Ovariectomy prevents the recovery of atrophied gastrocnemius skeletal muscle mass

Mitchell Sitnick,1 Andrea M. Foley,2 Marybeth Brown,2,3,4 and Espen E. Spangenburg5,6

1Molecular, Cellular, and Integrative Physiology Graduate Group, University of California Davis; 2Physical Therapy Program, 3Center for Gender Physiology, and 4Biomedical Sciences, University of Missouri, Columbia, Missouri; 5Section of Neurobiology, Physiology, and Behavior, Division of Biological Sciences, and 6Department of Physiology and Membrane Biology, School of Medicine, University of California, Davis, California

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Address for reprint requests and other correspondence: E. Spangenburg, Univ. of California, One Shields Ave., Davis, CA 95616 (e-mail: eespanenburg@ucdavis.edu).

Skeletal muscle atrophy occurs during a number of conditions, foremost among them, reduced physical activity (7, 32). Because skeletal muscle is integral to successful locomotion, respiration, balance, and other tasks necessary for maintenance of functional independence, it is critical to understand the mechanisms that are necessary for the recovery of atrophied muscle mass. In young healthy animals, atrophied skeletal muscle mass can be recovered after an episode of tail suspension or immobilization through normal regular reloading of the muscle (6, 9, 13). It appears that a number of mechanisms contribute to this regrowth, which includes activation of satellite cells, increased gene transcription, and enhanced protein synthesis (8, 11, 13). Recently, it has been demonstrated that in aged male animals there is failure to recover atrophied muscle mass with normal loading of the muscle (11). A number of potential causes for this failure have been hypothesized, some of which include reduced circulating hormones, upregulation of inflammatory cytokines, and a failure to increase necessary growth factors.

Changes in the hormonal control of protein synthesis by estrogens or testosterone could also play a role in the failure to regrow atrophied muscle mass. Both of these hormones are known to influence skeletal mass and function, and their deficiencies can potentially lead to muscle weakness and size loss (2, 15, 23, 44, 46). At this time, a few studies have demonstrated the importance of circulating testosterone in muscle growth (23); however, no studies have critically examined the importance of ovarian hormones in muscle growth. Several studies have shown that women who are experiencing menopause develop sarcopenia earlier than men with respect to age and also fail to respond to therapy designed to induce muscle growth (45, 46). At this time, no studies have attempted to identify the impact of ovariectomy surgery on the recovery of skeletal muscle from a bout of muscle atrophy.

The initiation of protein synthesis in muscle is thought to be regulated by the Akt/mammalian target of rapamycin (mTOR) signaling pathway (20). Activation of the Akt/mTOR pathway can be stimulated either through increased muscle loading, IGF-I, or insulin stimulation (3, 13, 50). Increased kinase activity of Akt allows for the inhibition of glycogen synthase kinase-3β (GSK3β) (4), thus inducing eukaryotic initiation factor-2 activation, which is thought to be a major regulator of translation initiation (4). mTOR acts in two potential mechanisms: first by inhibiting 4E-BP1 and secondly through activation of p70S6K (4). When mTOR phosphorylates 4E-BP1, it frees eukaryotic initiation factor-4e from 4E-BP1, allowing for an increase in the rate of protein synthesis (4). mTOR activation of p70S6K results in the phosphorylation of ribosomal protein S6, which increases the translation of mRNA that contain a series of pyrimidine residues near the 5' cap structure (4). Proteins encoded by these mRNA include ribosomal proteins, translation elongation factors, and poly(A) binding protein (4).

The Akt/mTOR pathway has been shown to be upregulated during muscle growth and downregulated during muscle atrophy (13, 43). In a hypertrophic model, synergist ablation, there is an increased activation of Akt and p70S6K (3). This hypertrophy can be blocked by using rapamycin, a selective inhibitor of mTOR. The Akt/mTOR pathway has been shown to be upregulated during muscle growth and downregulated during muscle atrophy (13, 43). In a hypertrophic model, synergist ablation, there is an increased activation of Akt and p70S6K (3). This hypertrophy can be blocked by using rapamycin, a selective inhibitor of mTOR.
of mTOR (3). With overexpression of p70S6K there is an increased cell size (18), and with the genetic removal of p70S6K there is reduction in muscle mass (37). During muscle regrowth, there is increased activation of the p70S6K signaling protein associated with the onset of muscle mass recovery (13); however, in aged animals this mechanism appears to be attenuated (35). Taken together these data implicate the Akt-mTOR pathway and more specifically p70S6K as a possible target for skeletal muscle growth. Ovarian hormones, particularly circulating estrogens, have been shown to activate the Akt-mTOR signaling pathway in a variety of tissues, including cardiac muscle (40, 41). However, at this time very little is known about the impact of these ovarian hormones on Akt-mTOR signaling pathway during skeletal muscle growth.

At this time, very little is known about the role of sudden changes in hormonal status, their role in the regulation of muscle mass, and the possible interactions ovarian hormones may have with cellular mechanisms that are thought to induce muscle growth. Therefore, two hypotheses were tested: 1) the removal of circulating ovarian hormones, through ovarietomy, would alter the recovery of atrophied muscle in young animals; and 2) ovarietomy would alter the activation of the Akt/mTOR signaling pathway during the regrowth process.

**EXPERIMENTAL PROCEDURES**

**Animals.** Thirty-six mature female virgin Sprague-Dawley rats, 6 mo of age with average body weights of 278 ± 21 g, were used. All animal care and use protocols were approved by the Institutional Animal Care and Use Committee at the University of Missouri-Columbia and were consistent with National Institutes of Health guidelines. Animals were housed in a temperature-controlled room (19–21°C) with a 12:12-h light-dark cycle. Rats were provided unlimited access to standard rat chow (Purina 5008) and water.

**Ovariectomy.** Animals were anesthetized with isoflurane and placed in right lateral recumbency on a water-jacketed heating pad to maintain body temperature. The flank region was clipped and aseptically prepped with Chlorohexadine. A stab incision was made through the skin, subcutaneous tissues, and body wall ~1 cm caudal to the last rib and 2 cm ventral to the transverse process of the lumbar vertebra. The right ovary was exteriorized, and a circumferential suture was placed around the ovarian artery and vein. An incision was made just distal to the suture, and the right ovary was removed. The body wall, subcutaneous tissue, and skin were closed with 4-0 Vycril. The process was repeated on the left side. In the Sham groups, the same procedure as described was performed with the exception of ligating the ovarian artery and vein. Both ovaries were exteriorized and then replaced into the abdominal cavity. Closure proceeded as described above.

**Hindlimb unloading.** Approximately 14 days postsurgery, when body weights returned to presurgery values, muscle atrophy was induced via hindlimb unloading (HLU) via tail suspension for 28 days according to previously described methodology (34). Sham-operated and ovarietomized (Ovx) animals were divided into two groups: control or HLU. In brief, HLU animals were suspended removing load from their hindlimbs for 28 days. During this time, the animals had access to water and rat chow ad libitum. After cessation of the unloading period animals were divided into two separate groups; one group was euthanized without the limbs ever bearing any mechanical load and the second group was allowed to freely ambulate around their 18” × 18” cages for 14 days. After 14 days, the recovery animals were euthanized. Animals were ultimately divided into six groups: Sham control (Sham-Cont, n = 6), Sham-HLU (n = 6), Sham recovery (Sham-Rec, n = 6), Ovx control (Ovx-Cont, n = 6), Ovx-HLU (n = 6), and Ovx recovery (Ovx-Rec, n = 6). On the day each animal was euthanized, the medial gastrocnemius muscle was carefully excised, weighed, and immediately frozen in liquid nitrogen. The HLU and Rec animals all had pair-fed ambulatory control animals that were killed at the same time point as the HLU and Rec animals. No statistical differences were found for any measure (body mass, muscle mass, or estrogen levels) among the control animals, so all of the control animals were folded together (data not shown). The medial gastrocnemius of rat was chosen because this muscle contains a similar percentage of slow and fast fibers, making it similar to the majority of human muscle with respect to fiber-type percentages (1, 16). However, it is very possible that these results may differ if collected on muscles that are dominated by fast fibers (e.g., extensor digitorum longus) or the slow fibers (soleus).

**Serum collection and estradiol measurements.** At terminal experiment, rats were deeply anesthetized with isoflurane. Blood was collected and centrifuged at 1,500 g at 4°C and serum obtained. Serum samples were stored at −80°C until assayed for estradiol. Serum 17β-estradiol was quantified using a commercially available radioimmunoassay (RIA) kit, 3rd Generation Estradiol RIA (Diagnostic Systems Laboratories, Webster, TX).

**Protein extraction and concentration measurements.** Medial gastrocnemius muscles were homogenized on ice in buffer that contained 50 mM HEPES (pH 7.4), 4 mM EGTA, 20 mM EDTA, 15 mM sodium pyrophosphate, 100 mM β-glycerocephosphate, 0.1% Triton X-100, 25 mM NaF, 5 mM NaVO₄, 10 mg/ml leupeptin, 1.75 mg/ml aprotinin, and 1 mM PMSF. The samples were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was saved and the pellet discarded. After centrifugation, all samples were stored at −80°C. The protein concentration was determined in triplicate by the Bradford method (Bio-Rad protein assay, Hercules, CA).

**Myofibril isolation for mTOR detection.** To detect mTOR protein expression, the methodology from above was modified because in our hands mTOR expression was undetectable in the supernatant fraction (data not shown). Previously, Reynolds et al. (43) suggested that mTOR may comigrate with myosin heavy chain, thus preventing mTOR detection through normal immunoblotting. To counteract this dilemma, the investigators utilized an affinity purification method to measure mTOR through normal immunoblotting. Gastrocnemius muscles were homogenized on ice in a series of three buffers: homogenization buffer (HB) (250 mM sucrose, 100 mM KCl, 5 mM EDTA, 5 mM EGTA, 20 mM Tris, 25 mM NaF, 5 mM NaVO₄, 10 mg/ml leupeptin, 1.75 mg/ml aprotinin, and 1 mM PMSF), wash buffer (WB) (175 mM KCl, 2 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 20 mM Tris, 25 mM NaF, 5 mM NaVO₄, 10 mg/ml leupeptin, 1.75 mg/ml aprotinin, and 1 mM PMSF) and resuspension buffer (RB) (150 mM KCl, 20 mM Tris, 5 mM EDTA, 5 mM EGTA, 25 mM NaF, 5 mM NaVO₄, 10 mg/ml leupeptin, 1.75 mg/ml aprotinin, and 1 mM PMSF).

Muscle samples were first homogenized on ice in HB. Homogenates were centrifuged for 10 min at 1,000 g to pellet the myofibrils. The supernatants were removed and saved (see J on Fig. 1). The pellets were resuspended in WB, homogenized briefly, and then centrifuged again for 10 min at 1,000 g. Again, the supernatants were saved (see 2 on Fig. 1) and the pellets were resuspended in WB. After a brief homogenization, the pellets were spun for 10 min at 1,000 g. Supernatants were saved (see 3 on Fig. 1), and the pellets were resuspended in RB. A brief homogenization was followed by another 10 min spin at 1,000 g. The final supernatants were saved (see 4 on Fig. 1), and the pellets were discarded. After the isolation all supernatants were stored at −80°C. Protein concentrations were measured in triplicate using the Bradford method (Bio-Rad protein assay). It was determined that the majority of the mTOR was detected in the second supernatant (2, Fig. 1), and therefore this part of the procedure was utilized for the rest of the experiments (see Fig. 3).

**SDS-PAGE, Western blotting, and immunodetection.** Homogenates were solubilized in sample buffer (250 mM Tris-HCl pH 6.8, 30%
glycerol, 8% SDS, 10% /H9252-mercaptoethanol, and 0.02% bromophenol blue) and allowed to boil at 100°C for 5 min. Seventy-five micrograms of protein of each sample were loaded onto 7.5% SDS-PAGE gels with the following exceptions: 100 /H9262 g of protein were used for total p70 S6 kinase, Ser 235/236 phos-S6 ribosomal protein, Ser 240/244 phos-S6 ribosomal protein, and total S6 ribosomal protein. All gels run for mTOR detection were 5.0% SDS-PAGE gels. All gels were run at 150 V for 1.25 h to allow for adequate protein separation. The proteins were then transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) at 50 V for 1 h. To confirm complete transfer and equal loading of the samples, the membranes were stained in Ponceau S solution. The membranes where then blocked for 1-h rocking in 5% nonfat dry milk (NFDM) in Tris-buffered saline with 0.1% Tween-20 added (TBS-T) with the exception of p70 S6 kinase, Ser235/236 phos-S6 ribosomal protein, Ser240/244 phos-S6 ribosomal protein, and S6 ribosomal protein, which used 3% NFDM in TBS-T. Blots were then serially washed (3/5 min) and then incubated with primary antibody overnight at 4°C. The next day, the blots were serially washed again (3 × 5 min) and incubated for 1 h with secondary antibody in 5% NFDM in TBS-T. After another serial wash with TBS-T, the blots were then incubated with enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL) to detect horseradish peroxidase activity on Kodak XAR-5 autoradiographic film. Films were exposed long enough to keep integrated optical densities in a linear, nonsaturated range for each band on every membrane. Bands were then quantified by using ImageQuant densitometry Software (Amersham Biosciences, Sunnyvale, CA).

Antibodies. The primary antibodies for Ser473 phos-Akt, (1:500 dilution), Akt (1:1,000), Ser2481 phos-mTOR (1:500), mTOR (1:500), Thr389 phos-p70 S6 kinase (1:500), p70 S6 kinase (1:500) Ser235/236 phos-S6 ribosomal protein (1:100), Ser240/244 phos-S6 ribosomal protein (1:1,000), and S6 ribosomal protein (1:2,000) were purchased through Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (1:1,000–2,000).

Statistical analysis. All data are expressed as means ± SE. Statistical significance was determined by using a two-way analysis of variance for multiple comparisons followed by a Tukey’s post hoc test. A P value of <0.05 was considered significant.

RESULTS

Muscle mass measurements. Medial gastrocnemius wet weight was significantly different between the control Sham and control Ovx groups (Table 1). Sham-HLU muscle wet weight measurements were significantly reduced by 27% compared with the Sham-Con weights. After 14 days of cage ambulation, the medial gastrocnemius wet weight of the Sham-

Table 1. Body and muscle mass characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>Condition</th>
<th>Body Weight, g</th>
<th>MG, mg</th>
<th>Protein Content, mg/muscle</th>
<th>Normalized MG, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont</td>
<td>Sham</td>
<td>270±5.4</td>
<td>1,645±44.0</td>
<td>637±52.5</td>
<td>6.1±0.1</td>
</tr>
<tr>
<td>HLU</td>
<td>Sham</td>
<td>260±12.0</td>
<td>1,200±66.0*</td>
<td>374±40.7*</td>
<td>4.6±0.2*</td>
</tr>
<tr>
<td>Rec</td>
<td>Sham</td>
<td>292±8.4</td>
<td>1,613±64.0*</td>
<td>570±75.9*</td>
<td>5.5±0.1**</td>
</tr>
<tr>
<td>Cont</td>
<td>Ovx</td>
<td>311±8.1</td>
<td>1,917±30.0*</td>
<td>643±34.9</td>
<td>6.2±0.1</td>
</tr>
<tr>
<td>HLU</td>
<td>Ovx</td>
<td>314±11.3</td>
<td>1,513±31.0*</td>
<td>463±51.2*</td>
<td>4.8±0.1‡</td>
</tr>
<tr>
<td>Rec</td>
<td>Ovx</td>
<td>302±11.9</td>
<td>1,502±72.3*</td>
<td>457±20.4*</td>
<td>4.9±0.2‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. MG, medial gastrocnemius; Cont, control; HLU, hind limb unloaded; Rec, recovery; Ovx, ovariectomized. *Statistically different (P < 0.05) from the Sham-Cont group. †Statistically different (P < 0.05) from the Sham-HLU group. ‡Statistically different (P < 0.05) from the Ovx-cont group.
Rec group returned toward control levels. Gastrocnemius wet weight for Ovx-HLU muscle was reduced significantly by 21% compared with the Ovx-Cont group. After 14 days of cage ambulation, the Ovx-Rec medial gastrocnemius wet weight still was significantly reduced by 21% compared with the Ovx-Cont group.

The Ovx-Cont group demonstrated a significant gain in overall body weight by 15% compared with the Sham-Cont group. Therefore, medial gastrocnemius muscle mass was also normalized to body weight to compare among groups. No differences in normalized muscle mass (muscle mass/body mass) were detected between the Sham-Cont and Ovx-Cont groups. Sham-HLU saw a significant 24% decrease in normalized medial gastrocnemius muscle mass from the Sham-Cont group (Table 1). After 14 days of recovery, the Sham-Rec normalized medial gastrocnemius muscle mass was significantly reduced compared with the Sham-Cont levels by 9% but significantly elevated by 19% compared with the Sham-HLU group. Therefore, in the Sham animals when normalized to body weight, the medial gastrocnemius muscle mass did not totally recover after 14 days of cage ambulation, but animals still demonstrated significant regrowth of the medial gastrocnemius muscle compared with the Sham-HLU group. With respect to the Ovx groups, normalized medial gastrocnemius mass of Ovx-HLU animals was significantly reduced by 22% compared with the Ovx-Cont group. Furthermore, normalized medial gastrocnemius mass of the Ovx-Rec group was significantly reduced by 19 and 9% compared with both the Ovx-Cont and Sham-Rec groups, respectively.

Total protein content was measured in the medial gastrocnemius as another marker of muscle mass. The Sham-HLU group demonstrated significant reductions of 41% in total protein compared with Sham-Cont group, whereas the Sham-Rec group exhibited no differences from the Sham-Cont group (Table 1). With Ovx, both HLU and Recovery groups’ protein levels were significantly diminished from Ovx-Cont values as evident by a 28 and 29% drop in protein levels.

Estradiol measurements. Serum estradiol in Ovx rats were significantly lower (67%) compared with the sham-operated animals. There was no significant difference in serum estradiol levels between sham-operated animals subjected to HLU or recovery or those kept ambulatory (data not shown).

Akt phosphorylation. Phosphorylation of Akt/mTOR signaling pathway was measured to determine whether mechanisms that regulate protein synthesis during muscle regrowth were fully functional with and without ovariectomy surgery. Akt phosphorylation was determined through immunoblotting for the phosphorylation status of residue Ser-473 on Akt. No differences were detected between the phosphorylation status and total Akt expression of the Sham groups of animals (Fig. 2). However, Ovx animals that were HLU had a significant decrease of 60 and 61% in Akt phosphorylation compared with the Sham-Cont or Sham-Rec groups, respectively. Akt phosphorylation was also reduced by 51 and 64% in the Ovx-Rec group compared with the Sham-Cont and Sham-Rec groups, respectively. No differences were detected for total Akt content among any of the groups.

When the changes in Akt were normalized as a ratio (phosphorylated/total), the same trend was observed, in that there was a nonsignificant decrease in Akt in Sham-HLU compared with the Sham-Cont and Sham-Rec groups (data not shown).

No difference was detected between the Sham-Cont and Sham-Rec groups. However, there was a significant ~69% decrease in the Akt ratio in the Ovx-HLU, which remained significantly depressed by 63% in the Ovx-Rec group compared with the Ovx-Cont group (data not shown).

mTOR phosphorylation. mTOR phosphorylation was determined through immunoblotting for the phosphorylation status of residue Ser-2481 on mTOR. Most studies have measured phosphorylation of Ser-2448 on mTOR, which has been thought to be phosphorylated by Akt (38, 39, 43). However, two recent studies by independent laboratories have suggested that Ser-2481 is phosphorylated by p70S6K and not by Akt (12, 25). In contrast, in a prior investigation, mutation of the Ser-2481 site of mTOR (preventing phosphorylation of the site) resulted in decreased p70S6K activity, suggesting that this residue on mTOR may regulate action upstream of p70S6K (42). Therefore, phosphorylation of site 2481 was determined. No differences were detected in the ratio of mTOR (phosphorylated/total) between any of the Sham or Ovx groups of animals (Fig. 3). However, Ovx animals that were HLU had a significant decrease in total mTOR compared with the Ovx-Cont group (data not shown).

p70S6K phosphorylation. The p70S6K phosphorylation was determined through immunoblotting for the phosphorylation status of residue Thr-389 on p70S6K. No significant differences were detected in p70S6K phosphorylation levels between the Sham-Cont group and the Sham-HLU group; however, there were significant increases of 230% in the Sham-Rec group compared with the Sham-Cont group (Fig. 4). In the Ovx groups, there were significant decreases in the levels of p70S6K phosphorylation in Ovx-HLU and Ovx-Rec animals compared with the Ovx-Cont group, a decline of 79 and 58%, respec-
tively. More so, Ovx-HLU and Ovx-Rec groups were both significantly lower by 91 and 82% compared with the Sham-Rec group, respectively. No significant different were detected between groups in the total content p70s6k.

When the changes in p70s6k were normalized as a ratio (phosphorylated/total), a similar trend was observed, in that there was a nonsignificant increase in p70s6k in Sham-HLU and a significant increase in the Sham-Rec compared with the Sham-Cont (data not shown). However, there was no change in the p70s6k ratio in the Ovx-HLU and significant depression by 60% in the p70s6k ratio of the Ovx-Rec group compared with the Ovx-Cont group (data not shown).

**S6 phosphorylation.** Ribosomal protein S6 phosphorylation was measured as an indicator of p70s6k activity, because S6 is a major substrate of p70s6k (5). Sham-HLU led to a significant 73% decrease in Ser240/244 phosphorylation of S6 ribosomal protein (Fig. 5) compared with Sham-Cont levels. However, no significant differences were detected in Ser235/236 phosphorylation levels of S6 between the Sham-Cont and Sham-HLU. The Sham-Rec group demonstrated significant increases of 99 and 54% in both Ser235/236 and Ser240/244 phosphorylation levels of S6 ribosomal protein compared with the Sham-Cont animals. Conversely, the Ovx-HLU group exhibited decreases of 45 and 64% for Ser235/236 and Ser240/244 phosphorylation levels compared with the Ovx-Cont group animals. Moreover, the Ovx-Rec group failed to increase Ser235/236 and Ser240/244 phosphorylation levels compared with the Ovx-HLU group, and these phosphorylation levels remained significantly less than that detected in the Ovx-Cont group. With respect to total S6 content, both Ovx-HLU and Ovx-Rec had significantly higher levels of total S6 ribosomal protein content compared with both the Ovx-Cont and Sham-Cont.

**DISCUSSION**

Here we demonstrate for the first time that the presence of ovarian hormones is critical for the regrowth of atrophied skeletal muscle in mature female animals. Specifically, we found that animals that had undergone Ovx surgeries failed to recover atrophied muscle mass induced by HLU. This failed recovery of atrophied muscle mass was associated with reduced activation of the Akt, p70s6k, and downstream substrates of p70s6k. These data suggest that surgical removal of the ovaries may alter the optimum physiological environment, thus preventing muscle regrowth from a bout of atrophy. This failed regrowth is associated with incomplete activation of the necessary signaling proteins that are critical for the initiation of protein translation.
In young women estrogen deficiency is rarely a problem, although in some cases it is necessary for women to undergo ovariectomy at a young age for various reasons such as ectopic pregnancy, endometriosis, cancer, or pelvic inflammatory disease. However, as women age they enter menopause, resulting in decreased production of circulating estrogens by the ovaries, and the Ovx model in rodents is commonly used to mimic menopause in women (2, 33, 49, 51). The data presented here would suggest that ovariectomy surgery may result in adverse consequences on the physiological function of the skeletal muscle. The loss of circulating ovarian hormones due to the Ovx results in a failure of reloading to activate the Akt-mTOR pathway in the gastrocnemius muscle, thus preventing muscle regrowth. Also, the Ovx surgery results in enhanced growth of medial gastrocnemius muscle mass in absolute terms, which agrees with a prior publication (19). This enhanced growth is not present when normalized to body mass, indicating that this growth is due to increases in overall body mass, which agrees with the prior publication (19). Finally, these data suggest that this growth is occurring independent of the Akt-mTOR signaling pathway in that no changes in phosphorylation levels were detected between the Sham-Cont and Ovx-Cont groups.

Unfortunately, some published data indicate that postmenopausal women do not always respond to exercise training regimens designed to improve strength and muscle mass (44, 45). In addition, multiple studies have found that estrogen therapy combined with increased levels of physical activity counteracted the development of age-related sarcopenia better than physical activity alone (15). Thus hormone therapy combined with mechanical loading of the muscle appears to prevent sarcopenia in women; however, the mechanisms that underlie the actions of estrogens in the recovery from a bout of atrophy remain untested. Although there are two types of estrogen receptors, alpha and beta, skeletal muscle specifically expresses the alpha subtype (29–31). The role of the estrogen receptor in skeletal muscle function is unclear; it does appear that estrogens do not affect mouse or rat satellite cell proliferation or differentiation (27), therefore suggesting that if estrogens play a role in muscle growth, it is independent of satellite cell physiology. It should be noted that removal of the ovaries may result in the reduction of other hormones released by the ovaries, such as inhibin, activin, or follistatin, that could have a direct impact on satellite cell function. If these hormones affect satellite cell function, the regrowth process could be altered by failed activation of the satellite cells. Activin and/or follistatin expression have been detected in cultured satellite cells (28), and recent data suggest that follistatin may directly affect satellite cell function during muscle regeneration (26).

The loss of circulating estrogens due to the Ovx surgery appears to have a direct impact on skeletal muscle function, and this is supported by the fact that the estrogen receptor alpha is expressed in muscle.

Recently, a number of investigations have begun to suggest that estrogens may in fact exert their effects through non-genomic events (14). Indeed, estrogens are capable of activating Akt- and MAPK-dependent signaling in various cancer lines (10, 17). With respect to striated muscle, estradiol has been suggested to prevent apoptosis in cardiac myocytes by activating Akt-dependent signaling (40), and raloxifene, an estrogen receptor modulator, has been shown to alter cardiac function by influencing Akt activity (36). Here, we find that load-induced activation of p70^6k is compromised when circulating estrogens are low. The reduction in p70^6k activation does not appear to be due to failed mTOR activation; thus this would suggest that failed recovery of muscle mass is not due to changes in mTOR activity. Although there does appear to be a loss of mTOR expression in the Ovx-HLU animals, the physiological consequence of this effect is unclear at the moment. The p85 subunit of PI3-K has been shown to interact with the estrogen receptor alpha in endothelium cells regulating the activation of PI3-K (47); therefore it is possible that a reduction in PI3-K activity may result in reduced p70^6k activity independent of mTOR. This possibility is not without precedence in that other investigations have found p70^6k activation without concurrent mTOR activation (5). However, it is equally possible that mTOR activation may be time dependent and we have not chosen the appropriate time point for measurement and simply missed the activation of mTOR. In addition, this may be true for the other signaling proteins as well, in that time points chosen for the specific measurements may have not been optimal for every signaling protein measured. Furthermore, it is possible that mTOR activity is being modulated by one of its binding partners, such as RAPTOR, although this remains to be tested at this time. Although it is unclear how estrogens are affecting the phosphorylation of p70^6k, it does appear that it may involve the reduction in upstream signaling through the estrogen receptor alpha. One may speculate that the presence of circulating estrogens provides a potential “priming” mechanism that is necessary for complete activation of p70^6k under conditions of increased mechanical loading. This suggestion is not completely unreasonable, because other investigators have suggested that a priming mechanism may exist for p70^6k and this mechanism may be critical for complete activation of the kinase (22). The reduction in phosphorylation of p70^6k was coupled with a reduced phosphorylation of ribosomal protein S6, which is a substrate of p70^6k (5). However, it is possible that the activity of other kinases has been affected by the Ovx surgery, resulting in reduced phosphorylation of S6. For example, in the Sham animals only one phosphorylation (Ser240/244) site showed a reduction in phosphorylation levels during HLU, suggesting that the same kinase may not be responsible for the phosphorylation of both sets of residues on S6. This is plausible because other kinases can phosphorylate S6 as well. However, it should be noted that in the Ovx-HLU animals both sets of residues demonstrated reduced levels of phosphorylation, indicating the potential importance of the ovarian hormones to muscle growth.

These data also demonstrate minimal changes in Akt, mTOR, or p70^6k phosphorylation after 28 days of hindlimb unloading in the Sham animals. With respect to the literature, these findings are contradictory in some cases (3, 13, 48), but other studies do in fact support the lack of a decrease in phosphorylation of Akt, mTOR, or p70^6k during spinal cord isolation (21, 48). Therefore, it appears at this time that the published results indicate that the changes in the Akt-mTOR signaling pathway during muscle atrophy are somewhat equivocal, although it is not unreasonable to speculate that these changes may be dependent on the model chosen to induce muscle atrophy and/or the time point at which the measures were made. Specifically, a number of these studies have used very different models (e.g., denervation, hindlimb suspension, immobilization) for different lengths of time, all of which may
account for the differences detected in these studies. Also, the results could be dependent on the muscle in which the measurements were made in that a majority of the studies made the measurements in the soleus, whereas our measurements were made in the medial gastrocnemius. In support of this, Hilder et al. (24) found significant reductions in Akt phosphorylation in the soleus muscle after hindlimb suspension and no changes in Akt phosphorylation in the gastrocnemius muscle. Interestingly, our data suggest that, because there was a reduction in phosphorylation of Akt and p70^S6k in the Ovx-HLU group compared with the Sham-HLU group, ovarian hormones may play a role in the maintenance of the phosphorylation levels of these signaling proteins during the onset of muscle atrophy.

This study found that Ovx surgery resulted in a failed recovery of atrophied muscle mass. In addition, this failed recovery appears to be associated with lack of activation of the Akt-p70^S6k signaling proteins. These data suggest that important considerations should perhaps be made in women who had ovariectomy surgeries or in postmenopausal women when prescribing therapy treatments aimed at recovery muscle mass in women who may have undergone long-term bouts of physical inactivity.

**GRANTS**

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