Protective Effect of Estrogens Against Oxidative Damage to Heart and Skeletal Muscle *In Vivo* and *In Vitro* (44463)

ADAM M. PERSKY,*[,1] PATTIE S. GREEN,† LEIGHANN STUBLEY,† CYNTHIA O. HOWELL,* LARISSA ZAULYANOV,† GAYLE A. BRAZEAU,* AND JAMES W. SIMPKINS†

Department of Pharmaceutics, University of Florida, College of Pharmacy, Gainesville, Florida 32610; and †Center for the Neurobiology of Aging and the Department of Pharmacodynamics, College of Pharmacy, University of Florida, Gainesville, Florida 32610

Abstract. **Estrogen has been shown to protect skeletal muscle from damage and to exert antioxidant properties. The purpose of the present study was to investigate the antioxidant and protective properties of estrogens in rodent cardiac and skeletal muscle and H9c2 cells. Female Sprague-Dawley rats were separated into three groups, ovariectomized (OVX), ovariectomized with estrogen replacement (OVX + E2), and intact control (SHAM), and were assessed at two time periods, 4 and 8 weeks. Rodents hearts were analyzed for basal and iron-stimulated lipid peroxidation in the absence and presence of β-estradiol (βE2) by measuring thiobarbituric acid reactive species (TBARS). Isolated soleus (SOL) and extensor digitorum longus (EDL) were analyzed for creatine kinase (CK) efflux. Using H9c2 cells, the *in vitro* effects of βE2 and its isomer α-estradiol were investigated under glucose-free/hypoxic conditions. TBARS assay was also performed on the H9c2 in the presence or absence of βE2. The results indicate that OVX rodent hearts are more susceptible to lipid peroxidation than OVX + E2 hearts. OVX soleus showed higher cumulative efflux of CK than OVX + E2. Furthermore, H9c2 survival during oxidative stress was enhanced when estrogen was present, and both OVX hearts at 4 weeks and H9c2 cells particularly were protected from oxidative damage by estrogens. We conclude that estrogen protects both skeletal and cardiac muscle from damage, and its antioxidant activity can contribute to this protection.** [P.S.E.B.M. 2000, Vol 223]

The importance of estrogen in protecting tissues is currently receiving increasing attention. Estrogen is a fat-soluble hormone that can contribute to membrane fluidity by direct interactions with phospholipids. It has also been suggested that estrogen can suppress free radical–induced peroxidation chain reactions because of the similarity in structure to vitamin E, namely the presence of the hydroxyl group on the phenolic A ring (1, 2). By the age of 55, women normally pass through menopause resulting in ovarian exhaustion of follicles and a precipitous decline in ovarian steroids. Women can live more than a third of their lives in an estrogen-deficient state; however, overwhelming evidence suggests that postmenopausal estrogen loss can have negative effects on the brain (3, 4), bone (5, 6) and cardiovascular system (7). Despite this evidence, less than 25% of postmenopausal women receive estrogen replacement therapy (ERT) largely because of the fear that ERT increases the risk of uterine cancer and perhaps mammary tumors.

The normal endogenous production of free radicals creates both beneficial and detrimental effects. Recent evidence suggests a link in the over-production of free radicals and/or decreases in antioxidant capacity with the development of disease (e.g., cancer, atherosclerosis, and Alzhei-

This work was supported by NIH PO1-A610485, a Merck/AFAR Research Scholarship in Geriatric Pharmacy (C.O.H.), and NIAAA T32 AA07561 (A.M.P.).

[1] To whom requests for reprints should be addressed at Department of Pharmaceutics, University of Florida, College of Pharmacy, 100494 J.H.M.H.C., Gainesville, FL 32610. E-mail: apersky@ufl.edu

Received February 17, 1999. [P.S.E.B.M. 2000, Vol 223]
Accepted July 27, 1999.

0037-9727/00/2231-0059$14.00/0

mer's) (8). Likewise, it has been suggested that free radical–induced processes may occur postmenopausal, thereby stimulating research into the role of these molecules during this physiological state. One consequence of the overproduction of free radicals is lipid peroxidation and damage to membranes. Free radicals can also cause damage to protein and mitochondrial and nuclear DNA. Endogenous antioxidant systems attempt to prevent or stop free radical damaging cascades to help maintain cellular integrity. The importance of free radical production and antioxidant status is evident in the negative correlation between the production of free radicals and longevity (9). Since it is believed that estrogen can act as an antioxidant, a decrease in endogenous levels can increase free radicals, thereby potentially causing adverse effects in a variety of tissues in postmenopausal women.

Similar to the negative effects that estrogen deprivation has on the brain, bone, and cardiovascular system, estrogen deprivation could increase vulnerability of skeletal muscle to damage. The increased vulnerability may lead potentially to muscle wasting and decreased strength and can partially account for the increased incidence of falls in elderly women and a general decline in the quality of life (10–12). In addition, estrogen's role in protecting skeletal muscle has been associated with exercise-induced muscle damage by reducing serum creatine kinase levels (13) and reducing delayed-onset muscle soreness (14). Tiidus (15) assessed estrogen's role in diminishing exercise-induced muscle damage and proposed that estrogens may exert their protective effects *via* direct antioxidant or membrane stabilization actions. Like skeletal muscle, cardiac muscle is also prone to free radical–induced damage (16) and could be an additional tissue responsive to estrogens.

The objective of these studies was to investigate the antioxidant and protective properties of estrogens in rodent cardiac and skeletal muscle. Furthermore, the protective role of estrogen was investigated using the H9c2 cell line, a cell line that demonstrates both skeletal and cardiac muscle properties (17). The overall hypothesis is that estrogen deprivation increases oxidative stress resulting in lipid peroxidation in muscle cells. Conversely, estrogen supplementation can offset the toxic effects in the rodent heart, skeletal muscle, and H9c2 cell line.

Materials and Methods

Animals and Treatments. All animal protocols were approved by the University of Florida Institutional Animal Care and Use Committee in accordance with NIH guidelines. Two-week-old female Sprague-Dawley rats (Harlan, Indianapolis, IN) were divided into three groups of eight animals: 1) intact controls (SHAM); 2) ovariectomized (OVX); and 3) ovariectomized animals receiving 17β-estradiol (βE2) supplementation (OVX + E2). Bilateral ovariectomy was done using the dorsal approach as described by Singh *et al.* (18). SHAM animals underwent the same surgical procedures without the removal of the ovaries. Five-millimeter Silastic implants containing either βE2 (mixed 1:1 with cholesterol; OVX + E2 group) or cholesterol (SHAM and OVX groups) were prepared as previously described by Singh *et al.* (18) and implanted under the skin concurrent with the ovariectomy procedure. Every 2 weeks, the Silastic implants were repositioned to maintain steroid diffusion from the implant.

At 4 or 8 weeks postovariectomy, animals were anesthetized with methoxyflurane, while a vaginal lavage was performed and blood obtained *via* cardiac puncture. The animals were then decapitated, and the heart, soleus (SOL) muscles, and extensor digitorum longus (EDL) muscles were harvested. SOL and EDL muscles were analyzed immediately for *in vitro* enzyme release. Blood samples were centrifuged, and the plasma fraction stored at −80°C until analyzed for creatine kinase, progesterone, and estrogen levels.

Effects of Estrogen on Hypoxia and Glucose Deprivation in the H9c2 Cell Line. H9c2 cells (culture passages 11–15, ATCC, Rockville, MD) were maintained in DMEM media (GIBCO, Gaithersburg, MD) supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone, Logan, UT) at 37°C under 10% CO_2/90% air using standard culture techniques.

Hypoxia experiments were initiated when cells were ≈ 50% confluent in Nunc 35-mm dishes (Fisher Scientific, Orlando, FL). The medium was changed into glucose-free, serum-free DMEM with hydroxypropyl-β-cyclodextrin (HPCD) encapsulated βE2 (Sigma Chemical Co., St. Louis, MO), HPCD encapsulated 17α-estradiol (αE2; Steraloids, Wilton, NH) or HPCD as the vehicle control. Steroids were used at a final concentration of 2 n*M* or 200 n*M*. Dishes were placed immediately in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA). The chamber was flushed with 100% N_2 for 15 min to achieve hypoxic conditions, and the cells were incubated in the chamber for 12 hr at 37°C. Cells were then returned to 10% CO_2/90% air for 12 hr before viability assessment. HPCD encapsulation of αE2 has been described previously (19). Viability was assessed by exposing cells to 1 μ*M* Calcein AM and 1 μg/ml propidium iodide (Molecular Probes, Eugene, OR) in PBS (pH 7.4) for 15 min. Cells were visualized using a fluorescent Nikon microscope, and two random fields were photographed. Live cells were distinguished by the presence of a bright green fluorescence and the absence of nuclear staining by propidium iodide.

Cumulative CK Activity from Isolated Muscles. EDL and SOL muscles were placed into a Teflon-coated basket and immersed in 9 ml of carbogenated (95% O_2/5% CO_2) balanced salt solution (BSS) at pH 7.4 as described in earlier work (20). After the solutions were placed in the bath, the BSS was drained from the incubation vessels at 30-min intervals followed by the addition of fresh medium over a 4-hr period. These drained solutions at each period were analyzed for CK. Myotoxicity is calculated from the

cumulative sum of the creatine kinase values determined at 30-min intervals from 30 to 240 min. This value is expressed as the cumulative release of creatine kinase (U/l) over the 240-min period.

Thiobarbituric Acid Reactive Substances (TBARS). Heart tissue or intact H9c2 cells were homogenized (PowerGen 125, Fischer Scientific, Pittsburgh, PA) in ice-cold 0.9% NaCl at pH 7 (20%w/v). TBARS were determined as previously described (21) by reacting the homogenate with 1.0% 2-thiobarbituric acid (TBA) solution in the presence of 12.5 *M* trichloroacetic acid (TCA) and 0.8 *M* HCl for 10 min at 100°C. For $FeCl_3$-stimulation of TBARS, the homogenate was incubated with 0.4 m*M* $FeCl_3$ at 37°C for 15 min in a water bath, and 1 m*M* desferrioxamine was added to the TBA reaction. In some studies, homogenates were incubated with either 10 μ*M* βE2, 0.1% ethanol (vehicle control), or normal saline for 1 hr prior to $FeCl_3$ treatment. Samples were centrifuged at 3000 r.p.m. at 4°C for 10 min, and the absorbance of the supernatant was read spectrophotometrically at 532 nm. The amount of TBARS was calculated from a standard curve using 1,1,3,3,-tetraethoxypropane in reagent-grade ethanol diluted with 0.9% normal saline and reacted with 0.8 *M* HCl/12.5% TCA and 1% TBA for 10 min at 100°C and absorbance read at 532 nm.

Plasma Creatine Kinase Activity (CK). Blood was centrifuged immediately, and the plasma fraction was frozen at −80°C until analysis. Plasma total CK levels and cumulative CK activity from isolated muscles were measured spectrophotometrically (Beckman DU-7400, Beckman Instruments, Fullerton, CA) at 340 nm using commercially available kits (Sigma Chemical Company, St. Louis, MO).

Circulating Estrogen and Progesterone. Serum concentrations of βE2 and progesterone were determined using solid phase radioimmunoassay kits (Diagnostics Products, Inc., Los Angeles, CA) according to the manufacturer's instructions.

Statistics. Mean and standard error of the mean (SEM) were calculated for all data. One-way analysis of variance (ANOVA) or Student's *t* test was performed to detect differences between treatments. A Tukey honest significant difference test was performed when significant differences were detected. Statistical significance was set at $P < 0.05$.

Table I. Physical and Hormonal Characteristics of 4-week and 8-week Old Animals According to Treatment Group

	SHAM	OVX	OVX + E2
Weight (g)			
4 weeks	252 ± 4 (7)	310 ± 5 (8)[a,b]	244 ± 2 (7)
8 weeks	285 ± 5 (8)	334 ± 8 (8)[a,b]	280 ± 15 (8)
Heart weight (g)			
4 weeks	0.92 ± 0.05 (8)	0.99 ± 0.03 (8)	0.91 ± 0.03 (8)
8 weeks	0.97 ± 0.03 (8)	1.1 ± 0.03 (8)[a,b]	0.95 ± 0.01 (8)
% heart mass			
4 weeks	0.37 ± 0.007 (7)	0.32 ± 0.006 (8)[a,b]	0.37 ± 0.004 (7)
8 weeks	0.34 ± 0.008 (8)	0.32 ± 0.01 (8)	0.34 ± 0.001 (8)
Soleus weight (mg)			
4 weeks	251 ± 59 (4)	306 ± 85 (4)	288 ± 45 (4)
8 weeks	211 ± 12 (4)	210 ± 15 (4)	207 ± 9 (4)
Soleus length (cm)			
4 weeks	2.5 ± 0.14 (4)	2.7 ± 0.29 (4)	2.8 ± 0.39 (4)
8 weeks	2.7 ± 0.13 (4)	2.8 ± 0.27 (4)	3.2 ± 0.49 (4)
EDL weight (mg)			
4 weeks	184 ± 46 (4)	219 ± 39 (4)	184 ± 29 (4)
8 weeks	207 ± 15 (4)	240 ± 31 (4)	197 ± 14 (4)
EDL length (cm)			
4 weeks	3.7 ± 0.38 (4)	3.9 ± 0.10 (4)	3.6 ± 0.37 (4)
8 weeks	4.0 ± 0.06 (4)	4.2 ± 0.28 (4)	4.1 ± 0.15 (4)
Estradiol (pg/ml)			
4 weeks	16.2 ± 2.5 (7)[c]	12.9 ± 1.4 (8)[a]	23.7 ± 1.3 (8)
8 weeks	12.3 ± 0.9 (8)	9.6 ± 0.80 (8)	29.1 ± 11.0 (8)
Progesterone (ng/ml)			
4 weeks	10.5 ± 3.0 (7)	9.4 ± 2.9 (8)	8.8 ± 3.0 (8)
8 weeks	44.2 ± 9.2 (8)[c]	9.4 ± 1.1 (8)[b]	8.6 ± 2.1 (8)
CK (U)			
4 weeks	103 ± 27 (8)	131 ± 43 (7)	59 ± 15 (8)
8 weeks	98 ± 19 (8)	62 ± 17 (7)	83 ± 26 (5)

Note. Data represented as means ± SEM with sample sizes in parentheses.
[a] $P < 0.05$ for OVX vs OVX + E2.
[b] $P < 0.05$ for OVX vs SHAM.
[c] $P < 0.05$ for SHAM vs OVX + E2.

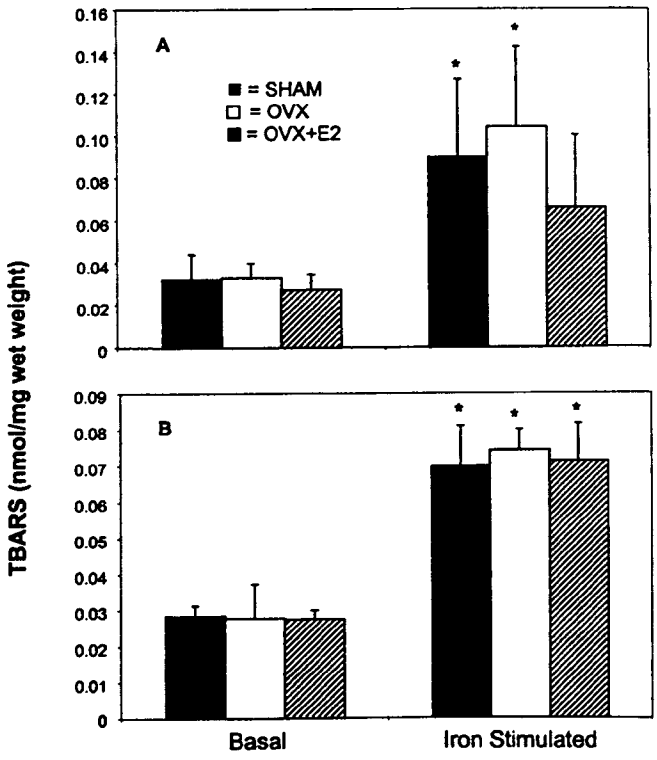

Figure 1. TBARS formation in the heart for (A) 4-week and (B) 8-week treatment animals comparing basal levels and iron-stimulated TBARS formation for SHAM (■), OVX (□), and OVX + E2 (▨). Statistical significance is expressed as follows: $^*P < 0.05$ for iron-stimulated vs basal for respective condition. Data are represented as mean ± SEM for $n = 8$ animals per treatment.

Results

The physical and hormonal characteristics for the 4-week and 8-week-old animals are shown in Table I. The OVX animals were 27% heavier at 4 weeks and 19% heavier at 8 weeks than the OVX + E2 animals. Heart weight in OVX animals increased by 12% at 8 weeks when compared to OVX + E2. Additionally, the percentage heart weight relative to total body weight as calculated by (heart weight/body weight)*100 was 15% less in the OVX animals when compared to OVX + E2 and 12% less than SHAM animals at 4 weeks, indicating a disproportionate growth of heart to body mass in OVX animals. There were also hormonal differences between the OVX and OVX + E2 groups. OVX animals had lower estrogen levels by 45% when compared to OVX + E2 at 4 weeks. There was a near significant difference in estrogen between OVX and OVX + E2 animals at 8 weeks ($P = 0.089$), but when an outlier was removed, the difference became significant. Progesterone levels in OVX animals were almost five times lower than SHAM animals at 8 weeks. Estrogen and progesterone levels were not unexpected because during most of the estrous cycle, estradiol concentrations are low (10–20 pg/ml), so the ovarectomy would not be expected to cause a dramatic reduction in circulating estradiol concentration. A possible sampling time effect could explain the small difference between the OVX and SHAM groups. Because biological indications of persistent estrogen exposure are more reliable than serum estradiol concentration, we performed vaginal lavages on all animals and examined uteri at the time of necropsy. For all of the OVX rats, expected results were seen; vaginal lavages were acellular, and uteri were atrophied. By contrast, all OVX + E2 rats showed vaginal smears predominated by cornified epithelial cells and hypertrophied uteri.

Thiobarbituric acid reacting species (TBARS) assay can be used as a general marker of lipid peroxidation (22). Comparisons were made between basal (unstimulated) levels of TBARS and during iron-stimulated conditions (Fig. 1) for 4-week animals (Panel A) and 8-week animals (Panel B). Iron (II) can initiate lipid peroxidation and give maximal TBARS formation, and these levels can be affected by antioxidant levels (22). Therefore, we used iron-stimulated TBARS as a measure of susceptibility to lipid peroxidation and an induct measure of antioxidant status. There was approximately three times more stimulated TBARS formation than unstimulated TBARS for the 4-week animals and a 2.5-fold increase in the 8-week animals. There were no significant differences between the three groups within the iron-stimulated condition for either time period. Although there was no statistical significance, there is a trend at 4 weeks in that βE2-treated animals showed lower levels of stimulated TBARS (55%), indicating less susceptibility to oxidative damage in the hearts of animals with high estrogen levels.

Individual muscles appear to be protected from membrane damage by the presence of estrogens as indicated by higher levels of CK efflux in estrogen-deprived animals (Fig. 2). Creatine kinase activity is a marker of muscle damage, and the cumulative CK activity from the SOL of the 4-week-old animals (Fig. 2, panel A) was higher in the OVX animals than in the OVX + E2. The 8-week SOL showed a similar pattern to the 4-week SOL with the OVX having a higher CK efflux. However, the EDL had no significant differences between conditions at either time period.

To examine the antioxidant properties of estrogen on muscles, 10 μ*M* βE2 was added to homogenate of OVX hearts and H9c2 cells (Fig. 3). There was a 22% reduction in stimulated TBARS formation for βE2-treated heart homogenate and a 20% decrease in stimulated TBARS in βE2-treated H9c2 cells when compared to the vehicle control. Only the heart homogenate showed significant reduction in TBARS when compared to the saline control, whereas the H9c2 cells showed near significance ($P = 0.07$). Both the cell line and animal tissue were protected from oxidative damage by the presence of βE2.

Exposure of H9c2 cells to hypoxic conditions in the absence of glucose resulted in a 61%–84% reduction in live cell number (Fig. 4). Treatment with βE2 concurrently with stressful conditions caused a concentration-dependent increase in live cell number with βE2 attenuating the decrease in viable cells by 38% and 71% with a 2-n*M* and a 200-n*M*

dose, respectively. This appears to be cytoprotective rather than a mitogenic effect of the steroid as neither concentration of βE2 tested altered cell number in the absence of the toxicity (data not shown). The ability of αE2, an inactive isomer of βE2, was examined and found to attenuate the toxic effects of glucose-free/hypoxic conditions. αE2 similarly protected 29% of the cells at a 2-n*M* concentration and 50% of the cells at a 200-n*M* concentration (Fig. 4). Glucose deprivation under normal atmospheric conditions (10% CO_2/90% air) killed 32%–57% of the H9c2 cells (data not shown). This reduction in live cell number was almost completely blocked by concurrent treatment with 200 n*M* of either steroid. βE2 and αE2 blocked 74% and 67% of this cell death, respectively (data not shown). Hypoxia in the presence of glucose did not alter cell viability (data not shown).

Discussion

Estrogen and estrogen therapy have demonstrated beneficial effects on neurons, bone, cardiovascular system, and body composition. Estrogen's effects on lipid profiles have been documented by increasing circulating HDL and lowering LDL (23). In the presence of estrogen there is also decreased oxidation of LDL, a hypothesized starting point to atherosclerotic lesions. Various neuroneal cell types have been shown to be protected against β-amyloid-induced toxicity by the presence of estrogen (24). This protection may lower the incidence of Alzheimer's disease in postmenopausal women on ERT. Additionally, numerous reports show that loss of bone mineral density is slowed when estrogen therapy is given (25, 26). The protection of estrogen against skeletal muscle damage has also been shown using an exercise model (13), although the mechanism by which estrogens offer this protection is unclear. Furthermore, there is insufficient data to indicate estrogen's protective effect on cardiac tissue independent of the deleterious effects of atherosclerosis.

The present study provides evidence that supraphysiologic concentrations of estrogen are capable of reducing skeletal muscle damage and lipid peroxidation in the heart as well as increasing survival of a cardiac and skeletal muscle–related cell line when exposed to noxious conditions. These findings, along with the protective effects of estrogens on brain, bone, and cardiovascular system found by other investigators, provide compelling evidence of the benefits of ERT. The protective effects of estrogen can be mediated through genomic effects *via* the classical estrogen receptor (ER) or by its antioxidant properties. The findings from this study suggest that the antioxidant capabilities of estrogen may be, in part, responsible for the protection of skeletal and cardiac muscle.

When hearts from ovariectomized animals were preincubated with estrogen, there was a significant decrease in markers of lipid peroxidation. It is unlikely that there was ER-based protection due to the short time course of incubation and physical status of the cells. Furthermore, the antioxidant properties were demonstrated by the protection of α-estradiol (αE2) against cell death when the H9c2 cells were exposed to glucose deprivation and anoxia. The αE2 does not effectively activate genomic estrogen receptor mechanisms and may therefore offers its protection through its antioxidant structure, capable of absolving free radicals and stabilizing cell membranes. Moreover, Kume-Kick (4) found that after 3 weeks of gonadectomy, plasma levels of ascorbate and brain levels of ascorbate and glutathione did not change, lending further evidence that other antioxidants may remain intact without the influence of sex hormones.

Under the conditions used in this present study, estrogen treatment appears to be more protective in the slow-twitch, high oxidative muscles (e.g., the soleus). This muscle group showed greater CK efflux when estrogen was

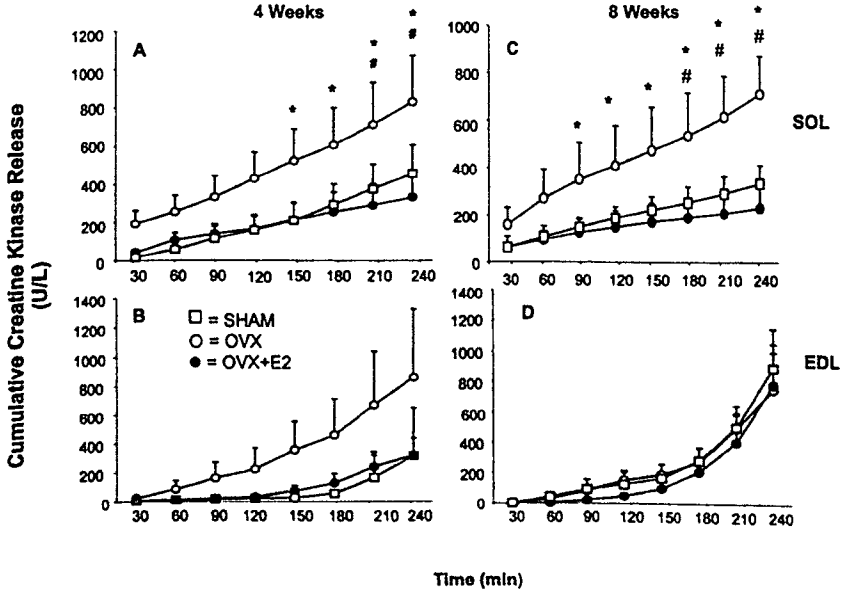

Figure 2. Cumulative creatine kinase release from 4-week treatment animals: (A) soleus and (B) extensor digitorum longus; and 8-week animals: (C) soleus and (D) extensor digitorum longus. Symbols represent: OVX (O), OVX + E2(●), SHAM (□). Statistical significance is expressed as follows: $^{*}P < 0.05$ for OVX vs OVX + E2; $^{\#}P < 0.05$ for OVX vs SHAM. Data are represented as mean ± SEM for $n = 4$ animals per treatment.

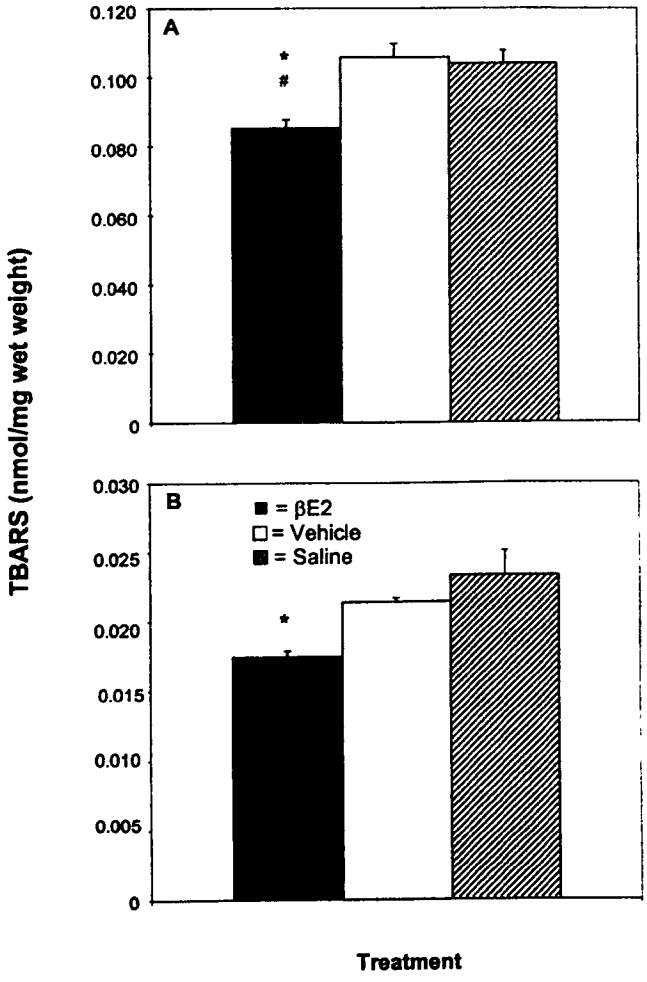

Figure 3. TBARS formation for hearts taken from (A) 4-week OVX animals ($n = 4$) and (B) H9c2 cell line ($n = 4$) after 1-hr incubation with 10 μM 17β-estradiol (■), 0.1% ethanol vehicle (□), or 0.9% NaCl (▨). Statistical significance is expressed as follows: $^{*}P < 0.05$ for estradiol vs vehicle; $^{\#}P < 0.05$ for estradiol vs saline. Data are represented as mean ± SEM for $n = 4$.

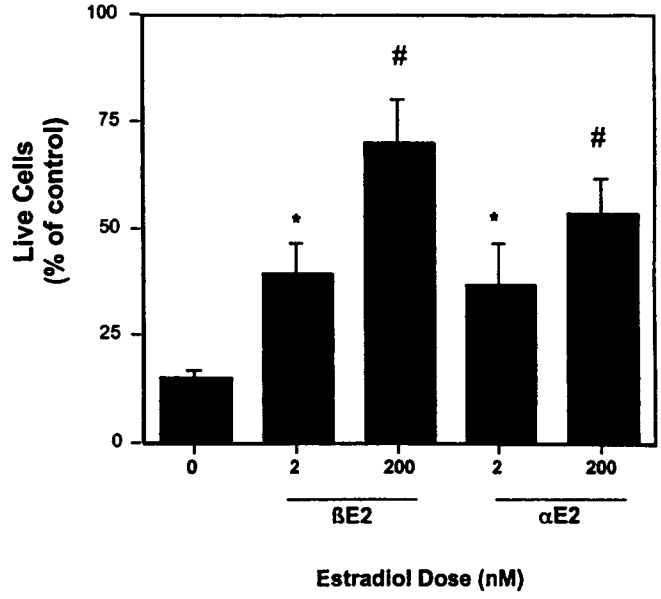

Figure 4. Percentage cell survival for H9c2 cell line when exposed to either 17β-estradiol (βE2) or 17α-estradiol (αE2) in glucose-deprived/hypoxic conditions. Cell number was normalized as percentage of the control (normal media, normal atmosphere) group. Statistical analysis was performed on raw data, and significance was expressed as follows: $^{*}P < 0.05$ vs the no steroid (O) group and $^{\#}P < 0.05$ vs both groups and the respective 2 nM dose. Data are represented as mean ± SEM for three wells per group.

not present indicating increased susceptibility to membrane damage and possibly myofibril damage. These data suggest that oxidative muscles like the SOL incur more damage in estrogen deprivation than the more glycolytic EDL muscle. We suggest two possible mechanisms for preferential damage, each potentially causing increased free radical production.

The first mechanism of preferential protection may be differences in muscle recruitment. The soleus and other slow-twitch fibers are postural muscles whereas the fast-twitch, glycolytic fibers (e.g., extensor digitorum longus) are mainly responsible for movement. When there is physical inactivity, as seen in ovariectomized animals, there is less metabolic demand placed on EDL-type muscles; however, the slow-twitch muscles are still recruited to maintain posture. Additionally, the increased body weight of ovariectomized animals, which is consistent with other studies (27–29), may increase the metabolic load on postural muscles to support the additional body weight since the increase in total body weight did not reflect changes in muscle weights. The continuous recruitment to support the body leads to a higher metabolic load and therefore a higher oxygen consumption and free radical production. As such, our paradigm of assessing animals under sedate conditions revealed an effect of estrogen deprivation on these slow-twitch muscles. However, we propose that fast-twitch muscles might also be affected by estrogen deprivation during exercise-induced stress.

The second mechanism of preferential protection may be the metabolic differences in muscle fibers. Slow-twitch fibers have a great supply of mitochondria, a high reliance on oxygen, and the ability to use fatty acids as a major energy source. This combination of conditions causes slow-twitch muscles to produce more free radicals. During estrogen deprivation, this condition is exacerbated because of a decline in the transport of glucose into the muscle cells (30) associated with a decreased insulin sensitivity (31), increased fasting glucose (31), and increased fasting insulin (32). Further, reduced glucose availability can increase the reliance of skeletal muscle on lipid oxidation.

In healthy tissue, the most important source of free radicals is the leak of electrons in the mitochondrial electron transport system (ETS) (33). Increased lipid oxidation and increased intracellular fatty-acid concentration, as may be seen in estrogen-deprived animals, places a greater metabolic pressure on mitochondria and decreases coupling efficiency, thus enhancing free radical production. High intracellular concentrations of fatty acids have the ability to act like a protonophore and cause a loss of the electrochemical gradient (34). Additionally, high concentrations of in-

tracellular fatty acids have an ability to dissociate the F_0F_1 ATPase complex (34).

These interruptions in the mitochondria can cause mitochondrial swelling, loss of the electrochemical gradient, and changes in electron flux through the electron transport complexes causing uncoupling between electron flux and oxidative phosphorylation and leading to free radical production. The increased production of free radicals further compromises mitochondrial function by depressing aerobic enzyme activity (e.g., aconitase), damaging mitochondrial DNA, and damaging mitochondrial membranes. The leak of CK from the muscle following damage can further interrupt energy metabolism by decreasing the ability to regenerate ATP and phosphocreatine (PCr) stores. Loss of CK can also weaken muscle structure because it helps form the tight lattice in the M-region of sarcomeres, and loss of CK can deteriorate the stability of contracting filaments. A decrease in the ability to regenerate PCr and disruptions in sarcomere structure can alter the shortening capacity of muscle and decrease the time to fatigue.

In this study skeletal muscle, especially the high oxidative type 1 fiber-rich muscles, cardiac muscle, and cardiac-related cell line showed less indications of membrane damage in the presence of β-estradiol or its isomer α-estradiol. The preferential protection of highly oxidative tissues may be related to the amount of free radical production from oxidative metabolism. Further investigation into the area of skeletal muscle and cardiac muscle's protection *via* estrogen is needed to enhance the therapeutic usage of estrogen replacement therapy or possible alternatives that could enhance antioxidant capacity such as antioxidant supplementation or aerobic exercise.

We would like to thank Ms. Ilene Monck, Ms. Nancy deFibre, Sue Glennon and Sandeep Patel for their technical assistance.

1. Sugioka K, Shimosegawa Y, Nakano M. Estrogens as natural antioxidants of membrane phospholipid peroxidation. FEBS Lett **210**:37–39, 1987.
2. Green PS, Gordon K, Simpkins JW. Phenolic A ring requirement for the neuroprotective effects of steroids. J Steroid Biochem Mol Biol **63**:229–235, 1997.
3. Simpkins JW, Green PS, Gridley K. A fundamental role for estrogens in cognition and neuroprotection. In: Brioni JD, Decker MW, Eds. Pharmacological Treatment of Alzheimer's Disease: Molecular and Neurobiological Foundations. New York: Wiley-Liss, p503, 1997.
4. Kume-Kick J, Ferris DC, Russo-Menna I, Rice ME. Enhanced oxidative stress in female rat brain after gonadectomy. Brain Res **738**:8–14, 1996.
5. Dargent-Molina P, Favier F, Grandjean H, Baudoin C, Schott AM, Hausherr E, Meunier PJ, Breart G. Fall-related factors and risk of hip fracture: The EPIDOS prospective study. Lancet **348**:145–149, 1996.
6. Warren GL, Lowe DA, Inman CL, Orr OM, Hogan HA, Bloomfield SA, Armstrong RB. Estradiol effect on anterior crural muscles-tibial bone relationship and susceptibility to injury. J Appl Physiol **80**:1660–1665, 1996.
7. Samaan SA, Crawford MH. Estrogen and cardiovascular function after menopause. J Am Coll Cardiol **26**:1403–1410, 1995.
8. Halliwell B. Free radicals and oxidative damage in biology and medicine: An introduction. In: Reznick AZ, Ed. Oxidative stress in skeletal muscle. Basel, Boston: Birkhäuser Verlag, p1, 1998.
9. Ku HH, Brunk UT, Sohal RS. Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. Free Radic Biol Med **15**:621–627, 1993.
10. Phillips SK, Rook KM, Siddle NC, Bruce SA, Woledge RC. Muscle weakness in women occurs at an earlier age than in men, but strength is preserved by hormone replacement therapy. Clin Sci (Colch) **84**:95–98, 1993.
11. Cauley JA, Petrini AM, LaPorte RE, Sandler RB, Bayles CM, Robertson RJ, Slemenda CW. The decline of grip strength in the menopause: Relationship to physical activity, estrogen use, and anthropometric factors. J Chronic Dis **40**:115–120, 1987.
12. Aloia JF, McGowan DM, Vaswani AN, Ross P, Cohn SH. Relationship of menopause to skeletal and muscle mass. Am J Clin Nutr **53**:1378–1383, 1991.
13. Bar PR, Amelink GJ, Oldenburg B, Blankenstein MA. Prevention of exercise-induced muscle membrane damage by oestradiol. Life Sci **42**:2677–2681, 1988.
14. Thompson HS, Hyatt JP, De Souza MJ, Clarkson PM. The effects of oral contraceptives on delayed onset muscle soreness following exercise. Contraception **56**:59–65, 1997.
15. Tiidus PM. Can estrogens diminish exercise-induced muscle damage? Can J Appl Physiol **20**:26–38, 1995.
16. Somani SM, Frank S, Rybak LP. Responses of antioxidant system to acute and trained exercise in rat heart subcellular fractions. Pharmacol Biochem Behav **51**:627–634, 1995.
17. Heads RJ, Latchman DS, Yellon DM. Stable high level expression of a transfected human *HSP70* gene protects a heart-derived muscle cell line against thermal stress. J Mol Cell Cardiol **26**:695–699, 1994.
18. Singh M, Meyer EM, Millard WJ, Simpkins JW. Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague-Dawley rats. Brain Res **644**:305–312, 1994.
19. Zaulyanov L, Green PS, Simpkins JW. Glutamate receptor requirement for neuronal death from anoxia-reoxygenation: An *in vitro* model for assessment of the neuroprotective effects of estrogens. Cell Mol Neurobiol **19**:705–718, 1999
20. Brazeau GA, al-Suwayeh S, Peris J, Hunter B, Walker DW. Creatine kinase release from isolated EDL muscles in chronic ethanol-treated rats. Alcohol **12**:145–149, 1995.
21. Dawson R, Tang E, Shih D, Hern H, Hu M, Baker D, Eppler B. Taurine inhibition of iron-stimulated catecholamine oxidation. In: Schaffer S, Lonbardini JB, Huxtable RJ, Eds. Taurine 3: Cellular and Regulatory Mechanisms. New York: Plenum Press, p155, 1998.
22. Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med **9**:515–540, 1990.
23. LaRosa JC. Metabolic effects of estrogens and progestins. Fertil Steril **62**:140S–146S, 1994.
24. Simpkins JW, Green PS, Gridley KE, Singh M, de Fiebre NC, Rajakumar G. Role of estrogen replacement therapy in memory enhancement and the prevention of neuronal loss associated with Alzheimer's disease. Am J Med **103**:19S–25S, 1997.
25. Gaumet N, Braillon P, Seibel MJ, Pointillart A, Coxam V, Davicco MJ. Influence of aging on cortical and trabecular bone response to estradiol treatment in ovariectomized rats. Gerontology **44**:132–139, 1998.
26. Kohrt WM, Ehsani AA, Birge SJ, Jr. HRT preserves increases in bone mineral density and reductions in body fat after a supervised exercise program. J Appl Physiol **84**:1506–1512, 1998.
27. Wagner JD, Martino MA, Jayo MJ, Anthony MS, Clarkson TB, Cefalu WT. The effects of hormone replacement therapy on carbohydrate

metabolism and cardiovascular risk factors in surgically postmenopausal cynomolgus monkeys. Metabolism **45:**1254–1262, 1996.
28. Tarttelin MF, Gorski RA. The effects of ovarian steroids on food and water intake and body weight in the female rat. Acta Endocrinol (Copenh) **72:**551–568, 1973.
29. Goldberg DI, Rumsey WL, Kendrick ZV. Exercise activation of myocardial lipoprotein lipase in male and estrogen-treated female rats. Metabolism **33:**964–969, 1984.
30. Bishop J, Simpkins JW. Estradiol enhances brain glucose uptake in ovariectomized rats. Brain Res Bull **36:**315–320, 1995.
31. Senoz S, Direm B, Gulekli B, Gokmen O. Estrogen deprivation, rather than age, is responsible for the poor lipid profile and carbohydrate metabolism in women. Maturitas **25:**107–114, 1996.
32. Poehlman ET, Toth MJ, Gardner AW. Changes in energy balance and body composition at menopause: A controlled longitudinal study. Ann Intern Med **123:**673–675, 1995.
33. Herrero A, Barja G. Sites and mechanisms responsible for the low rate of free radical production of heart mitochondria in the long-lived pigeon. Mech Ageing Dev **98:**95–111, 1997.
34. Wojtczak L, Schonfeld P. Effect of fatty acids on energy coupling processes in mitochondria. Biochim Biophys Acta **1183:**41–57, 1993.