Exercise training normalizes altered calcium-handling proteins during development of heart failure

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Exercised training normalizes altered calcium-handling proteins during development of heart failure. Our laboratories on a canine model of pacing-induced heart failure revealed significantly improved hemodynamic parameters in dogs with heart failure after long-term exercise training (34, 35). Although improved endothelium-mediated vasodilator function may contribute to preserving resting hemodynamics, the salutary effect of exercise training on systemic hemodynamics was only partially blocked by nitric oxide synthase antagonism with nitro-L-arginine (35), suggesting that additional mechanisms other than endothelial function are involved in the benefits of long-term exercise training. Using an isolated heart preparation, we further demonstrated that changes in cardiac mechanical function may contribute to the beneficial hemodynamic effects of exercise training in heart failure (34). Small changes in systolic properties and larger changes in diastolic properties were observed in response to exercise training. This resulted in a significant net improvement in overall left ventricular pump function (34). These results also support the hypothesis that there are other mechanisms involved in the beneficial effects of long-term exercise training on hemodynamics and cardiac function in the heart failure state. Because mechanical pump function is implicated in these changes, exercise training may lead to the modulation of calcium homeostasis (5, 9, 24).

Several calcium-handling proteins are involved in the maintenance of normal cardiac calcium homeostasis and contractile function. Among these proteins, cardiac sarcoplasmic reticulum calcium-ATPase (SERCA2a) and cardiac sodium/calcium exchanger (NCX1) are responsible for the control of both systolic and diastolic cytosolic calcium levels. SERCA2a promotes calcium sequestration into the sarcoplasmic reticulum against a substantial concentration gradient in an energy-requiring process, thus facilitating cardiac relaxation and reloading the sarcoplasmic reticulum calcium stores for the next cycle (14, 23). The NCX1 is located on hemodynamics and cardiac function in the heart failure state. Because mechanical pump function is implicated in these changes, exercise training may lead to the modulation of calcium homeostasis (5, 9, 24).

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on the sarcolemma and extrudes intracellular calcium, accounting for up to 30% of calcium removal during diastole (32, 36). A third calcium-handling protein, RyR2 (ryanodine receptor), mediates the systolic release of calcium from the sarcoplasmic reticulum (17, 36). Abnormal calcium homeostasis due to perturbations in the expression and function of these major calcium-regulating proteins have been described in the setting of clinical and experimental heart failure (5, 9, 24). Furthermore, Zhang et al. (39–41) demonstrated the beneficial effects of exercise training on calcium homeostasis in a rat myocardial infarction and cardiac dysfunction model. The alterations of calcium-handling proteins due to exercise training in a canine heart failure model have not been studied.

Accordingly, the purpose of this study was to test whether long-term exercise training alters the mRNA and protein levels of three calcium-handling proteins responsible for cardiac contractile function (SERCA2a, NCX1, and RyR2) during the development of heart failure.

MATERIALS AND METHODS

Pacing-induced heart failure model and physical training protocol. A total of 15 mongrel dogs from the same source, all matched for body weight (28–32 kg), age (1–3 years), and gender, were used in the study. Ten animals underwent chronic instrumentation surgery and were randomly assigned to either the rapid cardiac pacing alone group or the rapid cardiac pacing plus exercise training group. Methods used to create the pacing-induced heart failure model and the performance of physical training have been described previously (34, 35). Briefly, mongrel dogs were anesthetized (5–7 mg/kg iv thiopental followed by 1.5–2.0% inhaled isoflurane after endotracheal intubation) and prepared for sterile surgery. The heart was approached by thoracotomy in the left fifth intercostal space. A screw-type unipolar myocardial pacing lead was placed on the left ventricle. A Tygon catheter (Cardiovascular Instrument) was inserted into the descending thoracic aorta, and a solid state pressure gauge (6.5, Konigsberg Instruments, Pasadena, CA) was placed into the left ventricular chamber via the apex. All wires and catheters were tracked subcutaneously to the back of the dog. The thoracotomy was closed in layers, and a chest tube was inserted to reduce the remaining pneumothorax. The dogs were allowed to recover fully from surgery for at least 10 days and were trained to lie quietly on a laboratory table. Five additional animals were used for collecting myocardial tissue samples as molecular assay controls because no animal could serve as its own control for this purpose. These five animals underwent deep anesthesia, thoracotomy, and harvesting of their hearts.

Experimental design. After full recovery from the instrumentation surgery, baseline studies were performed for measurements of resting hemodynamics in an awake state. Rapid left ventricular pacing was then initiated at 210 beats/min for 3 wk, followed by an additional week of pacing at 240 beats/min with an external pacemaker (EV543, Pace Medical). These animals were assigned to one of two groups: 1) 4-wk rapid cardiac pacing alone with the pacemaker turned off for 2 h/day (n = 5) or 2) 4-wk rapid cardiac pacing plus daily exercise training (n = 5) with the pacemaker off for a total of 2 h/day. The daily exercise training protocol consisted of running on a treadmill (Creative Horse Systems) at 5.1 ± 0.3 km/h for 1 h every morning and 1 h every afternoon for the entire 4-wk period. The pacemaker was turned off during the periods of exercise training. On the final day of the 4-wk period, resting hemodynamics were measured in an awake state. The animals were killed by an overdose of pentobarbital sodium (120 mg/kg), and the chest was opened to harvest the heart for assessment of calcium-handling proteins.

The surgical and experimental protocols used for these studies were approved by the Institutional Animal Care and Use Committee of the Columbia-Presbyterian Medical Center, and animals were cared in accordance with the *Guiding Principles for the Use and Care of Laboratory Animals* [DHEW Publication No. (NIH) 85–23, Revised 1985].

Hemodynamic measurements and tissue collection. Hemodynamic measurements were obtained with the use of the previously implanted catheters and pressure gauges. In brief, aortic arterial pressure was measured by attaching the previously implanted catheter to P23ID strain-gauge transducers (Statham Instruments), and mean arterial pressure was determined on-line using a 3-Hz averaging filter (DA26, Medtron Engineering). Left ventricular systolic and diastolic pressures were measured with the previously implanted solid-state pressure gauges, which were calibrated in vitro against an electronic signal of known amplitude and cross-calibrated in vivo with measurements of pressure from the fluid-filled left ventricular catheter. The first derivative of left ventricular pressure (LVdP/dt) was derived from left ventricular pressure. Hemodynamic data were recorded on an eight-channel thermal writing chart recorder (30-V8808–10, Gould Electronics). Drift in the pressure gauges and chart recorder was eliminated by frequent calibration during the experiment.

Transthoracic echocardiography was performed in an awake state. Mean area ejection fraction (AEF, %) was calculated by averaging at least five steady-state cardiac cycles. These measurements were performed before rapid cardiac pacing or rapid cardiac pacing plus daily exercise training and 4 wk after our rapid cardiac pacing and exercise training regimen.

After euthanasia, ~150 mg of left ventricular myocardium (without visible connective or adipose tissue) were obtained per dog from random locations in the left ventricle and immediately flash frozen in liquid nitrogen, followed by storage at −80°C until use.

RNA isolation and Northern analyses. Total cellular RNA was purified from dog left ventricular samples using RNazol (Biotec Laboratories) according to the manufacturer’s instructions. RNA was quantified by spectrophotometry at 260 nm. The ratio of absorbance at 260 nm to that at 280 nm was >1.8 for all samples. Total cellular RNA (20 μg) was denatured at 65°C for 5 min and size fractionated on 6% formaldehyde–1% agarose gels, followed by overnight transfer of RNA onto nitrocellulose filters. The filters were fixed by ultraviolet cross-linking and prehybridized at 42°C overnight in a buffer containing 5× SSC (0.75 M sodium chloride and 0.075 M sodium citrate, pH 7.0), 5× Denhardt’s solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, and 0.1% BSA), 0.025 M sodium phosphate (pH 6.8), 1 mg/ml salmon sperm DNA, 0.1% SDS, and 50% (vol/vol) formamide. Blots were hybridized with random-primed cDNA probes in the same buffer mixture overnight at 42°C. Filters were washed in 0.2× SSC-0.1% SDS at 65°C and exposed on X-ray film at −80°C.

The SERCA2a probe used in this study was a 2.1-kb EcoRI fragment of the rat SERCA2a cDNA (19). The NCX1 probe was a 3.7-kb EcoRI/PstI fragment of the human cardiac NCX1 cDNA (18). A 4.9-kb fragment of the mouse glyceral-
dehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Ambion) was used as the GAPDH probe. All probes were uniformly labeled with random primers using Klenow and [α-32P]dCTP to a specific activity of >106 counts-min⁻¹-μg DNA⁻¹. GAPDH mRNA bands were used for internal control and normalization of mRNA values.

Protein isolation and Western analyses. Lysates of dog left ventricular tissue were made by sonication of 10 mg of each left ventricular sample in a sevenfold volume of lysis buffer (20 mmol/l sodium-HEPES, 4 mmol/l EGTA, and 1 mmol/l dithiothreitol, pH 7.4) in the presence of proteinase inhibitors (0.1 mmol/l leupeptin and 0.3 mmol/l phenylmethylsulfonyl fluoride) for 1 min on ice. The protein concentration was determined according to the method of Lowry et al. (20), using BSA as a standard. Samples (50 μg) denatured at 95°C were size fractionated with the use of SDS-PAGE under reducing conditions. To determine the levels of SERCA2a, NCX1, and tubulin, SDS-PAGE was performed with 7.5% separating and 4% stacking gels; for RyR2, 5% separating and 5% stacking gels; for SERCA2, 5% separating and 5% stacking gels. Electrophoresis was performed in a MiniProtean II cell (Bio-Rad) followed by transfer of proteins (4°C for 2 h at 100 V) onto nitrocellulose in a mini trans-blot transfer cell (Bio-Rad) filled with transfer buffer (25 mmol/l Tris·HCl, pH 8.3, 150 mmol/l glycine, and 20% methanol).

BLOTS were blocked overnight at 4°C in 5% nonfat milk diluted in Tris-buffered saline (20 mmol/l Tris·HCl, pH 7.6, and 150 mmol/l NaCl) with 0.1% Tween 20 (TBS-T). BLOTS were then incubated with primary antibody diluted in TBS-T (anti-SERCA2a, 1:8000, mouse monoclonal antibody (Novoceastra Laboratories, Benton Lane, UK); anti-NCX1, 1:5000, rabbit antiserum (Swant, Bellinzona, Switzerland); anti-RyR2, 1:4000, mouse monoclonal antibody (Upstate Biotechnology); and anti-tubulin, 1:6000, mouse monoclonal antibody (Sigma)) for 2 h at room temperature. After they were washed in TBS-T, BLOTS were incubated in the presence of a peroxidase-labeled secondary antibody (Amersham) diluted 1:10000 at room temperature. Blots were washed again and incubated with enhanced chemiluminescence reagent (Amersham) for 1 min, followed by autoradiography.

Optical densities of mRNA and protein signals on X-ray films were determined by a laser scanning densitometer (Molecular Dynamics) for quantification of mRNA and protein levels. Expression levels of SERCA2a and NCX1 mRNA were expressed relative to GAPDH, and all three protein levels were expressed relative to tubulin. However, RyR2 mRNA bands were normalized to the intensity of the corresponding GAPDH mRNA band for each sample and expressed in arbitrary units, with the control value set at 1 unit because the signals of RyR2 mRNA were very light.

Data analysis. Data are expressed as means ± SE. Changes in hemodynamic parameters between pre- and post-pacing values were compared by one-way ANOVA. For measurements of gene expression and protein levels, an unpaired two-tailed Student’s t-test followed by a Bonferroni correction was used. P < 0.05 was considered statistically significant.

RESULTS

Resting hemodynamic measurements in awake state. Resting hemodynamics were similar in the two groups of dogs after the instrumentation surgery and before the pacing regimen was started (baseline, Table 1). In the group of animals with rapid cardiac pacing alone, severe heart failure developed, as evidenced by the presence of pulmonary edema, ascites, shortness of breath, and depressed appetite. In contrast, these overt signs were diminished in dogs with the same cardiac pacing regimen plus daily exercise training. Hemodynamic measurements from these two groups of awake animals with the pacemaker off for at least 40 min are summarized in Table 1. All baseline hemodynamic measurements obtained at rest from dogs with cardiac pacing plus daily exercise training were significantly different from those obtained from the group of dogs with cardiac pacing alone. Improved cardiac contractility in the dogs with exercise training was demonstrated by a significant increase in both LV dP/dt and left ventricular systolic pressure.

Echocardiography measurements. The dogs with rapid cardiac pacing plus daily exercise training demonstrated significant improvement not only in resting hemodynamics but also in pump function of the left ventricle, as shown by the significant changes in mean AEF measured by echocardiography. Before rapid cardiac pacing, AEF was comparable between the exercise trained and untrained groups (52.4 ± 3.9% vs. 55.1 ± 3%, P > 0.05). After 4 wk of rapid cardiac pacing, AEF was 48 ± 4% in exercise trained animals and 32 ± 5% in untrained animals (P < 0.05).

Alterations of SERCA2a. Figure 1A shows a representative Northern blot of SERCA2a from normal hearts, failing hearts due to rapid pacing, and hearts from dogs with rapid cardiac pacing plus daily exercise training. Compared with normal hearts, there was a 33% reduction (P < 0.05) in mRNA expression in pacing-induced failing hearts but only a 9% reduction in animals with daily exercise training (P > 0.05). The changes in protein level of SERCA2a were consistent with the changes in message level (Fig. 1B): there was a 65% reduction in failing hearts (P < 0.05) and a 32% reduction in exercise-trained animals (P < 0.05 from failing hearts).

Alterations of NCX1. In contrast to the alterations of SERCA2a, mRNA expression of NCX1 increased by 44% (P < 0.05) in failing hearts compared with normal hearts but only increased by 22% in hearts from dogs with rapid cardiac pacing plus daily exercise training.
DISCUSSION

In this study, we found that long-term exercise training in dogs with rapid cardiac pacing-induced heart failure is associated with relatively preserved expression of three genes and proteins involved with calcium handling. As in many prior studies, rapid cardiac pacing produced hemodynamic abnormalities, including decreased myocardial contractility. In addition, significant abnormalities of both SERCA2a and NCX1 regulation were noted. SERCA2a protein was significantly downregulated and NCX1 protein was significantly upregulated in failing hearts. Rapid cardiac paced dogs subjected to daily exercise training, however, had improved SERCA2a and NCX1 myocardial protein levels and improved hemodynamic parameters. mRNA levels of SERCA2a and NCX1 paralleled the changes seen with the corresponding protein levels. Importantly, normalized levels of these calcium-handling proteins were accompanied by improved pump function of the left ventricle, as indicated by improved resting hemo-

Fig. 1. A: Northern analysis indicates that myocardial sarcoplasmic reticulum calcium-ATPase protein (SERCA2a) mRNA levels were significantly decreased in failing hearts (chronic heart failure [CHF]) compared with normal hearts (Normal). Long-term exercise training (Ex) partially normalized the decreased mRNA levels of SERCA2a (Pacing+Ex). B: myocardial SERCA2a protein levels were examined by Western analysis. The loading of total protein was normalized by tubulin. SERCA2a protein levels were significantly decreased in failing hearts compared with normal myocardium. The decreased SERCA2a protein level was partially normalized by 4 wk of exercise training. Kb, kilobase; KD, kilodalton; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 2. A: Northern analysis shows that mRNA levels of myocardial Na+/Ca2+ exchanger protein (NCX1) were significantly increased in failing hearts (CHF) compared with normal hearts; the upregulated NCX1 mRNA levels were partially normalized by long-term exercise training (Pacing+Ex). B: NCX1 protein levels (in 2 bands) were examined by Western analysis. In contrast to SERCA2a, the protein levels of myocardial NCX1 were significantly increased in the failing heart state; this upregulated NCX1 level was normalized by long-term exercise training (B). Reducing gel condition was used in our study. Four bands were detected (2 major bands at 70 and 120 kDa; 2 faint bands at 140 and 160 kDa). Because readings from these 2 faint bands by densitometer were insignificant, arbitrary numbers from 2 major bands were used for statistics.
dynamics and AEF. No significant changes were detected in the expression of RyR2 protein in hearts from sedentary, heart failure, and exercised dogs. These findings provide important information regarding the possible molecular mechanisms underlying the beneficial effects of exercise training on the progression of heart failure.

Our findings regarding the alterations of myocardial calcium-handling proteins in the heart failure state are consistent with reports from the majority of investigators. Several groups have reported the downregulation of both SERCA2a mRNA and protein levels, as well as the decreased sarcoplasmic reticulum calcium pump activity in rapid cardiac pacing-induced failing canine hearts (9, 16, 24). Data regarding the expression of the cardiac NCX1 in heart failure are somewhat limited compared with the data available for SERCA2a because of the broad diversity reported in the experimental literature. For instance, several groups reported an upregulation of NCX1 in the failing state in both animal models (13, 32) and human hearts (31), but other groups showed decreases in NCX1 in experimental heart failure (38) and human cardiomyopathy (12). In particular, Yao et al. (38) demonstrated that rapid cardiac pacing-induced heart failure resulted in a decrease in NCX1 in rabbits. The discrepancy between our results and other reports can be due to the differences of experimental protocol, heart failure model, pathogenesis, and species. Regarding the expression patterns of RyR2 in heart failure, several groups have reported no significant difference in RyR2 expression in failing explanted human hearts compared with non-failing control tissue (26, 28). These findings have been disputed, however. Brillantes et al. (6) showed decreased RyR2 mRNA levels in ischemic cardiomyopathy but not in dilated cardiomyopathy, and Go et al. (11) observed RyR2 mRNA downregulation in both ischemic and dilated cardiomyopathy. Nevertheless, we further confirmed the abnormalities of calcium-handling proteins in the heart failure state.

The hemodynamic benefits of exercise in the heart failure state are supported by the observations of several groups over the past two decades (4, 8, 10, 22, 27, 34, 35). Much of the functional improvement with exercise training in heart failure may be due to enhanced endothelial function, resulting in improved vasodila-

![Fig. 3. A: Northern analysis indicates that mRNA levels of ryanodine receptor were not altered in either condition of chronic rapid cardiac pacing or rapid cardiac pacing plus exercise training. B: Western analysis further confirmed the unaltered protein levels of this receptor in either condition.](image)

![Fig. 4. Statistical data show that mRNA levels of SERCA2a were significantly decreased and those of NCX1 were significantly increased in failing hearts compared with normal hearts. However, the downregulated SERCA2a and upregulated NCX1 were normalized by long-term exercise training. Ryodidine receptor protein (RyR2) mRNA remained unchanged (A). Changes in protein levels of these 3 calcium-handling proteins are parallel to the changes in their mRNA levels (B).](image)
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function, mainly via normalization of diastolic myocardial stiffness (34). The beneficial effects of exercise training on diastolic function in the failing heart may derive from an improvement in calcium homeostasis during diastole, allowing more efficient myocardial relaxation. Both SERCA2a and NCX1 are responsible for the maintenance of normal diastolic calcium level. However, the major contribution to diastolic calcium lowering is made by SERCA2a (3, 14) and only 20–40% of calcium extrusion during the relaxation phase is made by NCX1 (29, 37). The present study demonstrated that exercise training normalizes the downregulated SERCA2a and the upregulated NCX1 in a canine model of cardiac pacing-induced heart failure. The normalization of these two calcium-handling proteins by exercise training may be associated with significant hemodynamic improvement in the heart failure state. A series of studies by Zhang et al. (41) suggest mechanisms for this improvement (39, 40). With the use of fura 2 fluorescence to measure the calcium transients in single myocytes from myocardial infarction rats with exercise training, they found that sarcoplasmic reticulum calcium uptake was improved and NCX1 current was normalized. They concluded that improved myocyte contractile function due to exercise training was mediated by normalization of calcium homeostasis (39) and that phospholamban may also play a role (40).

Our study was limited to the pacing-induced model. Although Zhang et al. (39–41) reported that exercise training improves calcium homeostasis in postinfarction rat myocytes, it remains to be determined whether the effects of exercise training on calcium-handling proteins in heart failure are a general phenomenon. We used a canine heart failure model of rapid pacing for a number of reasons. First, we had previously demonstrated the benefits of exercise training when applied to dogs during a period of rapid ventricular pacing. Second, the pacing model of heart failure in dogs is a reliable and reproducible method for the induction of cardiac failure, leading to reproducible and consistent hemodynamic and biochemical abnormalities and thus reliable conclusions may be drawn. In contrast, studies of myopathic human ventricular tissue, although arguably more directly clinically applicable than the dog pacing model, have revealed more inconsistent results. These inconsistencies may be the result of differing etiologies of cardiomyopathy in the human samples, varying severity of disease, and differences in other factors, producing a mixed-study population. Our study was also limited by the fact that we have not addressed the molecular mechanisms responsible for the linkage between exercise training, normalization of abnormal calcium-handling proteins, and improved cardiac pump function. In this sense, the results of this study are somewhat descriptive. We also did not examine the possible alterations of phospholamban expression, although this protein and its state of phosphorylation may play a role in the beneficial effects of exercise training on cardiac performance in the setting of myocardial infarction (40). Despite these limitations, our results clearly indicate the striking normalization of SERCA2a and NCX1 due to exercise training during development of heart failure.

Although it has been reported that exercise training facilitates calcium transport mechanisms (30) and enhances extracellular calcium availability for cardiac contractile cycles (33), the fundamental link between the alterations of calcium-handling proteins and exercise training during development of heart failure is unknown. Potential candidates include exercise training-induced alterations in circulating hormone levels, which have been reported by several laboratories to be altered in both heart failure and exercise-trained states. For example, in neonatal myocytes, regulation of cardiac calcium channel expression, such as the α1-subunit of the L-type Ca2+ channel, is affected by catecholamine levels (21). Similarly, both levels of SERCA2a mRNA and protein are elevated in experimental hyperthyroid hearts (2, 7). Exercise training does result in improved catecholamine levels in patients with stable heart failure (10). The effects of altered hormone levels due to exercise training on calcium-handling proteins in the heart failure state merit further examination.

In conclusion, we have demonstrated that long-term exercise training partially prevents hemodynamic abnormalities and normalizes the downregulated SERCA2a and upregulated NCX1 in both mRNA and protein levels during development of heart failure. In the absence of exercise training, significant hemodynamic deterioration and abnormalities of SERCA2a and NCX1 were observed in dogs with rapid cardiac pacing-induced heart failure. Thus long-term physical training may ameliorate cardiac deterioration during development of heart failure in part via normalization of myocardial calcium-handling proteins.

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