in vivo [10, 11], indicating that acetylation of FoxO1 plays an important role in maintaining skeletal muscle mass. However, whether acetylation of FoxO1 participates in resistance training to alleviate hypoxia-induced atrophy and whether autophagy is involved remains unclear.

Accordingly, here we first evaluated the ability of resistance training to alleviate hypoxia-induced muscle atrophy, followed by detection of potential changes in the expression of autophagy-related genes, and final characterization of the expression and localization of FoxO1 and acetylated FoxO1 (Ac-FoxO1).

Methods

Animals and experimental design

Twenty-eight male Sprague-Dawley (SD) rats, each weighing approximately 200 g, were kept at 25±2 ℃ on a 12 h day-night cycle in the Beijing Sports University SPF Animal Laboratory (Beijing, China) and provided with food and water *ad-libitum*. Three or four rats were housed in each cage, and these animals divided randomly into four groups of 7 each: normoxic control group (group C), normoxic resistance-training group (group R), hypoxic control group (group H), and hypoxic resistance-training group (group HR). All experiments were conducted with the pre-approval of the Animal Ethical Committee of Beijing

Sport University (IACUC 2017009A) and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

Hypoxia exposure and resistance training

Groups C and R were placed in a normoxic environment; Groups H and HR were placed in a hypoxic chamber with a 12.4% oxygen concentration (simulated altitude of 4,000 m); Group R and HR performed incremental-load resistance-ladder training every other day, with pre-training for one week in normoxia to allow adaptation to the ladder. The 1.2-m ladder was placed at an angle of 85° to the ground, giving an actual climbing height of 1 m. The load was adjusted by changing the number of steel balls in the centrifuge tubes attached to the tail of the rats (Fig. 1A). Daily training involved 3 sessions of 5 climbs each, with a one-min rest between sessions, with this intervention lasting 4 weeks. This method of resistance training, the number of groups, and the incremental load are based on the research model of Lee S and colleagues [12], illustrated in Fig. 1A and documented in Tab. 1.

Table.1 Loading weights of daily resistance training (g) on rats.

Day (d)	Pre-training	Formal training								
	1-7	1	3	5	7	9	11	13	15	17-27
Load (weigh%)	0-50%	50%	60%	70%	80%	90%	100%	110%	120%	130%
Group R load (g)	0-121	121	153	188	217	257	294	323	376	422-485
Group HR load (g)	0-115	115	145	177	196	234	272	310	353	395-442

Body composition and muscle wet weight

The body weight and food intake of each group were recorded every other day. After the four-week intervention, the lean body mass (LBM) was determined by dual-energy X-ray absorptiometry (DEXA). The rats were anesthetized by injection with 3% sodium pentobarbital (3 mL/kg, i.p.) and then sacrificed by drawing blood from the abdominal aorta. The *extensor digitorum longus* (EDL) was dissected out; one side was weighed and then fixed, whereas the other side was divided into two parts, as follows: one part was wrapped in tin foil and placed at -80°C; and the other part was stored in RNA later.

Hematoxylin-eosin (HE) staining

EDLs were fixed in 4% paraformaldehyde overnight and were then trimmed into 5-mm^3 tissue blocks. Paraffin blocks were then prepared after dehydration, clearing, and wax impregnation. Sections (5 μm each) were cut with a rotary microtome, deparaffinized in xylene, and stained with hematoxylin and eosin. Images of the EDL sections from three rats in each group were recorded digitally under a bright-field microscope and their fiber cross-sectional area (FCSA) determined using Image J software.

Western blotting (WB)

After extracting EDL protein into radio immunoprecipitation assay (RIPA) lysates, protein concentrations were determined by the BCA (bicinchonininc acid) procedure, and protein loading was adjusted to 20 μg. Electrophoresis was performed in a running buffer (MES Running Buffer (B0002) and tris-acetate SDS running buffer (LA0041, Invitrogen)) using a gradient gel (4%–12% Bis-Tris (NW04125) and 3%–8% Tris Acetate (EA03755, Invitrogen)). Subsequently, the separated protein bands were transferred from the gel to nitrocellulose (NC) filter membranes using a semi-dry transblot module (iBlot 2, Life Technologies). These membranes were then blocked with Odyssey blocking buffer (LI-COR) for 1 h, washed with TBST (Tris-buffered Saline Tween-20), and probed with primary antibodies (heavy chain myosin, ab124205, Abcam; muscle-atrophy Fbox protein (MAFbx/Atrogin1), ab74023, Abcam; muscle-specific ring finger (MuRF1), ab172479, Abcam; autophagy-related gene 7 (Atg7), ab133528, Abcam; Sequestosome 1 (SQSTM1/p62), ab109012, Abcam; Coiled-coil myosin-like BCL2-interacting protein (Beclin1), ab207612, Abcam; microtubule associated protein light chain 3 (LC3), NB100-2220, Novusbio; FoxO1, ab52857, Abcam; Ac-FoxO1, AF2305, Affinity; Ubiquitin/Ub, sc-8017, Santa Cruz; α-tubulin, T6074, Sigma) overnight at 4℃; washed again with TBST; and incubated for 1 h in secondary antibodies (926-68071 and 926-32210, LI-COR). After a final wash, the bands were detected with a Near-Infrared Spectroscopy Detection System (Odyssey CLX, LI-COR) and were quantified by Image Studio software to determine relative protein expression.

Immunofluorescence (IF) staining

After dewaxing, dehydration and antigen retrieval, and addition of a quencher of spontaneous fluorescence, the paraffin sections were blocked with serum, and thereafter individually incubated with primary anti-FoxO1, Ac-FoxO1 or LC3 antibodies at 4℃ overnight, followed by incubation with fluorescent secondary antibody (GB21303, Servicebio) for 50 min at room temperature, and counterstaining of nuclei with 4,6-diamidino-2-phenylindole (DAPI). After washing and sealing, the sections were examined under a fluorescence microscope (Leica, Germany) and Image Pro-Plus (IPP) 6.0 software used to calculate the optical densities and cellular localization.

Autophagy PCR array

The expression of 84 autophagy-related genes (ATGs) of EDL rats was examined by PCR array (n=3). Total RNA was extracted with Trizol (Invitrogen); the concentration and purity of the RNA obtained assessed by NanoDrop® ND1000, cDNA synthesized by reverse transcription and, finally, high-throughput fluorescent quantitative PCR detection carried out. The autophagy PCR array (sp-TWCPART-0002, Qiyin Biotechnology, Shanghai) was read by a real-time quantitative PCR (RT-qPCR) instrument and the fold change in expression calculated by the $2^{-\Delta\Delta CT}$ procedure (employing GAPDH as an internal reference). The differentially expressed genes of groups R/C, H/C, and HR/H were screened. The screening criteria were set to $P<0.05$ and a fold change≥1.5. Subsequent functional analysis of the selected differential genes was performed.

Statistical analysis

All results are presented as the mean ± standard error of the mean (SEM). Two-way analyses of variance (ANOVAs) were used for comparisons among more than two groups. Simple-effect tests were used if there was an interaction between the two factors. Least-significant difference (LSD) tests were used for inter-group tests. Independent sample t tests were used to compare two groups, and $P<0.05$ was considered a statistically significant difference. Pearson correlation analysis was used to calculate the correlations among variables, and correlation coefficients are expressed by "*r*." All statistical analyses were performed using SPSS 19.0 software.

Results

Skeletal muscle morphology and atrophy-related protein expression in rats

In the early stages of exposure to hypoxia, the food intake of group H and HR decreased (14.0% and 9.6%, respectively), gradually recovering from the fifth day onward. After 25 days of intervention, the food intake of each group had gradually approached similar values (30.6 g in group C, 29.8 g in group R, 29.6 g in group H, and 28.9 g in group HR). The weight of the rats in each group increased continuously, less so in the case of the two hypoxic than the two normoxic groups (Fig. 1B).

The body weight and LBM in group H were decreased by 9.6% and 12.9%, respectively, compared to those of group C, and the LBM percentage (LBM/body mass, LBM%) also decreased significantly ($P < 0.05$); the LBM of group HR increased by 8.5% compared with that of group H, with LBM% being higher as well ($P < 0.05$). There was no significant difference in the fat mass among the groups (Fig. 1C).

The EDL wet weight of group H was 13.0% less than in group C, with a lower percentage EDL wet weight (EDL wet weight/LBM, EDL%). The EDL wet weight in HR group increased by 7.5% compared to that of group H, with a higher EDL% as well ($P < 0.05$; Fig. 1D). The FCSA of EDL in group H was 17.4% lower than in group C (the criterion for achieving muscle atrophy was that the FCSA was reduced by more than 10% [13]; $P < 0.05$; Fig. 1E).

Myosin, the main skeletal muscle constituent protein, is a dynamic source of muscle contraction [14]. Elevated skeletal muscle-specific E3 ubiquitin protein ligases, MuRF1 and Atrogin1 are hallmarks of muscle atrophy [15]. The EDL levels of Myosin and Atrogin1 in group H were significantly lower than those in group C and the level of Atrogin1 in group HR significantly lower than in group H ($P < 0.05$; Fig. 1F).

Expression of the autophagy-related proteins Atg7, SQSTM1/p62, Beclin1, and LC3

After the intervention, there was no difference in the level of ubiquitinylated protein among the groups (Fig. 2A). The level of Atg7, an important regulator of Ac-FoxO1-mediated autophagy [16, 17], was significantly lower in group R than group C, higher in group H than group C, and lower in group HR than group H ($P < 0.05$) (Fig. 2B). p62, Beclin1 and LC3 are three major proteins involved in autophagy. The levels and integrated optical density (IOD; positive expression levels) of LC3 and the LC3II/LC3I ratio were

all higher in group H than group C, and lower in group HR than group H (Fig. 2C3, D1 and D2), which is opposite to the level of p62 (Fig. 2C1). In addition, the level of the Beclin1 protein was higher in group H than group C (Fig. 2C2; $P < 0.05$). The level of p62 was positively correlated with EDL%; the level of Beclin1 positively correlated with the LC3II/LC3I ratio ($P < 0.05$); and the level of Beclin1 and LC3II/LC3I ratio negatively correlated with EDL% and the level of p62 protein [18] ($P < 0.05$; Fig. 2E).

Autophagy differential gene expression and functional analysis

Relative to group C (R/C), 11 genes were up-regulated and 21 down-regulated in group R. The down-regulated genes were primarily Genes Involved in Autophagic Vacuole Formation, Co-Regulators of Autophagy and Apoptosis and Autophagy in Response to Other Intracellular Signals. Relative to group C (H/C), 21 genes were up-regulated (the main functions were same as those down-regulated in group R) and 8 down-regulated in group H. Relative to group H (HR/H), 5 genes were up-regulated and 50 down-regulated in group HR. The genes down-regulated coded primarily for Co-Regulators of Autophagy and Apoptosis, Co-Regulators of Autophagy and the Cell Cycle, Autophagy in Response to Other Intracellular Signals, Autophagic Vacuole Formation and Protein Transport (Fig. 3A).

The potential interactions between autophy-related genes and FoxO1 were analyzed using the STRING functional protein association networks database (https://string-db.org/). There was a first-order interaction between ATGs and FoxO1 (direct regulatory genes of FoxO1) in group-R/C down-regulated (Fig. 3B1), group-H/C up-regulated (Fig. 3B2), and group-HR/H down-regulated genes (Fig. 3B3). Pathway analysis of FoxO1 primary-autophagy-regulated differentially expressed genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) functional-annotation bioinformatics microarray analysis database (https://david-d.ncifcrf.gov/). The autophagy pathway shown in the Fig. 3C was divided into three phases: (1) initiation (phagocytic/capsule formation); (2) autophagosome formation (membrane completely surrounds the substance to be degraded to form an autophagosome); and (3) autophagosomal/lysosomal fusion and autolysosome degradation [19–21]. Pathway analysis found that FoxO1 directly regulated group-R/C down-regulated, group-H/C up-regulated, and group-HR/H down-regulated genes enriched in the autophagy pathway; all the ATGs were mainly enriched at the first and second stages of the autophagic pathway (Fig. 3C).

Protein expression and localizations of FoxO1 and Ac-FoxO1

The protein levels of FoxO1 and Ac-FoxO1 were higher in group H than group C, while the level of FoxO1 was lower in group HR than group H ($P < 0.05$; Fig. 4A). The IOD values for FoxO1 and Ac-FoxO1 were higher in group H than group C and the IOD of FoxO1 higher in group HR than group H ($P < 0.01$). The percentage of FoxO1 localized in the nucleus was higher in group H than group C and lower in group HR than group H. In the case of Ac-FoxO1, this percentage was lower in both groups R and H than group C and higher in group HR than group H ($P < 0.05$; Fig. 4B and C).

Discussion

In the present study, our results demonstrated that resistance training attenuated muscle atrophy by reducing hypoxia-induced high autophagic levels, which may have been related to concomitant changes in FoxO1 and Ac-FoxO1.

Hypoxia induces muscle atrophy, which can be attenuated by resistance training

Here, a four-week exposure to 12.4% oxygen induced atrophy of the EDL (a typical fast-twitch muscle), and resistance ladder training attenuated this atrophy. We found that hypoxia reduced LBM, EDL wet weight and FCSA, while resistance training counteracted these changes (Fig. 1C–E).

Changes in the circumference and number of muscle fibers are known to affect skeletal muscle mass [22]. However, after maturity has been reached, the number of skeletal muscle fibers remains essentially unchanged, and resistance training promotes an increase in circumference (hypertrophy); hence, FCSA is a key regulator of muscle mass [23]. The different types of muscle fibers differ greatly with respect to morphology, metabolism and contractile properties [24]. Most reports indicate that hypoxia induces atrophy selectively in fast-twitch muscle fibers [25, 26], perhaps because of the fewer number of capillaries in type II muscle fibers along with their higher expression of hypoxia-inducible factor 1α (HIF1α). Indeed, resistance training is 50% more likely to produce hypertrophy of fast- than slow-twitch muscles [27, 28]. Studies have also shown that four-week moderate-intensity resistance ladder training significantly promotes hypertrophy of type-II muscle fibers and increases muscle strength (loads up to 150% of body weight) [29]. In addition, resistance training is one of the most powerful ways to stimulate skeletal muscle, while weight-bearing ladder training represents a method to promote skeletal muscle hypertrophy in rats and closely emulates human resistance training [30].

Furthermore, in the present study, we also observed and assessed muscle atrophy at the molecular level. Hypoxia induced a decrease in the expression level of the muscle-strength-related protein, myosin, and an increase in the muscle atrophy-associated protein, Atrogin1, whereas resistance training significantly reduced the expression of Atrogin1 (Fig. 1F). Myosin is an important protein involved in the formation of thick muscle filaments, and changes in myosin content reflect concomitant changes in muscle mass and tension [31]. As E3 ubiquitin ligases, atrogin1 and MuRF1 ubiquitinate and label skeletal muscle-specific proteins to target hydrolysis by 26S proteases, which were once considered to be key proteins for activating the ubiquitin-proteasome pathway (UPP). However, recent studies have identified additional E3 ubiquitin ligases, and Atrogin1 and MuRF1 have since become more recognized as markers of muscle atrophy due to their skeletal muscle specificity. MuRF1 can cause muscle atrophy by directly degrading sarcomeric crude fiber and causing proteolysis of myosin, which is more closely related to the UPP. Whether Atrogin1 is involved in protein ubiquitination in muscle atrophy is not clear, suggesting that the UPP may not be the primary means by which hypoxia-induced muscle atrophy is ameliorated by resistance training.

Resistance training alleviates hypoxia-induced muscle atrophy by attenuating autophagy

Increased synthesis of muscle protein may be an important source of hypertrophy in healthy organisms, but in connection with atrophy inhibition of protein degradation may play a more important role in maintaining skeletal muscle mass. Furthermore, such inhibition may be related to oxidative stress, apoptosis, inflammatory responses, autophagy and mitochondrial dynamics [32]. The UPP and ALP are the two major processes by which proteins are degraded [33, 34], but we observed no significant differences in the total level of ubiquitinylated protein between the groups (Fig. 2A). In agreement, although exposure of rats to 10.7% oxygen increases the level of E3 ubiquitin ligase mRNA, the corresponding protein level is not changed [35].

Characterization of the expression of important autophagy-related genes revealed that hypoxia induce muscle atrophy by promoting autophagy, whereas resistance training may inhibit autophagy. Hypoxia elevated levels of Atg7 and Beclin1, the LC3II/I ratio and the IOD of LC3 and decreased the level of p62 (Fig. 2B, C, D), with the percentage of EDL wet weight exhibiting a negatively correlation to the levels of autophagy-related proteins (Fig. 2E). Autophagy, a process that degrades cytoplasmic components, is regulated by many cellular pathways and evolutionarily conserved ATGs, among which Beclin1, LC3, and p62 represent three major components [5]. Beclin1 is involved in the initiation of this process forming a trimer with Vps34 and Atg14 to recruit autophagy-associated proteins [36]. The LC3 precursor can be cinverted into soluble LC3I by Atg14 and then linked by Atg3 and Atg7 to phosphatidylethanolamine (PE) to form the lipid-soluble LC3II-PE, involved in the fusion of autophagic lysosomes. Thus, the LC3II/LC3I ratio is a key indicator of autophagy [37]. p62 binds to ubiquitinylated protein and subsequently combines with LC3II to form a complex, that is degraded in lysosomes and the level of p62 is decreased after LC3I is converted to LC3II [38]. Some earlier reports support our present findings. When 11 individuals were exposed to normoxia and their identical twins to hypoxia (10.7%O2), the extent of autophagy in the latter group increased (i.e., the level of LC3II and LC3II/I ratio rose, while the p62 level declined) [39, 40]. Moreover, the levels of LC3 and Beclin1 in C2C12 myotubes are enhanced by exposure to the hypoxic modulator CoCl2 and inhibitors of autophagy promote the expression of myogenin and formation of myotubes [41]. Furthermore, similar to our observations, another investigation found that 8 weeks of resistance ladder training under normoxia reduces the LC3II/LC3I ratio and elevates the level of p62 [42]. Thus, regular long-term repetitive training may increase the threshold for activation of autophagy by stress, thereby protecting the muscle [43–45].

To explore the role of autophagy in regulating skeletal muscle mass in greater depth, we characterized PCR array. ALP is a complex protein network in which ATGs form various complexes in a non-linear manner to participate in different steps of the autophagy process [46, 47]. We observed here that hypoxia regulates autophagy primarily by up-regulating the expression of ATGs, in particular those whose products are involved in autophagic vacuole formation (Fig. 3A). In contrast, resistance training primarily down-regulates the levels of ATGs that encode mainly co-regulators of autophagy and apoptosis. Autophagy and apoptosis interact, with some specific stresses activating both, and this interaction plays an important role in regulating protein homeostasis and muscle atrophy and cell death [48, 49]. In addition, the ubiquitin-associated domain (UBA) of p62 can recruit ubiquitinylated proteins, so that p62 is

not only involved in activation of autophagy, but also serves as an important regulator of caspase 8-mediated apoptosis [50].

Mediation of autophagy by Ac-FoxO1 is involved in the alleviation of hypoxia-induced muscle atrophy by resistance training

Our interaction analysis between differentially expressed ATGs and FoxO1 showed that FoxO1 plays an important role in autophagy-mediated muscle atrophy (Fig. 3B). Such ATGs in group HR/H exhibit a primary regulatory relationship to FoxO1, being mainly involved in initiation of autophagy and autophagosome formation, which suggests that alleviation of hypoxia-induced autophagy by resistance training may be regulated by FoxO1. FoxO1 is the member of the FoxOs family that is most central to autophagy initiation, vesicle nucleation and extension and FoxO1 siRNA or FoxO1 inhibitors block the autophagic process. FoxO1 not only directly promotes autophagy by up-regulating the expression of ATGs but also indirectly promotes autophagy by regulating sestrin 3 (SESN3) and stimulating the tuberous sclerosis protein 1–2 (TSC1-2) complex to inhibit the mTOR pathway [51]. Accordingly, resistance training in normoxia could inhibit age-related muscle atrophy by attenuating the expression of FoxO1 and other ATG [52].

The role of FoxO1 in regulating autophagy may be closely related to its acetylation. Recently, FoxO1 was found to play a role to induce/enhance autophagy in the cytoplasm, and this role may be mediated by acetylation/deacetylation [17]. Here, hypoxia enhanced the levels of FoxO1 and Ac-FoxO1, as well as the nuclear localization of FoxO1, and cytoplasmic localization of Ac-FoxO1; whereas resistance training reduced the level and nuclear localization of FoxO1, as well as the level and cytoplasmic localization of Ac-FoxO1 (Fig. 4). Autophagy mediated by Ac-FoxO1 requires the participation of Atg7, a key regulator of autophagosome formation that forms thioester bonds to activate Atg12 and Atg8, which are required for the formation of LC3II and, thus, the early autophagy phase [53]. Our findings indicate that hypoxia elevates the level of cytoplasmic Ac-FoxO1, which then up-regulates the expression of Atg7 to promote the early autophagy phase, while resistance training decreases the levels of nuclear FoxO1, cytoplasmic Ac-FoxO1and Atg7.

Taken together, our present study revealed that hypoxic exposure induced muscle atrophy that was ameliorated via resistance training. Two primary discoveries have emerged from these findings. First, we found that autophagy played an important role in this process, especially during the early autophagy phase. This finding may help improve or design more effective resistance training methods for athletes or plateau tourists and aid in the development of targeted “sports pills” via various stage-specific autophagy inhibitors. Second, we found that FoxO1 played a novel role in the cytoplasm via acetylation regulation. Additionally, we found that Ac-FoxO1 was involved in the regulation of muscle mass by resistance training and hypoxic exposure. Our findings broaden the understanding of FoxO1 function and provide novel therapeutic targets for the prevention of hypoxia-induced muscle atrophy.

Conclusions

Exposure to hypoxia at a 12.4% oxygen concentration for four weeks induced EDL atrophy in rats, whereas four-week incremental weight-bearing ladder resistance training effectively attenuated this muscle atrophy. This attenuation may be related to nuclear FoxO1/cytoplasmic Ac-FoxO1-induced autophagy, especially during the early autophagy phase.

Abbreviations

ALP: autophagy-lysosome pathway; FoxO1: Forkhead box protein O1; Ac-FoxO1: acetylated FoxO1; LBM: lean body mass; EDL: extensor digitorum longus; FCSA: fiber cross-sectional area; Atg7: autophagy-related gene 7; SQSTM1/p62: Sequestosome 1; Beclin1: Coiled-coil myosin-like BCL2-interacting protein; LC3: microtubule associated protein light chain 3; UPP: ubiquitin-proteasome pathway

Declarations

Acknowledgments

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Ethical approval

Animal care and experimental procedures were approved by the Animal Ethical Committee of Beijing Sport University and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on a reasonable request.

Authors' contributions

All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript. HU Y designed the study and went through the whole project. FU P wrote the main manuscript and analyzed the data. YU J performed the animal trial. ZHU R and JIA J performed the experiments. KONG Z, GONG L, Hans-Christer H and LI Y reviewed the manuscript. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

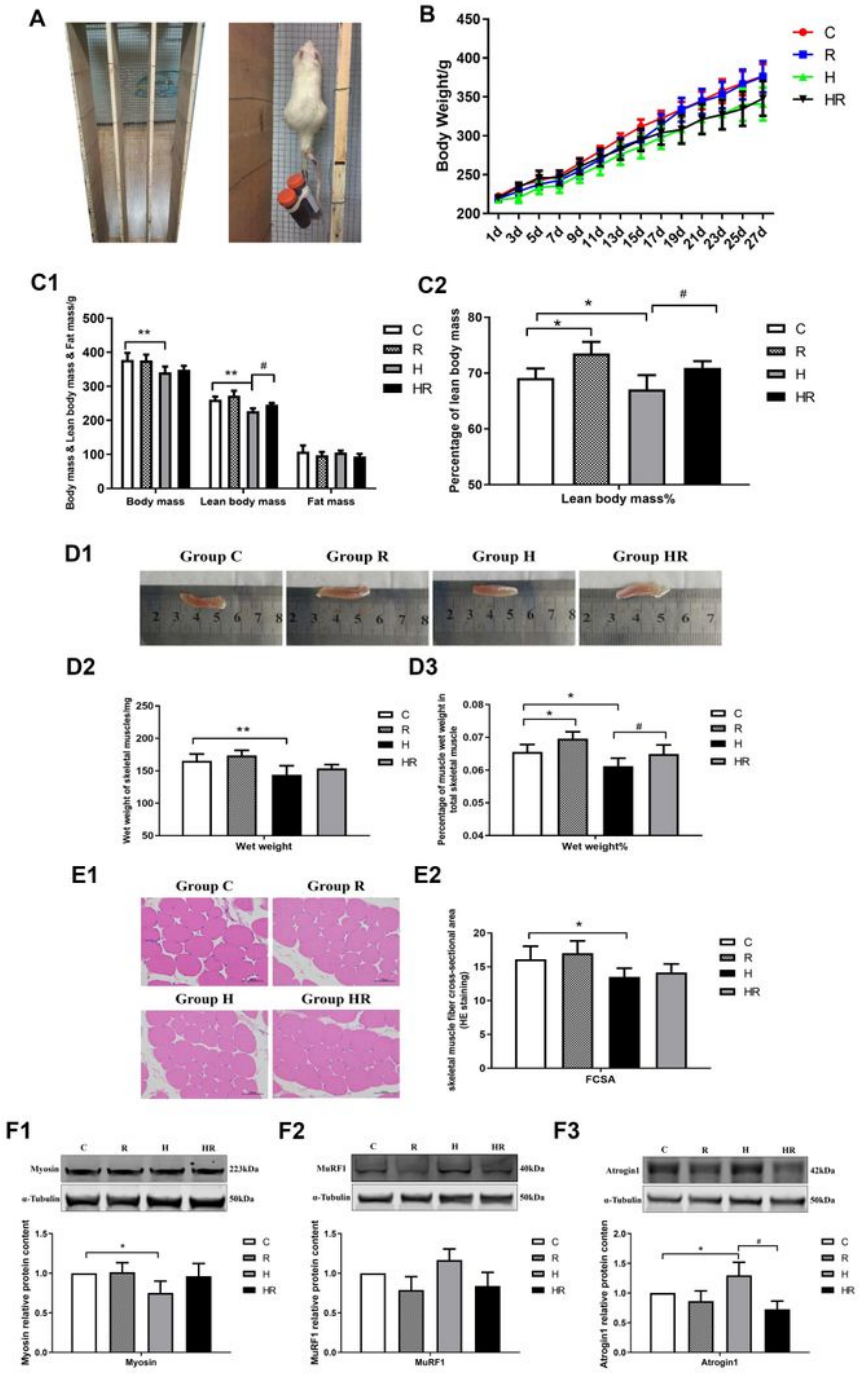

Figure 2

The skeletal muscle morphology and atrophy-related protein expression by the EDL of rats in the different groups. (A) Illustration of the procedure for resistance training; (B) Changes in body weight in each group during the intervention (n=7). (C1) Body, lean body, and fat mass after intervention; (C2) the percentage of lean body mass (LBM / body mass) (n=7); (D1) EDL morphology, (D2) EDL wet weight and (D3) EDL wet weight percentage (EDL wet weight / LBM, EDL%) after the intervention (n=7). (E1) HE staining of muscle

fibers. Nuclei are stained blue. 400-fold magnification (scale bar=50 μm). (E2) The fiber cross-sectional area (FCSA) (n=3). Myosin (F1), MuRF1 (F2), and Atrogin1 (F3) protein levels in the EDL after the intervention (n=7). *P<0.05, **P<0.01 compared with group C; #P<0.05 compared with group H.

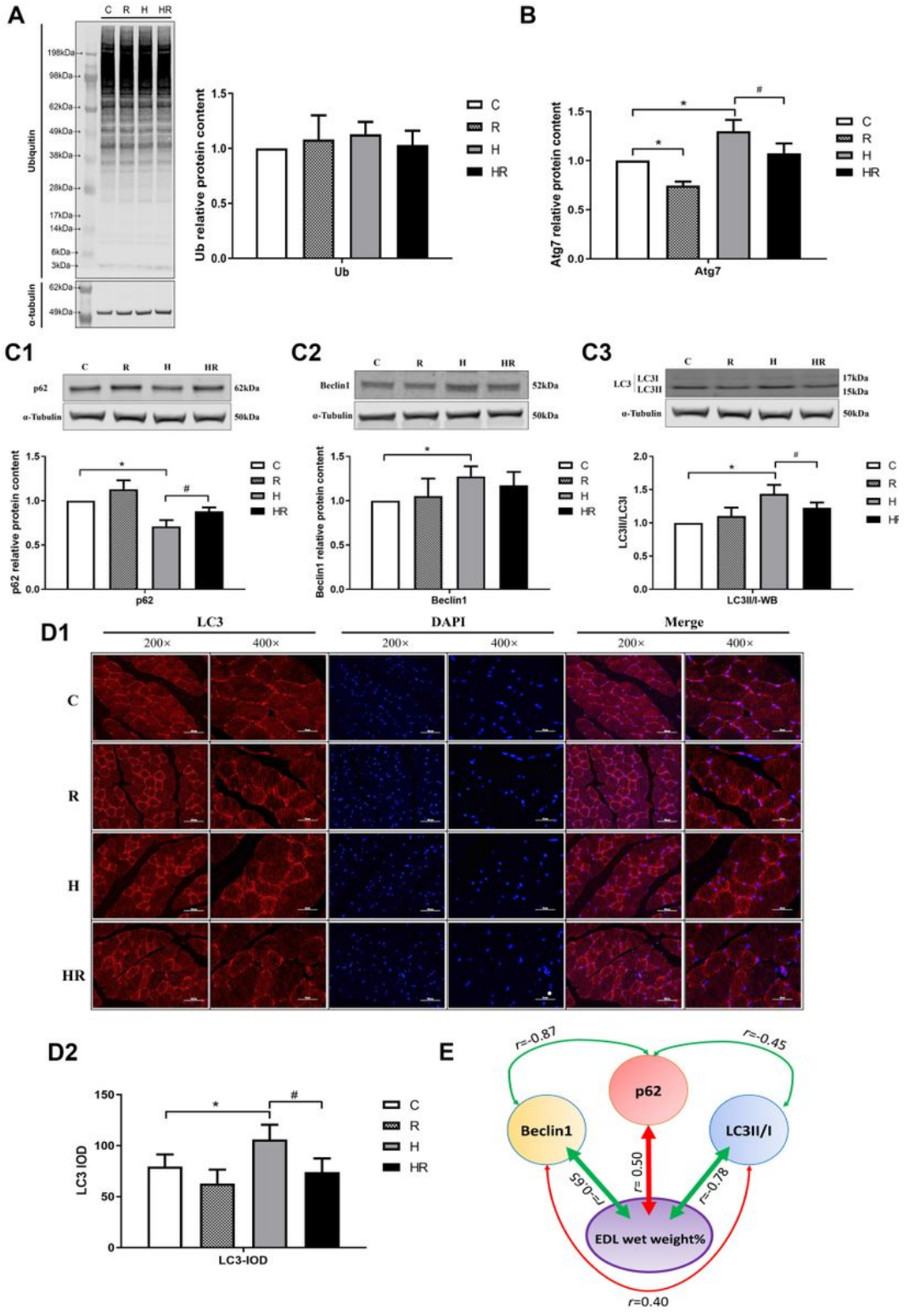

Figure 3

The expression of levels of key autophagy-related proteins by the EDL of rats in the different groups. (A) The level of total ubiquitinylated protein (Ub) (n=3); The levels of Atg7 (B), p62 (C1), Beclin1 (C2), and the

LC3II/LC3I ratio (C3) (n=7); immunofluorescent staining of LC3 (red). × = -fold magnification. Nuclei are stained blue with DAPI: (D1) merged image (n=3) and (D2) IDO value (n=7); (E) Correlations between EDL% and the levels of autophagy-related proteins. “r” is the correlation coefficient; the red arrow indicates a positive correlation and the green arrows negative correlations (n=7). *P<0.05 compared with group C; #P<0.05 compared with group H.

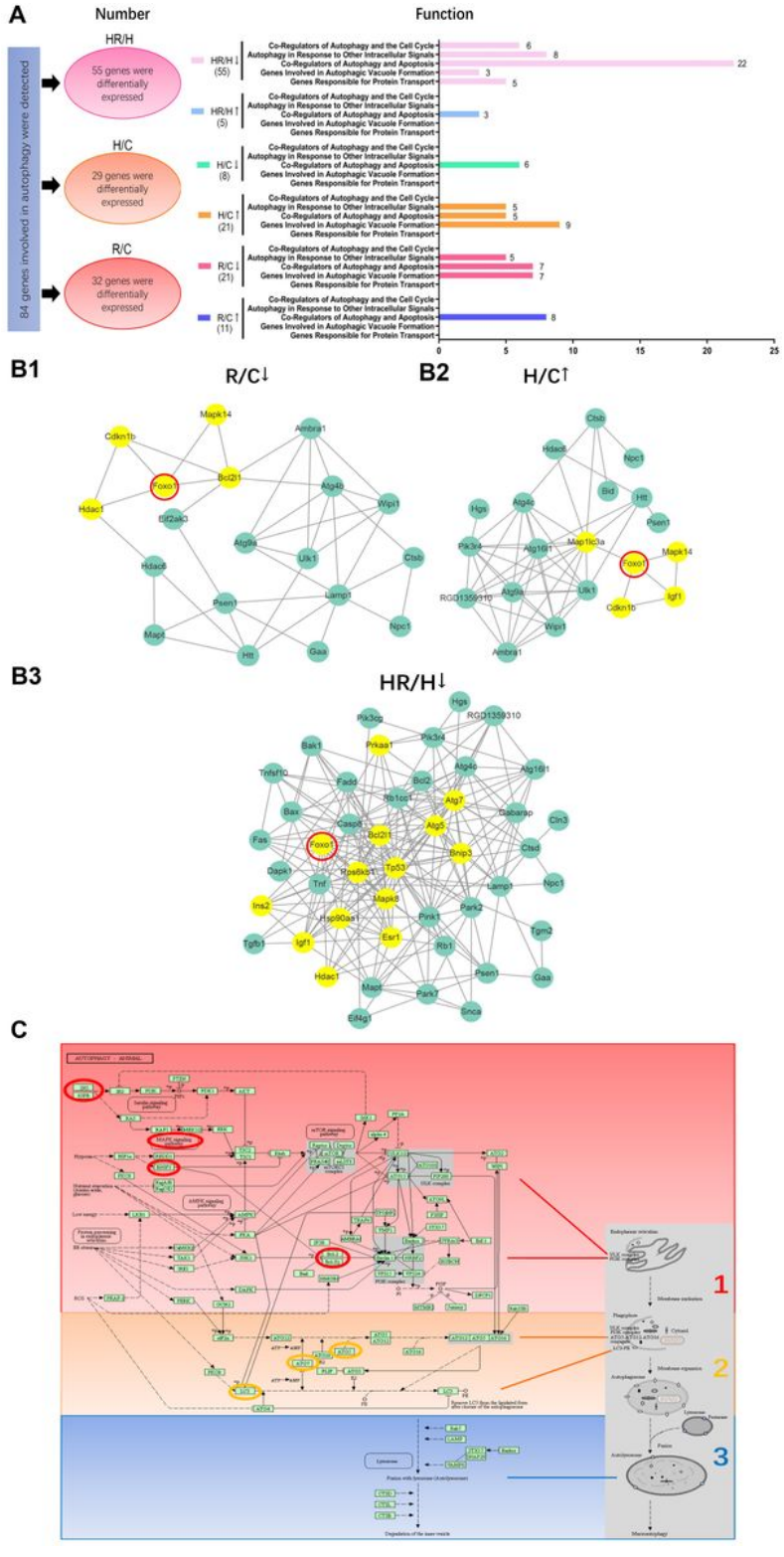

Figure 5

Analysis of the expressed differentially autophagy-related genes (ATGs) in the EDLs of the different groups of rats, together with Interaction and Pathway analysis of these genes and FoxO1. (A) The number of differentially expressed genes of group R/C, group H/C, and group HR/H; the screening criteria were $P<0.05$, and fold-changes ≥ 1.5. The main functions of these genes are also indicated. The interactions between the ATGs down-regulated in groups R/C (B1), up-regulated in H/C (B2), and down-regulated in HR/H (B3). The yellow circles indicate genes regulated directly by FoxO1. (C) Pathway analysis of the expressed differentially ATGs and regulated directly by FoxO1. The red and yellow circles represent the ATGs regulated by FoxO1. The 1, 2, and 3 labels represent the three stages of autophagy.

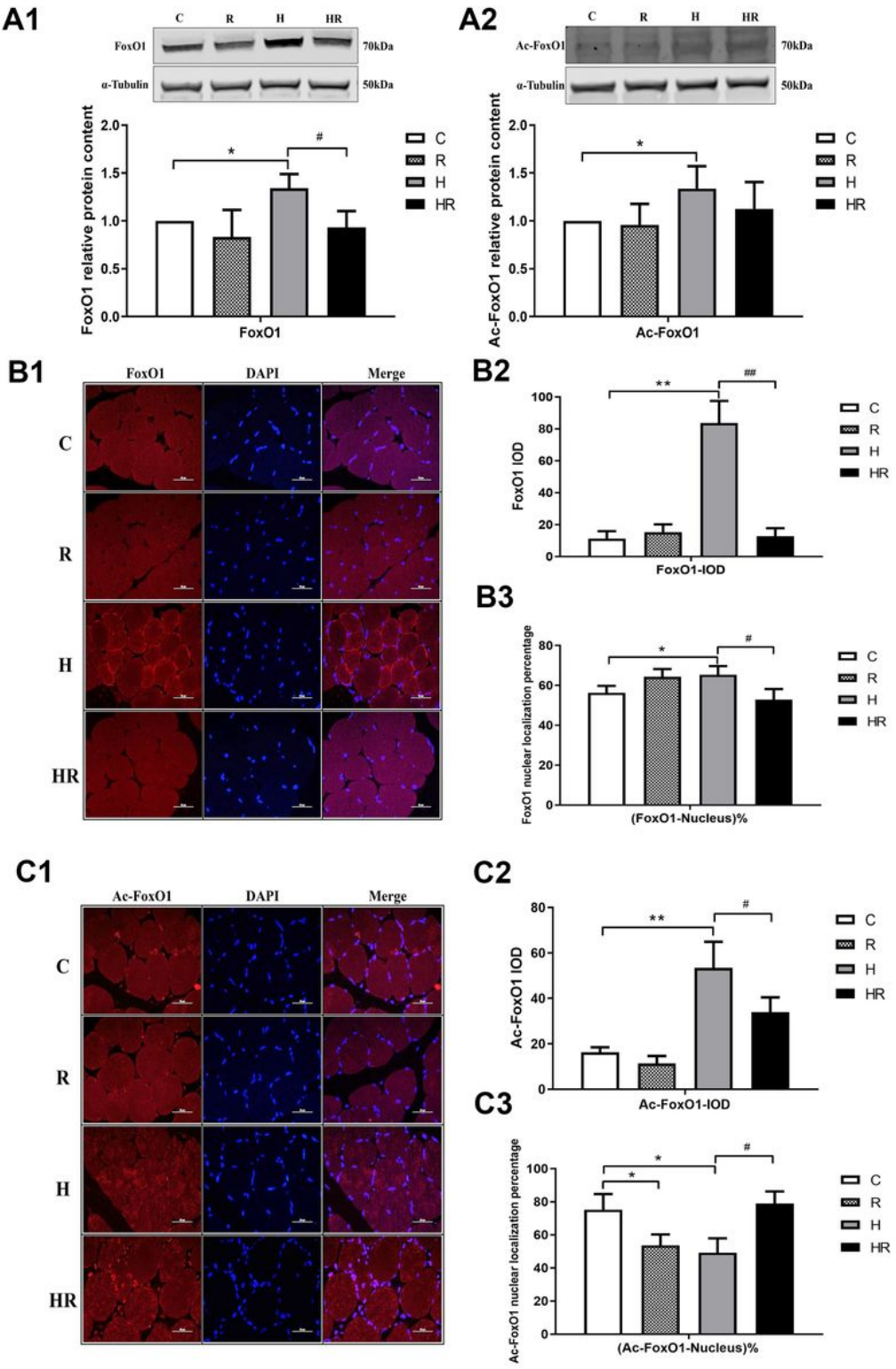

Figure 7

Protein expression and localization of FoxO1 and Ac-FoxO1 in the EDLs of the different groups of rats. Relative levels of FoxO1(A1) and Ac-FoxO1 (A2) (n=7); immunofluorescent staining (red) of FoxO1 (B1) and Ac-FoxO1 (C1). Nuclei are stained blue with DAPI. 400-fold magnification (scale bar =50 μm); IOD of FoxO1 (B2) and Ac-FoxO1 (C2), nuclear localization (percentage) of FoxO1 (B3) and Ac-FoxO1 (C3) (n=3). *P<0.05, **P<0.01 compared with group C; #P <0.05, ##P <0.01 compared with group H.