Effects of ovariectomy and estrogen on ischemia-reperfusion injury in hindlimbs of female rats

NICOLE STUPKA AND PETER M. TIIDUS

Department of Kinesiology and Physical Education, Wilfrid Laurier University, and
Department of Kinesiology, University of Waterloo, Waterloo, Ontario, Canada N2L 3C5

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Stupka, Nicole, and Peter M. Tiidus. Effects of ovariectomy and estrogen on ischemia-reperfusion injury in hindlimbs of female rats. J Appl Physiol 91: 1828–1835, 2001.—The effects of estrogen and ovariectomy on indexes of muscle damage after 2 h of complete hindlimb ischemia and 2 h of reperfusion were investigated in female Sprague-Dawley rats. The rats were assigned to one of three experimental groups: ovariectomized with a 17β-estradiol pellet implant (OE), ovariectomized with a placebo pellet implant (OP), or control with intact ovaries (R). It was hypothesized that following ischemia-reperfusion (I/R), muscle damage indexes (serum creatine kinase (CK) activity, calpain-like activity, inflammatory cell infiltration, and markers of lipid peroxidation (thiobarbituric-reactive substances]) would be lower in the OE and R rats compared with the OP rats due to the protective effects of estrogen. Serum CK activity following I/R was greater (P < 0.01) in the R rats vs. OP rats and similar in the OP and OE rats. Calpain-like activity was greatest in the R rats (P < 0.01) and similar in the OP and OE rats. Neutrophil infiltration was assessed using the myeloperoxidase (MPO) assay and immunohistochemical staining for CD43-positive (CD43+) cells. MPO activity was lower (P < 0.05) in the OE rats compared with any other group and similar in the OP and R rats. The number of CD43+ cells was greater (P < 0.01) in the OP rats compared with the OE and R rats and similar in the OE and R rats. The OE rats had lower (P < 0.05) thiobarbituric-reactive substance content following I/R compared with the R and OP rats. Indexes of muscle damage were consistently attenuated in the OE rats but not in the R rats. A 10-fold difference in serum estrogen content may mediate this. Surprisingly, serum CK activity and muscle calpain-like activity were lower (P < 0.05) in the OP rats compared with the R rats. Increases in serum insulin-like growth factor-1 content (P < 0.05) due to ovariectomy were hypothesized to account for this finding. Thus both ovariectomy and estrogen supplementation have differential effects on indexes of I/R muscle damage.

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Addresses for reprint requests and other correspondence: P. M. Tiidus, Dept. of Kinesiology & Physical Education, Wilfrid Laurier Univ., Waterloo, ON, Canada N2L 3C5 (E-mail: ptiidus@wlu.ca).
METHODS

Animals

This study was approved by the University of Waterloo Committee on Animal Ethics and Care and was performed in accordance with the guiding principles of the Canadian Council on Animal Care. Regular (with ovaries) and ovariectomized Sprague-Dawley rats (Harlan) were housed in cages of five to six animals in an environmentally controlled room with reversed light-dark cycles. The ovariectomy was performed by the animal supplier at 8 wk of age. Animals were allowed free access to food and water and fed an AIN-93-purified rodent diet. All animals were weighed before death.

Experimental Design

After 1 wk of acclimatization, the ovariectomized animals were implanted with either a 0.25-mg, 21-day-release 17β-estradiol pellet (n = 16; E-121, Innovative Research of America) or a corresponding, 21-day-release placebo pellet (n = 16; C-111, Innovative Research of America). The pellets were implanted subcutaneously (neck skin fold) under halothane anesthesia using a 10-gauge trochar needle (MP182, Innovative Research of America). The pellets were implanted subcutaneously (neck skin fold) under halothane anesthesia using a 10-gauge trochar needle (MP182, Innovative Research of America). The pellets were implanted subcutaneously (neck skin fold) under halothane anesthesia using a 10-gauge trochar needle (MP182, Innovative Research of America). Two weeks later, at ~12 wk of age, all of the ovariectomized animals underwent the 2 h of complete hindlimb ischemia and 2 h of reperfusion protocol (I/R). After the 2 h ischemia period, reperfusion was initiated by immediate reduction of cuff pressure to zero. Similar I/R protocols have been previously reported to influence skeletal muscle leukocyte accumulation and oxidative stress (e.g., Ref. 32).

R animals, also ~12 wk old, were assigned to one of three experimental groups: no intervention (R-sham; n = 10, 2 h of complete ischemia only (R-I only, n = 10), and 2 h of complete ischemia and 2 h of reperfusion (R-I/R; n = 16). The purpose of the R-sham group was to provide baseline values for CK, MPO, thiobarbituric-reactive substances (TBARS), calpain activity, and muscle neutrophil content. Because we have previously seen no effect of ovariectomy and estrogen replacement on these parameters before intervention (39, 41), the measures in R-sham group could be used as a baseline for the OE and OP groups as well. Preliminary power calculation estimates had suggested that the number of animals used in this study would be adequate for statistical power.

One hindlimb from the I/R or I-only animals was selected to undergo the experimental procedure. The animals were anesthetized via intraperitoneal injection of pentobarbital sodium (100 μg/100 g body wt) (Somnotol, MTC Pharmaceuticals, Cambridge, ON). Anesthesia was confirmed with the loss of withdrawal reflex from toe web pinch and maintained by pentobarbital sodium injections as required. The animals were kept warm with a heating pad, and vital signs were monitored throughout the procedure. Total ischemia was induced by placing a finger tourniquet (DigiKit, Kinetikos Medical, San Diego, CA) over the upper hindlimb and inflicting the cuff to a pressure of 350 mmHg (13) for 2 h of ischemia and 2 h reperfusion or 2 h of ischemia only. Previous studies have demonstrated significant skeletal muscle neutrophil infiltration within 1–2 h after exercise or I/R-induced damage (3, 32, 41). After I/R intervention, blood was drawn from the descending aorta of the rats and allowed to clot in a glass test tube. The gastrocnemius, tibialis anterior (TA), and plantaris (PLT) were excised from the experimental hindlimb, rinsed in saline, blotted, and snap frozen in liquid nitrogen. The soleus (Sol) was allotted for immunohistochemical analysis. Sol is composed primarily of slow-twitch muscle fiber types and thus differs from the gastrocnemius, TA, and PLT, which are of mixed-fiber type. It is currently not known whether muscle fiber type can influence post-I/R neutrophil infiltration. After removal, the Sol was frozen in isopentane cooled with liquid nitrogen after being placed on a block with embedding medium (OCT). The tourniquet remained in place and inflated while the hindlimbs were excised from the I-only animals to avoid reperfusion, after which blood was drawn from the descending aorta. All muscle samples and serum were stored at −70°C until the time of analysis.

Analysis

Serum. Serum was assayed for 17β-estradiol and insulin-like growth factor-1 (IGF-1) content using a commercially available radioimmunoassay kit (TEK21, Diagnostic Products, Los Angeles, CA, and 022-IGF-R20, Alpco Diagnostics, Windham, NH, respectively). Serum was also assayed spectrophotometrically (wavelength = 340 nm) for CK activity using a commercially available kit (DG147-UV, Sigma Diagnostics, Columbus, OH).

Calpain-like activity. Calpain-like, calcium-dependent proteolytic activity in the PLT was determined by a microplate reader (SpectraMax Plus 384, Molecular Devices) assay using a 1:100 dilution of the PLT extract, 2 mg/ml casein, 50 mM Tris (pH 7.5), 20 mM dithiothreitol, and either 5 mM free calcium or 5 mM EDTA. The PLT extract was incubated at 30°C. After 30 min, a 40-μl aliquot from each sample was assayed for calcium-dependent proteolysis using 20 μl of a concentrated Bio-Rad protein dye reagent (500-0006, Bio-Rad Laboratories, Hercules, CA) diluted in 120 μl of double-distilled H2O2. Calpain-like activity was determined by calculating the difference between the absorbance values (at 595 nm) from the reaction mixture containing calcium and that containing EGTA and expressed as caseinolysis per gram tissue wet weight over 30 min (2).

MPO. MPO activity was determined at a concentration of 6 mM H2O2 by continuously monitoring the oxidation of o-dianisidine dihydrochloride at 480 nm at 37°C using a recording spectrophotometer (3, 40). One unit of MPO activity was defined as a change in 1.0 unit of absorption at 480 nm/min expressed per gram tissue wet weight.

CD43+ cells. CD43 is expressed by polymorphs and has been used to detect the presence of neutrophils in skeletal muscle following muscle damage (26) or hindlimb unloading (37). The frozen, OCT-embedded Sol was serially cross sectioned to 5-μm thickness using a cryostat (Microm HM 500 OM, Microm International, Walldorf, Germany). Negative control sections were included in all analyses. The slides were dried overnight and stored at −80°C until analysis. Slides were fixed in cold acetone for 15 min. Endogenous peroxidase activity was blocked using a liquid diaminobenzidine substrate kit (00-2007, Zymed Laboratories, Walldorf, Germany). The slides were blocked with 1% goat serum (D3002S, Dako Diagnostics Canada, Mississauga, ON) for 15 min to control for nonspecific binding. The primary mouse anti-rat CD43 antibody (MCA54R, Serotect) was diluted 1:50 in 1% goat serum, and positive slides were incubated for 60 min. Slides were then incubated with a secondary goat anti-mouse antibody (65-6440, Zymed Laboratories) for 45 min and with peroxidase (95-6543-B, Zymed Laboratories) for an additional 15 min. A kit (00-2007, Zymed Laboratories) was used for color development. All slides were counterstained with hematoxylin solution (MHS-16, Sigma Chemical).
EFFECTS OF OVARIECTOMY AND ESTROGEN ON MUSCLE DAMAGE

Table 1. Animal characteristics

<table>
<thead>
<tr>
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<th>Weight, g</th>
<th>Days With Pellet</th>
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<tr>
<td>R-sham</td>
<td>237.9 ± 3.4</td>
<td></td>
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<tr>
<td>R-I only</td>
<td>236.2 ± 4.0</td>
<td></td>
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<tr>
<td>R-I/R</td>
<td>241.1 ± 2.9</td>
<td></td>
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<tr>
<td>OP-I/R</td>
<td>274.2 ± 3.2*</td>
<td>15.8 ± 0.5</td>
</tr>
<tr>
<td>OE-I/R</td>
<td>213.8 ± 2.1†</td>
<td>15.5 ± 0.7</td>
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Values are means ± SE. R-Sham, normal ovaries and no ischemia/reperfusion (I/R); R-I only, normal ovaries with 2 h ischemia and no reperfusion; R-I/R, normal ovaries with 2 h of ischemia and 2 h of reperfusion; R, normal ovaries; OP, ovariectomized with placebo pellet implant and 2 h of ischemia and 2 h of reperfusion; OE, ovariectomized with estrogen pellet implant and 2 h of ischemia and 2 h of reperfusion. The weights of the rats in the R-sham, R-I only, and R-I/R groups at the time of death were similar. The average number of days with the pellet implant was similar for the OE and OP groups. The animals in the *OP-I/R group weighed more and the rats in the †OE group weighed less compared with the other groups (P < 0.05).

The number of CD43+ cells in muscle cross section were counted manually and expressed as number of positive cells per square millimeter of muscle. Only those CD43+ cells that were surrounded by muscle fibers were counted, and those localized to the lumen of blood vessels or connective tissue were excluded from analysis. Entire muscle cross sections were captured with a digital camera (Spot, v2.2, Diagnostic Instruments, Sterling Heights, MI), and area was quantified using a computerized image analysis system (ImagePro Plus, v4.0, Media Cybernetics, Silver Spring, MD). The count variability was <10%.

TBARS. To estimate levels of lipid peroxidation in the TA, the TBARS assay developed by Ohkawa et al. (25) was used. Fresh TA homogenates (20:1 volume of 0.154 M KCl buffer/g wet wt) were added to a reaction mixture containing 0.2 ml of 0.28 M SDS, 1.5 ml of 3.5 M acetic acid (pH 3.5), and 55.5 mM thiobarbituric acid solution and heated at 95°C for 1 h. The resultant TBARS were extracted using a n-butanol-pyridine (15:1 vol/vol) mixture, and the absorbance was determined spectrophotometrically at 532 nm. The level of TBARS in the TA was measured using 0.5- to 5.0-nmol samples of 1,1,3,3-tetramethoxypropane as standards, and tissue lipid peroxidation levels were calculated as nanomoles TBARS per gram tissue wet weight.

Lactate and glycogen. Analysis of lactate and total glycogen were done on freeze-dried, perchloric acid-extracted TA muscle using standard fluorometric techniques (15).

Statistics

Data were analyzed using one-way ANOVA with five factors (R-sham, R-I only, R-I/R, OP-I/R, and OE-I/R). A Newman-Keuls post hoc analysis was performed if a significant main effect was obtained to identify which groups differed (Statistica v6.0, Statsoft, Tulsa, OK). The level of significance was P < 0.05. All data are presented as means ± SE.

RESULTS

Animal Characteristics

There was no significant difference in weight at the time of death among the three groups of female rats with intact ovaries (R-sham, R-I only, and R-I/R). However, these animals were significantly heavier compared with the OE animals (P < 0.05) and significantly lighter compared with the OP animals (P < 0.05) (see Table 1). The average number of days with pellet implant was similar among the OE and OP rats (see Table 1).

Estrogen

Serum estrogen content was significantly greater in the OE animals compared with the OP and R animals (P < 0.05). However, serum estrogen content was similar in OP animals and the R animals (See Table 2). This lack of difference in estrogen content could not be accounted for by the cessation of estrus in R rats. Estrus may cease if female rats are housed together and if there are no mature, breeding male rats in the neighboring cages. To confirm the presence of estrus, four R-sham rats were randomly chosen to undergo vaginal cell smears for 4 consecutive days before death (the average length of a rat estrus cycle) (10). These cells were examined under a light microscope. Changes in cell type and number were observed in all four of the animals during the 4 days, suggesting that the rats were indeed cycling (10).

IGF-1

The OP rats had a significantly greater serum IGF-1 content compared with the R and OE rats (P < 0.05). The OE group had the lowest serum IGF-1 content (P < 0.05).

Serum CK Activity

Serum CK activity was elevated after I/R in all groups compared with R-sham and R-I only (P < 0.01). The CK elevation following I/R was greater in the R group (P < 0.05) than in the OP or OE groups (see Fig. 1).

Calpain

Calpain-like activity in the PLT was significantly greater in the R-I only rats and R-I/R rats compared with R-sham rats (P < 0.05). Calpain-like activity was lower in the OE and the OP rats following I/R compared with the R-I only rats (P < 0.05) and the R-I/R rats (P < 0.05). After I/R, calpain-like activity was similar among the OE and OP rats (P = 0.57) (see Fig. 2).

MPO

MPO activity in the PLT was similar among the R-sham, R-I only, and R-I/R rats. There was also no

Table 2. Circulating 17β-estradiol and IGF-1 content

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<thead>
<tr>
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<th>17β-Estradiol, pg/ml</th>
<th>IGF-1, pg/ml</th>
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<tbody>
<tr>
<td>R</td>
<td>20.5 ± 1.15</td>
<td>759.8 ± 26.7</td>
</tr>
<tr>
<td>OP</td>
<td>15.3 ± 1.67</td>
<td>941.3 ± 18.7</td>
</tr>
<tr>
<td>OE</td>
<td>209.1 ± 50.3*</td>
<td>599.6 ± 15.3</td>
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Values are means ± SE. IGF-1, insulin-like growth factor-1. *The OE rats had significantly greater 17β-estradiol content compared with the OP and R rats (P < 0.05). However, there was no significant difference in 17β-estradiol content between the OP and R rats. †OP rats had greater serum IGF-1 content compared with the R and OE rats (P < 0.05). ‡R rats had greater serum IGF-1 content compared with the OE rats (P < 0.05).
significant difference in MPO activity in the OP rats and the R rats. MPO activity in the PLT of the OE animals was significantly lower compared with any other experimental group following I/R (P < 0.05) (see Fig. 3).

**CD43**+ Cells

The number of CD43+ cells/mm² tissue was greater in the OP group following I/R compared with any other experimental group. The number of CD43+ cells were significantly elevated in the OP group compared with R-sham animals (P < 0.05), and there was a trend toward an increase in the OE (not significant, P = 0.08) and R (not significant, P = 0.10) groups following I/R. Also, the number of CD43+ cells was greater in the R, OP, and OE groups after I/R compared with the R-I only group. Thus neutrophil infiltration occurred during reperfusion only and not during ischemia (see Fig. 4).

**TBARS**

TBARS content in the TA did not increase in response to ischemia only or I/R as it was similar among the R-sham, R-I only, and R-I/R rats. TBARS content was significantly greater in the OP-I/R group compared with any other experimental group (P < 0.05). TBARS content was ~1.5 times greater in the R rats compared with the OE rats (not significant, P = 0.12) (see Fig. 5).

**Muscle Lactate and Glycogen**

Lactate and glyogen were run as confirmational markers for I/R. As expected, in the R rats, muscle lactate increased significantly after 2 h of ischemia and returned to baseline values after 2 h of reperfusion (P < 0.05). After I/R, muscle lactate content was similar in the R, OP, and OE rats. Ischemia decreased

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**Fig. 1.** Serum creatine kinase (CK) activity. R-sham, normal ovaries with no ischemia-reperfusion (I/R); R-2I only, normal ovaries with 2 h of ischemia only; R-I/R, normal ovaries with 2 h of ischemia and 2 h of reperfusion; OP-I/R, ovariectomized with placebo pellet and I/R; OE-I/R; ovariectomized with estrogen pellet and I/R. *Serum CK activity was significantly elevated compared with R-sham and R-2I only. + Serum CK activity was significantly greater in the R-I/R rats compared with the OP-I/R and OE-I/R rats following I/R. Significance was set at P < 0.05.

**Fig. 2.** Muscle calpain-like activity. ww, Wet wt. Calpain-like activity was significantly greater in the R-2I only and R-I/R rats compared with R-sham rats. Calpain-like activity was significantly lower in the OP-I/R and OE-I/R rats compared with the R-I/R and R-2I only rats. Significance was set at P < 0.05. Results are from plantaris muscle.

**Fig. 3.** Muscle myeloperoxidase (MPO) activity. *MPO activity was significantly lower in the OE-I/R rats after I/R compared with any other group (P < 0.05). Results are from plantaris muscle.

**Fig. 4.** Muscle CD43-positive cells. *Number of CD43-positive cells per mm² of muscle was significantly greater in the OP-I/R rats following I/R compared with any other experimental group (P < 0.05). **Number of CD43-positive cells per mm² of muscle was greater in the OP-I/R and OE-I/R rats compared with the R-2I only rats (P < 0.05). Results are from soleus muscle.
cause a decrease in spontaneous cage activity, hence the greater body weight in OP animals (11, 12). Furthermore, estrogen can downregulate the growth hormone-IGF-1 axis (11, 12). There was a positive relationship between serum IGF-1 content and body weight. Serum IGF-1 content was greatest in the OP rats and lowest the OE rats, which incidentally also had the greatest serum estrogen content. The serum IGF-1 values obtained by us are similar to those reported by others in normal and ovariectomized rats (12).

Although serum estrogen content was ~10-fold greater in the OE animals compared with the OP and R animals (P < 0.05), there was no significant difference among the OP animals and the R animals. This lack of difference could not be attributed to the cessation of estrus in the R rats. Circulating estrogen content in OP rats has been reported to be similar to the low end of physiological estrogen fluctuations in R rats (27, 43). The estrogen values seen in our OP rats are similar to those in other studies (27, 41). However, a wide range of circulating estrogen values for normal, female rats has been reported in the literature. Similar to our findings, Persky et al. (27) found normal female rats (equivalent to our R rats) to have circulating estrogen values of 15–20 pg/ml. Zhai et al. (46) measured mean estrogen values of ~60 pg/ml, whereas others have reported values as high as 285 pg/ml (23). These discrepancies have, to our knowledge, not been fully addressed and may influence markers of muscle damage following ischemia and reperfusion.

Serum CK activity was elevated in the R-I/R rats but not in the R-I only rats. The latter is likely due to circulatory occlusion limiting CK efflux from muscle. After I/R, serum CK activity was greater in the R rats compared with the OE and OP rats. The protective effects of both estrogen and IGF-1 may account for this finding. IGF-1 has been shown to attenuate CK release from cardiac muscle following I/R (14) and from muscle that has been oxidatively stressed (with 2,4-dinitrophenyl) in vitro.

In the R rats, calpain-like activity was increased after both ischemia only and I/R relative to R-sham. Prolonged ischemia and I/R have been shown to disrupt calcium handling and increase intracellular calcium content in cardiac and skeletal muscle (46). In-
creases in intracellular calcium can activate calpain (2, 38). Elevated µ-calpain and m-calpain activities following I/R have been measured in cardiac muscle (42, 45). Skeletal muscle calpain activity has also been reported to be elevated after acute exercise (3, 41). This is the first report, to our knowledge, that demonstrates increased calpain activity in skeletal muscle in vivo following hindlimb I/R.

Both ovariectomy and the estrogen pellet implant appear to attenuate calpain-like activity following I/R. Calpain-like activity was lower in the OE and the OP rats following I/R compared with the R-I only rats ($P < 0.05$) and the R-I/R rats ($P < 0.05$). Following I/R, calpain-like activity was similar in the OE and OP group ($P = 0.57$). Decreased calpain-like activity in the OP rats compared with the R rats may be due to increased IGF-1 expression following ovariectomy. In a murine model of muscular dystrophy, IGF-1 administration (in conjunction with a high-protein diet) resulted in an attenuation in skeletal muscle calpain activity (44). The mechanism by which estrogen could attenuate calpain activity is unknown. Estrogen may act directly on muscle membranes to preserve ultrastructural stability and intracellular homeostasis (39, 41). We have previously reported that estrogen replacement in ovariectomized rats attenuates postexercise calpain activity in skeletal muscle (41).

Inflammatory cell infiltration into skeletal muscle was assessed using the MPO assay and immunohistochemical staining for CD43. MPO activity was similar among the R-sham, R-I only, R-I/R, and OP rats, but it was significantly lower in the OE rats ($P < 0.05$). Estrogen has been shown to attenuate leukocyte adhesion in cerebral blood vessels both under resting conditions and after I/R (31). Furthermore, after exercise, estrogen administration has been shown to attenuate MPO activity and inflammatory cell infiltration in male and ovariectomized female rats (40, 41). However, others have suggested that estrogen does not attenuate inflammatory cell infiltration; rather, it merely delays it (35). Estrogen decreases adhesion molecule expression and increases endothelial and neuronal NO synthase content (5). Nitric oxide produced by endothelial and neuronal NO synthase has potent anti-adhesive properties (5). Infiltrating neutrophils as well as leukocyte adhesion may contribute to total MPO activity. Thus the attenuation in MPO activity observed in the OE group may also reflect decreased leukocyte adhesion to blood vessels due to estrogen administration.

MPO activity did not increase in the R rats following ischemia only or I/R. There are conflicting reports in the literature about whether MPO activity does increase in skeletal muscle after I/R. Increases in MPO activity have been measured after 4 h of hindlimb ischemia and 1 h of reperfusion in mongrel dogs (33) and after 2 h of ischemia and 2 h of reperfusion in male rats (28). Others, however, did not detect elevations in MPO activity after 30 min of ischemia and 1 h of reperfusion in female rats (17) and after 4 h of ischemia and 4 h of reperfusion in male rats (9).

The number of CD43+ cells/mm² tissue in the R, OP, and OE rats was significantly elevated after I/R compared with that shown in the R-I only rats. Similar to our MPO results, the number of CD43+ cells was lower in the OE rats compared with the OP rats. Thus estrogen appears to attenuate leukocyte infiltration into skeletal muscle following I/R. However, other factors may confound the relationship between estrogen, ovariectomy, and inflammatory cell infiltration. Circulating estrogen values were similar in the OP rats and the R rats; however, after I/R, the number of CD43+ cells was almost twofold greater in the OP rats compared with the OE rats. Furthermore, the number of CD43+ cells was similar in the OE rats and the R rats, despite an ~10-fold difference in circulating estrogen content. Although circulating IGF-1 levels were elevated in the OP rats, the effect of this on post-I/R skeletal muscle leukocyte infiltration cannot be determined from this study.

Our MPO data (as an index of muscle neutrophil infiltration) did not always correlate exactly with our CD43+ immunohistochemically determined neutrophil counts in muscle tissue. Although MPO data have often been used as indirect markers of muscle neutrophil infiltration (3, 33, 40, 41), these data may not be as accurate as direct histochemical determination of neutrophil presence in muscle (32). We have also previously seen larger changes in postexercise muscle neutrophil infiltration via direct count using histochecmistry than inferred by changes to muscle MPO activity (41). It is also possible that differences in muscle fiber type may influence post-I/R neutrophil infiltration and account for some of the discrepancy in our MPO data [determined in PLT (mixed muscle)] and CD43+ data [determined in Sol (slow muscle)].

A compelling reason for measuring both calpain-like activity as well as neutrophil infiltration is the hypothesis put forward by Belcastro et al. (4), which suggests that calpain may stimulate neutrophil infiltration following skeletal muscle damage. A positive correlation between elevations in calpain-like and MPO activities in cardiac and skeletal muscle has been observed in rats after 1 h of treadmill running (24). Furthermore, cleaved peptide fragments of calpain have been shown to have neutrophil chemotactic potential (19, 20). We have previously reported a correspondence between estrogen administration to ovariectomized rats and attenuation of both calpain activity and neutrophil infiltration 1 h after running exercise in female rats (41). OE rats did have attenuated muscle calpain activity compared with OP rats following I/R. However, with overall results using the rodent hindlimb I/R model, no correlation between calpain-like and MPO activities or CD43+ cells was observed. It appears that there are important differences between exercise-induced muscle damage and that caused by I/R as it relates to induction of calpain-like activity.

Lipid peroxidation was assessed using the TBARS assay. In the R rats, TBARS content in muscle did not

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**REFERENCES**

2. Calpain-like activity was lower in the OE and the OP rats compared with the R-I only rats ($P < 0.05$) and the R-I/R rats ($P < 0.05$). Following I/R, calpain-like activity was similar in the OE and OP group ($P = 0.57$). Decreased calpain-like activity in the OP rats compared with the R rats may be due to increased IGF-1 expression following ovariectomy. In a murine model of muscular dystrophy, IGF-1 administration (in conjunction with a high-protein diet) resulted in an attenuation in skeletal muscle calpain activity (44). The mechanism by which estrogen could attenuate calpain activity is unknown. Estrogen may act directly on muscle membranes to preserve ultrastructural stability and intracellular homeostasis (39, 41). We have previously reported that estrogen replacement in ovariectomized rats attenuates postexercise calpain activity in skeletal muscle (41).

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The number of CD43+ cells/mm² tissue in the R, OP, and OE rats was significantly elevated after I/R compared with that shown in the R-I only rats. Similar to our MPO results, the number of CD43+ cells was lower in the OE rats compared with the OP rats. Thus estrogen appears to attenuate leukocyte infiltration into skeletal muscle following I/R. However, other factors may confound the relationship between estrogen, ovariectomy, and inflammatory cell infiltration. Circulating estrogen values were similar in the OP rats and the R rats; however, after I/R, the number of CD43+ cells was almost twofold greater in the OP rats compared with the OE rats. Furthermore, the number of CD43+ cells was similar in the OE rats and the R rats, despite an ~10-fold difference in circulating estrogen content. Although circulating IGF-1 levels were elevated in the OP rats, the effect of this on post-I/R skeletal muscle leukocyte infiltration cannot be determined from this study.

Our MPO data (as an index of muscle neutrophil infiltration) did not always correlate exactly with our CD43+ immunohistochemically determined neutrophil counts in muscle tissue. Although MPO data have often been used as indirect markers of muscle neutrophil infiltration (3, 33, 40, 41), these data may not be as accurate as direct histochemical determination of neutrophil presence in muscle (32). We have also previously seen larger changes in postexercise muscle neutrophil infiltration via direct count using histochecmistry than inferred by changes to muscle MPO activity (41). It is also possible that differences in muscle fiber type may influence post-I/R neutrophil infiltration and account for some of the discrepancy in our MPO data [determined in PLT (mixed muscle)] and CD43+ data [determined in Sol (slow muscle)].

A compelling reason for measuring both calpain-like activity as well as neutrophil infiltration is the hypothesis put forward by Belcastro et al. (4), which suggests that calpain may stimulate neutrophil infiltration following skeletal muscle damage. A positive correlation between elevations in calpain-like and MPO activities in cardiac and skeletal muscle has been observed in rats after 1 h of treadmill running (24). Furthermore, cleaved peptide fragments of calpain have been shown to have neutrophil chemotactic potential (19, 20). We have previously reported a correspondence between estrogen administration to ovariectomized rats and attenuation of both calpain activity and neutrophil infiltration 1 h after running exercise in female rats (41). OE rats did have attenuated muscle calpain activity compared with OP rats following I/R. However, with overall results using the rodent hindlimb I/R model, no correlation between calpain-like and MPO activities or CD43+ cells was observed. It appears that there are important differences between exercise-induced muscle damage and that caused by I/R as it relates to induction of calpain-like activity.

Lipid peroxidation was assessed using the TBARS assay. In the R rats, TBARS content in muscle did not
change in response to ischemia or I/R. TBARS content was significantly greater in the OP group following I/R compared with any other group. The high number of infiltrating neutrophils (CD43+ cells) observed in the OP rats following I/R and their potential contribution to increased oxygen radical production (32) may contribute to this finding. After I/R, TBARS content was ~40% lower in the OE rats compared with the R rats (not significant, P = 0.12). Estrogen has been shown to have antioxidant properties (36) and, as also reported by others, may diminish lipid peroxidation following muscle damage (27). Lower TBARS content has been reported in female rowers compared with male rowers (8), and hydroxyl radical production was attenuated in canine hearts treated with conjugated equine estrogen (22).

After I/R, the OE rats had sustained the least amount of skeletal muscle disruption, as characterized by serum CK activity, muscle inflammatory cell infiltration, calpain-like activity, and lipid peroxidation, compared with the R and OP rats. However, contrary to our initial hypothesis, serum CK activity, skeletal muscle MPO activity, and calpain-like activity in the R rats were greater than or equal to results observed in the OP rats.

We conclude that estrogen supplementation in ovariectomized rats can attenuate neutrophil infiltration and indexes of muscle damage following I/R. We also suggest that ovariectomy and its associated physiological changes (possibly related to increased circulating IGF-1 levels) can also influence indexes of muscle damage following I/R in female rats.

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