Estrogen receptor-α and -β and aromatase knockout effects on lower limb muscle mass and contractile function in female mice

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Submitted 14 August 2008; accepted in final form 27 January 2009

Brown M, Ning J, Ferreira JA, Bogener JL, Lubahn DB. Estrogen receptor-α and -β and aromatase knockout effects on lower limb muscle mass and contractile function in female mice. Am J Physiol Endocrinol Metab 296: E854–E861, 2009. First published January 27, 2009; doi:10.1152/ajpendo.90696.2008.—Estrogen (E2) exerts its effects through estrogen receptor-α (ERα) or -β (ERβ) and -mediates its effects. The need to identify the subtype of ER associated with muscle function motivated this research.

Two ER subtypes, ERα and ERβ, mediate E2 signaling, and they function as ligand-dependent transcription factors (11, 14, 35–37, 45). In addition to ligand-dependent ER activation, the ER can also be activated independently of E2, e.g., by growth factors (14). For example, IGF-1 phosphorylation of Akt is dependent on ERα in breast cancer cells (52).

Both ERs have been identified in skeletal muscle (19–21, 26–28, 50), but their role in the control of skeletal muscle function is poorly understood. To date, there are no studies investigating the loss of ERα on skeletal muscle contractile function and only one study (13) that explored the effect of ERβ knockout (KO) on skeletal muscle. Thus the potential ERα influence on muscle mass and contractile function is unknown, and results evaluating ERβ loss on muscle tissue are limited. Consequently, the primary purpose of the present study was to examine the potential consequences of ERα and ERβ loss on muscles with different architecture, fiber type, and role: soleus (Sol), plantaris (Plan), gastrocnemius (Gast), and tibialis anterior (TA) in mature female mice.

Kahlert et al. (20) reported that skeletal myoblasts contain E2 receptors that, when stimulated by estrone, show significant growth; both estrone and 17β-estradiol induce expression of transcription factors. Accordingly, we hypothesized that ERs play a role in skeletal muscle by ligand-dependent activation. However, Kahlert et al. (20) did not identify how estrone interacted with ERs, binding either one receptor type or both. Based on the previous reports that ERβ is highly expressed in many nonclassical E2 target tissues (46) and that ERα is highly expressed at the mRNA level but not the protein level in skeletal muscle (50), we hypothesized that ERα KO may not influence skeletal muscle and that muscle contractile properties and muscle mass in ERα KO mice would be comparable with those of corresponding wild-type (WT) mice. In addition, we hypothesized that muscle mass, fiber areas, tetanic tension, and protein in ERβ KO mice would be diminished compared with the WT control mice.

It has been reported that ERα and ERβ KO animals have twofold or more serum E2 than WT mice (16). As it is possible

**ESTROGENS (E2)** exert diverse physical effects in many tissues, including the female reproductive tract, mammary tissues, and cardiovascular, immune, nervous, and skeletal systems (14, 41, 42). Human and animal studies (6, 8, 24) have revealed estrogen receptor (ER) involvement in female sexual development and behavior, reproductive function, immune function, regulation of the neuroendocrine and cardiovascular systems, and bone metabolism.

In contrast to other tissues, relatively little is known about the effects of E2 on skeletal muscle (3) and the effects of E2 on skeletal muscle have been controversial. Previous studies (1, 7, 23, 47) have shown that E2 reduces skeletal muscle damage, hypothetically by stabilizing the muscle membrane. Other researchers (33, 39, 48) suggest that E2 has an anabolic effect on muscle. Conversely, several groups (18, 22, 32, 44) have reported that E2 administration to previously ovariectomized (OVX) rats decreases muscle mass and fiber size and reduces the maximal isometric contraction force.

Findings from our laboratory, and one other, indicate that E2 is important (12) and necessary for the restoration of atrophic muscle (2, 31, 43). For E2 to be considered seriously as an adjunct in rehabilitation as an anabolic agent for atrophic muscle, its safety must be assured. Hence, the development of a compound with the anabolic properties of E2 without the detrimental side effects requires understanding which ER mediates its effects. The need to identify the subtype of ER associated with muscle function motivated this research.

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It has been reported that ERα and ERβ KO animals have twofold or more serum E2 than WT mice (16). As it is possible
that E2 exerts its effects on skeletal muscle via indirect pathways that bypass the ER, we also chose to examine the consequences of E2 deficiency in mature aromatase (Ar) KO and WT control mice, which have been examined only to a limited extent by one group of investigators (30). Aromatase is an enzyme of the cytochrome P450 superfamily and functions to aromatize androgens to produce E2.

METHODS

Animals. Mature female ERα, ERβ, and ArWT and ArKO mice bred and maintained by the Lubahn laboratory in a C57BL6/J background were used for this study. Mice were 5–8 mo of age at the time of study to avoid the confound of characterizing animals in the rapid growth phase of development. The protocols used to generate the KO models have been published (17, 25, 29). Briefly, for ERα mice a Neo gene was targeted to the N-terminal of exon 2 to disrupt the reading frame of the o-receptor in embryonic stem cells. The KO mice were made on a 129 background. Because of genetic variations that result from difference in the number of backcrosses into the C57BL6 background, we always compare KO mice to WT littermates or WT mice from the same genotype. For ERβ mice, a similar process was used in that a Neo gene driven by a PGK promoter was inserted in the reverse orientation into the Pst I site in the first zinc finger in exon 3 and completely removes all E2 response element binding activity. ArKO mice were created by deletions of exons 1 and 2 of the cyp19 gene. Since this is a complete KO of all enzyme activity and there is only one aromatase gene in mice, this mouse is completely devoid of estradiol. KO and WT status was repeat verified at the end of all experiments using DNA from tail snips.

The protocols used for this study complied with the guidelines of the American Physiological Society. The study was approved by the University of Missouri Institutional Animal Care and Use Committee (protocol no. 4179).

Contractile properties. As type I fiber dominant muscles are reported to have more ER than type II dominant muscles (27), muscles with different fiber type proportions, function, and likely distribution of ER were chosen for study. To determine contractile properties, mice were anesthetized with pentobarbital sodium (0.15 ml pentobarbital with 0.85 ml saline) with 0.15 ml as first injection and anesthesia being and was determined for right unstimulated muscles. To do the experiment using DNA from tail snips.

For contractile testing, the left hindlimb and mouse torso were rigidly immobilized and muscles were attached in the order of Sol→Plan→Gast→Sol because of their lower Pn/CSA and for ERα Plan because of the significantly higher Po. Before the muscle samples were loaded, the protein homogenates from the BCA protein assay were diluted with equal parts of Laemmli buffer containing β-mercaptoethanol and heated to 100°C for 3 min as described by Moran et al. (34). Ten micrograms of total protein from each muscle were separated on a Bio-Rad mini gel (7.5% SDS-PAGE Tris·HCl) with an actin myosin control (Bio-Rad) as a standard. Gel electrophoresis was performed in SDS running buffer (12.1 g Tris, 11.3 g glycine, and 1 g SDS) until the tracking dye ran off the gel (Laemmli). The gel was stained overnight with Coomassie blue (0.1% Coomassie blue R 250, 40% methanol, and 10% glacial acetic acid) and destained with Coomassie destain (40% methanol and 10% glacial acetic acid). Gels were dried overnight using gel drying materials (Promega gel drying kit). Dried
gels were scanned using HP Scan Jet software and subsequently analyzed using the Kodak 1D 3.6 program for densitometric analysis.

Statistical analysis. Because the research question of interest was focused on differences between WT and KO groups, mean differences were analyzed using the two-tail Student’s t-test. Statistical significance was set at \( P < 0.05 \). When muscle weights were used to derive ratio data (e.g., muscle weight-to-body weight ratios), weights from the right (unstimulated) muscle were entered.

RESULTS

Body weight and muscle mass. ER\( \alpha \) KO animals had \( \sim 10\% \) higher body weight (\( P < 0.005 \)) than WT mice (Fig. 1). ArKO mice were \( \sim 20\% \) larger than ArWT, which was also significant (\( P < 0.001 \)).

TA muscle mass was \( \sim 15\% \) higher in ER\( \alpha \) KO mice (\( P < 0.05 \)) than in WT mice. There were no significant differences in ER\( \beta \) KO Gast, TA, Plan, or Sol muscle mass compared with WT litter mates. Consistent with the larger body mass, muscles from ArKO mice tended to be larger (7–14%) but differences were not statistically significant (Fig. 2).

Muscle weight-to-body weight ratio. Although ER\( \alpha \) KO status increased absolute TA muscle mass, the ratio of muscle mass to body mass eliminated the significant difference. Gast and TA in Ar KO mice had smaller ratios than WT (Fig. 3). In most animals, muscle size was a reflection of body size, but for Ar KO mice, general body growth did confer comparable muscle growth for the TA or Gast (\( P < 0.05 \)).

In situ contractile properties. Twitch tension, +dP/dt, absolute \( P_o \), and \( P_o/\text{anatomical aCSA} \) for Sol, Plan, Gast, and TA are presented in Table 1 and in Figs. 4 and 5. Plantaris \( P_o \) was significantly higher in ER\( \alpha \) KO (\( \sim 30\% \)) than in WT controls, but this significant difference disappeared when \( P_o/\text{aCSA} \) was examined. The nearly significant reduction in ER\( \alpha \) KO Gast \( P_o \) (\( P = 0.059 \)) did become significant when tension was expressed per aCSA (Fig. 5) and when expressed per fiber area (Table 2). TA \( P_o \) for ER\( \alpha \) KO approached significance (\( P = 0.084 \)) and became significant with the ratio of \( P_o/\text{aCSA} \) (\( P < 0.05 \)) and as the ratio of \( P_o/\text{fiber area} \) (Table 2; Fig. 5). These findings indicate that the ER\( \alpha \) KO Gast and TA could not produce as much force per unit area as a WT mouse. There were no additional ER\( \alpha \) KO effects on contractile properties.
For ERβ mice, there were no significant effects of KO status on contractile measures (Table 1; Figs. 4 and 5).

Although ArKO mice had significantly higher body masses, absolute peak tensions did not reflect greater muscle demand (Fig. 4). The ArKO Gast and TA had significantly lower Po/aCSA values compared with WT, which was not reflected in Po/fiber area (Table 2).

A summary of contractile properties is given in Table 3.

Muscle protein content. There was no significant difference between KO mice and their WT controls for muscle total (g/g) protein content (data not shown).

Myosin content. Even though there was a decline in tension/aCSA for ERβ TA and Gast and ArKO Gast and an increase in ERβ KO Pla, there were no significant differences in myosin content for any of the KO groups compared with WT (Fig. 6).

**DISCUSSION**

Several investigators (12, 30, 34) have found a loss in muscle mass and/or contractile tension in E2-deficient animals, suggesting that E2 may affect quality and quantity of the contractile proteins actin and myosin. Findings from this study support these results in that lack of E2 had a detrimental effect on muscle contractile quality but not for every muscle studied. We expected to find clear differences in muscle function

### Table 1. Twitch tension and −dP/dt

<table>
<thead>
<tr>
<th>Muscle</th>
<th>ERα WT (n = 13)</th>
<th>ERβ WT (n = 17)</th>
<th>ERβ KO (n = 11)</th>
<th>ArWT (n = 8)</th>
<th>ArKO (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>P₀, g</td>
<td>5.1±0.5</td>
<td>3.6±0.3</td>
<td>2.8±0.4</td>
<td>3.3±0.5</td>
<td>3.8±0.4</td>
</tr>
<tr>
<td>dP/dt, g/s</td>
<td>2.24±0.84</td>
<td>2.533±0.72</td>
<td>2.539±124</td>
<td>2.686±105</td>
<td>2.404±98</td>
</tr>
<tr>
<td>Plantaris</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P₀, g</td>
<td>9.4±0.9</td>
<td>11.4±0.8</td>
<td>8.3±1.1</td>
<td>8.1±1.0</td>
<td>7.6±1.1</td>
</tr>
<tr>
<td>dP/dt, g/s</td>
<td>2.705±100</td>
<td>2.698±82</td>
<td>2.711±123</td>
<td>2.883±115</td>
<td>2.436±92</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>P₀, g</td>
<td>29.8±3.1</td>
<td>28.1±2.2</td>
<td>23.3±3.2</td>
<td>26.4±2.3</td>
<td>22.7±2.6</td>
</tr>
<tr>
<td>dP/dt, g/s</td>
<td>4.072±214</td>
<td>3.815±167</td>
<td>3.414±251</td>
<td>3.402±251</td>
<td>3.975±215</td>
</tr>
<tr>
<td>Tibialis anterior</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P₀, g</td>
<td>21.9±1.6</td>
<td>20.5±1.6</td>
<td>17.1±1.8</td>
<td>16.2±1.8</td>
<td>17.0±1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. ER, estrogen receptor; Ar, aromatase; P₀, twitch tension; −dP/dt, peak rate of tension development; WT, wild type; KO, knockout.
among one or more groups of KO mice, but the interpretation of our current results shows that the story is more complex. Recently, Moran et al. (34) demonstrated in the mouse extensor digitorum muscle that the loss of E2 diminished $P_o$, the consequence of altered actin:myosin interaction. Subsequently, Moran et al. (33) restored $P_o$ and actin-myosin binding characteristics in OVX E2-deficient mice by giving them estradiol. These findings strongly support the need for E2 to maintain optimal muscle contractile function. Our results also indicate that E2 has effects on peak tension that may be mediated through the α-receptor.

The presence of ER in skeletal muscle suggests that skeletal muscle is a target tissue for E2 (9), but whether E2 impacts skeletal muscle tissue through ERα or ERβ or through other

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**Fig. 4.** Absolute peak tetanic tension (g) in soleus, plantaris, gastrocnemius, and tibialis anterior. *$P < 0.05$.

**Fig. 5.** Peak tetanic tension ($P_o$) expressed per derived anatomical cross-sectional area (CSA; g/cm²). *$P < 0.05$.
When IGF-1 levels are low, there is an increase in body and muscle mass (growth hormone) for the increase in body and muscle mass in the mouse, indicating that a different mechanism (e.g., increased IGF-1) may be responsible for the observed effects on contractile function in ERα KO female mice. Changes in ERα-to-ERβ ratios or lacking ERα and ERβ heterodimer formation (15) in ERα KO mice could have effects on Po. However, these may not be the only factors explaining the decreased Po/aCSA in ERα KO mice, as only two muscles of four were different between the ERα KO and WT groups (Table 3). Potentially, the elevated circulating estradiol levels in ERα KO female mice (4, 5) may generate signaling via the remaining ERβ or through other ERs (10, 38) to regulate skeletal muscle contractile tension.

Table 2. Gast and TA muscle fiber area and P./fiber areas

<table>
<thead>
<tr>
<th>Muscle</th>
<th>ERα WT (n = 12)</th>
<th>ERα KO (n = 8)</th>
<th>ER WT (n = 6)</th>
<th>ERβ KO (n = 6)</th>
<th>ArWT (n = 3)</th>
<th>ArKO (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gast</td>
<td>2.207±0.007</td>
<td>2.576±0.007</td>
<td>2.760±0.009</td>
<td>2.237±0.009</td>
<td>1.674±0.013</td>
<td>1.377±0.011</td>
</tr>
<tr>
<td>Muscle area, μm²</td>
<td>0.050±0.001</td>
<td>0.037±0.005</td>
<td>0.036±0.001</td>
<td>0.038±0.012</td>
<td>0.045±0.013</td>
<td>0.047±0.011</td>
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<tr>
<td>TA</td>
<td>1.914±0.001</td>
<td>2.052±0.001</td>
<td>2.412±0.001</td>
<td>2.003±0.001</td>
<td>1.707±0.001</td>
<td>1.674±0.005</td>
</tr>
<tr>
<td>Muscle area, μm²</td>
<td>0.040±0.001</td>
<td>0.027±0.001</td>
<td>0.027±0.006</td>
<td>0.027±0.006</td>
<td>0.039±0.010</td>
<td>0.039±0.009</td>
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Values are means ± SE. P., peak tetanic tension; Gast, gastrocnemius; TA, tibialis anterior. Area differences were not significant. P./fiber area: *P < 0.05.

Table 3. Summary of muscle contractile function differences in KO mice compared with WT controls

<table>
<thead>
<tr>
<th>Muscle</th>
<th>ERα KO</th>
<th>WT KO</th>
<th>ERα KO</th>
<th>WT KO</th>
<th>ERα KO</th>
<th>WT KO</th>
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<tbody>
<tr>
<td>Soleus</td>
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<tr>
<td>Gastrocnemius</td>
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<td>Tibialis Anterior</td>
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</table>

Table 4. Summary of myosin content differences in KO mice compared with WT controls

<table>
<thead>
<tr>
<th>Muscle</th>
<th>ERα KO</th>
<th>WT KO</th>
<th>ERα KO</th>
<th>WT KO</th>
<th>ERα KO</th>
<th>WT KO</th>
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<tbody>
<tr>
<td>Gast</td>
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<tr>
<td>TA</td>
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Fig. 6. Total myosin protein in arbitrary units. There were no differences in total myosin content between groups.
Although loss of ERα decreased Gast and TA muscle P0/αCSA and P0/fiber area, total protein and myosin content were similar in ERα KO and WT mice, which is consistent with the findings of Moran et al. (34). They revealed in OVX rats a lower extensor digitorum longus P0 resulting from a smaller fraction of strongly bound myosin, but total myosin levels were comparable for OVX and intact mice. Their finding suggests that loss of ERα in our current study may have affected the fraction of strong-binding myosin in the fast-fibered Gast and TA muscles. Alternatively, the loss of ERα may have altered calcium dynamics in muscle, an entirely different mechanism that could affect muscle function, particularly myosin binding (33).

There are other factors that could affect contractile quality that should be examined in future studies, such as diminished extracellular matrix proteins, alterations in the structural framework of the sarcolemma or Z-line, and an increase in intramuscular fat and/or connective tissue.

**ERβ KO effects on skeletal muscle tissue.** No variables were affected by the loss of ERβ. The only muscle of the four we examined that has been studied heretofore in ERβ mice is the Sol. Contractile function results from the present study are compatible with the previous report of Glenmark et al. (13), who indicated that there were no Sol contractile tension differences between WT and ERβ KO female mice. The lack of ERβ effect suggests that ERα can compensate for the loss and/or muscle ERβ is involved in other functions. Further studies are needed to verify these possibilities.

**Aromatase−/− deficiency effects on skeletal muscle.** Of the three conditions studied, loss of aromatized E2 had the greatest effect on body and muscle mass, which is consistent with our prior and other prior findings for OVX rats (12, 40). Our findings are not, however, consistent with those of MacLean et al. (30), who reported no differences for female ArKO mice in either body or muscle mass, when KO mice were compared with WT controls. It is probable that the 9-wk-old mice used in the Maclean et al. (30) study had not yet reached their mature body or muscle mass. It is unclear whether the cre-lox method used by MacLean et al. (30) to produce an ArKO mouse had a differential mass effect than the breeding process used in this study.

Our mice exhibited an increase in body and muscle size consistent with general body growth and suggestive of increased IGF-1 (12). Although ERα KO mice were found to have lower IGF-1 values (49), IGF-1 values for ArKO mice were unchanged (30). Regardless of the mechanism for the increase in size, there was no advantage conferred by this increase, as muscle mass normalized to body mass in ArKO was comparable with WT mice.

Why the Gast and TA muscles were more sensitive to E2 loss than the other muscles examined is unknown, but possibilities include a higher ER density, greater dependence on E2 for contractile protein synthesis (e.g., actin, myosin) than other hormones, and higher susceptibility to E2 mediated actin-myosin interaction. We expected to observe greater deficits in contractile tension in ArKO mice than we did, which suggests that growth factors other than E2 may have affected the study outcomes.

In summary (see Table 3), loss of ERα resulted in increased body mass and TA muscle mass, a higher force production in Pla, and a reduction in Gast and TA muscle force per anatomical CSA. Knocking out ERβ had no effects on any of the variables studied. Loss of aromatized E2 resulted in a large body mass increase with a concomitant increase in muscle size such that muscle mass normalized to body mass was comparable with WT mice. ArKO Gast and TA had lower P0/αCSA. There were no changes in total protein or myosin, but contractile tension results for TA and Gast suggest altered cross-bridge mechanics.

Taken together, our results indicate a complex effect of the removal of ERα and aromatase on skeletal muscle with a clear need for further investigation. Our data support the hypothesis that ligand-dependent and ligand-independent effects of ERs are responsible for the complex outcomes observed. Pharmacological intervention to attenuate the loss of muscle mass and strength with menopause will be complex.

**ACKNOWLEDGMENTS**

Funding from this study came from the Missouri Spinal Injuries Research Board, the University of Missouri Research Board, National Institute of Child Health and Human Development Grant HD-058834, National Institute of Environmental Health Sciences Grant PO1 ES-10535, the National Center for Complementary and Alternative Medicine RO1 AT-002978, and the University of Missouri Center for Phytonutrient and Phytochemical Studies.

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