Estrogen attenuates postexercise HSP70 expression in skeletal muscle

ZAIN PAROO, ELIZABETH S. DIPCHAND, AND EARL G. NOBLE
Faculty of Health Sciences, School of Kinesiology, and Lawson Health Research Institute, University of Western Ontario, London, Ontario, Canada N6A 3K7

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Paroo, Zain, Elizabeth S. Dipchand, and Earl G. Noble. Estrogen attenuates postexercise HSP70 expression in skeletal muscle. Am J Physiol Cell Physiol 282: C245–C251, 2002. First published October 3, 2001; 10.1152/ajpcell.00336.2001.—Exercise has been demonstrated as a physiological inducer of heat shock protein (HSP70). Many of the proposed signals of this response exhibit sexual dimorphism. Thus the present objectives were to determine whether HSP70 induction after exercise exhibits gender specificity and to elucidate the mechanisms underlying such a phenomenon. Postexercise HSP70 induction in skeletal muscle was greater in male than female rats at the level of protein and mRNA (P = 0.005). Moreover, placebo-treated ovariectomized animals demonstrated a greater HSP70 response to exercise than those treated with estrogen (P = 0.015 and 0.019 for protein and mRNA, respectively). These findings indicate that the gender-specific HSP70 response to exercise is mediated by the female-specific hormone estrogen. Compounds structurally related to 17β-estradiol, the major endogenous estrogen, but which do not activate the estrogen receptor, also attenuated HSP70 induction with exercise (P < 0.01), indicating a nongenomic hormonal mechanism. These findings highlight a specific example of the biological differences between males and females and reiterate the physiological effects of sex hormones extending beyond their role in reproductive function.

HEAT SHOCK, or stress, proteins (HSPs) are the products of rapidly inducible, highly conserved, ubiquitous transcriptional units. The induction of HSPs is believed to be mediated through proximal, contiguous, inverted repeats of the sequence nGAAn, termed heat shock elements (HSEs; Ref. 17). Expression of the major inducible HSP, HSP70, is autoregulated primarily at the level of transcription (16, 31). In the unstressed cell, HSPs are associated with the primary heat shock transcription factor, HSF1 (1). In response to a variety of stressors, intracellular accumulation of nonnative proteins requires HSP binding, to ameliorate misfolding and aggregation and facilitate proper refolding and/or targeting for degradation (11, 14). This chaperoning activity of HSPs allows HSF1 to acquire HSE-DNA binding activity and transcriptional competence through trimerization and phosphorylation events, respectively (27). As hsp transcripts (hsp mRNA) are translated, the level of HSPs rises, resulting in sequestration of HSF1 and, consequently, a tapering of the response (16).

One of the landmark studies in the area of gender-specific responses to exercise demonstrated that after a 1-h bout of stationary cycling at a given relative workload men demonstrated greater levels of circulating creatine kinase (CK), an indicator of exercise-induced muscle damage, than women (33). Of all the possible factors that could underlie this gender-specific response, one of the more obvious differences between males and females is in the content of sex hormones. After establishing this gender-specific response in a rodent model, Amelink and Bärr and coworkers (2, 3, 7) provided evidence for the involvement of the female-specific hormone estrogen in this response. Male and ovariectomized female rats exhibited higher indices of exercise-induced muscle membrane damage relative to intact females, and estrogen administration in these animals reversed this effect (7). Komulainen et al. (18) later directly demonstrated gender-specific exercise-induced skeletal muscle damage through histochemical assessment.

Our laboratory uses exercise as a physiological inducer of HSP70 (23). Although the biological events associated with exercise that signal HSP induction have not been established, many of the putative factors involved (22), including production of reactive oxygen intermediates, exercise-induced tissue damage, elevated body temperature, substrate depletion, calcium cycling, and adrenergic stimulation, demonstrate sexual dimorphism (6, 41). Because of the importance of intracellular denatured proteins in signaling HSP induction, exercise-induced skeletal muscle damage may be particularly important in signaling the HSP response to exercise. Because gender-specific exercise-induced skeletal muscle damage has been established, it was hypothesized that males and females differentially express HSP70 in skeletal muscle after exercise. In a preliminary study (30), we reported gender dimorphism in postexercise expression of HSP70. The
present series of experiments was undertaken to examine the sex-specific HSP70 response to exercise in a more comprehensive manner and to elucidate the mechanisms by which this phenomenon is mediated.

MATERIALS AND METHODS

The study was approved by the University of Western Ontario Committee on Animal Care and was performed in accordance with the guiding principles of the Canadian Council on Animal Care.

Experimental Design

Male, gonadally intact female, and ovariectomized female (major source of estrogen removed) rats were purchased from Charles River. At 8 wk of age, ovariectomized animals were implanted with subcutaneous 21-day hormone release pellets (Innovative Researchers of America) containing either 0.25 mg of 17β-estradiol, the major estrogen in mammalian systems, or placebo. Male and intact female rodents 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 (13, 15, 35, 39) indicating a required 40 µg·kg⁻¹·day⁻¹ dose, or ~0.25 mg over 21 days. Efficacy of hormone treatment was verified by plasma estradiol measurement with the Bayer Centaur automated chemiluminescence system performed by the Division of Endocrinology, London Health Sciences Center.

In an additional series of experiments designed to elucidate the mechanism by which estrogen influences HSP induction, animals were treated with a series of compounds structurally related to 17β-estradiol. Intact females were treated with 21-day hormone release pellets containing either 35 mg of tamoxifen, an estrogen receptor antagonist (25), or placebo. This dose of tamoxifen was selected because it was shown previously to exert antiestrogen effects in the rat (19). Ovariectomized females were treated with hormone pellets containing placebo, 0.25 mg of 17β-estradiol, 0.25 mg of 17α-estradiol, a stereoisomer of 17β-estradiol that does not activate the estrogen receptor (9), or 35 mg of tamoxifen.

At 11 wk of age, animals were randomly assigned to control or exercise groups. All animals were familiarized with the rodent treadmill 5 and 3 days before the experimental session. Exercise consisted of treadmill running at 30 m/min for 60 min, a moderate exercise intensity requiring ~75% of maximal oxygen uptake for both males and females (8). Animals were anesthetized via intraperitoneal injection of Somnitol (60 mg/kg) and were killed by exsanguination either 30 min or 24 h after exercise for analysis of hsp70 mRNA and HSP70, respectively. The pattern of gender-specific hormone-mediated HSP70 expression with exercise reported here was generally evident across skeletal muscles recruited with this exercise protocol, including the red and white portions of the gastrocnemius muscle and, in previous preliminary experiments, in red and white portions of the vastus muscle (30). For uniformity, all data presented here are from biochemical analyses performed on the white portion of the gastrocnemius muscle.

Biochemical Analysis

Serum CK activity. Serum CK activity was assessed using a commercially purchased kit (Sigma).

Western blotting. Frozen tissue samples were homogenized in 20 vols of 600 mM NaCl, 15 mM Tris (pH 7.5), and protein concentration was determined using a bicinchoninic acid technique modified for microplate use. Homogenates (100 µg) were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with a polyclonal antibody specific for the inducible isoforms of HSP70 as previously described (Stress Gen; Ref. 29). HSF1 content was determined with 200 µg of homogenate separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with a polyclonal antibody specific for HSF1 (Affinity Bioreagents).

Slot blotting. Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (10). RNA samples (5 µg) were blotted onto Zeta Probe membrane (Bio-Rad) with a Schleicher & Schuell manifold, cross-linked, and prehybridized at 50°C as described previously (24). Blots were probed overnight with a 32P end-labeled oligonucleotide specific for inducible hsp70 transcripts. Blots were washed at 55°C with 0.1% SDS and 0.1× SSC and exposed to Biomax MS film (Kodak). Blots were subsequently stripped and probed for the inducible isoform of heme oxygenase, HO-1 (see below), and 28S rRNA with an end-labeled oligonucleotide.

For HO-1 mRNA analysis, the EcoRI-HindIII fragment (828 base pairs) was isolated from the rat HO-1 cDNA clone (pRHO-1; Ref. 32) and labeled with [α-32P]dCTP by random prime labeling (Prime-a-Gene labeling system, Promega). Probe specificity was verified by Northern blot analysis.

Statistical Analysis

All quantification of blots was carried out using Scion image analysis software (National Institutes of Health). Except where indicated, data are reported as means ± SE and were compared by analysis of variance among treatment groups. Pairwise comparisons were conducted using a Tukey post hoc test, where the minimum level of significance was assigned as P < 0.05.

RESULTS

Animal Characteristics

At 11 wk of age, all experimental groups were of significantly different body weights (P < 0.001; Table 1). These data are in line with previous work demonstrating that male rats have greater body mass than females of the same age and that estrogen administration to estrogen-naive animals results in reduced body weight gain and lower body mass than those receiving placebo treatment (20). Age-matched animals were chosen for the present study because young adult male and female rats have similar maximal oxygen uptakes per unit body mass (8). Weight matching between the sexes (after puberty) would involve using females of significantly higher age than males. Thus the present

Table 1. Animal characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Mass, g</th>
<th>Plasma Estrogen, pg/ml</th>
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<tbody>
<tr>
<td>Male</td>
<td>409 ± 9</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>Female</td>
<td>216 ± 10</td>
<td>57 ± 11†</td>
</tr>
<tr>
<td>Placebo</td>
<td>319 ± 15</td>
<td>13 ± 8</td>
</tr>
<tr>
<td>Estrogen</td>
<td>242 ± 12</td>
<td>103 ± 21†</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6–8 animals/group. All groups had significantly different body weights (P < 0.001). †Estrogen-treated ovariectomized rodents had greater plasma estrogen levels than all other groups (P < 0.001). ‡Intact females had greater plasma estrogen levels than male and placebo-treated ovariectomized animals (P < 0.001).
Sex-Specific HSP70 Induction

As would be expected, intact females had greater plasma estradiol levels than male and placebo-treated ovariectomized rodents ($P < 0.001$; Table 1). Estrogen treatment of ovariectomized animals resulted in plasma estradiol levels greater than those for all other groups. The selection of the 21-day-release 0.25-mg hormone pellets in the present study was based on previously established work in which physiological hormone levels were used (13, 15, 39). Here, the estradiol levels observed for all animals were within previously established normative values for female rats (35). Because the intact females used in this investigation were chosen at random, that is, without controlling for ovarian cycle phase, the values obtained for this group likely represent an average of estradiol levels over the 4-day cycle in the normal female rat. Thus the difference in plasma estrogen levels between intact and estrogen-treated ovariectomized females likely reflects differences between the normal hormonal cycling of gonadally intact females versus the constant-release nature of the hormone pellets.

There were no differences in preexercise colonic temperatures among any of the experimental groups, and all groups demonstrated increases in core body temperature with exercise ($P < 0.001$). After exercise, males had higher colonic temperatures than intact females and 17β-estradiol- and tamoxifen-treated ovariectomized animals (Table 2; $P < 0.02$). Placebo- and 17α-estradiol-treated ovariectomized animals had postexercise temperatures that were higher than those for intact females ($P < 0.01$). These data are in line with previous work demonstrating the positive thermoregulatory effect of estrogen (4, 5, 38).

To determine whether the present exercise model resulted in lower postexercise skeletal muscle damage in estrogen-positive animals relative to estrogen-naïve animals, serum CK activity was assessed immediately after exercise (7). Indeed, ovariectomized females demonstrated greater serum CK activity with exercise than intact females (Fig. 1; $P < 0.001$).

### Exercise Induction of HSP70

After exercise, males demonstrated greater HSP70 and hsp70 mRNA than females (Fig. 2). A similar effect was observed between placebo- and estrogen-treated ovariectomized rodents, because placebo-treated ovariectomized animals had higher HSP70 and hsp70 mRNA content than estrogen-treated ovariectomized animals. Graphical representation of these data clearly illustrates that with exercise males demonstrate a greater HSP response than females, removal of the ovaries in females results in a response similar to that observed for males, and estrogen administration to these animals reverses this effect (Fig. 3; $P < 0.05$). Furthermore, these data indicate that the effect of estrogen in attenuating HSP induction with exercise is independent of the endogenous or exogenous source of the hormone.

HSP1-HSE DNA binding, as assessed by gel mobility shift assay, was only faintly detectable for all experimental groups, with only a minor tendency for greater signal in males and placebo-treated ovariectomized animals versus intact females and estrogen-treated ovariectomized animals (data not shown). Figure 4 illustrates similar HSF1 levels in skeletal muscle of males and females. These observations suggest that the gender-specific HSP response is not due to differences in the capacity to mount the stress response.

### Hormonal Mechanism

To determine the mechanism by which estrogen influenced postexercise HSP70, a series of compounds structurally related to 17β-estradiol were used. Treatment of intact females with tamoxifen, an estrogen receptor antagonist (25), did not alter the HSP re-

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Table 2. Postexercise colonic temperatures

<table>
<thead>
<tr>
<th>Group</th>
<th>Preexercise Temp., °C</th>
<th>Postexercise Temp., °C</th>
</tr>
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<tbody>
<tr>
<td>Male</td>
<td>38.0 ± 0.4</td>
<td>40.7 ± 0.5</td>
</tr>
<tr>
<td>Female</td>
<td>38.1 ± 0.4</td>
<td>38.7 ± 0.9*</td>
</tr>
<tr>
<td>Placebo</td>
<td>37.8 ± 0.4</td>
<td>40.0 ± 0.6*</td>
</tr>
<tr>
<td>Estrogen</td>
<td>37.8 ± 0.5</td>
<td>39.2 ± 0.3*</td>
</tr>
<tr>
<td>Female + TX</td>
<td>38.0 ± 0.5</td>
<td>40.1 ± 0.3*</td>
</tr>
<tr>
<td>OVX + α-estradiol</td>
<td>38.9 ± 0.5</td>
<td>39.9 ± 0.6*</td>
</tr>
<tr>
<td>OVX + TX</td>
<td>38.1 ± 0.6</td>
<td>39.6 ± 0.7*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6–8 animals/group. Placebo, placebo-treated ovariectomized (OVX); Estrogen, 17β-estradiol-treated OVX; TX, tamoxifen. All groups demonstrated increased colonic temperature with exercise ($P < 0.001$). *Postexercise rectal temperature greater than male ($P < 0.02$). †Postexercise rectal temperature greater than intact female ($P < 0.01$).
response to exercise relative to those treated with placebo, indicating that the hormonal mechanism is not receptor mediated (Fig. 5). In ovariectomized animals, 17α-estradiol and tamoxifen attenuated postexercise HSP70 levels relative to placebo, similar to the effect of 17β-estradiol (P < 0.01). Because of the potential antioxidant action of estrogen, expression of HO-1, induction of an oxidative stress-inducible enzyme was examined (32). Although HO-1 transcript levels were undetectable under control conditions, six of eight placebo-treated ovariectomized animals showed induction with exercise whereas none of their estrogen-treated counterparts exhibited any such response (Fig. 6).

DISCUSSION

The major finding of the present investigation was a gender-specific HSP response, with males demonstrating greater induction of HSP70 in skeletal muscle after exercise than females. Furthermore, this differential response appears to be mediated by the ovarian hormone estrogen, because placebo-treated ovariectomized rodents exhibited greater HSP70 levels with exercise than those treated with estrogen. Moreover, the results suggest that the mechanism by which estrogen is influencing the response is through non-genomic membrane stabilization.

Sex hormones classically exert their effects through interaction with intracellular receptors, with these hormone-receptor complexes acting as transcription factors on target genes (26). However, tamoxifen, an estrogen receptor antagonist, did not alter the ability of estrogen to protect against skeletal muscle damage in response to electrical stimulation (19). Moreover, tamoxifen treatment alone attenuated contraction-induced damage.

Although the present work does not directly assess the involvement of exercise-induced skeletal muscle damage on induction of HSP70, if a causal relationship

**Fig. 2.** Effect of gender (A) and estrogen (B) on exercise induction of skeletal muscle heat shock protein (HSP70). Animals were subjected to treadmill running at 30 m/min for 60 min and killed 24 h and 30 min after exercise for analysis of HSP70 and hsp70 mRNA, respectively. Top, 100 µg of homogenate from control and exercised male (M), intact female (F), and placebo (P)- and estrogen (E)-treated OVX animals were electrophoresed on SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, and incubated with a polyclonal antibody specific for HSP70. Bottom, 5 µg of total RNA was isolated from control and exercised male, intact female, and placebo- and estrogen-treated ovariectomized animals, slot blotted, and hybridized with an oligonucleotide probe specific for hsp70 mRNA. Membranes were stripped and rehybridized with a 28S rRNA oligonucleotide probe. Representative blots from 8 animals/group are shown.

**Fig. 3.** Skeletal muscle HSP70 content (A) and hsp70 mRNA expression (B) in male, intact female, and placebo- and estrogen-treated OVX animals. HSP70 content was assessed by immunoblotting for control animals and those killed 24 h after exercise. Gels were loaded with 100 µg of white gastrocnemius homogenate. hsp70 mRNA levels were assessed by slot blot hybridization for control animals and those killed 30 min after exercise. Membranes were blotted with 5 µg of total RNA and successively hybridized with oligonucleotide probes specific for hsp70 mRNA and 28S rRNA. Data are means ± SE. No differences in constitutive expression of HSP70 or hsp70 mRNA were observed among treatment groups. *Greater induction in estrogen-naive (males and placebo) vs. estrogen-positive (female and estrogen) animals (P < 0.05; n = 8 animals/group).
exists, estrogen-positive animals, which demonstrate reduced postexercise injury relative to estrogen-naive animals, should show an attenuated HSP response to exercise. Indeed, intact females and estrogen-treated ovariectomized animals demonstrated lower HSP70 induction than males and placebo-treated ovariectomized rodents. Similar to the series of studies outlined above, this effect was not receptor mediated, because tamoxifen treatment in intact females did not alter the response relative to those treated with placebo. Furthermore, 17α-estradiol and tamoxifen treatment of ovariectomized animals inhibited induction of HSP70 similar to the effect of 17β-estradiol, further implicating a nongenomic mechanism. The finding that these compounds, which exert different effects at the estrogen receptor, mitigate HSP induction in a fashion similar to that for the endogenous hormone suggests a mechanism of action common to these compounds. These lipophilic agents have been shown to reduce membrane fluidity and attenuate lipid peroxidation and thus are considered antioxidant membrane-stabi-

lizing molecules (9, 43). The greater induction of the oxidative stress-inducible enzyme HO-1 in placebo-treated ovariectomized animals relative to those treated with estrogen is consistent with this proposed antioxidant membrane-stabilizing mechanism of hormone action (6, 32, 43).

It has become increasingly apparent that estrogen exerts physiological effects extending far beyond its role in reproductive function. As such, this steroid has recently become the subject of numerous studies across a wide range of scientific disciplines. The most prominent shortcoming in these studies, however, has been the use of hormone treatments that are orders of magnitude greater than physiological concentrations. The chemical structure of 17β-estradiol indicates a phenol ring containing a potentially donatable hydroxyl group (37). This feature of the steroid hormone suggests a possible chain-breaking antioxidant mechanism of action, similar to that of vitamin E, for example (36). The micro- and millimolar hormone concentrations used in many investigations are conducive to such a direct antioxidant effect. However, the picomolar concentrations of circulating estrogen in physiological systems, such as those demonstrated here, are too low to exert any significant effect on cellular redox state. This has been verified in our model, because postexercise reduced glutathione levels were similar among male, intact female, and ovariectomized female animals (unpublished observations). Moreover, tamoxifen, which attenuated HSP70 induction with exercise in ovariectomized animals, similar to the effect of estradiol isomers, is not a phenolic compound (42) and therefore does not influence biological activities via direct antioxidant activity. Thus the mechanism by which estrogen attenuates HSP70 induction with exercise may be mediated through its indirect antioxidant properties by stabilizing cellular membranes. Future studies using membrane-stabilizing compounds other than those structurally related to estrogen and direct assessment of membrane integrity will be helpful in resolving the importance of disruption to cellular membranes in signaling HSP induction.

**Fig. 5.** Attenuation of postexercise HSP70 content by estrogen is not receptor mediated. Postexercise HSP70 expression, as assessed by immunoblotting, in the white gastrocnemius of placebo- and tamoxifen-treated intact females (P and TX, respectively) and placebo-, 17β-estradiol-, 17α-estradiol-, and tamoxifen-treated OVX animals (P, 17β, 17α, and TX, respectively) is shown. Administration of these compounds did not significantly affect constitutive HSP70 expression. As such, data are expressed as a percentage of the corresponding control group. *Greater HSP70 expression vs. all other groups (P < 0.05; n = 6–8 animals/group).
Many biological events that occur with exercise have independently been shown to induce HSP synthesis, and thus several candidate factors may signal the HSP response to exercise. The gender-specific hormone-mediated phenomenon reported here serves as a model by which this may be addressed. That is, because estrogen ablates HSP70 induction with exercise, the factors that play predominant roles in this response are likely those that are mediated by estrogen. These results suggest that exercise-induced tissue injury, particularly at cellular membranes, may be important in signalng the HSP response to exercise. Indeed, loss of integrity of plasma membrane-associated proteins has been shown as an early event in the HSP response to nonhyperthermic stress (12).

The most studied inducer of the HSP response has been temperature. Although evidence for temperature mediation of the HSP response to exercise has been offered (34, 40), the current investigation indicates that temperature does not play a predominant role. Although males had greater postexercise rectal temperatures and HSP70 content than intact females and estrogen-and tamoxifen-treated ovarioectomized animals, temperatures among male, tamoxifen-treated intact female, and α-estradiol-treated ovarioectomized rosters were not different despite lower postexercise HSP70 levels for the latter two groups. Moreover, tamoxifen-treated intact females and α-estradiol-treated ovarioectomized rats had significantly higher temperatures than placebo-treated intact females, yet HSP70 content postexercise was similar among these groups. Thus, although temperature may play a role, clearly there are other factors that are important in the HSP response to exercise.

The present investigation demonstrates greater HSP70 induction in males than females after physiological stress. This gender-specific response is mediated by the ovarian hormone estrogen through a non-genomic mechanism, perhaps involving stabilization of cellular membranes. Because HSP70 is stress inducible, the lower induction observed in females relative to males may be interpreted as a protective hormonal effect. However, this protection may attenuate cellular adaptive signals, such as HSP induction, that would otherwise lead to longer term adaptation (21, 28). This work serves as a specific example of the physiological differences between males and females, a simplicity often overlooked in biological research, and emphasizes that effects exerted by sex hormones extend far beyond their role in reproductive function.

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