17β-estradiol attenuates exercise-induced neutrophil infiltration in men

Lauren G. MacNeil,1 Steven K. Baker,2 Ivan Stevic,3 and Mark A. Tarnopolsky2

Departments of 1Kinesiology, 2Pediatrics and Medicine and 3Medical Sciences, McMaster University, Hamilton, Ontario, Canada

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MacNeil LG, Baker SK, Stevic I, Tarnopolsky MA. 17β-estradiol attenuates exercise-induced neutrophil infiltration in men. Am J Physiol Regul Integr Comp Physiol 300: R1443–R1451, 2011. First published March 2, 2011; doi:10.1152/ajpregu.00689.2009.—17β-estradiol (E2) attenuates exercise-induced muscle damage and inflammation in some models. Eighteen men completed 150 eccentric contractions after random assignment to placebo (Control group) or E2 supplementation (Experimental group). Muscle biopsies and blood samples were collected at baseline, following 8-day supplementation and 3 h and 48 h after exercise. Blood samples were analyzed for sex hormone concentration, creatine kinase (CK) activity and total antioxidant capacity. The mRNA content of genes involved in lipid and cholesterol homeostasis [forkhead box O1 (FOXO1), caveolin 1, and sterol regulatory element binding protein-2 (SREBP2)] and antioxidant defense (SOD1 and -2) were measured by RT-PCR. Immunohistochemistry was used to quantify muscle neutrophil (myeloperoxidase) and macrophage (CD68) content. Serum E2 concentration increased 2.5-fold with supplementation (P < 0.001), attenuating neutrophil infiltration at 3 h (P < 0.001) and 48 h (P < 0.001), and the induction of SOD1 at 48 h (P = 0.02). Macrophage density at 48 h (P < 0.05) and SOD2 mRNA at 3 h (P = 0.01) increased but were not affected by E2. Serum CK activity was higher at 48 h for both groups (P < 0.05). FOXO1, caveolin 1 and SREBP2 expression were 2.8-fold (P < 0.05), 1.4-fold (P < 0.05), and 1.5-fold (P < 0.001) and higher at 3 h after exercise with no effect of E2. This suggests that E2 attenuates neutrophil infiltration; however, the mechanism does not appear to be lesser oxidative stress or membrane damage and may indicate lesser neutrophil/endothelial interaction.

MATERIALS AND METHODS

Subjects and anthropometrics. Sample size estimates were calculated by using data from a similarly designed study (62). Using α = 0.05 and power = 0.8, calculations of CK activity and leukocyte common antigen positive cells indicated that eight participants per group was necessary. Participants were recruited, and 18 young men successfully completed the study. All subjects were prescreened with...
a questionnaire to ensure that they did not have any known illnesses or health problems, they were not taking medications or dietary supplements, and they had not regularly participated in resistance exercise in the preceding 6 mo. They were given an information sheet describing all of the testing procedures before providing written consent to participate. The study conformed to the standards outlined in the Declaration of Helsinki and was given approval by the Research Ethics Board of McMaster University. Body composition was measured using dual-energy X-ray absorptiometry scans (model QDR 1,000 W; Hologic, Waltham, MA). The thigh muscle cross-sectional area was calculated using anthropomorphic measurements of midheight circumference and skinfold thickness (36). The subject demographics were (means ± SD): age, 21 ± 2 yr; height, 181 ± 5 cm; and weight, 76.9 ± 12.8 kg.

Supplementation protocol. Subjects were assigned in a randomized, double-blind manner to either a Control group (n = 9) or Experimental group (n = 9). Control subjects consumed 400 mg glucose polymer (Pycose; Abbott Laboratories, Ross Division, St. Laurent, QB, Canada) for 10 days. Experimental subjects consumed ~300 mg glucose with 1 mg E2 (Estrase; Shire BioChem, St. Laurent, QB, Canada) for 2 days followed by 2 mg E2 for 8 days, similar to a protocol previously used by our group (20). Glucose and E2 tablets were concealed in gelatin capsules. On the morning of the 9th day, subjects reported to the laboratory and performed the exercise protocol. Supplementation continued until the day of the final biopsy and blood collection to maintain serum E2 concentrations throughout the collection protocol. Subjects in both groups were instructed to take one pill at the same time each day and return any unused pills. All subjects reported 100% compliance.

Exercise protocol and tissue collection. Muscle damage was induced with a previously developed eccentric exercise protocol (8). Approximately 2 wk before the exercise protocol, subjects were given a familiarization session with a Biodex isokinetic dynamometer (System 3; Biodex Medical Systems, Ronkonkoma, NY) in which they performed 3 to 5 submaximal contractions through the entire range of motion. On the testing day, following a short warm-up (10 min of light cycling), subjects were seated in the dynamometer with their right leg strapped to a lever arm. The lever arm was programmed to descend with a previously developed eccentric exercise protocol (8). Prior to each tissue collection, subjects abstained from any other forms of physical exertion (within 48 h), and avoided alcohol (within 48 h), caffeine (within 48 h), and any form of physical exertion (within 72 h), to ensure that the muscle damage would be the only variable to differentially affect the outcomes between biopsies (75).

Muscle biopsies were taken from the vastus lateralis of the control (left) leg before the familiarization session (baseline) and after 8 days of supplementation and the exercised (right) leg 3 h and 48 h after exercise, in anatomically distinct sites ~6 cm apart (45). The postexercise collection times were chosen because they represent two distinct phases of recovery from muscle damage (16). Blood was drawn from the antecubital vein at the same collection times. Muscle and blood samples were processed and stored for future measurements.

Blood enzyme and hormone concentrations. Serum CK activity was measured by the core laboratory at Hamilton Health Sciences Centre in batches containing all samples for each individual. Serum E2 (Fertigyn-E2-EASIA; Biosource, Nivelles, Belgium) and testosterone (Fertigyn-TESTO-EASIA, Biosource) concentrations were measured by enzyme amplified-sensitivity immunosorbent assays according to manufacturer’s specifications using baseline and post-supplementation blood collections. All hormone measurements were done in duplicate.

Total antioxidant capacity. Plasma samples collected at baseline and post-supplementation were analyzed for TAC using an assay based on the decolorizing of a solution of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cations (ABTS·−) by antioxidant solutions (76). After preparing stock solutions of ABTS (5.00 × 10−4 M) and sodium persulfate (6.89 × 10−3 M) (Sigma Aldrich, St. Louis, MO) in PBS (pH = 7.4), 1 ml of sodium persulfate solution was added to 99 ml of ABTS solution and allowed to equilibrate in the dark for 16 h. All measurements were taken in triplicate by adding 40 μl of each sample or standard to 160 μl ABTS−, incubating at 37°C for 30 min and measuring for absorbance at 734 nm using a Benchmark Plus 96-well microplate reader (Bio-Rad Laboratories, Hercules, CA). Serial dilutions of the vitamin E analog Trolox (Sigma Aldrich) were prepared in ethanol and used to generate a standard curve. Plasma samples were diluted 80-fold to ensure they were within the linear portion of the standard curve. All results are expressed as millimole Trolox equivalents.

Immunohistochemistry. Frozen muscle was serially cross sectioned to 5-μm thickness using a cryostat (Microm International, Walldorf, Germany), dried overnight, and stored until analysis. Negative control sections were included in all analyses. Slides were fixed in cold acetone for 15 min. Endogenous peroxidase activity was blocked using a liquid substrate kit (cat. no. 00-2014; Zymed Laboratories, San Francisco, CA). The slides were blocked with 1% goat serum (cat. no. D3002S; Dako Diagnostics, Mississauga, ON, Canada) for 15 min. The primary antibody was diluted in goat serum and positive slides were incubated for 30 min. The slides were then incubated with secondary goat anti-mouse antibody (cat. no. 95-6543-B; Zymed Laboratories) for 15 min and with peroxidase (cat. no. 95-6543-B; Zymed Laboratories) for an additional 15 min. A kit (cat. no. 00-2007; Zymed Laboratories) was used for color development. The primary antibodies used were monoclonal mouse anti-human myeloperoxidase (cat. no. M0748, Dako) at a 1:300 dilution for neutrophil detection and monoclonal mouse anti-human CD68 (cat. no. M0814, Dako) at a 1:100 dilution for macrophage detection.

Table 1. Primer and probe sequences for membrane homeostasis, antioxidant defense, and housekeeping genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Left Primer</th>
<th>Right Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD1</td>
<td>ctcaggaagacatcattgctca</td>
<td>cagcgtttccgttgggtctact</td>
<td>cttcatctccacctttgcccagattcat</td>
</tr>
<tr>
<td>SOD2</td>
<td>gacaaacatcctggcctacaa</td>
<td>cgcctcagcttccctaaac</td>
<td>agcccaaacattgcccccttctt</td>
</tr>
<tr>
<td>FOX1</td>
<td>aatccctcctggaggtgctaa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SREBP2</td>
<td>tggcaggaggaagccctctat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caveolin 1</td>
<td>ccgctgcattcgcctcgctct</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β2M</td>
<td>gctctccagcgtctcctaa</td>
<td></td>
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</table>

Sequences are 5’/3’.
Neutrophils and macrophages in the total cross-sectional area were counted and expressed as number of positive cells per square millimeter of muscle. This method has been previously published by our laboratory (63).

RNA extraction. The total RNA was extracted from the frozen skeletal muscle biopsy as described previously in detail by our group (45). Briefly, ~30 mg of skeletal muscle was homogenized on ice in 2 ml of Trizol reagent (cat. no. 15596; Life Technologies, Gaithersburg, MD). The homogenate was incubated for 10 min at room temperature, followed by phase separation using 200 μl of chloroform and precipitation of the total RNA from the aqueous phase using 500 μl of isopropyl alcohol. The RNA pellet was then washed three times in 75% ethanol and resuspended in 15 μl DEPC-treated water, aliquoted, and stored at −86°C. The concentration and purity of the RNA was determined using a UV spectrophotometer (Shimadzu UV-1201; Mandel Scientific, Guelph, ON, Canada) at the absorbance of 260/280 nm. Measurements were done in duplicate and had an average coefficient of variation of < 10%. The average purity (OD260/OD280) of the samples was 1.7 before DNase treatment. RNA integrity was assessed in a randomly chosen subset of samples using agarose gel electrophoresis, and the OD ratio of 28S to 18S rRNA was consistently > 1 for each sample.

DNase treatment. Prior to real-time quantitative RT-PCR analysis, the isolated RNA samples were treated with DNA-free recombinant DNase I (Ambion, Austin, TX) according to the manufacturer’s instructions to remove any potential genomic DNA contamination.

Real-time RT-PCR analysis. Changes in gene expression relative to baseline values were measured using real-time RT-PCR. Cu/Zn superoxide dismutase (SOD1) and Mn SOD (SOD2) were chosen for their role in oxidative stress management. Forkhead box O1 (FOXO1), caveolin 1 and sterol regulatory element binding protein 2 (SREBP 2) were selected for their involvement in cholesterol and lipid homeostasis and potential contribution to the mechanisms of membrane homeostasis and repair (46). The selected housekeeping gene was β2-microglobulin. It has been shown in previous work to remain constant following eccentric exercise (45), and this was again confirmed in the present study. The primer and probe sequences for these genes can be found in Table 1.

RT-PCR was completed using a TaqMan real-time method. The primers and a probe to each target gene were designed based on the cDNA sequence in GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene) with Primer 3 designer (http://frodo.wi.mit.edu/primer3). All target gene probes were labeled with FAM at their 5’ ends and BHQ-1 at their 3’ ends. Duplex RT-PCR was performed on an iCycler real-time PCR system (Bio-Rad Laboratories) in the One-step TaqMan RT-PCR Master Mix Reagents (Roche, Branchburg, New Jersey) according to the manufacturer’s instruction with target gene primers, target probe, housekeeping gene primers, and housekeeping gene probe in the same reaction (48). Determination of significant gene expression change was done as previously described (48). The genes of interest were normalized to the housekeeping gene, β2-microglobulin by following the standard method. Briefly, critical threshold (Ct) values of the housekeeping gene were subtracted from the Ct values of the gene of interest giving a ΔCt. This is equivalent to the log2 difference between endogenous control and the target gene (12). Values were then normalized to baseline, ΔΔCt. All samples were run in triplicate, fluorescence emission was detected using FAM and TAMRA filters, and Ct was automatically calculated.

Western blot analysis. Muscle biopsy samples were homogenized and prepared for PAGE using methods previously described (66). Briefly, frozen skeletal muscle tissue samples (~30 mg) were hand homogenized in 25 μl of phosphate buffer [50 mM KPi, 5 mM EDTA, 0.5 mM DTT, 1.15% KCl (wt/vol)] per milligram of tissue. A protease inhibitor cocktail (Sigma) was added to the phosphate buffer immediately prior to use at a ratio of 1:1,000. Samples were centrifuged at 600 g for 10 min at 4°C, and the supernatant was aliquoted for analyses. Protein concentrations of each sample were determined using the method described by Lowry et al. (43).

Samples were loaded on 10% SDS-polyacrylamide gels and transferred to a PVDF membrane. Membranes were blocked with 5% BSA (wt/vol) in Tris-buffered saline with 0.1% Tween (vol/vol) (TBST) and incubated overnight at 4°C in primary antibody: total ERK1/2 (cat. no. 9102, 1:1,000; Cell Signaling Technology, Danvers, MA); ERK1/2 Thr202/Tyr204 (cat. no. 9101, 1:1,000; Cell Signaling Technology); total AKT (cat. no. 9272, 1:1,000; Cell Signaling Technology); Akt Ser473 (cat. no. 9271, 1:1,000; Cell Signaling Technology). After blots were washed in TBST, membranes were incubated in horseradish peroxidase-linked anti-rabbit IgG secondary antibody (cat. no. NA934V, 1:6000; Amersham Biosciences, Piscataway, NJ), washed with TBST, and developed using ECL (cat. no. RP21206; Amersham Biosciences). Densitometry was performed on scanned images of X-ray film (Biomas XAR; Kodak).

Table 2. Subject and eccentric exercise trial characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Experimental Group</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>21.1 ± 2.4</td>
<td>20.9 ± 2.6</td>
<td>0.77</td>
</tr>
<tr>
<td>Height, cm</td>
<td>181.6 ± 5.6</td>
<td>180.9 ± 4.3</td>
<td>0.79</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>73.4 ± 11.5</td>
<td>80.4 ± 13.7</td>
<td>0.35</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>14.7 ± 5.2</td>
<td>20.1 ± 7.3</td>
<td>0.16</td>
</tr>
<tr>
<td>Quadriceps CSA, cm²</td>
<td>74.4 ± 10.0</td>
<td>78.5 ± 12.2</td>
<td>0.47</td>
</tr>
<tr>
<td>Work, kJ</td>
<td>24.9 ± 8.8</td>
<td>25.1 ± 4.0</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Values are means ± SD. CSA, cross-sectional area; Experimental group, 17β-estradiol supplementation.

Table 3. Serum hormone concentrations after 8 days of supplementation with either placebo (Control) or supplementation (Experimental) groups

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Experimental Group</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-Estradiol, pg/ml</td>
<td>38.4 ± 8.4</td>
<td>95.4 ± 35.2</td>
<td>***</td>
</tr>
<tr>
<td>Testosterone, nmol/l</td>
<td>14.6 ± 4.6</td>
<td>10.8 ± 4.7</td>
<td>***</td>
</tr>
</tbody>
</table>

Values are means ± SD. **P < 0.01 and ***P < 0.001 compared with control (n = 9/group).
Rochester, New York) using Image J version 1.40 g software (National Institutes of Health, Bethesda, Maryland).

Statistical analysis. Student’s unpaired t-tests were used to analyze subject characteristics, total work, and TAC. Two-way repeated-measures ANOVAs (supplementation group/time) were used to analyze CK activity, E2 concentration, testosterone concentration, neutrophil infiltration, macrophage infiltration, and phosphorylation status, and the linear 2^{-ΔΔCt} data sets of gene expression were measured with RT-PCR. When statistical significance was achieved, Tukey’s honestly significant difference post hoc test was used to determine the significance among the means. Statistica for Windows 5.0 (Statsoft, Tulsa OK) was used to perform t-tests and ANOVAs. The threshold for significance was set at $P < 0.05$. Data are presented as means ± SD, unless otherwise indicated.

RESULTS

Subject and work characteristics. Both groups were similar in age, weight, height, body fat percent, and thigh cross-sectional area (Table 2). All subjects completed the required 150 eccentric contractions. The total work completed was not different between groups ($P = 0.47$), suggesting that E2 supplementation did not affect muscle function.

E2 supplementation altered serum E2 and testosterone concentrations. Serum levels of E2 and testosterone following 8 days of supplementation are presented in Table 3. E2 concentrations were 2.5-fold higher ($P < 0.001$) and testosterone concentrations were 26% lower ($P = 0.01$) in the Experimental group than the Control group. Although the magnitude of change was smaller than previous research (20, 32, 74), the direction of change was similar. E2 and testosterone concentrations were not different between groups at baseline and remained unchanged in the Control group.

Baseline E2 concentrations are within the expected range of values for men and are similar to lower values found in women in the follicular phase (40–200 pg/ml); elevated values in the Experimental group are similar to the lower concentrations found in luteal phase women (100–150 pg/ml) (31).

E2 supplementation affected exercise-induced muscle inflammation. Neutrophil infiltration increased at 3 h (4.3-fold; $P < 0.05$), and 48 h (7.1-fold; $P < 0.001$), postexercise in the Control group (Fig. 1). The neutrophil infiltration values for the Experimental group did not change from baseline.

Macrophage infiltration increased 2.6-fold ($P < 0.05$) 48 h following exercise (Fig. 2). There was no difference between groups for macrophage infiltration.

Signaling proteins were not affected by E2 or eccentric exercise. Phosphorylation of the signaling proteins ERK1/2 (Thr202/Tyr204) and Akt (Ser473) were not significantly different from baseline at any time or between supplementation groups (Fig. 3).

TAC and SOD2 mRNA are not affected by E2. TAC was not affected by E2 supplementation (Table 4).

SOD1 mRNA content was greater in the Control group than the Experimental group 48 h after exercise ($P < 0.02$) (Fig. 2). SOD2 mRNA content was 2.3-fold higher 3 h after exercise, regardless of group ($P = 0.01$) (Fig. 4).
showed increases in the number of cells containing leukocyte common antigen; however, there was a very strong trend \( (P = 0.052) \) for less infiltration in women than men \( (0.74 \pm 0.59 \text{ cells/mm}^2 \text{ vs. } 2.70 \pm 2.18 \text{ cells/mm}^2) \) \( (62) \). Neutrophils are rapidly responding leukocytes that eliminate the cellular debris resulting from damage \( (28, 49, 68) \), whose adhesion and infiltration is primarily regulated by cytokines and cell adhesion molecules \( (18, 52) \). Based upon our hypothesis we believed that the difference in neutrophil density may have resulted from the membrane stabilizing and antioxidant properties of E2 in maintenance of calcium homeostasis and attenuation of the signal for inflammation \( (70) \). However, further investigation revealed that E2 did not influence CK efflux nor did it improve plasma TAC, suggesting that other mechanisms link E2 concentration and neutrophil response.

Neutrophil recruitment is influenced by endothelial nitric oxide synthase (eNOS) activity following damage \( (40, 58) \). Independent of gene transcription, E2 increases eNOS phosphorylation via two signaling proteins in a biphasic manner \( (58) \). The initial increase is mediated by MAPK/ERK, acutely activating eNOS within 5 min \( (15) \). Twenty minutes after exercise, a second increase in eNOS phosphorylation is mediated by protein kinase Akt \( (33, 58) \). As a result, mice implanted with E2 have a 3.2-fold increase in eNOS activity and attenuated leukocyte infiltration into muscle tissue following damage \( (58) \). To address this possibility, we measured ERK1/2 and Akt activation status and found that both were unchanged regardless of time or intervention in our study. It is important to note that the timing of the first biopsy following exercise \( (3 \text{ h}) \) occurred after the reported peak activities of these signaling proteins and phosphorylation states may have returned to baseline. Although not identified here, it is possible that E2 altered eNOS activity immediately after the cessation of exercise, thereby preventing neutrophil infiltration. The latter two possibilities need to be further explored in subsequent studies with earlier time points.

Skeletal muscle macrophage infiltration increased 48 h after exercise, regardless of E2 concentration. This is in agreement with previous results by our group and others that indicate an approximate 2–3-fold increase in macrophage density in the 24–48 h after a single bout of eccentric exercise \( (8–9, 47, 63) \). Of those studies, one compared men and women, and although men showed a greater increase in density, the trend was not

**DISCUSSION**

Research conducted on the potential influence for E2 to alter exercise-induced muscle damage and inflammation has produced inconsistent results, particularly in human studies. We examined the in vivo effects of E2 on markers of membrane damage, antioxidant capacity, inflammation, and gene expression following a single bout of eccentric exercise. In an attempt to minimize other influences of sex per se (i.e., XX, XY chromosomes, body fat differences, etc.), we recruited only men and altered their hormone concentration with supplementation in a repeated-measures design such that the only manipulated parameter was serum E2 concentration. Exercise-induced neutrophil infiltration and SOD1 induction were both attenuated by E2, while CK activity, plasma TAC, macrophage infiltration, and expression of the other genes examined were unaffected.

E2 eliminated the elevated skeletal muscle neutrophil abundance that occurred following a single bout of eccentric exercise, an observation supported by animal studies that report either partial (60%) \( (73) \) or complete attenuation (37, 70) of neutrophil accumulation with E2 in the 24 h after exercise. When a comparison was made between men and women following a single bout of eccentric exercise, both sexes

**Table 4. Plasma total antioxidant capacity (TAC) in Control and Experimental groups**

<table>
<thead>
<tr>
<th></th>
<th>Plasma TAC, mM Trolox equivalent</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>Control group</td>
<td>7.28 ± 1.07</td>
</tr>
<tr>
<td>Experimental group</td>
<td>6.48 ± 0.57</td>
</tr>
</tbody>
</table>

Values are means ± SD. Control \((n = 9)\) or Experimental \((n = 9)\) groups.

**Eccentric exercise-induced muscle damage.** Serum CK activity was elevated 4.6-fold \( (P = 0.04) \) 48 h after exercise (Table 5). The Experimental group values did not differ from the Control group values at any time.

FOXO1, caveolin 1, and sterol regulatory element binding protein-2 (SREBP2) were upregulated 3 h after exercise 2.8-fold \( (P = 0.002) \), 1.4-fold \( (P = 0.016) \), and 1.5-fold \( (P = 0.007) \), respectively (Fig. 5). The mRNA abundance changes induced by acute exercise were similar for both groups.

**Fig. 4. Expression fold change of oxidative stress genes in the Control group (open bars, \( n = 9 \)) and Experimental group (gray bars, \( n = 9 \)) from baseline after supplementation and exercise protocols. A: SOD1; B: SOD2. \(*P < 0.05 \text{ main effect of time compared with } 48H. \dagger P < 0.05 \text{ between treatments}. \) Values are means ± SD.
significant (63). Macrophages typically infiltrate damaged cells following neutrophils to regulate the immune response to injury and release growth factors important for regeneration and repair (68). Early responding neutrophils release cytokines important for the subsequent accumulation of macrophages, suggesting that an attenuated neutrophil response would result in attenuated macrophage recruitment. However, myogenic precursor cells initiate monocyte recruitment following damage when neutrophil infiltration is eliminated (14, 49), maintaining the growth response.

Differences in exercise-induced muscle damage between sexes may be due to the antioxidant properties of E2 that reduce oxidative stress and lipid peroxidation of the cell membrane (25, 38, 60). By donating a hydrogen atom from its phenolic hydroxyl group (11, 65), E2 can terminate peroxidation chain reactions, inhibit lipid oxidation in microsomes, liposomes, and macrophages and enhance plasma TAC (27, 65). However, our plasma TAC measurements were not affected by a 2.5-fold increase in circulating E2. Although this is not consistent with the proposed antioxidant properties of E2, plasma TAC measurements also remain unchanged in women across the menstrual cycle (10, 23). This may be because reports on the antioxidant effect of E2 typically use higher concentrations (up to 10–20 μM) (65) than our supplementation protocol (350.1 ± 129.2 pM) and the normal human physiological range (~150–750 pM) (31). As well, vitamin E and glutathione are primary reducing agents in plasma and muscle with concentrations several orders of magnitude higher than E2 (2, 30), which when changed may have influenced differences observed in TAC (27). Although plasma TAC can be used to measure systemic redox status, further work is needed to determine whether E2 directly affects either nonenzymatic or enzymatic antioxidant status in skeletal muscle.

E2 attenuated the late induction of SOD1 mRNA but did not affect SOD2 expression. The acute induction of SOD1 and SOD2 following exercise assists in the conversion of reactive oxygen species (ROS) to less active molecules (34–35). ROS associated with exercise are generated by two main sources: increased O2 consumption during exercise (29, 50) and release from activated neutrophils for apoptotic and chemotactic sig-

### Table 5. Serum creatine kinase activity following 150 eccentric contractions in Control and Experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Postsupplementation</th>
<th>3 h</th>
<th>48 h*</th>
</tr>
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<tbody>
<tr>
<td>Control group</td>
<td>113.6 ± 58.3</td>
<td>104.1 ± 52.4</td>
<td>255.7 ± 127.5</td>
<td>489.3 ± 193.2</td>
</tr>
<tr>
<td>Experimental group</td>
<td>207.1 ± 95.0</td>
<td>165.3 ± 84.3</td>
<td>268.5 ± 152.2</td>
<td>966.5 ± 1501.8</td>
</tr>
</tbody>
</table>

Values are mean ± SD. *P < 0.05 main effect for group compared with baseline. Control (n = 7), Experimental (n = 8) groups.

Fig. 5. Expression fold change of membrane homeostasis genes in the Control group (open bars, n = 9) and the Experimental group (gray bars, n = 9) from baseline after supplementation and exercise protocols. A: forkhead box O1 (FOXO1); B: caveolin 1; C: sterol regulatory element binding protein-2 (SREBP2). *P < 0.05 main effect of time compared with 48H. Values are means ± SD.
naling following muscle damage (68, 80). As E2 did not alter SOD1 or SOD2 expression at 3 h and did not affect plasma TAC, it likely did not act as an antioxidant buffering the ROS generated during exercise. Instead, the attenuation in SOD1 mRNA in the Experimental group at 48 h may have resulted from the E2-attenuated neutrophil infiltration that occurred at the same time, eliminating their potential contribution of mRNA to the total content and reducing the amount of ROS released for inflammatory signaling and transcriptional activation.

Membrane stabilizing properties are a second mechanism by which E2 may protect muscle from exercise-induced damage. E2 improves membrane fluidity in cell culture (69) and is associated with a lower efflux of CK from damaged muscle following exercise (3–5, 19, 57, 63); however, this is not a consistent result. Although CK activity increased 4.6-fold following exercise, indicating that the exercise successfully induced membrane damage, we did not find a difference with E2. Equal absolute (4-fold) (26) and relative (5-fold) (59) increases in CK activity between men and women following downhill running support the both magnitude of damage and lack of protection we observed. These inconsistencies may be due to differences in study design as early studies identifying a lower CK with E2 were conducted with rats after a 2-h flat running protocol (3–4, 7), while human-based research using eccentric exercise typically demonstrate no differences with E2 (26, 55, 59, 67). As well, improvements in membrane stability in liposomes only occurred with concentrations much higher (4–40 μM) than the in vivo concentrations mentioned earlier (77–78). The use of CK activity as an index of muscle damage is also negatively affected by the large intersubject variability commonly reported (16). This variability, evident within our Experimental group, and sample size may have prevented the detection of an effect on serum CK level. However, any effect on the cell membrane was not beneficial and may have reduced stability. E2 may affect cell membranes in animals or at high concentration, but our results indicate that changing E2 alone does not reduce CK release from muscle after high-intensity eccentric exercise.

The genes selected for their role in cholesterol and lipid homeostasis were induced early after exercise and although not significantly altered by E2, we may have been underpowered to address its effect on FOXO1 and SREBP2. The activation of caveolin 1 and SREBP2 at 3 h is in agreement with results previously reported by our laboratory in which they were both measured 3.2-fold higher with DNA microarray screening following a similar eccentric exercise protocol (46). All three genes are involved in the production of elements that may be important for membrane biosynthesis (46); FOXO1 and SREBP2 increase the production of lipids (1,22) and caveolin 1 is a lipid-binding protein in the plasma membrane (42,54). Although not a specific measure of membrane damage and repair, the synchronized upregulation of these mRNA species after exercise indicates an early pretranslational level of control following exercise to provide material to maintain lipid and cholesterol homeostasis, a modification not affected by E2.

Perspectives and Significance

This is the first study to investigate the effects of E2 on serum CK activity, plasma TAC, inflammation, and gene expression in men following eccentric exercise. Although it has been proposed that E2 protects muscle tissue from exercise-induced damage with antioxidant and membrane stabilizing properties, we did not observe differences that would support either of these effects. We did find an attenuation of neutrophil infiltration, which may have resulted from direct neutrophil/endothelial interaction though further investigation is needed to confirm or refute this possibility.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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