Exercise training delays cardiac dysfunction and prevents calcium handling abnormalities in sympathetic hyperactivity-induced heart failure mice

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Medeiros A., Rolim NP, Oliveira RS, Rosa KT, Mattos KC, Casarini DE, Irigoyen MC, Krieger EM, Krieger JE, Negrão CE, Brum PC. Exercise training delays cardiac dysfunction and prevents calcium handling abnormalities in sympathetic hyperactivity-induced heart failure mice. J Appl Physiol 104: 103–109, 2008. First published November 1, 2007; doi:10.1152/japplphysiol.00493.2007.—Exercise training (ET) is a coadjuvant therapy in preventive cardiology. It delays cardiac dysfunction and exercise intolerance in heart failure (HF); however, the molecular mechanisms underlying its cardioprotection are poorly understood. We tested the hypothesis that ET would prevent Ca2+ handling abnormalities and ventricular dysfunction in sympathetic hyperactivity-induced HF mice. A cohort of male wild-type (WT) and congenic α2A/α2C-adrenoceptor knockout (α2A/α2CARKO) mice with C57BL6/J genetic background (3–5 mo of age) were randomly assigned into untrained and exercise-trained groups. ET consisted of 8-wk swimming session, 60 min, 5 days/wk. Fractional shortening (FS) was assessed by two-dimensional guided M-mode echocardiography. The protein expression of ryanodine receptor (RyR), phospho-Ser2809-RyR, sarcoplasmic reticulum Ca2+ ATPase (SERCA2), Na+/Ca2+ exchanger (NCX), phospholamban (PLN), phospho-Ser16-PLN, and phospho-Thr17-PLN were analyzed by Western blotting. At 3 mo of age, no significant difference in FS and exercise tolerance was observed between WT and α2A/α2CARKO mice. At 5 mo, when cardiac dysfunction is associated with lung edema and increased plasma norepinephrine levels, α2A/α2CARKO mice presented reduced FS paralleled by decreased SERCA2 (26%) while it restored the expression of phospho-Ser2809-RyR to WT levels. Collectively, we provide evidence that improved net balance of calcium handling proteins paralleled by a decreased sympathetic activity on ET are, at least in part, compensatory mechanisms against deteriorating ventricular function in HF.

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IT IS WIDELY RECOGNIZED that exercise training is effective in reducing a number of cardiovascular risk factors (9, 32). Moreover, accumulated evidence shows that exercise training is an important strategy for the prevention and treatment of cardiovascular diseases (11, 37). In heart failure (HF), exercise training improves patient outcome by increasing exercise tolerance (20, 25) and reducing dyspnea and fatigue (18). However, whether exercise training has any effect in developing HF is less studied. Indeed, cellular adaptations of the exercised myocardium are not fully understood, but increased myocyte contractility and calcium sensitivity have been associated with an improved heart function (22, 34).

Several Ca2+ handling proteins are involved in the maintenance of normal cardiac Ca2+ homeostasis and contractile function. Among these proteins, sarcoplasmic reticulum Ca2+ ATPase (SERCA2), ryanodine receptor (RyR), and Na+/Ca2+ exchanger are responsible for the balance between sarcoplasmic Ca2+ uptake and release, and extrusion by sarcolemma, respectively (3, 4, 23). Ca2+ uptake by SERCA2 is regulated by a phosphorylatable protein, phospholamban (PLN), which in its dephosphorylated form inhibits SERCA2 activity (15).

Abnormal Ca2+ homeostasis by perturbation in the expression or function of these major Ca2+-regulating proteins has been described in severe HF (2, 6, 15, 33). In a recent study, we found that exercise training improved the net balance of cardiac Ca2+ proteins involved in transsarcolemmal flux and sarcoplasmic reticulum reuptake of Ca2+ in mice lacking both α2A/α2C-adrenoceptors (α2A/α2CARKO), in which sympathetic hyperactivity causes HF (7, 34). At 7 mo of age, when α2A/α2CARKO mice display severe HF, exercise training restored cardiac Na+/Ca2+ exchanger expression levels, and increased SERCA2, and phosphorylated PLN at both residues Ser16 and Thr17 expression levels, which resulted in improvement in left ventricular function. These findings suggest that the improvement in intracellular Ca2+ regulation is a molecular mechanism underlying the benefits of exercise training on overall ventricular function in severe HF (34). Just as important, one might expect that exercise training before the development of HF would display a protective role preventing Ca2+ handling abnormalities and preserving cardiac function. However, the potential involvement of Ca2+ handling proteins in exercise-induced cardioprotection remains to be elucidated.

The present investigation was undertaken to test the hypothesis that exercise training would decrease sympathetic activity and delay the onset of ventricular dysfunction in α2A/α2CARKO mice. In addition, exercise training would prevent the alterations in the expression of Ca2+ handling proteins involved in transsarcolemmal Ca2+ flux, Ca2+ reuptake, and release by sarcoplasmic reticulum in developing HF in this genetic model. Briefly, we found that exercise training mark-
edly delayed the onset of cardiac dysfunction in α2A/α2CARKO mice. The molecular basis for this cardioprotection includes a positive balance of cardiac Ca²⁺ handling proteins, which might be favored by the decreased sympathetic hyperactivity after exercise training in this genetic model.

MATERIALS AND METHODS

Sampling

Animal care. A cohort of male wild-type (WT) and congenic α2A/α2CARKO mice with C57Bl6/J genetic background aged 3–5 mo were studied. In α2A/α2CARKO mice, cardiac function is preserved until 3 mo of age with no signs of lung water retention, whereas at 5 mo, cardiomyopathy is associated with a modest but significantly increased water retention in lungs (25% vs. 5-mo-old WT, P < 0.05), which suggests pulmonary edema at this age. Genotypes were determined by PCR on genomic DNA obtained from tail biopsies using primers to detect the intact and disrupted genes.

Mice were maintained in a light-controlled (12-h light cycle) and temperature-controlled (22°C) environment and were fed a pellet rodent diet (Nuvital Nutrients S/A, Curitiba, PR Brazil) ad libitum and had free access to water. WT and α2A/α2CARKO mice were randomly assigned into untrained and exercise-trained groups. This study accorded to Ethical Principles in animal research adopted by Brazilian College of Animal Experimentation (www.cobea.org.br). In addition this study was approved by the University of Sao Paulo Ethical Committee (CEP no. 004).

Measurements and Procedures

Exercise training protocol. Exercise training consisted of 5 day/wk swimming sessions with gradually increased duration to 60 min for 8 wk in a swimming warmed-water (30–32°C) apparatus adapted for mice (10). The training sessions were performed during the dark cycle of the mice. Untrained mice were placed in the swimming apparatus for 5 min twice a week to mimic the water stress associated with the experimental protocol and handling. This swimming protocol has been characterized previously as low to moderate intensity and long duration due to improvement in muscle oxidative capacity and resting bradycardia (10).

Graded treadmill exercise test. Exercise capacity, estimated by total distance run, was evaluated using a graded treadmill exercise protocol for mice. After being adapted to treadmill exercises over 1 wk (10 min of exercise session), mice were placed in the treadmill streak and allowed to acclimatize for at least 30 min. Exercise began at 6 min/m and no grade and increased by 3 m/min every 3 min thereafter until exhaustion. The graded treadmill exercise test was performed in WT and α2A/α2CARKO mice before and after the exercise training period.

Cardiovascular measurements. Heart rate (HR) was determined noninvasively using a computerized tail-cuff system (BP 2000 Visitech Systems) described elsewhere (17). Mice were acclimatized to the apparatus during daily sessions over 6 days, 1 wk before starting the experimental period. HR measurements were obtained serially in WT and α2A/α2CARKO mice once a week throughout the 8 wk of experiment.

Noninvasive cardiac function was assessed by two-dimensional guided M-mode echocardiography, in halothane-anesthetized WT and α2A/α2CARKO mice, before and after the experimental period. 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 using an Acuson Sequoia model 512 echocardiographer equipped with a 14-MHz linear transducer. Left ventricle systolic function was estimated by fractional shortening as follows:

\[
\text{fractional shortening (\%)} = \frac{[(\text{LVEDD} - \text{LVESD})/\text{LVEDD}] 	imes 100}{100}
\]

where LVEDD means left ventricular end-diastolic dimension, and LVESD means left ventricular end-systolic dimension.

Plasma norepinephrine levels. Plasma norepinephrine was measured by HPLC using ion-pair reverse-phase chromatography coupled with electrochemical detection (0.5 V) as described by Monte et al. (29).

Antibodies. Mouse monoclonal antibodies to SERCA2 (1:2,500), PLN (1:5,000), RyR1 (1:5,000), and Na⁺/Ca²⁺ exchanger (1:2,000) were obtained from Affinity Bioreagents (Golden, CO); rabbit polyclonal phospho-Ser²⁸⁰⁹-RyR2 (1:2,000), phospho-Ser⁴⁸⁶-PLN (1:5,000), and phospho-Thr¹⁷-PLN (1:5,000) were obtained by Badrilla (Leeds, UK). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:2,000) was obtained from Advanced Immunnochemical (Long Beach, CA). Targeted bands were normalized to cardiac GAPDH.

Western blot analysis. Left ventricular homogenates were analyzed by Western blotting to compare SERCA2, PLN, phospho-Ser⁴⁸⁶-PLN, phospho-Thr¹⁷-PLN, Na⁺/Ca²⁺ exchanger, RyR1, and phospho-Ser²⁸⁰⁹-RyR2. Briefly, liquid nitrogen-frozen ventricles isolated from WT and α2A/α2CARKO mice were homogenized in a buffer containing 50 mM potassium phosphate buffer (pH 7.0), 0.3 M sucrose, 0.5 mM DTT, 1 mM EDTA (pH 8.0), 0.3 mM PMSF, 10 mM NaF, and phosphatase inhibitor cocktail (1:100, Sigma-Aldrich; Saint Louis, MO). Samples were subjected to SDS-PAGE in polyacrylamide gels (6% or 10% depending on protein molecular weight). After electrophoresis, proteins were electrotransferred to nitrocellulose membrane (Amersham Biosciences; Piscataway, NJ). Equal loading of samples (50 μg) and even transfer efficiency were monitored with the use of 0.5% Ponceau S staining of the blot membrane. The blotted membrane was then blocked (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 incubated with specific antibodies overnight at 4°C. Binding of the primary antibody was detected with the use of peroxidase-conjugated secondary antibodies (rabbit or mouse depending on the protein, 1:10,000, for 1 h:30 min at room temperature) and developed using 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).

Statistical Analysis

Data are presented as means ± SE. Two-way ANOVA for repeated measurements 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 α2A/α2CARKO) on plasma norepinephrine levels and protein expression levels. Statistical significance was considered achieved when the value of P was <0.05.

RESULTS

Effect of Exercise Training on Exercise Tolerance, Cardiac Contractility, and Plasma Norepinephrine Levels

At 3 mo of age, there was no difference in distance run and fractional shortening between WT and α2A/α2CARKO mice (Fig. 1). However, at 5 mo of age, α2A/α2CARKO mice displayed exercise intolerance and systolic dysfunction compared with age-matched WT mice. Exercise training in α2A/α2CARKO mice not only suppressed the decrease in exercise tolerance, but also increased it towards exercise-trained WT mice (Fig. 1A). In addition, exercise training prevented the systolic dysfunction in α2A/α2CARKO mice (Fig. 1B).

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α2A/α2CARKO mice displayed baseline tachycardia compared with age-matched WT mice (Fig. 2A). Exercise training significantly decreased baseline HR in both WT and α2A/α2CARKO mice from the 4th week to the end of the exercise training period. The reduction of HR in α2A/α2CARKO was so remarkable that it reached untrained WT levels at the 5th week of training (Fig. 2A).

Expression of Proteins Involved in Transsarcolemmal Flux and Sarcoplasmic Reuptake of Ca2+

Since downregulation of cardiac SERCA2 expression is associated with clinical signs of HF (12), we investigated whether sympathetic hyperactivity in our genetic model would alter the expression of SERCA2, Na+/Ca2+ exchanger, PLN, phospho-Ser16-PLN, and phospho-Thr17-PLN. Additionally, we tested whether exercise training would prevent these alterations in α2A/α2CARKO mice.

GAPDH protein levels were not different among the four groups studied. The expression of SERCA2 and Na+/Ca2+ exchanger was reduced in untrained α2A/α2CARKO mice by 26% and 34%, respectively (Fig. 4, A–C) compared with untrained WT. Exercise training caused no effect on Na+/Ca2+ exchanger expression levels in α2A/α2CARKO mice (Fig. 4, A and C) but significantly increased SERCA2 (Fig. 4, A and B) in α2A/α2CARKO mice compared with age-matched WT mice (Fig. 3, A and C). Phospho-Ser2809-RyR expression levels remained unchanged in exercise-trained WT mice. However, exercise training in α2A/α2CARKO mice decreased phospho-Ser2809-RyR expression toward WT levels (Fig. 3, A and C).

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Fig. 2. Heart rate (HR, A) and plasma norepinephrine levels (NE, B) in untrained and exercise-trained wild type (WTUN and WTT, respectively), and untrained and exercise-trained α2A/α2C-adrenoceptor knockout (α2A/α2CARKO) mice (DKOUN and DKOT, respectively). Note that exercise training caused no effect on Na+/Ca2+ exchanger expression levels in α2A/α2CARKO mice (Fig. 4, A and C) but significantly increased SERCA2 (Fig. 4, A and B).

Expression of Proteins Involved in Sarcoplasmic Ca2+ Release

As hyperphosphorylation of RyR is associated with deleterious effect of cardiac sympathetic hyperactivity (6, 33), we investigated whether RyR is hyperphosphorylated in our α2A/α2CARKO mice, and whether exercise training by decreasing sympathetic activity would restore RyR phosphorylation to WT mice levels.

While RyR expression remained unchanged among the four groups studied (Fig. 3, A and B), the phospho-Ser2809-RyR expression levels were significantly increased by 49% in α2A/α2CARKO mice compared with age-matched WT mice (Fig. 3, A and C). Phospho-Ser2809-RyR expression levels remained unchanged in exercise-trained WT mice. However, exercise training in α2A/α2CARKO mice decreased phospho-Ser2809-RyR expression toward WT levels (Fig. 3, A and C).

Fig. 1. Exercise capacity (A) represented by maximal distance run, and fractional shortening (FS, B) used as an index of systolic function, were evaluated at 3 and 5 mo of age in untrained and exercise-trained wild-type mice (WTUN and WTT, respectively), and untrained and exercise-trained α2A/α2C-adrenoceptor knockout (α2A/α2CARKO) mice (DKOUN and DKOT, respectively). Note that exercise training significantly improved distance run and FS in α2A/α2CARKO mice. Data are presented as means ± SE. *P < 0.05 vs. 3 mo of age; †P < 0.05 vs. WTUN group.

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expression. This increase in SERCA2 expression was so dramatic that it reached untrained WT mice levels. In WT mice, exercise training did not change SERCA2 or Na\(^+\)/H\(^+\) exchanger expression levels.

To evaluate the effect of exercise training on the balance between Ca\(^2+\) reuptake by SERCA2 and Ca\(^2+\) transsarcolemmal elimination by Na\(^+\)/Ca\(^2+\) exchanger, we calculated the SERCA2-to-Na\(^+\)/Ca\(^2+\) exchanger ratio (SERCA2:NCX ratio). SERCA2:NCX ratio was similar in untrained \(\alpha_{2A}/\alpha_{2C}\)ARKO and WT mice (Fig. 4, A and D). In \(\alpha_{2A}/\alpha_{2C}\)ARKO mice, exercise training significantly increased SERCA2:NCX ratio (Fig. 4, A and D). In WT mice, exercise training caused no change in SERCA2:NCX ratio.

As PLN controls the apparent Ca\(^2+\) affinity of SERCA2 (24), we additionally evaluated the expression of PLN and phosphorylated PLN at both Ser\(^{16}\) and Thr\(^{17}\) residues in \(\alpha_{2A}/\alpha_{2C}\)ARKO mice. PLN and phospho-Thr\(^{17}\)-PLN expressions were similar among all groups studied (Fig. 5, A, B, and D). Phospho-Ser\(^{16}\)-PLN was increased by 76% in untrained \(\alpha_{2A}/\alpha_{2C}\)ARKO mice compared with untrained WT mice (Fig. 5, A and C). Although exercise training had no impact on PLN and phospho-Thr\(^{17}\)-PLN expression levels in either WT or \(\alpha_{2A}/\alpha_{2C}\)ARKO mice, its effect on phospho-Ser\(^{16}\)-PLN expression levels was remarkable. Exercise training...
increased phospho-Ser$^{16}$-PLN expression in both WT and $\alpha_{2A}/\alpha_{2C}$ARKO mice (Fig. 5, A and C).

DISCUSSION

A wealth of data indicates that regular exercise can protect individuals from a host of cardiovascular and metabolic diseases (11, 19, 37). Indeed, exercise training is emerging as a key intervention for preventive cardiology (13, 36). However, the mechanisms by which exercise training can delay the onset of cardiovascular diseases are not completely understood. In the present investigation we demonstrate that exercise training in sympathetic hyperactivity-induced HF mice decreased plasma norepinephrine levels, and prevented Ca$^{2+}$ handling abnormalities and cardiac dysfunction.

Sympathetic hyperactivity plays a prominent role in the pathogenesis and evolution of cardiovascular diseases (8). Therefore, the decreased plasma norepinephrine levels and resting heart rate paralleled by improved fractional shortening in exercise-trained $\alpha_{2A}/\alpha_{2C}$ARKO mice suggest the positive impact of exercise training in the progression of HF. In fact, therapies that reduce sympathetic tone have been associated with a decreased risk factor for cardiovascular disease (8, 28) and an improved prognosis (8).

The key findings of the present study are that exercise training in $\alpha_{2A}/\alpha_{2C}$ARKO mice restored the phosphorylation of RyR at Ser$^{2809}$ to WT levels and increased the phosphorylation of PLN at Ser$^{16}$. These results suggest that the mechanisms underlying the amelioration in ventricular function include the prevention of cardiac Ca$^{2+}$ handling abnormalities by changing phosphorylation status of proteins involved in sarcoplasmic Ca$^{2+}$ release and reuptake.

The increased RyR phosphorylation at Ser$^{2809}$ in untrained $\alpha_{2A}/\alpha_{2C}$ARKO mice was somehow expected since hyperphosphorylation of RyR by cAMP- and Ca$^{2+}$/calmodulin-dependent protein kinases (PKA and CAMKII, respectively) are commonly observed in the hyperadrenergic state (6, 33, 39). The reduced expression of phospho-Ser$^{2809}$-RyR in exercise-trained $\alpha_{2A}/\alpha_{2C}$ARKO mice toward WT levels seems to be beneficial because chronic hyperphosphorylation of RyR is associated with diastolic Ca$^{2+}$ leak, leading to arrhythmogenicity (31, 35), and cardiac dysfunction. On the basis of the fact that the reduction in phospho-Ser$^{2809}$-RyR expression in exercise-trained $\alpha_{2A}/\alpha_{2C}$ARKO mice paralleled the decreased plasma norepinephrine levels, it is reasonable to speculate that reduced RyR phosphorylation at Ser$^{2809}$ is due to a decreased sympathetic drive.

Our study regarding the expression of proteins involved in intracellular Ca$^{2+}$ decline suggests that the increased phospho-Ser$^{16}$-PLN and decreased Na$^+/Ca^{2+}$ exchanger expression levels in untrained $\alpha_{2A}/\alpha_{2C}$ARKO mice may represent a compensatory mechanism against deteriorating cardiac function at the stage of sympathetic hyperactivity-induced cardiomyopathy presently studied. However, the compensatory mechanism eventually fails in the more advanced-stage cardiomyopathy, as we previously demonstrated by further deteriorated cardiac function associated with reduced phosphorylation of PLN at Ser$^{16}$ and increased Na$^+/Ca^{2+}$-exchanger expression in older $\alpha_{2A}/\alpha_{2C}$ARKO mice (7 mo of age), when sympathetic hyperactivity is associated with severe HF and 50% mortality rate (34). In fact, increased activity of Na$^+/Ca^{2+}$ exchanger (16,
22, 38) and reduced cardiac phosphorylation of Ser<sup>16</sup>-PLN together with increased Thr<sup>17</sup>-PLN have been reported in end-stage HF (21, 27, 34).

Despite the fact that exercise training reduced plasma noradrenaline levels in α<sub>2A/α2C</sub>-ARKO mice, the expression of phospho-Ser<sup>16</sup>-PLN was further increased while no changes were observed in phospho-Thr<sup>17</sup>-PLN expression. This result suggests that daily exercise stimulus is able to increase phosphorylation status of PLN at Ser<sup>16</sup> independent of cardiac sympathetic drive. Although the intracellular pathways involved in this response remain unknown, local regulation of PLN phosphorylation through a variety of phosphatases, kinases, and kinase-anchoring proteins could be considered as alternative mechanisms.

Recent studies have demonstrated that prior exercise training improves hypertension-induced HF (9) and ischemia-reperfusion injury (14) outcome, despite no changes or subtle increase in SERCA2 expression. These results suggest that the mechanism underlying exercise-induced cardioprotection may be influenced by factors such as training regimen and HF etiology. Our study shows that in a genetic model of sympathetic hyperactivity-induced HF, exercise training restored SERCA2 expression to control levels. In addition, Na<sup>+</sup>/Ca<sup>2+</sup> exchanger expression remained decreased in exercise-trained α<sub>2A/α2C</sub>-ARKO mice. Under this scenario, one may consider that exercise training favors Ca<sup>2+</sup> reuptake by sarcoplasmic reticulum, preventing Ca<sup>2+</sup> extrusion by sarcolemma.

**Study Limitations**

Our study shows that exercise training prevents both cardiac dysfunction and Ca<sup>2+</sup> handling abnormalities in developing HF. However, it does not provide direct evidence to support the cause-effect relationship between Ca<sup>2+</sup> handling protein expression and cardiac function. Although Ca<sup>2+</sup> transients tend to parallel changes in the expression of cardiac Ca<sup>2+</sup> handling proteins and ventricular function are attributed to an improvement of cardiac Ca<sup>2+</sup> transients.

The fractional shortening values of WT mice observed in the present study were lower than observed in our previous work (7). This was a matter of anesthesia used in the present study (halothane presently used vs. isoflurane), since a fractional shortening of 35% was observed in untrained WT mice when echocardiography evaluations were performed under isoflurane anesthesia. Thus the results obtained under halothane anesthesia are reliable, since we could reproduce the same results using isoflurane anesthesia, which has less impact than halothane on cardiac contractility.

**Conclusions**

Our findings support the idea that in a setting of developing HF, exercise training can delay cardiac dysfunction, which can be attributed, at least in part, to a positive balance of cardiac proteins involved in sarcoplasmic Ca<sup>2+</sup> release and reuptake. Altogether, we provide new insights on the molecular mechanisms whereby exercise training can contribute to delay cardiac dysfunction in a genetic model of sympathetic hyperactivity-induced HF.

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