Aerobic exercise training upregulates skeletal muscle calpain and ubiquitin-proteasome systems in healthy mice

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Cunha TF, Moreira JB, Paixão NA, Campos JC, Monteiro AW, Bacurau AV, Bueno CR Jr., Ferreira JC, Brum PC. Aerobic exercise training upregulates skeletal muscle calpain and ubiquitin-proteasome systems in healthy mice. J Appl Physiol 112: 1839–1846, 2012. First published March 29, 2012; doi:10.1152/japplphysiol.00346.2011.—Aerobic exercise training (AET) is an important mechanical stimulus that modulates skeletal muscle protein turnover, leading to structural rearrangement. Since the ubiquitin-proteasome system (UPS) and calpain system are major proteolytic pathways involved in protein turnover, we aimed to investigate the effects of intensity-controlled AET on the skeletal muscle UPS and calpain system and their association to training-induced structural adaptations. Long-lasting effects of AET were studied in C57BL/6J mice after 2 or 8 wk of AET. Plantaris cross-sectional area (CSA) and capillarization were assessed by myosin ATPase staining. mRNA and protein expression levels of main components of the UPS and calpain system were evaluated in plantaris by real-time PCR and Western immunoblotting, respectively. No proteolytic system activation was observed after 2 wk of AET. Eight weeks of AET resulted in improved running capacity, plantaris capillarization, and CSA. Muscle RING finger-1 mRNA expression was increased in 8-wk-trained mice. Accordingly, elevated 26S proteasome activity was observed in the 8-wk-trained group, without accumulation of ubiquitinated or carbonylated proteins. In addition, calpain abundance was increased by 8 wk of AET; whereas no difference was observed in its endogenous inhibitor calpastatin. Taken together, our findings indicate that skeletal muscle enhancements, as evidenced by increased running capacity, plantaris capillarization, and CSA, occurred in spite of the upregulated UPS and calpain system, suggesting that overactivation of skeletal muscle proteolytic systems is not restricted to atrophying states. Our data provide evidence for the contribution of the UPS and calpain system to metabolic turnover of myofibrillar proteins and skeletal muscle adaptations to AET. 

proteolysis; cross-sectional area; calpastatin; skeletal muscle plasticity

OVER THE LAST DECADES, the contribution of proteolytic pathways to skeletal muscle plasticity upon different stimuli has been widely investigated (13, 14, 24). Skeletal muscle calpains and the ubiquitin-proteasome system (UPS) are major intracellular proteolytic systems known to be affected by exercise (28, 30, 32, 39, 42, 43). The UPS is responsible for most of intracellular protein degradation (>80%), including sarcromeric proteins (7, 20); however, the proteasome could not degrade intact myofibrillar proteins until they are disassembled and removed from the myofibril (36). In turn, the calpain system, which is composed of several Ca2+-dependent pro-
teases (i.e., the calpains) and their endogenous inhibitor calpastatin, is responsible for the degradation of proteins involved in keeping those myofibrillar proteins properly assembled into myofibrils (17), making them available for degradation by the proteasome. Thus strong evidence suggests that orchestrated function of these systems is an important step in the process of metabolic turnover of myofibrillar proteins (12, 16, 28), calling for further investigation of its contribution to skeletal muscle adaptation after mechanical interventions.

Among the variety of stimuli able to induce skeletal muscle proteolytic systems adaptation, mechanical interventions must be highlighted, since reductions in muscle use commonly lead to myofiber atrophy by increasing muscle protein degradation over synthesis (11). The process of protein breakdown in atrophying states has been widely investigated, resulting in the identification of important roles played by the calpains and the UPS under such conditions (34, 35). In contrast, skeletal muscle growth in response to mechanical stimuli is known to require sarcomeric rearrangement and protein turnover (1, 15, 33), which may also rely on significant activity of both the calpains and the UPS.

It is well established that resistance training promotes skeletal muscle hypertrophy by increasing protein synthesis to a greater extent than degradation, resulting in larger and stronger fibers (33). Aerobic exercise is also recognized as an important stimulus, able to promote skeletal muscle enhancements (9); however, little is known about the role played by intracellular proteolytic systems on skeletal muscle protein turnover in response to aerobic exercise training (AET). In addition, the knowledge regarding the effects of AET on skeletal muscle trophy is not been clarified since controversial findings has been reported (22, 40).

The aim of the present study was to assess the time course of activation of proteolytic pathways (calpain and UPS) in response to exercise, as well as to AET, in skeletal muscle of healthy C57BL/6J mice.

EXPERIMENTAL PROCEDURES

Study Population

A cohort of C57BL/6J mice was housed five/cage in a temperature-controlled room (21°C) with a 12:12-h dark-light cycle with free access to standard laboratory chow (Nuvital Nutrientes S/A, Brazil) and tap water. All animal experimental procedures were according to ethical principles in animal research, as adopted by the Brazilian College of Animal Experimentation (www.cobea.org.br) and were approved by the Ethical Committee of the University of São Paulo (2007/28).
Time course of acute effects of exercise on skeletal muscle proteolytic systems. A subset of 5-mo-old male C57BL/6J mice was assigned randomly into one of the following groups: 1) untrained (UNTR), 2) trained for 2 wk, or 3) trained for 8 wk (n = 10/group). All mice were killed at the same age (7 mo old). Mice, submitted to 2 wk of AET, remained sedentary during the 6 wk before AET started, and the UNTR group remained sedentary during the 8 wk of protocol. All animals were killed 48 h after the last exercise session.

Cardiovascular Measurements

Resting heart rate (HR) was determined noninvasively using a computerized tail-cuff system (BP-2000, Visitech Systems, Apex, NC). Mice were acclimatized to the apparatus in daily sessions over 5 days before the measurements.

Treadmill Exercise Test

Animals allocated into the training study were submitted to graded exercise testing on a motor treadmill adapted to experimental models before and after the experimental period (2 or 8 wk of training). After being adapted to treadmill exercises for over 1 wk (10 min/day), mice were placed in the treadmill and allowed to acclimatize for at least 30 min. Treadmill speed started at 6 m/min and was increased by 3 m/min every 3 min at 0% grade until exhaustion, where mice could no longer maintain running speed for over 3 min.

Maximum Lactate Steady-state Workload Determination

Maximum lactate steady-state workload (MLSSw), a reliable marker of endurance capacity, was determined according to a previous study of our group (10). Eight-week-trained and UNTR mice were submitted to subsequent constant-load tests, performed with workload intensities varying from 9 to 21 m/min. The highest workload that could be maintained over 28 min of running without continual blood lactate accumulation (blood lactate varying by <1 mmol/l from 7 to 28 min) was considered as MLSSw. A single constant-load test was performed in the 8-wk-trained group after 4 wk of exercise training to adjust training intensity. All UNTR mice were exposed to treadmill exercise during 5 min, three times/wk, to become accustomed to exercise protocol and handling. Efficacy of training protocol was demonstrated by the distance covered in the graded treadmill exercise test and by the occurrence of resting bradycardia (10).

Skeletal Muscle Cross-sectional Area and Capillary to Fiber Ratio

Plantaris muscle was harvested, snap frozen in melting isopentane at L0 length, and stored in liquid nitrogen. Plantaris muscle was used due to the high prevalence of type II fibers, known to display a greater response to mechanical overload than type I fibers. Frozen muscles were cut into 10 μm-thick sections through the proximal-to-distal region using a cryostat (Cryostat Microm HM505E, Microm International GmbH, Walldorf, Germany). Muscle sections were then incubated for myofibrillar ATPase activity after alkali (pH 10.3) preincubation. Whole muscle cross-sectional area (CSA) was evaluated at ×200 magnification and analyzed further on a digitalizing unit connected to a computer (Image-Pro Plus, Media Cybernetics, Bethesda, MD). Skeletal muscle CSA was normalized to body mass. The same histological preparation was used for evaluation of capillary:fiber ratio, which was quantified in five nonoverlapping fields at 400× magnification. The total number of capillaries was divided by the total number of fibers counted in the same field. Only vessels with a diameter <10 μm were counted, which would largely be comprised of capillaries but may also include terminal arterioles or venules. All analyses were conducted by a single observer (A.V.N.B.), blinded to the mice’s identity.

Real-time PCR

RNA was isolated from plantaris muscle with Trizol reagent (Invitrogen, Carlsbad, CA). After assessment of RNA integrity (electrophoresis in agarose gels, followed by staining with ethidium bromide), purity (260:280 nm ratio), and concentration (ng/μl), we calculated RNA sample volume to obtain a final concentration of 50 ng/μl. cDNA was synthesized using oligo dT (50 μM) and SuperScript III RNase H- (Invitrogen) at 42°C for 50 min, and real-time PCR was performed. The primers used for gene amplification were: atrogin-1/muscle atrophy F-box (MAFbx) sense, 5'-TATCTAAGAGGCCCATGGAATCT-3', atrogin-1/Mafbx antisense, 5'-GTGGATCTCTTGGAAATCCAGGAT-3'; muscle RING finger-1 (MuRF1) sense, 5'-GGTGGAAGGGTCTTCTTGATCT-3', MuRF1 antisense, 5'-ACATCGCTCCTCCCTCCT-3'; cyclin B1 sense, 5'-AAATCTGGGACAAACACAAAC-3', cyclin B1 antisense, 5'-CC-TTCTTTACCTTCCAAAG-3'. Real-time PCR for target genes and endogenous reference gene cyclophilin were run separately, and amplifications were performed with an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) by using SYBR Green PCR Master Mix (Applied Biosystems). Specificity was tested by melting curve analysis. Results were expressed using the comparative cycle threshold (Ct) method, as described by the manufacturer. The ΔCt values were calculated in every sample for each gene of interest as Ctgene of interest – Ctcontrol gene. The calculation of the relative changes in the expression level of one specific gene (ΔΔCt) was performed by subtraction of the ΔCt from the control group (“Rest” group was used as a calibrator for acute exercise protocol; UNTR group was used as a calibrator for AET protocol) to the corresponding ΔCt from the acute exercise groups (at 0, 24, and 48 h) and AET groups (at 2 and 8 wk). The values and ranges given (see Figs. 1, A and B, and 2, A and B) were determined as follows: 2^-ΔΔCt with ΔΔCt. The control levels were arbitrarily set to 1.

Western Blot Analysis

20S proteasome, polyubiquitinated proteins, calpain, calpastatin, and carbonylated protein abundance were evaluated by Western

Study Design

Time course of acute effects of exercise on skeletal muscle proteolytic systems. A subset of 7-mo-old male C57BL/6J mice was assigned randomly into a rest or exercise protocol (exercise protocol is described below). Exercised mice were killed 0, 24, or 48 h after the exercise session (n = 15/group) and were compared with the resting control group (n = 10). Exercise consisted of a single session of maximal running on a treadmill. Treadmill speed started at 6 m/min and was increased by 3 m/min every 3 min at 0% grade until exhaustion, where mice could no longer maintain running speed for over 3 min.

Tissue and Biochemical Measurements

Blood lactate concentration was analyzed using an electroenzymatic method with a lactate analyzer (YSI 2300 STAT analyzer, Yellow Springs Instruments, Yellow Springs, OH).

Aerobic Exercise Training

Exercise training was performed on a motor treadmill, 5 days/wk, during 2 or 8 wk (10). Running speed and duration of exercise sessions were progressively increased to elicit MLSSw (~60% of maximal speed achieved during a graded treadmill exercise protocol) for 60 min at the 4th wk. An additional incremental load test was performed. 20S proteasome, polyubiquitinated proteins, calpain, calpastatin, and carbonylated protein abundance were evaluated by Western

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immunoblotting in total plantaris muscle extracts of UNTR and trained mice. Frozen muscles were homogenized in a buffer containing 1 mM EDTA, 1 mM EGTA, 2 mM MgCl₂, 5 mM KCl, 25 mM HEPES, pH 7.5, 100 µM PMSF, 2 mM DTT, 1% Triton X-100, and protease inhibitor cocktail (1:100; from Sigma-Aldrich, St. Louis, MO). Centrifugation was performed for 15 min at 10,000 g and 4°C, pellet was discarded, and supernatant (cytosolic proteins) was used. Samples were subjected to SDS-PAGE (6–15%), depending on protein molecular weight. After electrophoresis, proteins were electrotransferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Equal loading of samples and transfer efficiency were monitored with the use of 0.5% Ponceau S staining of the blot membrane. The blotted membrane was then blocked (5% BSA, 10 mM Tris-HCl, at pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature and then incubated overnight at 4°C with specific antibodies against 20S proteasome (α5/α7, β1, β5, β7 subunits; Abcam, Cambridge, MA; #ab22673), polyubiquitinated proteins (Biomol, Enzo Life Sciences, Farmingdale, NY; #BML-PW0930), calpain (52 subunit; Abcam; #ab28241-100), calpastatin (Abcam; #ab28252-100), and GAPDH (Advanced ImmunoChemical, Long Beach, CA; #2RGM2). Protein carbonylation was assessed by measuring the levels of carbonyl groups using the OxyBlot protein oxidation detection kit (Millipore, Billerica, MA; #7150). Carbonyl groups at the protein side-chains were derivatized from 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine. Derivatized protein samples were subjected to SDS-PAGE and electrotransferred to nitrocellulose membranes following the manufacturer’s instructions. Binding of the primary antibody was detected with the use of peroxidase-conjugated secondary antibodies (rabbit or mouse, depending on the protein, for 2 h at room temperature) and developed using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), detected by autoradiography. Quantification analysis of blots was performed with the use of Scion Image software (Scion based on NIH Image). Samples were normalized to relative changes in GAPDH and expressed as percentage of the UNTR group.

26S Proteasome Activity Assay

Chymotrypsin-like activity of the proteasome was assayed using the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-MCA; Biomol, Enzo Life Sciences). Assays were carried out in a microtiter plate by diluting 25 µg cytosolic protein into 200 µl 10 mM MOPS, pH 7.4, containing 25 µM LLVY-MCA, 2.5 mM ATP, and 5.0 mM MgCl₂. The rate of fluorescent product formation was measured with excitation and emission wavelengths of 350 and 440 nm, respectively. Peptidase activities were measured in the absence and presence (20 µM) of the proteasome inhibitor epoxomicin, and the difference between the two rates was attributed to the proteasome. Proteasome activity was linear for 30 min under the conditions of the assays (6).

Statistical Analysis

Data are presented as mean ± SE. Shapiro-Wilk normality test was used to verify data normal distribution. Unpaired Student’s t-test was used to compare the physiological effects of exercise training between UNTR and 8-wk-trained groups. For evaluation of acute effects of exercise and long-term effects of AET on skeletal muscle proteolytic systems, we used one-way ANOVA, followed by Duncan post hoc test. Statistical significance was considered achieved when the value of P was <0.05.

RESULTS

Time Course of Acute Effects of Exercise on Skeletal Muscle Proteolytic Systems

To evaluate the time-dependent effects of a single exercise session on skeletal muscle proteolytic systems, we measured MuRF1 and atrogin-1/MAFbx mRNA levels, proteasomal activity, and calpain and calpastatin protein levels in plantaris muscle of resting or exercised animals, killed after 0, 24, or 48 h, after a bout of running exercise. As shown in Fig. 1, MuRF1 mRNA levels, proteasome activity, and calpain and calpastatin protein levels were increased significantly, immediately after the exercise session. Twenty-four hours after the session, MuRF-1 mRNA levels and calpain levels returned to normal values, measured under resting conditions. However, the proteasomal activity remained high. In a very interesting and similar fashion, levels of all components were similar to between the resting group and the group killed 48 h after an exercise session.

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Fig. 1. Time course of proteolytic systems activation after a single bout of exercise. A: muscle RING finger-1 (MuRF1) and B: atrogin-1/muscle atrophy F-box (MAFbx) mRNA levels. C: 26S proteasomal activity. D: calpain and E: calpastatin protein levels, and F: representative blots of plantaris muscle in resting and exercised C57BL/6J mice, killed at different time points; n = 10–12/group. Data are presented as mean ± SE and were analyzed by 1-way ANOVA, followed by Duncan post hoc test. *P < 0.05 from “Rest” group; #P < 0.05 from “0 h” group; &P < 0.05 from “24 h” group.

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Time Course of AET Effects on Skeletal Muscle Proteolytic Systems

To evaluate the time-dependent effects of AET on skeletal muscle proteolytic systems, we measured MuRF1 and atrogin-1/MAFbx mRNA levels, proteasomal activity, and calpain and calpastatin protein levels in plantaris muscle of UNTR, 2-wk-trained, and 8-wk-trained mice. We observed increased MuRF1 mRNA levels, proteasomal activity, and calpain protein abundance in 8-wk-trained mice (Fig. 2, A, C, and D). On the other hand, data shown in Fig. 2C demonstrate that skeletal muscle proteasomal activity was reduced in 2-wk-trained mice. Proteasomal subunit expression was not changed, indicating that the proteasomal activation in the 8-wk-trained mice was probably due to post-translational modifications of the protease (Fig. 3A). Polyubiquitinated protein levels were evaluated to verify the effects of exercise training on skeletal muscle protein labeling for degradation. Of interest, polyubiquitinated protein abundance was not different between 8-wk-trained mice and UNTR counterparts (Fig. 3B), and protein carbonylation trended to be reduced by AET (P = 0.07; Fig. 3C).

Effects of Exercise Training on Running Performance and Plantaris Muscle Phenotype

To study the effect of exercise training on running performance, we evaluated total distance run achieved in a graded treadmill exercise test and MLSSw during subsequent constant-load tests. Eight-week-trained mice presented higher total distance run and MLSSw than UNTR ones (Table 1), confirming enhanced endurance performance after 8 wk of AET. Trained mice also displayed significant resting bradycardia (Table 1). Body weight, distance run, and resting HR were similar between groups before experimental protocol (data not shown). Interestingly, histological analysis showed that 8 wk of AET increased plantaris muscle CSA (Fig. 4A) and capillarization (Fig. 4B). Representative histological pictures are presented below the charts (Fig. 4C).

DISCUSSION

In this study, we evaluated the time course of activation of skeletal muscle proteolytic systems (UPS and calpain system) by a single exercise bout, as well as the long-term response to AET at distinct endpoints (2 or 8 wk of protocol) in healthy mice. Our data on the acute effects of exercise demonstrate that both systems were activated immediately after the exercise bout, which was no longer observed in the group studied 48 h after the exercise session. Moreover, we also show that 8 wk of moderate-intensity AET promoted long-lasting activation of the UPS and calpain system, which was not observed in mice submitted to only 2 wk of training, suggesting that observed effects occurred due to the long-term intervention (i.e., 8 wk of AET).

Mammalian skeletal muscle contains four proteolytic systems that could be involved in protein turnover: the lysosomes, the calpains, the caspase system, and the UPS. The contribution of lysosomal proteases to metabolic turnover of myofibrillar proteins is probably small, since low pH is required for optimal activity, and engulfment of myofibrils would be necessary. It is also unlikely that the caspase system displays significant activity in normal-functioning muscle cells, since the caspases are activated by events that initiate apoptosis. Therefore, the calpain and the UPS are likely to be the main proteolytic pathways involved in metabolic turnover of myofibrillar proteins in such a way that calpains initiate the process by disassembling and releasing myofilaments, which would be later degraded to small peptides or amino acids by the UPS (3, 16, 21, 27).

Studies in human and animal models demonstrated that alterations in skeletal muscle loading by space flight, limb suspension, and acute bouts of resistance or aerobic exercise may exert effects in both calpains and the UPS (4, 19, 29, 44); however, the effects of chronic aerobic exercise (i.e., AET) on calpains and UPS have not been addressed yet. In this context, our study is the first to provide insights on the effects of intensity-controlled AET in skeletal muscle calpains and UPS, which play a crucial role in skeletal muscle protein turnover.

Fig. 2. Time course of aerobic exercise training (AET)-induced activation of skeletal muscle proteolytic system. A: MuRF1 and B: atrogin-1/MAFbx mRNA levels, C: 26S proteasomal activity, D: calpain and E: calpastatin protein levels, and F: representative blots of plantaris muscle in untrained (UNTR) and trained C57BL/6J mice; n = 10–12/group. Data are presented as mean ± SE and were analyzed by 1-way ANOVA followed by Duncan post hoc test. *P < 0.05 from age-matched UNTR group; #P < 0.05 from the group trained for 2 wk.

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Here, we report that elevated mRNA and protein expression of main components of both systems, combined with increased 26S proteasome activity, were observed in skeletal muscle of healthy C57BL/6J mice after 8 wk of moderate-intensity AET.

Our data, regarding the acute effects of an exercise session, demonstrated a nice, time-dependent activation profile. All components, except for atrogin-1/MAFbx, displayed an up-regulation immediately after the exercise session, which suggests that the sarcomeric derangement, promoted by the recent mechanical stimulus, activates the proteolytic systems, probably to eliminate myofibrils damaged by exercise. Twenty-four hours after the exercise session, we observed that the only component that remained above resting levels was the proteasome, with a significant reduction observed on all other components. This finding suggests that already-tagged proteins were still being degraded by the proteasome; however, no E3 ligase activation was observed at that time point, indicating no remaining, untagged, damaged proteins. Activation of proteolytic systems went back to resting levels, 48 h after the exercise session, which is of vital importance for the discussion about long-term exercise training effects; since we killed mice 48 h after the last exercise testing, any remaining effect of this last session could have compromised a correct interpretation of our data.

Data shown in Table 1 provide strong evidence that the proposed exercise training protocol was highly efficient. Resting bradycardia and increased exercise tolerance are widely accepted as markers of aerobic conditioning (26). Furthermore, MLSSw was also improved after 8 wk of training, suggesting improved balance between lactate production and clearance, leading to enhanced aerobic capacity (38). Plantaris muscle capillarization was also increased, indicating increased blood supply to exercising muscles. These data provide sufficient functional, structural, and biochemical information to ensure that mice submitted to the intervention were in fact aerobically trained after 8 wk of training.

Regarding proteolytic pathways, we observed augmented, chymotrypsin-like activity of 26S proteasome, paralleled by increased mRNA expression of E3 ligase MuRF1, only after 8 wk of AET. MuRF1 and atrogin-1/MAFbx are muscle-specific ubiquitin ligases involved in the last step of protein labeling before degradation by the proteasome (20) and known to be required for skeletal muscle atrophy (4). However, we found that 8 wk of AET increased E3 ligase mRNA expression and proteasome activity in plantaris muscle, which was accompanied by skeletal muscle hypertrophy.

Corroborating our data on the acute effects of exercise, other studies have also demonstrated an upregulation of MuRF1 and atrogin-1/MAFbx mRNA levels after a single bout of resistance exercise (25, 31, 44), supporting the idea that UPS may also play an important role in skeletal muscle turnover and not only in atrophying states, which could explain our findings regarding long-term exercise training effects.

The only phase-dependent effect of AET was a decreased proteasome activity after 2 wk of AET, since no further, significant findings were observed at this time point (2 wk). These results indicate that what we observed on the 8-wk-trained group was in fact a chronic and long-lasting (>48 h) effect of AET.

We also verified that 8 wk of exercise training increased calpain protein levels and exerted no effect on calpastatin

### Table 1. Physiological and functional parameters of untrained and 8-wk-trained mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>UNTR (n = 10)</th>
<th>TRAINED (n = 10)</th>
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</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>27.5 ± 0.9</td>
<td>27.5 ± 0.8</td>
</tr>
<tr>
<td>Total distance run, m</td>
<td>248.1 ± 21.0</td>
<td>616.8 ± 48.0*</td>
</tr>
<tr>
<td>MLSSw, m/min</td>
<td>15.0 ± 0.9</td>
<td>21 ± 0.5*</td>
</tr>
<tr>
<td>Resting heart rate, bpm</td>
<td>585 ± 5</td>
<td>518 ± 5*</td>
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</table>

Body weight, total distance achieved in a graded treadmill running test, maximal lactate steady-state workload (MLSSw), and resting heart rate in untrained (UNTR) and 8-wk-trained C57BL/6J mice. Data are presented as mean ± SE and were analyzed by Student’s t-test. *P < 0.05 vs. age-matched UNTR.
expression. Thus increased protease abundance with no changes in its endogenous inhibitor expression suggests that exercise training activated the calpain system above UNTR levels. Accordingly, studies in human and animal models demonstrated overactivation of the calpain system by increased muscle load (30, 37, 41), indicating that increased muscle use stimulates this pathway. It was also suggested that increased calpains-mediated proteolysis might be necessary for the release of filaments disrupted by exercise, cleaning the area for a subsequent remodeling process (30).

When skeletal muscles undergo increased contractile activity (e.g., exercise), fibers are exposed to mechanical and metabolic challenges, leading to damage of intracellular proteins. Thus continuous degradation of damaged proteins and de novo protein synthesis are necessary to maintain normal contractile function. UPS is well known by its role in removing damaged proteins and for its major contribution to intracellular protein quality control (23); therefore, the nonaccumulation of polyubiquitinated or carbonylated proteins is probably due to increased 26S proteasome activity observed in exercise-trained mice, providing proper removal of potentially toxic agglomerates (e.g., oxidized proteins).

Skeletal muscle remodeling is dictated by the balance between the rates of protein synthesis and breakdown, and resistance exercise training is known to increase both simultaneously, albeit the change in the latter is smaller than the former, resulting in overall positive accumulation of muscle protein (33). In contrast, very little is known about the effects of AET on proteolytic pathways, and contradictory findings have been reported regarding skeletal muscle mass. Studies suggested that muscle atrophy might happen (40), whereas others presented results similar to ours, reporting skeletal muscle hypertrophy in human (18) and other animals (8, 22) after AET protocols. Despite controversial findings, it seems likely that when intensity-controlled AET is performed in adequate volume, skeletal muscle hypertrophy may occur (22), differing from exhaustive training regimens performed by high-performance endurance athletes, when individuals undergo significant energy deficit, ultimately leading to catabolic outcomes (40).

It is also important to mention that structural adaptations were accompanied by functional improvements, and we demonstrated recently that the same experimental model displays also enhanced skeletal muscle calcium handling and redox status after AET (9), which we also observed in a mouse model of heart failure, submitted to same exercise-training protocol (2, 5). Taken together, these findings provide evidence that overactivated proteolytic pathways did not impair skeletal muscle structure or function in the present animal model.

Although proteolytic pathways seem to have been overactivated, exercise-trained mice displayed plantaris muscle hypertrophy; therefore, it was reasonable to suppose that anabolic pathways might also have been upregulated by AET. This is supported by the fact that IGF-1 protein levels are higher in plantaris muscles of the trained than the UNTR counterparts (data not shown). Detailed evaluation of protein synthesis pathways was beyond the scope of the present study, but it is undoubtedly an interesting topic to be addressed in future studies.

Study Limitations

Our study shows that the calpain system and UPS are overactivated by AET when plantaris muscle is analyzed. One might argue that muscles with different fiber-type composition may react differently to the same training intervention (e.g., soleus). However, plantaris muscle was chosen due to the high prevalence of type II fibers, known to display greater response to mechanical stimuli than type I fibers.

In the present study, the acute effects of exercise were evaluated after a bout of exhaustive exercise, whereas the AET was performed at an intensity below the lactate threshold. However, it is important to highlight that even under higher exercise intensity (known to elicit prolonged postexercise effects), activation of proteolytic systems went back to resting levels, 48 h after the acute exercise session. This is a crucial finding for the interpretation of long-term exercise training effects, since the samples were collected 48 h after the last training session.

Finally, it will be important to explore further whether the present findings are reproducible in human muscle biopsies,
not only in healthy subjects but also in patients expected to present an elevated catabolic drive at baseline, such as in cancer cachexia and heart failure.

**Conclusion**

Altogether, our results suggest that 8 wk of intensity-controlled AET induced overactivation of calpain and proteasome systems in skeletal muscle of healthy C57BL/6J mice. Therefore, it is important to highlight that overactivation of calpains and UPS in skeletal muscle is not restricted to atrophying processes. Here, we suggest that this upregulation favors increased protein turnover in skeletal muscle, contributing to plantaris hypertrophy by intensity-controlled AET.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


