Exercise Improves Postischemic Cardiac Function in Males but Not Females
Consequences of a Novel Sex-Specific Heat Shock Protein 70 Response

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Abstract—Exercise is a physiological inducer of the cardioprotective heat shock protein, Hsp70. The putative biological events involved in signaling this response exhibit sexual dimorphism. Thus, it was hypothesized that exercise-mediated induction of Hsp70 would demonstrate sex specificity. After treadmill running, male rats exhibited 2-fold greater levels of cardiac Hsp70 relative to the levels in gonadally intact female rats ($P<0.001$). Ovariectomized female rats exhibited exercise-mediated induction of Hsp70 similar to that observed for male rats, and estrogen treatment in these female rats reversed this effect ($P<0.001$). Attenuation of Hsp70 signaling by estrogen was non–receptor-mediated, possibly involving a cellular membrane–stabilizing mechanism of action. The physiological importance of this sex-specific hormone-mediated stress response is underscored by the disparity in functional adaptation in response to exercise between male rats and female rats. Exercise markedly improved posts ischemic left ventricular developed pressure, the maximal rate of contraction, and maximal rate of relaxation, and it reduced left ventricular end-diastolic pressure in male rats ($P<0.001$). No such benefit of exercise was observed in intact female rats. A causal role for Hsp70 in this sex-specific cardioprotective adaptation was indicated, inasmuch as ablation of Hsp70 induction with antisense oligonucleotides designed against Hsp70 transcripts attenuated improvement in the recovery of cardiac function in exercised male rats ($P<0.05$). Thus, the sex-specific hormone-mediated Hsp70 response to exercise results in cardioprotective adaptation, preferentially in male rats relative to female rats. These findings suggest that exercise may be more important for males than for females in defending against the effects of heart disease and offer a novel manner by which males may reduce the sex gap in susceptibility to adverse cardiac events. (Circ Res. 2002;90:911-917.)

Key Words: antisense oligonucleotides ■ heat shock proteins ■ hormones ■ sex ■ stress

Cardiovascular disease is the leading cause of death in the industrialized world. As such, cardioprotective measures are of considerable interest. The major inducible heat shock protein, Hsp70, has been proposed as a potential cardioprotective agent. The induction of Hsp70 is mediated through the interaction of the primary heat shock transcription factor, HSF1, with proximal promoter heat shock elements (HSEs) on Hsp transcriptional units (see review1). In the quiescent state, HSF1 is maintained as an inactive monomer by a complex of Hsps. In response to proteotoxic stress, Hsps carry out their protein-chaperoning function to maintain cellular homeostasis, relieving HSF1 of its negative regulation, allowing trimerization and DNA-binding competency.

Currie et al2 first demonstrated that heat shock enhanced ventricular recovery after ischemia/reperfusion and subsequently demonstrated a close temporal relationship between the kinetics of Hsp70 accumulation and recovery from myocardial ischemia/reperfusion.3 Direct evidence for Hsp70-mediated cardioprotection has been provided by studies using transgenic models, in which animals overexpressing Hsp70 exhibited increased tolerance to myocardial trauma.4–6 Although these perturbations have been useful in demonstrating the potential for Hsp70-mediated cardioprotection, the applicability of such interventions to human populations is limited at present.

Our laboratory was the first to report an exercise model of Hsp induction.7 Although unknown, many of the putative biological events involved in signaling the Hsp70 response to exercise demonstrate sexual dimorphism, disparities largely attributed to estrogen, a female-specific hormone.8,9 As such, the present series of experiments were undertaken to address the following hypotheses: (1) Males demonstrate greater exercise-induced cardiac Hsp70 than do females. (2) The sex-specific Hsp70 response to exercise is due to attenuated signaling of Hsp70 induction by estrogen. (3) The sex-specific hormone-mediated Hsp70 response to exercise results in exercise-conferred cardioprotection, preferentially in males relative to females.
Materials and Methods

The present study was approved by the University of Western Ontario Council on Animal Care and was performed in accordance with the guiding principles of the Canadian Council on Animal care. Male, gonadally intact female, and ovarioctomized female (with the major source of estrogen removed) Sprague-Dawley rats were purchased from Charles River Laboratories, St Constant, Quebec. At 8 weeks of age, ovarioctomized animals were implanted with subcutaneous 21-day hormone-release pellets (Innovative Researchers of America) containing either 0.25 mg 17β-estradiol, the major estrogen in mammalian systems, or placebo. Male and intact female rats were implanted with placebo pellets. The dose of estrogen selected in the present investigation was based on previous work done with physiological estrogen supplementation in the rat model. Efficacy of hormone treatment was verified by plasma estradiol measurement with the use of the Bayer Centaur Automated Chemiluminescence System performed by the London Health Sciences Center, Division of Endocrinology. All animals demonstrated circulating estradiol levels within normative values for female rats (mean ± SD values are as follows: male rats, 10 ± 6 pg/mL; intact female rats, 57 ± 11 pg/mL; placebo-treated ovarioctomized female rats, 13 ± 8 pg/mL; and estrogen-treated ovarioctomized female rats, 103 ± 21 pg/mL). In an additional series of experiments designed to elucidate the mechanism by which estrogen influenced Hsp70 induction, animals were treated with a series of compounds structurally related to 17β-estradiol. Intact female rats were treated with 21-day hormone-release pellets containing either 35 mg tamoxifen, an estrogen receptor antagonist, or placebo. This dose of tamoxifen was selected because it has previously been shown to exert antiestrogen effects in the rat. Ovarioctomized female rats were treated with hormone pellets containing placebo, 0.25 mg 17β-estradiol, 0.25 mg 17α-estradiol, a stereoisomer of 17β-estradiol that does not activate the estrogen receptor, or 35 mg tamoxifen. At 11 weeks of age, the animals were randomly assigned to control or exercise groups. All animals were familiarized on the rodent treadmill at 3 and 5 days before the experimental session. Exercise consisted of treadmill running at 30 m/min for 60 minutes, a moderate exercise intensity requiring ~75% of maximal oxygen uptake for both male and female rats. Animals were anesthetized with sodium pentobarbital (60 mg/kg) or urethane (1.5 g/kg) and euthanized either 30 minutes after exercise for analysis of HSF1-HSE DNA binding and Hsp70 mRNA or 24 hours after exercise for analysis of Hsp70.

Western Blotting

Heart samples were homogenized in 20 vol of 600 mmol/L NaCl and 15 mmol/L Tris (pH 7.5), and protein concentration was determined by using a bicinchoninic assay modified for microplate use. Homogenates (50 µg) were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with a polyclonal antibody specific for inducible isoforms of the Hsp70 gene (StressGen Biotechnologies Corp) as previously described. All immunoblots for Hsp70 were run with an internal standard (40 pg/mL; H9262 and H11032). HSF1 content was determined with myocardial homogenates separated on full-sized SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with a polyclonal antibody specific for HSF1 (Affinity Bioreagents, Inc).

Slot Blotting

Total RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction. RNA samples (5 µg) were blotted onto a Zeta Probe membrane (Bio-Rad) by using a Schleicher & Schuell manifold, cross-linked, and prehybridized at 50°C as described previously. Blots were washed overnight with a 10× SSC probe hybridization solution containing 5% (w/v) SDS, 1× SSC, and 0.1× SSC and exposed to Biomax MS film (Kodak). Specificity of oligonucleotide probes was verified by Northern analyses (data not shown).

Gel Mobility Shift Assay

Cardiac samples were homogenized in 15 vol extraction buffer as per Locke et al, and protein concentration was determined by using a Bio-Rad assay modified for microplate analysis. Myocardial extracts (200 µg) were incubated with 1 ng 32P-labeled self-complementary ideal HSE oligonucleotide and separated on full-sized nondenaturing polyacrylamide gels. Gels were subsequently dried and exposed to autoradiographic film overnight at ~80°C.

Ischemia/Reperfusion Protocol

Twenty-four hours after exercise, the rats were euthanized by decapitation; their hearts were isolated, placed in cold Krebs-Henseleit buffer, and mounted on a cannula by the aorta for retrograde perfusion with the use of a modified Langendorff procedure. Perfusion was maintained at 10 mL/min with 95% O2/5% CO2–gassed Krebs-Henseleit buffer at 37°C. A latex water-filled balloon connected to a pressure transducer was inserted into the left ventricle to measure left ventricular developed pressure (LVEDP). By adjusting the volume of the intraventricular balloon, left ventricular end-diastolic pressure (LVEDP) was set at 5 mm Hg at the start of each experiment. Maximal rate of contraction and relaxation (+dP/dt and −dP/dt, respectively) were obtained with a differentiator. Hearts were paced at 300 bpm throughout the experiment. Functional measures were obtained online on a Pentium 586 computer with the use of a Biopac data analysis system (Biowin Synthesis Equipment). Hearts were equilibrated for 30 minutes to determine baseline function. Global ischemia was induced by terminating flow for 30 minutes (male and intact female rats), followed by a reperfusion period of 30 minutes. With this protocol, hearts from control males and intact female rats demonstrated an ~20% recovery of developed pressure. In follow-up experiments performed at a later date, hearts from control ovarioctomized animals exhibited upwards of 60% recovery after a 30-minute ischemic period. Such high recovery under control conditions would mask the effect of any perturbation designed to improve postischemic heart function. Thus, to obtain a level of functional recovery in control ovarioctomized rats similar to that observed for control male and intact female rats, the ischemic period for these animals was increased to 40 minutes.

Manipulation of Hsp70 Expression

To elucidate a causal relationship between the observed sex-specific Hsp70 response to exercise and sex-specific cardioprotective adaptation to exercise, Hsp70 expression was directly and specifically manipulated by using an antisense oligonucleotide approach. Phosphorothioate antisense oligonucleotides (18-mer) designed against the translation initiation start site of Hsp70 mRNA (5′-TGTTTTCTTTGCGCATTGTCG-3′, GenBank accession No. X742711) or nonsense oligonucleotides, those of a random sequence but with the same base composition as per antisense constructs (5′-GTCCGTATTTGTTCCAGTGTCG-3′), were dissolved in physiological saline and administered at a dose of 50 mg/kg. Immediately after exercising, male rats were treated with antisense, nonsense, or sham (equal volume of saline) via intraperitoneal injection. A previous report has indicated the efficacy of such a treatment in manipulating rodent cardiac gene expression.

CK Activity

Creatine kinase (CK) activity was assessed by using a commercially purchased kit (Sigma Chemical Co). Perfusion samples were collected just before ischemia and at 2, 5, 10, 20, and 30 minutes of reperfusion. Average CK activity was calculated by mathematical addition of CK values for perfusate samples at the intervals indi-
Statistical Analysis

All quantification of blots was carried out by using Scion image analysis software (NIH). Hsp70 mRNA was normalized to 28S rRNA. Data for Hsp70 variables are reported as percentage of corresponding controls (mean±SE) and compared by ANOVA among treatment groups, with pairwise comparisons made by using a Tukey post hoc test. Cardiac function data are reported as percentage of preischemic values (mean±SE) and compared by Student t tests; the minimum level of significance was assigned as *P<0.05.

Results

Sex-Specific Hormone-Mediated Hsp70 Induction

After exercising, male rats demonstrated myocardial Hsp70 levels that were 2-fold greater than those observed for intact female rats (Figures 1A and 1E). To elucidate the role of the ovarian hormone estrogen in this phenomenon, ovariectomized rodents were treated with either placebo or 17β-estradiol, the major endogenous estrogen in mammalian systems. Placebo-treated animals demonstrated myocardial Hsp70 levels that were 2-fold greater than those of their estrogen-treated counterparts (Figures 1B and 1E). Moreover, this sex-specific hormone-mediated induction of Hsp70 was reflected at the level of mRNA (Figures 1C, 1D, and 1F). Graphic representation of these data clearly illustrates that with exercise, male rats exhibit greater Hsp70 induction than do intact female rats; removal of the ovaries in rats (P group) resulted in Hsp70 induction similar to that observed in male rats, and estrogen replacement in these rats (E group) reversed this effect. *P<0.05 vs intact female and E exercise groups (n=8 per group). G, Representative autoradiogram from gel mobility shift assays indicating greater exercise-induced myocardial HSF1-HSE DNA binding in estrogen-naive rats (M and P groups) relative to estrogen-positive rats (F and E groups). C indicates control condition.
transcription factor content. However, myocardial HSF1 levels were similar for male and female rats (Figure 1H), indicating a similar capacity to mount the stress response. Moreover, male and female rats demonstrated similar induction of Hsp70 after whole-body heat shock (authors’ unpublished data, 2002), indicating that the lower postexercise level of Hsp70 in estrogen-positive relative to estrogen-naive animals is not due to any general inhibitory effect of the hormone on the transcriptional, posttranscriptional, or translational processing of Hsp70. Thus, the sex-specific Hsp70 response to exercise is at least partly due to attenuated Hsp70 transcriptional signaling by estrogen.

**Hormonal Mechanism**

Although cardiac cells contain functional estrogen receptors, treatment of gonadally intact female rats with tamoxifen, an estrogen receptor antagonist, did not alter the Hsp70 response relative to the response of placebo-treated rats, indicating that the mechanism by which estrogen is influencing the response is not receptor-mediated (Figure 2). Moreover, treatment of ovariectomized animals with 17α-estradiol, a synthetic stereoisomer of 17β-estradiol that does not activate the estrogen receptor, resulted in postexercise Hsp70 levels similar to those found with 17β-estradiol treatment. Ovariectomized rats treated with tamoxifen also showed an attenuated Hsp70 response relative to the response in rats treated with placebo. That 17β-estradiol, 17α-estradiol, and tamoxifen, which all exert different effects on the estrogen receptor, attenuated Hsp70 induction with exercise provides further evidence against a receptor-mediated mechanism of action. These molecules share a common property in that they are lipophilic and have been well characterized as cellular membrane–stabilizing agents, binding to phospholipid side chains in these structures. Indeed, denaturation of membrane-bound proteins has been established as a key signal for Hsp synthesis during nonhyperthermic stress. Thus, although the mechanism by which estrogen attenuates the exercise-mediated induction of Hsp70 is currently unknown, the present findings indicate that such an effect is mediated in a receptor-independent manner, possibly involving the stabilization of cellular membranes.

**Physiological Importance**

To determine the physiological significance of this sex-specific hormone-mediated Hsp70 response, cardiac function after ischemia/reperfusion was assessed in control and exercised male rats, intact female rats, and ovariectomized female rats. Ischemia/reperfusion is an experimental model of pathological processes related to cardiovascular disease that is widely used to assess resistance to the effects of such pathologies. Exercise improved postischemic LVDP (Figure 3A), the maximal rate of contraction (Figure 3B), and the maximal rate of relaxation (Figure 3C), and it reduced LVEDP in male rats (Figure 3D). No such benefit of exercise was observed in intact female rats. However, exercise improved all measures of cardiac function after ischemia/reperfusion in ovariectomized female rats; this effect was similar to the that observed in male rats. These functional outcomes appeared to be related to myocardial structural integrity, as much as exercised male and ovariectomized female rats demonstrated reduced CK efflux during reperfusion relative to that found in control and intact female rats (data not shown).

Figure 4 illustrates the close association between myocardial Hsp70 content and posts ischemic cardiac function. Male and ovariectomized female rats, which displayed markedly induced Hsp70 with exercise, demonstrated greater recovery after ischemia than did intact female rats, which exhibited a relatively low Hsp70 response. Thus, the induction of Hsp70 and improved recovery of posts ischemic cardiac function with exercise demonstrate sexual dimorphism.

**Manipulation of Hsp70 Expression**

To determine whether the disparity in postischemic cardiac function between male and female rats was directly related to the sex-specific Hsp70 response to exercise, an antisense oligonucleotide approach was developed to manipulate Hsp70 expression. Treatment of exercised male rats with antisense oligonucleotides against inducible Hsp70 transcripts ablated the Hsp70 induction associated with exercise and attenuated the improvement in recovery of postischemic cardiac function (Figure 5). This direct and specific manipulation of Hsp70 expression indicates that the protective effects of exercise on cardiac function after ischemia/reperfusion are, at least in part, Hsp70 dependent. Therefore, the sex-specific Hsp70 response to exercise results in greater posts ischemic cardiac function in male rats than in female rats.

**Discussion**

Ours are the first findings of a sex-specific Hsp response. After exercising, male rats, compared with intact female rats,
demonstrated a 2-fold greater cardiac Hsp70 content. Removal of the ovaries, the major endogenous source of estrogen, resulted in postexercise Hsp70 levels that were similar to those observed in male rats, and estrogen replacement in these animals reversed this effect. Thus, the sex-specific Hsp70 response to exercise is mediated by the female-specific hormone estrogen. The mechanism by which estrogen attenuated Hsp70 signaling was nongenomic and, although unknown at present, may involve stabilization of the cellular membranes. The physiological importance of this sexual dimorphism is reflected in the finding that exercise improved postischemic cardiac function in male rats and ovariectomized female rats, which exhibited marked induction of Hsp70 with exercise, but not in gonadally intact female rats, which demonstrated relatively low postexercise Hsp70 expression. Direct and specific manipulation of Hsp70 expression by using an antisense oligonucleotide approach indicated that the greater postischemic cardiac function in male rats relative to female rats was causally related to the sex-specific expression of Hsp70. Thus, the sex-specific induction of Hsp70 with exercise resulted in cardioprotective adaptation in male rats but not in intact female rats.

Estrogen has generally been considered a protective factor against cardiovascular disease. Much of this belief is based on epidemiological data indicating a lower incidence of coronary heart disease among women relative to men, at least until menopause, after which this disparity diminishes.25 Furthermore, postmenopausal women receiving estrogen replacement therapy are found to have reduced rates of cardiovascular disease.26 Moreover, a plethora of experimental work has characterized potential biological mechanisms for these observations.

However, the association between estrogen and heart health is being revisited. For example, when lifestyle factors are accounted for, the disparity in coronary heart disease rates between men and women is reduced.27 Furthermore, randomized clinical trials to determine more discriminantly the influence of hormone replacement therapy on coronary heart disease have yielded equivocal results.28,29 Also, much of the basic research outlining cardiovascular factors positively influenced by estrogen is derived from studies using pharmacological hormone concentrations.

Indeed, clinical data suggest that estrogen may have marked negative consequences, because premenopausal
women, after myocardial infarction, exhibit higher rates of mortality relative to their male counterparts. This dimorphism has been outlined in an experimental model in which estrogen-treated ovariectomized rats exhibited increased myocardial infarct size relative to those treated with placebo. Interestingly, the authors postulated that estrogen might inhibit the activity of protective factors that would otherwise be upregulated with infarction. Thus, among cardiovascular clinicians and researchers, the association between estrogen and heart health is a subject of increasing controversy.

The results of the present investigation are such that the effects of estrogen can be interpreted as either advantageous or disadvantageous. Because Hsp70 is stress inducible, the lower levels of Hsp70 after exercise in estrogen-positive animals relative to estrogen-naive animals suggest a beneficial effect of the hormone. Indeed, a membrane-stabilizing mechanism of action would aid in maintaining normal cellular function with exercise stress. However, this hormone-mediated maintenance of homeostasis with physiological stimuli attenuated requisite signals for organismal adaptation. Thus, on subsequent severe stress, this initial protective action of estrogen was manifested as a disadvantage; ie, although male and ovariectomized female rats demonstrated high cellular stress with exercise (as reflected by the marked induction of Hsp70), this stress signaled the induction of adaptive mechanisms to increase the defense against subsequent ischemia/reperfusion. Intact females, which demonstrated relatively low levels of cellular stress with exercise (as indicated by relatively low Hsp70 induction), were more susceptible to ischemia/reperfusion.

The present findings indicate the importance of stress and/or injury in signaling cardiac adaptive processes. Attenuation of the stress/injury response, although seemingly protective initially, may prove disadvantageous on subsequent stress. The most immediate use of a stress-based approach in therapeutics may be in the treatment of central nervous system injury, such as spinal cord transection. As a protective mechanism, injury to the central nervous system, which has generally been considered irreversible, is not accompanied by any appreciable stress/injury response. However, introducing stress and/or injury factors such as macrophages to transected spinal cords has been reported to result in significant recovery of motor function. In contrast to the traditional “protective” approach of cardiovascular research and medicine, our findings indicate the importance of the stress/injury response in cardioprotective adaptation, suggesting the possibility of a stress-based approach in the development of cardiac therapies.

Exercise is perhaps the most widely prescribed treatment and preventive measure for heart disease. However, the mechanisms by which exercise improves cardiovascular disease profile have not been fully defined. In the present study, a direct role for Hsp70 is supported. Although a number of studies using transgenic and gene transfection approaches have also established Hsp70 as a cardioprotective agent, it would be naive to assume that a whole-body perturbation, such as treadmill running, influences the activity of only one biological defense system. Thus, it is of interest whether other endogenous defense mechanisms also demonstrate sexual dimorphism. Still remaining is the question of whether protective perturbations, other than exercise, exhibit similar sex- and hormone-related specificity. The novel, sex-specific, hormone-mediated stress response reported in the present study suggests that exercise may be more important for males than for females in defending against the effects of cardiovascular disease and that exercise induction of

![Figure 4](image-url) **Figure 4.** Association between recovery of LVDP after ischemia and left ventricular (LV) Hsp70 content. Hatched bars represent data from control rats, and solid bars represent data from exercised rats. *P<0.03 vs control group (n=8 to 10 per group).

![Figure 5](image-url) **Figure 5.** Improved recovery of postischemic cardiac function (LVDP) with exercise is Hsp70 dependent. C indicates data from sham-treated control male rats; Ex, data from sham-treated exercised male rats; AS, data from exercised male rats treated with antisense oligonucleotides against inducible Hsp70 transcripts; and NS, data from exercised male rats treated with nonsense oligonucleotides. *P<0.05 vs C group (n=8 per group).
Hsp70 may represent a potential mechanism by which males may reduce the sex gap in susceptibility to adverse cardiac events.

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