Aerobic exercise training improves skeletal muscle function and Ca\textsuperscript{2+} handling-related protein expression in sympathetic hyperactivity-induced heart failure


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HEART FAILURE (HF) is a clinical syndrome characterized by a marked decrease in exercise tolerance and a distinct muscular weakness. HF patients are commonly limited by exertional fatigue during both normal daily activities and maximal exercise testing (23). Over the last decades, skeletal muscle fatigue in HF patients has been presumed to be a consequence of reduced blood flow secondary to decreased cardiac output (31). However, severity of exercise intolerance has been poorly correlated with some cardiovasculor indexes such as stroke volume, ejection fraction, and maximal cardiac output in HF (10, 30). Additionally, increases in cardiac function and oxygen delivery to skeletal muscle by oxygen supplementation failed to increase exercise tolerance and fatigue resistance in HF (29). These observations suggest that muscle fatigue, at least in some HF patients, may be due to intrinsic skeletal muscle changes rather than insufficient skeletal muscle blood flow.

Among several skeletal muscle abnormalities detected in HF patients, such as muscle atrophy, impaired metabolic response, and reduced mitochondrial function, alterations in excitation-contraction coupling have been proposed to explain the muscle fatigue process seen during HF. Previous studies have suggested that depressed sarcoplasmic Ca\textsuperscript{2+} levels and diminished rate of sarcoplasmic reticulum Ca\textsuperscript{2+} release and reuptake contribute to increased fatigue in HF. In fact, reduced sarcoplasmic reticulum Ca\textsuperscript{2+} release and reuptake (12) were observed in fiber bundles and intact fibers from fast-twitch muscle in severe HF rats. Skeletal muscle of post-myocardial infarction rats displayed ryanodine receptor (RyR) hyperphosphorylation and impaired Ca\textsuperscript{2+} release. In contrast, sarcoplasmic vesicles isolated from skeletal muscle of moderate HF rats displayed accelerated Ca\textsuperscript{2+} reuptake (22, 28).

Aerobic exercise training is considered an efficient adjuvant therapy for HF, and its impact on skeletal muscle is remarkable. In fact, exercise training was able to reverse skeletal muscle myopathy by preventing muscle atrophy, capillary rarefaction associated with improved skeletal muscle oxidative capacity, and decreased oxidative stress in HF rodents (1, 11). We previously demonstrated (6) that aerobic exercise training in nonfailing mice improves the Ca\textsuperscript{2+} handling of skeletal muscles comprising different fiber compositions. Similar results were recently demonstrated in biopsied vastus lateralsis of healthy humans (16) submitted to leg extension. However, the effect of aerobic exercise training on skeletal muscle Ca\textsuperscript{2+} handling in HF still needs to be better understood.

We previously reported (1, 2, 7) that mice lacking \alpha\textsubscript{2A} and \alpha\textsubscript{2C} adrenoceptors (\alpha\textsubscript{2A}/\alpha\textsubscript{2C}-ARKO) develop sympathetic hyperactivity-induced HF related to exercise intolerance, established skeletal muscle myopathy, and cardiac dysfunction with clinical signs of HF at 7 mo of age. The present study was undertaken to evaluate the effect of aerobic exercise training on expression of proteins involved in Ca\textsuperscript{2+} release and reuptake by sarcoplasmic reticulum of skeletal muscles comprising different fiber compositions in \alpha\textsubscript{2A}/\alpha\textsubscript{2C}-ARKO mice. We tested the hypothesis that exercise training would improve exercise tolerance and skeletal muscle force in HF mice associated with...
an improved net balance of Ca\textsuperscript{2+} handling proteins in both soleus and plantaris muscles.

**MATERIALS AND METHODS**

**Study population.** A cohort of male congenic α\textsubscript{2A}/α\textsubscript{2C}-ARKO mice in a C57BL/6J genetic background and their wild-type (WT) controls aged 5–7 mo were studied. At 5 mo of age, α\textsubscript{2A}/α\textsubscript{2C}-ARKO mice display dysfunction associated with exercise intolerance (15). At 7 mo of age, they present severe cardiac dysfunction associated with exercise intolerance, established skeletal muscle myopathy, and increased mortality rate (1, 2, 7). Mice were maintained in a 12:12-h light-dark cycle and a temperature-controlled environment (22°C) with free access to standard laboratory chow (Nuvital Nutrients, Curitiba, PR, Brazil) and tap water. This study was in accordance with Ethical Principles in animal research adopted by the Brazilian College of Animal Experimentation (www.cobea.org.br). In addition, this study was approved by the University of São Paulo Ethical Committee (CEP no. 2007/028). The experimental design is shown in Fig. 1.

**Exercise training protocol.** To verify whether exercise training could improve skeletal muscle Ca\textsuperscript{2+} handling in 7-mo-old α\textsubscript{2A}/α\textsubscript{2C}-ARKO mice, we performed exercise training in α\textsubscript{2A}/α\textsubscript{2C}-ARKO and WT mice aging from 5 to 7 mo of age. Exercise training consisted of 8 wk of running on a motor treadmill (ESD model 01 FUNBEC), 5 days/wk, for 60 min at maximal lactate steady-state workload, as described elsewhere (8). All untrained mice were exposed to treadmill exercise (5 min) three times a week to become accustomed to the exercise protocol and handling.

**Graded treadmill exercise test.** Exercise capacity, estimated by total distance run, correlates with skeletal muscle work capacity, and it is a method used for detecting exercise intolerance in HF. Exercise tolerance was evaluated with a graded treadmill exercise protocol for mice as previously described (8). Briefly, after being adapted to treadmill exercises over a week (10 min of exercise session), mice were placed in the treadmill streak and allowed to acclimatize for at least 30 min. Intensity of exercise was increased by 3 m/min (6–33 m/min) every 3 min at 0% grade until exhaustion. The graded treadmill exercise test was performed in WT and α\textsubscript{2A}/α\textsubscript{2C}-ARKO mice after completion of exercise training protocol at 7 mo of age.

**Cardiovascular measurements.** Resting blood pressure (BP) and heart rate (HR) were determined noninvasively with a computerized tail-cuff system (BP 2000 Visitech Systems, Apex, NC) described elsewhere (4). Mice were acclimatized to the apparatus during daily sessions over 4 days, 1 wk before starting the experimental period.

Noninvasive ventricular function was assessed by two-dimensional echocardiography in halothane-anesthetized WT and α\textsubscript{2A}/α\textsubscript{2C}-ARKO mice at 7 mo of age. Briefly, mice were positioned in the supine position with front paws wide open, and an ultrasound transmission gel was applied to the precordium. Transthoracic echocardiography was performed with an Acuson Sequoia model 512 echocardiographer (Acuson, Mountain View, CA) equipped with a 14-MHz linear transducer. Left ventricle systolic function was estimated by fractional shortening as follows: fractional shortening (%) = [(LVEDD – LVESD)/LVEDD] × 100, where LVEDD is left ventricular end-diastolic dimension and LVESD is left ventricular end-systolic dimension.

**Skeletal muscle functional assessment.** To verify whether exercise training would improve motor ability in HF mice, we performed motor ability tests in trained and untrained 7-mo-old WT and α\textsubscript{2A}/α\textsubscript{2C}-ARKO mice. Mice were submitted to the following tests: 1) the inclined plane test measured the maximal angle of a wood board on which the animal was placed until it slipped; 2) the ambulation test determined the mean length of a step, measured in hind foot ink prints while mice ran freely in a corridor (length, 50 cm; width, 8 cm; height of lateral walls, 20 cm) (27); and 3) Rotarod (IITC Life Science, Woodland Hills, CA), in which the mice were placed on the rod, which was rotating at an initial speed of 1 rpm, the speed was gradually increased from 1 to 40 rpm over a period of 5 min, and the time that the mice stayed on the rod was recorded. The mice were subjected to three successive trials, and the performance of each animal was measured as its best individual performance over the three trials (26).

**Skeletal muscle protein expression.** Immunoblots of untrained and exercise-trained 7-mo-old WT and α\textsubscript{2A}/α\textsubscript{2C}-ARKO mouse soleus and plantaris muscle homogenates were performed according to Towbin et al. (25). Briefly, liquid nitrogen-frozen muscles were homogenized in a buffer containing (in mM) 1 EDTA, 1 EGTA, 2 MgCl\textsubscript{2}, 5 KCl, 25 HEPES (pH 7.5), and 2 DTT, with 100 μM PMSF, 1% Triton X-100, and protease inhibitor cocktail (1:100; Sigma-Aldrich, St. Louis, MO). Samples were loaded and subjected to SDS-PAGE in polyacrylamide gels (10%). After electrophoresis, proteins were electrotransferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Equal loading of samples (25 μg) and even transfer efficiency were monitored with the use of 0.5% Ponceau S staining of the blotted membrane. The blotted membrane was then incubated in a blocking buffer (5% nonfat dry milk, 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature and then incubated with a specific antibody overnight at 4°C. Mouse monoclonal antibodies to sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA1) (1:2,500), SERCA2 (1:2,500), Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) (1:100), dihydropyridine receptor (DHPR)\textsubscript{α1} subunit (1:500), DHPR\textsubscript{β} subunit (1:500), and RyR1 (1:500) were obtained from Affinity BioReagents (Golden, CO) and rabbit polyclonal parvalbumin (1:2,000) from Sigma-Aldrich. Binding of the primary antibody was detected with the use of peroxidase-conjugated secondary antibodies (anti-rabbit, 1:3,000, for 1.5 h at room temperature) and developed with enhanced chemiluminescence (Amersham Biosciences) detected by autoradiography. Quantification analysis of blots was performed with the use of Scion Image software (Scion based on NIH Image). Targeted bands were normalized to α-tubulin antibody (1:1,000; Santa Cruz Biotechnology).

**Statistical analysis.** Data are presented as means ± SE. Two-way ANOVA with post hoc testing by Tukey (Statistica software, StatSoft, Tulsa, OK) was used to compare the effect of training (untrained and exercise trained) and genotype (WT and α\textsubscript{2A}/α\textsubscript{2C}-ARKO) on fractional shortening, HR, BP, body weight, lung wet-to-dry ratio, distance run, step length, fall angle, Rotarod, and protein expression levels. Statistical significance was considered achieved when the value of P was <0.05.

**RESULTS**

Exercise training reverses skeletal muscle dysfunction in heart failure mice. Physiological parameters of untrained and exercise-trained WT and α\textsubscript{2A}/α\textsubscript{2C}-ARKO mice are presented in Table 1. Untrained α\textsubscript{2A}/α\textsubscript{2C}-ARKO mice displayed lower fractional shortening, tachycardia, and increased lung wet...
Exercise training restores expression level of proteins involved in sarcoplasmic Ca\(^{2+}\) release in skeletal muscle from heart failure mice. Since impaired Ca\(^{2+}\) release from the sarcoplasmic reticulum has been identified as a contributor to skeletal muscle fatigue in HF (24), we investigated whether the expression of different DHPR subunits and RyR are altered in soleus and plantaris of our \(\alpha_{2A}/\alpha_{2C}\) ARKO mice and whether exercise training would change their expression profile.

DHPR\(\alpha_1\) subunit expression levels were decreased in soleus of untrained \(\alpha_{2A}/\alpha_{2C}\) ARKO mice compared with WT mice (Fig. 3B), while no changes in plantaris were observed among groups (Fig. 4B). DHPR\(\alpha_2\) levels were significantly reduced in plantaris of untrained \(\alpha_{2A}/\alpha_{2C}\) ARKO mice (Fig. 4C). DHPR\(\beta_1\) subunit expression levels were significantly reduced in both soleus and plantaris of untrained \(\alpha_{2A}/\alpha_{2C}\) ARKO compared with WT control mice (Figs. 3D and 4D). RyR expression levels were significantly decreased in soleus of untrained \(\alpha_{2A}/\alpha_{2C}\) ARKO mice compared with WT control mice (Fig. 3E). No changes in plantaris RyR levels were observed between untrained \(\alpha_{2A}/\alpha_{2C}\) ARKO and WT groups (Fig. 4E). Of interest, exercise training reestablished the expression of altered Ca\(^{2+}\) handling proteins in soleus and plantaris of \(\alpha_{2A}/\alpha_{2C}\) ARKO mice toward untrained WT values (Figs. 3 and 4). Exercise training also increased DHPR\(\beta_1\) levels in plantaris (Fig. 4D) and RyR levels in both soleus and plantaris (Figs. 3E and 4E) of trained WT mice. These data suggest that exercise training efficiently reverses changes in expression level of proteins involved in sarco-

Table 1. Physiological parameters in untrained and exercise-trained wild-type and \(\alpha_{2A}/\alpha_{2C}\) ARKO mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WTun (n = 6)</th>
<th>WTtr (n = 5)</th>
<th>KOun (n = 5)</th>
<th>Kotr (n = 6)</th>
</tr>
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<tbody>
<tr>
<td>FS, %</td>
<td>31.2 ± 1.4</td>
<td>31.3 ± 2.1</td>
<td>24 ± 1.3†‡</td>
<td>30.7 ± 1.3†‡</td>
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<tr>
<td>HR, beats/min</td>
<td>585 ± 6</td>
<td>518 ± 5*</td>
<td>687 ± 5*‡</td>
<td>598 ± 6†‡</td>
</tr>
<tr>
<td>BP, mmHg</td>
<td>112 ± 6</td>
<td>113 ± 5</td>
<td>113 ± 5</td>
<td>108 ± 6</td>
</tr>
<tr>
<td>BW, g</td>
<td>28.6 ± 0.9</td>
<td>27.2 ± 0.4</td>
<td>26.4 ± 1.1</td>
<td>25.9 ± 0.5</td>
</tr>
<tr>
<td>Lung wet-to-dry ratio</td>
<td>5.5 ± 0.1</td>
<td>5.8 ± 0.1</td>
<td>6.6 ± 0.5†‡</td>
<td>5.8 ± 0.2†‡</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE for n mice. WT, wild-type mice; KO, \(\alpha_{2A}/\alpha_{2C}\) adrenoceptor knockout (ARKO) mice; un, untrained; tr, trained; FS, fractional shortening; HR, heart rate; BP, blood pressure; BW, body weight. *P < 0.05 vs. WTun; †P < 0.05 vs. KOun; ‡P < 0.05 vs. WTtr.

to-dry ratio compared with WT mice. No changes in BP and body weight were observed among groups. Exercise training increased cardiac function, decreased resting HR, and reduced lung wet-to-dry ratio in \(\alpha_{2A}/\alpha_{2C}\) ARKO mice toward the values in WT mice. Of interest, untrained \(\alpha_{2A}/\alpha_{2C}\) ARKO mice also displayed exercise intolerance, shorter step length, reduced fall angle, and diminished time in the Rotarod test, which are good markers of skeletal muscle dysfunction, compared with WT mice (Fig. 2). As expected, exercise training improved exercise capacity and skeletal muscle function in \(\alpha_{2A}/\alpha_{2C}\) ARKO mice (Fig. 2). Trained WT mice improved exercise tolerance compared with the untrained WT group.

Fig. 2. Exercise training increases skeletal muscle function of heart failure mice. Exercise capacity represented by maximal distance run (A), step length (B), fall angle (C), and Rotarod test (D), used as indexes of skeletal muscle function, were evaluated at 7 mo of age in untrained and exercise-trained WT and \(\alpha_{2A}/\alpha_{2C}\) ARKO mice. Sample size is indicated in the columns. Note that exercise training significantly improved distance run, step length, and fall angle in \(\alpha_{2A}/\alpha_{2C}\) ARKO mice. Data are presented as means ± SE. *P < 0.05 vs. untrained WT mice; †P < 0.05 vs. untrained \(\alpha_{2A}/\alpha_{2C}\) ARKO mice.

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Exercise training improves expression level of proteins involved in sarcoplasmic Ca\(^{2+}\) release in skeletal muscle from heart failure mice. Considering that a reduction in magnitude and a slowing of sarcoplasmic reticulum Ca\(^{2+}\) reuptake are associated with reductions in force and skeletal muscle fatigue in HF (19), we investigated whether the expression of SERCA1, SERCA2, NCX, and parvalbumin are altered in soleus and plantaris of our \(\alpha_{2A}/\alpha_{2C}\)ARKO mice and whether exercise training would change their expression profile.

As depicted in Fig. 5, untrained \(\alpha_{2A}/\alpha_{2C}\)ARKO mice displayed a prominent reduction in SERCA2 and SERCA1 expression levels in soleus (Fig. 5B) and plantaris (Fig. 5E), respectively, compared with WT control mice. NCX levels were reduced in both soleus and plantaris of untrained \(\alpha_{2A}/\alpha_{2C}\)ARKO mice compared with the WT group (Fig. 5, C and F). In addition, the expression levels of parvalbumin, a well-known cytosolic Ca\(^{2+}\) buffer, were significantly elevated in untrained \(\alpha_{2A}/\alpha_{2C}\)ARKO mice compared with the untrained WT group (Fig. 5G). Interestingly, exercise training notably increased SERCA2 and SERCA1 expression levels in soleus and plantaris of \(\alpha_{2A}/\alpha_{2C}\)ARKO mice, respectively, toward untrained WT levels (Fig. 5, B and E). Exercise training also elevated SERCA2 and SERCA1 levels in the trained WT group. NCX expression levels were significantly improved in trained \(\alpha_{2A}/\alpha_{2C}\)ARKO and WT groups compared with genotype-matched untrained mice (Fig. 5, C and F). Exercise training had a significant effect in parvalbumin expression levels in plantaris of trained WT mice (Fig. 5G). These results suggest that HF-induced changes in skeletal muscle expression levels of proteins involved in sarcoplasmic Ca\(^{2+}\) reuptake are reversed by exercise training in soleus and plantaris muscles.
DISCUSSION

Our results, based on a well-established aerobic exercise training protocol, show that exercise training-induced changes in the net balance of skeletal muscle Ca\(^{2+}\) handling proteins contribute to improved exercise tolerance and muscle performance in sympathetic hyperactivity-induced HF mice. The main findings of the present study are that exercise training reestablished the expression levels of proteins involved in sarcoplasmic Ca\(^{2+}\) release (DHPR\(_{\alpha 1}\) subunit, DHPR\(_{\alpha 2}\) subunit, DHPR\(_{\beta 1}\) subunit, and RyR) and reuptake (SERCA1, SERCA2, and NCX) in soleus and plantaris muscles of \(\alpha_{2A}/\alpha_{2C}\) ARKO mice toward untrained WT values (see Fig. 6, a schematic illustration of the main results of the present study).

Large-scale epidemiologic studies demonstrated that low aerobic exercise capacity in subjects with cardiovascular disease is a stronger predictor of mortality than other established risk factors (3, 5). Therefore, the mechanisms underlying exercise intolerance in HF are of main interest. Accumulated evidence indicates skeletal muscle myopathy as a main contributor to reduced exercise capacity in HF. Muscles are often atrophied with reduced muscle oxidative capacity and impaired contractile properties, which further culminates in muscle fatigue. We previously demonstrated (1) that sympathetic hyperactivity-induced HF in mice is associated with skeletal muscle myopathy, and here we provide evidence for an imbalanced expression of Ca\(^{2+}\) handling protein-related muscular dysfunction in HF mice.

The process involved in excitation-contraction coupling in skeletal muscle is initiated when T tubules are depolarized, leading to conformational changes in the DHPR-RyR complex, leading to Ca\(^{2+}\) ion flow to the sarcoplasm, which triggers contraction. After contraction, SERCA performs the critical function of promoting muscle relaxation by sequestering Ca\(^{2+}\) from the sarcoplasm at the expense of ATP hydrolysis.
We showed that sympathetic hyperactivity-induced HF mice displayed significant decrease in expression profile of proteins involved in both sarcoplasmic reticulum Ca\(^{2+}\) release (different DHPR subunits and RyR) and reuptake (SERCA and NCX) in skeletal muscles comprising different fiber type compositions. This phenomenon was associated with muscle weakness and exercise intolerance. However, other studies observed accelerated Ca\(^{2+}\) release and reuptake (22, 28) or even increased RyR and SERCA protein levels that were not paralleled by changes in Ca\(^{2+}\) homeostasis and tetanic force (13) in moderate HF rats. These apparent contrasting results might be related to HF etiology or severity. In fact, some studies reported that during the time course of myocardial infarction-induced cardiac dysfunction in rats increased SERCA expression and activity in the early stage was reversed to decreased SERCA expression and activity and impaired Ca\(^{2+}\) homeostasis in the late stage associated with HF (13, 21, 28). Our data in severe HF mice support, at least in part, the hypothesis that decreases in expression of sarcoplasmic reticulum Ca\(^{2+}\)-related proteins are associated with muscle weakness and fatigue in chronic HF.

Aerobic exercise training is a potent adjuvant therapy for HF, with positive impact in both cardiac and skeletal mus-
Similar to increased levels of Ca\(^{2+}\) release-related protein, changes in Ca\(^{2+}\) reuptake-related protein levels were also observed after aerobic exercise training in HF mice. SERCA levels were downregulated in HF mice, and aerobic exercise training reestablished SERCA expression to WT levels. This is of particular interest since skeletal muscle is highly dependent on sarcoplasmic reticulum Ca\(^{2+}\) stores for a proper contraction.

To our knowledge, this study is the first to show that Ca\(^{2+}\) handling proteins are differentially regulated in muscles of HF mice comprising different fiber types, and aerobic exercise training plays an interestingly homeostatic role by reestablishing the protein expression to WT levels. Previous studies have reported that in HF slow-twitch muscles (e.g., soleus) are more susceptible to fatigue and reduction in muscle force than fast-twitch muscles (e.g., plantaris) (14). These responses might be related to specific properties of proteins embedded in sarcoplasmic reticulum of slow- and fast-twitch muscles. In fact, we showed that soleus (but not plantaris) of HF mice displayed a significant reduction in DHPR\(_{\alpha 1}\) (main subunit of DHPR pore) and RyR levels compared with WT mice. These changes would contribute to disrupted signal transduction through transverse tubule/sarcoplasmic reticulum junction (e.g., DHPR-RyR complex) and abrogated RyR opening during excitation-contraction coupling in slow-twitch muscles, probably anticipating fatigue response. It worth mentioning that slow-twitch muscles are more susceptible to mitochondrial Ca\(^{2+}\) overload since sarcoplasmic buffers, such as parvalbumin, are missing. In plantaris muscle, an upregulation of parvalbumin was observed in HF mice, probably as a compensatory response to increased sarcoplasmic Ca\(^{2+}\) levels, and aerobic exercise training kept parvalbumin levels increased. Of note, aerobic exercise training also increased parvalbumin expression levels in WT mice, which may increase sarcoplasmic Ca\(^{2+}\) buffer capacity, rendering a better contraction–relaxation synchronism.

In summary, the present findings provide evidence for the first time that sympathetic hyperactivity-induced HF mice display skeletal muscle dysfunction paralleled by impaired net balance of proteins involved in sarcoplasmic Ca\(^{2+}\) release and reuptake in muscles comprising different fiber types. Interestingly, aerobic exercise training, by rearranging the network of Ca\(^{2+}\) handling proteins, improved exercise tolerance and muscle performance in HF mice. However, we may not exclude that exercise training, by improving ventricular function and muscle perfusion, could also collaborate in the increased muscle performance presently observed. Altogether these results provide new insights on intracellular Ca\(^{2+}\) regulatory mechanisms underlying improved skeletal muscle contractility by aerobic exercise training in HF. Further studies on other intracellular targets are warranted.

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DISCLOSURES

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

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