Estrogen status and skeletal muscle recovery from disuse atrophy

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Estrogen status and skeletal muscle recovery from disuse atrophy. J Appl Physiol 100: 2012–2023, 2006. First published February 23, 2006; doi:10.1152/japplphysiol.01583.2005.—Although estrogen loss can alter skeletal muscle recovery from disuse, the specific components of muscle regrowth that are estrogen sensitive have not been described. The primary purpose of this study was to determine the components of skeletal muscle mass recovery that are biological targets of estrogen. Intact, ovariectomized (OVX), and ovariectomized with 17β-estradiol replacement (OVX+E2) female rats were subjected to hindlimb suspension for 10 days and then returned to normal cage ambulation for the duration of recovery. Soleus muscle mass returned to control levels by day 7 of recovery in the intact animals, whereas OVX soleus mass did not recover until day 14. Intact rats recovered soleus mean myofiber cross-sectional area (CSA) by day 14 of recovery, whereas the OVX soleus remained decreased (42%) at day 14. OVX mean fiber CSA did return to control levels by day 28 of recovery. The OVX+E2 treatment group recovered mean CSA at day 14, as in the intact animals. Myofibers demonstrating central nuclei were increased at day 14 in the OVX group, but not in intact or OVX+E2 animals. The percent noncontractile tissue was also increased 29% in OVX muscle at day 14, but not in either intact or OVX+E2 groups. In addition, collagen Ia mRNA was increased 45% in OVX muscle at day 14 of recovery. These results suggest that myofiber growth, myofiber regeneration, and extracellular matrix remodeling are estrogen-sensitive components of soleus muscle mass recovery from disuse atrophy.

steroid hormone; hypertrophy; muscle regeneration; fibrosis; extracellular matrix

ALTHOUGH THE CAPACITY FOR estrogen synthesis varies throughout a female’s lifetime, the consequences these hormonal changes have on skeletal muscle’s ability to regulate plasticity and mass are just beginning to be understood. Skeletal muscle myoblasts, myotubes, and mature fibers express functional estrogen receptors, suggesting that skeletal muscle is sensitive to estrogen steroid hormone signaling (33, 55). Estrogens can regulate skeletal muscle mass in developing livestock (60) and rats (37, 58). The depletion of ovarian hormones due to ovariectomy induces an anabolic environment characterized by increased circulating growth factor levels and a positive energy balance (23, 63). This environment manifests itself in pronounced increases in female rat organ weight, skeletal muscle mass, and body mass (23). Estradiol administration attenuates the ovariectomy-induced body mass and muscle protein accretion (54). Estrogen replacement in ovariectomized rats subjected to hindlimb disuse attenuates bone atrophy, but soleus muscle atrophy is not prevented (23, 34). After extended periods of hindlimb disuse, ovariectomy decreases rat skeletal muscle mass recovery, and estrogen replacement benefits attenuated muscle mass recovery (13, 57). These facts suggest an interaction between load-sensing mechanisms and estrogen signaling pathways that benefits skeletal muscle growth. However, estrogen-sensitive components of muscle mass recovery have not been clearly identified.

Skeletal muscle gene regulation is modulated by the load placed on the muscle (6). Several days of disuse can initiate biochemical and morphological changes related to muscle atrophy, protein isoform shifts, and metabolic enzyme alterations (21, 61). Normal weight-bearing activity is a therapeutic treatment for muscle mass restoration after extended periods of disuse (42). During the initial return to normal ambulation processes related to protein accretion, myofiber damage and subsequent regeneration are induced (31, 49). Characteristics of regenerating muscle during recovery from atrophy include centralized nuclei, small sized myofibers, and embryonic myosin heavy chain protein expression (17, 31). Skeletal muscle satellite cells, which become quiescent during hindlimb suspension (19), are activated during recovery from disuse (49) and provide a critical myonuclei source for muscle mass recovery (8). Recovery of muscle mass is an extremely rapid process in the healthy adult and is normally complete after 14 days, even when disuse periods are extended significantly beyond 10 days (10, 11, 49). However, processes related to complete regeneration can extend out to 5 wk (31).

Extracellular matrix remodeling is a critical process related to both skeletal muscle growth and regeneration from injury (36). Extracellular matrix components provide critical structural support, scaffolding for cellular mobility, elasticity, and initiate intracellular signaling cascades in skeletal muscle (36). During periods of extended disuse, the muscle’s relative proportion of extracellular matrix volume remains constant (48), whereas the extracellular matrix composition is altered. Atrophying soleus muscle undergoes a collagen isoform shift from type I to type III (48). Reloading after disuse induces alterations in the expression of the extracellular matrix proteins tenascin-C and fibronectin, which serve structural and elastic functions in skeletal muscle (25). Fibrosis, overexpansion of the extracellular matrix, can attenuate skeletal muscle mass recovery and myofiber regeneration after injury-induced damage (56). Fibroblasts contribute to the proportion of mitotically active cells during periods of increased loading and are primarily responsible for extracellular matrix protein synthesis (18, 25). Fibrosis and extracellular matrix remodeling can be regulated by hormone, growth factor, and inflammatory cyto-

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kine signaling (38). Additionally, the transforming growth factor-β (TGF-β) signaling pathway appears to be critical for regulation of extracellular matrix remodeling (38). Although estrogen is a modulator of TGF-β signaling (43), estrogen’s effect on fibrosis during skeletal muscle regeneration has not been established.

Estrogen has the capability to regulate several processes associated with skeletal muscle mass accretion and regeneration, which point to its potential for modulating muscle recovery from disuse atrophy (17, 39). Components of overall muscle mass recovery that are potentially estrogen sensitive include extracellular matrix remodeling, myofiber regeneration and growth, inflammation, and sarcolemma damage (39). Although estrogen loss can alter skeletal muscle recovery from disuse (13, 57), the components of wet mass recovery that are estrogen sensitive are not known. The primary purpose of this study was to determine the specific components of soleus muscle mass recovery that are biological targets of estrogen. An additional purpose was to determine whether estrogen-sensitive alterations occur specifically during the atrophy or recovery process. We hypothesized that estrogen loss would attenuate myofiber growth, extracellular matrix remodeling, and indexes of muscle regeneration during muscle recovery from disuse. Understanding the targets of estrogen in recovering skeletal muscle may lead to enhanced therapeutic treatments for muscle mass restoration after disuse in many clinical settings.

METHODS

Animals and housing. Intact and ovariectomized (OVX) female Sprague-Dawley rats (~200 g) were acquired from Harlan rodent colony (Indianapolis, IN) and randomly divided into four separate day-of-recovery treatment groups (n = 6/group; Fig. 1). These groups included 1) ground control (Con), 2) hindlimb suspension (Sus), 3) suspension with 7 days of reload (Sus+7), and 4) suspension with 14 days of reload (Sus+14). To determine whether OVX female animals exhibit delayed or deficient skeletal muscle regrowth after disuse, a subset of animals were randomly divided into ground control (n = 6) and suspension with 28 days of reloading (n = 6; Sus+28). The role of estrogen (OVX+E2) during both suspension and reload in recovery from atrophy was determined using six groups of OVX females: 1) OVX ground control receiving placebo (Con), 2) OVX ground control with exogenous estrogen for suspension and 14 days of reload (Con All), 3) OVX suspension with estrogen (Sus+E2), 4) OVX suspension with estrogen for suspension and 14 days of reload (Sus+14 All), 5) OVX ground control with estrogen for 14 days of reload (Con Rld), and 6) OVX suspension with estrogen for 14 days of reload (Sus+14 Rld). Animals were housed individually and kept on a 12:12-h light-dark cycle and given ad libitum access to normal rodent chow (intact female treatment groups) or phytoestrogen free rodent chow (Purina Test Diet, Richmond, IN; OVX treatment groups) and water for the duration of the study at the fully accredited animal care facilities at the University of South Carolina, Columbia. At the conclusion of the treatment, animals were killed under anesthesia and the soleus muscle was frozen in liquid nitrogen for analyses. Daily vaginal smears (5 days in length) were performed on all animals at the onset of the study to verify estrous cycling in intact females and a lack of estrous cycling in OVX animals. All procedures were approved by the University of South Carolina Institutional Animal Care and Use Committee.

Hindlimb suspension disuse and reload. Hindlimb suspension-induced disuse and reload recovery were performed as previously described (45), after the initial week of animal monitoring.

Estrogen replacement. The appropriate OVX treatment groups were implanted with subcutaneous 60-day hormone release pellets.
(Innovative Research of America) containing 0.25 mg of 17β-estradiol, the major estrogen in mammalian systems. Appropriate control OVX females were implanted with placebo pellets. The dose of estrogen selected in the present investigation was based on previous work done with physiological estrogen supplementation in the rat model and is designed to provide a constant 40 μg·kg body wt⁻¹·day⁻¹ (52, 59).

Plasma estradiol analysis. Plasma estradiol was extracted from 500 μl of plasma and measured by use of a commercially available double antibody radioimmunoassay kit (Diagnostic Products, Los Angeles, CA) as per manufacturer’s recommendations. Duplicate determinations were made for plasma samples from all treatment groups. The intra- and interassay coefficients of variation were 6.2 and 14.3%, respectively.

Muscle dry weights and water content. The soleus muscle was analyzed for dry muscle mass and water content as previously described (51) with the following modifications. Briefly, ~30 mg of total soleus muscle wet mass was weighed, subjected to a drying protocol of 70°C, and weighed every 30 min until a stable dry weight was obtained.

Muscle myofibrillar protein. Total and myofibrillar protein were determined as previously described (1) with the following modifications. Frozen muscle was homogenized in ice-cold buffer A (100 mM KCl, 20 mM imidazole, and 5 mM EDTA (pH 6.8) for 20 s by using a Polytron homogenizer on high speed. One-third of the homogenate was stored at −80°C until used for analysis of total muscle protein, and the remaining two-thirds were used for isolation of myofibrils, as follows. The muscle homogenate was centrifuged at 1,000 g for 10 min at 4°C. The supernatant was discarded, and the resulting myofibril pellet was resuspended in 5 ml of buffer B (175 mM KCl containing 0.5% Triton X-100, pH 6.8). After centrifugation at 1,000 g for 10 min at 4°C, the pellet was washed twice with buffer B to remove cellular membranes. The resulting pellet was washed twice in buffer C (150 mM KCl, 20 mM imidazole, pH 7.0) to remove residual Triton-X and then resuspended in 0.5 ml of buffer D, an aqueous solution of 5 mM EDTA, pH 7.4/100 mg original muscle powder. Myofibril suspensions were stored at −80°C until analyzed. The protein content of total muscle and myofibril suspensions was determined as previously described (51) with the following modifications. Total and myofibrillar protein were determined as previously described (1) with the following modifications. Frozen muscle was homogenized in ice-cold buffer A (100 mM KCl, 20 mM imidazole, and 5 mM EDTA (pH 6.8) for 20 s by using a Polytron homogenizer on high speed. One-third of the homogenate was stored at −80°C until used for analysis of total muscle protein, and the remaining two-thirds were used for isolation of myofibrils, as follows. The muscle homogenate was centrifuged at 1,000 g for 10 min at 4°C. The supernatant was discarded, and the resulting myofibril pellet was resuspended in 5 ml of buffer B (175 mM KCl containing 0.5% Triton X-100, pH 6.8). After centrifugation at 1,000 g for 10 min at 4°C, the pellet was washed twice with buffer B to remove cellular membranes. The resulting pellet was washed twice in buffer C (150 mM KCl, 20 mM imidazole, pH 7.0) to remove residual Triton-X and then resuspended in 0.5 ml of buffer D, an aqueous solution of 5 mM EDTA, pH 7.4/100 mg original muscle powder. Myofibril suspensions were stored at −80°C until analyzed. The protein content of total muscle and myofibril suspensions was determined using the Bradford assay (Bio-Rad).

Morphological analyses. For cross-sectional area (CSA; μm²), centralized nuclei, and noncontractile tissue percentage (%NCT) analyses, four distinct digital images from hematoxylin-eosin-stained muscle sections (10 μm) from the midbelly of the soleus muscle at an objective magnification of ×20 were obtained and analyzed as previously described (2, 47).

Crude protein extracts. Crude protein extracts were made from frozen soleus muscles by homogenization in Mueller buffer on ice with a Polytron homogenizer using 3 × 15-s pulses at a low setting as previously described (46).

Western blot analysis. p70s6k protein and phosphorylation levels were determined in soleus skeletal muscle as previously described (46). After transfer, membranes were probed with p70s6k primary antibody for total protein level (Santa Cruz, C-18) and phosphorylated p70s6k (Thr 389) protein primary antibody (Cell Signaling Technology, no. 9205), diluted in 1% milk-TBS-Tween. The horseradish conjugated anti-rabbit secondary antibody, diluted in 1% milk-TBS-Tween (1:6,500) and conjugated with alkaline phosphatase, was visualized by chemiluminescence (ECL, Amersham Life Sciences) as per manufacturer instructions and quantified by densitometry scanning (Scion Technologies, Frederick, MD).

Total RNA isolation and cDNA synthesis. Total RNA was isolated by using TRIzol reagent (Life Technologies, Grand Island, NY) as per manufacturer’s instructions.

Northern blot analysis. Northern blot analysis was performed as previously described (10). Briefly, 15 μg of total RNA were fractionated on a denaturing 1% agarose gel (1 × MOPS, 6.7% formaldehyde) and then transferred to a nylon membrane by capillary action. The collagen type Ia probe was a kind gift from Dr. Wayne Carver (Department of Developmental Anatomy and Biology, University of South Carolina School of Medicine). Membranes were then visualized by autoradiography (−80°C, 3–40 h), and quantified by densitometry scanning (Scion Image, Frederick, MD) obtaining an integrated optical density (IOD), which was used to calculate mRNA abundance. mRNA abundances were corrected for 18S IOD calculated from digital gel images before transfer and subsequently normalized to intact female ground control values.

Immunohistochemical analysis. For the analysis of soleus muscle embryonic myosin heavy chain (eMHC), transverse sections (10 μm) were cut from the midbelly of the soleus muscle on a cryostat at −20°C and mounted on coverslips. Immunohistochemistry staining was performed as previously described (27–29). Briefly, muscle sections were air-dried and fixed in acetone, and endogenous peroxidase activity was quenched by incubation in 0.6% peroxide. Sections were blocked with 4% horse serum and exposed to anti-eMHC antibody radioimmunoassay kit (Diagnostic Products, Los Angeles, CA). Control sections were incubated with 1× PBS only. Sections were washed in 1× PBS and anti-mouse secondary antibody (1:200) (Vector Laboratories) was incubated on all sections (control and treatment) for 90 min at 37°C. Sections were then incubated with the Vectastain ABC reagent (Vector Laboratories) per manufacturer instructions. Sections were then exposed to diaminobenzidine tetrahydrochloride with nickel (Vector Laboratories). Positive fibers were counted under a light microscope and expressed as the total number of eMHC-positive fibers per square millimeter muscle.

Data analysis. Differences within treatment control groups due to recovery day (10-day Sus, Sus+7, Sus+14, or Sus+28), circulating 17β-estradiol, and differences in estrogen-replacement controls or reloaded females due to the day of estrogen administration (All days or Rd days) were analyzed by one-way ANOVA. Where no significant effects occurred in treatment controls due to day or in estrogen-replacement controls and reloaded females due to the day of estrogen administration, respective groups were pooled. All other variables were analyzed by two-way ANOVA for main effects (estrogen treatment or recovery day) or interactions (estrogen treatment × recovery day). Where main effects existed devoid of significant interactions, values are presented as pooled. Where significant interactions existed, Bonferroni post hoc analyses were used between groups. Values are presented as means ± SE. Significance was set at P < 0.05.

RESULTS

Plasma estradiol. Ovariectomy (3 ± 1 pg/ml) decreased circulating estradiol 85% compared with intact values (20 ± 7 pg/ml). Ten days of hindlimb suspension did not alter circulating estradiol (32 ± 1 pg/ml) in intact females. Estrogen replacement with 17β-estradiol administration increased circulating estradiol (67 ± 27 pg/ml) above intact and ovariectomy values.

Analysis of day of controls and estrogen administration timing. The experimental design of the present study had cage-control rats for each estradiol treatment at each day of reload. In control rats, within intact, ovariectomy, or ovariectomy plus estradiol treatments, there were no effects of recovery day for any morphological or biochemical measurement analyzed in the present study. Control data within treatments were combined for all further analysis.

The experimental design also had two estrogen-replacement treatments given to ovariectomized rats, which varied by the time the estrogen was administered. The first estrogen-replacement treatment had estrogen administration given for the entire experimental time course (disuse and recovery), whereas the...
second treatment was administered at the time of atrophy recovery after the completion of hindlimb disuse. There were also no observed effects of estrogen treatment timing (entire time course or given at recovery) for any morphological or biochemical measurement analyzed in the present study. As such, data were combined for all further analysis and discussion.

Muscle mass recovery after disuse. Muscle mass was significantly altered by estrogen manipulation and recovery day, which had a significant interaction on muscle wet weight (Table 1). Ovariectomy alone increased soleus muscle wet weight 28% from intact controls and estrogen replacement attenuated this increase (Table 1). There was significant atrophy of the soleus muscle with hindlimb disuse in all treatments groups. After 10 days of disuse soleus wet weight decreased 27% in intact, 44% in ovariectomy, and 41% with estrogen-replacement treatments (Table 1). Muscle wet weight recovery was accomplished by the 7th day of recovery in intact animals. With ovariectomy, soleus wet weight remained decreased 34% at day 7 and remained 12% less than control values after 14 days of recovery. However, ovariectomized soleus wet weight was fully restored (123 ± 6 mg) by day 28 of recovery.

Because ovariectomy can induce changes in body mass, tibia lengths (mm) were used to correct for alterations in body size. There was no interaction of estrogen treatment and recovery day on tibia lengths (35.3 ± 1.6 mm). Soleus mass was then corrected for tibia length (mass/tibia). Estrogen treatment and recovery day had a significant interaction on the mass-to-tibia ratio (Table 1). Ovariectomy alone increased mass/tibia length 29% compared with intact animals and estrogen replacement attenuated this increase. Hindlimb disuse decreased the muscle mass-to-tibia ratio 25% in intact, 42% with ovariectomy, and 38% in estrogen-replacement muscle (Table 1). The muscle mass-to-tibia ratio was restored by 7 days of recovery in intact females, but in ovariectomized females it remained decreased 31 and 8% from ground control at days 7 and 14 of recovery, respectively. Ovariectomized rat mass-to-tibia ratio was restored to control values by the 28th day (3.4 ± 0.1 mg/mm) of recovery.

Muscle dry weight, water content, and total muscle myofibrillar protein. Estrogen status did not alter muscle dry weight (mg). However, there was a main effect of recovery day on muscle dry weight. Suspension (15.9 ± 1.3 mg) decreased dry weight 40% compared with control (26.6 ± 1.3 mg) values. Dry weight remained decreased 39% from control values after 7 (17 ± 4 mg) or 14 days of recovery (20.7 ± 1.2 mg).

Estrogen status altered soleus muscle water content. Soleus muscle from ovariectomized rats had a 39% increase in water content compared with the intact control muscle (Table 1). Estrogen replacement returned muscle water content to intact control levels. Intact muscle water content decreased 21% with suspension but was restored to control values at days 7 and 14 of recovery. In ovariectomized rats, soleus water content decreased 44% with suspension and remained decreased 32% after 7 days of recovery, but it returned to control values by day 14 of recovery. Estrogen replacement did not affect muscle water loss with suspension, as suspension decreased muscle water content 43%.

Estrogen status did not alter the loss or recovery of myofibrillar protein in the present study (Table 1). Suspension decreased myofibrillar protein 60% in the intact animals and 57% in the ovariectomized animals. Myofibrillar protein remained decreased until day 14 of recovery in both treatment groups. Animals receiving estrogen replacement also had a similar loss (50%) of myofibrill protein with suspension, which was restored after 14 days of recovery. There was a main effect of treatment day on soleus muscle total protein content. Hindlimb suspension (13 ± 2 mg) decreased muscle total protein content 54% from control (25 ± 2 mg), and it remained decreased 35% with 7 (16 ± 3 mg) and 26% with 14 days (19 ± 1 mg) of recovery. There was no interaction of estrogen status and recovery day on muscle total protein content.

Myofiber cross-sectional area. Muscle fiber cross-sectional areas were not altered from intact values by OVX or estrogen replacement treatments (Table 1). Muscle total protein content analyzed in the present study. As such, data were combined for all further analysis and discussion.

Table 1. Muscle wet weights, wet weights corrected for tibia lengths, myofibrillar protein, water content, and percent noncontractile tissue in intact, ovariectomized, and ovariectomized with estrogen replacement rat soleus muscle recovering from hindlimb suspension disuse

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Muscle Wet Wt, mg</th>
<th>Muscle Wet Wt/ Tibia Length, mg/mm</th>
<th>Myofibrillar Protein Content, mg/soleus</th>
<th>Water Content, mg</th>
<th>% NCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>102 ± 5</td>
<td>2.8 ± 0.1</td>
<td>15 ± 2</td>
<td>75 ± 5</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>Sus</td>
<td>75 ± 3a</td>
<td>2.1 ± 0.1b</td>
<td>6 ± 1b</td>
<td>59 ± 5b</td>
<td>32 ± 2a</td>
</tr>
<tr>
<td>Sus + 7</td>
<td>96 ± 6b</td>
<td>2.8 ± 0.2b</td>
<td>6 ± 1b</td>
<td>75 ± 5a</td>
<td>29 ± 2a</td>
</tr>
<tr>
<td>Sus + 14</td>
<td>97 ± 2b</td>
<td>2.8 ± 0.1b</td>
<td>18 ± 2b,c</td>
<td>73 ± 4b</td>
<td>18 ± 1b,c</td>
</tr>
<tr>
<td>OVX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>131 ± 2d,e</td>
<td>3.6 ± 0.1d,e</td>
<td>14 ± 1</td>
<td>104 ± 5d</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>Sus</td>
<td>74 ± 3a</td>
<td>2.1 ± 0.1a</td>
<td>6 ± 1c</td>
<td>58 ± 2a</td>
<td>35 ± 3a</td>
</tr>
<tr>
<td>Sus + 7</td>
<td>86 ± 7a</td>
<td>2.5 ± 0.2a</td>
<td>9 ± 1c</td>
<td>70 ± 3a</td>
<td>31 ± 1a</td>
</tr>
<tr>
<td>Sus + 14</td>
<td>116 ± 5b,c</td>
<td>3.3 ± 0.1b,c</td>
<td>11 ± 2a</td>
<td>96 ± 5b,cd</td>
<td>26 ± 1b,c</td>
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<tr>
<td>OVX + E2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>98 ± 8</td>
<td>2.9 ± 0.1</td>
<td>14 ± 1</td>
<td>73 ± 2</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>Sus</td>
<td>58 ± 1a</td>
<td>1.8 ± 0.1a</td>
<td>7 ± 2a</td>
<td>42 ± 10a</td>
<td>34 ± 3a</td>
</tr>
<tr>
<td>Sus + 14</td>
<td>95 ± 3b</td>
<td>2.9 ± 0.1b</td>
<td>12 ± 2b</td>
<td>73 ± 3b</td>
<td>23 ± 2b</td>
</tr>
</tbody>
</table>

Values are means ± SE. % NCT, percent noncontractile tissue; OVX, ovariectomized; OVX + E2, ovariectomized with estrogen replacement; Con, ground control; Sus, hindlimb suspended; Sus + 7, 7-day reload; Sus + 14, 14-day reload. *Significantly different (P < 0.05) from treatment-matched Con. Significantly different (P < 0.05) from treatment-matched Sus + 7. Significantly different (P < 0.05) from Intact Con. Significantly different (P < 0.05) from OVX + E2 Con.

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replacement in control muscle (Fig. 2A). However, there was an interaction of estrogen treatment and recovery day on muscle cross-sectional area. Hindlimb disuse decreased fiber cross-sectional area similarly (40–55%) in all treatment groups (Fig. 2A). After 7 days of recovery, intact and ovariectomized cross-sectional areas remained decreased 42 and 44%, respectively, from control values. After 14 days of recovery, cross-sectional area from intact rats was restored to control values, whereas ovariectomized rats maintained a 42% deficit. Cross-sectional area did return to control values by the 28th day of recovery in ovariectomized females, demonstrating fiber growth after the second week of recovery.

During remodeling and growth, the distribution of myofiber cross-sectional areas within the muscle can provide important information beyond mean area alone. However, in control muscle, ovariectomy increased small (<500 μm²) fiber incidence 251%, and estrogen replacement attenuated this increase (Fig. 2B). With suspension, intact muscle small myofiber incidence increased 154% and was further elevated after 7 (508%) and 14 days (405%) of recovery. Muscle from ovariectomized rats did not demonstrate a change in small myofiber incidence with suspension or 7 days of recovery. However, after 14 days of recovery small fiber incidence increased 97%, returning to baseline after 28 days of recovery (9.2 ± 1.3%). In controls, large (>4,000 μm²) myofiber incidence was not altered from intact values by ovariectomy or estrogen replacement (Fig. 2C). Regardless of estrogen status, suspension ablated the incidence of large myofibers (Fig. 2C). In intact animals, large fiber incidence was returned to control values at day 14 of recovery, whereas large fiber incidence in ovariect-
increased Thr389 phosphorylation 115% compared with intact ovariectomized muscle total p70S6kinase abundance to control with intact animals (Fig. 3B). Estrogen replacement returned B-control muscle total p70s6k protein abundance by 36%, compared with phosphorylation in control muscles. Ovariectomy reduced contraction, 7 days, or 14 days of recovery (Fig. 3B). However, in muscle from ovariectomized rats, central nuclei incidence returned to control values at day 7 of recovery but was then elevated 271% from control values at day 14 of recovery. Estrogen replacement reduced the incidence of myofibers with central nuclei at day 14 of recovery. There was a main effect of recovery day on the incidence of eMHC-positive myofibers. eMHC-positive fibers peaked after 7 days of recovery and remained elevated after 14 days of recovery (Fig. 6B). However, there was no estrogen treatment and recovery day interaction on the occurrence of eMHC positively stained fibers.

Noncontractile tissue. The composition and volume of the extracellular matrix is thought to contribute to the regulation of skeletal muscle remodeling processes (15, 16). Extracellular matrix expansion can influence muscle mechanical properties and is important in the pathology of fibrosis (17, 40). There was an interaction of estrogen treatment and recovery day interaction on the volume %NCT. Suspension increased soleus muscle %NCT in intact (53%), ovariectomized (74%), and estrogen-replacement (58%) soleus muscle. Seven days of recovery increased the number of central nuclei containing fibers in intact animals 120%, which returned to control levels at day 14. However, in muscle from ovariectomized rats, central nuclei incidence returned to control values at day 7 of recovery but was then elevated 271% from control values at day 14 of recovery. Estrogen replacement reduced the incidence of myofibers with central nuclei at day 14 of recovery. There was a main effect of recovery day on the incidence of eMHC-positive myofibers. eMHC-positive fibers peaked after 7 days of recovery and remained elevated after 14 days of recovery (Fig. 6B). However, there was no estrogen treatment and recovery day interaction on the occurrence of eMHC positively stained fibers.

Myofiber regeneration. Centrally localized nuclei (Fig. 4) and positive staining for eMHC (Fig. 5) are indicators of myofiber regeneration in skeletal muscle (17). There was an interaction of estrogen treatment and recovery day on the incidence of myofibers demonstrating central nuclei (Fig. 6A). Suspension decreased central nuclei incidence in intact (76%), ovariectomized (90%), and estrogen-replacement (72%) soleus muscle. Seven days of recovery increased the number of central nuclei containing fibers in intact animals 120%, which returned to control levels at day 14. However, in muscle from ovariectomized rats, central nuclei incidence returned to control values at day 7 of recovery but was then elevated 271% from control values at day 14 of recovery. Estrogen replacement reduced the incidence of myofibers with central nuclei at day 14 of recovery. There was a main effect of recovery day on the incidence of eMHC-positive myofibers. eMHC-positive fibers peaked after 7 days of recovery and remained elevated after 14 days of recovery (Fig. 6B). However, there was no estrogen treatment and recovery day interaction on the occurrence of eMHC positively stained fibers.

Collagen I is highly expressed in postural skeletal muscle and mediates the muscle’s resistance to stretch (14). In control

Fig. 3. p70s6k protein expression in suspended and reloaded ovariectomized female rat soleus muscle. A: representative Western immunoblot of total p70s6kinase in OVX ground Con, Sus, Sus+7, and Sus+14 muscle. B: soleus total p70s6k protein expression in intact (black bars), OVX (gray bars), and OVX+E2 (hatched bars) Con, 10-day Sus, Sus+7, and Sus+14 treatment groups. Values are presented as means ± SE and normalized to intact female soleus ground control. *Significantly (P < 0.05) different from treatment-matched Con. †Significantly (P < 0.05) different from treatment-matched 10-day Sus. ‡Significantly (P < 0.05) different from treatment-matched Sus+7. #Significantly (P < 0.05) different from intact ground control. *Significantly (P < 0.05) different from OVX+E2 ground control.

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muscle, estrogen administration decreased collagen 1α abundance 60 and 76% from intact and ovariectomized values, respectively (Fig. 7). Intact muscle collagen 1α mRNA abundance was not altered by either hindlimb suspension or 7 days of recovery but was decreased 70% at day 14 of recovery. In ovariectomized muscle, suspension decreased collagen 1α mRNA abundance 62% from control and returned to control levels after 7 days of recovery. Collagen 1α mRNA abundance was increased 46% above control values in ovariectomized muscle after 14 days of recovery. In ovariectomized animals administered estrogen, suspension had no effect on collagen 1α mRNA abundance. Interestingly, recovery for 14 days with estrogen administration resulted in increased collagen 1α mRNA abundance compared with treatment control values.

**DISCUSSION**

Estrogen loss has been shown to alter skeletal muscle wet weight and protein content during recovery from disuse atrophy (57). A variety of processes within skeletal muscle contribute to disuse mass recovery including edema, myofiber regeneration and growth, and extracellular matrix remodeling. The results of the present study demonstrate that estrogen is an important regulator of soleus muscle fiber growth and extracellular matrix remodeling. The effects of estrogen on muscle recovery from disuse skeletal muscle fiber cross-sectional area during the first 2 wk of reload. However, estrogen replacement at physiologically relevant levels restored the ability of muscle fibers to recover cross-sectional area over this same 2-wk period. Another important finding was that the beneficial effect of estrogen replacement on muscle recovery was independent of when the hormone was administered, before disuse or at the time of recovery. Therefore, to our knowledge, this paper demonstrates for the first time that estrogen has a direct effect on the recovery of soleus muscle myofiber size after atrophy. Although ovarian hormone loss dramatically affected myofiber growth during the first 2 wk of recovery, myofiber cross-sectional area was able to fully recover after 28 days, regardless of hormone status. This finding extends previous work with ovarian hormone loss and muscle mass recovery and demonstrates that the soleus muscle can still regain functional muscle mass without ovarian hormones, but the process is significantly delayed.

In the present study, ovarian hormone status induced alterations in soleus muscle wet weight. Fluctuations in muscle wet weight can be accounted for by a variety of muscle components and are not totally dependent on myofiber size (35). This study and others clearly demonstrate that ovariectomy is a potent stimulus for increased rat soleus muscle mass that is independent of increased myofiber cross-sectional area (23, 24). Interestingly, despite this basal increase in soleus muscle mass with ovariectomy, soleus myofibers from these animals had an altered capacity for plasticity related to loading stimuli. The ovariectomized rats in the present study demonstrated a nearly complete restoration of muscle mass during 2 wk of recovery from disuse without a corresponding increase in myofiber area. These findings are in agreement with previous work (13). Initial changes in skeletal muscle mass during disuse recovery are attributed primarily to edema and characterized by deficits in both force production and mean cross-sectional area (11, 31, 45). The time course of gene expression induced early during reloading is a highly coordinated process related to muscle damage and repair, oxidative metabolism, and muscle regeneration (26). In the present study, the time course of skeletal muscle growth after disuse followed a sequential pattern that first involved edema and then was followed by increased dry weight, myofibrillar protein accretion, and myofiber cross-fig. 4. Representative soleus muscle H and E staining of intact ground control (A), intact with 10 days of hindlimb suspension and 14 days of reloading (B), OVX ground control (C), and OVX with 10 days of hindlimb suspension and 14 days of reloading (D).
sectional area restoration. Estrogen has the potential to regulate several important processes related to this sequence of muscle recovery from atrophy, and the present study demonstrates a direct effect of estrogen on myofibers. Specifically, estrogen administration rescued soleus mean fiber cross-sectional area and large myofiber incidence in ovariectomized animals during 2 wk of recovery.

Sitnick et al. (57) recently demonstrated that ovariectomy attenuates the recovery of rat gastrocnemius muscle mass during a 14-day recovery period from 28 days of hindlimb suspension and established a role for ovarian hormones in the activation of Akt and p70s6kinase (57). These proteins are critical to the regulation of protein synthesis capacity and/or efficiency (5). The present study also found alterations in p70s6kinase phosphorylation and total protein expression with ovariectomy during a shorter period of hindlimb suspension and during recovery in the postural soleus muscle. We have now expanded on these findings to confirm that the loss of the specific ovarian hormone estrogen is sufficient to induce these alterations. Coinciding with p70s6kinase phosphorylation and total expression, estrogen replacement also rescued ovariectomy-induced alterations in myofiber growth and small myofiber occurrence. Increased p70s6kinase protein expression at day 14 of recovery in ovariectomized animals provides further evidence for protein accretion associated with myofiber growth at later time points of recovery. Soleus myofiber cross-sectional area in ovariectomized rats returned to control levels between the 2nd and 4th weeks of recovery. Myofiber growth is complex and involves many processes related to the myofiber’s microenvironment. The delay in fiber growth with ovariectomy may demonstrate the importance of the resolution of early processes related to damage and inflammation so that subsequent myofiber growth can occur. Candidates for estrogen-sensitive manipulation of the fiber environment during muscle recovery from disuse include extracellular matrix remodeling, satellite cell regulation, and inflammatory processes (39).

The extracellular matrix has a critical role in muscle function and homeostatic regulation that is related to both its protein composition and cellularity (36). Skeletal muscle extracellular matrix remodeling can be modulated by mechanical stress, growth factor, hormone, and cytokine signaling (44, 53). The present study demonstrates both disuse and the subsequent recovery dramatically affect extracellular matrix volume. However, the composition, as it relates to collagen 1α mRNA
expression, was not sensitive to disuse and recovery. Although not affecting extracellular matrix volume, ovarian hormone status did alter muscle collagen 1a expression. Ovariectomy can induce rat hepatic extracellular matrix expansion, which is characterized by increases in types I and III procollagen mRNA abundance (62). However, in the present study, the regulation of muscle collagen 1a expression was not estrogen sensitive and may be regulated by other ovarian hormones. Ovariectomy may alter extracellular matrix volume indirectly through other hormones or growth factors. IGF-I overabun-

Fig. 6. Soleus muscle fiber regeneration with suspension and reloading in ovariectomized female rats. A: occurrence of centralized nuclei in intact (black bars), OVX (gray bars), and OVX+E2 (hatched bars) Con, Sus, Sus+7, and Sus+14 treatment groups. Transverse sections (10 μm) were cut from the midbelly of the soleus muscle on a cryostat at −20°C and mounted on coverslips. Hematoxylin and E staining was performed to determine the percentage of fibers with central nuclei. B: soleus fiber embryonic myosin heavy chain (eMHC) expression. Transverse sections (10 μm) were cut from the midbelly of the soleus muscle on a cryostat at −20°C and mounted on coverslips. Immunohistochemistry staining was performed for eMHC and 4 digital images were taken from each muscle section at a ×20 magnification to determine myofiber expression per mm² of muscle area. There was a main effect of recovery day on eMHC expression per mm² of soleus muscle area. Values are presented as means ± SE. *Significantly (P < 0.05) different from treatment-matched Con. †Significantly (P < 0.05) different from treatment-matched Sus. ‡Significantly (P < 0.05) different from treatment-matched Sus+7.

Fig. 7. Alterations in collagen mRNA with suspension and reloading in ovariectomized female rats. Ten to 15 μg of total RNA were fractionated on a denaturing 1% agarose gels (1 × 3 MOPS, 6.7% formaldehyde) and then transferred to a nylon membrane by capillary action. Membranes were visualized by autoradiography (−80°C, 3–40 h) and quantified by densitometry scanning. A: representative Northern blot of collagen 1a mRNA abundance and 18S and 28S mRNA in ovariectomized Con and Sus+14 treatments. B: collagen 1a mRNA expression in intact, OVX, and OVX+E2 Con, 10-day Sus, Sus+7, and Sus+14 treatment groups. Values are presented as means ± SE and normalized to intact female soleus ground control. *Significantly (P < 0.05) different from treatment-matched Con. †Significantly (P < 0.05) different from treatment-matched Sus. ‡Significantly (P < 0.05) different from treatment-matched Sus+7. #Significantly (P < 0.05) different from intact ground control. εSignificantly (P < 0.05) different from OVX+E2 ground control.
dence is thought to contribute to fibrosis in chronically strained skeletal muscle (20), and estrogen depletion due to ovariec-
tomy increases circulating growth hormone and IGF-1 (12, 32).

Muscle loading and ovarian hormone loss had an additive
effect on volume of noncontractile tissue and collagen 1α
mRNA abundance, suggesting that a hormone and mechanical
signaling interaction may underlie extracellular matrix remodel-
ing. Endomysial fibroblasts are the primary source of extra-
cellular matrix protein synthesis (25), and they express estro-
gen receptors (30). TGF-β is a profibrotic cytokine, and intra-
cellular signaling related to this pathway has been shown to
interact with the estrogen receptor. Specifically, estrogen re-
cipients can physically bind and inhibit the action of Sma and
MAD-related protein 3 (Smad-3), a downstream component of
TGF-β signaling (43). Skeletal muscle recovery from either
laceration or cardiotoxin injury is inhibited by TGF-β-induced
expression of profibrotic proteins (40). Estrogen effects on
TGF-β signaling may also have implications for satellite cell
activity. In myoblasts, Smad-3 functions in TGF-β-mediated
suppression of myoblast differentiation through interaction
with myogenic regulatory factor MyoD (41).

There is the possibility that during disuse ovariec
tomized muscle had a greater depletion of both the myogenic precursor
pool and myofiber nuclei, because both of these pools are
decreased in atrophying skeletal muscle (49). However, the
present study demonstrates that estrogen effects on muscle
recovery are more likely related to events during the recovery
process rather than during the period of disuse. Estrogen
replacement in ovariec
tomized animals at the time of reloading
was as effective for rescuing muscle mass and myofiber size by
day 14 of recovery as when estrogen was supplied during both
the atrophy and recovery phases. This finding suggests that
estrone’s contribution for successful muscle recovery may be
related to myogenic activation upon reloading rather than
myoblast protection during atrophy. A retarded ability of myo-
genic precursor cells to activate and differentiate occurs after
prolonged atrophic conditions (50). Disuse combined with estro-
gen loss appears to require longer recovery periods, which would
allow precursor cells to reach a critical population necessary for
both myofiber growth and successful regeneration. Ovariectomy
appears to delay the local expression of cellular markers respon-
sible for differentiation in this population of cells.

The resolution of inflammation and edema may be critical in
signaling associated with myofiber growth. Bondesen et al. (9)
suggest that the attenuation of inflammation may be critical for
myofiber growth owing to its regulation of immune cell phago-
cytosis of necrotic tissue. When the muscle’s inflammatory
response is manipulated, recovery from muscle damage is
delayed, but not ablated (9). Prolonged or exaggerated edema
and inflammation due to ovarian hormone loss could delay
regeneration, and ultimately myofiber growth. In the present
study, ovariec
tomy prolonged the demonstration of histologi-
cal markers for regeneration during reloading. Small myofiber
incidence, a morphological marker of ongoing muscle regen-
eration (17), was induced after 7 days of recovery in the intact
female, and ovarian hormone loss suppressed this induction.
There is a strong possibility that the estrogen-sensitive mecha-
nisms delaying muscle regeneration may also be attenuating
myofiber growth. Estrogen accelerates cutaneous wound heal-
ing (3), and hormone replacement therapy prevents chronic
wound development in postmenopausal women (7). Ashcroft
et al. (4) suggested that estrogen receptor regulation of mac-
rophage inhibitory factor is responsible for the suppression of
excessive proteolysis during cutaneous wound healing. A key
characteristic of estrogen’s action on wound healing appears to
be the suppression of extracellular matrix component deposi-
tion at the site of injury (3, 43). Immediate early gene expres-
sion in muscle can be regulated by growth factor, cytokine, and
estrogen receptor signaling interactions (22). Estrogen signal-
ing has the potential to interact with both growth factor and
inflammatory cytokine signaling pathways. Estradiol, IGF-1,
and the inflammatory cytokine TNF-α are independently capa-
cable of activating serum response element promoter regions
on reporter genes in MCF-7 cells, but the addition of constitut-
tively active estrogen receptor-α markedly enhances this
activation. Because inflammatory signaling is critical for the
response of muscle precursor cells during regeneration (9),
interactions between these pathways could amplify the impor-
tance of estradiol availability during regeneration. Further
work is needed to determine whether potential alterations in the
inflammatory response at the onset of reloading after disuse are
sensitive to estrogen.

In summary, successful muscle recovery from disuse atro-
phy requires the integration of many signaling pathways that
serve to first initiate and then sustain muscle regeneration and
growth. The present study demonstrates that ovarian hormones
can have a significant impact on muscle fiber size and extra-
cellular matrix remodeling during reloading after disuse. Es-
trogen may aid muscle recovery by suppressing extracellular
matrix remodeling. Determining the importance of estrogen
regulation of muscle growth and remodeling processes during
reloading after atrophy could provide novel therapeutic strat-
egies targeted at the maintenance and recovery of muscle mass.

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