Effects of ovariectomy and hindlimb unloading on skeletal muscle

JONATHAN S. FISHER,1 EILEEN M. HASSER,2 AND MARYBETH BROWN1

1Program in Physical Therapy, Washington University School of Medicine, St. Louis, 63108; and 2Department of Veterinary Biomedical Sciences, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65211

RECEPTORS FOR THE STEROID HORMONE ESTROGEN have been identified in mammalian skeletal muscle (8, 26). An anabolic effect of estrogen on developing skeletal muscle in cattle and sheep has been described (29), but in growing young rats estrogen has been reported to inhibit skeletal muscle development (15, 28). Endogenous estrogen in women falls substantially at menopause, and it appears that there is a related loss of lean body mass (18). Muscle force per unit cross-sectional area was observed to be lower in postmenopausal compared with premenopausal women and in age-matched postmenopausal women who had been taking hormone replacement therapy (HRT) (17). In addition, back extensor strength was found to be greater in postmenopausal women taking estrogen than in those taking placebo (11).

The purpose of this study was to examine the effects of ovariectomy on skeletal muscle function under control conditions and after 2 wk of reduced muscle use (hindlimb unloading). We chose mature (7–8 mo) rats to remove the rapid growth of previously studied young animals (15, 28) as a possible confounding factor in the experimental design. If ovarian hormones play a role in the maintenance of muscle mass and/or function, a reduction in circulating levels of hormones should have a negative effect on skeletal muscle mass and/or function. Additionally, if ovarian hormones play a role in the maintenance of muscle mass, the deleterious effects of muscle disuse should be exaggerated when hormone levels are reduced. Thus we hypothesized that hindlimb unloading-related atrophy would be greater in ovariectomized rats than in intact animals.

METHODS

Animals. Virus-free female Sprague-Dawley rats, 6–7 mo of age, were obtained from Charles River (Wilmington, MA). Skeletal characteristics that might affect muscle length and mass have stabilized in rats this age (14); 6-mo-old rats have passed the initial, rapid skeletal growth stage that occurs in young rats. Upon receipt, animals were placed in a virus-free environment, two per cage, and maintained in a barrier room where temperature (22–23°C) and hours of light/dark were controlled (12:12-h light-dark cycle). Food and water were available ad libitum. After 7–10 days of acclimatization to the animal facility, rats were placed randomly into one of four groups: intact (Int, n = 8), ovariectomized (Ovx, n = 10), intact-hindlimb unloaded (Int-HU, n = 10), and ovariectomized-hindlimb unloaded (Ovx-HU, n = 12).

Ovariectomy. After an intraperitoneal injection (80 μl/100 g body wt) with a mixture of ketamine HCl (75 mg/ml) and promazine HCl (12.5 mg/ml), the surgical site was shaved and cleansed thoroughly with Betadine. A midline abdominal incision was made, and the junction between the ovaries and uterus was located and tied with 2.0 silk. Ovaries were then removed bilaterally. Rats were allowed 2 wk to recover from the surgery and during that time were monitored to ensure that they were eating properly, gaining weight, and moving about normally.

The efficacy of the ovariectomy was confirmed after the terminal experiment by visual inspection for marked uterine atrophy in ovariectomized animals.

Hindlimb unloading. Two small casts were applied to HU animals, one around the tail and the other around the upper thorax, to prevent animals from chewing at the tail cast. Wires from the tail and thorax casts were fed through a leader wire to the cage. A midline abdominal incision was made, and the junction between the ovaries and uterus was located and tied with 2.0 silk. Ovaries were then removed bilaterally. Rats were allowed 2 wk to recover from the surgery and during that time were monitored to ensure that they were eating properly, gaining weight, and moving about normally.

The purpose of this study was to examine the effects of ovariectomy on skeletal muscle function under control conditions and after 2 wk of reduced muscle use (hindlimb unloading). We chose mature (7–8 mo) rats to remove the rapid growth of previously studied young animals (15, 28) as a possible confounding factor in the experimental design. If ovarian hormones play a role in the maintenance of muscle mass and/or function, a reduction in circulating levels of hormones should have a negative effect on skeletal muscle mass and/or function. Additionally, if ovarian hormones play a role in the maintenance of muscle mass, the deleterious effects of muscle disuse should be exaggerated when hormone levels are reduced. Thus we hypothesized that hindlimb unloading-related atrophy would be greater in ovariectomized rats than in intact animals.
between controls with and without casts, so control animals in the present study were not casted. Int and Ovx animals were placed in single cages during the 2-wk period in which other animals underwent hindlimb unloading.

Given the time the animals spent to acclimatize to the new animal facility, to recover from surgery, to accommodate to the single cages, and 2 wk of hindlimb unloading or normal cage activity, the rats were between 7 and 8 mo of age when studied.

Contractile properties. Rats were anesthetized with pentobarbital sodium (50 mg/kg body wt), and anesthesia was maintained with an additional 2.5–5 mg/kg dose given every ~45–60 min. Rats were placed on a 39°C water-jacketed heating pad to maintain body temperature. The soleus (Sol), extensor digitorum longus (EDL), peroneus longus (Per), and plantaris (Plan) muscles were surgically exposed at their distal ends. The distal tendon of each muscle was attached in turn to a Grass force transducer with 2.0 silk, and the exposed portions of the muscles were bathed with 37°C rat Ringer solution. Sol (antigravity, slow-twitch), EDL (nonpostural, fast-twitch), Per (nonpostural, fast-twitch), and Plan (locomotor, fast-twitch) muscles were selected to permit study of muscles with different proportions of fast and slow fiber types and different anatomic functions. The tibial and peroneal nerves were isolated, placed on a bipolar stimulating electrode, and covered with mineral oil at 37°C. The left hindlimb was rigidly immobilized. Before contractile properties were obtained, animals were allowed to thermoequilibrate for ~30 min.

Muscles were studied in the following order: Plan, Sol, Per, and EDL. A length-tension curve for each muscle was obtained, and muscles were adjusted to optimal length. Peak isometric twitch tension was obtained with supramaximal 0.5 ms square-wave pulses (Grass Instruments S48). Peak isometric twitch tension, time to peak twitch tension (TPT), and one-half relaxation time (RT1/2) were determined. Peak tetanic tension (Po) was elicited by 0.5 ms supramaximal pulses delivered at 100 Hz (400-ms duration) for Sol and at 150 Hz for EDL (250-ms duration), Plan (300-ms duration), and Per muscles (350-ms duration). For each muscle, the adjustment to optimal length and the subsequent measurement of Po, were performed only once. Several minutes passed between tests of muscles innervated by the same nerves (Plan/Sol, Per/EDL), so fatigue probably was not a factor in measurement of Po (Po has previously been found to be reproducible in our laboratory).

Histology. After contractile characteristics were obtained, the Sol, EDL, Per, Plan, and gastrocnemius (Gast) muscles were removed and weighed. Muscles were pinned at their in situ length, embedded in OCT tissue-freezing medium, frozen in liquid nitrogen, and placed in a −80°C freezer until analysis. Muscles were sectioned at 8 μm, preincubated at 4.3 and 4.55 pH, and incubated for ATPase activity for determination of major fiber types I, IIa, and IIb (5). ATPase-stained sections were used for determination of fiber areas. The areas of 50 type I and all type I fibers were measured in Sol by using a computer digitizing package (EasyDigit). The areas of 50 type Iib and Ila fibers were measured in the Per, Gast, Plan, and EDL. As there were few type I fibers in the white portion of the Gast studied and few type I fibers in the Per, EDL, and Plan, all type I fibers in the photos obtained from these muscles were digitized (~25–50 fibers).

Animal treatment. All animal procedures were approved by the animal studies committees of both Washington University School of Medicine and the University of Missouri-Columbia. All procedures met recommendations for animal care published by The American Physiological Society.

Data management. Data were analyzed by using a 2 × 2 analysis of variance to determine effects of surgery (Int vs. Ovx), treatment (control vs. HU), or Ovx × HU interaction. Bonferroni post hoc tests were performed if interaction effects were statistically significant (P < 0.05).

RESULTS

Body and muscle mass. Ovx animals had 18% higher body weight (P < 0.05) than did Int rats (Fig. 1). Body weight was similar to Int in both Int-HU and Ovx-HU groups (Ovx × HU interaction, P < 0.05).

Sol and Plan muscle masses increased with ovariection and decreased with hindlimb unloading. Sol mass in Ovx group was ~22% greater than in Int rats, and Sol mass was ~18% lower in both HU groups than in Int rats (Ovx × HU interaction, P < 0.05). There was a similar trend in Plan muscle (Ovx × HU interaction, P < 0.07). Plan mass in Ovx group was ~18% greater than in Int animals, whereas Plan in both HU groups was ~20% smaller than in Int rats.

Although the pattern of Gast mass was similar to the pattern for Sol and Plan muscles, there was no Ovx × HU interaction. Main effects of ovariection (growth) and hindlimb unloading (atrophy) were both statistically significant for Gast mass (P < 0.05).

There were no ovariection or hindlimb-unloading effects on muscle mass in the nonpostural muscles, Per and EDL.

Muscle weight-to-body weight ratio. Although ovariection resulted in an increase in body weight and muscle mass, the ratio of muscle weight to body weight was unaffected by ovariection (Table 1). Muscle weight-to-body weight ratios were similar between Int and Ovx animals and also not different between Int-HU and Ovx-HU groups. Muscle weight-to-body weight ratios were lower in HU animals than in controls for Sol, Plan, and Gast muscles (P < 0.05), but not for Per. Muscle weight-to-body weight ratios were higher in HU animals than in controls for EDL (P < 0.05).

Contractile characteristics. Effects of ovariection on Po and specific tension were inconsistent. Po of Per muscle (Table 2) in Int was higher than in Ovx group (P < 0.05). In contrast, EDL Po was lower in Int than in Ovx rats. However, Per and EDL Po values were not different between Int-HU and Ovx-HU groups (HU × Ovx interactions, P < 0.05). Although Sol and Plan Po values were unaffected by ovariection, both were lower in HU animals than in controls (P < 0.05).

Plan-specific tension (Table 2) was lower in Ovx than in Int group (P < 0.05). The opposite occurred for EDL muscle: EDL-specific tension was greater in Ovx than in Int group (P < 0.05). There were no differences in Plan- or EDL-specific tension between Int-HU and Ovx-HU animals (HU × Ovx interactions, P < 0.05). Sol-specific tension was lower in HU animals than in controls (P < 0.05) but was unaffected by Ovx.

Neither ovariection nor hindlimb suspension had an effect on Per-specific tension.

Contraction times were generally faster in Ovx than in Int rats. TPT was shorter in Ovx than in Int animals for EDL (P < 0.05). TPT tended to be shorter in Ovx
than in Int animals for Sol, Plan, and Per muscles, but differences between groups were not statistically significant. RT1/2 was shorter in Ovx than in Int animals for all four muscles studied ($P < 0.05$).

Fiber type distribution and fiber areas. Sol type I percentages were 88$\pm$4, 96$\pm$1, 94$\pm$2, and 95$\pm$1% for Int, Ovx, Int-HU, and Ovx-HU groups, respectively (main effect of ovariectomy, $P < 0.05$). For Plan muscle, type I percentages were 8$\pm$2, 6$\pm$1, 11$\pm$2, and 11$\pm$1% (main effect for hindlimb unloading, $P < 0.05$). There were no other ovariectomy or hindlimb-unloading effects on fiber type distribution.

In Plan, type IIb fiber areas (Table 3) were larger in Ovx animals than in controls (ovariectomy main effect, $P < 0.05$). There were no other statistically significant ovariectomy effects on fiber areas. There was hindlimb unloading-related atrophy in all fiber types for the three plantar flexors ($P < 0.05$), but not in EDL or Per.

**DISCUSSION**

Estrogen has been found to inhibit skeletal muscle growth in growing rats (15, 28). Conversely, the rate of skeletal muscle growth is greater in young ovariectomized rats compared with controls (3, 15, 28). However, the effects of estrogen in mature rats have not previously been examined. Therefore, we investigated the effects of ovariectomy on skeletal muscle mass and contractile function under control conditions and after 2 wk of hindlimb unloading. Our major findings were that 1) there was substantial ovariectomy-related body and plantar flexor muscle growth; 2) ovariectomy-related skeletal muscle growth may have been prevented by hindlimb unloading, or, alternatively, 3) there may have been greater hindlimb unloading-related atrophy in Sol and Plan muscles of Ovx animals than in Int rats; and 4) contraction times (TPT and
RT1/2) were faster in Ovx animals compared with Int
animals. Estrogen probably affects rat skeletal muscle indi-
crectly through regulation of growth hormone (GH) and
insulin-like growth factor I (IGF-I). After ovariectomy in
the rat, GH release and IGF-I expression both increase (4, 13).
Estrogen were to act on muscle mass through direct interac-
tion with its receptor in skeletal muscle, Sol would be
expected to respond differently to ovariectomy than
Plan or Gast, which have been found to contain more
estrogen-binding sites than Sol (20). In contrast, fast-
twitch nonpostural muscles, Per and EDL, were una-
fected by ovariectomy. It appears that changes in
muscle mass subsequent to ovariectomy in rats are
related to muscle function (i.e., antigravity or locomotor
vs. nonpostural), rather than fiber type or estrogen-
binding site distribution. It has been shown that there
is a synergistic effect of resistance exercise and GH or
IGF-I on muscle mass in rats (10, 19). Perhaps, there-
foro, the ovariectomy-related plantar flexor growth
present study, as opposed to the younger, growing
animals studied by others (3, 4, 15, 28). However, it
appears that the rapid ovariectomy-related growth
observed in younger animals (3, 4, 15, 28) occurs in
mature animals as well. Despite differences in muscle
mass between control and ovariectomized rats, muscle
mass-to-body weight ratios were similar in control
and Ovx animals in the present study and in others (3, 21).
The pronounced growth of ovariectomized animals has
been found to cause a general increase in organ weights
(skeletal muscle, heart, kidney, spleen) in Ovx animals
(3), and does not appear to be limited to muscle.
Therefore, greater muscle mass in Ovx animals com-
pared with controls might be explained by hormonal
regulation of body growth, rather than by direct effects
of estrogen on skeletal muscle.

Table 3. Fiber cross-sectional areas

<table>
<thead>
<tr>
<th></th>
<th>Int</th>
<th>Ovx</th>
<th>Int-HU</th>
<th>Ovx-HU</th>
</tr>
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<tbody>
<tr>
<td>Sol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>3,866±183</td>
<td>3,483±202</td>
<td>2,013±170</td>
<td>2,070±111</td>
</tr>
<tr>
<td>II</td>
<td>3,195±126</td>
<td>3,267±256</td>
<td>2,161±122</td>
<td>1,942±194</td>
</tr>
<tr>
<td>Plan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1,961±230</td>
<td>2,161±120</td>
<td>1,420±114</td>
<td>1,560±67</td>
</tr>
<tr>
<td>II</td>
<td>1,917±124</td>
<td>2,213±87</td>
<td>1,601±125</td>
<td>1,630±1201</td>
</tr>
<tr>
<td>Per</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>3,113±201</td>
<td>4,009±215</td>
<td>2,662±103</td>
<td>2,780±212</td>
</tr>
<tr>
<td>II</td>
<td>3,056±370</td>
<td>3,093±262</td>
<td>2,131±211</td>
<td>2,144±131</td>
</tr>
<tr>
<td>EDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1,449±189</td>
<td>1,314±182</td>
<td>1,164±181</td>
<td>1,205±79</td>
</tr>
<tr>
<td>II</td>
<td>1,547±108</td>
<td>1,632±156</td>
<td>1,438±82</td>
<td>1,467±81</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed in µm². *Main effect for
hindlimb unloading (P < 0.05); †main effect for ovariectomy (P < 0.05).
loading-related atrophy and apoptosis have been found to be attenuated by treatment with exercise, IGF-I, and GH, and there appears to be a synergistic effect of GH and exercise in unloaded muscle (1, 10, 19).

Hindlimb unloading appeared to prevent ovariectomy-related increase in muscle mass in both Plan and Sol in the present study. It has been reported that GH release falls during hindlimb unloading (31), and this change in GH release might have counteracted the ovariectomy-related increase in GH/IGF-I expression (4). Alternatively, if muscle activity is required to elicit the hypertrophic effects of GH/IGF-I (10, 19), the reduction of activity by hindlimb unloading might have prevented GH/IGF-I-dependent muscle growth. If, as it appears, the ovariectomy-related muscle growth is a GH/IGF-I-mediated phenomenon in the rat (4), suppression of ovariectomy-related growth by hindlimb unloading could be dependent on modifications in GH/IGF-I expression, decreased muscle responsiveness to GH/IGF-I, or both.

The seemingly greater effect of hindlimb unloading on Sol and Plan of Ovx animals compared with Int rats may also suggest a protective role of ovarian hormones during skeletal muscle disuse. Estrogen is known to prevent exercise-induced muscle damage in rats and humans (2, 25), and estrogen has also been found to be related to lower efflux of muscle proteins from undamaged muscles at rest (2, 25). In the rat, muscle protein breakdown, quantified by measurement of urinary 3-methylhistidine excretion, was lower in absolute terms and relative to body weight in estrogen-replaced Ovx rats than in control Ovx animals (21). The results of the present study suggest that estrogen may also have a protective effect in skeletal muscle during disuse that may attenuate the unloading-related loss of muscle proteins.

In the present study, the effects of ovariectomy on specific tension (P₀/muscle mass) were inconsistent. In an earlier study on rat diaphragm, specific tension was similar in Ovx rats, Ovx rats replaced with estrogen (Ovx+E₂), and Ovx+E₂ rats that were also given progesterone (12). However, specific tension was significantly lower in Ovx rats given only progesterone (12). In humans, specific tension in the adductor pollicis muscle was found to be higher in premenopausal than in postmenopausal women (17). Furthermore, in postmenopausal women undergoing HRT, specific tension was not different from that of premenopausal women (17). When maximum quadriceps isometric force was measured throughout the menstrual cycle in women, strength was found to be greatest during the ovulatory phase, when estrogen levels are the highest (22). Postmenopausal women who took estradiol, but not those who took estradiol with medroxyprogesterone, had greater back extensor strength than postmenopausal women on placebo (11). However, other studies have found no effects of HRT on strength measurements (6, 7, 24). It is possible that differences in HRT dose and composition (E₂ with or without progesterone) could explain conflicting findings in studies of human strength and HRT.

In the present study, TPT in EDL and RT₁/₂ for all four muscles examined were slower in Int than in Ovx animals. This pattern supports the previous finding of extended relaxation times in diaphragm in frog muscle fibers after treatment with diethylstilbestrol (a synthetic estrogen), in human quadriceps during the ovulatory phase of the menstrual cycle (22), and in diaphragm of Ovx+E₂ rats compared with Ovx animals (12). The estrogen-related increase in muscle relaxation times in the present study cannot be explained by differences in fiber type and suggests an effect of estrogen on the sarcoplasmic reticulum (SR). In the present study, relaxation times were greater in Int rats than in Ovx animals, even after treatment by hindlimb unloading. Although, in the present study, we did not observe the usual slow-to-fast change in fiber distribution, hindlimb unloading has been suggested to increase SR Ca²⁺ uptake speed by increasing the density of the SR Ca²⁺-ATPase or increasing the proportion of fast Ca²⁺-ATPase (27). In contrast, it has been reported that diethylstilbestrol directly inhibited rabbit muscle SR Ca²⁺-ATPase by interfering with Ca²⁺-binding sites (16). The seemingly additive effects of ovariectomy and hindlimb unloading on relaxation are consistent with the reported mechanisms of hindlimb unloading and estrogen-related changes in relaxation times.

In conclusion, plantar flexor muscles were larger in Ovx rats than in Int controls. Hindlimb unloading may have prevented or modified the usual endocrine response to ovariectomy that may mediate growth in ambulatory ovariectomized animals. The apparently greater hindlimb unloading-related atrophy in Sol and Plan muscles of Ovx animals compared with Int animals suggests a possible protective effect of ovarian hormones on muscle mass during disuse. Faster relaxation times in Ovx animals are consistent with the reported inhibition of skeletal muscle SR Ca²⁺ uptake by estrogen.