Exercise training improves the net balance of cardiac Ca\(^{2+}\) handling protein expression in heart failure

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There is convincing evidence for the benefits of regular exercise training in heart failure (HF) patients (33). The improvement in peak oxygen uptake, quality of life, and functional class after exercise training in HF patients strongly suggests that this non-pharmacological strategy plays an important role for the treatment of HF (16, 33). However, the underlying mechanism by which exercise training improves clinical outcome in HF patients is still under investigation. In a previous study we demonstrated a significant reduction in muscle sympathetic nerve activity after exercise training in chronic HF patients (33). Other studies show that exercise training caused a significant increase in endothelial function (40). These neural and vascular improvements result in less vasocostriction and increased peripheral oxygen supply (38), possibly leading to a decreased intracellular TNF-\(\alpha\) and interleukin-1 expression (11). The consequence of this peripheral adaptation is the increase in muscle oxidative capacity and exercise tolerance in HF patients.

The cardiac effects of exercise training in HF are less understood. Some investigators have reported that exercise training has no effect in cardiac output (39, 26). In contrast, recent studies have shown that exercise training increases stroke volume and, hence, cardiac output in patients with HF (7, 9).

Ventricular function is highly coupled with calcium transients in the heart, and myocardial dysfunction observed in severe HF is caused mainly by alterations in phosphorylation status of sarcomeric proteins (19) and a diminished sarcoplasmic reticulum Ca\(^{2+}\) load that arises from enhanced activity and expression of Na\(^+\)-Ca\(^{2+}\) exchanger (41), reduced sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA2) function (32), and increased diastolic Ca\(^{2+}\) leak via ryanodine receptors (1, 22). In addition, alterations of SERCA2 activity have been attributed to a phosphorylatable protein, phospholamban (PLN) (8, 13). which in its dephosphorylated form, decreases the apparent Ca\(^{2+}\) affinity of SERCA2. PLN protein levels are unchanged in HF (25) patients. However, PLN phosphorylation at Ser16 by protein kinase A seems to be decreased, while Thr24 phosphorylation by Ca\(^{2+}\)-calmodulin-dependent protein kinase II is increased in aortic stenosis-induced HF (1, 24). Thus, Ca\(^{2+}\) handling protein abnormalities often accompany the development of HF and may be considered markers or potential new therapeutic targets.

We have previously reported that mice lacking both \(\alpha_{2A}/\alpha_{2C}\)-adrenoceptors (\(\alpha_{2A/\alpha_{2C}}\)ARKO) develop sympathetic hyperactivity-induced HF (4). Therefore, these mice provide a model system for better understanding the mechanisms underlying the cardiac deleterious effect of sympathetic hyperactivity, as well as, to test different therapeutic strategies for HF.

In the present study, we tested three hypotheses: 1) that ventricular dysfunction of \(\alpha_{2A/\alpha_{2C}}\)ARKO is associated with abnormalities in the cardiac expression of Ca\(^{2+}\) handling proteins. 2) that exercise training can increase overall cardiac function in a genetic model of sympathetic hyperactivity-induced HF, and 3) that exercise training can improve the net balance of cardiac Ca\(^{2+}\) handling proteins involved in transsarcomembral flux and sarcoplasmic reticulum reuptake of Ca\(^{2+}\) in this genetic model of HF.
CARCICD Ca HANDLING IN TRAINED HEART FAILURE MICE

MATERIALS AND METHODS

Sampling

Animals' care. A cohort of male wild-type (WT) and congenic α2α2α2α2ARKO mice in a C57BL/6J genetic background were studied from 2 to 7 months of age. At this age, α2α2α2α2ARKO mice display advanced stage of cardiomyopathy at previously described (4). Genotypes were determined by polymerase chain reaction on genomic DNA obtained from tail biopsies using primers to detect the intact and disrupted genes.

Mice were maintained in a light (12-h light cycle) and temperature (22°C)-controlled environment and were fed a pellet rodent diet (Nuvital Nutrimentos, Curitiba, PR Brazil) ad libitum and had free access to water. WT and α2α2α2α2ARKO mice were randomly assigned into untreated and exercise-trained groups. This study was conducted in accordance with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (www.cobea.org.br).

Measurements and Procedures

Gated treadmill exercise test. Exercise capacity, estimated by total distance run, was evaluated with 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 exercise streak and allowed to acclimatize for at least 30 min. Exercise began at 6 m/min with no grade and increased by 3 m/min every 3 min thereafter until exhaustion. WT and α2α2α2α2ARKO mice performed the graded treadmill exercise test before and after the experimental period.

Exercise training protocol. Low intensity exercise training was performed on a motor treadmill over 8 wk, 5 days/wk (4–5). The running speed and duration of exercise were progressively increased to elicit 60% of maximal speed, achieved during a graded treadmill exercise protocol, for 60 min at the fourth week. This intensity was maintained during the rest of the 8-wk training period. All untrained mice were exposed to treadmill exercise (5 min) three times a week to become accustomed to the protocol and handling.

Cardiovascular measurements. Heart rate (HR) was determined noninvasively with a computerized tail-cuff system (BP 2000 Visitech Systems) described elsewhere (15). Mice were acclimatized to the apparatus during daily sessions over 6 days, 1 wk before the experimental period started. HR measurements were obtained serially in WT and α2α2α2α2ARKO 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 α2α2α2α2ARKO 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 echocardiograph equipped with a 14-MHz linear transducer. Left ventricle systolic function was estimated by fractional shortening as follows: Fractional Shortening (%) = [(LVEDD – LVESD)/LVEDD] X 100, where, LVEDD means left ventricular end-diastolic dimension, and LVESD means left ventricular end-systolic dimension.

Estimation of cardiac sympathetic tone. To confirm that α2α2α2α2ARKO mice have increased cardiac sympathetic tone, we measured the HR (electrocardiogram) after pharmacological blockade of muscarinic receptors with methylatropine (1 mg/kg, Sigma Chemical) and β-adrenergic receptors with propranolol (3 mg/kg, Sigma Chemical) at the end of the experimental protocol. The sympathetic tone was analyzed as the difference between the maximum HR after methylatropine injection and the intrinsic HR (HR after muscarinic and β-adrenergic receptor blockade) (10, 21, 28).

Structural analysis. Twenty-four hours after the last exercise training session, untrained and exercise-trained α2α2α2α2ARKO and WT control mice were killed and their tissues harvested. Cardiac chambers were then fixed by immersion in 4% buffered formalin and embedded in paraffin for routine histological processing. Sections (6 μm) were stained with hematoxylin and eosin for examination by light microscopy. Only nucleated cardiac myocytes from areas of transversely cut muscle fibers were included in the analysis. Quantification of left ventricular fibrosis was achieved by Sirius red staining. Cardiac myocyte width and ventricular fibrosis were measured in the LV free wall with a computer-assisted morphometric system (Leica Quantimet 500, Cambridge, UK).

Antibodies. Mouse monoclonal antibodies to SERCA2 (1:2500), PLN (1:500), and Na+-Ca2+ exchanger (1:2000) were obtained from Affinity BioReagents (Golden, CO); rabbit polyclonal antibody to protein phosphatase type 1 (PPI, 1:1000) was obtained from Upstate Biotechnology (Lake Placid, NY); phospho-Ser16PLN (1:5000) and phospho-Thr21PLN (1:5000) were obtained from Badrilla (Leeds, UK). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2000) was obtained from Advanced Immunoneal (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-Ser16PLN, phospho-Thr21PLN, PPI, and Na+-Ca2+ exchanger. Briefly, liquid nitrogen frozen ventricles isolated from WT and α2α2α2α2ARKO mice were homogenized in a buffer containing 50 mM potassium phosphate buffer (pH 7.0), 0.3 M sucrose, 0.5 mM EDTA (pH 8.0), 0.3 mM PMSE, 10 mM NaF, and phosphatase inhibitor cocktail (1:100, Sigma–Aldrich, St. Louis, MO). Samples were subjected to SDS-PAGE in polyacrylamide gels (6 or 10% depending on protein molecular weight). After electrophoresis, proteins were electro-transferred to nitrocellulose membrane (American Biosciences, Piscovit, NJ). Equal loading of samples (50 μg) and even transfer efficiency were monitored with the use of 0.3% 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 130 h at room temperature) and developed by enhanced chemiluminescence (American 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. For distance run, fractional shortening, and HR measurements, comparisons of untrained and exercise-trained α2α2α2α2ARKO and WT control mice were performed by two-way ANOVA for repeated measurements with post hoc testing by Duncan (Statistica software; StatSoft, Tulsa, OK). For cardiac structural analysis, cardiac sympathetic tonus, and protein expression levels, comparison among all groups were performed by two-way ANOVA with post hoc testing by Duncan (Statistica Software, StatSoft). Probability values <0.05 were considered statistically significant.

RESULTS

Effect of Exercise Training on Exercise Tolerance and HR

α2α2α2α2ARKO mice displayed exercise intolerance compared with WT control mice (288 ± 14 vs. 359 ± 9 m, P = 0.05). Exercise-trained α2α2α2α2ARKO mice increased exercise capacity toward untrained WT mice levels (365 ± 8 vs. 359 ± 9 m). As expected, exercise training increased exercise toler-
Cardiac Contractility and Myocyte Structure

Baseline fractional shortening was significantly lower in untrained α2A/α2C-ARKO mice compared with age-matched untrained WT control mice (Fig. 2). While exercise training did not change fractional shortening in WT mice, it significantly increased fractional shortening toward normal levels in α2A/α2C-ARKO mice (Fig. 2). The quantitative morphometrical analysis showed that cardiac myocyte cross-sectional diameter was significantly greater in untrained α2A/α2C-ARKO mice compared with untrained WT control mice (Fig. 3A). Cardiac myocyte cross-sectional diameter was significantly lower in exercise-trained α2A/α2C-ARKO mice than in untrained α2A/α2C-ARKO mice, but significantly greater than in untrained WT control mice (Fig. 3A). Increased cardiac myocyte cross-sectional diameter in untrained α2A/α2C-ARKO mice was paral-

Fig. 1. Heart rate (HR, A) and cardiac sympathetic tone (ST, B) in untrained and exercise-trained wild-type (WTUN and WTT, respectively) and α2A/α2C-adrenoceptor knockout (ARKO; DKOUN and DKOT, respectively) mice. Note that exercise training decreased HR and ST in α2A/α2C-ARKO mice to untrained WT control mouse levels. As expected, WTT displayed bradycardia. Data are presented as means ± SE; *P < 0.05 vs. baseline levels, †P < 0.05 vs. untrained group.

Fig. 2. Fractional shortening (FS) used as an index of systolic function evaluated at 5 (G) and 7 (b) mo of age in WTUN and WTT, respectively, and α2A/α2C-ARKO. Note that exercise training significantly improved FS in α2A/α2C-ARKO mice. Data are presented as means ± SE. *P < 0.05 vs. WT groups, †P < 0.05 vs. DKOUN group.

Fig. 3. Cardiac myocyte cross-sectional diameter (A) and cardiac collagen volume fraction (B) in untrained (G) and exercise-trained (b) wild-type (WT) and α2A/α2C-ARKO (DKO) mice. Exercise training partially reduced cardiac myocyte cross-sectional diameter in α2A/α2C-ARKO and had no impact on ventricular fibrosis as measured by collagen volume fraction. Data are presented as means ± SE. *P < 0.05 vs. WT groups, †P < 0.05 vs. untrained DKO group.
leled by an increased ventricular fibrosis, represented by a threefold increase in cardiac collagen volume fraction of α2A/α2C/AR KO mice when compared with untrained WT control mice (Fig. 3B). Exercise training tended to reduce left ventricular fibrosis in α2A/α2C/AR KO mice, but this result did not reach significantly values (Fig. 3B). Exercise trained WT mice did not change either myocyte cross-sectional diameter or cardiac collagen volume fraction when compared with untrained WT mice.

**Expression of Proteins Involved in Intracellular Ca2+ Regulation**

Since it has been demonstrated that the expression of calcium handling proteins is altered in HF (17, 37), we investigated whether the expression of these proteins is altered in our α2A/α2C/AR KO mice and whether exercise training would change their expression profile.

GAPDH protein levels remained unchanged among the three groups studied and were used to normalize the Ca2+ handling protein levels. SERCA2 expression levels were significantly reduced in untrained α2A/α2C/AR KO mice compared with untrained WT control mice (Fig. 4, A and B). In contrast, no significant differences were found in SERCA2 expression between exercise-trained α2A/α2C/AR KO mice and WT control mice. Na+·Ca2+ exchanger expression levels were significantly increased in untrained α2A/α2C/AR KO mice (Fig. 4, A and C). Exercise training significantly reduced Na+·Ca2+ exchanger expression in α2A/α2C/AR KO mice to same level observed in untrained WT control mice (Fig. 4, A and C). In contrast, exercise training in WT mice had no impact on either SERCA2 or Na+·Ca2+ exchanger expression levels. As the sarcoplasmic Ca2+ content depends on Ca2+ reuptake by SERCA2 relative to transsarcolemmal Ca2+ elimination by Na+·Ca2+ exchanger, we calculated the SERCA2/Na+·Ca2+ exchanger ratio for all mice studied (Fig. 4D). SERCA2/Na+·Ca2+ exchanger ratio tended to be reduced in untrained α2A/α2C/AR KO mice (P = 0.08). Exercise training increased SERCA2: Na+·Ca2+ exchanger ratio in α2A/α2C/AR KO mice to the level observed in WT control mice (Fig. 4D).

Despite the fact that SERCA2 activity is under PLN control, PLN expression levels were similar among the three groups studied (data not shown), while the expression of phosphorylated PLN was changed by both genotype and exercise training (Fig. 5). In untrained α2A/α2C/AR KO mice, the expression of phospho-Ser16-PLN normalized to total PLN was not significantly different from untrained WT control mice (Fig. 5, A and B). However, exercise training significantly increased phospho-Ser16-PLN in α2A/α2C/AR KO, while it did not change it in WT mice. The expression of phospho-Thr17-PLN normalized to total PLN was higher in untrained α2A/α2C/AR KO mice than in untrained WT control mice. Exercise training further increased phospho-Thr17-PLN expression levels in α2A/α2C/AR KO (Fig. 5, A and C). This response was also observed in exercise-trained WT mice, which displayed increased phospho-Thr17-PLN expression levels compared with untrained WT mice.

PLN phosphorylation is mainly regulated by PP1, and the expression and activity of PP1 seem to be increased in end-stage HF (29, 42). The expression of PP1 was increased in untrained α2A/α2C/AR KO mice compared with untrained WT control mice (Fig. 5, A and D). Exercise training significantly reduced PP1 expression to levels similar to WT control mice (Fig. 5, A and D), while no changes in PP1 expression levels were observed in exercise-trained WT mice.

**DISCUSSION**

A great body of evidence indicates that exercise training has a beneficial effect on the treatment of HF patients including an improvement in stroke volume and cardiac output (7, 9). However, the exact mechanism by which exercise training...
improves cardiac function remains elusive. In the present study we demonstrate first that this genetic model of HF is also accompanied by Ca^{2+} handling abnormalities, second, that exercise training improves ventricular function in these mice, and finally we provided direct evidence for the involvement of Ca^{2+} handling proteins in this process.

Symptomatic exacerbation is the hallmark of HF and has been associated with high morbidity and mortality (34). Usually this is a secondary phenomenon, but under the conditions of the present study sympathetic augmentation was directly achieved by the use of genetically engineered mice. Interestingly, even though the experimental conditions suppressed the effects of negative feedback signals, we still observed some degree of reduction in cardiac sympathetic tone upon exercise training.

The novel finding of the present study is that exercise training increases the cardiac expression of SERCA2 and phosphorylation of PLN at Ser^{16} and Thr^{17} in α_{2A}/α_{2C}ARKO mice. In addition, exercise training restored Na+-Ca^{2+} exchanger and PPI expression levels in these mice. Presumably, both changes tend to improve Ca^{2+} handling under this condition. Interestingly, the effect of exercise training on SERCA2, Na+-Ca^{2+} exchanger, and PPI was only observed in α_{2A}/α_{2C}ARKO mice; no changes were observed in WT mice. These responses suggest that training effects are restricted to proteins with altered baseline expression levels.

Previous studies have shown that exercise training improves sarcoplasmic reticulum Ca^{2+} uptake paralleled by a normalized Na+-Ca^{2+} exchanger current and increased expression of SERCA2 in ischemia-induced HF rats (20, 43). Our findings extend these observations to show that exercise training also is associated to an increased phosphorylation of PLN at Ser^{16} and Thr^{17} and decreased PPI expression levels in α_{2A}/α_{2C}ARKO mice with sympathetic hyperactivity-induced HF. Under this scenario one may consider the possibility that phosphorylation of PLN at either Ser^{16} or Thr^{17} removes the inhibitory effect of PLN on SERCA2. Furthermore, PLN acts as an integrator of β-adrenergic and Ca^{2+}-dependent signaling pathways to promote increased myocardial contractility. Thus, it is reasonable to suggest that exercise training-induced augmentation of phosphorylated PLN cardiac levels improves Ca^{2+} reuptake and fractional shortening in α_{2A}/α_{2C}ARKO mice. It is important to note that the phosphorylation of PLN at Ser^{16} or Thr^{17} is differentially affected by training and genotype. While the effects of exercise training on phosphorylation of PLN at Ser^{16} are specific to α_{2A}/α_{2C}ARKO mice, its effect at Thr^{17} occurred in both WT and α_{2A}/α_{2C}ARKO mice, even with the latter presenting higher phospho-Thr^{17}PLN baseline expression levels than WT controls. These responses suggest that the increased expression of phosphorylated PLN at Ser^{16} might play a role improving ventricular function of exercise trained α_{2A}/α_{2C}ARKO mice, since increased phospho-Thr^{17}PLN expression levels were not related to an improved fractional shortening in exercise-trained WT mice.

We also observed a reduction in PPI expression in exercise-trained α_{2A}/α_{2C}ARKO mice. PPI regulates the phosphorylation status of PLN by dephosphorylating PLN at both Ser^{16} and Thr^{17} residues. In HF, the overall activity and expression of PPI are exaggerated (1, 12). The consequence of this abnormal increased expression is a reduction in Ca^{2+} reuptake by SERCA2 followed by a decrease in sarcoplasmic reticulum Ca^{2+} load, which deteriorates ventricular function (36). Interestingly, we provide evidence that exercise training reverses the increased PPI expression levels in α_{2A}/α_{2C}ARKO to WT control mice levels. Moreover, it may contribute, at least in part, to the improvement in fractional shortening after exercise training.

Kubo et al. (18) demonstrated that treatment with β-blocker normalizes the abundance of cardiac myocyte Ca^{2+} regulatory proteins and Ca^{2+} handling. It is possible that our strategy
based on exercise training has similar effects. In this case, exercise training would restore the depressed β-adrenergic receptor signaling observed in persistent sympathetic activation, which, in turn, improves the balance of adrenergic-mediated regulation of kinases and phosphatases that control intracellular Ca²⁺ homeostasis.

HF is characterized by progressive myocardial remodeling associated with cardiac myocyte loss and ventricular fibrosis (2). In fact, we observed cardiac myocyte hypertrophy associated with increased collagen volume fraction in αSARKO mice. The mechanisms underlying the maladaptive cardiac remodeling in HF are not completely understood, but alterations in proteins that regulate Ca²⁺ homeostasis have been reported (14, 31). In addition, other subcellular abnormalities such as mitochondrial remodeling, apoptosis, changes in myosin isoform composition, and alterations in troponin phosphorylation and Ca²⁺-binding affinity have also been found (5, 6, 27, 35) in HF. Exercise training partially decreased cardiac myocyte cross-sectional diameter but had little impact on ventricular fibrosis. The mechanisms by which exercise training reverses cardiac ultrastructural abnormalities in αSARKO mice is beyond the scope of the present study, but it is undoubtedly an interesting topic for future investigations.

**Study Limitations**

Our study shows that exercise training causes both an increase in fractional shortening and alterations in the expression of Ca²⁺ handling proteins. However, it does not provide direct evidence to support the cause-effect relationship between Ca²⁺ handling proteins expression and cardiac function. Although Ca²⁺ transients tend to parallel changes in the expression of cardiac Ca²⁺ handling proteins and cardiac function (1, 3, 21, 30), we have not directly assessed Ca²⁺ transients. The data are consistent with the idea that the changes observed by exercise training in the expression of Ca²⁺ handling proteins and ventricular function can be attributed to an improvement of cardiac Ca²⁺ transient. Furthermore, it is important to emphasize that the exercise training protocol was efficient to improve exercise tolerance and elicit resting bradycardia, which paralleled the improvement in ventricular function in this genetic model of sympathetic hyperactivity-induced HF.

Even though the present model is characterized by a sympathetic hyperactivity as seen in human HF, one may argue that in this case the hyperactivity is not secondary, but primary, and the levels reached may be too high. However, HR that is highly influenced by sympathetic tone responded favorably to 8 wk exercise training, indicating that, although high, the sympathetic tone can still be modulated by a physiological intervention.

In conclusion, we provided evidence that Ca²⁺ handling is impaired in this HF model. Furthermore, the benefits of exercise training in this genetic model of sympathetic hyperactivity-induced HF include improvement in the net balance of myocardial Ca²⁺ handling proteins. These findings provide additional insight into the improvement in cardiac function associated with exercise training in HF.

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**REFERENCES**


MAXIMAL LACTATE STEADY STATE IN RUNNING MICE: EFFECT OF EXERCISE TRAINING

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SUMMARY

1. Maximal lactate steady state (MLSS) corresponds to the highest blood lactate concentration (MLSSc) and workload (MLSSw) that can be maintained over time without continual blood lactate accumulation and is considered an important marker of endurance exercise capacity. The present study was undertaken to determine MLSSw and MLSSc in running mice. In addition, we provide an exercise training protocol for mice based on MLSSw.

2. Maximal lactate steady state was determined by blood sampling during multiple sessions of constant-load exercise varying from 9 to 21 m/min in adult male C57BL/6J mice. The constant-load test lasted at least 21 min. The blood lactate concentration was analysed at rest and then at 7 min intervals during exercise.

3. The MLSSw was found to be 15.1 ± 0.7 m/min and corresponded to 60 ± 2% of maximal speed achieved during the incremental exercise testing. Intra- and interobserver variability of MLSSc showed reproducible findings. Exercise training was performed at MLSSw over a period of 8 weeks for 1 h/day and 5 days/week. Exercise training led to resting bradycardia (21%) and increased running performance (28%). Of interest, the MLSSw of trained mice was significantly higher than that in sedentary littermates (19.0 ± 0.5 vs. 14.2 ± 0.5 m/min; P = 0.05), whereas MLSSc remained unchanged (3.0 mmol/L).

4. Altogether, we provide a valid and reliable protocol to improve endurance exercise capacity in mice performed at highest workload with predominant aerobic metabolism based on MLSS assessment.

Key words: blood lactate, endurance capacity, exercise training, mice.

INTRODUCTION

Over the past few years, there has been a proliferation of genetically altered murine models of human cardiovascular diseases and metabolic disorders, increasing interest in how closely murine cardiovascular physiology resembles that of the human. Because exercise training has been associated with several metabolic, cardiovascular and neurovegetative benefits,1,2 it is important to determine an efficient exercise training protocol in mice matched to each individual's fitness level.

Among several training regimens used for improving performance, maximal lactate steady state (MLSS) workload is considered the best marker of aerobic endurance capacity.3 The MLSS is the highest workload that can be maintained over time without continual blood lactate accumulation. The physiological importance of MLSS workload is that it defines the exercise intensity above which there is a net contribution of energy associated with lactate accumulation owing to an increased rate of glycolysis that exceeds the rate of mitochondrial pyruvate utilization.4 At exercise workloads up to MLSS, there is a balance between lactate production and its removal. However, at workloads above MLSS the rate of lactate production exceeds the rate of lactate clearance. For that reason, direct determination of MLSS is considered the gold standard for evaluating aerobic/anaerobic metabolism transition.

The MLSS test has been applied successfully to determine the workload of aerobic/anaerobic transition in rats,5 whereas it is lacking for mice. Indeed, the effect of exercise training based on MLSS to improve the endurance capacity of mice has not yet been studied. Therefore, the present study was designed to determine the MLSS of mice submitted to running exercise at subsequent constant-load tests performed with different workloads (varying from 9 to 21 m/min) on different days. Exercise training based on MLSS was also performed in order to test whether exercise training in mice would shift the MLSS, as has been previously shown in rats6 and humans.6

METHODS

Study population

Five-month-old male C57BL/6J mice (n = 40), weighing 28.7 ± 0.8 g, were housed three to five per cage in a temperature-controlled room (22°C) with a 12:12 h dark-light cycle, with free access to standard laboratory chow (Nuvital Nutrients S/A, Curitiba, Brazil) and tap water. All animal experimental procedures were performed in accordance with the Brazilian College of Animal Experimentation (http://www.coebea.org.br).

Blood lactate concentration

Capillary blood samples (25 μL) were taken from the tail vein. Mice kept running while blood was being taken by gently holding the tail. Blood was
transferred to 1.5 mL tubes containing 50 µL sodium fluoride (1%). The blood lactate concentration was analysed using an electroenzymatic method with a lactate analyser (YSI 2300 Stat Analyzer, Yellow Springs Instruments, Yellow Springs, OH, USA).

Incremental load test
Mice were submitted to incremental exercise testing on a motor treadmill. The intensity of exercise was increased by 3 m/min (6–33 m/min) every 3 min at 0% grade until exhaustion. This test provided the total distance run and the peak workload was measured at the termination of the test. Based on the latter, individual workloads corresponding to 60% peak workload were determined.

Constant-load test
Each constant-load test session consisted of a warm-up period of 10 min at 6 m/min and a 28 min constant-load bout of exercise. Blood samples (25 µL) were taken from the tail vein every 7 min of running for further lactate measurements. The workload intensity for the first constant-load test was 60% of peak workload determined previously in the incremental load test. If, during the first constant-load test, a steady state or a decrease in blood lactate was observed, further subsequent 28 min tests with increased workload intensities (63 m/min increases) were performed on separate days. If the first constant-load test resulted in a clearly identifiable increase in blood lactate, further constant-load tests were conducted with subsequently reduced workload intensities.

The highest workload that could be maintained over 28 min of running without continual blood lactate accumulation (blood lactate varying by less than 1 mmol/L from 7 to 28 min) was considered the maximal lactate steady state workload (MLSSw). The maximal lactate steady state concentration (MLSSc) was calculated as the average blood lactate concentration measured at 7, 14, 21 and 28 min of the test.

Reproducibility, based on intra-observer variability of blood lactate concentrations, was determined using a test–retest assessment. Two sets of constant-load tests (workload intensities varying from 9 to 21 m/min) were randomly applied in the same mice at a 1 week interval and blood samples were taken by the same observer (JCBF). Blood lactate concentrations for the same constant workload were compared between tests. Interobserver variability of blood lactate concentrations was assessed by two observers (JCBF and NPLR), independently and in a blinded fashion. For this assessment, two sets of constant-load tests were applied at 1 week intervals, as described above.

Oxygen consumption
In order to verify the percentage oxygen consumption corresponding to the previously determined MLSSw, oxygen consumption was measured by means of a rapid-flow, open-circuit indirect calorimeter. This method was adopted, with some modification, from previous studies in rats. Oxygen consumption was measured continually by means of expired gas analysis during the incremental exercise testing (3 m/min increments every 3 min at 0% grade) performed on a motor treadmill. Peak oxygen consumption was defined as the highest oxygen consumption attained at the end of the exercise period when the mice could no longer maintain the running speed. Gas analysis was performed by means of carbon dioxide (CD-3A) and oxygen (S-3A/I) analysers (AMETEK, Pittsburgh, PA, USA).

Exercise training
Five-to-six-old mice were randomly assigned into sedentary (n = 17) and trained (n = 15) groups. Exercise training consisted of 8 weeks of running (from 5 to 7 months of age) on a motor treadmill (ESED model 01; FUNBEC, São Paulo, Brazil), 5 days a week, for 60 min at MLSSw intensity, as described above. Constant-load tests were performed in sedentary and trained mice before and after the training period and MLSSw intensity and MLSSc were determined. An additional constant-load test was performed in the trained group after 4 weeks of exercise training in order to adjust training intensity. The training sessions occurred during the cycle for the mice, which, in the present study, was between 0700 and 1900 hours. Sedentary mice were handled every day to habituate them to the experimental protocols. To evaluate the effect of exercise training on running performance, total distance run achieved during an incremental load test was calculated before and after the experimental protocol in sedentary and trained mice. In addition, resting blood pressure (BP) and heart rate (HR) were measured using a computerized tail-cuff system (BP 2000, Visitech Systems, Apex, NC, USA). This procedure was adopted in order to verify resting bradycardia, which has been considered a physiological marker of aerobic adaptation to exercise training.11

Experimental protocols
Protocol 1: MLSS determination
To determine MLSSw and MLSSc, eight mice were submitted to subsequent constant-load tests performed with workload intensities varying from 9 to 21 m/min. The workload intensity for the first constant-load test corresponded to 60% of peak workload obtained in the incremental load test. A single constant-load test was performed per day and the protocol covered a period of up to 4 days.

Protocol 2: Exercise conditioning after training based on individual MLSSw
To evaluate whether training would lead to a robust endurance conditioning by promoting a shift in MLSS workload to higher exercise intensity, we compared sedentary with exercise-trained mice. One day after the last exercise training session, HR was measured in order to verify the occurrence of resting bradycardia. Twenty-four hours later, mice were submitted to an incremental exercise testing until exhaustion in order to assess the running performance after exercise training and to determine the first workload intensity (60% of peak workload) to be applied in the constant-load test. Both MLSSw and MLSSc were determined after a series of constant-load tests, as described above.

Statistics
Results are presented as the mean±SEM. One-way ANOVA with post hoc testing using Tukey’s test (Statistica software; StatSoft, Tulsa, OK, USA) was used to compare changes in blood lactate concentrations between 7 and 28 min of constant-load tests performed at different exercise intensities. Correlation of blood lactate concentrations within and between observers was assessed by intraclass correlation coefficients. Two-way ANOVA for repeated measures was used to examine the effect of training (sedentary and exercise trained) and condition (before and after 8 weeks of exercise training) on peak speed, total distance run, resting HR, MLSSc and MLSSw. Statistical significance was defined as P<0.05.

RESULTS
Determination of MLSS
Figure 1a shows the response of blood lactate concentrations over time in running mice (constant workload intensities ranging from 9 to 18 m/min). A clear stabilization of blood lactate concentration was observed in running mice at 9, 12 and 15 m/min, which corresponded to blood lactate changes by less than 1 mmol/L after the 7 min of testing (Fig. 1b). At 18 m/min, continual blood lactate accumulation was observed over time and changes in blood lactate concentration exceeded 1 mmol/L within the 7 and 28 min of testing (Fig. 1b). Therefore, MLSSw was determined to be 15.1 ± 0.7 m/min and the blood lactate concentration achieved at this exercise
Maximal lactate steady state in mice

![Graph](image)

**Fig. 1** Time-course of blood lactate concentration assessed by submaximal constant-load tests. (a) The workload of the first constant-load test corresponded to 60% of maximal speed achieved during an incremental load test. (X), 9 m/min (n = 2); (b), 12 m/min (n = 7); (c), 15 m/min (n = 8); (d), 18 m/min (n = 6). (b) Changes in blood lactate concentration between 7 and 28 min of constant-load tests performed at different exercise intensities. Note that maximal blood lactate concentration (MLSS) is achieved at 15 m/min, the highest workload at which blood lactate concentration was measured. Values are the mean ± SEM.*P < 0.05 compared with 9, 12, and 15 m/min.

Baseline BP did not differ between sedentary and trained mice (107 ± 3 vs 110 ± 3 mm Hg). In contrast, HR decreased by 21% in trained mice compared with the pre-exercise training period (482 ± 13 vs 593 ± 21 b.p.m.), whereas HR was unchanged in the sedentary group throughout the study (573 ± 12 vs 572 ± 19 b.p.m.; $F_{1,37} = 11.15; P = 0.05$).

**Exercise training effects**

Bodyweight was significantly reduced in trained mice compared with sedentary mice (27.9 ± 0.8 vs 30.4 ± 0.3; $t = 3.2; P = 0.05$).
As shown in Fig. 4b, MLSSw increased in trained mice compared with the pre-exercise training period (504 ± 18 vs 407 ± 11 m post- vs pre-exercise training, respectively). Conversely, distance run decreased significantly after 8 weeks in sedentary mice (316 ± 23 vs 385 ± 16 m post- vs pre-experimental period; \( F_{1,16} = 11.72; P = 0.05 \)). In addition, MLSSc was similar between groups throughout the study (Fig. 4c). These data suggest that aerobic performance improved in trained mice, whereas similar levels of MLSSc were achieved at a higher MLSSw.

**DISCUSSION**

The relative lack of data regarding the cellular and molecular mechanisms of exercise-induced changes in cardiovascular and metabolic functions in mice calls for well-defined protocols of endurance training for this species. The present study provides a method for measuring endurance capacity based on the determination of MLSS in mice. It is well established that MLSS is a good marker of endurance exercise capacity and the determination of MLSS is used to design training programmes for athletes, cardiac patients and respiratory disease patients. The present results show that MLSSw and MLSSc, obtained from consecutive constant-load tests, were 15 m/min and 3 mmol/L, respectively. Of interest, MLSS occurred at 60% of maximal speed achieved in the incremental exercise testing. Furthermore, exercise training increased running performance and MLSSw, whereas MLSSc remained unchanged.

**Determination of MLSS**

Maximal lactate steady state is defined as the highest exercise intensity at which blood lactate concentration does not increase beyond the initial transient during constant-load exercise. That is, MLSS represents an equilibrium between lactate transport into the blood and its removal from the blood. Thus, MLSS seems to indicate an exercise intensity above which the rate of glycolysis exceeds the rate of mitochondrial pyruvate utilization, causing net lactate formation.

The commonly accepted criterion for determining MLSS is the highest exercise intensity for which blood lactate concentration varies by less than 1 mmol/L during the final 20 min of constant-load exercise lasting at least 30 min. Nevertheless, the method for the determination of MLSS must be reproducible and objective. In the present study we demonstrated that mice are able to maintain running speeds up to 15 m/min with changes in blood lactate concentration of no more than 1 mmol/L from 7 to 28 min of testing. In addition, the lower intra- and interobserver variability of MLSSc shows the robustness of the protocol proposed.

Billat et al. have recently used critical speed to assess endurance capacity in 2-month-old mice of different strains. Critical speed was determined by a regression line of a plot of the distance run and time to exhaustion in four constant-load runs (in a range of 18–51 m/min). The authors observed that the critical speed of C57BL/6J mice was achieved at 18 m/min, which is higher than the MLSSw of 15 m/min determined in the present study. These contrasting results are not surprising because it has been demonstrated previously that critical speed can be achieved at higher workloads when compared with MLSSw in humans, although the two variables are strongly correlated.

To our knowledge, this is the first study demonstrating that the determination of MLSSw using a submaximal constant-load test can
be applied to mice. The MLSSw occurs at 60% of maximal speed achieved in an incremental exercise testing, which enables its application in the evaluation of endurance performance. This is of particular interest for studying genetically modified mice with severe cardiovascular disorders.

Effect of exercise training on the cardiovascular system and MLSS

Resting bradycardia is considered a useful and reliable indicator of aerobic conditioning. Exercise training performed at MLSSw in mice significantly reduced resting HR by 21%. This effect is similar to those reported elsewhere.11 Indeed, exercise training increased total distance run in incremental exercise testing by 60%. Therefore, both resting bradycardia and increased running performance demonstrated the effectiveness of exercise training performed at MLSSw.

There was no difference in systolic blood pressure between exercise-trained and sedentary mice. These results are consistent with previous observations by our group11,22,23 and others24 showing that arterial pressure remains unchanged in exercise-trained normotensive animals and humans.

Exercise training in mice increased absolute MLSSw (from 15 to 19 m/min) without significant changes in MLSSc. Interestingly, exercise training maintained the relative MLSSw, which was observed at 60% of maximal speed achieved during incremental exercise testing. Similar results were observed in rats. Gobatto et al. reported a maintained MLSSc for sedentary and swimming-trained rats, whereas MLSSw was shifted towards a higher intensity (% of bodyweight overload).25 In humans, an increased absolute MLSSw by exercise training is also observed when compared with pre-exercise MLSSw.11 In addition, MLSSw is markedly increased in athletes compared with age-matched sedentary individuals.26 In fact, MLSSw is used to evaluate endurance capacity and to define training programmes for athletes of different modalities.11,21,24

The increased MLSSw after exercise training could be associated with a better regulation of lactate production and its clearance, which would lead to improved lactate turnover. Exercise training based on MLSSw is known to reduce glycolytic rate and is associated with less reduction in muscle glycogen, an increase in the rate of fat oxidation and improved mitochondrial oxidation of pyruvate at a given workload.26,27,28 These responses will culminate in an improved endurance capacity after exercise training.

If there is a clear and established relationship between MLSSw and endurance capacity, the same cannot be said for MLSSc. The MLSSc exhibits wide interindividual variability29 and seems to be influenced by either muscular mass involved or motor pattern of exercise.26 The same response has been observed in other species. In rats, blood lactate concentration seems not to be significantly affected by exercise training, whereas MLSSw or lactate threshold workload are increased in swimming30 and running training,31 respectively. The present results corroborate the findings in rats, because exercise-trained mice exhibited increased MLSSw without any significant change in MLSSc.

Conclusions

In conclusion, the present study demonstrated that a submaximal constant-load test can be used to determine MLSS in mice. Indeed, MLSSw is a valid and reliable method for measuring endurance capacity. The increase in MLSSw after exercise training showed the effectiveness of training based on MLSSw. Moreover, this method can be applied to match training intensity to individual endurance capacity. Finally, training did not change the relative exercise intensity of MLSSw in mice. Therefore, we propose that one way to achieve effective improvements in endurance capacity in mice is to train the mice at either 60% of a mouse’s maximal running speed or a blood lactate concentration of 3 mmol/L.

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REFERENCES


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Abstract:

Objective: We tested the hypothesis that exercise training (ET) would prevent Ca\(^{2+}\) handling abnormalities and onset of ventricular dysfunction in sympathetic hyperactivity-induced heart failure (HF) mice. Methods: A cohort of male wild type (WT) and congeneric \(\alpha_{2A}/\alpha_{2C}\)ARKO mice with C57BL6/J genetic background (3 to 5 months 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-Ser\(^{2809}\)-RyR, sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA2), Na\(^{+}\)-Ca\(^{2+}\) exchanger (NCX), phospholamban (PLN), phospho-Ser\(^{16}\)-PLN, and phospho-Thr\(^{17}\)-PLN were analyzed by Western blotting. Results: At 3 months of age, no significant difference in FS was observed between WT and \(\alpha_{2A}/\alpha_{2C}\)ARKO mice. At 5 months of age, when cardiac dysfunction is in an early-stage, \(\alpha_{2A}/\alpha_{2C}\)ARKO mice presented reduced FS paralleled by decreased SERCA2 and NCX. Conversely, \(\alpha_{2A}/\alpha_{2C}\)ARKO mice displayed increased phospho-Ser\(^{16}\)-PLN and phospho-Ser\(^{2809}\)-RyR. ET in \(\alpha_{2A}/\alpha_{2C}\)ARKO mice prevented exercise intolerance and ventricular dysfunction. ET significantly increased the expression of SERCA2 (58%) and phospho-Ser\(^{16}\)-PLN (30%) while it restored the expression of phospho-Ser\(^{2809}\)-RyR to WT levels. Conclusion: Collectively, we provided evidence that improved net balance of Ca\(^{2+}\) handling proteins upon ET is, at least in part, a compensatory mechanism against deteriorating ventricular function in the early-stage of sympathetic hyperactivity-induced HF.
Dear Dr. Piper,

We are submitting the original article entitled "Exercise Training Delays Onset of Heart Failure and Prevents Calcium Handling Abnormalities in Sympathetic Hyperactivity-Induced Heart Failure Mice" by Alessandra Medeiros, Natale P.L. Rolim, Rodrigo S.F. Oliveira, Kaleizu T. Rosa, Katt C. Mattos, Maria Claudia Irigoyen, Eduardo M. Krieger, José Eduardo Krieger, Carlos Eduardo Negrão, Patricia C. Brum. All authors have read and approved the manuscript submission to Cardiovascular Research. Moreover, the manuscript, or part of it, neither has been published nor is currently under consideration for publication by any other journal.

In this manuscript we studied the effect of exercise training on cardiac function and expression of Ca$^{2+}$ handling proteins during the progression of heart failure in a genetic model of sympathetic hyperactivity-induced heart failure in mice. Our findings support the idea that in a setting of developing heart failure, exercise training can delay cardiac dysfunction, which can be attributed, at least in part, to a positive balance of proteins involved in sarcoplasmic Ca$^{2+}$ release and reuptake. Altogether, we provided new insights on the molecular mechanisms whereby exercise training can contribute to delay the onset of heart failure. The molecular basis for this cardioprotection includes a positive balance of cardiac Ca$^{2+}$ handling proteins, which might be favored by the decreased sympathetic hyperactivity after exercise training.

We look forward to your response and are at your disposal for any further inquiries.

Best Wishes,

Patricia Chakur Brum

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Exercise Training Delays Onset of Heart Failure and Prevents Calcium Handling Abnormalities in Sympathetic Hyperactivity-Induced Heart Failure Mice

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Running title: Exercise training delays heart failure in mice

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Abstract

Objective: We tested the hypothesis that exercise training (ET) would prevent Ca\(^{2+}\) handling abnormalities and onset of ventricular dysfunction in sympathetic hyperactivity-induced heart failure (HF) mice.

Methods: A cohort of male wild type (WT) and congenic α\(_{2A}/α_{2C}\)ARKO mice with C57BL6/J genetic background (3 to 5 months of age) were randomly assigned into untrained and exercise-trained groups. ET consisted of 8-wk swimming session, 60min, 5 days/wk. Fractional shortening (FS) was assessed by two-dimensional guided M-mode echocardiography. The protein expression of ryanodine receptor (RyR), phospho-Ser\(^{2809}\)-RyR, sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA2), Na\(^+\)-Ca\(^{2+}\) exchanger (NCX), phospholamban (PLN), phospho-Ser\(^{16}\)-PLN, and phospho-Thr\(^{17}\)-PLN were analyzed by Western blotting.

Results: At 3 months of age, no significant difference in FS was observed between WT and α\(_{2A}/α_{2C}\)ARKO mice. At 5 months of age, when cardiac dysfunction is in an early-stage, α\(_{2A}/α_{2C}\)ARKO mice presented reduced FS paralleled by decreased SERCA2 and NCX. Conversely, α\(_{2A}/α_{2C}\)ARKO mice displayed increased phospho-Ser\(^{16}\)-PLN and phospho-Ser\(^{2809}\)-RyR. ET in α\(_{2A}/α_{2C}\)ARKO mice prevented exercise intolerance and ventricular dysfunction. ET significantly increased the expression of SERCA2 (58%) and phospho-Ser\(^{16}\)-PLN (30%) while it restored the expression of phospho-Ser\(^{2809}\)-RyR to WT levels.

Conclusion: Collectively, we provided evidence that improved net balance of Ca\(^{2+}\) handling proteins upon ET is, at least in part, a compensatory mechanism against deteriorating ventricular function in the early-stage of sympathetic hyperactivity-induced HF.

Key words – calcium, ventricular function, heart failure, exercise training.
Introduction

It is widely recognized that exercise training is effective in reducing a number of cardiovascular risk factors [1, 2]. Moreover, accumulated evidence shows that exercise training is an important strategy for the prevention and treatment of cardiovascular diseases [3, 4]. In heart failure (HF), exercise training improves patient outcome by increasing exercise tolerance [5, 6] and reducing dyspnea and fatigue [7]. 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 [10, 11].

Several Ca\(^{2+}\) handling proteins are involved in the maintenance of normal cardiac Ca\(^{2+}\) homeostasis and contractile function. Among these proteins, sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA2), ryanodine receptor (RyR) and Na\(^+-\)Ca\(^{2+}\) exchanger are responsible for the balance between sarcoplasmic Ca\(^{2+}\) uptake and release, and extrusion by sarcolemma, respectively [12-14]. Ca\(^{2+}\) uptake by SERCA2 is regulated by a phosphorylatable protein, phospholamban (PLN), which in its dephosphorylated form inhibits SERCA2 activity [15]. Abnormal Ca\(^{2+}\) homeostasis by perturbation in the expression or function of these major Ca\(^{2+}\) regulating proteins have been described in HF [15-18]. In a recent study, we found that exercise training improved the net balance of cardiac Ca\(^{2+}\) proteins involved in transsarcolemmal flux and sarcoplasmic reticulum reuptake of Ca\(^{2+}\) in mice lacking both \(\alpha_2A/\alpha_2C\)-adrenoceptors (\(\alpha_2A/\alpha_2C\)ARKO), in which sympathetic hyperactivity causes HF [10]. At 7 months of age, when \(\alpha_2A/\alpha_2C\)ARKO mice display severe HF, exercise training restored cardiac Na\(^+-\)Ca\(^{2+}\) expression levels, and increased SERCA2, and phosphorylated PLN at both residues Ser\(^{16}\) and Thr\(^{17}\) expression levels, which resulted in improvement in the left ventricular function. These findings suggest that the improvement in intracellular Ca\(^{2+}\) regulation is an new benefit of exercise.
training on overall ventricular function in severe HF [10]. Just as important, one might expect that exercise training prior to the development of HF would display a protective role preventing Ca\(^{2+}\) handling abnormalities and preserving cardiac function. However, the potential involvement of Ca\(^{2+}\) handling proteins in exercise-induced cardioprotection remains to be elucidated.

The present investigation was undertaken to test the hypotheses that exercise training would delay the onset of ventricular dysfunction in α\(_{2A}/α_{2C}\)ARKO mice. In addition, exercise training would prevent the alterations in the expression of Ca\(^{2+}\) handling proteins involved in transsarcolemmal Ca\(^{2+}\) flux, Ca\(^{2+}\) reuptake and release by sarcoplasmic reticulum in this experimental model of HF. Briefly, we found that exercise training markedly delayed the onset of HF and preserved ventricular function in α\(_{2A}/α_{2C}\)ARKO mice. The molecular basis for this cardioprotection includes a positive balance of cardiac Ca\(^{2+}\) handling proteins, which might be favored by the decreased sympathetic hyperactivity after exercise training in this genetic model.
Materials and methods

Sampling

Animals’ Care. A cohort of male wild type (WT) and congenic $\alpha_{2A}/\alpha_{2C}$ARKO mice with C57Bl6/J genetic background aged 3 to 5 months were studied. In $\alpha_{2A}/\alpha_{2C}$ARKO mice, cardiac function is preserved until 3 months of age while cardiomyopathy is an early-stage at 5 months as previously described [10, 19]. Genotypes were determined by polymerase chain reaction on genomic DNA obtained from tail biopsies using primers to detect the intact and disrupted genes.

Mice were maintained in a light- (12-h light cycle) and temperature- (22°C) controlled environment and were fed a pellet rodent diet (Nuvital Nutrientes S/A, Curitiba, PR Brazil) ad libitum and had free access to water. WT and $\alpha_{2A}/\alpha_{2C}$ARKO mice were randomly assigned into untrained and exercise-trained groups. This study was accorded to Ethical Principles in animal research adopted by Brazilian College of Animal Experimentation (www.cobea.org.br).

Measurements and Procedures

Exercise Training Protocol. Exercise training consisted of five day/week swimming sessions with gradually increased duration to sixty minutes, for eight weeks in a swimming warmed water (30-32°C) apparatus adapted for mice [20]. The training sessions were performed during the dark cycle of the mice. Untrained mice were placed in the swimming apparatus for 5 minutes twice a week to mimic the water stress associated to 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 [20].
**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 a week (10 min of exercise session), mice were placed in the exercise streak and allowed to acclimatize for at least 30 minutes. Exercise began at 6 m/min with no grade and increased by 3 m/min every 3 minutes thereafter until exhaustion. The graded treadmill exercise test was performed in WT and α2A/α2CARKO mice before and after exercise training period.

**Cardiovascular Measurements.** Heart rate (HR) was determined non-invasively using a computerized tail-cuff system (BP 2000 Visitech Systems) described elsewhere [21]. Mice were acclimatized to the apparatus during daily sessions over 6 days, one week before starting the experimental period. HR measurements were obtained serially in WT and α2A/α2CARKO mice once a week throughout the 8 weeks of experiment.

Non-invasive cardiac function was assessed by two-dimensional guided M-mode echocardiography, in halothane-anesthetized WT and α2A/α2CARKO mice, before and after 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:

\[ \text{Fractional Shortening} \%(\%)=\left[\frac{\text{LVEDD}-\text{LVESD}}{\text{LVEDD}}\right] \times 100 \]

where, LVEDD means left ventricular end-diastolic dimension, and LVESD means left ventricular end-systolic dimension.
Cardiac Sympathetic Tone Measures. To confirm that \( \alpha_{2A}/\alpha_{2C}\)ARKO mice have increased cardiac sympathetic tone, we measured the HR (electrocardiogram) after pharmacological blockade of muscarinic receptors with methylatropine (1 mg/kg, Sigma Chemical, USA), and \( \beta \)-adrenergic receptors with propranolol (3 mg/kg, Sigma Chemical, USA) at the end of experimental protocol. The sympathetic tonus was analyzed as the difference between the maximum HR after methylatropine injection and the intrinsic HR (heart rate after muscarinic and \( \beta \)-adrenergic receptor blockade) [22-24].

Antibodies. Mouse monoclonal antibodies to SERCA2 (1:2500), phospholamban (PLN, 1:500), ryanodine (RyR, 1:5000) and \( \text{Na}^+ -\text{Ca}^{2+} \) exchanger (NCX, 1:2000) were obtained from Affinity BioReagents (Golden, CO); rabbit polyclonal phospho-Ser\textsuperscript{2809}-RyR\textsubscript{2} (1:2000), phospho-Ser\textsuperscript{16}-PLN (1:5000), and phospho-Thr\textsuperscript{17}-PLN (1:5000) were obtained by Badrilla (Leeds, UK). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2000) was obtained from Advanced Immunochemical (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-Ser\textsuperscript{16}-PLN, phospho-Thr\textsuperscript{17}-PLN, NCX, RyR, and phospho-Ser\textsuperscript{2809}-RyR. Briefly, liquid nitrogen frozen ventricles isolated from WT and \( \alpha_{2A}/\alpha_{2C}\)ARKO 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 electro-transferred 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, 1:10000, for 1:30 h at room temperature) and developed using enhanced chemiluminescence (Amersham Biosciences; Piscataway, NJ) detected by autoradiography. Quantification analysis of blots was performed with the use of Scion Image software (Scion Corporation based on NIH image).

Statistical Analysis

Data are presented as mean ± SE. Two-way ANOVA for repeated measurements with post-hoc testing by Tukey (Statistica software, StatSoft, Inc., Tulsa, OK, USA) was used to compare the effect of training (untrained and exercise-trained) and genotype (WT and α2A/α2C ARKO) on distance run, fractional shortening, and HR measurements along the training period. Two-way ANOVA with post-hoc testing by Tukey (Statistica software, StatSoft, Inc., Tulsa, OK, USA) was used to compare the effect of training (untrained and exercise-trained) and genotype (WT and α2A/α2C ARKO) on cardiac sympathetic tonus 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 Heart Rate.

At 3 months of age, there was no difference in distance run and fractional shortening between WT and $\alpha_{2A}/\alpha_{2C}$ARKO mice (Fig. 1). However, at 5 months of age, when cardiomyopathy is in an early-stage, $\alpha_{2A}/\alpha_{2C}$ARKO mice displayed exercise intolerance and systolic dysfunction when compared to age-matched WT mice. Exercise training in $\alpha_{2A}/\alpha_{2C}$ARKO 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 $\alpha_{2A}/\alpha_{2C}$ARKO mice (Fig 1B).

$\alpha_{2A}/\alpha_{2C}$ARKO mice displayed baseline tachycardia when compared to age-matched WT mice (Fig 2A). Exercise training significantly decreased baseline HR in both WT and $\alpha_{2A}/\alpha_{2C}$ARKO mice from the fourth week to the end of exercise training period. The reduction of HR in $\alpha_{2A}/\alpha_{2C}$ARKO was so remarkable that reached untrained WT levels at the fifth week of training (Fig 2A). $\alpha_{2A}/\alpha_{2C}$ARKO mice displayed sympathetic hyperactivity compared to WT mice. Exercise training significantly decreased cardiac sympathetic tone in both $\alpha_{2A}/\alpha_{2C}$ARKO mice and WT mice when compared to their untrained littermates.

Expression of proteins involved in sarcoplasmic Ca$^{2+}$ release

As hyperphosphorylation of RyR is associated with deleterious effect of cardiac sympathetic hyperactivity [17, 18], we investigated whether RyR is hyperphosphorylated in our $\alpha_{2A}/\alpha_{2C}$ARKO 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 3A and 3B), the phospho-Ser\textsuperscript{2809} - RyR expression levels were significantly increased in α\textsubscript{2A}/α\textsubscript{2C}ARKO mice when compared to age-matched WT mice (Fig 3A and 3C). Phospho-Ser\textsuperscript{2809} - RyR expression levels remained unchanged in exercise-trained WT mice. However, exercise training in α\textsubscript{2A}/α\textsubscript{2C}ARKO mice decreased phospho-Ser\textsuperscript{2809} -RyR expression towards WT levels (Fig 3A and 3C).

**Expression of Proteins Involved in transsarcolemmal flux and sarcoplasmic reuptake of Ca\textsuperscript{2+}.**

Since downregulation of cardiac SERCA2 expression may precede clinical signs of HF [26], we investigated whether sympathetic hyperactivity in our genetic model would alter the expression of SERCA2, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, PLN, phospho-Ser\textsuperscript{16} -PLN, and phospho-Thr\textsuperscript{17} -PLN. Additionally, we tested whether exercise training would prevent these alterations in α\textsubscript{2A}/α\textsubscript{2C}ARKO mice.

GAPDH protein levels were not different among the four groups studied. The expression of SERCA2 and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger was reduced in untrained α\textsubscript{2A}/α\textsubscript{2C}ARKO mice by 26% and 34%, respectively (Fig 4A and 4B). Exercise training caused no effect on Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger expression levels in α\textsubscript{2A}/α\textsubscript{2C}ARKO mice, but significantly increased SERCA2 expression. This increase in SERCA2 expression was so dramatic that reached that untrained WT mice levels (Fig 4A and 4B). In WT mice, exercise training did not change SERCA2 or Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger expression levels.

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

As PLN control the apparent Ca\(^{2+}\) affinity of SERCA2 [27], 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. Phospho-Ser\(^{16}\)-PLN was increased by 76% in untrained \(\alpha_2A/\alpha_2C\)ARKO mice when compared to untrained WT mice (Fig. 5A, and 5B). 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. 5A and 5C).
Discussion

A wealth of data indicates that regular exercise can protect individuals from a host of cardiovascular and metabolic diseases [3, 4, 28]. Indeed, exercise training is emerging as a key intervention for preventive cardiology [29, 30]. However, the mechanisms by which exercise training can delay the onset of cardiovascular diseases are not completely understood. In the present investigation we demonstrated that exercise training in sympathetic hyperactivity-induced HF mice: 1) decreased cardiac sympathetic tone, 2) prevents Ca$^{2+}$ handling abnormalities and cardiac dysfunction.

Sympathetic hyperactivity plays a prominent role in the pathogenesis and evolution of cardiovascular diseases [31]. Therefore, the decreased sympathetic activity and resting heart rate paralleled by improved fractional shortening in exercise-trained $\alpha_{2A}/\alpha_{2C}$ARKO mice suggest 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 [31, 32] and an improved prognosis [31].

The key findings of the present study are that exercise training in $\alpha_{2A}/\alpha_{2C}$ARKO mice restores the phosphorylation of RyR at Ser$^{2809}$ to WT levels and increases the phosphorylation of PLN at Ser$^{16}$. Moreover, they 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 hyperadrenergic state [17, 18, 33]. The reduced expression of phospho-Ser$^{2809}$-RyR in exercise-trained $\alpha_{2A}/\alpha_{2C}$ARKO mice towards WT levels seems to be beneficial because
chronic hyperphosphorylation of RyR is associated with diastolic Ca$^{2+}$ leak leading to arrhythmogenicity [34, 35], and cardiac dysfunction. Based on the fact that the reduction in phopho-Ser$^{2860}$-RyR expression in exercise-trained α$\text{2A}$/α$\text{2C}$ARKO mice paralleled with a decreasing cardiac sympathetic tone, it is reasonable to speculate that reduced RyR phosphorylation at Ser$^{2860}$ is due to a decreased sympathetic drive.

Our study regarding the expression of proteins involved in intracellular Ca$^{2+}$ decline suggest that the increased phospho-Ser$^{16}$-PLN and decreased Na$^+$-Ca$^{2+}$ exchanger expression levels in untrained α$\text{2A}$/α$\text{2C}$ARKO mice may represent a compensatory mechanism against deteriorating cardiac function in the early-stage of sympathetic hyperactivity-induced cardiomyopathy. 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 untrained α$\text{2A}$/α$\text{2C}$ARKO mice even with sustained sympathetic hyperactivity [10]. In fact, increased activity of Na$^+$-Ca$^{2+}$ exchanger [11, 36, 37] and reduced cardiac phosphorylation of Ser$^{16}$-PLN together with increased Thr$^{17}$-PLN have been reported in end-stage HF [10, 38, 39].

Despite the fact that exercise training reduced cardiac sympathetic tone in α$\text{2A}$/α$\text{2C}$ARKO mice, the expression of phospho-Ser$^{16}$-PLN was further increased while no changes were observed in phospho-Thr$^{17}$-PLN expression. This result suggests that daily exercise stimulus is able to increase phosphorylation status of PLN at Ser$^{16}$ 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 kinases-anchoring proteins could be considered as alternative mechanisms.
Recent studies have demonstrated that prior exercise training improves hypertension-induced HF [1] and ischemia reperfusion injury [40] 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\(^+-\)Ca\(^{2+}\) exchanger expression remained decreased in exercise-trained \(\alpha_{2A}/\alpha_{2C}\)ARKO mice. Under this scenario, one may consider that exercise training favors Ca\(^{2+}\) reuptake by sarcoplasmic reticulum preventing Ca\(^{2+}\) extrusion by sarcolemma.

Study Limitations

Even though chronic sympathetic hyperactivity in HF is considered to be secondary to a prior cardiac insult, several cardiovascular risk factors such as, hypertension, obesity and insulin resistance are often accompanied by increased sympathetic activity even with no clinical signs of cardiac dysfunction [41]. Therefore, \(\alpha_{2A}/\alpha_{2C}\)ARKO mice are a suitable model for studying disease-stage related changes in developing HF associated with sympathetic hyperactivity. Nevertheless, therapies that decrease sympathetic tone are reported to improve prognosis [31, 32], which is the case of exercise training cardiac effects in the genetic model presently studied.

Our study shows that exercise training prevents both cardiac dysfunction and Ca\(^{2+}\) handling abnormalities in developing HF. However, it does not provide direct evidence to support the cause-effect relationship between Ca\(^{2+}\) handling proteins expression and cardiac function. Although Ca\(^{2+}\) transients tend to parallel changes in the expression of cardiac Ca\(^{2+}\) handling proteins and cardiac function [42-45], we did not directly assessed
Ca²⁺ transients. The data are consistent with the idea that the changes observed by exercise training in the expression of Ca²⁺ handling proteins and ventricular function are attributed to an improvement of cardiac Ca²⁺ transient.

Conclusion

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 proteins involved in sarcoplasmic Ca²⁺ release and reuptake. Altogether, we provided new insights on the molecular mechanisms whereby exercise training can contribute to delay the onset of HF.

Acknowledgments

The authors want to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo, São Paulo - SP (FAPESP # 2002/04588-8) for funding the present investigation. We also want to express our gratitude to Fundação Zerbini, São Paulo – SP, for the support in this study.

References


advanced chronic heart failure secondary to ischemic or nonischemic cardiomyopathy. Am J Cardiol 2005;95:136-140.


Figure Legends

Figure 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 (■) months of age in untrained and exercise-trained wild type (WTUN and WTT, respectively), and α₂A/α₂CARKO (DKOUN and DKOT, respectively) mice. Note that exercise training significantly improved distance and FS in α₂A/α₂CARKO mice. Data are presented as mean±SE. * P<0.05 vs 3 months of age, # P<0.05 vs. WTUN group.

Figure 2. Heart rate (HR, A) and cardiac sympathetic tone (ST, B) in untrained and exercise-trained wild type (WTUN and WTT, respectively), and α₂A/α₂CARKO (DKOUN and DKOT, respectively) mice. Note that exercise training decreased HR and ST in α₂A/α₂CARKO mice to untrained WT mice levels. As expected, WTT displayed resting bradycardia. Data are presented as mean±SE. * P<0.05 vs basal levels, # P<0.05 vs. untrained group, † P<0.05 vs WTUN group, ‡ P<0.05 vs WTT group.

Figure 3. Ryanodine (RyR) and Phospho-Ser²⁸⁰⁹-RyR expression normalized to total RyR in untrained (□) and exercise-trained (■) wild type (WT), and α₂A/α₂CARKO (DKO) mice. Data are presented as mean±SE. A: Representative blots of RyR and Phospho-Ser²⁸⁰⁹-RyR from untrained and exercise-trained WT and DKO mice. B and C: RyR and Phospho-Ser²⁸⁰⁹-RyR, respectively. * P<0.05 vs. untrained group, # P<0.05 vs WTUN group.

Figure 4. SERCA2 and Na⁺-Ca²⁺ exchanger (NCX) expression and SERCA:NCX ratio in untrained (□) and exercise-trained (■) wild type (WT), and α₂A/α₂CARKO (DKO) mice. Data are presented as mean±SE. A: Representative blots of SERCA2, NCX and GAPDH from untrained and exercise-trained WT and DKO mice. B, C, and D: SERCA2, NCX
expression and SERCA:NCX ratio, respectively. Targeted bands were normalized to cardiac GAPDH. * $P<0.05$ vs. untrained group, # $P<0.05$ vs WTUN group.

Figure 5. Phospholamban (PLN), and Phospho-Ser$^{16}$-PLN and Phospho-Thr$^{17}$-PLN expression normalized to total PLN in untrained (□) and exercise-trained (■) wild type (WT), and α$_{2A}$/α$_{2C}$ARKO (DKO) mice. Data are presented as mean±SE. A: Representative blots of PLN, Phospho-Ser$^{16}$-PLN, Phospho-Thr$^{17}$-PLN and GAPDH from untrained and exercise-trained WT and DKO mice. B, C, and D: PLN, Phospho-Ser$^{16}$-PLN and Phospho-Thr$^{17}$-PLN, respectively. Targeted bands were normalized to cardiac GAPDH. * $P<0.05$ vs. untrained group, # $P<0.05$ vs WTUN group.
Figure 1
Click here to download high resolution image
Figure 2
Click here to download high resolution image

A

B

WT UN (n=15)
WT T (n=22)
DK UN (n=16)
DK OT (n=15)

Basal 1 2 3 4 5 6 7 8

Weeks

ST (pm)

WT

DKO

Untrained
Trained

(7)

(7)

(7)

(6)
Figure 3
Click here to download high resolution image
Figure 4
Click here to download high resolution image

A

![Western blot images of SERCA2, NCX, and GAPDH proteins](image)

B

![Bar graph showing SERCA2 protein expression](image)

C

![Bar graph showing NCX protein expression](image)

D

![Bar graph showing SERCA2:NCX ratio](image)
Figure 5
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A

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B

![Bar chart](chart1.png)

C

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D

![Bar chart](chart3.png)
Duration-controlled swimming exercise training induces cardiac hypertrophy in mice

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Abstract

Exercise training associated with robust conditioning can be useful for the study of molecular mechanisms underlying exercise-induced cardiac hypertrophy. A swimming apparatus is described to control training regimens in terms of duration, load, and frequency of exercise. Mice were submitted to 60- vs 90-min session/day, once vs twice a day, with 2 or 4% of the weight of the mouse or no workload attached to the tail, for 4 vs 6 weeks of exercise training. Blood pressure was unchanged in all groups while resting heart rate decreased in the trained groups (8-18%). Skeletal muscle citrate synthase activity, measured spectrophotometrically, increased (45-58%) only as a result of duration and frequency-controlled exercise training, indicating that endurance conditioning was obtained. In groups which received duration and endurance conditioning, cardiac weight (14-25%) and myocardial ratio (13-20%) increased. The best conditioning protocol to promote physiological hypertrophy, our primary goal in the present study, was 90 min, twice a day, 5 days a week for 4 weeks with no overload attached to the body. Thus, duration- and frequency-controlled exercise training in mice induces a significant conditioning response qualitatively similar to that observed in humans.

Key words
- Exercise training
- Swimming
- Cardiac hypertrophy
- Mice
- Myocardium

Introduction

In response to a variety of mechanical, hemodynamic, hormonal and pathologic stimuli, the myocardium adapts to increased workloads through the hypertrophy of individual muscle cells (1). Cardiac hypertrophy can occur as an adaptive response to a physiological (exercise training) or pathological (valvular disease, hypertension, or obesity) increase in cardiac work (2,3). Different forms of cardiac hypertrophy arise as a result of a combination of genetic, physiologic, and environmental factors. The molecular mechanisms underlying exercise-induced physiological as well as pathological hypertrophy are poorly understood. In either concentric or eccentric pathological hypertrophy, the expression of cardiac embryonic genes such as those coding for natriuretic peptides is increased (4), but is unchanged in exercise-induced physiological hypertrophy (5). With the recent advances in transgenic and gene-targeting approaches, engineered mice have been used as a powerful tool to identify and validate genes and cellular pathways underlying different cardiovascular phenotypes such as hypertrophic (6) and di-
lated (7) cardiomyopathies, but the lack of standardized and reproducible exercise training protocols has limited the understanding of this complex biological process.

Exercise training leads to improvement of cardiovascular capacity which is associated with lower resting and submaximal heart rates, increased ventricular weights and volume, and myocyte hypertrophy (8.9). The adaptation to exercise training is dependent on factors such as training load, duration and frequency. Swimming is recognized for its efficiency in inducing myocardial hypertrophy and a significant increase in left ventricular end-diastolic volume in rats (10.11). In the present study, we developed a robust and reproducible exercise training protocol for the development of cardiac hypertrophy in mice. A swimming training apparatus was built and used to test different swimming programs regarding duration, frequency and load of physical exercise. Using this approach, we provided evidence that duration and frequency but not load-controlled swimming training regimens lead to significant endurance conditioning and myocardial hypertrophy in mice.

**Material and Methods**

**Study population**

Nine-week-old male C57/BL6 mice (N = 62) were maintained in a light- (12-h light cycle) and temperature- (22°C) controlled environment and were fed a pelleted rodent diet (Nuvital Nutrientes S/A, Curitiba, PR, Brazil) ad libitum and had free access to water. The training sessions were performed during the dark cycle of the mice which in the present study were kept from 7:00 to 19:00 h. The animals were randomly assigned to 3 sedentary (S1-S3, N = 19) and 7 exercise-trained (T1-T7, N = 43) groups according to the two protocols described below.

All animal experimental procedures followed Institutional guidelines and were approved by the Ethics Committee of the University of São Paulo Medical School.

**Training protocols**

Group assignment and number of mice used in each protocol are given in Table 1.

**Protocol 1**. The adaptation to exercise training depends on factors such as training load, duration and frequency. In protocol 1, duration, frequency and load of physical exercise were investigated. Training duration was associated with changes in length of exercise sessions (60 vs 90 min), while exercise with the addition of different workloads (2 or 4% of body weight) modified the training load. Training frequency was investigated by comparing the number of exercise training sessions per day (once vs twice a day). The aim was to optimize these parameters to induce robust cardiovascular effects, such as resting bradycardia and cardiac hypertrophy. The mice were randomly assigned to a sedentary (S1, N = 13) and five trained (T1-5, N = 29) groups. The T1, T2 and T3 groups were exercised at the same load but with different duration or frequency of exercise, with T1 and T2 being used as duration groups and T3 as the frequency group. In contrast, T4 and T5 groups (load groups) were trained with the same duration and frequency of exercise but with different loads.

<table>
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N = number of mice; bw = body weight; volume = duration of each session training in minutes.
The T1 group trained 5 days a week, twice a day with a gradual progression toward a 90-min session for 6 weeks. The T2 and T3 groups performed a swimming training protocol similar to that of the T1 group, except that exercise sessions were shorter (a 60-min session, twice a day). The training frequency of the T2 and T3 groups was also reduced (once a day, 90-min session). The T4 and T5 groups performed a swimming protocol similar to that of the T2 group (a 60-min session), except that they were submitted to swimming with a 2 or 4% body weight workload, respectively. All mice were weighed once a week and when necessary the workload (2 or 4% of body weight) was adjusted to body weight changes. Sedentary mice were placed in the swimming apparatus for 5 min twice a week to mimic the water stress associated with the experimental protocol.

Protocol 2. In this protocol the results obtained for group T1 were evaluated with regard to the duration (4 vs 6 weeks) of training. Mice were randomly assigned to 2 sedentary (S2 and S3, 4 vs 6 weeks, respectively, N = 6) and 2 exercise-trained (T6 and T7, 4 vs 6 weeks, respectively, N = 14) groups.

Swimming apparatus

We designed a swimming apparatus especially planned for exercise training of mice. The system consists of two coupled 200-L water glass tanks of different dimensions. The outer tank measures 60 cm in diameter, 100 cm in width and 50 cm in height. The inner tank is divided into 14 lanes with a surface area of 15 x 15 cm per lane and a depth of 35 cm to allow individual training. To prevent floating during the swimming session, water bubbling was produced by tubes connected to an air pump system. A heating system kept the water temperature between 30 and 32°C and a water filter with a flow capacity of 420 l/h was used to clean the swimming apparatus (Figure 1).

Resting systolic blood pressure and heart rate measurements

Tail-cuff systolic blood pressure and heart rate (HR) were determined during the 4- or 6-week-period of study using a computerized tail-cuff system (BP 2000 Visitech Systems, Apex, NC, USA) (12). Blood pressure values were determined for each animal by averaging blood pressure measurements obtained on two different days of the same week during the animal’s dark cycle.

Analysis of cardiac structure

Twenty-four hours after the last exercise training session, sedentary and exercise-trained mice were killed and tissues harvested. The weights of the heart and of the dissected chamber atria, right ventricle and left ventricle were measured. The dissected chambers were then fixed by immersion in 4% buffered formalin and embedded in paraffin for routine histologic processing. Sections (4 µm) were stained with hematoxylin and eosin for examination with a light microscope. Myocyte width was measured in the left ventricle free wall with a computer-
assisted morphometric system (Leica Quantimet 500, Cambridge, UK). For 10 myocytes containing a nucleus visible in the field, a single transverse measurement of width passing through the nucleus was determined. Myocyte diameter was determined for each animal by averaging the measured myocytes.

**Skeletal muscle oxidative enzyme activity**

Muscle samples were taken from the left and right soleus at the time of killing and frozen in liquid nitrogen for later processing. Citrate synthase activity was measured spectrophotometrically in whole muscle homogenates and the amount of the complex resulting from coenzyme A and oxaloacetate was determined (13).

**Statistical analysis**

Data are reported as means ± SEM. Data for the exercise-trained groups were compared to those for the sedentary groups using one-way ANOVA for repeated measures and two-way ANOVA followed by the Tukey post hoc test in Protocols 1 and 2, respectively. Statistical significance was set at P < 0.05.

**Results**

**Resting systolic blood pressure and heart rate during the exercise-training period**

Baseline blood pressure did not differ among the groups studied in Protocol 1 (Figure 2A). In contrast, after 3 weeks of exercise training, HR decreased significantly in all trained groups compared to the pre-exercise period (T1, 12.3%; T2, 12%; T3, 8.3%; T4, 10%, and T5, 8%) as well as to sedentary littermates (Figure 2B). In contrast, HR was unchanged in the sedentary group throughout the study. The development of resting bradycardia in the exercising mice indicates that aerobic conditioning was achieved with these training regimens (14).

In Protocol 2, reduction of exercise training from 6 to 4 weeks resulted in no change in blood pressure or HR responses (Figure 2C and D, respectively). Baseline blood pressure remained unchanged in all sedentary and exercise-trained groups, while signifi-

![Figure 2. Resting systolic blood pressure (mmHg) and heart rate (bpm) during physical training in Protocols 1 (A and B) and 2 (C and D). Data are reported as mean ± SEM. *P < 0.05 compared to the sedentary groups S1 (B) and S2 and S3 (D) at the same time. †P < 0.05 compared to the trained groups before and after physical training (two-way ANOVA test). Resting blood pressure did not change among groups after physical training; however, heart rate decreased in all trained groups after 2-3 weeks of physical training (P < 0.05). bet = before exercise training.](image-url)
cant resting bradycardia was achieved at both T6 (18%) and T7 (16.4%) 4 and 6 weeks of exercise training, respectively, compared to sedentary littermates (Figure 2D).

Cardiac structure analysis

The body weights of exercise-trained mice from the T1 to T4 groups were similar to those of sedentary mice (Table 2). In contrast, mice exercised at a higher load (T5) showed a significantly lower body weight than sedentary mice. In Protocol 2, the body weight of T6 mice did not differ from that of their sedentary littermates, while T7 mice had a significantly lower body weight than S3.

Cardiac hypertrophy was observed only in mice from duration (T1) and load groups (T4 and T5) in Protocol 1 (Table 2). The increased heart weight to body weight ratio was mainly due to an increase in left ventricle weight in the T1 and T4 groups. In the load group (T4), the normalized weight of the atria also contributed to the increased heart weight. In contrast, in the group exercised at a higher load (T5) the normalized increase in heart weight was associated mainly with a smaller body weight.

The magnitude of cardiac hypertrophy was not influenced by reduction of exercise training from 6 to 4 weeks. Both the T6 and T7 groups presented increased heart weight to body weight ratios mainly due to an increased left ventricle weight to body weight ratio (Table 2).

Changes in myocyte width paralleled the changes observed in the left ventricle to body weight ratios. A significant increase in myocyte width was observed in the exercise-trained groups (T1, T4, T6 and T7) compared to sedentary littermates in both protocols (Figure 3A,C).

Skeletal muscle oxidative enzyme activity

An increased muscle oxidative activity concomitant with an increase in aerobic work capacity is one of the hallmarks of cardiac muscle adaptation to aerobic conditioning. We measured the maximal activity of citrate synthase (an enzyme involved in the citric acid cycle) in the soleus muscle of all exercise-trained and sedentary mice as a marker of muscle oxidative activity.

Interestingly, in Protocol 1, the maximal activity of citrate synthase was significantly

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<td></td>
<td>T7</td>
<td>22.20 ± 0.82*</td>
<td>5.13 ± 0.13*</td>
<td>3.80 ± 0.10*</td>
<td>1.01 ± 0.04</td>
<td>0.29 ± 0.02</td>
</tr>
</tbody>
</table>

AT = atria, BW = body weight, H = heart, LV = left ventricle, RV = right ventricle, S = sedentary group, T = training group. Data are reported as mean ± SEM. *P < 0.05 compared to S1, **P < 0.05 compared to S2, and *P < 0.05 compared to S3 (two-way ANOVA test). As shown, cardiac hypertrophy was significant in groups T1, T4, T5, T6 and T7 due mainly to an increase in left ventricular and atrial weight.
higher only in exercise-trained mice from the duration group when compared with sedentary mice and other trained groups (Figure 3B). In Protocol 2, exercise-trained mice from the T6 and T7 groups showed a significant increase in citrate synthase activity when compared to their respective sedentary littermates (Figure 3D).

Discussion

In the current study we examined exercise conditioning, which represents one of the major cardiovascular adaptations to chronic cardiovascular stress, and provide evidence that duration- and frequency-controlled but not load-controlled exercise training regimens induce substantial endurance conditioning and myocardial hypertrophy in mice. These results suggest that the duration- and frequency-controlled training regimens can be useful to unravel the role of particular genes and pathways in exercise-induced cardiac hypertrophy in the context of the whole animal.

Exercise training model

Swimming rather than running was chosen as a model because of its efficiency in inducing myocardial hypertrophy and greater left ventricular end-diastolic volume in rats (10,11). Although most treadmill running studies have failed to show cardiac hypertrophy in rats (15,16), some investigators (17,18) have observed cardiac hypertrophy in mice trained in voluntary running protocols or intensity-controlled treadmill running. However, it is important to emphasize that conditioning associated with voluntary running in wheels is difficult to quantify because there is a wide variation in the amount of running among animals. Even though Kemi et al. (17) recently showed that graded running intensity results in major increments of ventricular mass than fixed running intensity, their protocol was probably restricted to healthy mice considering the intensity levels achieved during the exercise training period (85-90% VO$_{2\text{max}}$). Regarding swimming, most training protocols (3,19-21) have adopted group swimming, which promotes a vigorous response but adds stress as an important confounding variable. One exception is the study by Kaplan et al. (19) showing a swimming training-induced cardiac hypertrophy limited to female mice.

In the present study, we developed a swimming apparatus for mice with individual lanes and bubbling water to avoid animal floating, which facilitated the control of load, duration and frequency during the swim-
Swimming training and cardiac hypertrophy in mice

Swimming sessions. As previously described (19), minimal diving was observed in mice and their forelegs were relatively inactive during swimming, with almost all of the vigorous muscular activity being performed by the hindlegs.

The current study demonstrated that mice who are close to their limit of functional adaptation (very high peak oxygen uptake, intrinsic HR and cardiac sympathetic tone) develop cardiac hypertrophy and endurance conditioning by swimming.

Blood pressure and heart rate responses

Resting bradycardia is a useful and reliable indicator of endurance conditioning (22). The exercise-trained groups showed a lower resting HR when compared to the sedentary groups (P < 0.05). However, the magnitude of resting bradycardia was higher in the duration-controlled trained groups (12-18%) than in the load- and frequency-controlled trained groups (8-10%). The bradycardic response occurred two to three weeks after training in all groups. Although the magnitude of resting bradycardia did not differ significantly among the trained groups, mice from the T6 and T7 groups (duration-controlled training regimen, Protocol 2) tended to present a greater magnitude of resting bradycardia than T1 mice (duration-controlled training regimen, Protocol 1). This response might be related to the variability of resting HR values in this species. In a recent review, Bernstein (23) pointed out the wide variability in the definition of normal resting HR in mice, with reported values ranging from 212 to 690 bpm (24,25).

There was no difference in systolic blood pressure after all swimming training regimens when exercise-trained mice were compared with sedentary littermates. These results are consistent with previous observations by our group (26,27) and others (28) showing that arterial pressure remains unchanged in exercise-trained normotensive animals and humans.

Skeletal muscle oxidative capacity

The adaptation of skeletal muscle oxidative capacity induced by exercise training is well established and is also considered to be a good marker for exercise training efficiency (29). However, few swimming studies in rodents have documented an increased skeletal muscle oxidative capacity associated with exercise training (30). One of the purposes of the present study was to determine if skeletal muscle oxidative capacity would be affected by different swimming training regimens in mice. Interestingly, we observed a significant elevation in citrate synthase activity only in the duration-controlled exercise groups from Protocol 1 (T1, 58%), and Protocol 2 (T6, 45%, and T7, 54%) groups. In contrast, the lower duration (T2), frequency (T3), and load groups (T4 and T5) showed no significant changes in citrate synthase activity. These results suggest that duration-controlled exercise training is the most efficient regimen for inducing skeletal muscle adaptation in mice. In fact, duration of training has been identified as the main factor affecting skeletal muscle oxidative capacity after exercise training in rodents (31). In contrast, the effect of load exercise training on oxidative enzyme activity in rodents remains controversial (32,33).

Ventricular weights and left ventricular cardiac myocyte dimensions

Although an increase in heart weight-to-body weight ratio had been previously reported in mice (19,34,35), the present data are the first to demonstrate that the increase in heart weight is influenced by exercise training regimens. We found that the duration-controlled swimming training was the most efficient regimen for inducing cardiac hypertrophy in mice. Other studies with different physical training protocols such as...
voluntary wheel running (18) and treadmill running (17) have reported smaller cardiac hypertrophies (10 and 12.3%, respectively) compared to the present study (25%). The hypertrophic responses were accompanied by an increase in myocyte width. Interestingly, the time course of exercise training (4 vs 6 weeks) did not influence the magnitude of cardiac hypertrophy (groups T6 vs T7).

Limitations of the present study

Swimming has been suggested to be the most efficient type of exercise for inducing conditioning and cardiac hypertrophy in animals (36) even though it is associated with an important stress response (28). We placed the mice from the sedentary groups in the water for 5 min twice a week in an attempt to minimize the effect of stress when comparing sedentary vs trained groups. Furthermore, although the corticosterone levels were not assessed in these animals, the adrenal gland weight was determined and did not differ among groups (data not shown).

Earlier studies have demonstrated that exercise training by swimming increases the heart weight-to-body weight ratio by 12 to 31% in rats (16) and by 16 to 29% in mice (19,34). Cardiac hypertrophy is well known to occur in response to various stimuli, such as pressure and volume overload. Exercise training is mainly related to a volume overload-induced cardiac eccentric hypertrophy with predominant longitudinal myocyte growth (1). Although myocyte length has not been determined, we were able to detect an increase in myocyte width which paralleled the changes observed in left ventricle to body weight ratios. This is consistent with the idea that exercise training-induced eccentric hypertrophy can lead to proportional myocyte width and length growth (37).

In the present investigation, training regimens associating duration, load, and frequency of exercise were restricted to male mice. We cannot exclude a gender effect on this response although similar cardiovascular and metabolic responses to physical training have been shown in male and female rats (17).

In the present study we describe a swimming apparatus to produce exercise-induced cardiac hypertrophy in mice and provide evidence indicating that duration- and frequency-controlled but not load-controlled exercise is associated with physical conditioning in mice.

References

11. Oscai LB, Mole PA, Brie B & Holloszy JO (1971). Cardiac growth and respiratory enzyme levels in male rats subjected to a running


