Sphingomyelinase depresses force and calcium sensitivity of the contractile apparatus in mouse diaphragm muscle fibers

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Ferreira LF, Moylan JS, Stasko S, Smith JD, Campbell KS, Reid MB. Sphingomyelinase depresses force and calcium sensitivity of the contractile apparatus in mouse diaphragm muscle fibers. J Appl Physiol 112: 1538–1545, 2012. First published February 23, 2012; doi:10.1152/japplphysiol.01269.2011.—Diseases that result in muscle weakness, e.g., heart failure, are characterized by elevated sphingomyelinase (SMase) activity. In intact muscle, SMase increases oxidants that contribute to diminished muscle force. However, the source of oxidants, specific processes of muscle contraction that are dysfunctional, and biochemical changes underlying the weakness elicited by SMase remain unclear. We tested three hypotheses: 1) SMase-induced depression of muscle force is mediated by mitochondrial reactive oxygen species (ROS), 2) SMase depresses force and calcium sensitivity of the contractile apparatus, and 3) SMase promotes oxidation and phosphorylation of myofibrillar proteins. The mitochondrial-targeted antioxidant D-Arg-2′,6′-dimethyl-Tyr-Lys-Phe-NH2, decreased cytosolic oxidants and protected intact muscle bundles from weakness stimulated by SMase. SMase depressed maximal calcium-activated force by 20% in permeabilized single fibers (in kN/m2: control 117 ± 6; SMase 93 ± 8; P < 0.05). Calcium sensitivity of permeabilized single fibers decreased from 5.98 ± 0.03 (control) to 5.91 ± 0.02 (SMase; P < 0.05). Myofibrillar protein nitrotyrosines, carbonyls, and phosphorylation were unaltered by SMase. Our study shows that the fall in specific force of intact muscle elicited by SMase is mediated by SMase-induced muscle weakness. The exact source of ROS production elicited by SMase is unknown. In nonmuscle cells, SMase and ceramide stimulate ROS production by mitochondria (38, 56). In recent years, Szeto and Schiller (53) developed a mitochondria-targeted polypeptide with antioxidant action [D-Arg-2′,6′-dimethyl-Tyr-Lys-Phe-NH2 (SS-31)], which decreases skeletal muscle mitochondrial ROS emission (2) and protects from disuse-induced muscle weakness (44). To test hypothesis I, we determined the effects of the mitochondrial antioxidant SS-31 on cytosolic oxidants and force of skeletal muscle exposed to exogenous SMase.

Hypothesis I: SMase-induced depression of muscle force is mediated by mitochondrial ROS. SMase and its reaction product, ceramide, increase ROS in whole muscle and cultured muscle cells (20). The nonspecific antioxidant N-acetylcycteine exerted partial protection against SMase-induced depression of force, suggesting ROS as mediators of SMase-induced muscle weakness. The exact source of ROS production elicited by SMase is unknown. In nonmuscle cells, SMase and ceramide stimulate ROS production by mitochondria (38, 56). In recent years, Szeto and Schiller (53) developed a mitochondria-targeted polypeptide with antioxidant action [D-Arg-2′,6′-dimethyl-Tyr-Lys-Phe-NH2 (SS-31)], which decreases skeletal muscle mitochondrial ROS emission (2) and protects from disuse-induced muscle weakness (44). To test hypothesis I, we determined the effects of the mitochondrial antioxidant SS-31 on cytosolic oxidants and force of skeletal muscle exposed to exogenous SMase.

Hypothesis II: SMase depresses force and calcium sensitivity of the contractile apparatus. Heightened activity of SMase could disrupt any of the three main processes involved in muscle force production: 1) membrane depolarization, 2) sarcoplasmic reticulum calcium ion (Ca2+) release, and 3) Ca2+-activated force of the contractile apparatus. ROS, which are increased by SMase in skeletal muscle, are known to depress sarcoplasmic excitability (54) and force of the contractile apparatus (3, 8, 32, 35). Of the three main processes mentioned above, Ca2+-activated force of the contractile apparatus seems to be the most sensitive to physiological levels of ROS (3, 45, 48). Therefore, we hypothesized that SMase depresses force and calcium sensitivity of the contractile apparatus.

Hypothesis III: SMase promotes oxidation and phosphorylation of myofibrillar proteins. Oxidation of select myofibrillar proteins decreases force and Ca2+ sensitivity of the contractile apparatus (13, 29, 35, 43, 46). Similarly, the phosphorylation state of myofibrillar proteins modulates maximal force and Ca2+ sensitivity of the contractile apparatus (15, 40, 52). Ceramide activates protein kinases and phosphatases (25, 39). Activation of protein kinases and phosphatases that react with myofibrillar proteins can also be a secondary effect of ROS stimulated by SMase (5, 58). Hence, we hypothesized that SMase promotes oxidation and phosphorylation of myofibrillar proteins.

We used intact diaphragm bundles, chemically permeabilized single fibers, and myofibrillar proteins isolated from intact skeletal muscle bundles to test our hypotheses.

METHODS

Animals. We studied C57BL/6J mice (6–8 wk old; Harlan, Indianapolis, IN). Animals were maintained in a 12:12-h dark:light cycle...
and received water and food ad libitum. Each animal was deeply anesthetized by inhalation of isoflurane (Aerrane, Baxter Healthcare, Deerfield, IL) and killed by removal of vital organs after cervical dislocation. All procedures conformed to the regulations for use and care of laboratory animals in the United States and were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

**Experimental solutions.** In experiments using bundles of intact diaphragm fibers, we used bicarbonate-buffered physiological solution (in mM: 137 NaCl, 5 KCl, 1 MgSO₄, 1 NaH₂PO₄, 24 NaHCO₃, and 2 CaCl₂), equilibrated with 95% O₂–5% CO₂ (pH ~7.4). SS-31 (100 μM) was dissolved in the muscle buffer solution. For permeabilized single-fiber experiments, we used relaxing (in mM: 100 KCl, 20 imidazole, 4 ATP, 2 EGTA, and 7 MgCl₂; pH adjusted to 7.0 using KOH), permeabilizing (1% Triton X-100 in relaxing solution), and pCa solutions, where pCa = log₁₀[Ca²⁺]. pCa solutions (pH 7.0 at 22°C) contained free Ca²⁺, ranging from 1 nM (pCa 9.0) to 32 μM (pCa 4.5), added as CaCl₂, and (in mM) 20 imidazole, 14.5 creatine phosphate, 7 EGTA, 4 MgATP, and 1 free Mg²⁺. The pH of pCa solutions was adjusted to 7.0 at 22°C using KOH, and ionic strength was adjusted to 180 mM by adding KCl as needed. All chemicals were from Sigma-Aldrich (St. Louis, MO), except for CaCl₂ (Orion ISE Calibration Standard, Thermo Scientific, Hudson, NH), used in pCa solution, ATP (Roche, Indianapolis, IN), and Triton X-100 (Thermo Scientific).

**In vitro treatment.** We dissected two diaphragm fiber bundles/animal and mounted both separately for in vitro treatment, as described recently (20). Briefly, we tied the rib to a glass rod and attached the central tendon to a force transducer (BG Series, 100 g, Kulite, Leonia, NJ) using silk suture (4-0). The fiber bundle was placed in a water-jacketed organ bath containing Krebs buffer, continuously gassed with 95% O₂–5% CO₂. Prior to the experimental treatment, we positioned the fiber bundle at the length that elicited the highest twitch force (Fₒ). Fiber bundles were treated with SMase (0.5 U/ml) or divalent control (0.15% glycerol) in Krebs solution for 60 min at 37°C. For experiments using SS-31, muscles were pretreated with buffer plus vehicle (control) or buffer containing SS-31 for 15 min before adding recombinant SMase to the bath (total exposure: SMase 45 min; SS-31 or vehicle 60 min). SMase was purchased from Sigma-Aldrich (Staphylococcus aureus; Cat. No. S8633). SS-31 was purchased from the W. M. Keck Foundation (Large Scale Peptide Synthesis Laboratory, Yale University, New Haven, CT). After treatment, we processed fiber bundles using protocols described below to test each hypothesis.

**Cytosolic oxidant activity.** This technique was described in depth in our recent study (20). Briefly, we used the fluorochrome probe 2',7'-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Eugene, OR) at 20 μM to measure overall cytosolic oxidant activity (36, 47). The DCFH-DA crosses the cell membrane and is cleaved into DCFH by intracellular esterases. DCFH is oxidized into 2',7'-dichlorofluorescein (DCF), which emits the fluorescence signal in proportion to the oxidants in the cytosol. The measurement area is ~0.27 mm², and fluorescence from the entire field of view is quantified and recorded for statistical analysis (4). In our previous study, we observed that SMase increased cytosolic oxidants (20). In the present study, our focus was on SS-31 effects on cytosolic oxidants in diaphragm preparations exposed to SMase. Hemidiaphragms used in positive-control studies (SMase vs. vehicle) were incubated in DCFH for 30 min before SMase exposure (20). In experiments focused on the effects of SS-31 on DCF fluorescence, hemidiaphragms were incubated in DCFH (20 μM), with or without SS-31 (10 μM), for 15 min prior to the addition of SMase (0.5 U/ml). After 30 min of incubation with SMase, we measured the oxidized derivative (DCF; 480 nm excitation, 520 nm emissions) and corrected for background emissions using an epifluorescence microscope (Eclipse TE2000, Nikon Instruments, Melville, NY), a charge-coupled device camera (CoolSNAP ES, Photometrics, Tucson, AZ), and a computer-controlled shutter in the excitation light pathway.

**Contractile function of intact fiber bundles.** Fiber bundles were stimulated to contract using electrical field stimulation (supramaximal voltage, 0.25–0.30 ms pulse, and 250–300 ms train duration) via platinum electrodes at frequencies of 1–300 Hz. We measured force using a digital oscilloscope and computed cross-sectional area using muscle weight and Lₒ (14) to calculate specific force (in kN/m²). To test the role of mitochondrial ROS using SS-31, we measured skeletal muscle force at 37°C. All solutions used for measurement of muscle force contained D-tubocurarine (25 μM).

**Permeabilized single-fiber mechanics.** We prepared permeabilized single fibers using a technique similar to that described by Campbell and Moss (9), as adapted for diaphragm muscle by our group (27). Immediately after SMase exposure (see *In vitro treatment* above), diaphragm fiber bundles were immersed in ice-cold relaxing solution; bundles used for isolation of single fibers were not subjected to electrically stimulated contractions. Fiber bundles were subdivided, tied to glass capillary tubes, and placed in skinning solution for 3 h at 4°C to achieve chemical permeabilization. We stored permeabilized fiber bundles in 50% v/v glycerol/relaxing solution at ~20°C for up to 3 days before experiments. On the day of the experiment, we isolated single fibers from permeabilized bundles in ice-cold relax solution and mounted segments of individual fibers between a force transducer (403B, Aurora Scientific, Ontario, Canada) and a motor arm (312B, Aurora Scientific). After mounting the fiber, the temperature of the apparatus was set to 22°C and maintained throughout the experiment. Upon reaching the desired temperature, fibers were positioned at a sarcomere length of ~2.60 μm in relax solution. We excluded from analyses single fibers that had no visible striation pattern at pCa 4.5 or developed force at pCa 6.4 > 10% maximum force. The latter responses were observed in two fibers, were not evident in previous studies of mouse diaphragm (27), and may relate to expression of type I myosin heavy chain (22).

Fiber length and diameter were measured using video microscopy. The fiber was transferred to pCa 9.0. Calcium-activated force was elicited by immersing the fiber in pCa solutions (22°C), ranging from 6.4 to 4.5. In each solution, force was allowed to plateau. We then performed a quick-release step by rapidly shortening the fiber by 20% of the segment length for 20 ms before returning to the initial length. The force response to this procedure was fitted using a single exponential equation starting at the residual force (9) to determine the rate of tension redevelopment (kᵣ). Experiments were performed and analyzed using SIControl software (10). The force–pCa relationship of individual fibers was analyzed using a four-parameter Hill equation (Prism 5.0b, GraphPad Software, La Jolla, CA): $F = F_{pasp} + F_{p}(10^{-pCa_{50}})/(10^{-pCa_{50}} + (10^{-pCa_{50}})^{10})$, where $F_{p}$ is passive force, $F_p$ is maximal active force, and $pCa_{50}$ is the pCa that elicits half-maximal activation. $F_p$ was also calculated by subtracting baseline force (pCa 9.0) from maximal Ca²⁺-activated force (pCa 4.5). We determined fiber “rundown” as the decrease in force at pCa 4.5 (pCa 4.5) from the beginning to the end of the experiment. We studied three to five fibers/diaphragm fiber bundle (two fiber bundles/animal, control, and SMase; total of four mice). Data from individual fibers were fitted using the Hill equation, and parameters from fibers of the same bundle were averaged to yield a mean value for each animal.
standard rigor buffer (in mM: 75 KCl, 20 MOPS, pH 7.0, 2 MgCl₂, 2 EGTA, and 1 NaCl) plus Triton X-100 and inhibitors, followed by 15 min on ice and centrifugation at 16,100 g (4°C) for 5 min. P1 was then rinsed in standard rigor buffer and pelleted (P2) at 1,500 g (4°C) for 1 min. P2 was resuspended in K-60 (in mM: 60 KCl, 20 MOPS, and 2 MgCl₂, pH 7.0) plus BSA (1 mg/ml) and inhibitors and pelleted (P3) at 1,500 g (4°C) for 1 min. P3 was resuspended in 100 μl K-60, BSA and inhibitors, plus 100 μl of 2 mM DTT in K-60 solution mixed 1:1 with glycerol and stored at −80°C.

Western blot analysis and protein phosphorylation. To pellet myofibrils for analysis, P3 (see above) was thawed and centrifuged at 1,500 g for 2 min at 4°C. P3 was resuspended in 200 μl urea sample buffer (in M: 8 urea, 0.05 Tris, pH 6.8, 0.075 DTT, and 0.05% bromophenol blue) and heated at 98°C for 5 min. Myofibrillar proteins were fractionated on 15% Tris–HCl polyacrylamide gels (Criterion precast gels, Bio-Rad, Hercules, CA) at 200 V for 50 min (PowerPac HC, Bio-Rad). Gels were either transferred to membrane for Western blot analysis or fixed for testing protein phosphorylation. Total protein was measured by staining gels with SimplyBlue (Invitrogen, Life Technologies, Grand Island, NY).

Proteins were transferred to reduced–fluorescence polyvinylidene difluoride membrane (Immobilon-FL, Millipore, Billerica, MA) at 150 mA for ~18 h at 4°C. Membranes were blocked in Odyssey blocking buffer (LI-COR, Lincoln, NE) for 6–8 h at room temperature. Primary antibodies were diluted 1:1,000 in blocking buffer, mixed 1:1 with PBS plus 0.2% Tween, and incubated overnight at room temperature, followed by four, 5-min washes. Carboxyl groups on myofibrillar protein side chains were detected by derivatization to 2,4-dinitrophenylhydrazine (DNPH) prior to loading protein aliquots on the gel (OxyBlot protein oxidation kit, Millipore). Isolated myofibrils (100 μg) were resuspended in 25 μl of 20 mM HEPES (pH 7.4) plus 100 μl of 10 mM DNPH [adapted from Barreiro et al. (6)] and incubated for 15 min at room temperature. We used aliquots of myofibrillar protein extracts, which did not undergo DNPH derivatization, to test for nonspecific binding of the primary antibody. Membranes were probed with anti-DNPH (OxyBlot kit). Membranes were then incubated with fluorescence–conjugated secondary antibodies to detect phosphorylated myofibrillar proteins, gels were fixed in 10% glacial acetic acid/50% methanol (3:1, 25 min), stained in Pro-Q Diamond phosphoprotein gel stain (LI-COR), and incubated for 15 min at room temperature. The relationship between relative force (% maximal tetanic) and stimulation frequency was partial (Fig. 2). The relationship between relative force (% maximal tetanic) and stimulation frequency was partial (Fig. 2). The relationship between relative force (% maximal tetanic) and stimulation frequency was partial (Fig. 2). The relationship between relative force (% maximal tetanic) and stimulation frequency was partial (Fig. 2). The relationship between relative force (% maximal tetanic) and stimulation frequency was partial (Fig. 2). The relationship between relative force (% maximal tetanic) and stimulation frequency was partial (Fig. 2).
at pCa 4.5 tended to be slower with SMase treatment (in s⁻¹; control 54 ± 2; SMase 45 ± 3; P < 0.11).

Post-translational modifications of myofibrillar proteins. SMase exposure did not increase oxidation of myofibrillar proteins measured by DNPH-derivatized carbonyls (Fig. 5) or nitrotyrosines (data not shown). Enzyme-mediated modifications, phosphorylation (Fig. 6), and ubiquitin conjugation (data not shown) were also unaffected, nor did we observe changes in gel migration patterns of myofibrillar proteins stained with SimplyBlue.

DISCUSSION

In the present study, we sought to test three hypotheses: 1) SMase-induced depression of muscle force is mediated by mitochondrial ROS, 2) SMase depresses force and calcium sensitivity of the contractile apparatus, and 3) SMase promotes oxidation and phosphorylation of myofibrillar proteins. Our results support the first two hypotheses, but we found no evidence of carbonylation or phosphorylation of myofibrillar proteins as mechanisms for SMase-induced muscle weakness.

SS-31 lowers cytosolic oxidants and protects against depression of force caused by SMase. SMase increases ceramide content in muscle cells (20). Ceramide enhances ROS produc-

Fig. 2. SS-31 protects against SMase-induced depression of specific force (kN/m²). Force measured at 37°C, normalized for cross-sectional area; n = 5/group. Groups are control (○), SS-31/SMase (■), and SMase (●). *P < 0.05 for control or SS-31/SMase vs. SMase; #P < 0.05 for control vs. SS-31/SMase. Statistical analysis: repeated measures ANOVA and Bonferroni’s post hoc test.
Exogenous SMase increased cytosolic oxidants that lead to depression of muscle force (20). We found that SS-31 lowered cytosolic oxidants in SMase-treated muscles but not in controls. SS-31 preferentially localizes to the inner mitochondrial membrane and diminishes mitochondrial ROS in several cell types (2, 53, 59). The antioxidant effect of SS-31 arises from the presence of tyrosine (direct ROS scavenger) and facilitation of electron transport in the inner mitochondrial membrane (53, 55). SS-31 lowers mitochondrial ROS emission in skeletal muscle (2, 44) and has no effect on NADPH oxidase activity (44). SS-31 also prevented the increase in cytosolic oxidants and depression of force elicited by SMase—findings that support mitochondrial ROS as mediators of muscle weakness elicited by SMase. We cannot exclude the possibility that very low levels of cytosolic SS-31 might affect oxidant activity via a nonmitochondrial mechanism. However, SS-31 levels in the cytosol are two to three orders of magnitude lower than in mitochondria, which concentrate SS-31. This makes a cytosolic mechanism of action less likely. In combination, data from this study and our previous report (20) suggest that exogenous SMase increases ceramide in skeletal muscle, leading mitochondria to release ROS into the cytosol, which impairs myofibrillar protein function.

In our previous study, the antioxidant N-acetyl cysteine (NAC) exerted partial protection against depression of force elicited by SMase (20). The distribution of NAC to mitochondria is limited (53), and its antioxidant effects are dose dependent, which may explain the partial efficacy of NAC in protecting muscle force from SMase effects. SS-31 levels in the cytosol are two to three orders of magnitude lower than in mitochondria, which concentrate SS-31. This makes a cytosolic mechanism of action less likely. In combination, data from this study and our previous report (20) suggest that exogenous SMase increases ceramide in skeletal muscle, leading mitochondria to release ROS into the cytosol, which impairs myofibrillar protein function.

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drial ROS—likely a direct consequence of sphingomyelin hydrolysis on the biophysical properties of the membrane.

_SMase promotes weakness through impairment of muscle contractile apparatus._ SMase effects on muscle, mentioned above, were evident at the lower temperatures necessary for studies in permeabilized single fibers. Weakness of an intact muscle can stem from impairments of membrane excitability, Ca$^{2+}$ release, and/or myofibrillar protein function. Our experiments using permeabilized single fibers show that the primary determinant of weakness elicited by SMase is dysfunction of myofibrillar proteins (Fig. 3). The decrease in maximal calcium-activated force suggests a lower number of force-generating cross bridges and/or less force/cross bridge.

We also found a decrease in calcium sensitivity of the contractile apparatus. The change in pCa$_{50}$ indicates a 25% increase in Ca$^{2+}$ concentration necessary to elicit half-maximal force after SMase exposure. The mechanisms that control Ca$^{2+}$ sensitivity in skeletal muscle are complex and not well understood. One possibility is that bound cross bridges activate the thin filament through a cooperative mechanism that facilitates further cross-bridge binding. This cooperative mechanism would lead to a steep tension-pCa curve and a relatively high pCa$_{50}$ value (low Ca concentration). If this is the case, a reduction in the number of bound cross bridges in SMase-treated fibers could explain both the reduced force and lower pCa$_{50}$. Other potential mechanisms involving the troponin (Tn)-tropomyosin complex are possible as well.

Post-translation modifications of myofibrillar proteins. The decrease in maximal calcium-activated force and calcium sensitivity can be caused by covalent modifications, for example, oxidation (8, 29, 35), or phosphorylation of myofibrillar proteins (23, 34). Based on the SMase-induced increase in cysteolic oxidants and blunting of the depression of force by antioxidant treatment (20), we tested for oxidation of myofibrillar proteins. Several myofibrillar proteins are susceptible to oxidative and nitrosative modifications that impair function (12, 13, 29, 30, 42, 46). Notably, carbonylation of actin and tropomyosin are associated with myocardial contractile dysfunction in heart failure (11). In our experiments, the abundance of carbonyl groups or nitrotyrosines was unchanged in myofibrillar proteins of diaphragm bundles exposed to SMase for 1 h in vitro. A longer exposure to SMase may be necessary to elicit increases in myofibrillar protein carbonyls. However, our data indicate that myofibrillar protein carbonylation does not mediate the muscle weakness that we observed after SMase exposure.

Oxidation of thiol groups in myofibrillar proteins is an alternative cause for depression of maximal force and calcium sensitivity in single fibers (19, 32, 35). Disulfide exchange reactions e.g., S-glutathiolation (35)) and formation of intermolecular disulfide bridges are potential modifications documented in myofibrillar proteins (11). However, thiol oxidation could not be detected using the reducing conditions required for optimal isolation of myofibrillar proteins (12, 13). Our preliminary attempts to isolate myofibrillar proteins from diaphragm bundles under nonreducing conditions resulted in low myofibrillar protein yields that precluded resolution of thiol oxidation using physiological concentrations of H$_2$O$_2$ (unpublished observations). More sophisticated methods will be necessary to define the myofibrillar redox proteomics that underlie the decrease in calcium sensitivity and maximal force triggered by SMase.

Ceramide activates protein kinases and phosphatases (23, 35). In cardiac myocytes, phosphorylation of TnI lowers Ca$^{2+}$ sensitivity of the contractile apparatus (22), and dephosphorylation of both tropomyosin (48) and myosin-binding protein C (31) depresses maximal Ca$^{2+}$-activated force. These effects are similar to those elicited by SMase in diaphragm bundles, and it is therefore possible that some of the functional changes elicited in the current experiments may have been produced by...
phosphorylation or dephosphorylation of skeletal tropomyosin (28), TnI (49), and/or myosin-binding protein C (1). However, our measurements with Pro-Q Diamond did not reveal statistically significant differences in the phosphorylation state of any of these sarcomeric proteins.

Limitations and methodological aspects. Exogenous SMase acts on sphingomyelin localized on the extracellular leaflet of the plasma membrane (33, 50). This mechanism of action and downstream products will closely reflect the effects of secretory SMase in vivo (33, 50). However, the effects of SMase on skeletal muscle of patients will be a result of prolonged exposure to SMase. Our findings represent short-term effects of SMase on skeletal muscle (60 min). Whereas we anticipate that signaling pathways stimulated by SMase will be similar for acute vs. prolonged exposure to SMase, post-translational modifications undetected within 60 min might be seen after a prolonged period of heightened SMase activity. Thus our experiments may underestimate the impact of SMase on skeletal muscle contractile properties.

Conclusions. Mitochondrial ROS increase cytosolic oxidants in muscle exposed to SMase, leading to a decrease in specific force. Muscle weakness elicited by SMase occurs largely due to decreases in calcium sensitivity and calcium-activated force of the contractile apparatus. Changes in the carbonylation or phosphorylation state of myofibrillar proteins are not involved in the contractile impairment elicited by SMase. We speculate that SMase results in muscle weakness due to oxidation of thiol groups in myofibrillar proteins.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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