High- versus moderate-intensity aerobic exercise training effects on skeletal muscle of infarcted rats

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Moreira JB, Bechara LR, Bozi LH, Jannig PR, Monteiro AW, Dourado PM, Wisløff U, Brum PC. High-versus moderate-intensity aerobic exercise training effects on skeletal muscle of infarcted rats. J Appl Physiol 114: 1029–1041, 2013. First published February 21, 2013; doi:10.1152/japplphysiol.00760.2012. Poor skeletal muscle performance was shown to strongly predict mortality and long-term prognosis in a variety of diseases, including heart failure (HF). Despite the known benefits of aerobic exercise training (AET) in improving the skeletal muscle phenotype in HF, the optimal exercise intensity to elicit maximal outcomes is still under debate. Therefore, the aim of the present study was to compare the effects of high-intensity AET with those of a moderate-intensity protocol on skeletal muscle of infarcted rats. Wistar rats underwent myocardial infarction (MI) or sham surgery. MI groups were submitted either to an untrained (MI-UNT); moderate-intensity (MI-CMT, 60% VO2 max); or matched volume, high-intensity AET (MI-HIT, intervals at 85% VO2 max) protocol. High-intensity AET (HIT) was superior to moderate-intensity AET (CMT) in improving aerobic capacity, assessed by treadmill running tests. Cardiac contractile function, measured by echocardiography, was equally improved by both AET protocols. CMT and HIT prevented the MI-induced decay of skeletal muscle citrate synthase and hexokinase maximal activities, and increased glycogen content, without significant differences between protocols. Similar improvements in skeletal muscle redox balance and deactivation of the ubiquitin-proteasome system were also observed after CMT and HIT. Such intracellular findings were accompanied by prevented skeletal muscle atrophy in both MI-CMT and MI-HIT groups, whereas no major differences were observed between protocols. Taken together, our data suggest that despite superior effects of HIT in improving functional capacity, skeletal muscle adaptations were remarkably similar among protocols, leading to the conclusion that skeletal myopathy in infarcted rats was equally prevented by either moderate-intensity or high-intensity AET.

Recent efforts by researchers have identified possible intracellular mechanisms underlying the skeletal muscle abnormalities in cardiovascular diseases, and promising targets such as metabolic enzymes, calcium handling-related proteins, antioxidant scavengers, and inflammatory cytokines (9, 12, 39, 46). However, despite the identification of possible important players and advances in pharmacological treatment for HF over the last century, no available pharmacological therapy is able to prevent the onset of HF skeletal myopathy or to prevent or revert its consequences. Therefore, adjuvant therapies for treating skeletal muscle abnormalities in cardiovascular diseases must be emphasized and more deeply studied.

Following this rationale, it has been shown that aerobic exercise training (AET) not only improves cardiac function in patients with HF, but also promotes a broad range of beneficial skeletal muscle outcomes such as prevention of skeletal muscle atrophy, increase in type I muscle fiber percentage, and improvements in metabolic parameters (1, 4, 8, 18, 27), which altogether, improved functional capacity and led to better quality of life and longer survival. Such beneficial effects of AET on impaired skeletal muscle due to cardiovascular diseases have been reported for many years (1, 17, 27, 34), including important contributions from our group (4, 8, 9, 11), however, the optimal exercise intensity to elicit maximal outcomes is still a matter of debate among clinicians and researchers. Although moderate-intensity AET is undoubtedly a safe approach and known to improve ventricular function, skeletal muscle, and exercise capacity in patients with HF, recent studies suggest that high-intensity AET, achieved by interval training, promotes superior outcomes for cardiac patients (35, 60), including additional gains in cardiac function and aerobic capacity, which is the single best predictor of cardiovascular mortality (37). Nevertheless, a systematic comparison between the effects of moderate-intensity and high-intensity AET on skeletal muscle adaptations in cardiovascular diseases has never been reported in humans or animal models.

Therefore, in the present study, we compared the effects of high-intensity AET with those of a matched-volume, moderate-intensity protocol on skeletal muscle of rats submitted to myocardial infarction (MI), using morphological, metabolic, and intracellular parameters in skeletal muscles of distinct fiber type composition.

MATERIALS AND METHODS

Animal model and experimental design. Eight-week-old male Wistar rats (100 rats were initially purchased from Anilab LTDA, Paulinia, Brazil) were randomly assigned into MI or fictitious surgery (SHAM). Rats were deeply anesthetized with ketamine (50 mg/kg ip)
and xylazine (10 mg/kg ip), followed by left thoracotomy. The mediastinum was accessed by incision of intercostal muscles between the third and fourth ribs, the heart was carefully exteriorized, and the left anterior descending (LAD) coronary artery was occluded with 6/0 thread. After LAD ligation the thorax was closed, and lung collapse was prevented by rapid withdrawal of air from the pleural cavity. Sham-operated animals underwent similar left thoracotomy and cardiac exteriorization, with the exception of LAD ligation. Four weeks after surgical procedures, rats were submitted to echocardiographic examination and exercise testing for assessment of functional capacity, as described below. At this time point (the fourth week after surgery), experimental groups were divided in such a way that all parameters (i.e., functional capacity, cardiac structure, and function) were similar among the groups of similar cardiac condition (i.e., MI).

SHAM refers to the healthy, fictitiously operated control group, MI-UNT refers to the group of MI rats that remained untrained during the 8 wk of postinfarction protocol, MI-CMT refers to MI rats that were submitted to 8 wk of a continuous moderate-intensity AET protocol, and MI-HIT refers to MI rats that were submitted to 8 wk of high-intensity aerobic interval training. AET protocols are described in detail below. Rats were kept in a temperature-controlled (21°C) facility with a 12:12-h light:dark cycle and were housed five per cage, with free access to water and standard laboratory chow (Nuvital Nutrientes, Colombo, PR, Brazil). Rats were killed by decapitation and tissue was carefully removed and processed according to the desired experiment. All experiments were performed in soleus and plantaris muscle, which were chosen due to their contrasting structural metabolic characteristics (i.e., fiber type distribution and prevalent metabolism). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD) and were approved by the University of São Paulo’s Ethical Committee (#2008/40).

Echocardiographic evaluation. Rats underwent M-Mode echocardiographic examination before (4 wk after surgery) and after (12 wk after surgery) experimental protocols. Rats were anesthetized with ketamine (50 mg/kg ip) and xylazine (10 mg/kg ip) and were placed in a supine position. Cardiac dimensions were measured by a linear probe (14 Mhz) and left ventricular fractional shortening (LVFS) was calculated by the formula LVFS (%) = [(LVEDD - LVESD)/LVEDD] × 100, where LVEDD means left ventricular end-diastolic dimension and LVESD means left ventricular end-systolic dimension. Echocardiographic examination followed the recommendations of the American College of Echocardiography (45).

Exercise testing and measurement of aerobic capacity. Rats were submitted to exercise testing before (4 wk after surgery) and after (12 wk after surgery) experimental protocol. Animals were adapted to treadmill exercise over 5 days (10 min each day) before tests. Rats ran on a graded treadmill until exhaustion at 15° inclination; the speed started at 6 m/min and was increased by 3 m/min every 3 min until rats were unable to run (15). The same test protocol was also used to measure maximal oxygen uptake (VO2 max), when animals were placed on a treadmill mounted into a metabolic chamber connected through a tube to an air pump used to maintain airflow inside the chamber (3,500 ml/min). The tube was serially connected to an oxygen analyzer, which continuously measured partial oxygen pressure (pO2) throughout the exercise test. Oxygen uptake could then be calculated by the formula VO2 = (pO2 room air − pO2 during test) × F/m, where F is the airflow through the chamber (3,500 ml/min) and m is the rat’s body mass in kilograms. VO2 max was considered achieved when oxygen uptake no longer increased despite an increase in workload (i.e., intensity at which oxygen uptake leveled off = jVO2 max) and rats were no longer able to run. Tests were performed by an experienced observer (JBNM) who was blinded to rat identities.

Aerobic exercise training protocols. AET protocols started 4 wk after surgical procedures. Moderate-intensity AET was performed at a treadmill speed corresponding to 60% VO2 max, which was kept unchanged throughout the entire session (i.e., continuous moderate-intensity training, or CMT). High-intensity AET was performed in such a way that rats ran during 3 min at 60% VO2 max, followed by 4-min intervals at 85% VO2 max (i.e., high-intensity interval training, or HIT), which was repeated seven times, so each HIT session lasted for 49 min. CMT and HIT protocols were of matched volume, meaning that total running distances in each session of either CMT or HIT were identical; therefore, CMT session duration was adjusted to match HIT distance (20). A 5-min warmup at 40% VO2 max was performed by both trained groups before each AET session. AET protocols were performed at 15° inclination 5 days per week over 8 wk. At the end of the fourth week, animals were submitted to an additional exercise test to adjust running intensity. SHAM and MI-UNT animals were placed on the treadmill twice a week for 10 min each day at 40% VO2 max to maintain running skills.

Skeletal muscle fiber type distribution and cross-sectional area. Muscles frozen in liquid nitrogen were vertically mounted at L0 in fixed bases and serially sectioned in cryostat (10-μm sections). Sections were submitted to myosin ATPase staining after alkali preincubation (pH 10.6) (7). Fiber type distribution and cross-sectional area (CSA) were evaluated at 200× magnification and analyzed by a digitizing unit connected to a computer (Image Pro-Plus, National Institutes of Health). All analyses were conducted by a single observer (JBNM) who was blinded to rat identities.

Citrate synthase assay. Muscles were homogenized in phosphate buffer [50 mM sodium phosphate, 1 mM EDTA and protease inhibitor cocktail (Sigma-Aldrich, São Paulo, SP, Brazil) pH 7.4] and centrifuged for 15 min at 12,000g and 4°C. The pellet was discarded and the supernatant was used for the assay. The assay mixture contained 100 mM Tris, 1 mM EDTA, 0.2 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.1 mM acetyl-CoA, 1% (v:v) Triton X-100, sample (130 μg of soluble proteins per milliliter of total assay volume), and 0.5 mM oxaloacetate (added last), as originally described (2). Sample absorbance at 412 nm was monitored in 96-well plates for 10 min at 25°C, and maximal citrate synthase activity was measured within the linear range of the assay.

Hexokinase assay. Muscle homogenates were obtained as described in Citrate synthase assay. Hexokinase (HK) maximal activity assay was performed in the presence of 75 mM Tris–HCl, 7.5 mM MgCl2, 0.8 mM EDTA, 1.5 mM KCl, 4 mM mercaptoethanol, 0.4 mM NADP, 2.5 mM ATP, 1.4 units/ml creatine phosphate, 0.05% (v:v) Triton X-100, excess G6PDH, sample (750 μg of soluble proteins per milliliter of total assay volume) and 1 mM glucose as substrate (added last), as originally described (62). Sample absorbance at 340 nm was monitored in 96-well plates for 10 min at 25°C. HK activity was measured within the linear range of the assay.

Skeletal muscle glycogen content. Approximately 50 mg of tissue was digested in 400 μl of 30% (w:v) KOH (30 min at 100°C), followed by the addition of 800 μl of pure ethanol for glycogen precipitation (10 min at 100°C). Samples were cooled on ice and submitted to centrifugation (30 min at 3,000g and 4°C). The supernatant was carefully discarded and the glycogen pellet was suspended in 1 ml of 5% (v:v) trichloroacetic acid. Each sample (300 μl) was mixed with 600 μl of 0.2% anthrone (w:v in 95% sulfuric acid) and heated (10 min at 100°C). Sample absorbance was read at 620 nm in 10-ml cuvettes and compared with a standard glucose curve. Glycogen concentration was corrected by the exact tissue mass used in the assay, so glycogen content is expressed as micrograms of glycogen per milligram of tissue (μg glycogen/mg muscle).

Superoxide dismutase assay. Superoxide dismutase (SOD) maximal activity assay was based on the inhibition of xanthine/xanthine oxidase-driven cytochrome C reduction by SOD present in the sample. Muscle homogenates were obtained as described in Citrate synthase assay. Initially, the cytochrome C reduction rate was followed in the absence of the sample for 5 min at 550 nm (absorbance) in a mixture containing 1.18 mM xanthine, 19 mM cytochrome C and xanthine oxidase, diluted in sodium phosphate buffer (50 mM pH 7.8). Xanthine oxidase concentration was adjusted to obtain a standard rate.
of cytochrome C reduction of 0.025 units of absorbance per minute. Next, the cytochrome C reduction rate was measured in the presence of the sample (23). The difference between the two rates was attributed to SOD being present in the tested sample. SOD activity was measured within the linear range of the assay.

Catalase assay. Muscle homogenates were obtained as described in Citrate synthase assay. The rate of hydrogen peroxide (H$_2$O$_2$) decomposition by catalase was assessed by following the decay in sample absorbance at 240 nm (i.e., absorbance of H$_2$O$_2$) for 4 min in the presence of 10 mM H$_2$O$_2$. Catalase maximal activity was measured within the linear range of the assay, as described (57).

Glutathione redox status. Reduced (GSH), total (total GSH), and oxidized glutathione (GSSG) were measured in soleus and plantaris muscles with the Glutathione Fluorescent Detection Kit (K006-F5; Arbor Assay, Ann Arbor, MI) following the manufacturer’s instructions. Briefly, a proprietary compound binds to the free thiol groups of GSH present in the sample to yield a highly fluorescent product (510 nm and 390 nm were emission and excitation wavelengths, respectively). After reading the free GSH concentration, all GSSG in the sample was reduced to GSH so that total GSH could be assessed. GSSG was then calculated by the formula (Total GSH − Free GSH)/2.

Proteasomal activity. Muscles were homogenized in phosphate buffer without protease inhibitors (50 mM sodium phosphate, 1 mM EDTA pH 7.4) and centrifuged for 15 min at 12,000g and 4°C. The pellet was discarded and the supernatant (soluble proteins) was used for the assay. 26S proteasome activity was measured as the cleavage rate of a synthetic fluorescent peptide (Enzo Life Sciences, Farmingdale, NY), a specific substrate of the proteasome chymotrypsin catalytic site. The reaction mixture contained 25 mM Tris (pH 7.4), 5 mM MgCl$_2$, 25 µM ATP, 25 µM LLVY-AMC, and the sample (25 µg of soluble proteins). Fluorescent product formation was followed for 90 min (440 nm and 350 nm were emission and excitation wavelengths, respectively) at 37°C in the presence or absence of epoxomicin (20 µM), a highly specific inhibitor of chymotrypsin-like proteasome activity, and the difference between the two rates was considered as 26S proteasomal activity (chymotrypsin-site).

Quantitative real-time PCR. Messenger RNA (mRNA) levels of ubiquitin ligases Atrogin-1/MAFbox (atrogin-1) and Muscle RING-Finger-1 (MuRF1) were assessed in soleus and plantaris muscles by quantitative real-time polymerase chain reaction (qRT-PCR). RNA was isolated from muscles with Trizol reagent (Invitrogen, São Paulo, Brazil) following the manufacturer’s instructions. After assessment of RNA integrity [electrophoresis in agarose gels followed by staining with 4% w/v agarose gel (Sigma-Aldrich, São Paulo, Brazil), purity (260:280 nm) ratio, and concentration (ng/µl) (spectrophotometry by NanoDrop 2000; Thermo Scientific, Rockford, IL), reverse transcription was performed using the ReverTraid First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD). In summary, 2 µg of total RNA, oligo(dT) (0.5 µg), RiboLock RNase inhibitor (20 units), 1 mM dNTP mix, and ReverTraid Reverse Transcriptase (200 units) were added to a reaction buffer performing a 20·µl final solution (Fermentas), which was incubated at 42°C for 1 h plus 10 min at 70°C for reverse transcription termination. After cDNA synthesis, qRT-PCR for target genes and endogenous reference gene cyclophilin were run separately and amplifications were performed with an ABI Prism 7500 Sequence Detection System by using the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas). 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 Ct$_{\text{gene of interest}}$ minus Ct$_{\text{reporter gene}}$; cyclophilin was the control gene. Calculation of relative changes in mRNA levels (namely ΔΔCt) was performed by subtracting ΔCt value of SHAM group from ΔCt values of the other three groups (MI-SED, MI-CMT and MI-HIT). SHAM levels were arbitrarily set to 1. Primer sequences are shown in Table 1.

Protein ubiquitination. Muscle homogenates were mixed with 0.8% (w:v) SDS, 200 mM mercaptoethanol, 0.02% (w:v) bromophenol blue, and 40% (w:v) glycerol, loaded into polyacrylamide gels [25 µg of soluble proteins; 10% (w:v) acrylamide gels], submitted to electrophoresis (100 V constant, 1.5 h, room temperature), and electrotransferred to nitrocellulose membranes (100 V constant, 2 h, 4°C). Unspecific binding of antibodies was prevented by incubation of the membranes with 5% BSA (w:v) followed by incubation with the primary antibody against ubiquitin (sc8017; Santa Cruz Biotechnology, Santa Cruz, CA) [overnight under gentle shaking at 4°C, diluted in solution containing 5% (w:v) BSA, 10 mM Tris-HCl, 150 mM NaCl, and 0.1% (v:v) Tween-20, pH 7.6]. Membranes were washed three times (5 min each under gentle shaking at room temperature) with PBS-Tween solution and later incubated with horseradish-peroxidase (HRP)-conjugated secondary antibody from the same host as primary (2 h under gentle shaking at room temperature). Antibody detection was performed in a digitalizing unit (ChemiDoc; BioRad, São Paulo, Brazil) after incubation with luminol and hydrogen peroxidase as HRP substrate. Different membranes were compared by loading a standard reference sample in all gels and results were corrected to Ponceau red staining (0.5%, w:v) of the membrane. Skeletal muscle protein ubiquitination was analyzed in the broadest molecular weight range as possible. Data are presented as a percentage of the SHAM group (arbitrarily set as 100%). Representative images of the membranes are provided along with the data.

Protein carbonylation. Protein carbonylation was assessed by measuring the levels of carbonyl groups using the OxyBlot Protein Detection Kit (s7150; Millipore, Billerica, MA). Soluble proteins (25 µg) were denatured by 6% (w:v) SDS and derivatized by DNPH for 15 min. The reaction was stopped and proteins were submitted to electrophoresis and membrane blotting as described under Protein ubiquitination. Membranes were incubated with anti-DNP primary antibody and HRP-conjugated secondary antibody, followed by detection in a digitalizing unit (ChemiDoc; BioRad). Different membranes were compared by loading a standard reference sample in all gels, and results were corrected to Ponceau red staining (0.5%, w:v) of the membrane. Skeletal muscle protein carbonylation was analyzed in the broadest molecular weight range possible. Data are presented as a percentage of the SHAM group (arbitrarily set as 100%). Representative images of the membranes are provided along with the data.

Statistical analysis. Data are presented as mean ± standard error. Shapiro-Wilk tests were used to verify normal distribution of the data. Echocardiographic parameters, exercise performance, and aerobic capacity were compared among groups by one-way ANOVA for

<table>
<thead>
<tr>
<th>Ligase</th>
<th>Primer Sequence</th>
</tr>
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<tbody>
<tr>
<td>MuRF1 forward</td>
<td>5′GCCGCGAGGGTTGGTCTCCATCTC'</td>
</tr>
<tr>
<td>MuRF1 reverse</td>
<td>5′CCACCAACACAGCGGTCCCTC'</td>
</tr>
<tr>
<td>Atrogin-1 forward</td>
<td>5′TACTAGAGGCTACGCCATTCTCTCT'</td>
</tr>
<tr>
<td>Atrogin-1 reverse</td>
<td>5′TTGGAATCTTCTGTAAGCCAGAATG'</td>
</tr>
<tr>
<td>PDK4 forward</td>
<td>5′CATCTCCAGCTGCACAGGGCGTGAGC'</td>
</tr>
<tr>
<td>PDK4 reverse</td>
<td>5′GATCCTGTTCAGAAATCTGTTGAGTACAGCAGTG'</td>
</tr>
<tr>
<td>PPAR-delta forward</td>
<td>5′CCACCATGTCCTGATTGACGCGATGA'</td>
</tr>
<tr>
<td>PPAR-delta reverse</td>
<td>5′CTGAAAGGAGAGACAGAAGGAGAG'</td>
</tr>
<tr>
<td>TFAM forward</td>
<td>5′TTCGCGGCGTCGGTTGAGTACGACGCTAC'</td>
</tr>
<tr>
<td>TFAM reverse</td>
<td>5′GGAGCTGTCGGCAGGGAGGCTG'</td>
</tr>
<tr>
<td>PGC1-alp forward</td>
<td>5′GGCAACGCTGTTAAGCTGAAAGCAGACAG'</td>
</tr>
<tr>
<td>PGC1-alp reverse</td>
<td>5′AAGCTGCTCAGAGGCGGTCTG'</td>
</tr>
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</table>

MuRF1, Muscle RING-Finger-1; Atrogin-1, Atrogin-1/MAFbox; PDK4, pyruvate dehydrogenase isozyme 4; PPAR-delta, peroxisome proliferator-activated receptor-delta; TFAM, transcription factor A, mitochondrial; PGC1-alp, peroxisome proliferator-activated receptor gamma coactivator 1-alpha.
Table 2. Physiological parameters

<table>
<thead>
<tr>
<th></th>
<th>SHAM (n = 9–10)</th>
<th>MI-UNT (n = 8–11)</th>
<th>MI-CMT (n = 6–9)</th>
<th>MI-HIT (n = 9–11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>441 ± 41</td>
<td>423 ± 36</td>
<td>431 ± 18</td>
<td>407 ± 39</td>
</tr>
<tr>
<td>LVPWS, mm</td>
<td>1.30</td>
<td>1.40</td>
<td>1.33</td>
<td>1.33</td>
</tr>
<tr>
<td>Incidence of lung edema</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>4.82 ± 0.01</td>
<td>4.70 ± 0.1</td>
<td>4.60 ± 0.1</td>
<td>4.50 ± 0.1</td>
</tr>
<tr>
<td>IVSS, mm</td>
<td>1.76 ± 0.03</td>
<td>1.75 ± 0.03</td>
<td>1.75 ± 0.03</td>
<td>1.75 ± 0.03</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>110 ± 06</td>
<td>110 ± 06</td>
<td>110 ± 06</td>
<td>110 ± 06</td>
</tr>
</tbody>
</table>

BW, body weight; MI, myocardial infarcted; SHAM, healthy control; UNT, untrained; CMT, moderate-intensity training; HIT, high-intensity training. Data are presented as mean ± SE, except for incidence of lung edema, which is shown as a percentage of animals that displayed lung edema. Lung edema was considered when the value of lung wet-to-dry ratio was above the average of the SHAM group ± SD. MI extension was calculated as (endocardial + epicardial circumference of infarcted tissue)/(endocardial + epicardial circumference of entire left ventricle) and expressed as a percentage. *P < 0.05 vs. SHAM.

Table 3. Echocardiographic data

<table>
<thead>
<tr>
<th></th>
<th>SHAM</th>
<th>MI-UNT</th>
<th>MI-CMT</th>
<th>MI-HIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVFS, %</td>
<td>39.4 ± 1.5</td>
<td>39.9 ± 1.7</td>
<td>21.3 ± 1.7*</td>
<td>22.1 ± 3.8*</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>4.82 ± 0.40</td>
<td>4.94 ± 0.35</td>
<td>7.56 ± 0.38*</td>
<td>7.39 ± 0.62*</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>7.68 ± 0.39</td>
<td>8.05 ± 0.42</td>
<td>9.46 ± 0.32*</td>
<td>9.40 ± 0.45*</td>
</tr>
<tr>
<td>IVSS, mm</td>
<td>1.76 ± 0.22</td>
<td>1.89 ± 0.11</td>
<td>1.27 ± 0.07*</td>
<td>1.63 ± 0.15*</td>
</tr>
<tr>
<td>LVSD, mm</td>
<td>0.85 ± 0.10</td>
<td>1.15 ± 0.09</td>
<td>0.89 ± 0.05</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td>LVPWS, mm</td>
<td>2.26 ± 0.06</td>
<td>2.40 ± 0.14</td>
<td>2.08 ± 0.09</td>
<td>2.49 ± 0.17</td>
</tr>
<tr>
<td>LVPWD, mm</td>
<td>1.30 ± 0.05</td>
<td>1.38 ± 0.12</td>
<td>1.22 ± 0.10</td>
<td>1.34 ± 0.07</td>
</tr>
<tr>
<td>HR_mean, bpm</td>
<td>230 ± 8</td>
<td>245 ± 16</td>
<td>256 ± 18</td>
<td>258 ± 17</td>
</tr>
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</table>

LVFS, left ventricular fractional shortening; LVESD, left ventricular end-systolic diameter; LVEDD, left ventricular end-diastolic diameter; IVSS, interventricular septum thickness at systole; LVSD, interventricular septum at diastole; LVPWS, left ventricular posterior wall thickness at systole; LVPWD, left ventricular posterior wall at diastole; HR_mean, heart rate under anesthesia; SHAM, healthy controls; Pre, 4 wk after surgical procedure; Post, 12 wk after surgical procedure; MI, myocardial infarcted; UNT, untrained; CMT, moderate-intensity training; HIT, high-intensity training. *P < 0.05 vs. SHAM at the same time point (Pre or Post); †P < 0.05 vs. MI-UNT at the same time point; ‡P < 0.05 vs. same group at Preprotocol; $P = 0.06 vs. SHAM at the same time point.

RESULTS

Physiological parameters. Relevant physiological parameters are presented in Table 2. Body weight at the end of the protocol was similar among groups. Although lung wet-to-dry ratio did not differ among groups, 63% of untrained MI rats presented lung edema, whereas only 33% of animals in each trained group presented such signs of congestive HF. Soleus muscle weight was reduced in the MI-UNT group, which did not occur in either MI-CMT or MI-HIT. Plantaris weight did not differ among animals. Heart weight-to-body weight was increased in MI-UNT and MI-HIT groups. MI extension was similar among all infarcted group.

Cardiac structure and function. Table 3 shows that MI groups presented severe contractile dysfunction (reduced LVFS) 4 wk after surgery, to a similar degree among the three MI groups. Significant LV dilation at systole or diastole was observed in MI groups 4 wk after surgery, as was thinning of interventricular septum (Table 3), suggesting that cardiac remodeling occurred along with contractile dysfunction. CMT and HIT promoted significant and similar improvements in LVFS, while no temporal effect (Pre- vs. Postprotocol) was observed in the SHAM and MI-UNT groups (Table 3). No LV dilation was observed in MI-CMT and MI-HIT groups after the AET protocol. IVSS returned to SHAM values 12 wk after surgeries in all MI groups (Table 3). LV posterior wall thickness at systole increased in MI-UNT and MI-CMT groups between the fourth and twelfth weeks after surgeries (Table 3). No significant differences in echocardiographic parameters were observed between MI-CMT and MI-HIT groups. Heart rate under anesthesia did not differ among groups before or after the experimental protocol, indicating that deepness of anesthesia was similar among all groups during echocardiographic examination (Table 3).

Functional capacity. Functional capacity was assessed as maximal exercise performance in running tests until exhaustion and VO2max. Figure 1A shows that both AET protocols significantly increased running performance as measured by graded treadmill tests until exhaustion, whereas superior effects were observed after HIT. Similarly, although CMT and HIT improved aerobic capacity (VO2max) in MI rats, high-intensity AET elevated VO2max above that of the SHAM group, which did not occur after CMT (Fig. 1B). Running intensity at VO2max (iVO2max) also increased in both trained groups and was higher in MI-HIT than MI-CMT (Fig. 1C).

Skeletal muscle fiber type distribution and cross-sectional area. Myosin ATPase staining of skeletal muscle sections revealed that both moderate- and high-intensity AET increased slow-twitch fiber (i.e., type I) percentage in soleus muscle (Fig. 2A). CMT also increased type I fiber percentage in plantaris muscle, whereas a trend toward increase was observed after HIT (Fig. 2B; P =
Data are presented as mean ± SEM. *P < 0.05 vs. SHAM at the same time point; #P < 0.05 vs. MI-UNT at the same time point; §P < 0.05 vs. Preprotocol of same group; †P < 0.05 vs. all other groups at the same time point.

Fig. 1. Functional capacity. Total distance run in graded treadmill running tests (A), aerobic capacity (VO₂ max) (B), and running intensity at which VO₂ max was reached (iVO₂ max) (C) 4 (Preprotocol) and 12 wk (Postprotocol) after surgical procedures in healthy control (SHAM) and myocardial infarcted (MI) rats that remained untrained (UNT) or underwent either moderate-intensity training (CMT) or high-intensity training (HIT). VO₂ max was measured in 6 animals from each group because VO₂ max assessment requires individual testing of each animal. Data are presented as mean ± SEM. *P < 0.05 vs. SHAM at the same time point; #P < 0.05 vs. MI-UNT at the same time point; §P < 0.05 vs. Preprotocol of same group; †P < 0.05 vs. all other groups at the same time point.

0.07). We also measured skeletal muscle fiber CSA to verify the effects of AET protocols on skeletal muscle trophicity. MI-UNT animals presented atrophy of type I and type II fibers of soleus muscle (Fig. 2, C and E), compared with SHAM animals. Soleus muscle atrophy was not observed in any of the trained groups and no significant differences between AET protocols were observed (Fig. 2, C and E). On the other hand, type I fibers of plantaris were not affected by MI or AET (Fig. 2D). However, reduced CSA of plantaris type II fibers was observed in MI-UNT and MI-CMT groups, suggesting that only HIT prevented MI-induced atrophy of plantaris type II fibers (Fig. 2F).

Skeletal muscle metabolism. Citrate synthase is a pace-making enzyme of the Krebs cycle; therefore, it is used as an indicator of cellular aerobic metabolism. Maximal citrate synthase activity trended (P = 0.07) toward a reduction in soleus muscle homogenates of MI-UNT animals (Fig. 3A), whereas a significant decay was observed in the plantaris muscle of MI-UNT animals when compared with SHAM animals (Fig. 3B). CMT and HIT similarly increased maximal citrate synthase activity in both soleus and plantaris muscles (Fig. 3, A and B). In turn, hexokinase phosphorylates glucose as a first reaction of glycolysis; therefore, its maximal activity may reflect the capacity of anaerobic metabolism, thus we performed hexokinase assays in soleus and plantaris muscles. Untrained MI rats presented significant impairment of maximal hexokinase activity in soleus and plantaris muscles, which was prevented by CMT and HIT (Fig. 3, C and D). No differences were found between AET protocols for hexokinase activity (Fig. 3, C and D). Because skeletal muscle glycogen is an important source of long-term energy and its depletion rate was shown to be altered in HF (28, 36), we assessed soleus and plantaris glycogen content. Skeletal muscle glycogen content was not altered by MI, although both AET protocols substantially increased soleus and plantaris glycogen content (Fig. 3, E and F), without differences between AET protocols. Considering our findings on citrate synthase activity, we further performed qPCR to detect possible differences in mRNA levels of mitochondrial transcriptional factors and genes involved in mitochondrial biogenesis (Table 4). Despite no statistically significant differences being observed in the plantaris muscle, we found that transcription factor A mitochondrial (TFAM) mRNA levels were significantly reduced in soleus muscle of MI-UNT animals, and both AET protocols prevented this reduction. Interestingly, both training protocols reduced mRNA levels of pyruvate dehydrogenase kinase 4 (PDK4), an endogenous inhibitor of pyruvate dehydrogenase (PDH), when compared with the SHAM and MI-UNT groups.

Skeletal muscle redox balance. SOD maximal activity presented as being reduced in soleus muscle of untrained MI rats (Fig. 4A) and a decay trend (P = 0.06) was observed in the plantaris muscles of this group (Fig. 4B). CMT increased SOD maximal activity, whereas HIT prevented the MI-induced decrease in SOD maximal activity in soleus muscle (Fig. 4A). Catalase maximal activity was not different among groups in any of the muscles studied (Fig. 4, C and D). Because glutathione is a powerful antioxidant buffer, we assessed its redox state in soleus and plantaris muscles. Free glutathione concentration (i.e., GSH, reduced state) was unchanged among groups in soleus (Fig. 4E) and plantaris (Fig. 4F) muscles. We found an increased concentration of oxidized glutathione (i.e., gluta-
thione disulfide, or GSSG) in soleus muscle of MI-UNT rats (Fig. 4G), which did not occur in MI-CMT or MI-HIT rats. We did not find a statistical difference between MI-CMT and MI-HIT animals in any of the above-mentioned variables.

Skeletal muscle ubiquitin-proteasome system. Activity of 26S proteasome was evaluated in soleus and plantaris muscles as an index of the ubiquitin-proteasome system (UPS) activation. Chymotrypsin-like proteasomal activity was increased in soleus (Fig. 5A) and plantaris (Fig. 5B) muscles of MI-UNT rats, which was prevented by both AET protocols, and no difference was observed between CMT and HIT (Fig. 5A and B). Protein ubiquitination was also measured because it is the master signal for proteasomal degradation, and accumulation of ubiquitinated proteins is observed when excessive protein damage occurs. Figure 5C shows that MI increased protein ubiquitination in soleus muscle of untrained rats, which was equally prevented by CMT and HIT. Protein ubiquitination in plantaris (Fig. 5D) and protein carbonylation in soleus and plantaris muscles (data not shown) did not differ among groups. We also assessed mRNA levels of ubiquitin ligases required in muscle atrophy (6). Soleus atrogin-1 mRNA levels were reduced by HIT in MI rats (Fig. 5E). Similarly, MuRF1 mRNA levels were reduced in MI-CMT and MI-HIT rats compared with SHAM rats (Fig. 5F). When attention is turned to the plantaris muscle, only MI-UNT animals presented increased mRNA levels of atrogin-1 (Fig. 5G) compared with SHAM animals. Plantaris MURF1 mRNA levels were not altered in any of the MI groups (Fig. 5H).

DISCUSSION

Skeletal muscle abnormalities are known to contribute to exercise intolerance and low functional capacity in patients
with cardiac problems (14, 29); however, it is still an underestimated problem for a patient’s prognosis because no available medication is effectively able to counteract its consequences, which highlights the importance of AET as therapy for cardiovascular diseases.

In this sense, the major finding of this study was that CMT and HIT equally counteracted skeletal muscle maladaptation in infarcted rats. We show that skeletal muscle metabolic, structural, and intracellular abnormalities observed in infarcted rats were prevented by both AET protocols.

Echocardiographic data show that MI groups displayed similar degrees of contractile dysfunction and left ventricular enlargement 4 wk after the surgeries, compared with SHAM animals, which corroborates the histological evaluation of MI extension and indicates that induced cardiac damage was similar among infarcted groups before training intervention. Interventricular septum thinning was also depicted in all three MI groups in the preprotocol evaluation, which when taken together with contractile dysfunction and ventricular hypertrophy, demonstrates pathological remodeling after MI. More than 60% of MI-UNT rats presented lung edema along with contractile dysfunction, suggesting the development of congestive HF in this subset of animals. Only 33% of MI-CMT or MI-HIT animals presented such a sign, which was not accompanied by contractile dysfunction, because both training protocols substantially improved LVFS and reverted left ventricular enlargement in MI rats to values close to those of SHAM animals. Corroborating previous findings in human and animal models (20, 54, 60), we show here that HIT promoted superior improvements in running performance and aerobic capacity than CMT, in such a way that MI-HIT presented higher aerobic capacity than the SHAM group, which was not observed in the MI-CMT group. Workload at \( \overline{V}O_2 \max \) was also superiorly improved by HIT in MI rats. These data indicate that
HIU T promoted superior improvement of functional capacity than CMT in MI rats.

Depressed skeletal muscle aerobic capacity has already been reported in HF (52). Pioneer studies identified impaired metabolic capacity in skeletal muscle of patients with HF as a major alteration and correlating with exercise intolerance (30–32), disturbing the dogma that HF affects only the cardiac muscle. Our data suggest that these metabolic abnormalities were more pronounced in plantaris muscle, which probably occurred due to the reduced number of type I (oxidative) fibers in this muscle, compared with soleus. Reduction of citrate synthase maximal activity may compromise de novo ATP synthesis during exercise in MI rats; therefore, the higher maximal citrate synthase activity in MI-CMT and MI-HIT rats than in MI-UNT rats suggests aerobic metabolism improvements in both trained groups. Of particular interest, citrate synthase maximal activity in soleus muscle positively correlated with running performance (r = 0.46, P < 0.05), whereas the correlation between left ventricular contractile function and running performance was not significant (r = 0.22, P > 0.12). It is clear that these findings do not guarantee a causal relationship between skeletal muscle aerobic capacity and running performance in our model, but they corroborate previous reports from the literature, demonstrating a strong correlation between skeletal muscle parameters and functional capacity in patients with HF (24) and important relationships between metabolic skeletal muscle adaptations and training and improvements in performance (56). An increased percentage of type I (i.e., oxidative) fibers in soleus and plantaris muscles of both trained groups is further evidence that CMT and HIT enhanced skeletal muscle aerobic metabolism in infarcted rats. Our findings regarding aerobic metabolism corroborate a recent report by Bartlett and colleagues (5) showing that a single bout of high-intensity interval exercise or continuous exercise equally induce activation of signaling pathways associated with mitochondrial biogenesis. Due to this fact, we evaluated mRNA levels of different mitochondrial transcription factors and found that both training protocols prevented the reduction of TFAM gene expression (Table 4). Because TFAM plays a key role in mitochondrial genome replication, we also hypothesized that mitochondrial density would be reduced in soleus muscle of MI-UNT rats. To test this hypothesis, we performed immunoblotting to detect protein levels of the mitochondrial components ATP synthase, mitochondrial malate dehydrogenase 2 (MDH2), and pyruvate dehydrogenase (PDH). Surprisingly, protein expression of those components was unaltered in MI-UNT rats (data not shown), suggesting that TFAM reduction in MI-UNT rats did not result in reduced mitochondrial biogenesis in soleus muscle. Therefore, training protocols might control oxidative metabolism by modulating enzyme activity other than expression, which is further suggested by the fact that PDK4 mRNA levels was reduced in soleus muscle of both trained groups. In fact, a lower inhibitory drive over PDH would thereby result in a higher PDH activity, although its expression was unchanged among groups in both muscles.

Anaerobic metabolism was also impaired in MI-UNT rats as suggested by reduced hexokinase activity in both soleus and plantaris muscles of this group. CMT and HIT effectively increased hexokinase maximal activity in plantaris muscle, whereas only HIT improved such a parameter in the soleus muscle, reinforcing the importance of studying skeletal muscle comprised of fibers of different characteristics (i.e., oxidative vs. glycolytic), which may present distinct responses to pathological and physiological stimuli (10, 48). Skeletal muscle glycogen content was not altered by MI, which has already been reported in this animal model (36). However, it was also shown that the rate of glycogen depletion is more pronounced in patients with HF and in the infarcted rat animal model (28, 36); therefore, the increased glycogen content in both soleus and plantaris muscles of MI-CMT and MI-HIT groups indicates similar enhancement of metabolic capacity by moderate- or high-intensity AET in infarcted rats.

For decades, acknowledgeable studies reported skeletal muscle atrophy as a major aggravator of HF syndrome (3, 29, 52). Later, the related literature provided reports of distinct response to atrophic stimuli between different muscles, suggesting that oxidative muscles (e.g., soleus) are more susceptible to damage due to inactivity or unloading, whereas glycolytic muscles (e.g., plantaris or extensor digitorum longus) tend to be more affected in diseased conditions (42, 47, 51, 58, 59). Considering that HF is a degenerative and rather complex disease, there is no absolute agreement on which muscle will present the most pronounced damage after MI; therefore, we decided to evaluate both soleus and plantaris muscle due to their contrasting metabolic properties.

Skeletal atrophy in MI rats is demonstrated here by reduced CSA of type I and type II muscle fibers in MI-UNT rats. Either CMT or HIT was able to maintain normal CSA of soleus fibers in MI rats (i.e., similar to SHAM values). Interestingly, plantaris muscle presented a distinct response to pathological (i.e.,

| Table 4. mRNA levels of mitochondrial-related genes |
|---------------------------------|----------------|----------------|----------------|----------------|
|                                | SHAM (n = 8)   | MI-UNT (n = 10)| MI-CMT (n = 8) | MI-HIT (n = 10) |
| Soleus muscle                  |                |                |                |                |
| PDK4                           | 1.00 ± 0.16    | 0.96 ± 0.20    | 0.40 ± 0.09‡   | 0.48 ± 0.08‡   |
| TFAM                           | 1.00 ± 0.04    | 0.80 ± 0.04    | 0.93 ± 0.07    | 0.86 ± 0.07    |
| PGC-1α                         | 1.00 ± 0.18    | 0.98 ± 0.25    | 0.59 ± 0.09    | 0.53 ± 0.14    |
| PPARG-1-alpha                  | 1.00 ± 0.13    | 0.88 ± 0.17    | 0.61 ± 0.10    | 0.62 ± 0.12    |

Plantaris muscle

| PDK4                           | 1.00 ± 0.38    | 0.83 ± 0.22    | 0.54 ± 0.19    | 0.85 ± 0.15    |
| TFAM                           | 1.00 ± 0.11    | 1.07 ± 0.05    | 1.09 ± 0.09    | 1.12 ± 0.06    |
| PGC-1α                         | 1.00 ± 0.11    | 1.18 ± 0.13    | 0.94 ± 0.13    | 0.92 ± 0.12    |
| PPARG-1-alpha                  | 1.00 ± 0.20    | 1.14 ± 0.15    | 0.89 ± 0.07    | 0.91 ± 0.11    |

PDK4, pyruvate dehydrogenase isozyme 4; TFAM, transcription factor A, mitochondrial; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARG-1-alpha, peroxisome proliferator-activated receptor delta; SHAM, healthy controls; MI, myocardial infarcted; UNT, untrained; CMT, moderate-intensity training; HIT, high-intensity training. *P < 0.05 vs. SHAM; ‡P < 0.05 vs. MI-UNT.
MI) and physiological (i.e., AET) stimuli. Only type II fibers of plantaris muscle were atrophied in MI-UNT rats, whereas CMT presented absolutely no effect in type II CSA in this muscle and only a modest preventive effect of HIT was observed, because MI-HIT still presented a strong trend ($P < 0.07$) toward plantaris type II fiber atrophy. Neither MI nor AET protocols affected plantaris type I fiber CSA. This differential response to stimuli between type II and type I fibers might be explained by two main factors: blood supply and oxidative damage. Although capillary rarefaction in skeletal muscle in patients with HF has been earlier identified (38), Li and colleagues more recently demonstrated that type II fibers display a greater loss of capillarization than type I in an animal model of HF (26), so the greater lack of perfusion may aggravate atrophy in type II fibers. The second factor (i.e., oxidative damage) was described by Yu and colleagues in a recent study (61) when they suggested that glycolytic muscle fibers are subjected to increased damage after a cachectic

![Fig. 4. Skeletal muscle redox balance. Superoxide dismutase (SOD) maximal activity in soleus (A) and plantaris (B), catalase maximal activity in soleus (C) and plantaris (D), reduced glutathione (GSH) concentration in soleus (E) and plantaris (F), and oxidized glutathione (GSSG) concentration in soleus (G) and plantaris (H) muscles of SHAM and MI rats that remained untrained (UNT) or underwent either CMT or HIT. Data are presented as mean ± SEM. *$P < 0.05$ vs. SHAM; # $P < 0.05$ vs. MI-UNT.](http://jap.physiology.org/)

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stimulus due to its reduced production of nitric oxide, which is an important agent capable of inducing expression of antioxidant enzymes.

Thus considering the important role played by oxidative stress in skeletal muscle under catabolic states, we conducted experiments to verify the effects of the two AET protocols on pro- and antioxidant drive in infarcted rats. We found that AET protocols prevented the MI-induced impairment of SOD in both muscles studied, indicating a better scavenging capacity of reactive oxygen species in trained groups. In addition, we

![Fig. 5. Skeletal muscle ubiquitin-proteasome system. 26S proteasome activity in soleus (A) and plantaris (B), protein ubiquitination in soleus (C) and plantaris (D), atrogin-1 mRNA levels in soleus (E) and plantaris (F), and MuRF1 mRNA levels in soleus (G) and plantaris (H) muscles of SHAM and MI rats that remained untrained (UNT) or underwent either CMT or HIT. Representative blots of ubiquitin and Ponceau staining of the membrane are presented beneath C and D. Data are presented as mean ± SEM. *P < 0.05 vs. SHAM.](http://jap.physiology.org)
found an increased concentration of glutathione disulfide (GSSG) in soleus lysates from the MI-UNT group. Reduced glutathione (GSH) is a potent endogenous antioxidant; therefore, that MI-UNT rats presented increased levels of oxidized glutathione (GSSG) and normal levels of GSH, suggests a redox imbalance in soleus muscles of this group, which was not observed in MI-CMT or MI-HIT groups, whereas no differences were found between CMT and HIT.

Atrophying muscles are also known to display boosted catabolic signaling (16), and special attention is driven to the ubiquitin-proteasome system (UPS), which is responsible for selective degradation of damaged proteins and required for skeletal muscle atrophy after hind limb nerve injuries (6). Therefore, we also evaluated the activation of the UPS by measuring mRNA levels of two major ubiquitin ligases, protein ubiquitination (the master signal for proteasomal degradation), and maximal activity of 26S proteasome in its chymotrypsin site, the major determinant of overall proteolysis (22).

We observed increased proteasomal activity in both soleus and plantaris muscles of MI-UNT rats, which did not occur in MI-CMT and MI-HIT rats, indicating that training protocols equally prevented proteasomal overactivation. Interestingly, accumulation of ubiquitinated proteins was found only in the soleus muscle of MI-UNT, which was also prevented by both CMT and HIT, without significant difference between AET protocols. Messenger RNA levels of atrogin-1 and MuRF1 corroborate, at least in part, the data on protein ubiquitination and proteasomal activity. Protein ubiquitination was not changed in plantaris muscle, so we show once again a distinct response between soleus and plantaris muscles, which in this case might be explained by the fact that only type II fibers atrophied in plantaris muscle of MI-UNT rats, whereas in the soleus muscle the amount of damaged proteins was superior (suggested by atrophy of both type I and type II fibers), and even an increased protein breakdown (i.e., increased proteasomal activity) was not sufficient to restrain the accumulation of ubiquitinated proteins. The intriguing fact that soleus muscle in MI-UNT rats presented higher proteasomal activity and accumulation of ubiquitinated proteins despite unchanged MuRF1 and atrogin-1 mRNA levels led us to look for possible explanations. These two E3 ligases gained extraordinary attention after the discovery that mice lacking either of them would remain untrained, as we presented in the article. In addition, the degree of contractile dysfunction was similar among the three infarcted groups before training intervention, and so was MI extension, as evaluated by histology. Although the beneficial effects of both AET protocols on skeletal muscle are evidenced in most of our data, we cannot conclude whether AET protocols reverted or prevented skeletal myopathy in infarcted rats, due to methodological limitations. This issue can be solved only by taking skeletal muscle biopsies at different time points after MI, which is clearly not possible in this animal model, which calls for studies in human subjects (e.g., patients with HF post-MI). We used a synthetic substrate for assessment of proteasome activity, which does not allow us to verify which proteins were being degraded by this protease, but taken together with immunoblotting of ubiquitinated proteins, this assay provides an important indicator of UPS activation in skeletal muscle.

LIMITATIONS

As reported in several studies (33, 44, 50, 63), the cardiac and systemic responses to MI are reasonably variable among animals. This can be partially due to Wistar rats being an outbred lineage, thus presumably genetically diverse. In this study we did not observe signs of congestive HF in all infarcted rats; however, pathological cardiac remodeling (4 and 12 wk after surgeries) and lung edema were observed in a great number of infarcted rats, particularly those that remained untrained, as we presented in the article. In addition, the degree of contractile dysfunction was similar among the three infarcted groups before training intervention, and so was MI extension, as evaluated by histology. Although the beneficial effects of both AET protocols on skeletal muscle are evidenced in most of our data, we cannot conclude whether AET protocols reverted or prevented skeletal myopathy in infarcted rats, due to methodological limitations. This issue can be solved only by taking skeletal muscle biopsies at different time points after MI, which is clearly not possible in this animal model, which calls for studies in human subjects (e.g., patients with HF post-MI). We used a synthetic substrate for assessment of proteasome activity, which does not allow us to verify which proteins were being degraded by this protease, but taken together with immunoblotting of ubiquitinated proteins, this assay provides an important indicator of UPS activation in skeletal muscle.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS


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