Estradiol replacement reverses ovariectomy-induced muscle contractile and myosin dysfunction in mature female mice

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HORMONE REPLACEMENT THERAPY has been a common treatment for women to alleviate menopausal symptoms and to maintain bone mineral density. While the effects of estrogen on reproductive and skeletal tissues have been widely studied, much less is known about the effects of estrogen on skeletal muscle. It is known that estrogen receptors are present in mouse, rat, and human skeletal muscle, suggesting that it is a tissue sensitive to estrogen (6, 9, 12, 13, 35). Several studies have shown that skeletal muscle of young, growing female rodents are sensitive to estrogen status (11, 18, 33, 34), but few studies have been conducted on mature female rodents. Recently, we showed that skeletal muscle contractility was diminished following the loss of ovarian hormones in both the soleus and extensor digitorum longus (EDL) muscles of adult female mice (19). This loss of muscle function was not due to a decline in contractile protein content. Instead, we found that the loss was due to alterations in myosin function. Specifically, the fraction of strong-binding myosin during contraction, i.e., myosin heads that were generating force, was 15% lower in muscle from ovariectomized mice compared with controls (19).

Because several hormones are altered following removal of the ovaries, it is not clear which ovarian hormone(s) was (were) responsible for the observed declines in myosin and contractile function. Previously, Warren and coworkers (33) showed that estradiol replacement in immature ovariectomized mice preserved contractile function of the EDL muscle, suggesting that estradiol may be an important ovarian hormone regulating contractility during growth. In addition, recent studies have shown that estradiol is a key ovarian hormone regulating skeletal muscle recovery following disuse atrophy (17, 26) and that muscle-specific genes are downregulated in female mice lacking estrogen receptor-β (9). Thus the primary purpose of the present study was to determine if estradiol replacement following the removal of ovarian hormones could reverse skeletal muscle deficits in adult female mice. We hypothesized that estradiol treatment would reverse myosin structural alterations and force-generating decrements in soleus and EDL muscles from ovariectomized mice.

A secondary purpose of the study was to further investigate ovarian hormone-related changes in muscle that occur in addition to the alterations in force-generating properties (19, 20). For example, changes in wet muscle mass that occur in response to ovariectomy and estradiol replacement were studied further by measuring dry muscle mass and fiber cross-sectional areas. Also, the premise that soleus muscles from ovariectomized, estradiol-treated mice would be less susceptible to acute injury than those from mice without estradiol treatment was addressed. This premise was based on previous studies showing that estradiol administration to male and ovariectomized female rodents can reduce the postexercise serum creatine kinase level, a commonly used marker of muscle damage (1, 3). However, muscle function can be considered a better indicator of muscle injury (32), and there is no evidence indicating that circulating estradiol had a beneficial effect on female mouse skeletal muscle.

MORPHOMETRIC ASSESSMENT showed that skeletal muscle contractility was diminished following the loss of ovarian hormones in both the soleus and extensor digitorum longus (EDL) muscles of adult female mice (19). This loss of muscle function was not due to a decline in contractile protein content. Instead, we found that the loss was due to alterations in myosin function. Specifically, the fraction of strong-binding myosin during contraction, i.e., myosin heads that were generating force, was 15% lower in muscle from ovariectomized mice compared with controls (19).

METHODS

Animals and study design. Female C57BL/6 mice aged 3 mo (n = 69) were purchased from the National Institute on Aging.
aging colony. Mice were housed four or five per cage on a 12:12-h light-dark cycle and were given phytoestrogen-free commercial rodent chow (2019 Teklad Global 19% Protein Rodent Diet, Harland Teklad, Madison, WI) and water ad libitum on arrival (week 0; Fig. 1). At week 2 mice were housed individually. Animal use and care procedures were approved by the institutional animal care and use committee and complied with guidelines set by the American Physiological Society.

All 69 mice underwent surgery during week 3 (Fig. 1). At this time point mice were nearly 4 mo old, an age at which regular estrous cycles occur (7). One group of mice underwent sham operations (Sham; n = 20), which consisted of the same procedures as the ovariectomy except that the ovaries were not removed. Ovarian tissue was surgically removed from 49 mice as previously described (19). A subgroup of these mice immediately received a placebo treatment (OVX-placebo; n = 8), and another subgroup immediately received an estradiol treatment (OVX + E2; n = 8). Treatments consisted of 60-day time release pellets (Innovative Research of America, Sarasota, FL) that were implanted subcutaneously via a ∼3-mm incision on the dorsal aspect of the neck. Hormone pellets contained 0.18 mg of 17β-estradiol while placebo pellets contained the same matrix as the estradiol pellets but no hormone. The remaining mice that were ovarioctomized did not receive any treatment at this time (OVX; n = 33). All mice were returned to individual cages postsurgery and remained there for ∼5 wk.

Approximately 30 days postsurgery (∼week 7.5; range 33–35 days), a subset of Sham and OVX mice were killed (Fig. 1; 30d-Sham and 30d-OVX; n = 10 each). These mice were studied to determine muscle function at the time of estradiol or placebo replacements that began in another subset of OVX mice. Mice in the former subset were weighed and anesthetized by an intraperitoneal injection of pentobarbital sodium (100 mg/kg body wt) with supplemental doses given as required. After the muscles were harvested, mice were euthanized by exsanguination while under anesthesia. Mice in the latter subset were implanted during week 8 with placebo (n = 13) or estradiol (n = 10) pellets. During week 12, i.e., ∼60 days postsurgery (range 57–65 days), all remaining mice were killed as described above. Those mice included one Sham group (60d-Sham), two ovarioctomized, placebo-treated groups (60d-OVX + 30d-Placebo and 60d-OVX + 60d-Placebo), and two ovarioctomized, estradiol-treated groups (60d-OVX + 30d-E2 and 60d-OVX + 60d-E2) (Fig. 1). These mice were ∼6 mo of age, an age at which normal estrous cycles continue to occur in ovary-intact female C57BL/6 mice (7).

One soleus muscle from each of the 69 mice was assessed in vitro for contractility and subsequently for total noncollagenous protein and contractile protein contents. Soleus muscles contralateral to those used for determining in vitro contractility were used for histology (fiber typing and fiber cross-sectional area measurements). Ideally all assays would have been performed on soleus muscles, but electron paramagnetic resonance (EPR) measurements on mouse soleus muscles are currently not possible. Because EPR measurements are possible on mouse EDL muscles and because we have found EDL and soleus muscles to be affected similarly by ovariectomy (19), we used EDL muscles for EPR analyses of myosin structural dynamics.

**Plasma estradiol analysis.** Plasma was separated from the blood that was collected during exsanguination, immediately frozen in liquid nitrogen, and then stored at −80°C. Estradiol EIA kit, DSL-10-4300 (Diagnostic Systems Labs, Webster, TX), was used to assay plasma estradiol from 53 of the 69 mice. Estradiol levels from the remaining 16 plasma samples (n = 8 OVX and n = 8 OVX + E2) were assayed using Estradiol ELISA, RES0241 (Immuno Biological Laboratories, Minneapolis, MN) because of a recall on the DSL kit making additional kits unavailable. Plasma estradiol values from the two kits were not different within the OVX + E2 group (P ≥ 0.599), but within the OVX group, Immuno Biological Laboratories values were greater than those obtained using the DSL kit [13.5 pg/ml (SD 3.6) vs. 6.8 pg/ml (SD 3.4); P = 0.010]. Regardless, circulating estradiol levels in these OVX mice were considerably less than that in Sham or OVX + E2 mice (see RESULTS and Table 1).

**Soleus muscle in vitro contractility.** A soleus muscle from one hindlimb of each mouse was studied for contractile function. The isolated soleus muscle preparation used for testing contractility has

**Table 1.** Plasma estradiol, body and muscles masses, and protein contents from sham-operated, ovarioctomized, and ovarioctomized + 17β-estradiol-replaced mice

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>OVX</th>
<th>OVX+E2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol, pg/ml</td>
<td>19.8 (4.77)</td>
<td>9.96 (3.99) †</td>
<td>39.9 (12.6) †</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>21.9 (1.51)</td>
<td>25.5 (2.27) †</td>
<td>22.7 (1.62) †</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Soleus muscle mass, mg</td>
<td>6.98 (0.47)</td>
<td>8.39 (0.91) †</td>
<td>7.11 (0.64) †</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EDL muscle mass, mg</td>
<td>8.82 (0.85)</td>
<td>9.59 (0.34) †</td>
<td>8.71 (0.90) †</td>
<td>0.002</td>
</tr>
<tr>
<td>Total noncollagenous protein, mg</td>
<td>1.02 (0.19)</td>
<td>1.12 (0.23)</td>
<td>1.04 (0.15)</td>
<td>0.225</td>
</tr>
<tr>
<td>Contractile protein, mg</td>
<td>0.63 (0.10)</td>
<td>0.66 (0.15)</td>
<td>0.62 (0.11)</td>
<td>0.430</td>
</tr>
</tbody>
</table>

Values are means (SD). EDL, extensor digitorum longus. P, P value from one-way ANOVA with Holms-Sidak post hoc tests. †Significantly different from sham-operated (Sham) mice; ‡significantly different from ovarioctomized + 17β-estradiol-replaced (OVX+E2) mice.
been described in detail previously (19,31). After equilibrating in Krebs buffer for 15 min at 25°C, passive stiffness of the inactive muscle was determined by stretching the muscle sinusoidally from 97.5% \(L_o\) to 102.5% optimal muscle length (\(L_o\)) at 0.5 Hz and measuring the force response (300B-LR; Aurora Scientific, Aurora, ON, Canada). Maximal isometric tetanic force (\(P_o\)) was determined by stimulating muscles for 400 ms at 150 V and 120 Hz (S48 stimulator with SIU5D; Grass Telefactor, Warwick, RI). A second maximal tetanic contraction was elicited with a sinusoidal length oscillation of 0.01% \(L_o\) at 500 Hz to determine active muscle stiffness. The stimulator and servomotor system were controlled by computer using a KPCI-3108 interface board (Keithley Instruments; Cleveland, OH) and TestPoint software (Capital Equipment; Billerica, MA).

An eccentric contraction injury protocol was performed on a subset of soleus muscles following the in vitro baseline contractility measures. Three minutes following the active stiffness measure, an injury protocol consisting of a series of 10 eccentric contractions began. For these contractions, muscles were passively shortened from \(L_o\) to 0.9 \(L_o\) over 3 s, stimulated tetanically for 133 ms as the muscle lengthened to 1.1 \(L_o\) at 1.5 \(L/s\), and then passively returned to \(L_o\). Each eccentric contraction was separated by 3 min of rest to prevent fatigue. Previously, we have shown that as little as 2 min of rest between contractions is sufficient to prevent fatigue as isotropic force does not recover during a 2-h incubation period postinjury as would be expected if muscle fatigue occurred in addition to injury (15). Four minutes following the final eccentric contraction, \(P_o\) was remeasured to assess the extent of injury induced by the injury protocol. Lactate dehydrogenase (LDH) released into the bath was also assayed as a marker of membrane damage. Immediately before the first eccentric contraction, 5 \(\mu\)l of bath was sampled to determine baseline LDH activity. Immediately following the final eccentric contraction, 5 \(\mu\)l of the bath was resampled for LDH activity. LDH assays were conducted as described by Warren and coworkers (30) except the assays were done at 25°C. Each soleus muscle was removed from the bath assembly, trimmed, blotted dry, weighed, and then immediately frozen in liquid nitrogen and stored at \(-80^\circ\)C for subsequent protein analyses.

Myosin structural dynamics of EDL muscle. EDL muscles were studied by EPR spectroscopy to determine myosin function during contraction. Muscles were glycercinated and prepared for spectroscopy by spin labeling with 0.5 mM 4-(2-iodoacetamido)-2,2,6,6-tetramethyl-1-piperidinyloxy spin label (IASL; Sigma) specifically at Cys 707 (SH1) of the myosin head (14, 19). IASL-labeled fiber bundles were fixed in a glass capillary tube inside a TE102 cavity (4102ST/707 (SH1) of the myosin head (14, 19)). IASL-labeled fiber bundles were collected under conditions of rigor, relaxation, and contraction for that muscle. Force was monitored using bicinchoninic acid protein assay with albumin standards (Pierce Biotechnology, Rockford IL). Quantitative polyacrylamide gel electrophoresis was performed on those homogenates to determine contractile protein contents (10, 19). Contractile protein content is defined as the sum of myosin heavy chain (MHC) and actin contents.

Determination of fiber type and cross-sectional area. Serial cross sections (10 \(\mu\)m thick) from a subset of soleus muscles were cut on a microtome cryostat. Fiber types were determined based on MHC immunohistochemistry using MHC I antibody VP-M667 (Vector Laboratories, Burlingame, CA) and antibodies to MHC IIa and IIb generated from hybridomas (ATCC, SC-71 and BF-F3, respectively) (20, 25). Approximately 120 fibers from each soleus muscle were typed (range 100–130 fibers per muscle). Fiber cross-sectional areas were determined by measuring the circumference of those same fibers using ImageJ software (National Institutes of Health, Bethesda, MD). A mean fiber cross-sectional area for each fiber type and a mean fiber type percentage were calculated for each soleus muscle analyzed. The muscle means were then used for statistical analyses to determine if differences occurred among Sham, OVX, and OVX + E2 mice.

Statistical analyses. To determine if outcomes varied depending on treatment time (i.e., 30 or 60 days), contractile and protein content data from similar groups were analyzed and pooled when not different. Specifically, Student’s independent \(t\)-tests were used to determine if differences occurred between the 30d-Sham and 60d-Sham mice. No differences were found in the 12 measures examined (\(P > 0.069\)) so those two groups were pooled and are collectively referred to as Sham. One-way ANOVAs were used to determine if differences occurred among the 30d-OVX, 60d-OVX + 30d-Placebo, and 60d-OVX + 60d-Placebo mice. No differences were found (\(P > 0.125\)) so those three groups were pooled and are collectively referred to as OVX. In other words, the effects of ovariectomy were the same at 30 and 60 days post-surgery and were not affected by placebo treatment. Student’s independent \(t\)-tests were also used to determine if differences occurred between the 60d-OVX + 30d-E2 and 60d-OVX + 60d-E2 mice. No differences were found in the 12 measures examined between those groups having estradiol treatment for 30 and 60 days (\(P > 0.085\)) so those two groups were pooled and are referred to as OVX + E2. Groups that were pooled are identified by the symbols (i.e., *, †, ‡, #) in Fig. 1.

Pooled data were then analyzed by one-way ANOVAs to determine if differences occurred among Sham, OVX, and OVX + E2 mice. Holm-Sidak post hoc tests were performed if a difference was detected among the means by the omnibus \(F\)-test. When assumptions of normality or equal variance were violated, data were analyzed by Kruskal-Wallis one-way ANOVA on ranks with Dunn’s pairwise multiple comparison post hoc tests. Pearson correlations were used to determine if relationships occurred between muscle contractile parameters and circulating estradiol levels. Statistical analyses were done using SigmaStat version 3.1 (Systat Software; Point Richmond, CA) with an \(\alpha\)-level of 0.05. Values are reported as means (SD).

RESULTS

Plasma estradiol. Circulating estradiol levels were different among the three groups of mice, with each group being different from one another (Table 1). Mean plasma estradiol in OVX mice was 50% lower than the mean circulating level in Sham mice. OVX + E2 mice had estradiol levels ranging from 21.5 to 65.8 pg/ml with the mean level approximating the peak level attained during the estrous cycle in young, healthy female mice (21).

Body and muscle masses. Estradiol replacement eliminated the ovariectomy-induced increases in body and muscle masses (Table 1). Mean body mass for OVX mice was ~16% greater than that for Sham and OVX + E2 mice. Soleus muscle mass of OVX mice was, on average 19% greater than that of Sham and OVX + E2 mice. Mean EDL muscle mass of OVX mice was ~10% greater than that of Sham and OVX + E2 mice.
Protein contents. Total noncollagenous protein content of soleus muscles was measured to determine if the muscle mass increase in response to ovariecotomy and subsequent decrease with estradiol replacement were due to changes in noncollagenous protein content or to changes collagen and/or nonprotein contents. Total noncollagenous protein content of soleus muscles was not different among OVX, Sham, and OVX + E2 mice (Table 1), suggesting that muscle mass increases in the OVX mice were due to changes in collagen and/or nonprotein contents. Similarly, contractile protein content of soleus muscle was not different among OVX, Sham, and OVX + E2 mice (Table 1). MHC and actin contents were not different (P ≥ 0.404) among groups with the mean MHC and actin contents for all soleus muscles being 0.40 mg (SD 0.09) and 0.24 mg (SD 0.05), respectively. The mean ratio of MHC to actin in soleus muscle was 1.66 (SD 0.28). These data show that estradiol does not alter the quantity of the major contractile proteins in soleus muscle of mice.

To test the possibility that the increases in muscle masses following ovariecotomy were due to increases in fluid and not protein, dry muscle masses were measured from a subset of OVX and OVX + E2 mice (n = 8 each). Soleus muscle dry mass was not different between OVX and OVX + E2 mice [1.63 mg (SD 0.13) vs. 1.58 mg (SD 0.10); P = 0.355]. Similarly, EDL muscle dry masses were not different between OVX and OVX + E2 mice [2.30 mg (SD 0.30) vs. 2.10 mg (SD 0.33); P = 0.259]. Thus it appears that increased water content accounted for the OVX-induced increases in wet muscle masses.

Soleus muscle fiber cross-sectional areas and types. Muscle fiber cross-sectional areas were not affected by estradiol status irrespective of fiber type (P ≥ 0.422; Table 2). These data indicate that the changes in nonprotein mass found in the whole muscles occurred extracellularly. Previous studies on rat muscles have shown that the relative proportions of the four fiber types shift following the removal of ovarian hormones and subsequent replacement of estradiol (11, 23). Therefore MHC immunohistochemistry was performed to determine if fiber type was estrogen sensitive in mice. The percentage of fibers in soleus muscle that were identified as type I, IIA, IIX, and IIB did not change with ovarian hormone status (P ≥ 0.177; Table 2).

Soleus muscle contractility. Soleus muscles were studied in vitro to determine muscle contractile function and how that function was altered by the loss of ovarian hormones and subsequent replacement of estradiol. Overall, muscles from ovariecrotized mice displayed lower contractile capacity than control mice, i.e., Sham (Table 3). Furthermore, estradiol replacement reversed the declines in soleus muscle contractile function following ovariecotomy (Table 3). Both absolute P0 and P0 normalized to contractile protein content of soleus muscles from OVX mice were lower than those from Sham and OVX + E2 mice (Table 3 and Fig. 2). P0 was normalized by contractile protein content per muscle fiber length instead of by physiological cross-sectional area of muscle because the ovariecotomy-induced increase in nonprotein mass affects cross-sectional area (19). Thus specific P0 is an inaccurate and not reported. P0 expressed as a function of contractile protein content was affected by estradiol status (P = 0.003) being 9–16% less in soleus muscles from OVX mice compared with those from Sham and OVX + E2 mice (Table 3). Correlational analyses were performed to determine if the force generated by soleus muscles was related to circulating estradiol levels. A positive correlation was found (Fig. 3), suggesting that the force-generating capacity of mouse soleus muscle is related to the amount of the ovarian hormone, estradiol, in circulation.

Active stiffness was determined for soleus muscles as an indicator of myosin strongly bound to actin during contraction. Estradiol replacement eliminated the decline in active stiffness observed with ovariecotomy (Table 3) and was correlated with circulating estradiol (r = 0.329; P = 0.044). Passive stiffness, which reflects an inactive muscle’s resistance to lengthening, was also determined for all soleus muscles studied in vitro for contractility. Passive stiffness of soleus muscles from OVX mice was greater than that from Sham mice, and estradiol replacement tended to reverse the increase (Table 3).

EDL muscle myosin structural dynamics. To assess the possibility that actin–myosin interactions were affected by estradiol levels, the structural distribution of myosin during contraction in bundles of fibers from a subset of EDL muscles was measured (n = 5 each). EPR spectroscopy showed that the fraction of strong-binding myosin during contraction was affected by estradiol status (P = 0.045; Fig. 2). The relatively low fraction observed in OVX mice [0.263 (SD 0.034)] was reversed by estradiol replacement [OVX + E2 mice = 0.311 (SD 0.022)] to a fraction equivalent to that in Sham mice [0.300 (SD 0.024)]. These data reinforce the results of active stiffness on soleus muscle indicating that the dysfunction in

Table 2. Soleus muscle fiber cross-sectional areas and fiber types of sham-operated, ovariecrotized, and ovariecrotized+17β-estradiol-replaced mice

<table>
<thead>
<tr>
<th>Fiber CSA, μm²</th>
<th>Sham</th>
<th>OVX</th>
<th>OVX+E2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber Type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>1,714 (605)</td>
<td>1,772 (526)</td>
<td>1,704 (536)</td>
<td>0.983</td>
</tr>
<tr>
<td>Type IIA</td>
<td>1,664 (474)</td>
<td>1,695 (549)</td>
<td>1,544 (266)</td>
<td>0.882</td>
</tr>
<tr>
<td>Type IIX</td>
<td>1,846 (543)</td>
<td>1,403 (410)</td>
<td>1,762 (485)</td>
<td>0.422</td>
</tr>
<tr>
<td>Type IIB</td>
<td>2,003 (264)</td>
<td>2,056 (528)</td>
<td>2,061 (451)</td>
<td>0.978</td>
</tr>
<tr>
<td>Fiber types based on MHC immunohistochemistry, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>54 (9.7)</td>
<td>47 (9.5)</td>
<td>53 (7.8)</td>
<td>0.278</td>
</tr>
<tr>
<td>Type IIA</td>
<td>43 (5.3)</td>
<td>48 (3.3)</td>
<td>41 (9.0)</td>
<td>0.177</td>
</tr>
<tr>
<td>Type IIX</td>
<td>14 (13.8)</td>
<td>16 (13.5)</td>
<td>12 (7.8)</td>
<td>0.594</td>
</tr>
<tr>
<td>Type IIB</td>
<td>2 (1.6)</td>
<td>2 (1.8)</td>
<td>2 (1.4)</td>
<td>0.849</td>
</tr>
</tbody>
</table>

Values are means (SD). For fiber cross-sectional area (CSA), n = 4 per group. For fiber types, values are the percent expression from the total no. of fibers analyzed, and n = 6 per group. For each column, the percentages are greater than 100 because ~10% of the type I fibers in each of the 3 groups also stained positive for IIa myosin heavy chain (MHC). P, P value from one-way ANOVA.
actin-myosin interaction due to ovariectomy was reversed by estradiol replacement.

Susceptibility of soleus muscle to injury. Previous research on rodent skeletal muscle has indicated that the loss of estradiol increases a muscle’s susceptibility to injury (e.g., 29). To further address this issue, an eccentric contraction injury protocol was performed on a subset of isolated soleus muscles. Peak eccentric force generated by soleus muscle was unaffected by estradiol status (Table 3). Three variables were measured to assess acute muscle injury. First, the change in peak eccentric force from the first to the final eccentric contraction was measured to assess muscle injury. Second, peak eccentric force from the first to the final eccentric contraction was not affected by estradiol status (Table 3).}

### Table 3. In vitro contractile properties of soleus muscles from sham-operated, ovariectomized, and ovariectomized + 17β-estradiol replaced mice

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>OVX</th>
<th>OVX+E2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_o$, mN</td>
<td>180 (14.4)</td>
<td>168 (16.7)*</td>
<td>187 (17.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Normalized $P_o$, N·cm/kg</td>
<td>0.189 (0.032)</td>
<td>0.173 (0.028)**</td>
<td>0.206 (0.031)</td>
<td>0.003</td>
</tr>
<tr>
<td>Active stiffness, N/m</td>
<td>315 (16.8)</td>
<td>286 (24.6)*</td>
<td>305 (29.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Passive stiffness, N/m</td>
<td>9.87 (0.950)</td>
<td>10.7 (1.07)*</td>
<td>10.2 (0.690)</td>
<td>0.014</td>
</tr>
<tr>
<td>Eccentric force, mN</td>
<td>338 (43.8)</td>
<td>325 (45.3)</td>
<td>343 (46.5)</td>
<td>0.454</td>
</tr>
<tr>
<td>$P_o$, %</td>
<td>19.2 (5.41)</td>
<td>23.1 (6.33)</td>
<td>19.3 (6.59)</td>
<td>0.117</td>
</tr>
<tr>
<td>Passive stiffness, %</td>
<td>0.189 (0.032)</td>
<td>0.173 (0.028)**</td>
<td>0.206 (0.031)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Values are means (SD). $P_o$ maximal isometric tetanic force; normalized $P_o$, maximal isometric tetanic force normalized to contractile protein content per muscle fiber length; $\Delta P_o$, percent difference between the first and last eccentric contraction; $\Delta P_o$, percent difference between the pre- and postinjury $P_o$. $P$ value from one-way ANOVA with Holms-Sidak post hoc tests.

**Significantly different from Sham mice; †significantly different from OVX+E2 mice.

measured to assess membrane damage and was found to increase by ~42% following the injury protocol but again with no difference among the three groups of mice ($P = 0.410$). LDH activity was 8.06 U·g$^{-1}$·h$^{-1}$ (SD 1.59) for Sham, 7.84 U·g$^{-1}$·h$^{-1}$ (SD 1.49) for OVX, and 8.10 U·g$^{-1}$·h$^{-1}$ (SD 1.06) for OVX + E2 mice. These three indexes of muscle injury imply that estradiol status did not affect susceptibility of soleus muscle to an acute injury.

**DISCUSSION**

The removal of ovarian hormones from female mice via ovariectomy is detrimental to skeletal muscle contractile function (19, 33). In the present study, we have demonstrated that a specific ovarian hormone, estradiol, can reverse ovariectomy-induced contractile dysfunction, indicating that it is the key ovarian hormone regulating skeletal muscle function in mature female mice. One mechanism by which estradiol restored contractility was by increasing strong-binding myosin, as indicated by both EPR spectroscopy and active stiffness measures. Circulating estradiol levels were positively correlated with muscle force generation and myosin function, further supporting the contention that estradiol affects skeletal muscle. Previously, we showed that force generation was ~20% lower in soleus and EDL muscles from adult mice that were ovariectomized for 60 days compared with ovari-intact mice (19). Similar soleus muscle decrements were found in the present study for mice that were ovariectomized for 30 or 60 days (30d-OVX, 60d-OVX + 30d-Placebo, and 60d-OVX + 60d-Placebo). However, following ovariectomy, several hormones are altered so we could not be certain which ovarian hormone(s) was (were) responsible for the observed muscle dysfunction. In the present study, estradiol was replaced to systematically determine if it was the key ovarian hormone in terms of skeletal muscle function. We found that ~30 days of estradiol replacement starting ~30 days after ovariectomy reversed the decrement in $P_o$ (60d-OVX + 30d-E2). Similarly, immediate replacement of estradiol in ovariectomized mice prevented the loss in $P_o$ from occurring (60d-OVX + 60d-E2). These results indicate that estradiol is important for optimal skeletal muscle contractility, agreeing with some (8, 33, 34) but not all previous studies (5, 18). For example, Warren and coworkers (33) showed that $P_o$ of EDL

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**Fig. 2.** Effect of estradiol on force-generating capacity and myosin function. The left and middle sets of bars represent muscle and myosin functional results from whole intact soleus muscles, and the right set of bars myosin function of permeabilized extensor digitorum longus (EDL) muscle fibers via electron paramagnetic resonance spectroscopy. Maximal isometric tetanic force ($P_o$) was normalized to contractile protein content per fiber length. Bars represent means (SD) expressed as a percentage of Sham mice. *Significantly different from Sham mice; †significantly different from OVX + E2 mice.

**Fig. 3.** A positive relationship between plasma estradiol levels in mice and their soleus muscle’s maximal isometric tetanic force.
muscles from young, immature ovariectomized mice replaced with estradiol was 14% greater compared with estrogen-deficient mice of the same age, while McCormick and coworkers (18) reported that soleus muscle $P_o$ was not affected by ovariectomy or estrogen replacement in young, growing rats. It is noteworthy that these and most other rodent studies of estrogen effects on skeletal muscle have been conducted on young, growing rodents.

Our previous work (19) and single-fiber experiments conducted by Wattanapermpool and Resier (34) show that Ca$^{2+}$-activated force by soleus and EDL muscle fibers was reduced by $\sim$20% following ovariectomy in rodents. In addition, the fraction of strong-binding myosin during contraction (i.e., myosin that is generating force during contraction) was reduced in fibers from ovariectomized mice (19). This reduction occurred to the same extent as the reduction in force, indicating that myosin dysfunction could explain the ovariectomy-induced decrements in force generation. We have extended these observations in the present work by showing that the ovariectomy-induced reduction in strong-binding myosin can be reversed by estradiol replacement. This was measured directly in EDL muscle fibers by EPR spectroscopy, in conjunction with site-directed spin labeling of myosin, and indirectly by active stiffness measurements of intact soleus muscles. Previously, we found good agreement between EPR and active stiffness results, showing that active stiffness of whole muscle is a good indicator of strong-binding myosin (19). Collectively, these data implicate the major contractile protein, myosin, as the molecule that is somehow affected by estradiol status and accounts for the ovariectomized-induced loss of force-generating capacity.

In general, the function of estradiol in skeletal muscle has been studied minimally. Recently, it has been shown that estradiol plays a role in skeletal muscle recovery following hindlimb suspension in female rats, further implicating estradiol as a key ovarian hormone in skeletal muscle (17, 26). The failure of atrophied skeletal muscle to regrow in ovariectomized rats was associated with reduced activation of the Akt-p70<sup>60K</sup> signaling pathway, suggesting that proteins critical for initiating translation were negatively affected by removing ovarian hormones (17, 26). As in our studies showing that estradiol status affects myosin function, the link between estradiol and the downstream outcome (i.e., reduced muscle force or recovery from atrophy) is not clear. This “link” could occur through genomic or nongenomic mechanisms of estradiol.

The genomic estrogenic effects in skeletal muscle occur primarily through the nuclear estrogen receptors-α and -β (4, 35). Estrogen receptor-β is more responsible for the nonclassical, i.e., nonsexual, effects of estrogens in the central nervous and immune systems and likely skeletal muscle as well. Despite this, estrogen receptor-β-null mice have near normal muscle contractile properties (9). A possible nongenomic effect of estrogen on skeletal muscle is protection from oxidative damage since estradiol has antioxidant properties (22). With aging, oxidation of myosin perturbs its functional interaction with actin, resulting in reduced muscle force (14, 16, 24). Whether oxidation of myosin occurs as a result of removing the antioxidant, estradiol, has not been tested.

Estrogen status affects muscle size, and in turn muscle size can affect force generation. However, the loss of ovarian hormones can increase rodent hindlimb muscle masses, by $\sim$20% (this study and 8, 17, 19, 26), yet decrease the force generated by those muscles. Furthermore, estradiol replacement reverses these effects (this study and 17). The ovariectomy-induced increases in muscle mass were not due to increased muscle protein as protein contents and dry muscle masses were the same in muscles from ovariectomized and ovary-intact rodents (this study and 17, 19, 26). Similarly, contractile protein content, which should be directly related to force generation, is not affected by estrogen status. Instead, ovariectomy-induced increases in muscle mass appear to be due to accumulation of fluid (this study and 17). Muscle fiber cross-sectional areas were measured in the present study to determine if the increased fluid occurred intra- or extracellularly. Cross-sectional area of all types of fibers was unaffected by estradiol status, indicating that extracellular fluid accumulation caused the ovariectomy-induced increases in wet muscle mass.

Estradiol has been shown to reduce markers of muscle injury in rodents, but the marker most commonly used has been the serum level of intramuscular proteins, such as creatine kinase, which is indicative of muscle membrane damage (1–3, 27). Whether estradiol is protective for muscle contractile function is much less clear. To test the premise that estradiol reduces susceptibility to an acute muscle injury beyond membrane damage, an eccentric injury protocol was performed, and contractile function was reassessed postinjury. Estradiol replacement had no effect on the change in eccentric force from the first to the last eccentric contraction, the change in $P_o$, from pre- to post eccentric contractions, or the amount of LDH released into the bath. Previous studies have shown that estrogen protects skeletal muscle from membrane damage as indicated by higher release of intramuscular enzymes in estrogen-deficient states (e.g., following ovariectomy or in males relative to females) (1–3, 27), and Tiidus proposed that these effects are brought about by estrogen’s antioxidant capacity and its ability to stabilize membranes (28), although direct evidence for this is sparse (22). It is unclear why we did not find a protective effect of estradiol treatment in terms of LDH release, but in terms of muscle function it is not a unique observation (33). Two possible explanations for the discrepancy have been pointed out previously (33). First, intramuscular enzyme release was the only marker used to assess muscle injury in those studies showing a protective effect of estradiol (1–3, 27), whereas the extent of muscle contractile impairment is considered to be a better marker of muscle injury (32). Second, those studies showing a protective effect were conducted on rats, whereas our studies have been conducted on mice (this study and 33). Another point to take into consideration is that our injury-susceptibility measures were acute, i.e., made within a 30-min time period. Thus effects that estrogen may have on the immune system and its role in remediating muscle injury days to weeks postinjury were not assessed in this study.

In conclusion, estradiol replacement successfully reversed ovariectomy-induced declines in mouse hindlimb muscle contractility and myosin function. Restoration of $P_o$ following the removal estradiol was not due to changes in contractile protein content but rather to an increase in the fraction of strong-binding myosin during contraction. The mechanism by which estradiol affects myosin function is not known, but it could...
occur through genomic or nongenomic effects. Ovariectomy also resulted in increased muscle mass due to an accumulation of extracellular fluid, and estradiol replacement reversed the occurrence of this. Overall, estradiol proved beneficial to skeletal muscle of mature female mice.

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