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 unknown. 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. Our experiments included intact muscle bundles, permeabilized single fibers, and isolated 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 mitochondrial ROS and can be attributed largely to dysfunction of the contractile apparatus.

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-acetylcysteine 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 mitochondrial-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 sarcolemmal 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 (Aerane, 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). In experiments using bundles of intact diaphragm fibers, we used bicarbonate-buffered physiological 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 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 purchase 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 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 L₀ (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 electrophysiological stimulation contractions. Fiber bundles were subdivided, tied to glass capillary tubes, and placed in skinnning 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 function at the residual force (9) to determine the rate of tension redevelopment (kₑ). Experiments were performed and analyzed using SLC2ontrol 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 = Fₚₓₓₓₓ + Fₚₚₚₚ(10⁻ DriverManager⁺)/(10⁻ DriverManager⁺ + (10⁻ DriverManagerₚₚ₁₁). Where Fₚₚₚₚ is passive force, Fₓₓₓₓ is maximal active force, nH is the Hill coefficient, and DriverManager is the pCa that elicits half-maximal activation. Fₓₓₓₓ 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 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, 2 MgCl₂, 2 EGTA, and 1 NaCl) plus Triton and inhibitors, followed by 5 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-dinitrophenylhydrazone (DNPH) prior to loading protein aliquots on myofibrillar protein side chains were detected by derivatization to 2,4-dinitrophenylhydrazone (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 in 2 M HCl [adapted from Barreto 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 in Odyssey blocking buffer, mixed 1:1 with PBS–0.2% Tween plus 0.01% SDS for 30 min, followed by four, 5-min washes. Fluorescent secondary antibodies were used for detection (goat anti-rabbit IRD800, Rockland Antibodies & Assays, Gilbertsville, PA). After drying, membrane fluorescence was imaged, and results were quantified using the Odyssey infrared imaging system (LI-COR).

To detect phosphorylated myofibrillar proteins, gels were fixed in 10% glacial acetic acid/50% methanol (3×, 20 min), washed in water (6×, 5 min), stained in Pro-Q Diamond phosphoprotein gel stain (Molecular Probes/Invitrogen, Life Technologies) for 90 min, and de-stained (3×, 25 min in 15% 1,2-propanediol and 50 mM sodium acetate, pH 4). After transfer to fresh de-stain solution, gels were incubated overnight and washed in water (3×, 5 min). Gels were then scanned using a Typhoon imager (GE Healthcare, Piscataway, NJ), and images were analyzed using Odyssey software (LI-COR).

Statistics. All comparisons were performed using commercially available software (Prism 5.0b, GraphPad Software; SigmaPlot v. 11, Systat Software, San Jose, CA). We used repeated measures ANOVA and Bonferroni’s test for multiple comparisons to analyze the force-frequency relationship of intact fiber bundles. All other variables and parameters were analyzed using a paired t-test. Data from parameters describing the force–pCa relationship and k₀ of individual permeabilized single fibers were averaged within each hemidiaphragm to obtain a representative value for each animal under the two conditions (control and S/Mase; n = 4/group). These were used for statistical comparisons among muscles. We preferred this conservative approach for analysis of single-fiber data to avoid pseudoreplication and minimize the risks of type I error. However, the outcome was essentially the same as that obtained by testing data from all fibers individually. Data are presented as mean ± SE. Statistical significance was accepted when P < 0.05.

RESULTS

SS-31 decreases cytosolic oxidant activity in SMase-treated diaphragm. DCF fluorescence was unchanged by SS-31 under control conditions (SS-31 = 98 ± 8% control; P = 0.296 vs. control). However, DCF fluorescence of SMase-treated muscles decreased ~45% by SS-31 in muscles treated with SMase (Fig. 1), suggesting that mitochondrial ROS contribute to the rise in cytosolic oxidants stimulated by SMase.

SS-31 protects against depression of force caused by SMase. SMase decreased tetanic diaphragm force (Fig. 2; 30–150 Hz) results, consistent with our previous study (20). In muscles treated with SS-31/SMase, forces—elicited by stimulus frequencies, ranging from 1 to 150 Hz—were essentially the same as in vehicle controls. At 250 and 300 Hz, SS-31 protection of force was partial (Fig. 2). The relationship between relative force (% maximal tetanic) and stimulation frequency was unchanged by SMase and SS-31 (data not shown). These findings are consistent with our hypothesis that mitochondrial ROS cause depression of force after exposure to SMase. Subsequent results address potential targets and biochemical effects of ROS that might depress skeletal muscle force.

Mechanics of permeabilized single fibers. Permeabilized single fibers are unstable at 37°C, and experiments were performed at 22°C. In muscle, studies at room temperature depress cellular ROS production (4) and can blunt force depression via ROS-mediated mechanisms [ref. (17) and unpublished observations]. Therefore, in preliminary experiments, we tested for temperature dependence of SMase effects by measuring force of intact bundles at room temperature. The depression of force elicited by SMase was similar when force was measured at room temperature (data not shown) and 37°C (Fig. 2).

We performed experiments using a total of 31 permeabilized single fibers. Fibers accepted for formal analyses had segment lengths of 995 ± 91 μm (control) and 1,046 ± 34 μm (SMase). Cross-sectional areas were 870 ± 5% at 37°C (control) and 810 ± 5% at 22°C (SMase). Overall fiber rundown during the protocol was 10 ± 1.6% and 7.5 ± 0.4% in control and SMase, respectively. None of these variables differed between groups.

SMase treatment decreased specific force by 20 ± 5% at pCa 4.5 (Fig. 3). There was no difference in Fₙₐₛ (pCa 9.0) between control and SMase groups (Fig. 3). Thus maximal Ca²⁺-activated force (force at pCa 4.5 – force at pCa 9.0) decreased from 117 ± 6 kN/m² in control fibers to 93 ± 8 kN/m² in SMase-treated fibers (P < 0.05; n = 4 muscles; mean of three to five fibers/muscle). SMase also decreased calcium sensitivity of the contractile apparatus, as shown by lower pCa₅₀ values (Fig. 4). nH describes the slope of the sigmoidal force–pCa relationship and was unchanged by SMase treatment (control 2.82 ± 0.32; SMase 2.71 ± 0.21; n = 4 animals/group).

SMase exposure had marginal effects on fiber mechanics during the quick-release step. Residual force at pCa 4.5 tended to be less after SMase exposure (in kN/m²: control 62 ± 9; SMase 44 ± 4; P < 0.07). The kᵢᵣ following the short release
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-
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 protection against SMase-induced weakness was also partial at 250 and 300 Hz (Fig. 2). After SMase exposure, tetanic force displays a “descending limb” at stimulus frequencies higher than 150 Hz (Fig. 2). The decreasing force at high-stimulus frequencies, plateau region in controls, suggests that SMase impairs membrane excitability and voltage-dependent Ca\(^{2+}\) release. A similar response is expected from control diaphragm muscles if stimulus frequencies are increased progressively beyond 300 Hz, as electrical pulses will reach the sarcolemma during the membrane refractory period. Because SS-31 did not normalize the descending limb of the force-frequency relationship, we consider that this effect of SMase is independent of mito-
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,
Ca2+ release, and/or myofibrillar protein function. Our ex-
periments 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 pCa50 indicates a 25%
increase in Ca2+ concentration necessary to elicit half-maximal
force after SMase exposure. The mechanisms that control Ca2+
sensitivity in skeletal muscle are complex and not well under-
stood. 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
pCa50 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
pCa50. 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 cyto-
solic oxidants and blunting of the depression of force by
antioxidant treatment (20), we tested for oxidation of myofi-
brillar proteins. Several myofibrillar proteins are susceptible to
oxidative and nitrosoative modifications that impair function
(12, 13, 29, 30, 42, 46). Notably, carboxylation of actin and
tropomyosin are associated with myocardial contractile dys-
function in heart failure (11). In our experiments, the abun-
dance of carbonyl groups or nitryosines 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 carboxylation 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 inter-
membrane disulfide bridges are potential modifications docu-
mented 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 dia-
aphragm bundles under nonreducing conditions resulted in low
myofibrillar protein yields that precluded resolution of thiol
oxidation using physiological concentrations of H2O2 (unpub-
ilished observations). More sophisticated methods will be nec-

deecessary 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 Ca2+
sensitivity of the contractile apparatus (22), and dephosphory-
lation of both tropomyosin (48) and myosin-binding protein C
(31) depresses maximal Ca2+-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
elicted 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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