Exercise training improves myocardial tolerance to ischemia in male but not in female rats

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The major inducible heat shock protein, heat shock protein 70 (Hsp70), has been shown to be a cardioprotective agent. Currie et al. (10) were the first to show that whole body hyperthermia rendered the rodent myocardium more tolerant to ischemia-reperfusion (I-R) injury. These researchers later noted that the induction of Hsp70 coincided with the cardioprotection afforded by hyperthermia, although no direct relationship was established (19). Subsequent research using transgenic models of Hsp70 overexpression has provided direct evidence for the involvement of Hsp70 in cardioprotection (24, 34, 41).

It is widely accepted that exercise can lead to beneficial cardiovascular adaptations. Although its precise mechanisms on heart health have not been fully defined, chronic exercise is believed to influence a number of parameters, including improving blood-lipid profile, reducing sympathetic tone and enhancing endogenous defense systems [for a review, see Eriksson (12)]. Regarding endogenous defense mechanisms, several studies have reported augmented Hsp70 content and altered antioxidant status in the myocardium following exercise (6, 16, 23, 28, 29, 33, 42, 43). Further, acute exercise has been shown to protect the myocardium from I-R injury, as illustrated by improved recovery of all indices of left ventricle mechanical function following I-R (23, 33). Although several factors have been implicated in this model of exercise-induced cardioprotection, the use of an antisense oligonucleotide against Hsp70 by Paroo et al. (33) to partially abolish this response confirmed involvement of Hsp70 in males.

Scheuer and Stezoski (36) were among the first to report that extended exercise training leads to increased myocardial tolerance to I-R injury in rats. This finding has been observed several times since (3, 4, 7, 15). Harris and Starnes (15) have implicated enhanced expression of Hsp70 in the rat myocardium in the ischemic tolerance acquired through regular exercise.

The majority of the data examining exercise-acquired tolerance to I-R injury in rats comes from male animals. Observations from our laboratory have indicated that unlike in male rodents, Hsp70 expression is often not elevated in skeletal or cardiac muscle in females following acute exercise (32, 33). In agreement with the suggested role for Hsp70 in cardioprotection, hearts from female rodents did not demonstrate the same exercise-mediated improvement in ischemic tolerance observed in male hearts (33). Further, ovariectomized female rodents demonstrated male-like responses in regard to both increased Hsp70 content and acquired cardioprotection, thus supporting a role for estrogen in attenuating these responses (32, 33). This sexual discrepancy has also been observed using a model of hyperthermia to induce Hsp70 production and cardioprotection where estrogen attenuated Hsp70 production in a dose-dependent manner (37).

In addition to Hsp70, the antioxidant manganese superoxide dismutase (MnSOD) has been implicated in the cardioprotection offered by exercise. Studies using both acute (16, 43) and repetitive exercise training (6) have documented increased expression of MnSOD in the myocardium concomitant with improved tolerance to I-R. Further, exercise-acquired ischemic tolerance has been attenuated using antisense oligonucleotides...
to block MnSOD production (14). Others have reported that nitric oxide (NO), particularly NO derived from endothelial nitric oxide synthase (eNOS), is also associated with increased ischemic tolerance (8, 9, 39). Estrogen stimulates eNOS production in the rat myocardium (31) and vasculature (30), and so it is expected that female myocardial tissue will express a greater content of eNOS compared with males. The effects of exercise training on myocardial eNOS expression remain to be identified.

The purpose of the current study was to determine whether or not the repeated stress of regular exercise training would be sufficient to cause an increase in Hsp70 and MnSOD content of female cardiac muscle, as well as to investigate potential training-induced alterations in myocardial eNOS. Further, we aimed to determine whether exercise training would render the female myocardium more tolerant to I-R injury. We hypothesized that regular exercise training would lead to increased myocardial Hsp70 and MnSOD content in female hearts concomitant with an increased tolerance to I-R injury.

**MATERIALS AND METHODS**

**Animals.** All procedures were approved by the University of Western Ontario Council on Animal Care in accordance with the Guidelines of the Canadian Council on Animal Care. Adult (10 wk old) male and female Sprague-Dawley rats (n = 40, Charles River Laboratories, St. Constant, Quebec) were housed in standard rat cages. Room temperature (22 ± 1°C) and relative humidity (50%) were strictly maintained, with a 12:12-h light-dark cycle. Rats were fed standard rat chow and water ad libitum.

**Training protocol.** Twenty male and twenty female rats were used. Each sex was separated into two groups (n = 10 each), with one group performing the exercise training and the other serving as sedentary controls. Two animals died over the course of the study, leaving n = 9 in each control group. Control animals were handled regularly and performed normal ambulatory activity but were never exercised per se. Training was performed for 60 min/day, 5 days/wk for 14 wk at the following intensities: weeks 1–3: 24 m/min, weeks 4–6: 27 m/min, weeks 6–14: 30 m/min (all at a 2% grade).

**Heart perfusion and tissue harvesting.** Twenty-four hours following the last training session, animals were euthanized by decapitation. To match ages, control animals were killed at the same time as the trained group. Following a midline incision, the chest plate was reflected to expose the heart and lungs. The heart was lifted slightly and removed by cutting the arch of the aorta beyond the left subclavian artery (~1–1.5 cm from the heart). The heart was immediately arrested by placing it in ice cold Krebs-Henseleit buffer (KHB). Hearts were mounted on a stainless-steel cannula and inserted into the aorta for retrograde perfusion using a modified Langendorff procedure (25, 33). Perfusion was maintained at 37°C at a rate of 15 ml/min for male hearts and 10 ml/min for female hearts with KHB containing 120 mM NaCl, 4.63 mM KCl, 1.17 mM KH2PO4, 1.25 mM CaCl2, 1.2

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**Table 1. Body and heart weights**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight, g</th>
<th>Heart Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10</td>
<td>649.3±14.15†</td>
<td>3.00±0.22†</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>315.3±6.7</td>
<td>1.60±0.10</td>
</tr>
<tr>
<td>Trained</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10</td>
<td>427.7±17.4*</td>
<td>2.47±0.20*</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>330.8±13.4</td>
<td>1.67±0.07</td>
</tr>
</tbody>
</table>

Values are means ± SE. Males weighed more than females (*P < 0.001, female = 380.6) in both trained and control conditions. Trained males weighed significantly less than control males (†P < 0.001, F = 43.7). Training status had no effect on female body weight. Male hearts weighed more than female hearts (*P < 0.01, F = 24.63). Training had no effect on heart weight.
mM MgCl2, 20 mM NaHCO3, and 8 mM glucose gassed with 95% O2 and 5% CO2.

Hearts were equilibrated for 30 min to determine baseline function, at which point, global, no-flow ischemia was induced by terminating flow for 30 min. Hearts were subsequently reperfused for an additional 30 min. Measures of left ventricle mechanical function [left ventricular end-diastolic pressure (LVEDP), left ventricular end-diastolic pressure (+dP/dt), and −dP/dt] function were expressed as a percentage of preischemic values during reperfusion. Hearts were paced at 300 bpm throughout the experiment. Functional measures were obtained online using the dataanalyst software package (ver. 1.80.3, EMKA Technologies, Paris, France).

Immediately following the I-R protocol, the hearts were removed from the cannula, the atria and right ventricle were discarded, and the left ventricle was frozen in liquid nitrogen and stored at −70°C until analyzed. Plantaris muscle was also removed and frozen for analysis of citrate synthase activity to determine the efficacy of the training protocol.

Western blot analysis. Heart samples (65–80 mg) were cut from the frozen left ventricles and homogenized in 15 volumes of buffer containing 600 mM NaCl and 15 mM Tris (pH 7.5). Samples were centrifuged briefly to clarify debris in the homogenate. Protein concentration of the homogenates was determined using a bicinchoninic assay (Sigma, B9643). Following protein determination, heart samples (50 µg total protein for Hsp70 and MnSOD, 125 µg for eNOS) were separated by SDS-PAGE. Membranes were incubated with a polyclonal antibody specific for the inducible isoform of Hsp70 (SPA-812; StressGen Biotechnologies, Victoria, BC, Canada), MnSOD (SO-111; StressGen Biotechnologies), or eNOS (#610297; BD Biosciences, Franklin Lakes, NJ). Internal standards were loaded in the first lane of each gel (40 µg soleus protein for Hsp70 and 50 and 125 µg of myocardial protein for MnSOD and eNOS, respectively).

Membranes were then incubated with appropriate secondary antibody [goat anti-rabbit conjugated with alkaline phosphatase (#170-6518, Bio-Rad Laboratories, Hercules, CA) for Hsp70 and MnSOD, goat anti-mouse secondary antibody conjugated with horseradish peroxidase (#55400; BD Biosciences) for eNOS]. Hsp70 and MnSOD blots were developed in BCIP and p-nitro blue tetrazolium chloride color development agents (170-6532/170-6539; BioRad Laboratories) in 100 µl development buffer for <15 min. eNOS blots were developed by enhanced chemiluminescence (RPN2106; Amersham Biosciences, Piscataway, NJ) and exposed to film (Kodak Biomax). Western blot analysis bands were quantified (Scion Image, National Institutes of Health) and expressed as a percentage of the standard.

Citrate synthase. Samples (65–80 mg) were cut from the frozen plantaris muscles and homogenized in 15 volumes of buffer containing 600 mM NaCl and 15 mM Tris (pH 7.5). Citrate synthase activity was determined using a spectrophotometer (DU-640; Beckman Coulter) according to the methods described by Srere (38a).

Statistical analysis. All data are presented as means ± SE. Indices of left ventricle function at 0, 5, 10, 15, 20, 25, and 30 min of reperfusion, citrate synthase activity, myocardial Hsp70, MnSOD, and eNOS data were compared by two-way ANOVA among treatment groups. When significant differences were observed (P ≤ 0.05), pairwise comparisons were made using the Student-Newman-Keuls post hoc test. All statistical tests were performed using the Sigma Stat software package (ver. 2.03, SPSS, Chicago, IL).

RESULTS

Animal characteristics. Animal weights on the final day of training and heart weights at time of death are presented in Table 1. Males weighed more than females (P < 0.05) in both trained and control conditions. Trained males weighed significantly less than control males (P < 0.05). Training status had no effect on female body weight. Male hearts weighed more
than female hearts ($P < 0.05$) and training had no effect on heart weight.

**Myocardial Hsp70, MnSOD, and eNOS content.** Two-way ANOVA revealed a significant effect for sex (male > female, $P < 0.05$) and training status (trained > control, $P < 0.05$) for myocardial Hsp70 content, while no interaction between the two variables was detected ($P = 0.09$, Fig. 1). No differences were detected in myocardial MnSOD content (Fig. 2). There was a significant interaction detected between sex and training status for myocardial eNOS expression ($P < 0.05$). Pairwise comparisons showed that training lead to decreased myocardial eNOS content in females and that control female hearts demonstrated greater eNOS expression than male control hearts (Fig. 3).

**Citrate synthase activity.** Citrate synthase activity in the plantaris (a mixed muscle) was increased with training (41.33 vs. 34.44 $\mu$M·min$^{-1}$·g wet weight$^{-1}$, main effect for training $P = 0.027$, $F = 5.4$). No differences were observed between males and females. Increased citrate synthase activity indicates that the animals were aerobically trained as a result of the exercise protocol.

**Left ventricle mechanical performance.** Baseline measures of left ventricular function during the equilibration period are presented in Table 2. During reperfusion, control female hearts demonstrated better recovery of LVDP (Fig. 4A), maximal rate of contraction (+dp/dt; Fig. 4B), maximal rate of relaxation (−dp/dt, Fig. 4C), and LVEDP (Fig. 4D) than male control hearts ($P < 0.05$ at each time point indicated in Fig. 4, A–D).

Compared to control, exercise training led to improvements in post-ischemic LVDP (Fig. 5A), maximal rate of contraction (+dp/dt, Fig. 5B), maximal rate of relaxation (−dp/dt, Fig. 5C), and LVEDP (Fig. 5D) in male rat hearts ($P < 0.05$ at each
time point indicated in Fig. 5, A–D). No improvement in postischemic function was observed in the hearts from trained female animals compared with control (Fig. 6, A–D).

DISCUSSION

The major finding of the current study, that females do not show improved tolerance to I-R injury following exercise training, runs counter to our initial hypothesis. Under control conditions, female rat hearts demonstrated greater cardioprotection against I-R injury than hearts of their male counterparts. However, it appears that exercise training is more beneficial to male rather than female hearts in improving the ability of the myocardium to withstand I-R injury, with the male heart showing a significant improvement in ischemic tolerance, while the female hearts demonstrated no change.

It is important to note that this is not a model for prevention of cardiovascular (CV) disease or myocardial infarction (MI); rather it is a model for the prognoses following MI. To our knowledge, there are no data suggesting a sexual discrepancy in the preventive benefits of regular exercise. In fact, human females are at an advantage with respect to prevention of MI, as the beneficial effects of estrogen on cardiovascular health are well documented [for a review, see Mendelsohn and Karas (27)]. The actions of estrogen are partially responsible for the lower incidence of CV disease among premenopausal women relative to age-matched men (17, 20).

That exercise training results in adaptations that improve myocardial recovery from ischemia in males is not new. Scheuer and Stezoski (36) were among the first to report this phenomenon over 30 years ago. This finding has been reported many times since (3, 4, 7, 15) in an effort to identify what mechanisms might be responsible. There is mounting evidence supporting roles for Hsp70 and MnSOD as potential mediators of some of the cardioprotective effects of exercise. Elevated myocardial Hsp70 has been associated with increased ischemic tolerance in models of acute (one-bout) exercise (33), short-term (3–5 bouts) (23), and extended training (12 wk) (15). In an attempt to isolate the role of Hsp70,
animals run in a cold environment (thus preventing increased core body temperature) demonstrated that exercise-induced tolerance to I-R injury can occur in the absence of any increases in myocardial Hsp70 content (13, 40). However, hearts from animals that were exercised in a normothermic environment demonstrated greater tolerance to ischemia in conjunction with a substantial increase in myocardial Hsp70 compared with the animals run in the cold (40).

The antioxidant MnSOD has also been implicated in the ischemic tolerance conferred by exercise. Again, the results are equivocal. Yamashita et al. (43) reported that one bout of exercise leads to an increase in myocardial MnSOD content along with improved recovery from I-R 30 min and 48 h following exercise, a finding the same group later reproduced (16). Brown et al. have reported increased cardioprotection following exercise training both with (6) and without (7) increases in myocardial MnSOD content. The latter finding has been corroborated by Lennon et al. (22).

Nonetheless, investigators using antisense oligonucleotides to block the exercise induction of MnSOD (14, 43) or Hsp70 (33) report a loss of cardioprotection. Others report that both proteins are elevated following exercise training, with the suggestion that they are both involved in the acquired cardioprotection (35). The exact mechanisms involved in exercise-induced cardioprotection remain to be elucidated; however, it does appear that both MnSOD and Hsp70 are involved, depending on the experimental model employed. The current data suggest that the ischemic tolerance acquired through exercise training in male hearts is more closely associated with increases in Hsp70, as MnSOD content did not change.

While it seems that elevated myocardial Hsp70 content may be associated with cardioprotection in male rat hearts, it remains unclear what effect exercise has in mediating cardioprotection in the female myocardium. Demirel et al. (11) have reported increased Hsp70 in female hearts concomitant with cardioprotection following 5 consecutive days of exercise.
However, Jew and Moore (18) have reported increased Hsp70 content in the female rat myocardium following exercise training (more than 20 wk) with no improvement in postischemic cardiac function compared with control animals. Our previous report suggested that females do not demonstrate increased tolerance to I-R, nor enhanced expression of Hsp70 in the heart following a single bout of exercise (33). With respect to Hsp70, it is likely the differing reports are due to the exercise model used. As mentioned previously, our laboratory (33) and others (37) have illustrated that estrogen can limit the production of Hsp70 in the myocardium. The rodent estrus cycle is 4 days in length, with blood estrogen concentrations fluctuating throughout (38). Hence, in a model of repeated exercise (i.e., 5 days or greater), rats will perform exercise on days when blood estrogen is low. The increase in Hsp70 reported using models of extended exercise, such as the current study, may be due to accumulation on these days.

Regardless of myocardial Hsp70 expression, the current data indicate that female rats do not demonstrate improved myocardial tolerance to I-R following exercise training. Brown and colleagues (7) have also reported sex-dependent differences in exercise-induced cardioprotection. Using infarct size as their primary measure of myocardial damage, they found that male hearts adopted a protected phenotype after just one bout of exercise, while the female rats required 5 days of running to achieve a similar reduction of infarct size following I-R (7). Brown et al. (5) later reported that 12 wk of exercise training reduced the susceptibility of the female myocardium to ischemia as illustrated by a reduction in infarct size compared with control animals following I-R. With regard to mechanical function of the left ventricle, however, they found that hearts from trained females demonstrated improved LVDP only for the later half of the ischemic insult and the first 5 min of reperfusion. Compared with control, the exercise-trained hearts did not show any improvement in mechanical performance for the remaining duration of reperfusion (5). These observations, in addition to those of Jew and Moore (18), are in agreement with our current and previous (33) reports that exercise does not improve mechanical recovery of left ventricle function following a severe episode of ischemia in female hearts from rats.

Although both male and female hearts responded to the stress of exercise training with increased content of Hsp70, this adaptation did not provide any additional ischemic tolerance for the female heart. It is plausible that the female heart is already protected against ischemic stress, and there is little or no capacity for further defense against ischemia. The data contained herein lend support to this notion and show that under control conditions, female hearts are less susceptible to ischemic injury than male hearts. Neither MnSOD nor Hsp70 content differed between the male and female control hearts; therefore, it is unlikely that either of these two proteins is responsible for the difference in ischemic tolerance, which was observed. However, elevated myocardial levels of eNOS observed in the control female heart may be important in this regard.

Nitric oxide, particularly NO derived from eNOS, has often been associated with increased ischemic tolerance (8, 9, 39). Estrogen stimulates eNOS production in the rat myocardium (31) and vasculature (30). Therefore, eNOS may provide cardioprotection for the control female heart but may also attenuate cellular stress signals that would otherwise lead to adaptations (such as increased myocardial Hsp70) following acute exercise (33). Elevated expression of eNOS results in reduced beta-adrenergic signaling in the myocyte by way of increased cyclic-GMP activity, which leads to decreased cyclic-AMP, and thus diminished PKA activity [for a review, see Balligand (2)]. Taken together with the role for PKA in the myocardial Hsp70 response as documented by Melling et al. (26), it is plausible therefore that estrogen may attenuate acute exercise induction of myocardial Hsp70 via this mechanism. However, following exercise training, female hearts demonstrated a decrease in eNOS expression in the myocardium. It is plausible, then, that with exercise training, the female myocardium adopts a different blend with regard to mechanisms of cardioprotection (Fig. 7), one in which the inhibitory effect of eNOS on signaling leading to Hsp70 production is alleviated. Indeed, the current data indicate that the decrease in eNOS occurs
concomitantly with an increase in myocardial Hsp70 in female hearts. Under these circumstances, the increase in Hsp70 may indeed have functioned in a protective capacity, although no additional cardioprotection was afforded since eNOS expression was down regulated.

The question that remains is what role Hsp70 plays in mediating ischemic tolerance in the heart. Both Leger et al. (21) and Amrani et al. (1) report that increases in Hsp70 expression following hyperthermia in the myocardium are localized to the coronary vasculature. The suggestion would be that Hsp70 might help maintain vessel function, preserve coronary perfusion, and therefore confer ischemic tolerance. The role of estrogen in stimulating eNOS has a similar effect [for a review, see Mendelsohn and Karas (27)], therefore it is possible that control female hearts already have vascular protection. Although it has yet to be determined whether exercise-induced increases in Hsp70 also localize to the vasculature, adaptations in the coronary vessels with respect to cardioprotection warrant further investigation.

In conclusion, the current data show that exercise training enhanced myocardial tolerance to ischemia in male, but not female rat hearts. Further, while both males and females demonstrated an increase in content of the proposed cardioprotectant Hsp70, a decline in myocardial eNOS levels in the female rat heart was observed. This may have resulted in a rebalancing of potential protective mechanisms in the female heart, such that there was no improvement in left ventricular mechanical function following exercise training.

GRANTS

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