Intracellular \(\text{Ca}^{2+}\) transients in mouse soleus muscle after hindlimb unloading and reloading

CHRISTOPHER P. INGALLS, GORDON L. WARREN, AND R. B. ARMSTRONG

Muscle Biology Laboratory, Department of Health and Kinesiology, Texas A & M University, College Station, Texas 77843-4243

Ingalls, Christopher P., Gordon L. Warren, and R. B. Armstrong. Intracellular \(\text{Ca}^{2+}\) transients in mouse soleus muscle after hindlimb unloading and reloading. J. Appl. Physiol. 87(1): 386–390, 1999.—The objective of this study was to determine whether altered intracellular \(\text{Ca}^{2+}\) handling contributes to the specific force loss in the soleus muscle after unloading and/or subsequent reloading of mouse hindlimbs. Three groups of female ICR mice were studied: 1) unloaded mice (\(n = 11\)) that were hindlimb suspended for 14 days, 2) reloaded mice (\(n = 10\)) that were returned to their cages for 1 day after 14 days of hindlimb suspension, and 3) control mice (\(n = 10\)) that had normal cage activity. Maximum isometric tetanic force (\(P_o\)) was determined in the soleus muscle from the left hindlimb, and resting free cytosolic \(\text{Ca}^{2+}\) concentration ([\(\text{Ca}^{2+}\)]) \(_i\), tetanic [\(\text{Ca}^{2+}\)] \(_i\), and 4-chloro-m-cresol-induced [\(\text{Ca}^{2+}\)] \(_i\) were measured in the contralateral soleus muscle by confocal laser scanning microscopy. Unloading and reloading increased resting [\(\text{Ca}^{2+}\)] \(_i\), and specific force by 58% and 24%, respectively. Although unloading reduced \(P_o\) and specific force by 58% and 24%, respectively, compared with control mice, there was no difference in tetanic [\(\text{Ca}^{2+}\)], \(P_o\) specific force, and tetanic [\(\text{Ca}^{2+}\)] \(_i\) were reduced by 58%, 23%, and 23%, respectively, in the reloaded animals compared with control mice; however, tetanic [\(\text{Ca}^{2+}\)] \(_i\) was not different between unloaded and reloaded mice. These data indicate that although hindlimb suspension results in disturbed intracellular \(\text{Ca}^{2+}\) homeostasis, changes in tetanic [\(\text{Ca}^{2+}\)] \(_i\) do not contribute to force deficits. Compared with unloading, 24 h of physiological reloading in the mouse do not result in further changes in maximal strength or tetanic [\(\text{Ca}^{2+}\)] \(_i\).

Hindlimb unloading (i.e., suspension) in rodents appears to mimic the effects of human spaceflight on skeletal muscle size (6) and function (16, 28). Soleus muscles in rodents have been shown to lose >45% of their mass and strength after 2–4 wk of hindlimb unloading (5, 7, 31). Reductions in skeletal muscle mass primarily account for the decreases in maximal isometric tetanic force production (\(P_o\)) in hindlimb-unloaded muscle. However, observations in single-fiber and whole muscle preparations indicate that reductions in skeletal muscle mass or fiber cross-sectional area are less than the decrease in \(P_o\) (5, 7, 8, 19, 31). Thus hindlimb unloading results in decreases in \(P_o\) when normalized to cross-sectional area, i.e., specific force, of whole muscle and single myofibers.

Decreases in myofibrillar protein concentration (27, 30) and elevations in the relative interstitial fluid volume (14) of soleus muscles have been shown to occur after hindlimb unloading in the rat, which could contribute to the decreases in specific force. However, elevations in interstitial fluid volume cannot explain specific force decrements in single fibers. Disruptions in excitation-contraction (E-C) coupling, and hence attenuated release of \(\text{Ca}^{2+}\) from the sarcoplasmic reticulum (SR), could also contribute to the decreased specific force after hindlimb unloading. E-C uncoupling is the primary mechanism responsible for strength deficits associated with low-frequency fatigue (4, 32) and eccentric contraction-induced muscle injury (1, 12, 31). Therefore, the first objective of this study was to test the hypothesis that intracellular \(\text{Ca}^{2+}\) homeostasis in the soleus muscle is disturbed after unloading of mouse hindlimbs and that E-C uncoupling contributes to the decrease in specific force.

Significant injury (e.g., segmental necrosis, central corelike lesions, thick filament dissolution) to skeletal muscle fibers has been documented in mice and rats exposed to real and simulated microgravity (9, 17, 18, 20, 21). However, most of the damage to the skeletal muscle fibers appears to be due to the reloading of the hindlimbs (17, 18). The degree to which reloading-induced muscle injury compounds existing strength deficits incurred by hindlimb suspension is unknown, and the possible role of E-C uncoupling in the strength deficit has not been studied. Therefore, the second objective of this study was to test the hypothesis that E-C uncoupling and strength deficits are exacerbated when mouse soleus muscles are reloaded (i.e., injured) after hindlimb unloading.

MATERIALS AND METHODS

Animals and experimental design. Thirty-seven female ICR mice were divided into three groups: 1) control mice (\(n = 10\)) that had normal cage activity, 2) unloaded mice (\(n = 11\)) that were hindlimb suspended for 14 days, and 3) reloaded mice (\(n = 10\)) that were returned to their cages for 1 day after 14 days of hindlimb unloading. In each condition the mouse was anesthetized with pentobarbital sodium (100 mg/kg ip), and the soleus muscles from both hindlimbs were removed for study. The soleus muscle from the right hindlimb was used to make [\(\text{Ca}^{2+}\)] \(_i\) measurements, and the contralateral soleus muscle was used to study in vitro contractile mechanics. To characterize the extent of muscle damage caused by hindlimb unloading and reloading, both soleus muscles from six additional animals (\(n = 2\)/group) were used for histological analysis. All animal care and use procedures met the guidelines set by the American Physiological Society and were approved by the Institutional Animal Care and Use Committee.
Hindlimb suspension procedure. A modification of the tail harness model of Fitts et al. (7) was used to suspend the hindlimbs of mice, as previously described (31). Briefly, the tail harness consisted of a triangular-shaped wire (21 gauge), a 2.5 x 7.5 cm piece of fabric patch, cyanoacrylate glue, and elastic tape. While the mouse was anesthetized with methoxyflurane (~4 min), the tail was cleaned with alcohol, placed between a triangular-shaped wire, and secured to the wire with a fabric patch wrap and cyanoacrylate glue. Finally, the outer layer of the fabric patch was wrapped with elastic tape. With use of this method, the tail of the mouse remained in good health, presumably because the weight of the animal was equally distributed across three-fourths of the tail. By use of a swivel attached to the apex of the triangular-shaped wire, and fishing line, and an overhead support bar to suspend the mouse, was equally distributed across three-fourths of the tail. By use of a swivel attached to the apex of the triangular-shaped wire, and an overhead support bar to suspend the mouse, was equally distributed across three-fourths of the tail. By use of a swivel attached to the apex of the triangular-shaped wire, and secured to the wire with a fabric patch wrap and cyanoacrylate glue. Finally, the outer layer of the fabric patch was wrapped with elastic tape.

Histological analysis. The number of actively degenerating muscle fibers per muscle cross section was used to estimate the extent of the histopathology caused by hindlimb unloading and reloading. Soleus muscles were excised, cut transversely, embedded in Tissue Tek OCT, frozen in melting isopentane, and stored at -80°C. Cross sections (10 µm thick) were cut at each of four levels equally spaced along the length of each muscle in a microtome cryostat at -25°C. An Olympus BX-60 microscope and x20 [0.50 numerical aperture (NA)] UplanFl objective and BioQuant (version 3.000.6, R & M Biometrics) image-analysis software were used to determine the total fiber number and frequency of degenerating fibers from Gomori's trichrome-stained muscle sections.

Muscle mechanics. Soleus muscles were dissected free and studied at 37°C with use of an in vitro preparation, as previously described (31). Isometric twitch (P<sub>t</sub>) and tetanic (P<sub>o</sub>) 400-ms trains with 0.2-ms pulses at 150 Hz) contractions were initiated at 7 and 8 min into the incubation, respectively. Specifc force was calculated by normalizing P<sub>t</sub> to physiological cross-sectional area [i.e., muscle wet weight ÷ (muscle density x fiber length)]; muscle fiber length was assumed to be 71% of muscle length (3). The muscle lengths in the control, unloaded, and reloaded mice were 13.9 ± 0.3, 13.9 ± 0.3, and 14.1 ± 0.3 (SD) mm, respectively.

Muscle [Ca<sup>2+</sup>]<sub>i</sub> measurements. Measurement of [Ca<sup>2+</sup>]<sub>i</sub> was based on the ratiometric confocal laser scanning microscopy approach that used visible-wavelength Ca<sup>2+</sup>-sensitive dyes, as described previously (12). Soleus muscles were incubated with fluo 3-acetylmetohyl ester (AM) (20 µM) and fura red-AM (20 µM; Molecular Probes) in an oxygennated Krebs-Ringer solution containing 3 mg/ml of Pluronic F127 for 45 min at room temperature. The muscles were observed using a NO-RAN Odyssey XL confocal laser scanning microscope with a 50-mW argon-krypton laser attached to a Zeiss AxioSkop upright microscope. The dyes were excited using a 488-nm laser line, and the fluorescence in the two channels was collected through 515- to 545-nm (i.e., primarily that of fluo 3) and 365- to 465-nm (i.e., primarily that of fura red) band-pass filters, respectively. A Zeiss Achroplan x10 water-immersion objective (0.30 NA) was used to acquire the optical sections. Soleus muscles were mounted in a superfused preparation (Krebs-Ringer solution at 25°C), and sarcomere length was set to 3.2 ± 0.03 µm. Optical sections (i.e., 765 x 589 µm) were acquired from a standardized location on the muscle at rest and during tetanic (500-ms train with 0.2-ms pulses at 100 Hz) and 4-chloro-m-cresol (10 mM)-induced contractions after ~15, 20, and 35 min of perfusion, respectively. Depending on the muscle, -6-12 soleus muscle fibers were resolved in a given optical section. 4-Chloro-m-cresol is thought to be a novel and specific activator of the SR Ca<sup>2+</sup> release channel (10). Optical sections were acquired every 30-60 s during an 8-min incubation with 4-chloro-m-cresol; the time point that resulted in the highest fluo 3-to-fura red ratio was used to indicate peak [Ca<sup>2+</sup>]<sub>i</sub>. During contractions the fluo 3 and fura red channels were sampled at a rate of 15 Hz with a laser dwell time of 100 ns. After background subtraction, voxels in the fluo 3 channel were divided by the corresponding voxels in the fura red channel to obtain an eight-bit ratio image of [Ca<sup>2+</sup>]<sub>i</sub>. To estimate [Ca<sup>2+</sup>]<sub>i</sub>, an in vitro calibration was applied to the eight-bit gray-scale voxel intensity. After determination of intracellular fluo 3 and fura red concentrations, fluo 3 and fura red salts were added to [Ca<sup>2+</sup>]<sub>i</sub> standards (Molecular Probes). This in vitro calibration method does not take into account changes in dye properties when bound to intracellular proteins. Dye ratio values were converted to [Ca<sup>2+</sup>]<sub>i</sub> by using the Hill equation (R = R<sub>max</sub>[Ca<sup>2+</sup>]<sub>i</sub>/(Ca<sub>50</sub> + [Ca<sup>2+</sup>]<sub>i</sub>)), where R<sub>i</sub> is ratio, R<sub>max</sub> is maximum ratio, n is Hill coefficient, and Ca<sub>50</sub> is Ca<sup>2+</sup> concentration that produces maximal force.

Statistical analyses. Changes over time in body weight were assessed using a group (control, unloaded, reloaded)-by-time (before and after treatment) ANOVA with repeated measures on the time factor. Differences in muscle weight and mechanics were evaluated using individual one-way ANOVAs. The mean of the plateau values was used to determine differences in tetanic [Ca<sup>2+</sup>]<sub>i</sub> between groups. Because of the nature of the [Ca<sup>2+</sup>]<sub>i</sub> calibration, statistics (Kruskal-Wallis 1-way ANOVA on ranks) were performed only on the fluo 3-to-fura red ratio. When significant differences were detected, Student-Newman-Keuls and Dunn's post hoc tests were applied for parametric and nonparametric tests, respectively. An a-level of 0.05 was used for all statistical tests. Values are means ± SE unless otherwise noted.

RESULTS

Body weight. The mean body weight of the mice before hindlimb suspension was 30.5 ± 0.3 g (n = 31). After 2 wk, body weight increased 7% in the control mice, decreased 4% in the unloaded group, and did not change (-3%) significantly in the reloaded group. The final body weights of the mice are shown in Table 1.

Soleus muscle weight. The mean soleus muscle wet weight of the control mice was 8.2 ± 0.2 mg. Soleus muscle wet weights in the unloaded and reloaded mice were 45% and 43% less than in the control group, respectively (Table 1).

Table 1. Body weight, soleus muscle weight, and contractile properties in control and hindlimb-unloaded and -reloaded mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Unloaded</th>
<th>Reloaded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>32.4 ± 0.6</td>
<td>28.8 ± 0.6</td>
<td>30.1 ± 0.6</td>
</tr>
<tr>
<td>Soleus muscle wt, mg</td>
<td>8.2 ± 0.2</td>
<td>4.5 ± 0.1</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>Pt, mN</td>
<td>37.2 ± 1.3</td>
<td>28.9 ± 1.7</td>
<td>24.4 ± 1.3</td>
</tr>
<tr>
<td>P&lt;sub&gt;o&lt;/sub&gt;, mN</td>
<td>256.8 ± 12.6</td>
<td>107.2 ± 5.4</td>
<td>108.9 ± 4.5</td>
</tr>
<tr>
<td>N/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>33.0 ± 1.2</td>
<td>25.2 ± 0.8</td>
<td>25.3 ± 1.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. P<sub>t</sub>, peak isometric twitch force; P<sub>o</sub>, peak isotonic tetanic force. *Significantly different from control; †significantly different from control but not different from unloaded; ‡significantly different from control and unloaded (P < 0.05).
Histology. The numbers of muscle sections used to evaluate the extent of the histopathology in the control, unloaded, and reloaded mice were 20, 20, and 19, respectively. The average numbers of fibers per muscle cross section in control, unloaded, and reloaded mice were 481 ± 68, 597 ± 64, and 533 ± 50, respectively. The total numbers of actively degenerating muscle fibers observed in these sections were 4, 22, and 85 for the control, unloaded, and reloaded muscles, respectively.

In vitro soleus muscle contractile mechanics. The effects of unloading and reloading of the mouse hindlimbs on soleus muscle contractile mechanics are shown in Table 1. The mean $P_t$ of the control mice was 37.2 ± 1.3 mN. $P_t$ values in the unloaded and reloaded mice were 21 and 31% less than in the control group, respectively. The mean $P_o$ of the control mice was 256.8 ± 12.6 mN. $P_o$ values in the unloaded and reloaded mice were 58% less than in the control group. Specific force of the control group was 33.0 ± 1.2 N/cm². Specific forces in the unloaded and reloaded mice were 24% and 23% less than in the control group, respectively.

Resting $[Ca^{2+}]_i$. Two weeks of hindlimb suspension resulted in elevated resting $[Ca^{2+}]_i$ (Fig. 1). There was a 24% increase in the median ratio of fluo 3 to fura red in the unloaded soleus muscles. Twenty-four hours of reloading resulted in a significant reduction in the ratio of fluo 3 to fura red compared with the unloaded muscles, but it was still elevated 16% relative to control muscles. The resting $[Ca^{2+}]_i$ in the soleus muscle was estimated to be 17, 23, and 21 nM in the control, unloaded, and reloaded mice, respectively.

Tetanic and 4-chloro-m-cresol $[Ca^{2+}]_i$ transients. $Ca^{2+}$ transients during tetanic contractions and 4-chloro-m-cresol-induced contractures are shown in Fig. 2. There was no difference between the control and unloaded groups for tetanic $[Ca^{2+}]_i$ as estimated by the fluo 3-to-fura red ratios. The median ratios for control and unloaded soleus muscles over the 400-ms tetanic contractions were 1.15 and 1.11, respectively. However, the median ratio was reduced by 7% in the reloaded group, which was significantly less than that in the control muscles. The median fluo 3-to-fura red ratio was not different between the unloaded and reloaded muscles. The estimated median $[Ca^{2+}]_i$ levels during the tetanic contraction for the control, unloaded, and reloaded mice were 426, 375, and 330 nM, respectively. In contrast to the tetanic $[Ca^{2+}]_i$, the peak ratio during the 4-chloro-m-cresol-induced contracture for the unloaded (1.33) soleus muscles was reduced compared with the control muscles (1.47), whereas the peak ratio was not different from control in the reloaded (1.44) muscles. The estimated $[Ca^{2+}]_i$ levels during the 4-chloro-m-cresol-induced contractures for the control, unloaded, and reloaded mice were 1.6, 0.8, and 1.4 µM, respectively.

DISCUSSION

The primary purpose of the present study was to examine the possibility that reduced $[Ca^{2+}]_i$ transients during tetanic contractions might contribute to strength loss after simulated weightlessness and subsequent reloading of mouse hindlimb skeletal muscle. The results indicate that E-C uncoupling does not occur to any significant extent during tetanic contractions of mouse soleus muscle after 2 wk of hindlimb unloading. The median $[Ca^{2+}]_i$ (375 µM) during tetanic contractions in unloaded muscles was not different from that in control muscle (426 µM). This is in general agreement with the literature, because no major abnormalities have been noted in previous studies that examined
SR function from hindlimb-suspended soleus muscles (11, 15, 25). The primary finding in these previous studies was that the Ca\(^{2+}\) release and uptake components acquire biochemical (15) and functional (15, 25) characteristics similar to those of fast-twitch muscle. Therefore, although certain functional characteristics of the SR (i.e., peak 4-chloro-m-cresol-induced [Ca\(^{2+}\)]) appear to be altered because of hindlimb unloading, impairment of E-C coupling is not responsible for the observed strength deficits.

E-C coupling does appear impaired, albeit to a minor extent, after physiological reloading of mouse hindlimbs. It was estimated that reloading resulted in a 23% decrease in tetanic [Ca\(^{2+}\)], compared with control soleus muscles (Fig. 2). However, despite this decrease in availability of Ca\(^{2+}\) during tetanic contractions in the unloaded muscles, only the deficit in twitch force was compounded in the unloaded muscle compared with unloaded muscles. Peak tetanic [Ca\(^{2+}\)] and forces were not different between unloaded and reloaded muscles. Results suggest that the return to quadrupedal cage activity after 2 wk of hindlimb unloading imparts only minor impairments in E-C coupling and force output in mouse soleus muscle.

The histological data suggested that physiological unloading in mouse hindlimbs for 24 h after 2 wk of unloading was associated with greater muscle damage than hindlimb unloading alone (4.5 vs. 1.1 degenerating muscle fibers per section, respectively). Interestingly, there was no change in P\(e\) between the unloaded and reloaded muscles, despite the greater damage in the reloaded muscles.

Previously, our laboratory showed that 2 wk of hindlimb unloading in the mouse result in a greater susceptibility to contraction-induced injury of the soleus muscle (31). Compared with muscles from control animals, mouse soleus muscles unloaded for 2 wk exhibited significantly greater force decrements after isometric (−15 vs. +1%) and eccentric (−31 vs. −8%) contraction protocols (31). Therefore, it appears that the reloading employed in the present study was below the threshold required for inducing significant losses of maximum strength. Whether increased activation of the soleus muscle during physiological reloading (e.g., treadmill running) would result in greater skeletal muscle strength deficits remains to be tested experimentally.

The significance of the elevations (36%) in resting [Ca\(^{2+}\)] in the soleus muscle after hindlimb unloading is unclear (Fig. 1). However, several observations suggest that this change in intracellular Ca\(^{2+}\) homeostasis may play a role in initiating changes in skeletal muscle mass and/or phenotype during hindlimb unloading. A transient loss in intracellular Ca\(^{2+}\) homeostasis has been shown to precede changes in myosin heavy chain phenotype during chronic low-frequency stimulation (24). In addition, a loss of Ca\(^{2+}\) homeostasis is known to be associated with a number of pathological conditions in skeletal muscle (13). Increases in total muscle [Ca\(^{2+}\)] have been shown to occur after hindlimb immobilization (2). Presumably, it is the increase in resting free and total [Ca\(^{2+}\)] that serves to upregulate the Ca\(^{2+}\)-mediated neutral protease system (23, 26, 29) that has been reported after hindlimb suspension in rat skeletal muscle. Pharmacological blockade of the influx of extracellular Ca\(^{2+}\) during limb immobilization (22) as well as blockade of intracellular Ca\(^{2+}\) during hindlimb unloading (29) have been shown to attenuate skeletal muscle fiber atrophy and proteolysis, respectively.

In conclusion, factors other than E-C uncoupling must account for the majority of the decrease in soleus muscle specific force after hindlimb unloading in the mouse. Decreases in myofibrillar protein concentration (27, 30) and/or increases in the relative interstitial fluid volume (14) seem the most likely candidates to explain reductions in specific force. Physiological reloading results in a minor impairment of E-C coupling, but unloading-induced maximal strength deficits are not exacerbated by 24 h of reloading. Finally, future studies should examine whether the elevations in resting [Ca\(^{2+}\)] are responsible for initiating skeletal muscle mass and strength deficits during hindlimb unloading.

We thank J. osh Wenke for assistance with the histological analyses and Drs. Susan Hamilton and Michael Reid (Baylor College of Medicine) for input on this project.

This research was supported by a grant from the National Space Biomedical Research Institute and by the Omar Smith Chair.

Address for reprint requests and other correspondence: C. P. Ingalis, Dept. of Health and Kinesiology, 158 Read Bldg., Texas A & M University, College Station, TX 77843-4243.

Received 8 September 1998; accepted in final form 12 March 1999.

REFERENCES


