Cardiac anti-remodelling effect of aerobic training is associated with a reduction in the calcineurin/NFAT signalling pathway in heart failure mice

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Cardiomyocyte hypertrophy occurs in response to a variety of physiological and pathological stimuli. While pathological hypertrophy in heart failure is usually coupled with depressed contractile function, physiological hypertrophy associates with increased contractility. In the present study, we explored whether 8 weeks of moderate intensity exercise training would lead to a cardiac anti-remodelling effect in an experimental model of heart failure associated with a deactivation of a pathological (calcineurin/NFAT, CaMKII/HDAC) or activation of a physiological (Akt–mTOR) hypertrophy signalling pathway. The cardiac dysfunction, exercise intolerance, left ventricle dilatation, increased heart weight and cardiomyocyte hypertrophy from mice lacking α_{2A} and α_{2C} adrenoceptors (α_{2A}/α_{2C}ARKO mice) were associated with sympathetic hyperactivity induced heart failure. The relative contribution of Ca^{2+}–calmodulin high-affinity (calcineurin/NFAT) and low-affinity (CaMKII/HDAC) targets to pathological hypertrophy of α_{2A}/α_{2C}ARKO mice was verified. While nuclear calcineurin B, NFATc3 and GATA-4 translocation were significantly increased in α_{2A}/α_{2C}ARKO mice, no changes were observed in CaMKII/HDAC activation. As expected, cyclosporine treatment decreased nuclear translocation of calcineurin/NFAT in α_{2A}/α_{2C}ARKO mice, which was associated with improved ventricular function and a pronounced anti-remodelling effect. The Akt/mTOR signalling pathway was not activated in α_{2A}/α_{2C}ARKO mice. Exercise training improved cardiac function and exercise capacity in α_{2A}/α_{2C}ARKO mice and decreased heart weight and cardiomyocyte width paralleled by diminished nuclear NFATc3 and GATA-4 translocation as well as GATA-4 expression levels. When combined, these findings support the notion that deactivation of calcineurin/NFAT pathway-induced pathological hypertrophy is a preferential mechanism by which exercise training leads to the cardiac anti-remodelling effect in heart failure.

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Abbreviations Akt, protein kinase B; α_{2A}/α_{2C}ARKO mice, α_{2A} and α_{2C} adrenergic receptor knockout mice; β-MHC, β-myosin heavy chain; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; FS, fractional shortening; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GATA-4, GATA binding protein 4; HDAC 4 and 5, class II histone deacetylases; IGF-I, insulin-like growth factor; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; mTOR, mammalian target of rapamycin; NFATc3, nuclear factor of activated T-cells transcription factor; PI3K, phosphatidylinositol 3-kinase; SERCA2, sarcoplasmic reticulum Ca^{2+}-ATPase; WT, wild-type.

Cardiac hypertrophy is an adaptative response of the heart to a variety of pathophysiological stimuli, such as hypertension, myocardial infarction, valvular insufficiency, infectious agents, or mutations of contractile proteins. Pathological hypertrophy is associated with severe cardiac dysfunction, arrhythmias, sudden death and heart failure (Levy *et al.* 1990; Frey & Olson, 2003). Indeed, epidemiological studies have revealed that cardiac hypertrophy is an independent risk factor for heart failure development (Ho *et al.* 1993; Lorell & Carabello, 2000). However, not all forms of cardiac hypertrophy are pathological, since exercise training induces physiological

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cardiac hypertrophy associated with improved cardiac function in athletes (Naylor *et al.* 2008).

Several studies have reported Ca^{2+}-handling abnormalities in hypertrophied and failing myocardium in response to neurohumoral activation, stretch and pacing (Bustamante *et al.* 1991; Balke & Shorofsky, 1998; Rossman *et al.* 2004; MacDonnell *et al.* 2007; Rolim *et al.* 2007). In fact, decreased Ca^{2+} transient peak and prolonged Ca^{2+} decay with increased diastolic intracellular Ca^{2+} have been described in animal and human heart failure (Gwathmey *et al.* 1987; Schwinger *et al.* 1995; Seki *et al.* 2003; Bartholomeu *et al.* 2008), and are related to sustained activation of Ca^{2+} sensitive signal transduction pathways, such as calcineurin pathway. Calcineurin is a Ca^{2+}/calmodulin-dependent phosphatase that regulates hypertrophic response (Molkentin *et al.* 1998; Molkentin, 2000; Diedrichs *et al.* 2004; Wilkins *et al.* 2004). Once activated, calcineurin directly dephosphorylates members of nuclear factor of activated T-cells transcription factor family (NFATc3) in the cytoplasm, resulting in their nuclear translocation and activation of hypertrophic genes. In the nucleus, NFAT interacts specifically with a cardiac-restricted zinc finger protein, GATA-4, involved in pathological cardiac hypertrophic response (Molkentin *et al.* 1998). Calcineurin activity and expression are increased in failing hearts and are paralleled by increased NFATc3 translocation to the nucleus and higher GATA-4 expression levels (Diedrichs *et al.* 2004), with consequent reactivation of fetal genes (Molkentin *et al.* 1998). Although pharmacological and genetic inhibition of calcineurin or NFAT in rodents suffice for regression of pathological hypertrophy (Sussman *et al.* 1998; Meguro *et al.* 1999; Lim *et al.* 2000; Bueno *et al.* 2002; Wilkins *et al.* 2002; Bourajjaj *et al.* 2008), to date it is unknown whether exercise training, a physiological stimulus, is able to deactivate the calcineurin pathway associated with an anti-remodelling effect in heart failure.

In contrast to the proposed pathological role for calcineurin/NFAT signalling in the heart, the insulin-like growth factor (IGF-I)–phosphatidylinositol-3 kinase (PI3K)–protein kinase B (Akt)–mammalian target of rapamycin (mTOR) pathway has been reported to mediate physiological hypertrophy associated with exercise training, while it is deactivated in pressure-overload hypertrophy (Kemi *et al.* 2008*b*).

Here, we investigated the effect of moderate-intensity exercise training on the calcineurin and AKT/mTOR signalling pathways in a genetic model of sympathetic hyperactivity-induced heart failure (α_{2A}/α_{2C}ARKO). α_{2A}/α_{2C}ARKO mice display severe cardiac dysfunction associated hypertrophy, cardiac Ca^{2+}-handling abnormalities and clinical signs of heart failure by 7 months of age (Bartholomeu *et al.* 2008; Ferreira *et al.* 2008). Previously, we have demonstrated the beneficial effects of exercise training on cardiac function, Ca^{2+}-handling, and survival in α_{2A}/α_{2C}ARKO mice (Rolim *et al.* 2007). Therefore, the hypotheses of the present study were that moderated exercise training in α_{2A}/α_{2C}ARKO mice would: (1) deactivate the calcineurin signal pathway, (2) activate Akt/mTOR signal pathway, and (3) reduce cardiac mass and cardiac myocyte dimensions associated with a reduced expression of fetal genes.

Methods

Sampling

A cohort of male congenic α_{2A}/α_{2C}ARKO mice in a C57BL6/J genetic background and their wild-type controls (WT) were studied from 5 to 7 months of age. At 7 months of age, α_{2A}/α_{2C}ARKO mice present severe cardiac dysfunction associated with exercise intolerance and increased mortality rate (Brum *et al.* 2002; Rolim *et al.* 2007; Bartholomeu *et al.* 2008). Mice were maintained on a 12 : 12 h light–dark cycle in a temperature-controlled environment (22°C) with free access to standard laboratory chow (Nuvital Nutrientes, Curitiba, PR, Brazil) and tap water. This study was conducted in accordance with the ethical principles of animal research adopted by the Brazilian College of Animal Experimentation (www.cobea.org.br). The animal care and protocols in this study were reviewed and approved by the Ethical Committee of the Medical School of the University of Sao Paulo (174/06).

Graded treadmill exercise test

Exercise capacity, estimated by total distance run, was evaluated with a graded treadmill exercise protocol for mice as previously described (Ferreira *et al.* 2007). Briefly, after being adapted to treadmill exercises over 1 week (10 min each session), mice were placed in the exercise streak and allowed to acclimatize for at least 30 min. Exercise intensity was increased by 3 m min^{-1} (6–33 m min^{-1}) every 3 min at 0% grade until exhaustion. The graded treadmill exercise test was performed in wild-type (WT) and α_{2A}/α_{2C}ARKO mice before and after the experimental protocol to estimate exercise capacity, and at the fourth week of training in α_{2A}/α_{2C}ARKO to readjust exercise training intensity.

Exercise training protocol

α_{2A}/α_{2C}ARKO mice performed moderated intensity exercise training on a motor treadmill over 8 weeks (5th–7th month of age), 5 days per week. The running speed and duration of exercise were progressively increased to elicit 60% of maximal speed at the second week of training. At the fourth week of training, run

capacity was evaluated in order to readjust exercise training intensity. In untrained α_{2A}/α_{2C}ARKO and WT mice, treadmill running skills were maintained by treadmill running for 5 min, three times a week. This procedure was also performed in order to avoid any interference of treadmill stress on the variables studied. This latter activity did not seem to alter maximal exercise capacity (Fig. 1*A*).

Cardiovascular measurements

Non-invasive cardiac function was assessed by two-dimensional guided M-mode echocardiography, in halothane-anaesthetized WT and α_{2A}/α_{2C}ARKO mice before and after the experimental protocol. 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. The left ventricular end-diastolic and end-systolic dimension (LVEDD and LVESD, respectively), and the left ventricle systolic function by fractional shortening (FS) were evaluated. The FS was estimated as follows:

$$\mathrm{FS\%} = \mathrm{LVEDD} - \mathrm{LVESD}/\mathrm{LVEDD} \times 100$$

Structural analysis

Twenty-four hours after the last exercise training session, a subset of mice were killed by intravenous injection of sodium pentobarbital (120 mg kg^{-1}) and their tissues harvested. The heart was stopped at diastole (KCl, 14 mM) and dissected to obtain the left ventricle, which corresponds with the remaining organ upon removal of both atria and the free wall of the right ventricle. For morphometric analysis, left ventricle samples obtained from the free wall, at the level of papillary muscle, were fixed in 4% buffered formalin and embedded in paraffin, cut in 4 μm sections and subsequently stained with haematoxylin and eosin. Two randomly selected sections from each animal were visualized by light microscopy using an objective with a calibrated magnification (400×). Myocytes with visible nucleus and intact cellular membranes were chosen for diameter determination. The width of individually isolated cardiomyocyte displayed on a viewing screen was manually traced, across the middle of the nuclei, with a digitizing pad and determined by a computer assisted image analysis system (Quantimet 520; Cambridge Instruments, UK). For each animal approximately 15 visual fields were analysed.

RT-PCR

α_{2A}/α_{2C}ARKO mice present reactivation of fetal genes involved in cardiac remodelling and failure (Bartholomeu *et al.* 2008). Therefore, we further evaluated whether exercise training would decrease β-myosin heavy chain (β-MHC) gene expression. RNA was isolated from left ventricle tissue with Trizol reagent (GIBCO-BRL, Invitrogen Corp., Carlsbad, CA, USA). cDNA was synthesized using Superscript III reverse transcriptase (200 U ml^{-1}, Invitrogen) at 42°C for 50 min. Taq DNA Polymerase (Fermentas, Burlington, ON, Canada) was used for DNA amplification in the presence of specific primers for cardiac isoform of β-MHC. The specific primer sequences were: β-MHC sense, 5′-TGGCAAGACGGTGACTGTG-3′; β-MHC antisense, 5′-CTCAAGGAGCGCTACGCTT-3′. For each cDNA, the number of amplification cycles was that necessary for 50% saturation, as determined in preliminary assays. mRNA levels were normalized to that of β-actin mRNA (which is not changed in this model of HF) in the same assay.

Cellular fractionation

Immediately following the experimental protocol, mice were killed by cervical dislocation and hearts were minced and homogenized in ice-cold homogenization RIPA buffer (150 mM NaCl, 0.5% deoxycholate, 1% Triton X-100, 1 : 300 Sigma protease inhibitor cocktail and 50 mM Tris-HCl at pH 7.4). The homogenate was centrifuged at 100 *g* for 5 min (4°C). The resulting supernatant was centrifuged again at 600 *g* for 5 min (4°C) to obtain the nuclear pellet and the cytoplasmic extract (supernatant). The pellet was incubated with RIPA buffer containing 0.3% SDS and DNAse (1 mg ml^{-1}) for 30 min (4°C) and then centrifuged at 2000 *g* for 10 min (4°C) to obtain the nuclear extract (supernatant).

Western blot analysis

Calcineurin B, NFATc3 and GATA-4 expression levels were evaluated by Western blotting in total, cytoplasmic and nuclear extracts from WT and α_{2A}/α_{2C}ARKO hearts. In addition, Akt, p-Akt ser473, mTOR, p-mTOR ser2448, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), p-CaMKII thr286, class II histone deacetylases HDAC4 and HDAC5 expression levels were evaluated in cytoplasmic extracts. Briefly, samples were subjected to SDS-PAGE in polyacrylamide gels (8–12%) depending on protein molecular mass. After electrophoresis, proteins were electrotransferred to nitrocellulose membrane (Bio-Rad Biosciences; Piscataway, NJ, USA). Equal loading of samples and transfer efficiency were monitored with the use of 0.5% Ponceau S staining of the blot membrane. The blotted membrane was then blocked

(5% non-fat dry milk, 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature and then incubated overnight at 4°C with specific antibodies against calcineurin B (Upstate Biotechnology, Inc., Lake Placid, NY, USA), NFATc3, GATA-4, Lamin B, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and troponin I (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Akt, p-Akt Ser473, mTOR, p-mTOR ser2448, CaMKII, p-CaMKII thr286, HDAC4 and HDAC5 (Cell Signaling Technology, Inc., Beverly, MA, USA). Binding of the primary antibody was detected with the use of peroxidase-conjugated secondary antibodies (rabbit or mouse, depending on the protein, for 2 h at room temperature) and developed using enhanced chemiluminescence (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) detected by autoradiography. Quantification analysis of blots was performed with the use of Scion Image software (Scion based on NIH image). Samples were normalized to relative changes in Lamin B (nucleus) and GAPDH (cytoplasm) and expressed as a percentage of control.

Confocal microscopy

Isolated cardiomyocytes were analysed by confocal microscopy to compare the cellular localization of NFATc3 and GATA-4. Cardiomyocytes from WT and α_{2A}/α_{2C}ARKO mice were enzymatically isolated as previously described (Guatimosim *et al.* 2001). Briefly, the hearts were mounted on a Langendorff system, perfused for ∼5 min with calcium-free solution containing (in mM): 130 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 0.33 NaH_2PO_4, 1 $C_3H_5NaO_3$, 3 $C_3H_3NaO_3$, 22 glucose and 25 Hepes (pH 7.4). Afterwards, the hearts were perfused for 10–15 min with a solution containing 1 mg ml^{-1} collagenase type II (Worthington Biochemical Corp., Lakewood, NJ, USA) and 0.17 mg ml^{-1} protease type IX (Sigma-Aldrich Corp., St Louis, MO, USA). The digested heart was then removed from the cannula, and the ventricles were cut into small pieces. Single cells were isolated by mechanical trituration and stored in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil). Only Ca^{2+} tolerant, quiescent, rod-shaped myocytes showing clear cross striations were studied. Cells were then fixed with 4% paraformaldehyde and permeabialized as previously described (Guatimosim *et al.* 2008). Cardiomyocytes were stained with primary antibodies against NFATc3 and GATA-4 (1 : 100; Santa Cruz). Cells were then stained with secondary antibodies conjugated to Alexa (1 : 1000; Invitrogen), followed by visualization of signals by confocal imaging using the Zeiss Meta confocal microscope (Zeiss Germany) from CEMEL (Biological Sciences Institute, UFMG, Belo Horizonte, Brazil).

Cyclosporine treatment

To test whether calcineurin signalling pathway would be functionally involved in ventricular dysfunction and remodelling in this heart failure animal model, α_{2A}/α_{2C}ARKO mice (6 months of age) were treated for 4 weeks with cyclosporine (Sandimmum, Novartis, East Hanover, NJ, USA) delivered once a day at a rate of 25 mg kg^{-1} day^{-1} (I.P.). This dose was previously reported to inhibit calcineurin phosphatase activity in mice (Meguro *et al.* 1999).

Statistical analysis

Data are presented as means ± S.E.M. Two-way analysis of variance (ANOVA) with Duncan's *post hoc* test (Statistica software, StatSoft, Inc., Tulsa, OK, USA) was used to compare the effect of genotype (WT and α_{2A}/α_{2C}ARKO mice) and exercise training (untrained and trained) on data from Fig. 1. One-way analysis of variance (ANOVA) with Duncan's *post hoc* test (Statistica) was used to analyse data from Figs 2, 3, 4 and 5. Statistical significance was considered to be achieved when the value of P was < 0.05.

Results

Exercise training in α_{2A}/α_{2C}ARKO mice improves cardiac function and exercise tolerance associated with a cardiac anti-remodelling effect

α_{2A}/α_{2C}ARKO mice displayed exercise intolerance and lower basal FS when compared to WT control mice (Fig. 1*A* and *B*). Exercise training increased cardiac function and exercise capacity in α_{2A}/α_{2C}ARKO mice to WT levels. As expected, exercise training increased exercise capacity of WT mice with no changes in FS, heart weight and cardiomyocyte width. Consistent with left ventricle dysfunction, α_{2A}/α_{2C}ARKO mice presented increased heart weight, cardiomyocyte width, and left ventricular dilatation linked to increased LVESD and LVEDD (Fig. 1*C* and Table 1). Eight weeks of exercise training induced a significant cardiac anti-remodelling effect, reducing heart weight and cardiomyocyte width of α_{2A}/α_{2C}ARKO mice (Fig. 1). In addition, exercise training prevented lung water retention observed in α_{2A}/α_{2C}ARKO mice (Table 1).

Exercise training reduces calcineurin-mediated signalling pathway activation in failing hearts of α_{2A}/α_{2C}ARKO mice

Since calcineurin activation plays an important role in pathological cardiac hypertrophy (Molkentin *et al.* 1998;

Table 1. Structural parameters in WT and α_{2A}/α_{2C}ARKO mice

	WT	KO	KOt	KOc
LVEDD (mm)	3.74 ± 0.08 (8)	4.03 ± 0.06 (10)*	3.98 ± 0.03 (10)*	3.84 ± 0.11 (9)
LVESD (mm)	3.07 ± 0.08 (8)	3.44 ± 0.06 (10)*	3.22 ± 0.04 (10)	3.08 ± 0.10 (9)#
AT (mg mm^{-1})	0.42 ± 0.03 (10)	0.43 ± 0.04 (8)	0.39 ± 0.03 (13)	0.34 ± 0.02 (8)
RV (mg mm^{-1})	1.60 ± 0.16 (10)	1.52 ± 0.06 (8)	1.38 ± 0.13 (13)	1.45 ± 0.09 (8)
LV (mg mm^{-1})	5.64 ± 0.22 (10)	6.52 ± 0.25 (8)*	5.84 ± 0.20 (13)	5.95 ± 0.13 (8)
Lung wet/dry ratio	5.57 ± 0.13 (13)	6.44 ± 0.36 (7)*	5.27 ± 0.19 (16)#	5.52 ± 0.21 (9)#

LVEDD, left ventricular diastolic diameter; LVESD, left ventricular systolic diameter; AT, atria; RV, right ventricle; LV, left ventricle. AT, RV and LV weights were normalized by tibial length. Data are presented as means ± S.E.M. *$P < 0.05$ *vs.* WT; #$P < 0.05$ *vs.* KO. The number of animals studied in each group is showed in parentheses. Data were analysed by one-way ANOVA with Duncan's *post hoc* test.

Molkentin, 2000; Diedrichs *et al.* 2004; Wilkins *et al.* 2004), we evaluated expression levels of calcineurin B and its downstream targets, NFATc3 and GATA-4, in hearts of WT and α_{2A}/α_{2C}ARKO mice. No changes in cardiac calcineurin B total levels were observed in untrained and trained α_{2A}/α_{2C}ARKO mice compared with WT mice (Fig. 2*A* and *B*). Nuclear calcineurin B translocation was significantly increased in untrained α_{2A}/α_{2C}ARKO mice, whereas exercise training decreased it toward WT levels (Fig. 2*A* and *B*).

Even though total cardiac NFATc3 expression levels were similar among all three groups studied (Fig. 2*A* and *C*), we observed a significant increase of NFATc3 translocation to the nucleus in α_{2A}/α_{2C}ARKO mice (Fig. 2*A* and *C*). Interestingly, exercise training decreased nuclear expression of NFATc3 to WT levels, which suggests an inhibition of NFATc3 nuclear translocation after exercise training in α_{2A}/α_{2C}ARKO mice. Total and nuclear GATA-4 expression levels were elevated in α_{2A}/α_{2C}ARKO compared to WT mice (Fig. 2*A* and *D*). Exercise training reduced both GATA-4 expression levels and nuclear translocation in α_{2A}/α_{2C}ARKO mice to WT levels. Immunofluorescence experiments corroborated these findings in isolated ventricular myocytes (Fig. 2*E*).

The increased cardiac expression of GATA-4 and translocation of NFATc3 to the nucleus were paralleled by increased β-MHC mRNA levels (13%, $P < 0.05$) in α_{2A}/α_{2C}ARKO compared with WT mice. Of interest, exercise trained α_{2A}/α_{2C}ARKO mice had significantly reduced cardiac β-MHC gene expression (by 95%, $P < 0.05$), with levels similar to WT mice.

While calcineurin is a high-affinity target of Ca^{2+}–calmodulin, CaMKII is a low-affinity target of Ca^{2+}–calmodulin (Saucerman & Bers, 2008; Song *et al.* 2008) also involved in cardiac hypertrophy and dilatation (Maier, 2009). Therefore, we also evaluated

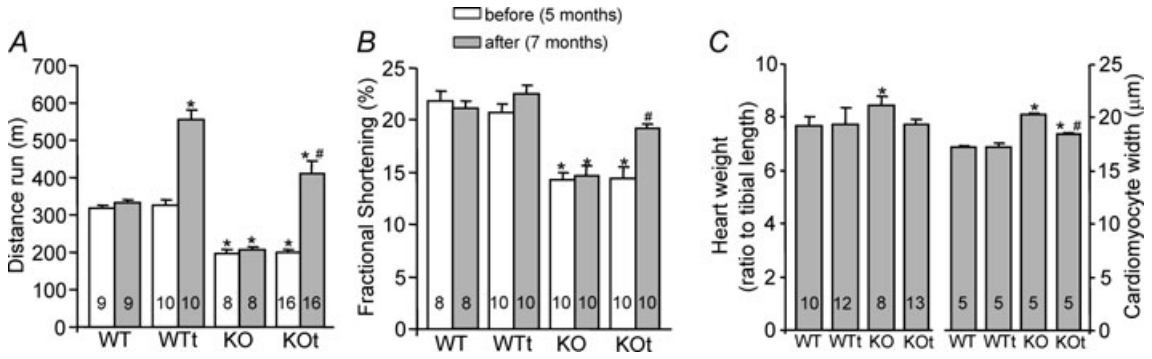

Figure 1. Exercise training re-establishes cardiac function and leads to a cardiac anti-remodelling effect in α_{2A}/α_{2C}ARKO mice

Total distance run (*A*), fractional shortening (*B*), heart weight and cardiomyocyte width (*C*) measurements were performed in untrained wild-type (WT), trained wild-type (WTt), untrained α_{2A}/α_{2C}ARKO (KO) and trained α_{2A}/α_{2C}ARKO (KOt) mice, before (open columns) and after (filled columns) exercise training protocol. Note that untrained KO mice presented exercise intolerance, cardiac dysfunction and cardiac hypertrophy. Exercise training also improved cardiac function and decreased cardiac mass and cardiomyocyte width in α_{2A}/α_{2C}ARKO mice. Data are presented as means ± S.E.M. *$P < 0.05$ *vs.* WT, #$P < 0.05$ *vs.* KO. *A* and *B* were analysed by two-way ANOVA for repeated measurements with Duncan's *post hoc* test. *C* was analysed by two-way ANOVA with Duncan's *post hoc* test.

the relative contribution of the CaMKII signalling pathway in cardiac remodelling of α_{2A}/α_{2C}ARKO mice. No changes in CaMKII pathway activation were observed in α_{2A}/α_{2C}ARKO mice. CaMKII and p-CaMKII expression levels as well as HDAC4 and HDAC5 cytoplasmic levels were similar among all mice studied (Fig. 3*A*–*C*). These results suggest that the CaMKII signalling pathway is not involved in exercise training anti-cardiac remodelling effect.

To further investigate whether a pro-survival Akt/mTOR pathway was involved in the cardiac anti-remodelling effect of exercise training in α_{2A}/α_{2C}ARKO mice, we evaluated expression levels of Akt and mTOR in heart homogenates from WT and α_{2A}/α_{2C}ARKO mice. Both cardiac Akt and mTOR expression and their phosphorylation levels at serine 473 and serine 2448, respectively, were similar among all mice studied (Fig. 3*D* and *E*). These results indicate that beneficial effects

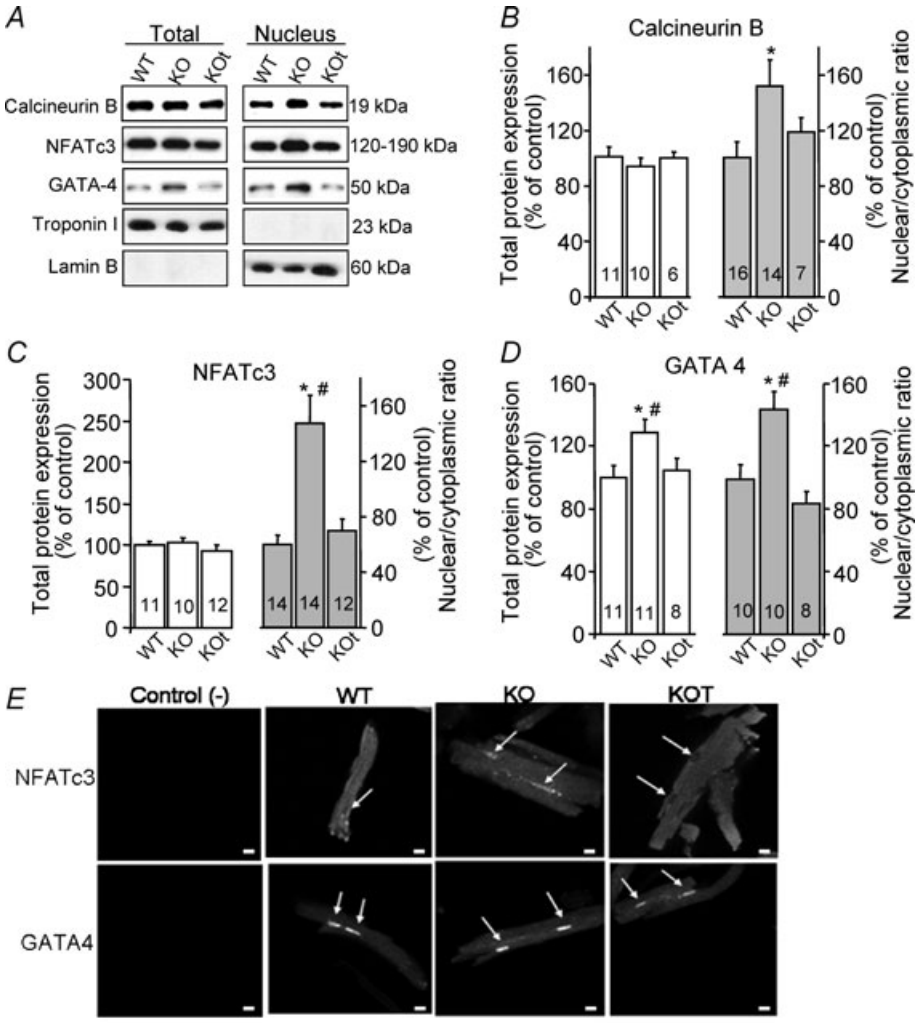

Figure 2. Cardiac anti-remodelling effect of exercise training in α_{2A}/α_{2C}ARKO mice is associated with decreased calcineurin signalling pathway activation

A, representative blots of calcineurin B, NFATc3 and GATA-4 in total and nuclear extracts from untrained wild-type (WT), untrained α_{2A}/α_{2C}ARKO (KO) and trained α_{2A}/α_{2C}ARKO (KOt) mice after exercise training protocol. Total and nuclear extracts were normalized by troponin I and laminin B, respectively. *B*–*D*, calcineurin B (*B*), NFATc3 (*C*) and GATA-4 (*D*) expression levels and nuclear/cytoplasmic ratio from WT, KO and KOt. *E*, confocal images showing immunofluorescence labelled cardiomyocytes stained with anti-NFATc3 or anti-GATA-4 antibodies. *F*, cytoplasmic and nuclear localization of NFATc3 and GATA-4 in untrained WT, untrained KO and trained KOt. Exercise training prevented nuclear calcineurin B, NFATc3 and GATA-4 translocation in α_{2A}/α_{2C}ARKO mice. Arrows indicate cell nucleus. Bar = 10 μm. Data are presented as means ± S.E.M. $^*P < 0.05$ *vs.* WT, $\#P < 0.05$ *vs.* KOt. Data were analysed by one-way ANOVA with Duncan's *post hoc* test.

exerted by exercise training are not related to Akt/mTOR signalling pathway activation.

Sustained calcineurin B inhibition re-establishes cardiac function and remodelling in α_{2A}/α_{2C}ARKO mice

To evaluate the relative contribution of calcineurin signalling pathway on ventricular dysfunction and remodelling in this heart failure model, we treated α_{2A}/α_{2C}ARKO mice with cyclosporine. As depicted in Fig. 4, cyclosporine treatment improved FS (Fig. 4*A*) and exercise capacity (Fig. 4*B*) of α_{2A}/α_{2C}ARKO mice to WT values. The increased left ventricular function was associated with a pronounced cardiac anti-remodelling effect, characterized by reduced heart weight (Fig. 4*C*) and LVEDD (Table 1). As expected, 4 weeks of cyclosporine treatment diminished nuclear translocation of calcineurin B, NFATc3 and GATA-4 in α_{2A}/α_{2C}ARKO mice to WT levels (Fig. 5*A*–*D*). No changes in pro-survival Akt/mTOR pathway were observed in cyclosporine treated α_{2A}/α_{2C}ARKO mice (Fig. 5*E* and *F*).

Discussion

Exercise training is a key intervention for prevention and therapy in cardiology (Roveda *et al.* 2003; Jonsdottir *et al.* 2006; Wisloff *et al.* 2007), and its effect on cardiac function and structure in heart failure includes improved net balance of Ca^{2+}-handling proteins, and improved left ventricular function associated with cardiac anti-remodelling (Lu *et al.* 2002; Rolim *et al.* 2007; Kemi *et al.* 2008*a*; Medeiros *et al.* 2008). However, the mechanisms underlying the exercise training-induced cardiac anti-remodelling effect in heart failure remain unknown. The key findings of the present study are that moderate-intensity exercise training reduced nuclear translocation of calcineurin B, NFATc3 and GATA-4 without significant changes of the CaMKII signalling pathway in hearts of α_{2A}/α_{2C}ARKO mice. These molecular changes were paralleled by diminished cardiac mass and reduced expression of fetal cardiac genes. Indeed, exercise training in α_{2A}/α_{2C}ARKO mice improved exercise tolerance and ventricular function while reducing lung water retention. Unexpectedly, these changes occurred without concomitant activation of the Akt-mTOR signalling pathway in this genetic model of heart failure.

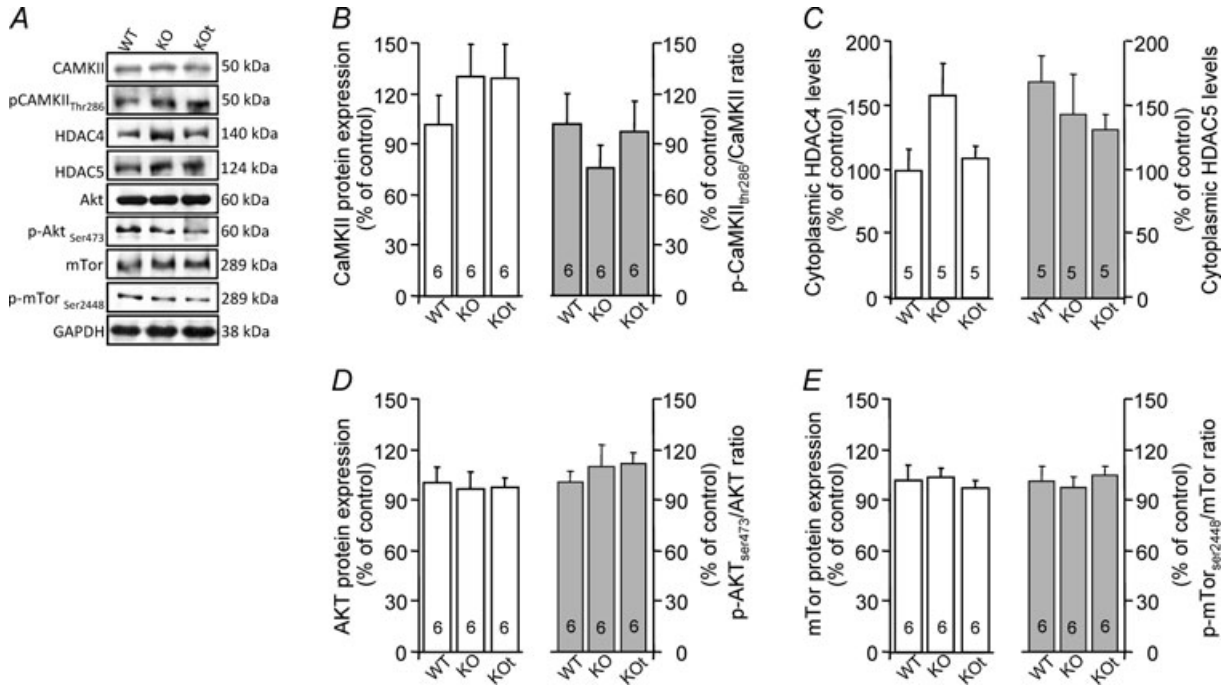

Figure 3. Exercise training has no effect on CaMKII and Akt signalling pathway activation in α_{2A}/α_{2C}ARKO mice

A, representative blots of CaMKII, p-CaMKII thr286, HDAC4, HDAC5, Akt, p-Akt ser473, mTOR, p-mTOR ser2448 in cytoplasmic fraction from untrained wild-type (WT), untrained α_{2A}/α_{2C}ARKO (KO) and trained α_{2A}/α_{2C}ARKO (KOt) mice after exercise training protocol. *B*–*E*, CaMKII and p-CaMKII thr286 (*B*), HDAC4 and HDAC5 (*C*), Akt and p-Akt ser473 (*D*), and mTOR and p-mTOR ser2448 (*E*) expression levels in cytoplasmic fraction from WT, KO and KOt mice. Note that cardiac CaMKII, Akt and mTOR expression levels and their phosphorylation at threonine 286, serine 473 and serine 2448, respectively, were similar among all mice studied. Data are presented as means ± S.E.M. Data were analysed by one-way ANOVA with Duncan's *post hoc* test.

Considering that both calcineurin and CaMKII are targets of Ca^{2+}–calmodulin, one might expect that both pathways could be involved in hypertrophic responses of cardiac myocytes in α_{2A}/α_{2C}ARKO mice. However, the preferential activation of the calcineurin pathway in our heart failure model might be explained by the higher affinity of Ca^{2+}–calmodulin for calcineurin than CaMKII. In fact Song *et al.* (2008) demonstrated that differential Ca^{2+}–calmodulin binding affinities of targets (e.g calcineurin *vs.* CaMKII) predict the selective activation of distinct Ca^{2+} signalling pathways in paced adult rabbit ventricular myocytes (Song *et al.* 2008).

Cardiac hypertrophy in α_{2A}/α_{2C}ARKO mice can be counteracted by cyclosporine treatment, which reinforces the involvement of the calcineurin pathway on cardiac remodelling in this heart failure model. As the therapeutic use of cyclosporine for reducing heart failure-associated cardiac remodelling has been tempered by its known side effects (Molkentin, 2000), moderate intensity aerobic exercise training emerges as an adjuvant therapy for the cardiac anti-remodelling effect by decreasing the calcineurin/NFAT signalling pathway that is exacerbated in heart failure. In fact Konhilas *et al.* (2006) reported that voluntary exercise decreased NFAT activity and reversed the cardiac disease phenotype in hypertrophic cardiomyopathy animal model (Konhilas *et al.* 2006). Our study extends the knowledge that exercise training deactivates the calcineurin/NFAT signalling pathway to heart failure. This is particularly important since heart failure is a common end-point of several cardiomyopathies.

The mechanisms by which exercise training decreases calcineurin/NFAT signalling pathways in heart failure might involve improved cardiac net balance of Ca^{2+}-handling proteins. In fact we previously reported that moderate intensity aerobic exercise training improved cardiac intracellular Ca^{2+} regulation of α_{2A}/α_{2C}ARKO mice in different stages of heart failure by increasing the expression of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2), and phosphorylation of phospholamban at both Serine16 and Threonine17 residues (Rolim *et al.* 2007; Medeiros *et al.* 2008). The impact of exercise training on SERCA2 and phosphorylation of phospholamban is positive for heart failure since both are decreased in human heart failure and associated with increased calcineurin phosphatase activity (Munch *et al.* 2002). An alternative mechanism associated with decreased calcineurin signalling pathway by exercise training might be related to its effect on sympathetic nerve activity and neurohormones. Sympathetic hyperactivity, currently achieved by the use of genetically engineered mice, plays a prominent role in cardiac remodelling and failure (Barki-Harrington *et al.* 2004) and β-adrenoceptor stimulation increases calcineurin activity in hearts of hypertensive rats (MacDonnell *et al.* 2007). Exercise training is efficient in reducing sympathetic nerve activity of heart failure patients (Roveda *et al.* 2003; Fraga *et al.* 2007). In addition, we have also observed decreased circulating noradrenaline (Medeiros *et al.* 2008), and cardiac angiotensin II (Pereira *et al.* 2009) levels in exercise trained α_{2A}/α_{2C}ARKO mice.

Exercise training-induced deactivation of the calcineurin signalling pathway in α_{2A}/α_{2C}ARKO mice was associated with improved ventricular function, reduced reactivation of cardiac fetal genes and decreased cardiomyocyte width, which highlights the role of exercise training in reversing pathological hypertrophy associated with heart failure in our model. However, one could expect that exercise training instead of deactivating a pathological pathway in cardiac remodelling would rather activate intracellular pathways involved in physiological cardiac hypertrophy, such as the Akt/mTOR signalling pathway. The Akt/mTOR signalling pathway has been

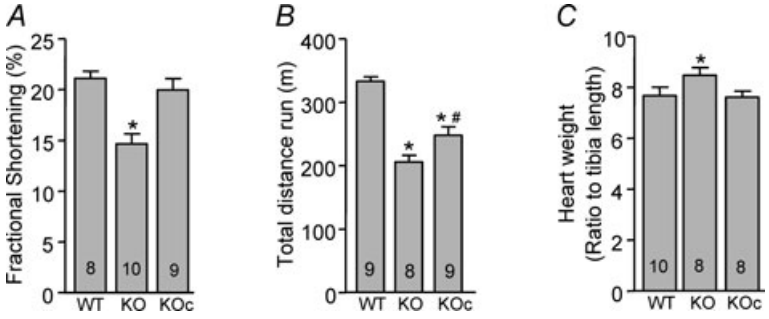

Figure 4. Sustained calcineurin B inhibition reestablishes cardiac function and leads to a cardiac anti-remodelling effect in α_{2A}/α_{2C}ARKO

Total distance run (*A*), fractional shortening (*B*) and heart weight (*C*) from wild-type (WT), α_{2A}/α_{2C}ARKO (KO) and cyclosporine-treated α_{2A}/α_{2C}ARKO (KOc) mice. Note that 4 weeks of cyclosporine treatment improved cardiac function by 28% and decreased heart weight to WT levels. No changes in exercise tolerance were observed in KO mice treated with cyclosporine. Data are presented as mean ± S.E.M. $^*P < 0.05$ *vs.* WT, $\#P < 0.05$ *vs.* KO. Data were analysed by one-way ANOVA with Duncan's *post hoc* test.

reported as a key mediator of cardiac physiological growth, since constitutive activation of Akt leads to physiological hypertrophy and increased cardiac contractility (Condorelli *et al.* 2002; McMullen *et al.* 2003). Moreover, physiological cardiac hypertrophy is blunted in Akt-1 knockout mice (DeBosch *et al.* 2006).

We observed no changes in Akt/mTOR expression levels, or in their phosphorylated levels in α_{2A}/α_{2C}ARKO when compared with WT and exercise trained α_{2A}/α_{2C}ARKO mice. This result was somehow unexpected, but factors such as exercise training intensity may contribute to this response. In fact, in parallel to observed skeletal muscle hypertrophy associated with Akt/mTOR signalling activation in high- but not low-intensity exercise training (Nader & Esser, 2001; Atherton *et al.* 2005), exercise-induced physiological cardiac hypertrophy has been primarily observed in high-intensity exercise training regimens (McMullen *et al.* 2003, 2007; Wilkins *et al.* 2004; Kemi *et al.* 2008*a*). Another factor worth mentioning is that the effect of exercise training on cardiac Akt/mTOR signalling pathway has been mainly studied in hearts of animals with preserved function (Kemi *et al.* 2008*a*), but not in animal models of cardiovascular disease. Therefore, we cannot exclude that the Akt/mTOR signalling pathway is not the main mechanism involved in the cardiac anti-remodelling effect of exercise training in failing hearts.

In the present study, we took advantage of a genetic model of sympathetic hyperactivity-induced HF to assess the effect of moderate intensity aerobic exercise training on cardiac structure and function and the involvement of the calcineurin/NFAT and Akt/mTOR signalling pathways in these responses. We cannot exclude that some of the results reported here may be influenced by exercise training intensity and stage of cardiomyopathy in α_{2A}/α_{2C}ARKO mice, but within this time window, moderate intensity aerobic exercise training in severe heart failure was effective in improving cardiac function with a cardiac anti-remodelling effect associated with deactivation of the calcineurin/NFAT signalling pathway. It will be important, however, to further explore whether a more intense exercise training regimen and earlier heart failure stages lead to different efficacy in reducing or preventing cardiac remodelling.

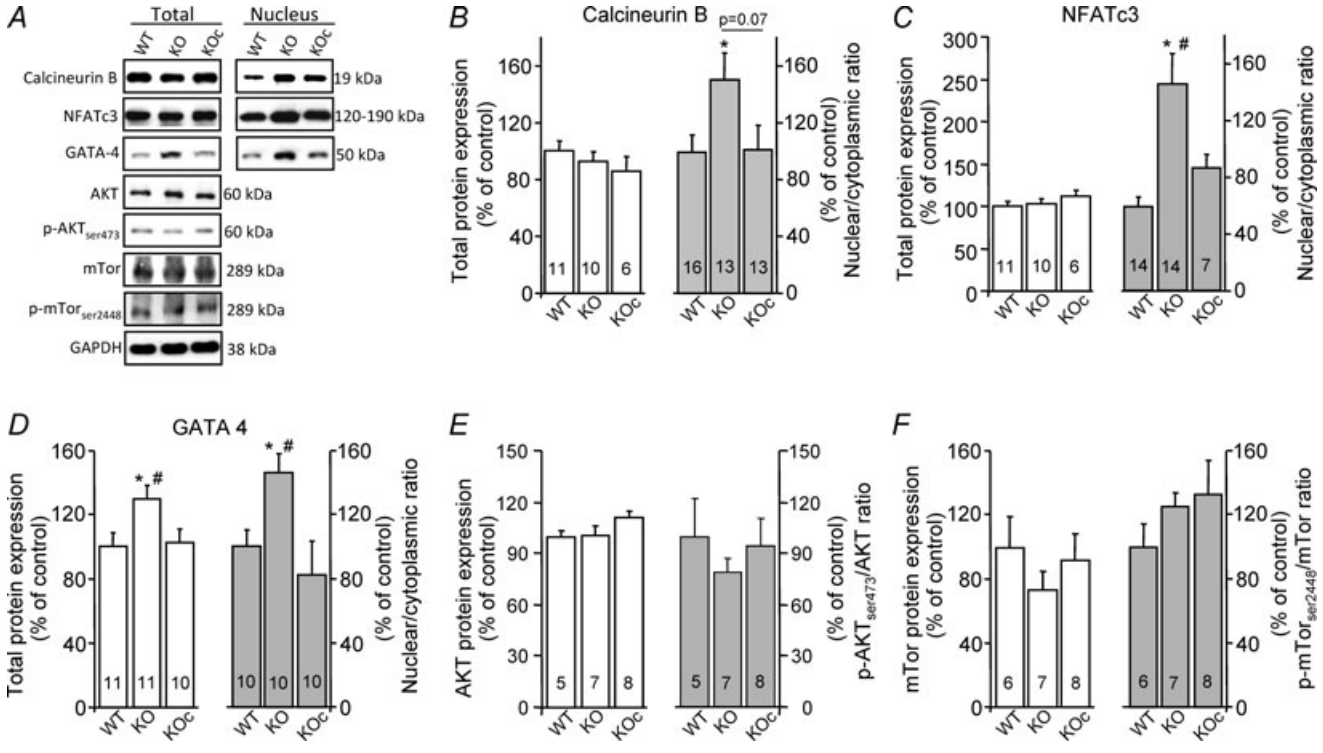

Figure 5. Sustained cyclosporine treatment decreases calcineurin/NFAT pathway in α_{2A}/α_{2C}ARKO mice
A, representative blots of calcineurin B, NFATc3 and GATA-4, in total and nuclear extracts and Akt, p-Akt ser473, mTOR and p-mTOR ser2448 in cytoplasmic extract from untrained wild-type (WT), untrained α_{2A}/α_{2C}ARKO (KO) and cyclosporine-treated α_{2A}/α_{2C}ARKO (KOc) mice. *B–D*, calcineurin B (*B*), NFATc3 (*C*) and GATA-4 (*D*) expression levels and nuclear/cytoplasmic ratio from WT, KO and KOc. *E* and *F*, cytoplasmic Akt and p-Akt ser473 (*E*), and mTOR and p-mTOR ser2448 (*F*) expression levels from WT, KO and KOc mice. Cyclosporine treatment prevented nuclear cyclosporine B, NFATc3 and GATA-4 translocation in α_{2A}/α_{2C}ARKO mice. $^*P < 0.05$ *vs.* WT, $\#P < 0.05$ *vs.* KOc. Data were analysed by one-way ANOVA with Duncan's *post hoc* test.

Conclusion

In summary, moderate intensity aerobic exercise training induced a cardiac anti-remodelling effect in heart failure mice associated with deactivation of the calcineurin/NFAT signalling pathway and decreased reactivation of fetal genes. These findings support the notion that deactivation of pathological hypertrophy signalling pathways is as preferential mechanism underlying the cardiac anti-remodelling effect of moderate intensity aerobic exercise training in heart failure.

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Author contributions

R.S.F.O. and J.C.B.F. contributed to study design and performed most part of the experiments. E.R.M.G. and S.G. collaborated

on experiments shown in Fig. 2*E*, N.A.P. collaborated on real time PCR experiments, and A.M. and N.P.L.R collaborated on fractional shortening evaluation and western blotting experiments. P.C.B. directed and designed the study, and wrote the manuscript. S.G. revised the manuscript critically.

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