SERCA1 overexpression minimizes skeletal muscle damage in dystrophic mouse models

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¹Department of Kinesiology, School of Public Health, University of Maryland, College Park, Maryland; ²Department of Orthopaedics, University of Maryland School of Medicine, Baltimore, Maryland; and ³Department of Pediatrics, University of Cincinnati, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio

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Mázala DA, Pratt SJ, Chen D, Molkentin JD, Lovering RM, Chin ER. SERCA1 overexpression minimizes skeletal muscle damage in dystrophic mouse models. Am J Physiol Cell Physiol 308: C699–C709, 2015. First published February 4, 2015; doi:10.1152/ajpcell.00341.2014.—Duchenne muscular dystrophy (DMD) is characterized by progressive muscle wasting secondary to repeated muscle damage and inadequate repair. Elevations in intracellular free Ca²⁺ have been implicated in disease progression, and sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase 1 (SERCA1) overexpression has been shown to ameliorate the dystrophic phenotype in mdx mice. The purpose of this study was to assess the effects of SERCA1 overexpression in the more severe mdx/Utr⁻/⁻ mouse model of DMD. Mice overexpressing SERCA1 were crossed with mdx/Utr⁻/⁻ mice to generate mdx/Utr⁻/⁻/+SERCA1 mice and compared with wild-type (WT), WT/+SERCA1, mdx/+SERCA1, and genotype controls. Mice were assessed at ~12 wk of age for changes in Ca²⁺ handling, muscle mass, quadriceps tone, markers of muscle damage, and response to repeated eccentric contractions. SERCA1-overexpressing mice had a two- to threefold increase in maximal sarcoplasmic reticulum Ca²⁺-ATPase activity compared with WT which was associated with normalization in body mass for both mdx/+SERCA1 and mdx/Utr⁻/⁻/+SERCA1. Torque deficit in the quadriceps after eccentric injury was 2.7-fold greater in mdx/Utr⁻/⁻/+SERCA1 mice and compared with WT mice, but only 1.5-fold greater in mdx/Utr⁻/⁻/+SERCA1 vs. WT mice, only 1.5-fold greater in mdx/Utr⁻/⁻/+SERCA1 vs. WT mice, but only 1.5-fold greater in mdx/Utr⁻/⁻/+SERCA1 vs. WT mice. Markers of muscle damage (% centrally nucleated fibers, necrotic area, and serum creatine kinase levels) were higher in both mdx and mdx/Utr⁻/⁻ vs. WT, and all were attenuated by overexpression of SERCA1. These data indicate that SERCA1 overexpression ameliorates functional impairments and cellular markers of damage in a more severe mouse model of DMD. These findings support targeting intracellular Ca²⁺ control as a therapeutic approach for DMD.

Duchenne muscular dystrophy; mdx; mdx/Utr⁻/⁻; calcium; SERCA1; injury

DUCHENNE MUSCULAR DYSTROPHY (DMD), the most common form of muscular dystrophy, is an X-linked disorder that was first described over a century ago (17a). DMD is caused by the absence of dystrophin, a structural protein found on the cytoplasmic surface of the sarcolemma (30), and is characterized clinically by severe, progressive, and irreversible loss of muscle function. While the genetic basis for DMD has been known since 1987 (30), the mechanism(s) responsible for the progressive muscle damage and decrease in muscle specific force production that occur secondary to the lack of dystrophin remain unclear. Two main theories have been proposed to explain the cellular basis for the marked and progressive muscle degeneration: 1) a mechanical instability theory and 2) an alteration in cell signaling theory (37). The mechanical instability hypothesis (20) suggests that, in the absence of dystrophin, the resulting mechanical weakness of either the sarcolemma or the cytoskeletal-sarcolemmal interface triggers a pathway of cellular damage, degeneration, and repair (38, 67). An increase in myofiber damage observed secondary to stress, such as after forceful lengthening (“eccentric”) contractions, supports the concept of mechanical instability as the initial cause of symptoms associated with dystrophic skeletal muscle (49). The second hypothesis focuses on alterations in molecular signaling processes within dystrophic myofibers. Specifically, alterations in myofiber calcium (Ca²⁺), nitric oxide, and reactive oxygen species have been identified in dystrophic myofibers.

Elevations in intracellular free Ca²⁺ concentration ([Ca²⁺]) and the downstream activation of protein degratory or necrotic pathways have been implicated in DMD disease progression (10, 22, 35). Although the precise mechanisms remain unclear, there is evidence to support increased activation of membrane Ca²⁺ influx channels in the activation of Ca²⁺-dependent proteases (i.e., calpains) (2, 5, 46, 66) and either cell necrosis or apoptosis (3, 17, 57, 63). Furthermore, there is evidence of impaired Ca²⁺ removal from the sarcoplasm due to reduced levels of the sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase 1 (SERCA1) expression or impaired Ca²⁺ pumping capacity of SERCA1 (16, 33, 59). Investigations targeting SERCA1 upregulation support the notion that increased levels of SERCA1 can mitigate the dystrophic pathology as well as contraction-induced muscle damage in mdx mice (26, 43).

One challenge in understanding the cellular basis of DMD as well as developing effective therapeutic strategies is that not all animal models closely mimic the disease. The most universally used laboratory animal model of DMD, the mdx mouse, has an X-linked recessive mutation in the dystrophin gene which resembles that seen in boys with DMD (6). This mouse model lacks dystrophin (61) and shows some hallmark features of the DMD pathology (6, 64). However, mdx mice live a near normal lifespan and the phenotype is transient and much less severe than that seen in patients with DMD, casting doubt on the mdx mouse as the most appropriate model for the disease (1). One reason that mdx mice do not display an equivalent pathology to DMD may be due to utrophin (Utr) (74), a homolog of dystrophin, that in mice is upregulated in the absence of dystrophin. This idea has been supported by the observation that mice lacking both dystrophin and utrophin (mdx/Utr⁻/⁻) have a much more severe myopathy (15, 27).
On the basis of studies demonstrating the role of impaired Ca\(^{2+}\) homeostasis and impaired SERCA1 in the dystrophic phenotype, we hypothesized that SERCA1 overexpression would mitigate the severe muscle pathology observed in \(mdx/Utr\) \(^{-/-}\) mice. The purpose of this study was to compare changes in Ca\(^{2+}\) handling, muscle size and histology, contractile function, and susceptibility to contraction-induced injury in wild-type (WT), \(mdx\), and \(mdx/Utr\) \(^{-/-}\) mice with and without overexpression of SERCA1. Our results show that overexpression of SERCA1 in dystrophic mouse models not only decreases markers of muscle damage, but also attenuates force loss after maximal eccentric contractions. This is the first report to show significant changes in the \(mdx/Utr\) \(^{-/-}\) mouse from SERCA1 overexpression. These novel findings suggest that improvements in control of \([\text{Ca}^{2+}]_i\) may be of importance in the development of therapies for DMD.

**MATERIALS AND METHODS**

*Ethical approval.* All procedures were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the University of Maryland, College Park.

*Animal breeding and genotyping.* A breeding pair of SERCA1-overexpressing (+SERCA1) mice were obtained from the Cincinnati Children’s Hospital Medical Center. Briefly, +SERCA1 mice were generated using a modified human skeletal muscle α-actinin promoter to selectively overexpress the fast muscle isofrom SERCA1 (26) in skeletal muscle. Breeders were then used to establish a colony of +SERCA1 mice at the University of Maryland Central Animal Research Facility. Animals were genotyped using the following forward and reverse primer sequences: 5’-CGA GAG TAG CAG TTG TAG TAG CTA-3’ (forward) and 5’-ACA AAG GCA GTG ACA GT3’ (reverse). The thermodcyclic polymerase chain reaction (PCR) conditions consisted of 5 min at 94°C followed by 34 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 60 s, and a final extension at 72°C for 5 min. Breeding pairs of \(mdx/Utr\) \(^{-/-}\) were previously obtained from Dr. Diego Fraidenraich of the University of Medicine and Dentistry of New Jersey (Newark) in collaboration with Dr. Robert Grange of Virginia Polytechnic Institute and State University (Blacksburg). Offspring from \(mdx/Utr\) \(^{-/-}\) mice were genotyped for \(Utr\) using a primer sequence and PCR conditions generously provided by Dr. Dawn A. Lowe from the University of Minnesota, Department of Physical Medicine and Rehabilitation (Minneapolis). Genotyping for dystrophin was performed according to a protocol previously published (60).

To establish the +SERCA1 dystrophic mice, +SERCA1 mice were bred with \(mdx/Utr\) \(^{-/-}\) mice. Offspring from +SERCA1 × \(mdx/Utr\) \(^{-/-}\) were genotyped and used to produce breeding pairs to generate \(mdx/+\) SERCA1 and \(mdx/Utr\) \(^{-/-}\)/+SERCA1 mice. All animals, including WT, WT/+SERCA1, \(mdx\), \(mdx/+\) SERCA1, \(mdx/Utr\) \(^{-/-}\), and \(mdx/Utr\) \(^{-/-}\)/+SERCA1 used in the present study were derived from our in-house animal colony. All mice were kept in the same room (typical ambient conditions 20.9% O\(_2\) and 22 ± 1°C) with equal access to food and water, bedding, and light cycles (12 h light/12 h dark). For all evaluations, we used animals at 3 mo of age based on previous work by Goonasekera et al. (26).

**Experimental procedures.** Two subsets of mice were used in the current study: 1) mice used to analyze muscle mass, protein expression, sarcomplasmic reticulum (SR) Ca\(^{2+}\)-ATPase activity, and \([\text{Ca}^{2+}]_i\); levels in single muscle fibers; and 2) mice used to evaluate quadriceps muscle function (torque and response to eccentric contractions) and muscle histology. In the subset of mice used to evaluate the effect of SERCA1 overexpression on muscle mass, \([\text{Ca}^{2+}]_i\), handling and protein expression, animals were euthanized by cervical dislocation and the tubialis anterior (TA), extensor digitorum longus (EDL), soleus, plantaris, heart, and quadriceps (QUAD) were quickly dissected, weighed, and snap frozen in liquid nitrogen and stored at −80°C. At the time of death, the flexor digitorum brevis (FDB) muscle was removed and single muscle fibers isolated for assessing \([\text{Ca}^{2+}]_i\) levels. In the subset of mice used to perform functional assessments, mice were anesthetized with isoflurane and maintained under anesthesia throughout the duration of the assessment for muscle torque and injury (details below). At the end of the experiments, animals were euthanized by cervical dislocation and muscles were obtained for histological assessments.

**Single muscle fiber isolation and free \([\text{Ca}^{2+}]_i\), measurements.** Detailed methods for single muscle fiber isolation and \([\text{Ca}^{2+}]_i\) measurements have been previously described (47). Briefly, single muscle fibers were obtained from the FDB muscle by collagenase digestion with type 2 collagenase (Worthington) in minimal essential medium (MEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen). After incubation at 37°C in 95% O\(_2\)-5% CO\(_2\), single muscle fibers were obtained by trituration. Subsequently, fibers were maintained in MEM solution with 10% FBS at 37°C, 95% O\(_2\)-5% CO\(_2\) until used for \([\text{Ca}^{2+}]_i\), assessment.

One day after dissection, fibers were loaded with fura-2 AM for 15 min. The fura-2 ratio was measured in response to varying stimuli (see protocol below) as an index of \([\text{Ca}^{2+}]_i\). Fibers loaded with fura-2 AM were placed in a stimulation chamber containing parallel electrodes, and the chamber was positioned on a Nikon TiU microscope stage. Muscle fibers were continuously perfused with a stimulating Tyrode solution (in mM: 121.0 NaCl, 5.0 KCl, 1.8 CaCl\(_2\), 0.5 MgCl\(_2\), 0.4 NaH\(_2\)PO\(_4\), 24.0 NaHCO\(_3\), and 5.5 glucose) with 0.2% FBS (11). This solution was bubbled with 95% O\(_2\)-5% CO\(_2\) to maintain a pH of 7.3 (11). Levels of \([\text{Ca}^{2+}]_i\) were assessed by the fura-2 fluorescence ratio using an IonOptix Hyperswitch system with dual excitation, single emission filter set for fura-2 (excitation 340 nm and 380 nm; emission 510 nm). Signals were captured and analyzed using the IonWizard software (IonOptix). Global fura-2 ratio was measured in muscle fibers using trains of stimuli at 10, 30, 50, 70, 100, 120, and 150 Hz for 350 ms with fibers resting 1 min between frequencies. Peak fura-2 \([\text{Ca}^{2+}]_i\) handling and frequency is determined by the average ratio in the last 100 ms of the 350 ms tetanus, when Ca\(^{2+}\) fura-2 should be at a steady state. All single muscle fibers were evaluated at room temperature.

**Maximal Ca\(^{2+}\)-ATPase activity.** Quadriceps muscles were homogenized and used to measure maximal SR Ca\(^{2+}\)-ATPase activity in whole muscle homogenates as described by Chin et al. (12). Briefly, frozen muscles were weighed and homogenized in 200 mM sucrose, 10 mM Na\(_2\)HPO\(_4\), 1 mM EDTA, and 40 mM l-histidine (pH 7.8), with a polystyron at 60% maximal power for three 10-s intervals, separated by at least 30 s (12). Aliquots of the initial homogenate were frozen for analysis of maximal SR Ca\(^{2+}\)-ATPase activity. Maximal SR Ca\(^{2+}\)-ATPase activity was measured as described previously (12, 62) with assay modified for a 96-well plate. Briefly, the ATPase reaction was measured in buffer containing 20 mM HEPES, 200 mM KCl, 15 mM MgCl\(_2\), 10 mM Na\(_2\)HPO\(_4\), 0.3 mM NADH, 10 mM phosphoenolpyruvate (PEP), 5 mM ATP, 4 μM calcium ionophore A23187, and 18 U/ml of both lactate dehydrogenase and pyruvate kinase (pH 7.0) and either 1 mM CaCl\(_2\) or 1 mM CaCl\(_2\) + cyplosporin A (CPA). CPA is a selective inhibitor of the SERCA2 (CPA) ATPase. SR Ca\(^{2+}\)-activated ATPase activity was measured in triplicate in a 96-well plate at 37°C, and maximum activity was determined as the difference between maximal activity (1 mM CaCl\(_2\)) and basal activity (1 mM CaCl\(_2\) + CPA).

**SERCA1 protein expression.** Total protein was isolated from QUAD by homogenization in lysis buffer (20 mM HEPES buffer, pH 7.5, 100 mM NaCl, 1.5 mM MgCl\(_2\), 0.1% Triton X-100, 20% glycerol) containing 1 mM DTT and protease inhibitor cocktail.

This article is the topic of an Editorial Focus by Arthur J. Cheng, Daniel C. Andersson, and Johanna T. Lanner (10a).
(complete mini EDTA-free protease inhibitor cocktail, Roche Diagnostics, Indianapolis, IN). After 20 min of incubation at 4°C, samples were centrifuged at 20,000 g and the supernatant was collected and frozen at −80°C until analyzed. Total protein concentration was determined using a BCA assay (Thermo Fisher Scientific, Rockford, IL). Samples were solubilized in loading buffer and denatured by heating at 100°C for 5 min. For immunoblotting for SERCA1 expression, 20 μg total protein was loaded on bis-acrylamide gels, separated by SDS-PAGE electrophoresis then transferred to PVDF membrane (EMD Millipore, Billerica, MA). Membranes were blocked with 5% nonfat milk at room temperature for 1 h followed by overnight incubation at 4°C in anti-SERCA1 antibody (1:2,500, Thermo Fisher Scientific). GAPDH (1:2,000, Thermo Fisher Scientific) was used as a loading control. Proteins were detected with Clarity western ECL substrate (Bio-Rad, Hercules, CA) and imaged using Image Lab system (Bio-Rad), and protein levels were quantified by densitometry. SERCA1 protein levels are expressed in arbitrary units. Membranes were also stained with MemCode Reversible Protein Stain Kit (Thermo Fisher Scientific) to confirm equal loading.

**Markers of skeletal muscle damage.** Histological analysis was completed on the basis of recommended standards from previously published protocols (TREAT-NMD: http://www.treat-nmd.eu/research/preclinical/dmd-sops/; see also Ref. 54). For histological analysis, transverse sections were cut from frozen muscle tissue on a cryostat (~8 μm thickness) and collected onto glass slides (SuperFrost Plus; VWR, West Chester, PA). After fixation in methanol for 5 min followed by a 30-s wash in H2O2, samples were incubated in Harris modified hematoxylin solution for 3 min. After washing in H2O2 and Scott’s tap water solution for 1 min each, slides were incubated in eosin for 2 min. Samples were then washed in a series of ethanol solutions (50% × 30 s, 75% × 30 s, 95% × 2 min, 100% × 2 min) and then xylene (2 washes for 3 min each). Sections were mounted in Permount and imaged using a Nikon Eclipse 50i microscope (×20 objective) and Nikon’s NIS-Elements Basic Research software. The percentage of centrally nucleated fibers (CNFs) was determined in a blinded fashion and was calculated as the number of fibers with central nuclei divided by the number of total fibers per muscle section. Muscle necrosis was determined by calculating the percentage area of the muscle with the presence of infiltrating inflammatory cells (basophilic staining), hypercontracted fibers, and degenerating fibers with scattered sarcotubules (28) as a percentage (area) of the whole muscle section. For both percentage of CNFs and necrotic area, sections were randomized and viewed at ×20 magnification. Optical fields contained an average of 183 ± 19 fibers, and >20 fields were counted per muscle.

For assessment of creatine kinase (CK) activity, blood was collected from animals through heart puncture immediately prior to euthanasia. CK levels were determined using a Creatine Kinase Fluorometric Assay Kit (catalog no. 700630, Cayman) as described by euthanasia. CK levels were determined using a Creatine Kinase Fluorometric Assay Kit (catalog no. 700630, Cayman) as described by (N·mm). The femoral nerve was stimulated via subcutaneous needle electrodes (36BTP, Jari Electrode Supply, Gilroy, CA). Proper electrode position was determined by a series of isometric Twitches and by observing isolated knee extension in the anesthetized animal. Length-tension and force-frequency curves were generated to ensure maximum isometric tension, and optimal length was set prior to initiating lengthening contractions. For inducing lengthening contractions, a custom program, written on commercial software (LabVIEW version 8.5, National Instruments, Austin, TX), was used to synchronize contractile activation and the onset of forced knee flexion. Injury resulted from 15 maximal lengthening contractions (150-Hz stimulation; 900/s) superimposed onto maximal QUAD isometric contractions through a 40°–100° arc of motion (full knee extension considered 0°) spaced 1 min apart. Maximal isometric torque was measured before lengthening contractions and 5 min after the last lengthening contraction and was used to calculate force deficits. Sham procedures (contractions without lengthening, or passive lengthening without contractions, both with knee immobilized) have been performed (50).

**Radiography.** Postural changes, such as excessive kyphosis, have been noted previously in the mdx/Utr−/− mice (7, 56, 71). To confirm those reports and assess any changes due to SERCA1 overexpression, radiographs (X-rays) were performed on a separate group of mice with a digital Faxitron radiography machine (Faxitron X-Ray, Lincolshire, IL). We used a previously established protocol to demonstrate the spine curvature of mice (the kyphotic index) by drawing two lines between the caudal margin of the seventh (cervical vertebra and the caudal margin of the sixth lumbar vertebra (34).

**Statistical analysis.** SPSS software (version 21.0; IBM, Somers, NY) was used for statistical analyses of data. A one-way analysis of variance (ANOVA) was used to evaluate differences between all groups, with the Scheffé’s test for post-hoc analyses. In cases where two independent groups were compared (i.e., mdx vs. mdx+/SERCA1 or mdx/Utr−/− vs. mdx/Utr−/−+SERCA1) we used a Student’s t-test. The statistical significance was accepted at P < 0.05. Values shown represent means ± SE.

**RESULTS**

**Effects of SERCA1 overexpression on body mass, muscle mass, and intracellular calcium handling.** The body and muscle mass of mice are shown in Table 1. The body mass of mdx was 13% greater than WT, while the body mass from mdx/Utr−/− was 35% lower than WT (P < 0.05; Table 1). Body mass of dystrophic mice overexpressing SERCA1 (mdx/+SERCA1 and mdx/Utr−/−+/SERCA1) was similar to WT mice, indicating that overexpression of SERCA1 was able to rescue the characteristic greater body mass of mdx mice and the lower body mass of the mdx/Utr−/− model. Absolute muscle mass was higher in TA, plantaris, soleus, and EDL of mdx mice compared with WT (Table 1) and this compensatory hypertrophy was attenuated with SERCA1 overexpression. Absolute muscle mass of QUAD and heart was significantly reduced in mdx/Utr−/− vs. WT, with the difference mitigated in mdx/Utr−/−+/SERCA1 for QUAD, indicating rescue in the larger proximal skeletal muscle.

Since muscle hypertrophy is a known early response in dystrophic mice, we also evaluated changes in relative muscle mass (Fig. 1). The relative mass of the TA, soleus, and plantaris from mdx was 22%, 27%, and 25% greater, respectively, compared with WT (P < 0.05; Fig. 1). Moreover, the TA (37%), soleus (28%), plantaris (38%), and EDL (46%) had greater relative masses in mdx/Utr−/− compared with WT (P < 0.05; Fig. 1). Overexpression of SERCA1 in both mdx mice (mdx/+SERCA1) and mdx/Utr−/− (mdx/Utr−/−+/SERCA1) rescued this pseudo-hypertrophy in TA, soleus, plantaris, and EDL muscles. When expressed relative to body mass, we did not observe any differences in QUAD (Fig. 1E) and heart muscle mass (Fig. 1F) between mdx and mdx/Utr−/− relative to WT. Interestingly, SERCA1 overexpression resulted in lower

**References**

1. C701 SERCA1 RESCUE OF DYSTROPHIC MUSCLE DAMAGE

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relative muscle mass for EDL and QUAD in WT mice ($P < 0.05$; Fig. 1), although no differences were found for TA, soleus, and plantaris. Finally, hearts from WT/+SERCA1 mice had greater relative muscle mass compared with WT mice ($P < 0.05$; Fig. 1F).

Aside from characterization of muscle mass, all experiments were performed using the QUAD muscle. This muscle was chosen because proximal muscles are affected earlier and to a greater extent in patients with DMD and the mdx mouse (14, 40, 51). The QUAD muscle was also chosen because 1) it is a large muscle that allows for better assessment of differences in contractility; 2) it is the only knee extensor; and 3) it is more crucial for gait than the plantar flexors and dorsiflexors (52). We performed Western blot analysis to determine the level of SERCA1 protein expression in the QUAD muscle of all genotypes (Fig. 2A). SERCA1 content was higher in QUADs from WT/+SERCA1 vs. WT (60%; $P < 0.05$), mdx/+SERCA1 vs. mdx (49%; $P < 0.05$), and mdx/Utr$\text{}^-$/+SERCA1 vs. mdx/Utr$\text{}^-$/ (36%; $P < 0.05$) (Fig. 2A). This is consistent with previous characterization of the skeletal $\alpha$-actinin-driven SERCA1 transgenic mouse showing increased expression of SERCA1 in QUAD, gastrocnemius, diaphragm, and soleus muscle (26). There was no difference in SERCA1 content between WT, mdx, and mdx/Utr$\text{}^-$/, in agreement with previous findings for the QUAD muscle (59). To determine whether SERCA1 overexpression resulted in an increase in function (i.e., greater SERCA1 activity), we measured maximal SR Ca$^{2+}$-ATPase activity in QUAD muscle homogenates. Our

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### Table 1. Effects of SERCA1 overexpression on body and muscle mass

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of animals</th>
<th>Body mass, g</th>
<th>TA, mg</th>
<th>Plantaris, mg</th>
<th>Soleus, mg</th>
<th>EDL, mg</th>
<th>QUAD, mg</th>
<th>Heart, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT + SERCA1</td>
<td>16</td>
<td>29.6 ± 0.7</td>
<td>14.3 ± 1.2</td>
<td>16.4 ± 0.4</td>
<td>6.4 ± 0.2</td>
<td>9.0 ± 0.4</td>
<td>271.5 ± 27.8</td>
<td>136.2 ± 5.1</td>
</tr>
<tr>
<td>mdx</td>
<td>14</td>
<td>34.1 ± 1.1</td>
<td>13.6 ± 3.7</td>
<td>12.9 ± 1.2</td>
<td>6.4 ± 0.9</td>
<td>5.8 ± 0.6</td>
<td>141.8 ± 31.0</td>
<td>153.6 ± 18.6</td>
</tr>
<tr>
<td>mdx/Utr$\text{}^-$/+SERCA1</td>
<td>5</td>
<td>31.1 ± 0.7</td>
<td>6.7 ± 3.2</td>
<td>22.7 ± 0.8</td>
<td>10.0 ± 0.9</td>
<td>12.7 ± 0.8</td>
<td>321.0 ± 16.8</td>
<td>165.6 ± 7.3</td>
</tr>
<tr>
<td>mdx/Utr$\text{}^-$/+SERCA1</td>
<td>8</td>
<td>19.2 ± 3.6</td>
<td>4.4 ± 2.6</td>
<td>14.7 ± 1.3</td>
<td>7.3 ± 0.4</td>
<td>6.8 ± 0.6</td>
<td>219.0 ± 19.0</td>
<td>165.8 ± 4.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. Total no. of animals, body mass, and muscle mass for mice of all genotypes used for all experiments. Muscles analyzed include tibialis anterior (TA; A), plantaris, soleus, extensor digitorum longus (EDL), quadriceps (QUAD), and heart (wild type (WT, n = 15), WT/+SERCA1 (n = 8), mdx (n = 8), mdx/+SERCA1 (n = 10), mdx/Utr$\text{}^-$/ (n = 5), and mdx/Utr$\text{}^-$/+SERCA1 (n = 5)). $^*$P < 0.05 vs. WT; $^*$P < 0.05 vs. mdx; $^*$P < 0.05 vs. mdx/Utr$\text{}^-$/.

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![Fig. 1. Sarcoplasmic/endo粗plasmic reticulum Ca$^{2+}$-ATPase (SERCA1) overexpression alters relative muscle mass in dystrophic mouse models. Muscle mass is shown relative to body mass (mg/g) for tibialis anterior (TA; A), soleus (B), plantaris (C), extensor digitorum longus (EDL; D), quadriceps (QUAD; E), and heart (F). Wild-type mice (WT), n = 15; WT/+SERCA1, n = 8; mdx, n = 8; mdx/+SERCA1, n = 10; mice lacking dystrophin/utrophin (mdx/Utr$\text{}^-$/), n = 5; mdx/Utr$\text{}^-$/+SERCA1, n = 5. $^*$P < 0.05 vs. WT; $^*$P < 0.05 vs. mdx; $^*$P < 0.05 vs. mdx/Utr$\text{}^-$/]. Data presented as means ± SE.
results demonstrate that QUAD muscles from animals overexpressing SERCA1 had greater maximal Ca$^{2+}$-activated ATPase activity in QUAD muscle. A, top: representative Western blot from WT, dystrophin-deficient (mdx), and dystrophin- and utrophin-deficient (mdx/Utr-) mice with and without SERCA1 overexpression. A, bottom: densitometry values for WT (n = 5), WT+/SERCA1 (n = 5), mdx (n = 3), mdx/+SERCA1 (n = 3), mdx/Utr$^{-/-}$ (n = 3), and mdx/Utr$^{-/-}$/+SERCA1 (n = 3). AU, arbitrary units. B: maximal SR Ca$^{2+}$-ATPase activity in QUAD muscle homogenates (WT (n = 3), WT+/SERCA1 (n = 3), mdx (n = 3), mdx/+SERCA1 (n = 3), mdx/Utr$^{-/-}$ (n = 3), and mdx/Utr$^{-/-}$/+SERCA1 (n = 3)). *P < 0.05 vs. WT; &P < 0.05 vs. mdx; YP < 0.05 vs. mdx/Utr$^{-/-}$. Data presented as means ± SE.

Fig. 2. SERCA1 overexpression increases SERCA1 protein levels and maximal sarcomplasmic reticulum (SR) Ca$^{2+}$-ATPase activity in QUAD muscle. A: top: representative Western blot from WT, dystrophin-deficient (mdx), and dystrophin- and utrophin-deficient (mdx/Utr-) mice with and without SERCA1 overexpression. A: bottom: densitometry values for WT (n = 5), WT+/SERCA1 (n = 5), mdx (n = 3), mdx/+SERCA1 (n = 3), mdx/Utr$^{-/-}$ (n = 3), and mdx/Utr$^{-/-}$/+SERCA1 (n = 3). AU, arbitrary units. B: maximal SR Ca$^{2+}$-ATPase activity in QUAD muscle homogenates (WT (n = 3), WT+/SERCA1 (n = 3), mdx (n = 3), mdx/+SERCA1 (n = 3), mdx/Utr$^{-/-}$ (n = 3), and mdx/Utr$^{-/-}$/+SERCA1 (n = 3)). *P < 0.05 vs. WT; &P < 0.05 vs. mdx; YP < 0.05 vs. mdx/Utr$^{-/-}$. Data presented as means ± SE.

**Effects of SERCA1 overexpression on markers of muscle damage.** The effect of SERCA1 overexpression on muscle damage was evaluated by histology and circulating levels of CK. Skeletal muscles from WT+/SERCA1 mice did not show any pathological features compared with WT (Fig. 4A), consistent with a previous report by Goonasekera et al. (26). Muscle sections from mdx and mdx/Utr$^{-/-}$ mice had a greater number of CNFs compared with WT (P < 0.01; Fig. 4, A and B), while mdx/+SERCA1 had 58% fewer CNFs compared with mdx mice (P < 0.05; Fig. 4, A and B), and mdx/Utr$^{-/-}$/SERCA1 had 40% less CNFs compared with mdx/Utr$^{-/-}$ (P < 0.05; Fig. 4, A and B). Muscle necrosis was determined by the presence of basophilic staining (infiltrating inflammatory cells) and fibers with disrupted sarcoplasm (54). The percentage of necrosis in both mdx and mdx/Utr$^{-/-}$ was greater compared with WT (P < 0.05 for mdx vs. WT, and P < 0.01 for mdx/Utr$^{-/-}$ vs. WT; Fig. 4C). Muscle sections from mdx/+SERCA1 had 50% less necrosis compared with mdx (P < 0.05; Fig. 4C), while muscle sections from mdx/Utr$^{-/-}$/SERCA1 had almost 90% less necrotic area compared with mdx/Utr$^{-/-}$ (P < 0.01; Fig. 4C).

Creatine kinase is frequently used as an indirect measure of muscle damage in DMD (23, 25, 26, 28, 79). Both mdx and mdx/Utr$^{-/-}$ had greater CK levels compared with WT mice (86% greater for mdx and 76% for mdx/Utr$^{-/-}$; P < 0.01; Fig. 4D). Overexpression of SERCA1 in mdx (mdx/+SERCA1) reduced CK levels by 60% compared with mdx mice (P < 0.05; Fig. 4D), results that support findings from Goonasekera et al. (26). The mdx/Utr$^{-/-}$/SERCA1 had a 50% reduction in blood CK levels compared with mdx/Utr$^{-/-}$ mice (P < 0.05; Fig. 4D).

**Effects of SERCA1 overexpression on muscle torque and susceptibility to injury.** To assess the effects of SERCA1 expression on skeletal muscle function, we used an in vivo model to assess QUAD muscle function before and after injury induced by repeated eccentric contractions. Without injury, muscle peak torque expressed relative to muscle mass (i.e., normalized peak torque) was ~43% lower in mdx compared with WT (P < 0.05, Fig. 5A), indicating a reduction in muscle quality in mdx mice. In the QUAD of mdx/Utr$^{-/-}$ mice, normalized peak torque was 56% lower compared with WT (P < 0.05; Fig. 5A). We did not observe differences in normalized peak torque between mdx and mdx/Utr$^{-/-}$, a finding different from previous studies that found differences in the posterior crural muscles (gastrocnemius, soleus, and plantaris), EDL, and sternomastoid (8, 15, 27, 36). In WT mice with SERCA1 overexpression, there was no difference in normalized peak torque, indicating that muscle quality was not altered in these mice (P = 0.14). SERCA1 overexpression also did not alter normalized peak torque in mdx mice (P = 0.21 vs. mdx/+SERCA1) or in mdx/Utr$^{-/-}$ mice (P = 0.19 vs. mdx/Utr$^{-/-}$/SERCA1) compared with their genotype controls (Fig. 5A). Overall, overexpression of SERCA1 did not rescue the deficit in normalized peak torque in either dystrophic mouse model.

To evaluate the possible protective effect of increased SERCA1 overexpression on the acute damage induced by eccentric contractions, we performed two different analyses to evaluate in vivo susceptibility to injury: 1) total percent loss in torque at the end of 15 eccentric contractions and 2) average percent force loss during the 15 contractions (from rep 1 to rep
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15). Lengthening contractions induced a greater deficit in torque in mdx and mdx/Utr−/− compared with WT mice (P < 0.05; Fig. 5, B and C). Susceptibility to injury was reduced by the overexpression of SERCA1 in mdx mice (mdx/+SERCA1) and in mdx/Utr−/− mice (mdx/Utr−/−/+SERCA1). Our findings are in agreement with Morine et al. (43), in which overexpression of SERCA1 ameliorated in vitro contraction-induced damage in the diaphragm muscle of mdx mice.

Effects of SERCA1 overexpression on skeletal deformity in dystrophic mice. Muscle weakness is a key contributor to joint contractures, growth retardation, and spinal curvature (i.e., kyphosis) in mdx and mdx/Utr−/− mice (34, 55). In mdx/Utr−/− mice the severe curvature (Fig. 6C) is reduced with SERCA1 overexpression (Fig. 6B). This is consistent with the increased body mass in mdx/Utr−/−/+SERCA1 mice, indicating increased growth secondary to an improvement in muscle mass and attenuated muscle damage with contractile activity.

DISCUSSION

In the present study, we used two different mouse models of DMD with varying degrees of pathology to show that overexpression of SERCA1 leading to a two- to threefold increase in SR Ca2+-ATPase activity was sufficient to 1) alter body and muscle mass to more closely resemble that of healthy animals; 2) decrease markers of muscle damage; and 3) protect the muscle from contraction-induced injury. More specifically, dystrophic mice overexpressing SERCA1 (mdx/+SERCA1 and mdx/Utr−/−/+SERCA1) had a lower incidence of CNFs and necrosis and lower circulating CK levels compared with mdx and mdx/Utr−/−. While neither mdx nor mdx/Utr−/− dystrophic mice had any increase in relative peak torque or change in excitation-contraction (E-C) coupling with SERCA1 overexpression, both mdx/+SERCA1 and mdx/Utr−/−/+SERCA1 were less susceptible to injury after eccentric contractions compared with mdx and mdx/Utr−/−. Overall, these results agree with and extend previous findings showing that SERCA1 overexpression can mitigate muscle damage in mouse models of DMD under both resting conditions and in response to eccentric contractions (26, 43). We hypothesize that this is due to the increased capacity of SERCA1 to remove cytosolic Ca2+ following influx with eccentric contractions. This is the first report to demonstrate that this approach can mitigate the disease phenotype in the mdx/Utr−/− mouse, a model with a more similar disease progression to that of DMD patients than the mdx mouse. These data support the notion that increased SERCA expression, or activity, could have therapeutic utility in DMD.

In the current study, we observed an attenuation of the pseudohypertrophy in limb muscles of mdx/+SERCA1 mice at 3 mo of age. Compared with WT, our observations of a 22–46% increase in muscle mass in mdx mice are consistent with previous reports showing a 20–30% hypertrophy in soleus and EDL muscle (13) and a 20% (53) and 58% (48) increase in TA muscle in mdx mice under 6 mo of age. This early hypertrophy is purportedly a compensatory effect, due to the muscle weakness and myocellular damage and consequent activation of satellite cells to induce new myofiber growth. The reduction in this hypertrophy with increased Ca2+ clearance capacity is consistent with the notion that Ca2+-dependent pathways, including calcineurin activation, contribute to the...
adaptive hypertrophy early in the developmental stages of mdx mice (9, 65). The increased muscle size in mdx mice, however, does not result in an equivalent increase in force production per unit area, with both soleus and EDL muscle showing a decrease in specific force by 5 mo of age (39), indicating that these muscles are larger but weaker when normalized to muscle volume. This is thought to be due to cellular malformations including myofiber branching (21, 37) and altered E-C coupling (37). We also report pseudohypertrophy in the mdx and mdx/mdx/Utr−/− mice. In both disease models, SERCA1 overexpression rescued this pseudohypertrophy effect, reducing relative muscle mass to WT levels.

Interestingly, SERCA1 overexpression also resulted in reduced muscle size in WT mice. It is possible that the increase in SR Ca2+-ATPase activity resulted in increased energy expenditure and thus a smaller, less energy-efficient muscle. It has recently been shown that treatment of muscle fibers with FK506 resulted in increased Ca2+ efflux from the ryanodine receptor with no significant increase in resting [Ca2+]i, but rather an increase in energy expenditure (oxygen consumption) due to increased SR Ca2+-ATPase activation (4). Thus it is plausible that the increased energy expenditure from increased ATPase activity led to development of smaller, less efficient muscles. It was not the aim of the current study to address this adaptation in WT muscle, but this issue warrants further investigation.

There was no improvement in QUAD normalized peak torque in either line of dystrophic mice, indicating no improvement in specific tension (Fig. 5A). This may be due to the fact that proximal muscles show an earlier and greater extent of damage in dystrophic mice (44). However, this study was underpowered for this endpoint (β < 0.80), a limitation of the study due to the challenges of breeding the mdx/mdx/Utr−/− mice (47). Still, SERCA1 overexpression profoundly reduced muscle damage and reduced the susceptibility to injury in both dystrophic mouse models. Morine et al. (43) previously showed an attenuation of eccentric-induced injury in diaphragm muscle with SERCA1 overexpression. However, we are the first to show a functional improvement in a hindlimb muscle group. Overall, SERCA1 overexpression results in a relatively smaller but more durable muscle, capable of withstanding injury from repeated lengthening contractions.

Impaired Ca2+ homeostasis contributes to disease pathology in dystrophic muscle (2, 24, 31, 42, 76), presumably due to both an increase in Ca2+ entry and reduced Ca2+ clearance. Given the role of increased cytosolic Ca2+ in muscle degradation/necrosis pathways (58), we postulate that increased expression of SERCA1 was critical in maintaining [Ca2+]i, homeostasis and in reducing muscle damage in both mdx and mdx/mdx/Utr−/− during the described compensatory hypertrophy phase of 3-mo-old mice. One mechanism that may contribute to increased sarcoplasmic Ca2+ entry in dystrophic muscle is store-operated calcium entry (19, 42). Another source of Ca2+ influx in dystrophic muscle is through stretch-activated Ca2+ channels (75, 78). Isolated muscles from dystrophic mice treated with stretch-activated channel blockers (streptomycin

Fig. 4. SERCA1 overexpression decreases markers of damage in dystrophic mouse models. A: hematoxylin and eosin staining of QUAD muscle sections from different mouse genotypes (scale bar, 100 μm). The yellow arrows indicate examples of central nuclei that were used for quantification of centrally nucleated fibers (CNFs). The yellow circles show examples of necrosis. B: percentage of CNFs in muscle sections from mouse genotypes [WT (n = 4), WT/+SERCA1 (n = 4), mdx (n = 5), mdx/+SERCA1 (n = 5), mdx/Utr−/− (n = 4), mdx/Utr−/−/+SERCA1 (n = 5)]. C: percentage of necrotic areas in mouse sections from different genotypes [WT (n = 4), WT/+SERCA1 (n = 4), mdx (n = 5), mdx/+SERCA1 (n = 5), mdx/Utr−/− (n = 4), and mdx/Utr−/−/+SERCA1 (n = 5)]. D: levels of creatine kinase (CK) in different mouse groups [WT (n = 4), WT/+SERCA1 (n = 4), mdx (n = 6), mdx/+SERCA1 (n = 6), mdx/Utr−/− (n = 3), and mdx/Utr−/−/+SERCA1 (n = 3)]. *P < 0.05 vs. WT; **P < 0.01 vs. WT; &P < 0.05 vs. mdx; ¥P < 0.05 vs. mdx/Utr−/−. Data presented as means ± SE.
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A

Torque per lengthening contraction. A total of 15 repetitions were performed for each animal. C: percent loss in torque throughout 15 lengthening contractions for all genotypes (WT (n = 5), WT/+SERCA1 (n = 6), mdx (n = 4), mdx/+SERCA1 (n = 5), mdx/Utr−/− (n = 3), and mdx/Utr−/−/+SERCA1 (n = 7)). Data presented as means ± SE.

Fig. 5. SERCA1 overexpression does not alter muscle torque production but protects DMD mouse models from eccentric contraction-induced injury. A: maximal isometric QUAD torque normalized to QUAD mass in all genotypes [WT (n = 5), WT/+SERCA1 (n = 6), mdx (n = 4), mdx/+SERCA1 (n = 5), mdx/Utr−/− (n = 3), and mdx/Utr−/−/+SERCA1 (n = 7)]. B: loss in maximal torque per lengthening contraction. C: percent loss in torque throughout 15 lengthening contractions for all genotypes [WT (n = 5), WT/+SERCA1 (n = 6), mdx (n = 4), mdx/+SERCA1 (n = 5), mdx/Utr−/− (n = 3), mdx/Utr−/−/+SERCA1 (n = 7)]. *P < 0.05 vs. WT; &P < 0.05 vs. mdx; YP < 0.05 vs. mdx/Utr−/−. Data

between WT and dystrophic muscle, findings consistent with results from the present study. However, SERCA1 is decreased in diaphragm muscle (59), which also has a greater susceptibility to damage (18, 43). While SERCA1 protein levels may not be different in limb muscles, SR Ca2+/ATPase activity in mdx/Utr−/− muscle relative to WT. One possible reason is that SERCA1 regulatory proteins (i.e., sarcolipin) are altered in mdx and mdx/Utr−/−, explaining the disconnect between SERCA1 expression level and SR Ca2+/ATPase activity (59). It is known that sarcolipin, an SR membrane protein that inhibits SERCA1 and SERCA2 activities, is upregulated in dystrophic muscle and correlates with decreased SR Ca2+ uptake velocity (59). A limitation in the current study is that we did not measure sarcolipin levels to determine whether it can explain the difference between our findings and those of Schneider and colleagues (59). We examined calsequestrin levels to examine changes in SR Ca2+ storage, but found no differences between mdx and mdx/Utr−/− vs. WT or with SERCA1 overexpression (data not shown). Certainly, it is surprising that an ~100% increase in SERCA1 protein and a two- to threefold increase in SR Ca2+/ATPase led to only minimal changes in muscle size and did not rescue preinjury force production. Future studies are required to fully explore the SERCA1 regulatory proteins that effect sarcoplasmic Ca2+ concentration in dystrophic muscle and cellular adaptations to increased SERCA1 levels.

Interestingly, we did not detect a difference in SR Ca2+/ATPase activity between dystrophic and WT mice, while the study from Schneider et al. (59) observed a decrease in Ca2+ uptake between these groups. Ca2+ uptake, an in vitro assessment of Ca2+ clearance capacity, was shown to be reduced in mdx (59) and δ-sarcoglycan-null mice (26); but in the current

or gadolinium) show improved muscle force production (75), while daily intraarterial injections of streptomycin decreased levels of blood CK and reduced cellular Evans blue dye uptake (41). Thus, current literature suggests multiple mechanisms that contribute to increased cytosolic Ca2+ levels in muscle of dystrophic mice and report evidence that inhibition of this Ca2+ influx attenuates the extent of muscle damage. Findings from the current study are consistent with these previous reports and reiterate the role of perturbations in [Ca2+]i, in the disease pathology in DMD and the ability of increased Ca2+ clearance mechanisms to attenuate muscle damage.

Dystrophic muscle has impairments in Ca2+ removal mechanisms such as the SERCA1 pump. Others (59) have shown that the expression of SERCA1 in the QUAD does not differ

Fig. 6. Representative radiographs showing spinal curvature from WT mouse (A), mdx/Utr−/−/+SERCA1 (B), and mdx/Utr−/− (C). Lines AB and CD are used to demonstrate the differences in spine curvature in different animal genotypes. Line CD is drawn perpendicular from the dorsal edge of the vertebra at the point of greatest curvature. An increase in the length of line CD indicates an increase in kyphosis.
study, SR Ca\textsuperscript{2+}-ATPase activity, an in vitro assessment of the maximum ATP catalytic rate of the SERCA pump, was not reduced in \textit{mdx} and \textit{mdx/Utr}\textsuperscript{−/−} vs. WT mice. SR Ca\textsuperscript{2+} uptake in vitro could plausibly be altered without a decrease in SR Ca\textsuperscript{2+}-ATPase activity in vitro if membrane integrity is not maintained since Ca\textsuperscript{2+} uptake measures require Ca\textsuperscript{2+} to be sequestered and maintained in membrane vesicles, whereas this is not the case with ATPase activity measurement. Thus, membrane leakiness, which is thought to be a problem with dystrophic muscle, may be responsible for this difference between measurements of Ca\textsuperscript{2+} uptake in vitro versus in vitro SR Ca\textsuperscript{2+}-ATPase activity per se.

Regardless of the cause, the inability to properly remove Ca\textsuperscript{2+} after contractions might be one of the factors contributing to increased sarcoplasmic Ca\textsuperscript{2+} levels, which leads to increased myofiber death through Ca\textsuperscript{2+}-dependent protease activation and mitochondrial Ca\textsuperscript{2+} overload in dystrophic muscle (2, 68, 76). Consistent with this notion, SERCA1 overexpression attenuated muscle pathology in both the \textit{mdx} and the δ-sarcoglycan-null mouse models of DMD (26). In the current study we report a two- to threefold increase in SR Ca\textsuperscript{2+}-ATPase activity in SERCA1-overexpressing mice. This is similar to the two- to fourfold increase in SR Ca\textsuperscript{2+} uptake in SERCA1-overexpressing δ-sarcoglycan-null mice (26), which also shows a rescue of their histopathology. Reduced markers of muscle damage and improved function after lengthening contractions with increased SERCA1 further supports the idea that Ca\textsuperscript{2+} clearance mechanisms are rate-limiting for maintaining muscle health with repeated eccentric contractions in dystrophic muscle. There are reports now indicating that an increased amount of SERCA1 protein and increasing total SR Ca\textsuperscript{2+} pump capacity can rescue some of the disease pathology (26, 43). Future studies will be required to determine whether SERCA1 regulatory proteins are also altered when SERCA1 is overexpressed and what their functional consequences are on Ca\textsuperscript{2+} pumping capacity.

A striking finding in the current study is the attenuation of contraction-induced injury by overexpression of SERCA1. Although traditional views that eccentric contraction-induced injury is due to structural damage of the muscle, resulting in disruptions of the sarcomere, more recent evidence indicates that loss of force production following eccentric contraction is due to disruptions in E-C coupling (32, 72, 73). The acute decrease in force-producing capacity with eccentric contractions (i.e., within 1 h) is both Ca\textsuperscript{2+}-dependent and dependent on the activation of Ca\textsuperscript{2+}-activated proteases (calpains) (81). This cellular change, following rupture of the plasma membrane, is due to an influx of Ca\textsuperscript{2+} through both transient receptor potential channel 1 and stretch-activated channels in the plasma membrane (80). Activated calpains are known to target components of the Z-disks, such as desmin and α-actinin, as well as titin and proteins located at the triad junction responsible for E-C coupling, such as junctophilin-1 and -2 (45, 70, 81). Therefore, overexpression of SERCA1 may decrease the extent of muscle damage due to its ability to buffer intracellular calcium following influx from the extracellular space. This, in turn, would minimize activation of calpains and the downstream degradation of proteins critical for E-C coupling, structural integrity of the muscle, and force production. This, in addition to healthier, less damaged muscle under resting conditions, would attenuate the damage due to eccentric contraction in \textit{mdx/Utr}\textsuperscript{−/−} + SERCA1 and \textit{mdx/Utr}\textsuperscript{−/−} + SERCA1 mice. Our data are consistent both with one previous report of reduced eccentric-induced force loss in diaphragm muscle from \textit{mdx} mice with SERCA1 overexpression (43) and with the attenuation of eccentric-induced force loss in single fibers from \textit{mdx} mice with inhibition of stretch-activated Ca\textsuperscript{2+} channels (75, 78).

In summary, we have shown that SERCA1 overexpression can mitigate the muscle hypertrophy, muscle damage, and susceptibility to contraction-induced injury in two models of DMD. These data indicate that increased SERCA1 can reduce the myocellular damage and improve functional outcomes in dystrophic mice. Furthermore, these data support the validation of SERCA1 as a therapeutic target for new drug therapies in DMD.

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DISCLOSURES

Eva R. Chin is the Founder and Chief Scientific Officer of MyoTherapeutics. No other conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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