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

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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 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.

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

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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 was maintained with a 0.05-ml injection given as needed. Each mouse was placed sidelying on a water-jacketed heating pad that maintained body temperature at 37°C. The left Sol, Plan, Gast, and TA muscles were surgically exposed only at their insertions. Sol, Plan, Gast, and TA muscles are uni- or multipennate; span one or more joints; vary from 40–95% type II myosin heavy chain distribution; function as locomotor, postural, or antigravity muscles; and likely have varying proportions of ER. The distal tendon of each muscle was attached in turn to Grass force transducer with 4.0 silk. Tibial and peronal nerves were isolated and placed in turn on a bipolar stimulating electrode. Preliminary study revealed 6V to be supramaximal for each type muscle. Force curves generated at 15, 50, 75, 100, and 125 Hz for each type muscle. The exposed tendon of each muscle and the nerves were bathed continuously with 37°C mineral oil.

For contractile testing, the left hindlimb and mouse torso were rigidly immobilized and muscles were attached in the order of Sol→Plan→Gast→TA to a force transducer by the distal tendon and adjusted in length so that passive tension was 0 g. A twitch was obtained at that position with the parameters: 0.5 ms, 0.3 Hz, at 6 V; subsequently, the micromanipulator was used to progressively lengthen each muscle to the point where peak twitch was attained (L₀). At optimal length, a peak tetanic contraction (P₀) was elicited by pulses delivered at 150 Hz, 300-ms duration, and an intensity of 6 V for each type muscle. Preliminary study revealed 6V to be supramaximal; the 300-ms duration was greater than what was required to achieve P₀. Force curves generated at 15, 50, 75, 100, and 125 Hz revealed that all muscles were maximally recruited usually by the time 100 Hz was reached. All data were collected using Power Lab. The peak rate of tension development (+dp/dt) was obtained from the steepest linear portion of the P₀ curve. The duration of contractile function testing was ~15 min.

In pilot studies, random testing of muscles was done, as well as testing in the order TA→Gast→Plan→Sol, and no differences in tension were observed, regardless of stimulation order. Repeat testing of Sol and Plan during preliminary study and subsequently during actual stimulation indicated the protocol did not result in a decrement of force.

Tissue harvest. After contractile characteristics were obtained, left and right Sol, Plan, Gast, and TA muscles were removed, cleaned of extraneous tissue, blotted, and weighed. Left-sided muscles, those that were electrically stimulated, were pinned at their in situ length, embedded in optimum cutting temperature tissue-freezing medium, frozen slowly in chilled 2-methylbutanol, and then placed in liquid nitrogen and stored at ~80°C. The right unstimulated muscles were snap frozen in liquid nitrogen and subsequently stored in a ~80°C freezer until analysis.

In subsequent experiments with additional mice, Sol, Plan, Gast, and TA muscles were removed, pinned at their in situ length, and immersed in 15% nitric acid for 24 h to digest connective tissue. After being rinsed, each muscle was placed in a 50% solution of glycerol/water. Under a dissecting microscope, multiple individual muscle fibers were teased out and fiber length was measured in ~25 fibers per muscle. Muscle lengths were averaged and used to derive anatomical cross-sectional areas (aCSA) for each muscle using the formula: aCSA = muscle mass/fiber length × muscle density (1.0562).

Histochemistry. Left-sided optimum cutting temperature prepared muscles from ER α mice were thawed to ~22°C in a microtome, oriented vertically, and sectioned at 10 μm. Sections were stained using traditional hematoxylin and eosin to reveal evidence of potential muscle damage or inflammation and with NADH. Fiber areas were obtained from NADH-stained sections. Photos were taken from multiple cross-sections to obtain a minimum of 200 fibers for fiber area measures. Area determinations were done using a calibrated pen by circling each fiber. Image J software (National Institutes of Health) was used to derive area data, which were subsequently copied into an Excel spreadsheet.

Due to a complete freezer failure with loss of all frozen tissues, fiber area measures were not obtained in sufficient numbers to permit determination of P₀/fiber area for all muscles. Fiber areas were obtained in sufficient sample numbers (n = 2–6 muscles) in each group for the Gast and TA, which are reported.

Total protein. Total protein is a general indicator of muscle well-being and was determined for right unstimulated muscles. To do the assay, muscle samples were homogenized in HES buffer (20 mM HEPES, 1 mM EDTA, and 250 mM sucrose, pH to 7.4) and standard samples (×3) of 10 μg were derived. Protein content was determined using the BCA protein assay (Sigma protein standard, microstandard, 1 mg BSA/ml in 0.15 M NaCl, and 0.05% NaN₃) and a spectrophotometer (Bausch and Lomb; Milton Roy: Spectronic 24).

Total myosin. Myosin content is closely associated with the contractile tension capabilities of muscle (41) and was measured for the Gast and TA because of their lower P₀/CSA and for ERα Plan because of the significantly higher P₀. 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. ERα KO animals had ~10% higher body weight ($P < 0.005$) than WT mice (Fig. 1). ArKO mice were ~20% larger than ArWT, which was also significant ($P < 0.001$).

TA muscle mass was ~15% higher in ERα KO mice ($P < 0.05$) than in WT mice (Fig. 2). There were no significant differences in ERβ 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 ERα 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 $P_o$/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 ERα KO (~30%) than in WT controls, but this significant difference disappeared when $P_o/aCSA$ was examined. The nearly significant reduction in ERα 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 ERα KO approached significance ($P = 0.084$) and became significant with the ratio of $P_o/aCSA$ ($P < 0.05$) and as the ratio of $P_o$/fiber area (Table 2; Fig. 5). These findings indicate that the ERα KO Gast and TA could not produce as much force per unit area as a WT mouse. There were no additional ERα 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).

Gast and TA muscle fiber area. Fiber CSA was determined for Gast and TA as tetanic tension/aCSA was lower in these muscles. Average fiber CSA for each group is presented in Table 2. There was no significant difference in Gast or TA fiber area between KO and WT groups.

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...
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 Po, the consequence of altered actin:myosin interaction. Subsequently, Moran et al. (33) restored Po 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...

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

Fig. 5. Peak tetanic tension (Po) expressed per derived anatomical cross-sectional area (CSA; g/cm²). *P < 0.05.
exists in the two species (49). When IGF-1 levels are low, growth hormone (for the increase in body and muscle mass in the mouse, indicating that a different mechanism (e.g., increased muscle mass) is involved. The results suggest that there are ligand-dependent and ligand-independent effects of ER that are muscle specific.

**ERα KO effects on skeletal muscle tissue.** Our major findings in the ERα group were that ERα KO resulted in 1) an increase in whole body weight and TA muscle mass; 2) a reduction in P₃/aCSA in Gast and TA muscles; and 3) a Plan P₃ that was significantly greater in ERα KO than in WT female mice but not when expressed per aCSA.

ERα KO mice had an ~13% higher body weight compared with WT animals. This finding is in agreement with previous reports (16, 49) demonstrating an ~14% increase in adult body weight in ERα KO female mice. There was also a concomitant increase in muscle TA mass. However, muscle mass-to-body weight ratios were not different, suggesting that the increase in muscle size was the consequence of general body growth or occurred in response to increased demand (larger body size). Fisher et al. (12) demonstrated that mature OVX rats had an ~18% increase in body mass and about the same percentile increase in both type I and type II myosin heavy chain dominant muscle mass compared with intact rats. Fisher et al. (12) found the increase in body size and muscle mass in rats was significantly correlated to IGF-1 values, which increased ~35%. IGF-1 reportedly does not increase with OVX in the mouse, indicating that a different mechanism (e.g., increased growth hormone) for the increase in body and muscle mass exists in the two species (49). When IGF-1 levels are low, there may be a compensatory increase in growth hormone as evidenced by Yakar et al. (51). However, muscle is an autocrine organ producing its own IGF-1; potentially, within tissue IGF-1, levels regulate muscle mass independently of whole body growth or growth hormone. Nonetheless, the exact mechanism by which muscle mass is regulated and the discovery of the molecular effects of ERs within muscle are a long-term goals of our laboratories.

There are still other factors that 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 P₃. However, these may not be the only factors explaining the decreased P₃/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 P₃/fiber areas

<table>
<thead>
<tr>
<th></th>
<th>ERα WT (n = 12)</th>
<th>ERα KO (n = 8)</th>
<th>ERWT (n = 6)</th>
<th>ERβ KO (n = 6)</th>
<th>ArWT (n = 3)</th>
<th>ArKO (n = 2)</th>
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</thead>
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<tr>
<td>Gast muscle area, μm²</td>
<td>2.207 ± 0.344</td>
<td>2.576 ± 0.177</td>
<td>2.760 ± 0.165</td>
<td>2.237 ± 0.226</td>
<td>1.674 ± 0.206</td>
<td>1.377 ± 0.204</td>
</tr>
<tr>
<td>Gast P₃/fiber area</td>
<td>0.050 ± 0.007</td>
<td>0.037 ± 0.005*</td>
<td>0.036 ± 0.009</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 muscle area, μm²</td>
<td>1.914 ± 0.233</td>
<td>2.052 ± 0.258</td>
<td>2.412 ± 0.332</td>
<td>2.003 ± 0.174</td>
<td>1.707 ± 0.223</td>
<td>1.674 ± 0.205</td>
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<tr>
<td>TA P₃/fiber area</td>
<td>0.040 ± 0.010</td>
<td>0.027 ± 0.006*</td>
<td>0.022 ± 0.011</td>
<td>0.027 ± 0.006</td>
<td>0.039 ± 0.010</td>
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Values are means ± SE. P₃, peak tetanic tension; Gast, gastrocnemius; TA, tibialis anterior. Area differences were not significant. P₃/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>Soleus</th>
<th>Plantaris</th>
<th>Gastrocnemius</th>
<th>Tibialis Anterior</th>
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<tr>
<td>Absolute tetanic tension</td>
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<tr>
<td>ERα KO</td>
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<td>ArKO</td>
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<tr>
<td>Tetanic tension/anatomical CSA</td>
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<td>ERα KO</td>
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<tr>
<td>+dP/dt</td>
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| CSA, cross-sectional areas; - condition of study had no effect; +, significant condition effect; ↑ and ↓, direction of significant effect. |

**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/aCSA and Pₐ/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/aCSA. 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**

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