Fast Twitch Skeletal Muscle Remodeling by Prolonged Endurance Exercise is Associated with Crosstalk between Anabolic and Catabolic Signaling Pathways in Mice

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Abstract

Endurance exercise (EXE) is a potent inducer of both muscle fiber transformation as well as autophagy in skeletal muscles. However, it has remained unknown whether autophagy is associated with EXE-induced muscle fiber transformation. Thus, we examined autophagy responses in fast muscle (tibialis anterior; TA) tissues after six-week long term treadmill EXE by assessing a series of autophagy signaling pathways and muscle fiber transformation through Western blot analysis and fluorescence microscopy. First, we confirmed that EXE caused slow muscle phenotypes in TA muscle, evidenced by reduction in cross-sectional areas of muscle fibers, increases in type I and II fibers, and upregulation of mitochondrial proteins. Subsequently, our results showed that transformation of muscle fiber types concurred with autophagy upregulation (e.g., an increase in LC3-II, LC3-I/I ratio, and BNI3P); however, intriguingly, inductive signaling of autophagy (e.g., phosphorylation of AMPK and BCL2) were suppressed. Moreover, anabolic signaling (e.g., an increase in phosphorylation levels of AKT, mTOR, p70S6K, and FOXO3), which typically serves as anti-autophagy factor were significantly elevated. Our findings suggest that although autophagy levels were sustained higher in TA of EXE-trained mice compared to sedentary mice, concomitant potentiation of anabolic signaling by EXE may serve as a negative feedback to prevent excessive catabolism induced by autophagy. We also suggest that this anabolic response may be necessary for remodeling of anaerobic fast muscle fibers into aerobic muscle fibers and mitochondrial biogenesis.

Keywords: Endurance exercise; Autophagy; Mitochondria; Skeletal muscle; Anabolic signalling; Muscle fiber type

Introduction

Skeletal muscle is a highly dynamic, plastic tissue and thus quickly responds to cellular stimuli such as mechanical stress caused by physical exercise. Chronic endurance exercise has been known to modulate morphological (e.g., reduction in cross-sectional area) and biochemical (e.g., muscle fiber-type shifts from fast muscle fibers to slow muscle fibers and mitochondrial biogenesis) changes. These changes contribute to improvement of muscular endurance and metabolic efficiency [1-6]. A recent study has shown that endurance exercise (EXE)-induced biophysical adaptations in skeletal muscle is associated with protein turnover: the balance between muscle protein synthesis and muscle protein breakdown [7]. Given a study showing that prolonged transition time of intracellular calcium during endurance muscle contraction and relaxation plays an important role in mediating aerobic phenotypes in fast muscle tissues [8,9], calcium-mediated signaling has long been considered as a potential mechanism associated with EXE-mediated muscular adaptation. For example, increased calcium transition during EXE allows calcium-activated protease CALPAIN to cleave structural and contractile proteins, which are removed by an ubiquitin-proteasome system (UPS), resulting in downsizing muscle mass [10]. Aside from the UPS in association with induction of aerobic phenotypes of skeletal muscle [11], autophagy has emerged as a new potential regulator of protein turnover [12].

Autophagy is a catabolic process involved in removal of cellular cargos such proteins, lipids, and small organelles, which requires the several sequential signaling cascades, including initiation of the isolated membrane, nucleation, elongation, maturation, and degradation [13,14]. Autophagy levels are generally determined by changes in the amount of lipidated form of microtubule-associated protein light chain 3-II (LC3-II as well as p62 and BCL2/adenoenovirus E1B 19kDA protein-interacting protein 3 (BNI3P) and other ATG proteins including BECLIN-1, ATG7. To terminate the whole autophagy process, the consecutive interplay between autophagosomes and lysosomes via lysosome-associated membrane protein and CATHEPSINS are required [15,16]. Recent studies have provided strong evidence that EXE induces autophagy in skeletal muscle tissues [17] and play a potential role in skeletal muscle homeostasis [18]. However, the functional role of autophagy in transformation of muscle fiber types and metabolic adaptation in fast muscle tissues in response to EXE remains poorly understood.

Although autophagy plays an important role in maintaining muscular homeostasis, excessive autophagy can result in muscular atrophy and degeneration [19]. Thus, proper operation of autophagy with precise orchestration of numerous molecular interactions is critical. Adenosine monophosphate (AMP)-activated kinase (AMPK), a master regulator of metabolism in many tissues including skeletal muscle is a critical regulator of autophagy under numerous cellular stress conditions [20-25]. Activated AMPK phosphorylates its downstream signaling molecule ULK1 at Ser555 and thus potentiates a class III PI3 kinase to induce autophagy. By contrast, activation of a
mammalian target of rapamycin (mTOR) via anabolic signaling cascade by AKT antagonizes the autophagic process by phosphorylating ULK1 at a different phosphorylation site (Ser757) [26]. Thus, modulation of AMPK and mTOR activation status determines autophagy initiation and abolition. Although a recent study has reported that EXE-induced autophagy is correlated with transformation of muscle phenotypes from fast twitch fiber to slow twitch fibers [27], complex signaling nexus of EXE-induced autophagy pathways (AMPK vs mTOR) and its association with muscle fiber type shifting in fast muscle tissues remain to be elucidated.

In this study, we sought to examine potential relationships between endurance exercise-induced autophagy and muscular phenotype changes in tibialis anterior (TA) muscle and investigate a potential adaptive mechanism of autophagy regulation in response to long-term EXE. Our study showed that EXE-induced autophagy occurs despite the disrupted induction process of autophagy and is associated with aerobic phenotypes in TA muscle. Furthermore, the present study provides potential evidence that enhanced anabolic signaling cascade may be necessary for prohibiting excessive activation of autophagy but for promoting protein synthesis for the conversion of muscle fiber types and mitochondrial biogenesis.

Materials and Methods

Animals

Male C57BL/6 mice (N=28, 8 weeks old) were purchased from ENVIGO company (Tampa, FL, USA) and housed in a temperature-controlled room under a 12 h light:12 h dark cycle with a standard chow diet and water ad libitum. All experiments were conducted according to the Declaration of Helsinki regulations addressing the welfare of experimental animals and were approved by the Institutional Animal Care and Use Committee of the University of West Florida (#2015-002). After one-week of acclimation period, the animals were divided into two groups: sedentary control group (CON, n=14) and endurance exercise group (EXE, n=14).

Endurance exercise protocol

Endurance exercise was performed using a modified motor-driven animal treadmill. The animals assigned to EXE were allowed to be familiarized with treadmill running with slow speed (at 9 meter/minute for 30 min), and then the speed was gradually increased up to 15 meter/minute throughout the one-week acclimation period. After the adaptation, the animals assigned to the EXE group performed treadmill running exercise at 15 meter/minute at 0% incline for 60 min to sedentary control group were exposed to the same degree of environmental stress (e.g., noise from treadmill).

Tissue extraction

The animals were sacrificed by cervical dislocation, and tibialis anterior (TA) muscle was excised right after 90 min of the last session of endurance exercise training to examine the peak autophagy response according to recent studies [12,23]. We chose TA muscle because this muscle consists of mostly fast-twitch fibers (type IIB and IIa) without slow-twitch fiber (type I) in C57BL/6 mice [28], such that adaptive responses of fast muscle fibers to endurance exercise would be optimally observed. Five animals were used for tissue histology and nine animals for biochemical assays for each group. Once animals reached a surgical plane of anesthesia, the TA muscles were excised quickly. Muscle tissues assigned for the histological analysis were embedded with OCT freezing medium and frozen by submerging it in isopentane chilled in liquid nitrogen. Other tissues were stored in cryogenic storage tubes and kept at -80°C until needed for Western blot analysis.

Skeletal muscle fiber-type composition and cross-sectional area

Frozen TA muscles were cross-sectioned at 10 µm in the middle section with a cryostat microtome (LEICA CM 1860, Germany), and the sectioned samples were collected on glass slides. The tissue samples were air-dried for 30 min at room temperature (RT) prior to histological processing. Then, they were permeabilized with 0.5% Triton X-100 mixed with phosphate-buffered saline (PBS) for 30 min at RT, followed by 5 min of washing with PBS. After the wash, each tissue sample was delimated in a circle drawn using ImmEdge™ pen (H-4000, Vector Laboratories, USA). The tissues were blocked with 5% normal goat serum blocking solution (50602Z, ThermoFisher Scientific, USA) for 1 h at RT, followed by wash (3 x 5 min) with PBS. Thereafter, two different Fab blockers (AffiniPure Fab Fragment Goat Anti-Mouse IgG, #115-007-003, Jackson ImmunoResearch, USA and AffiniPure Fab Fragment Goat Anti-Mouse IgM, µ Chain Specific, #115-007-020, Jackson ImmunoResearch, USA) were applied to the tissues to eliminate endogenous IgG and IgM cross-activity. After 1 h incubation at RT, the tissue sections were washed (3 x 5 min) with PBS, and designated primary antibodies, dystrophin (1:100, #PAI-21011, ThermoFisher, USA), myosin heavy chain type I (1:10, #A4.840, Developmental Studies Hybridoma Bank, USA), and myosin heavy chain type IIa (1:25, #SC-71, Developmental Studies Hybridoma Bank, USA) were incubated for 1 h at RT. The tissue sections were then washed with PBS (3 x 5 min), and secondary antibodies (Alexa Fluor 488, 1:50, #A11049, Jackson ImmunoResearch, USA; AMCA 350, 1:50, #A115-155-020, Jackson ImmunoResearch); and Cy3 1:50, #115-165-071, Jackson ImmunoResearch) were applied and incubated for 1 h at RT under dark environment. After washing (3 x 5 min) with PBS, the tissue sections were mounted with VECTASHIELD® Hard +Set Mounting Medium (H-1400, Vector Laboratories, USA) under cover glasses, and sealed with a nail polisher. The tissues’ fluorescence images were visualized and captured using EVOS Auto inverted fluorescence microscope (Life Technologies™, #AMAFD1000) provided with three filters (DAPI, GFP, and RFP).

An analysis of cross-sectional area (CSA) of muscle fibers was conducted in accordance with published references [27]. Briefly, the values of CSA were obtained by measuring circumferences (green-colored dystrophin) of total 60 random muscle fibers acquired from five random fields, using Image J software (NIH, USA). Also, the composition of myosin heavy chain (MHC) isoforms were assessed by counting the number of each muscle fiber: type I (blue), type IIa (red), and type IIx+b (dark).
Detection of autophagy positive muscle fibers

Determination of autophagy-positive muscle fibers was based upon counting the number of fibers displaying accumulation of green fluorescence-tagged LC3 puncta in TA muscle tissues using immunofluorescence microscopy. Muscle tissues were sectioned (10 μm thick) using a cryostat and were fixed with 4% formaldehyde made with PBS (pH 7.4) on the ice for 20 min. The cross-sectioned tissues were washed for 10 min with PBS and permeabilized in 0.3% Triton X-100 with 10 mM sodium citrate solution (pH=6.0) for 20 min at room temperature. After being washed with PBS for 10 min, the tissues were blocked with 5% normal goat-serum for 30 min at room temperature and incubated with LC3 antibodies (Cell signaling, #4108, 1:200) prepared in 5% normal goat-serum in PBS at 4°C overnight. Next day, the tissues were washed with PBS for 15 min and incubated with goat anti-rabbit Alexa Fluor® 488 (Jackson ImmunoResearch, #111-545-144, 1:200) for 30 min at RT. The tissues were washed for 10 min with PBS and incubated with NucBlue® Live Cell Stain Ready Probes™ reagent (Lifetecnologies, #R10477) for 2 min at RT. The tissues were washed for 10 min with PBS and mounted with VECTASHIELD® Mounting medium solution (VECTOR, #H-1400).

Green fluorescence puncta were probed using EVOS Auto fluorescence microscope (Life Technologies™, #AMAFD1000). The percentage of LC3-positive puncta in muscle fibers was calculated.

Western blot analysis

TA muscles were homogenized 1:20 w/v in T-PER® buffer (Thermo Scientific, #78510, Waltham, MA) containing a Halt® Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, #78446, Waltham, MA), using a Polytron™ PT 2500E Homogenizer (Kinematica, #08-451-320, Bohemia, NY). Tissue homogenates were centrifuged at 10,000 x g for 15 min for tissue protein extraction. Protein concentrations were measured using a Pierce™ Coomassie Plus Assay Kit (Thermo Scientific, #23236, Waltham, MA). For Western blot assays, 50 μg of proteins were separated by SDS-PAGE using Bolt® 4-12% Bis-Tris Plus Gels (Invitrogen, #NW04125BOX, Carlsbad, CA) for 1 h at 80 volt and then transferred to PVDF membranes for 1 h at 30 volt in a cold condition. Non-specific proteins were blocked for 60 min at room temperature in a blocking solution (5% BSA in Tris-buffered saline containing 0.1% tween-20), and then the membranes were incubated with designated primary antibodies overnight at 4°C. The antibodies used are as follows: LC3 (#4108, 1:1000), p62 (#5114, 1:1000), BNIP3 (#3769, 1:1000), AMPKa (#2532, 1:1000), p-AMPKa Thr172 (#2535, 1:1000), BECLIN-1 (#3738, 1:1000), ATG7 (#2631, 1:1000), AKT (#9272, 1:1000), p-AKTSer473 (#9271, 1:1000), mTOR (#1222, 1:1000), p-ULK1Ser757 (#14202, 1:1000) from Cell Signaling Technology; LAMP-2 (#PA9-655, 1:1000), DRP1 (#PAI-16987, 1:500), p-DRP1Ser671 (#PA5-37534, 1:500), and OPA1 (#MA5-16149, 1:1000) from ThermoFisher Scientific; CATHEPSIN L (#133641, 1:500), MitoProfile® Total OXPHOS (#110413, 1:1000), MFN2 (#56889, 1:200), and NRF-1(#175932, 1:1000) from Abcam; BCL2 (#7382, 1:500), p-BCL2Ser70 (#377576, 1:500), p70S6K (#8418, 1:500), p-p70S6K (#8416, 1:500), and TFAM(#376672, 1:500) from Santa Cruz Biotechnology; FOXO3a (#NB2-16521, 1:1000), p-FOXO3aSer233 (#NBP1-19927, 1:1000), and PGC-1a (#NBPI-04676, 1:1000) from Novus Biologicals. After the overnight incubation, the membranes were washed three times of 10 min with 1X TBS-T and then incubated with designated secondary antibodies conjugated with HRP for 1 h at room temperature: goat anti-rabbit (#1148960, 1:4000), rabbit anti-goat (#811620, 1:2000) from ThermoFisher Scientific; goat anti-mouse (#2005, 1:5000) from Santa Cruz Biotechnology. Then, the membranes were washed 3 times of 10 min in 1X TBS-T, after which immunoreactivity was detected with SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific, #34076, Waltham, MA). The digital images were acquired using ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA), and band intensities were quantified by using Image Lab™ software version 5.2.1 (Bio-Rad, Hercules, CA). The digital intensity of all targeted protein images was normalized by the intensity of Ponceau (#P7170, Sigma-Aldrich, St. Louis, MO)-stained proteins on the same membrane to verify equal loading and quality of transfer.

Statistical analysis

All data were analyzed using the SPSS 22.0 software package (SPSS, Chicago, IL, USA). Values in the table and bar graphs were expressed as means ± SEM and fold changes, respectively. Independent, unpaired t-test was performed to examine the significant difference between two groups (control vs. exercise group). A statistical significance level was established at p<0.05.

Results

Endurance exercise elicits slow muscle phenotypes in TA muscles

To examine whether long-term endurance exercise (EXE) induces aerobic morphological adaptation in TA muscle tissues, a ratio of TA muscle mass to body weight and muscle mass (wet weight) were measured. As presented in (Table 1). TA muscle weight was significantly decreased in EXE (93.9 ± 1.61 g) group compared with CON group (101.7 ± 3.06 g), without significant changes in body weight and relative muscle mass between groups.

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<thead>
<tr>
<th></th>
<th>CON</th>
<th>EXE</th>
<th>P</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>29.6 ± 0.87</td>
<td>27.9 ± 0.74</td>
<td>0.079</td>
</tr>
<tr>
<td>TA muscle mass (mg)</td>
<td>101.7 ± 3.06</td>
<td>93.9 ± 1.61</td>
<td>0.021*</td>
</tr>
<tr>
<td>TA muscle mass/ body weight (mg/g)</td>
<td>3.46 ± 0.14</td>
<td>3.38 ± 0.09</td>
<td>0.329</td>
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Table 1: Effects of six weeks of endurance exercise in changes of body weight, muscle mass of TA, and a ratio of muscle mass of TA to body weight. Values are expressed as a mean ± SEM. * indicates significant difference compared to CON at p<0.05.

Next, we assessed whether EXE alters the cross-sectional area (CSA) of individual muscle fiber and induces a shift of fiber types. Data from immunofluorescence microscopy showed that the CSA of TA muscle fibers after EXE was significantly diminished compared with CON group (Figures 1a-1c). In addition, EXE acquired highly oxidative slow fibers (type IIa, red), whereas the number of highly intermediate fibers (type IIx and IIb, black) was significantly reduced (Figures 1d-1f).
Endurance exercise sustains high levels of autophagy in TA muscle despite attenuated AMPK activities

Since we observed a significant reduction in muscle mass, and autophagy is a potential catabolic process, we explored a question of whether exercise-induced autophagy is associated with the reduced fiber cross-sectional area. We assessed a key autophagy index protein LC3-II and found that EXE significantly increased LC3-II (Figures 2a and 2b) as well as the LC3-II to I ratio (Figures 2a and 2c) in EXE group compared to CON group. To confirm if the increase in LC3-II is due to an enhanced autophagy flux, we next measured levels of p62 since it has been reported to be a marker for autophagy flux. Interestingly, p62 levels were not changed (Figures 2a and 2d) but a potent autophagy inducer, BNIP3, was significantly elevated in the EXE group, compared with CON group (Figures 2a and 2e).

Given that AMPK activity has been suggested to regulate induction of autophagy, we next measured the levels of phosphorylated AMPK. Our data showed that phosphorylation levels of AMPK were remarkably suppressed in response to exercise compared with those in CON group (Figures 2d and 2f).

Endurance exercise interferes in autophagy induction processes without modulating lysosomal proteins in TA muscle

Since dissociation of BECLIN-1 from a BCL2-BECLIN-1 complex upon phosphorylation of BCL2 is an upstream signaling process for the induction of autophagy [12], we measured levels of phosphorylated BCL2 at serine 87 (p-BCL2 <sub>Ser87</sub>), total BCL2, and BECLIN-1. Interestingly, both p-BCL2 <sub>Ser87</sub> and BCL2 levels were diminished, resulting in significant reduction in p-BCL2 <sub>Ser87</sub>/BCL2 ratio in the absence of BECLIN-1 modulation in the EXE group (Figures 3a-3c). Moreover, ATG 7, a rate limiting enzyme involved in autophagy induction was repressed in response to EXE (Figures 3a and 3d). Next, we measured lysosome associated membrane protein-2 (LAMP-2) and CATHEPSIN L, both of which are linked to lysosomal fusion with autophagosomes and final degradation, respectively and found no significant changes after EXE (Figures 3a and 3e-3g).
Endurance exercise promotes anabolic activities in TA muscle

Elevated anabolic signaling pathways have been known to interfere in autophagy induction; for example, mTOR-induced phosphorylation of Unc-51 Like Kinase 1 (ULK1) prohibits class III PI3K activation and results in interrupting autophagy induction. To examine if EXE modulates anabolic signaling paradigms that may regulate activation or deactivation of autophagy, we assessed a series of canonical anabolic signaling molecules (e.g., AKT-mTOR-p70S6K axis). Our data revealed that phosphorylation levels of AKT\(^{\text{Ser473}}\), mTOR\(^{\text{Ser2448}}\), and p70S6K\(^{\text{Ser411}}\) (Figures 4a-d) were significantly elevated in response to EXE. Furthermore, phosphorylation levels of ULK1\(^{\text{Ser757}}\) by mTOR were increased (Figures 4a and 4e). We also observed that FOXO3, a key transcription factor associated with catabolism and autophagy, was hyperphosphorylated (inactivated) in response to exercise (Figures 4a and 4f).

Endurance exercise induces mitochondrial biogenesis in TA muscle

To ascertain whether exercise-induced morphological modification (e.g., muscle mass reduction and fiber type shifts) is associated with mitochondrial biogenesis as well as mitochondrial turnover, we examined changes in five key mitochondrial proteins (complex I through IV), except complex V, were significantly upregulated (Figures 5a and 5b). We next examined key mitochondrial biogenesis-related transcription factors. Intriguingly, neither peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) nor mitochondrial transcription factor A (TFAM) were changed; however, levels of nuclear respiratory factor-1 (NRF-1), another key transcription factor associated with mitochondrial
mitochondrial biogenesis, as evidenced by upregulated mitochondrial proteins (e.g., electron transport chain: complex I-IV). Generally, mitochondrial biogenesis emerges with upregulation of key transcription factors such as PGC-1α, NRF1, and TFAM [39,40]; interestingly, however, we did not observe upregulation of those proteins except for NRF1. Although it is not clear why upregulation of PGC-1α and TFAM are not observed in the current study, a recent study provides evidence that mitochondrial biogenesis can occur independent of PGC-1α [41]. It can be also presumed that duration of total training (e.g., acute vs. short-term vs. long-term) may affect the levels of those transcription factors; for example, if EXE-induced aerobic adaptation of muscle tissues reaches the pinnacle of mitochondrial biogenesis, transcription factors for mitochondrial biogenesis will return to basal levels to maintain cellular homeostasis. This may be one of possible explanations for the results of our study. Further studies are needed to examine the exact time periods of adaptive responses of mitochondrial biogenesis and homeostatic regulatory functions of key transcription factors of mitochondrial biogenesis.

Mitochondrial turnover (e.g., fusion and fission) is a critical element that is linked to mitochondrial biogenesis and elimination [42,43]. Although it is still unclear whether fission or fusion or both of them are linked to exercise-induced mitochondrial biogenesis, our findings, showing upregulation of mitochondrial fission protein DRP1, with fusion proteins OPA1 and MFN2 remaining unchanged, suggest that mitochondrial fission may be necessary for the biogenesis of mitochondria. In line with our data, recent studies also have revealed that endurance exercise enhances DRP1 expression without the alteration of MFN2 levels [30,42,44,45]. Nevertheless, given that mitochondria are highly dynamic organelles, and frequently undergoing fusion and fission processes in various stress conditions, our results (DRP1 upregulation) may not describe the entire adaptive outcomes of mitochondrial biogenesis in response to long-term EXE.

In conclusion, our findings support the general notion that long-term EXE is a key stimulator of transformation of muscular phenotypes: shifts from slow to fast muscle fibers, reduction in cross-sectional area, and mitochondrial biogenesis in fast muscle tissues. Importantly, our results reveal that while long-term EXE retains increased autophagy (an increased in LC3-II and BNIIP3 protein levels), autophagy induction is suppressed in parallel with enhanced anabolic signaling cascades (AKT-mTOR-p70S6K). This improved anabolic signaling cascade is associated with upregulation of mitochondrial biogenesis, which concurs with an increase in mitochondrial fission protein DRP1 in the absence of modulation of fusion proteins. Consequently, our findings provide an important insight into an adaptive mechanism of EXE-mediated autophagy in fast muscle tissues, supposing an anabolic signaling cascade as a possible feedback system necessary for guiding the suitable rate of autophagy in response to long-term EXE.

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References


