Sex differences in intracellular Ca\(^{2+}\) accumulation following eccentric contractions of rat skeletal muscle in vivo

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Summary

Sonobe T, Inagaki T, Sudo M, Poole DC, Kano Y. Sex differences in intracellular Ca\(^{2+}\) accumulation following eccentric contractions of rat skeletal muscle in vivo. Am J Physiol Regul Integr Comp Physiol 299: R1006–R1012, 2010. First published July 14, 2010; doi:10.1152/ajpregu.00623.2009.—It is commonly believed that sex differences in skeletal muscle damage following eccentric exercise are due to sex differences in estrogen and sex influences. However, the cause of the sex differences in the response of muscle damage to eccentric exercise remains to be resolved. The mechanistic bases for this sex-specific phenomenon remain to be resolved. The muscle damage has been linked to loss of Ca\(^{2+}\) homeostasis and resultant intramyocyte Ca\(^{2+}\) accumulation; therefore, we tested the hypothesis that the greater eccentric exercise-induced muscle damage in males would be associated with more pronounced [Ca\(^{2+}\)]\(_i\) accumulation. The intact spinotrapezius muscle of adult Wistar rats [male, female, and ovariectomized (OVX)—to investigate the effects of estrogen] was exteriorized. Tetanic eccentric contractions (100 Hz, 700-ms duration, 20 contractions/min for a total of 10 sets of 50 contractions) were elicited by electrical stimulation during synchronized muscle contractions of rat skeletal muscle in vivo following muscle contractions. In male rats, that investigation (36) demonstrated that ECC contractions induced a more rapid and exaggerated [Ca\(^{2+}\)]\(_i\) accumulation than isometric contractions. The in vivo spinotrapezius preparation used in conjunction with ECC contractions is well suited to address the question as to whether the presence of normal estrogen levels is not obligatory for the difference between the sexes.

Materials and Methods

Animals

Wistar male (n = 17), female (n = 14), and ovariectomized female (OVX, n = 16) rats (Japan SLC, Shizuoka, Japan) were used in this study. The ovariectomy was performed at 10 wk of age, and experiments were undertaken 4 wk afterward to permit recovery from the surgical intervention and allow for restabilization of lower estrogen levels. A subset of the data reported for the male rats has been published previously (36). Rats were maintained on a 12:12-h light-dark cycle and received food and water ad libitum. All experiments were conducted under the guidelines established by the Physiological Society of Japan and were approved by University of Electro-Communications Institutional Animal Care and Use Committee. The rats were anesthetized using pentobarbital sodium (60 mg/kg ip), and supplemental doses of anesthesia were administered as needed. At the end of experimental protocols, animals were killed by pentobarbital sodium overdose.

Muscle Preparation

All experimental techniques, including the spinotrapezius muscle preparation, were performed as described previously (36). Exterior-
zation of the right spinotrapezius muscle, accessed through a midline incision, was performed with as little disruption as possible to minimize tissue damage. The exposed spinotrapezius muscle with principal vascular and neural pathways maintained was attached to a wire horseshoe around the caudal periphery by six equidistant sutures placed around the caudal perimeter (29, 40). For the contraction protocols, electrodes were placed on the dorsal spinotrapezius surface along the caudal periphery, facilitating whole muscle contractions. The muscle surface was kept moist by superfusing with warmed Krebs-Henseleit Buffer (KHB; 132 NaCl, 4.7 KCl, 21.8 NaHCO3, 2 MgSO4, 2 CaCl2 mM) equilibrated with 95% N2-5% CO2 and adjusted to pH 7.4, at 37°C.

Fluorescence Ca2+ indicator fura-2 AM (5 mM; Dojindo Laboratories, Kumamoto, Japan) was dissolved in DMSO and Pluronic F-127 and dispersed into KHB solution at a final concentration of 20 μM. The muscles were incubated in fura-2 AM/KHB solution for 30 min on a 37°C hotplate. After incubation, muscles were rinsed with dye-free KHB solution to remove nonloaded fura-2.

Microscopy and Fluorescence Measurement

As shown in Fig. 1, the spinotrapezius muscles, loaded with fura-2 AM, were mounted on the 37°C glass hotplate (Kitazato Supply, Shizuoka, Japan) and observed by fluorescence microscopy using a ×10 objective lens (0.30 numerical aperture; Nikon, Tokyo, Japan). After ensuring that the muscle was not grossly damaged and supported blood flow, a sampling area (~880 × 663 μm) was selected using branching vessels as landmarks, and bright-field images were captured. Thereafter, 340-nm and 380-nm wavelength excitation light was delivered using a Xenon lamp equipped with appropriate fluorescent filters, and pairs of fluorescence images were captured through the 510-nm emission wavelength filter for ratiometry.

Image Analysis

Images were captured by a charge-coupled device digital camera (DP70, Olympus, Tokyo, Japan) using image-capture software (DP Control; Olympus). After selecting an appropriate region of interest, which included multiple muscle fibers, the spinotrapezius fluorescence was observed. At first, an image excited by 340-nm wavelength was captured at 5-s exposure time, and following manual switching of excitation filters from 340 to 380 nm, a second image was captured. Images were converted to 340/380 ratio (F340/F380: R) image by ImageJ software (National Institutes of Health, Bethesda, MD), and the ratio image data, indicating Ca2+ levels, were averaged over the whole area sampled. The fluorescence intensity of serial ratio images was normalized to the starting point (i.e., precontraction, R0) of each experiment (R/R0).

Experimental Protocols

Each group of animals (male, female, and O VX ) was divided into two subsets: 1) in vivo control and 2) in vivo ECC contractions. Muscle stimulations were performed as described in detail previously (36).

In vivo control group. During the resting (nonstimulated) control experiment, sequential fluorescence images were captured every 5 min for 90 min. Each pair of F340 and F380 images was analyzed and quantified for evaluation of time-course effects.

In vivo ECC contractions group: Muscle-lengthening contractions in in vivo spinotrapezius muscle. The motor device coupled with electrostimulator (model RU-72; NEC Medical Systems, Tokyo, Japan) delivered a stretch (lengthening) of 10% of resting sarcomere length (i.e., to ~3.0 μm). The muscle was stimulated tetanically at resting spinotrapezius sarcomere length (frequency of 100 Hz, 5–8 V, total stimulus duration of 700 ms) every 3 s for 2.5 min (i.e., 50 contractions). Muscle lengthening was started 200 ms after initiation of electrical stimulation and immediately returned to resting length at the end of electrical stimulation. The lengthening contraction was performed 50 times sequentially and repeated for 10 sets interspersed by 5-min rest/recovery periods. Pairs of fluorescence images (R) were captured preconcentration and immediately after each set of contractions, as well as at the end of the 5-min between-set recovery (immediately prior to initiation of the subsequent set of contractions). Immediately after the 10th set of ECC contractions, the apparent fiber width perpendicular to the longitudinal muscle fiber axis was measured for each muscle fiber in which both sarcolemmal boundaries were visible on the screen, and the expanded regions were identified as hypercontracted. Muscle fiber damage associated with hypercontracted regions was expressed as a percentage of the total muscle fibers counted.

17β-Estradiol Measurement

Blood was sampled at the end of the observation period via the postcaecal vein (~5 ml) and immediately centrifuged at 3,000 rpm and 4°C for 10 min. Five-hundred microliters of fresh plasma was dispensed into microtubes, and these samples were stored at −80°C until analysis. Plasma concentrations of 17β-estradiol were determined using a sandwich-EIA Kit (Cayman Chemical, Ann Arbor, MI). All techniques and materials used in this analysis were in accordance with the manufacturer’s protocol. Optical density was quantified on a
RESULTS

Control [Ca\(^{2+}\)]\(_i\) was not different among groups and was unchanged over the 90-min observation period in control (i.e., non-ECC contracted) muscles.

Plasma 17β-estradiol levels were 38.2 ± 7.2 (range of 21.0 to 57.7) and 21.3 ± 3.1 (range of 12.6 to 31.7) pg/ml in female and OVX rats, respectively (P < 0.05).

Direct in vivo observation permitted discrimination of individual muscle fibers and identification of the presence of blood flow in almost all vessels (feed artery, arterioles, capillary, venules). This ensured that alterations in [Ca\(^{2+}\)]\(_i\) were not the product of, or exacerbated by, ischemia induced by surgical manipulation (Fig. 2, bright-field images). Figures 2 and 3 illustrate that elevation of [Ca\(^{2+}\)]\(_i\) in male rat muscle did not present uniformly across all fibers but rather occurred in close proximity to the hypercontracted regions (Fig. 3 arrows). In marked contrast to muscles from males, females and OVX muscles had few hypercontracted fibers even after 10 sets of the ECC contractions protocol (Fig. 2). The incidence of hypercontracted fibers following ECC contractions was significantly higher in a male compared with female and OVX rats (P < 0.01, Fig. 3B).

In vivo [Ca\(^{2+}\)]\(_i\) changes and relative force reduction of the spinotrapezius muscle for ECC contractions condition are depicted in Fig. 4 and Fig. 5, respectively. F<sub>340/F<sub>380</sub> ratio values after the final contraction set (no. 10) were elevated 42.8 ± 5.3% (P < 0.05) above precontraction baseline in male rat muscles (Fig. 4), whereas female and OVX rats showed only a 9.4 ± 3.5% and 17.6 ± 1.2% elevation of [Ca\(^{2+}\)]\(_i\), respectively (both P < 0.05 compared with males, Fig. 4). Although the [Ca\(^{2+}\)]\(_i\) value of OVX rat muscles tended to be higher than those from intact female rats (n.s.), there remained a significantly lower [Ca\(^{2+}\)]\(_i\) compared with male rat muscles over sets 4 to 10 (P < 0.05).

Figure 5 shows the changes of relative tetanic force. Isometrically evoked active force was significantly decreased within each set of ECC contractions for all groups (Male: −66.1%, Female: −42.8%, OVX: −36.3% at 46–50 of 10th set vs. the

Statistical Analysis

All statistical analyses were performed in Prism version 4.0 (GraphPad Prism, San Diego, CA). A two-way repeated-measures ANOVA and Bonferroni post-hoc test was used for male vs. female or OVX comparisons. A one-way repeated-measures ANOVA and Bonferroni post hoc test were used for relative force comparison and calpain-3 autolysis. Measured values are presented as means ± SE. Significance was established at P < 0.05.

Force Measurement

The wire horseshoe that was attached to the spinotrapezius muscle was connected by fine wire to a strain gauge. Torque (0–10 mN/m, full-scale deflection) was monitored by computer using Mac Lab/8s (AD Instruments, Colorado Springs, CO) via a strain gauge-linked motor device (Model RU-72, NEC Medical Systems, Tokyo, Japan) during all contraction protocols. The muscle force during electrical stimulation (700 ms) was resolved as active force (isometric phase, 0–200 ms) and passive force (eccentric phase, 200–500 ms), respectively. Among the 10 sets of 50 contractions, the first 5 and last 5 contractions of set numbers 1, 5, and 10 were averaged and plotted graphically as an index of fatigue.

Calpain-3 Autolysis Measurement

To determine whether calpain-3 autolysis was elicited by the ECC contractions protocol, the spinotrapezius muscle was removed immediately after the 10th set of ECC contractions and homogenized (male: n = 5, female: n = 5, OVX: n = 6). Homogenates were incubated at 4°C for 20–60 min, and an aliquot was kept for protein analysis (DC Protein Assay Kit, Bio-Rad, Hercules, CA). A further dilution of the homogenate was made with extraction buffer (1:10 vol/vol), which was then added (2:1 vol/vol) to SDS loading buffer (50 mM Tris-HCl, 10% SDS, 2% glycerol, 20% mercaptoethanol, 0.001% bromophenol blue, pH 6.8). Samples were heated to 100°C for 5 min and analyzed by Western blot analysis. Protein from the total muscle extracts (~20 μg) was separated on an 8% SDS-PAGE gel and transferred to polyvinylidene fluoride membranes. Membranes were probed with antibody against calpain-3 (1:100, mouse monoclonal, Novocastra monoclonal 12A2), after anti-mouse horseradish peroxidase (1:1,000; AP1254) was added to the membranes. The signals were developed by ECL plus Western Blotting Detection System (RPN2132; Amersham Biosciences, Piscataway, NJ). The resulting bands were quantified as OD × band area using a CS analyzer (ATTO). The size of the immunodetected proteins was verified by means of standard molecular-weight markers (Bio-Rad Laboratories).

Statistical Analysis

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Male rats (*P < 0.01) had higher in male rats than that in female and OVX rats, but the amount of autolyzed calpain-3 tended to increase after the 10th set of ECC contractions (Fig. 6). Calpain-3 exists in sarcolemma and neighboring myocytes, which may lead to further degenerative structural and functional changes over the ensuing hours and days (26, 27).

There was a marked sex specificity of ECC contractions to evoke muscle damage. Using the downhill running exercise model, Komulainen et al. (30) demonstrated that histological disruption, associated with loss of submembrane dystrophin and swollen fibers, was present immediately following exercise in male but not female rats. In agreement with these latter findings, the present investigation found locally hypercontracted myocytes in male (Figs. 2 and 3) but not female and OVX spinotrapezius muscles following ECC contractions.

In an apparent contradiction, we found that, while isometrically evoked active force decreased more in male than female and OVX rats after all ECC sets (P < 0.01), there was no difference in passive force production among groups (Fig. 5). According to the classic force-velocity relationship, lengthening (i.e., passive) tension is proportional to the level of isometrically evoked active tension (17). Therefore, we consider that the sustained passive force in male rat compared with active force level might result from enhanced muscle stiffness. It has been reported that after ECC contractions, there is a rise in muscle stiffness (11, 21) and passive tension (46, 47).

We have also shown that high [Ca\textsuperscript{2+}]i accumulation areas correspond spatially with hypercontracted parts in male muscle fibers (Figs. 2 and 3). In the in vitro study of single skeletal muscle fibers, Balnave et al. (5) demonstrated that the resting [Ca\textsuperscript{2+}]i was higher after lengthening contractions, whereas the distribution of [Ca\textsuperscript{2+}]i within the resting fibers appeared to be spatially homogeneous. However, they pointed out the possibility that these findings may have resulted from low spatial resolution and lack of focus due to thick specimens and a restricted observation window. One advantage of the present methodology is the ability to observe [Ca\textsuperscript{2+}]i changes in each of 10–20 muscle fibers in vivo in real time. Heterogeneous [Ca\textsuperscript{2+}]i accumulation patterns among fibers were observed in male but not female muscles under our experimental conditions.

Source of Elevated [Ca\textsuperscript{2+}]i, and Putative Mechanism of Myocyte Damage

There are two major potential sources of Ca\textsuperscript{2+}: intracellular sarcoplasmic reticulum (SR) and the extracellular space.

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**DISCUSSION**

The principal original finding of this investigation was that the rise in [Ca\textsuperscript{2+}]i characteristic of male rats following repeated ECC contractions was markedly lower in female rats. Moreover, this sex difference was not prevented in OVX rats where the ECC contractions-induced [Ca\textsuperscript{2+}]i increase was also substantially less than found in their male counterparts.

**Histological Muscle Damage, Distribution of Elevated [Ca\textsuperscript{2+}]i, and Force Production**

ECC contractions lead to a disruption in muscle ultrastructure and focal myocyte damage (16). Specifically, immediately following ECC contractions, there was a myocyte “hypercontracture” and swelling, which compressed surrounding capillaries and neighboring myocytes, which may lead to further degenerative structural and functional changes over the ensuing hours and days (26, 27).

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In an apparent contradiction, we found that, while isometrically evoked active force decreased more in male than female and OVX rats after all ECC sets (P < 0.01), there was no difference in passive force production among groups (Fig. 5). According to the classic force-velocity relationship, lengthening (i.e., passive) tension is proportional to the level of isometrically evoked active tension (17). Therefore, we consider that the sustained passive force in male rat compared with active force level might result from enhanced muscle stiffness. It has been reported that after ECC contractions, there is a rise in muscle stiffness (11, 21) and passive tension (46, 47).

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**Fig. 3.** The presence of hypercontracted muscle fibers and [Ca\textsuperscript{2+}]i, increased after the in vivo ECC protocol in male rats. A: local [Ca\textsuperscript{2+}]i, increased in hypercontracted muscle fiber areas (arrows). Compared with male, female and ovariectomized (OVX) muscle had very few hypercontracted fibers or regions with high [Ca\textsuperscript{2+}], even after 10 sets of the ECC contractions protocol. Also, some fibers demonstrated a slight increase of [Ca\textsuperscript{2+}]i, that was distributed quite uniformly along the fiber (#). Scale bar = 50 μm. B: incidence of hypercontracted fibers consequent to ECC contractions was significantly higher in male compared with female and OVX rats (*P < 0.01).

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**Fig. 4.** Effects of sex differences on in vivo [Ca\textsuperscript{2+}]i, following ECC contractions in spinotrapezius muscles. Fluorescence intensity (R) was measured after 50th contraction of each set and ratiometric quantified data were graphed as changes from precontraction (before 1st set) levels (R0). Values shown are means ± SE. *Significantly different (P < 0.05) from initial level for each condition. *Significant difference (P < 0.05) between male and either female or OVX for the same time points.

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Yeung and colleagues (48–50) demonstrated that stretch-activated ion channel (SAC) blocker treatment prevented an ECC contractions-induced increase in the subsequent resting \([\text{Ca}^{2+}]_{\text{i}}\) in isolated single fibers from the \textit{mdx} mouse, a model of human Duchenne muscular dystrophy. Moreover, in healthy male rats, we have shown that the elevated \([\text{Ca}^{2+}]_{\text{i}}\) accompanying/following ECC contractions could be prevented, or at least substantially reduced, by SAC blockers (36). These investigations provide strong support that the majority of the ECC contraction-induced \([\text{Ca}^{2+}]_{\text{i}}\) accumulation constitutes influx resulting from increased effective sarcolemmal permeability (SACs) rather than SR damage.

The loss of intramyocyte \([\text{Ca}^{2+}]_{\text{i}}\) homeostasis and resultant elevation of \([\text{Ca}^{2+}]_{\text{i}}\) is considered to represent a primary factor in the process of myocyte degeneration subsequent to ECC contractions (8, 51). One plausible mechanistic link between the proteolysis of cytoskeletal proteins and elevated \([\text{Ca}^{2+}]_{\text{i}}\) is activation of \([\text{Ca}^{2+}]_{\text{i}}\)-activated neutral proteases (calpains) (7, 8, 19, 45). Skeletal muscle fibers contain several different kinds of the ubiquitous calpains (μ-calpain, m-calpain, and calpain-3) (7, 19). In particular, calpain-3 autolysis is strongly \([\text{Ca}^{2+}]_{\text{i}}\) dependent (33). The ECC contractions protocol of the present investigation induced calpain-3 autolysis in all groups immediately after ECC contractions. Moreover, the amount of autolyzed calpain-3 tended to be higher in male rats than that in female and OVX rats (Fig. 6). It has been shown that calpain-3 binds not only to titin (33) but also interacts with the \([\text{Ca}^{2+}]_{\text{i}}\) release ryanodine receptor (31). Therefore, the hypercontracted regions observed following ECC contractions were, quite likely, the result of calpain-myofibril interactions: A response expected to cause concomitant autolysis of calpain-3 (45, 51).

**Estrous Cycle and OVX Model**

An estrous cycle of ~4 to 5 days is present in intact female rats (10, 20). During this estrous cycle, blood 17β-estradiol fluctuates between ~17 ± 2 and 88 ± 2 pg/ml, and thus, muscles are exposed to periodically high concentrations of estradiol (10). Although different 17β-estradiol levels were observed in female rats after repeated ECC contractions (21 to 58 pg/ml), ECC contraction-induced \([\text{Ca}^{2+}]_{\text{i}}\) changes were not significantly different between intact female and OVX groups (Fig. 4). The \([\text{Ca}^{2+}]_{\text{i}}\) elevation in OVX animals following ECC contractions was slightly, but not significantly, higher than intact females but remained significantly lower than observed in males. These data suggest that elevated circulating 17β-estradiol levels are not the primary reason that intramyocyte \([\text{Ca}^{2+}]_{\text{i}}\) elevation—and associated myocyte damage—are far less pronounced in female than male rat muscles.

Fig. 5. Changes of relative active and passive force during ECC contraction protocols. A: representative force output captured by computer during activation. B: force normalized to initial (1st set) denotes the average of first 5 and last 5 contractions of sets 1, 5, and 10 from all groups. Values shown are expressed as means ± SE. *Significantly different (\(P < 0.05\)) from initial level for each condition.

Fig. 6. Effect of ECC contractions on the autolysis of calpain-3 proteins. Representative Western blot images for calpain-3 after 10 sets of ECC contractions. The amount of autolyzed calpain-3 (56 kDa) tended to be higher in male rats than that in female and OVX rats (\(P = 0.16\)).
Many studies have supported the notion that estrogen could effect changes in muscle damage and repair in response to various stimuli (6, 28, 39, 41, 42, 44). Specifically, Tiidus et al. (44) have consistently demonstrated estrogen dependence effects using OVX female rats with estrogen replacement. An estrogen-induced attenuation of postdamage inflammation-related neutrophil infiltration of skeletal muscle is also present in male, as well as OVX rats (44). Tiidus et al. have also demonstrated the attenuation of postexercise muscle calpain activity and suggested that the potential of estrogen to preserve sarcolemmal stability following ECC exercise may help maintain muscle [Ca$^{2+}$], homeostasis (42). These latter findings, indicating that estrogen exerts a protective influence on muscle integrity following ECC exercise, were conducted using downhill running exercise, whereas the present investigation evaluated ECC contractions elicited by electrical stimulation. In contrast to the single-muscle electrical stimulation paradigm herein, running exercise represents a whole-body stressor with an integrative more complex effect on the body’s oxidative, metabolic, and hormonal milieu. Thus, it is not inconceivable that estrogen may serve an antioxidative, or other, role rather than serve to maintain [Ca$^{2+}$], homeostasis per se. In support of this hypothesis and the present results, Moran et al. (32) recently reported that estradiol was not effective in protecting muscle damage due to ECC contractions induced by electrical stimulation.

With regard to the potential mechanism for estrogenic muscle damage preservation, skeletal muscle possesses both α- and β-estrogen receptors (25). Therefore, the possibility must be considered that the estrogen influence on postexercise muscle damage may be mediated via receptor-mediated mechanisms. However, this appears not to be the case as Enns et al. (14) have recently demonstrated that the ability of estrogen to attenuate exercise-induced muscle damage is not a receptor-mediated event. Elucidation of the mechanism(s) for these estrogen effects may help clarify the sex differences with respect to muscle inflammation and injury after ECC exercise. Further mechanistic studies designed specifically to investigate the potential for estrogen to inhibit muscle damage will doubtless be insightful for clarifying the discrepancies identified from animal and human studies (22, 43).

**Potential Mechanisms Regulating Ca$^{2+}$ Influx**

The causes of sex differences in the Ca$^{2+}$ influx mechanism(s) in skeletal muscle following ECC contractions remain unclear. In smooth muscle, many studies (9, 12, 24, 34) have shown that L-type Ca$^{2+}$ current density is significantly greater in males compared with females. For example, membrane depolarization by high KCl-induced Ca$^{2+}$ entry is greater in the aorta of male than female rats (12). This finding is consistent with the observation that decreased estrogen increases the number of cardiac L-type Ca$^{2+}$ channels in heart muscle (24). If estrogen does act to inhibit Ca$^{2+}$ influx in skeletal muscle, [Ca$^{2+}$], accumulation should be influenced by circulating estrogen level. That a substantial reduction in estrogen levels in the present investigation did not reverse the sex-associated protection from post-ECC [Ca$^{2+}$], elevation and myocyte damage, at least under the conditions studied, suggests that the presence of normal estrogen levels is not an obligatory facet of the protection conferred by sex.

**Perspectives and Significance**

In conclusion, our data demonstrate clearly that the rise in [Ca$^{2+}$], characteristic of male rats following repeated ECC contractions was markedly lower in females and that the ability for female rats to better maintain [Ca$^{2+}$], homeostasis during and following ECC contractions may be important for protecting against ECC-induced muscle damage. This sex specificity of [Ca$^{2+}$], accumulation did not appear to be controlled in any simple fashion by circulating estrogen levels, and this suggests a pressing need for further investigations to resolve the mechanistic bases for altered control of post-ECC [Ca$^{2+}$], regulation in females.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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SEX DIFFERENCE IN MUSCLE Ca^{2+} AFTER CONTRACTIONS IN VIVO

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