Mitochondrial-targeted antioxidants protect skeletal muscle against immobilization-induced muscle atrophy

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Mitochondrial-targeted antioxidants protect skeletal muscle against immobilization-induced muscle atrophy. *J Appl Physiol* 111: 1459–1466, 2011. First published August 4, 2011; doi:10.1152/japplphysiol.00591.2011.— Prolonged periods of muscular inactivity (e.g., limb immobilization) result in skeletal muscle atrophy. Although it is established that reactive oxygen species (ROS) play a role in inactivity-induced skeletal muscle atrophy, the cellular pathway(s) responsible for inactivity-induced ROS production remain(s) unclear. To investigate this important issue, we tested the hypothesis that elevated mitochondrial ROS production contributes to immobilization-induced increases in oxidative stress, protease activation, and myofiber atrophy in skeletal muscle. Cause-and-effect was determined by administration of a novel mitochondrial-targeted antioxidant (SS-31) to prevent immobilization-induced mitochondrial ROS production in skeletal muscle fibers. Compared with ambulatory controls, 14 days of muscle immobilization resulted in significant muscle atrophy, along with increased mitochondrial ROS production, muscle oxidative damage, and protease activation. Importantly, treatment with a mitochondrial-targeted antioxidant attenuated the inactivity-induced increase in mitochondrial ROS production and prevented oxidative stress, protease activation, and myofiber atrophy. These results support the hypothesis that redox disturbances contribute to immobilization-induced skeletal muscle atrophy and that mitochondria are an important source of ROS production in muscle fibers during prolonged periods of inactivity.

proteases; oxidative stress; mitochondria

SKELETAL MUSCLE ATROPHY commonly occurs during prolonged periods of inactivity due to limb immobilization or prolonged bed rest. This inactivity-induced muscle atrophy results in a decrease in muscle force-generating capacity and increases the risk for subsequent health problems such as bone fractures, osteoporosis, and increased risk of falls (7, 16, 25). Therefore, understanding the signaling pathway(s) responsible for disuse muscle atrophy is an important first step toward developing a therapeutic approach to delay or prevent skeletal muscle atrophy.

It is well established that disuse muscle atrophy occurs as a result of a reduction in protein synthesis and increased proteolysis (4). In this regard, proteolysis appears to play a major role in the loss of muscle protein during prolonged inactivity in rodents (11). Furthermore, growing evidence suggests that inactivity-induced oxidative stress is an important activator of key proteases (e.g., calpain and caspase-3) in skeletal muscle

(22, 28). However, the primary sources of disuse-induced oxidant production in limb skeletal muscle remain unknown.

Previous work from our group has demonstrated that NADPH oxidase and xanthine oxidase are contributors to reactive oxygen species (ROS) production in inactive respiratory muscles (14, 27). Nonetheless, the small amount of ROS production from these two sources suggests that other sites of ROS production exist (14, 27). Furthermore, recent work by our group reveals that mitochondria are an important source of ROS production in diaphragm muscle during prolonged mechanical ventilation (17). Nonetheless, the primary source of ROS production in inactive locomotor skeletal muscles remains unknown and forms the basis for the present experiment. Guided by our preliminary studies, we formulated the hypothesis that mitochondrial ROS production plays a dominant role in immobilization-induced ROS production and oxidative stress in skeletal muscle. To test this hypothesis, we treated mice with a novel mitochondrial-targeted antioxidant (SS-31) and exposed the animals to 14 days of hindlimb immobilization. SS-31 was chosen because of its selective targeting to the inner mitochondrial membrane (29).

Our results reveal that prevention of inactivity-induced increases in mitochondrial ROS production in locomotor skeletal muscles protects slow- and fast-twitch muscle fibers against oxidative damage, mitochondrial dysfunction, and fiber atrophy.

METHODS

Experiment 1

Animals. Twelve adult male C57BL/6 mice (20–28 wk old, 26.85 ± 0.34 g body wt) were maintained on a 12:12-h light-dark cycle, with food (AIN-93 diet) and water provided ad libitum throughout the experimental period. The Institutional Animal Care and Use Committee of the University of Florida approved these experiments.

Experimental design. Experiment 1 was performed to determine the effect of a mitochondrial-targeted antioxidant (SS-31) in normal ambulatory animals. Briefly, animals (*n* = 7/group) were randomly assigned to one of two experimental groups: the control group was injected subcutaneously with saline daily for 14 days, and the SS-31 group was injected with SS-31 (1.5 mg/kg sc) daily for 14 days. At the completion of the 14-day treatment period, we measured muscle-to-body weight ratio, fiber cross-sectional area (CSA), and mitochondrial function [respiratory control ratio (RCR)]. Our results reveal that, compared with control, treatment with SS-31 did not alter any of these dependent measures (see RESULTS). Therefore, *experiment 2* was performed using SS-31 to determine the role of mitochondrial ROS production in prolonged inactivity-induced skeletal muscle atrophy.

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Experiment 2

Animals. Seventy-two adult male C57BL/6 mice (21–28 wk old, 26.44 ± 0.54 g body wt) were maintained on a 12:12-h light-dark cycle, with food (AIN-93 diet) and water provided ad libitum throughout the experimental period. The Institutional Animal Care and Use Committee of the University of Florida approved these experiments.

Experimental design. To test the hypothesis that mitochondrial ROS production plays a critical role in immobilization-induced skeletal muscle atrophy, mice were randomly assigned to one of three experimental groups ($n = 24$/group): *1*) no treatment (control group), *2*) 14 days of hindlimb immobilization (cast group), and *3*) 14 days of hindlimb immobilization + SS-31 treatment (cast + SS group). Note that the cast group received daily saline injections, whereas the animals in the cast + SS group were treated with SS-31 (1.5 mg/kg sc) daily during the immobilization period.

Experimental procedures. IMMOBILIZATION. Mice were anesthetized with gaseous isoflurane (3% for induction, 0.5–2.5% for maintenance). Anesthetized animals were cast-immobilized bilaterally, with the ankle joint in the plantar-flexed position to induce maximal atrophy of the soleus and plantaris muscle. Both hindlimbs and the caudal fourth of the body were encompassed by a plaster of paris cast. A thin layer of padding was placed underneath the cast to prevent abrasions. In addition, to prevent the animals from chewing on the cast, one strip of fiberglass material was applied over the plaster. The mice were monitored on a daily basis for chewed plaster, abrasions, venous occlusion, and problems with ambulation.

SS-31 ADMINISTRATION. SS-31 dissolved in saline (1.5 mg/kg sc) was administered daily via injections (neck and shoulder area) during the immobilization period.

Biochemical Measures

Preparation of permeabilized muscle fibers. We measured mitochondrial ROS production and respiration in permeabilized muscle fibers. This technique has been adapted from previously published methods (10, 23). Briefly, small portions ($\sim$25 mg) of soleus and plantaris muscle were dissected and placed on a plastic petri dish containing ice-cold *buffer X* (60 mM K-MES, 35 mM KCl, 7.23 mM K_2EGTA, 2.77 mM CaK_2EGTA, 20 mM imidazole, 0.5 mM DTT, 20 mM taurine, 5.7 mM ATP, 15 mM phosphocreatine, and 6.56 mM $MgCl_2$, pH 7.1). The muscle was trimmed of connective tissue and cut down to fiber bundles (4–8 mg wet wt). Under a microscope and with a pair of extra-sharp forceps, the muscle fibers were gently separated in ice-cold *buffer X* to maximize surface area of the fiber bundle. To permeabilize the myofibers, each fiber bundle was incubated in ice-cold *buffer X* containing 50 μg/ml saponin on a rotator for 30 min at 4°C. The permeabilized bundles were then washed in ice-cold *buffer Z* (110 mM K-MES, 35 mM KCl, 1 mM EGTA, 5 mM K_2HPO$_4$, 3 mM $MgCl_2$, 0.005 mM glutamate, 0.02 mM malate, and 0.5 mg/ml BSA, pH 7.1).

Mitochondrial respiration in permeabilized fibers. Respiration was measured polarographically in a respiration chamber (Hansatech Instruments) maintained at 37°C. After the respiration chamber was calibrated, permeabilized fiber bundles were incubated with 1 ml of respiration *buffer Z* containing 20 mM creatine to saturate creatine kinase (21, 26). Flux through complex I was measured using 5 mM pyruvate and 2 mM malate. The ADP-stimulated respiration (state 3) was initiated by addition of 0.25 mM ADP to the respiration chamber. Basal respiration (state 4) was determined in the presence of 10 μg/ml oligomycin to inhibit ATP synthesis. RCR was calculated by dividing state 3 by state 4 respiration.

Mitochondrial ROS production. Mitochondrial ROS production was determined using Amplex red (Molecular Probes, Eugene, OR). The assay was performed at 37°C in 96-well plates with succinate as the substrate. Specifically, this assay was developed on the concept that horseradish peroxidase catalyzes the H_2O_2-dependent oxidation of nonfluorescent Amplex red to fluorescent resorufin red, and it is used to measure H_2O_2 as an indicator of superoxide production. SOD was added at 40 U/ml to convert all superoxide to H_2O_2. Using a multiwell-plate reader fluorometer (SpectraMax, Molecular Devices, Sunnyvale, CA), we monitored resorufin formation at an excitation wavelength of 545 nm and a production wavelength of 590 nm. The level of resorufin formation was recorded every 5 min for 15 min, and H_2O_2 production was calculated with a standard curve.

Western blot analysis. Protein abundance was determined in skeletal muscle samples via Western blot analysis. Briefly, soleus and plantaris tissue samples were homogenized 1:10 (wt/vol) in 5 mM Tris (pH 7.5) and 5 mM EDTA (pH 8.0) with a protease inhibitor cocktail (Sigma) and centrifuged at 1,500 g for 10 min at 4°C. After collection of the resulting supernatant, muscle protein content was assessed by the method of Bradford (Sigma, St. Louis, MO). Proteins were separated using electrophoresis via 4–20% polyacrylamide gels containing 0.1% sodium dodecyl sulfate for $\sim$1 h at 200 V. After electrophoresis, the proteins were transferred to nitrocellulose membranes and incubated with primary antibodies directed against the protein of interest. 4-Hydroxynonenal (4-HNE; Abcam) was probed as a measurement indicative of oxidative stress, while proteolytic activity was assessed by cleaved (active) calpain-1 (Cell Signaling) and cleaved (active) caspase-3 (Cell Signaling). After incubation, membranes were washed with PBS-Tween and treated with secondary antibody (Amersham Biosciences). A chemiluminescent system was used to detect labeled proteins (GE Healthcare), and membranes were developed using autoradiography film and a developer (Kodak). The resulting images were analyzed using computerized image analysis to determine percent change from control. Finally, to control for protein loading and transfer differences, membranes were stained with Ponceau S. Ponceau S-stained membranes were scanned, and the lanes were quantified using the 440CF Kodak Imaging System (Kodak, New Haven, CT) to normalize Western blots to protein loading.

Histological Measures

Myofiber CSA. Sections from frozen soleus and plantaris samples (supported in OCT compound) were cut at 10 μm using a cryotome (Shandon, Pittsburgh, PA) and stained for dystrophin, myosin heavy chain (MHC) type I, and MHC type IIa proteins for fiber CSA analysis, as described previously (13). CSA was determined using Scion Image software (National Institutes of Health).

Statistical Analysis

Comparisons between groups for each dependent variable were made by a one-way ANOVA, and, when appropriate, Tukey's honestly significant difference test was performed post hoc. Significance was established at $P < 0.05$. Data are presented as means $\pm$ SE.

RESULTS

SS-31 Does Not Alter Myofiber CSA and Mitochondrial Function in Ambulatory Animals

To determine the effect of the mitochondrial antioxidant SS-31 on muscle-to-body weight ratio, fiber CSA, and mitochondrial respiratory function (RCR), we treated animals with the same dose of SS-31 that was provided to the immobilized animals for 14 days. Our results show that, compared with control animals, treatment with SS-31 does not alter muscle-to-body weight ratio, myofiber size, and mitochondrial respiratory function (Table 1). Collectively, these data indicate that treatment of healthy ambulatory animals with SS-31 does not alter body weight, muscle fiber size, or mitochondrial function.

Table 1. *Muscle-to-body weight, CSA, and mitochondrial function in soleus and plantaris muscle from control and SS-31-treated animals*

	Control	SS-31
	Muscle-to-body weight ratio, mg/g	
Soleus	0.38 ± 0.01	0.37 ± 0.01
Plantaris	0.73 ± 0.02	0.76 ± 0.01
	CSA, μm²	
Soleus fiber		
Type I	1,690 ± 176.6	1,893 ± 292.1
Type IIa	1,346 ± 195.5	1,525 ± 290.2
Type IIb/x	1,762 ± 299.0	1,951 ± 359.9
Plantaris fiber		
Type IIa	1,090 ± 87.2	1,018 ± 80.2
Type IIb/x	2,243 ± 97.1	2,058 ± 209.9
	Mitochondrial respiratory function	
Soleus		
State 3 respiration, nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$	16.54 ± 0.93	18.95 ± 0.84
State 4 respiration, nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$	2.72 ± 0.26	3.40 ± 0.22
RCR	6.25 ± 0.41	5.95 ± 0.55
Plantaris		
State 3 respiration, nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$	10.32 ± 0.57	10.37 ± 0.60
State 4 respiration, nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$	1.72 ± 0.09	1.59 ± 0.03
RCR	5.57 ± 0.30	6.52 ± 0.35

Values are means ± SE. CSA, cross-sectional area; RCR, respiratory control ratio. There were no significant differences in muscle-to-body weight ratio; CSA, or mitochondrial respiratory function between control (saline-injected) and SS-31-treated animals.

Physiological Responses to 14 Days of Immobilization

We measured the muscle-to-body weight ratio after 14 days of immobilization. Muscle-to-body weight ratio in soleus and plantaris muscles decreased significantly after 14 days of immobilization compared with control (Fig. 1). More importantly, SS-31 administration abolished the decrease in the muscle-to-body weight ratio in soleus and plantaris muscles.

SS-31 Impedes Immobilization-Induced ROS Production From Mitochondria in Skeletal Muscles

We determined the effects of SS-31 on prevention of immobilization-induced ROS production from hindlimb skeletal muscles by measuring mitochondrial ROS production under basal (state 4) conditions. Indeed, treatment with SS-31 prevented the immobilization-induced increase in mitochondrial H_2O_2 production in soleus and plantaris muscles compared with the cast group (Fig. 2). Thus we have shown that treatment with SS-31 during prolonged immobilization of skeletal muscles effectively inhibits mitochondrial ROS production in the skeletal muscle mitochondria.

Lipid Hydroperoxides Are Elevated in Mitochondria After Prolonged Immobilization

β-Unsaturated 4-HNE-conjugated cytosolic proteins were measured as an indicator of lipid peroxidation to determine if mitochondrial ROS production is required for oxidative stress in immobilized skeletal muscles. Compared with control, 14 days of immobilization resulted in a significant increase in 4-HNE in soleus and plantaris muscles (Fig. 3). Our results reveal that treatment of animals with SS-31 protected the skeletal muscles against the ROS-induced increase in 4-HNE-conjugated cytosolic proteins associated with prolonged immobilization. These findings indicate that increased mitochondrial ROS production is a requirement for immobilization-induced oxidative damage to proteins in skeletal muscles.

Skeletal Muscle Mitochondrial Oxidative Phosphorylation

To determine if treatment of animals with SS-31 protects mitochondria from immobilization-induced mitochondrial uncoupling, we measured mitochondrial RCR after 14 days of immobilization. As shown in Fig. 4, 14 days of immobilization significantly reduced the RCR of mitochondria in soleus and plantaris muscles when pyruvate/malate was used as the substrate. Treatment of animals with SS-31 prevented immobilization-induced mitochondrial uncoupling.

Increased Mitochondrial ROS Production Is Required for Immobilization-Induced Fiber Atrophy

Myofiber CSA was evaluated to determine the role of mitochondrial ROS production in prolonged immobilization-induced muscle atrophy. Prolonged immobilization for 14 days resulted in significant atrophy of type I, IIa, and IIb/IIx myofibers. Importantly, treatment with SS-31 attenuated atrophy in all myofiber types after 14 days of immobilization (Fig. 5). Our data suggest that prevention of the immobilization-induced
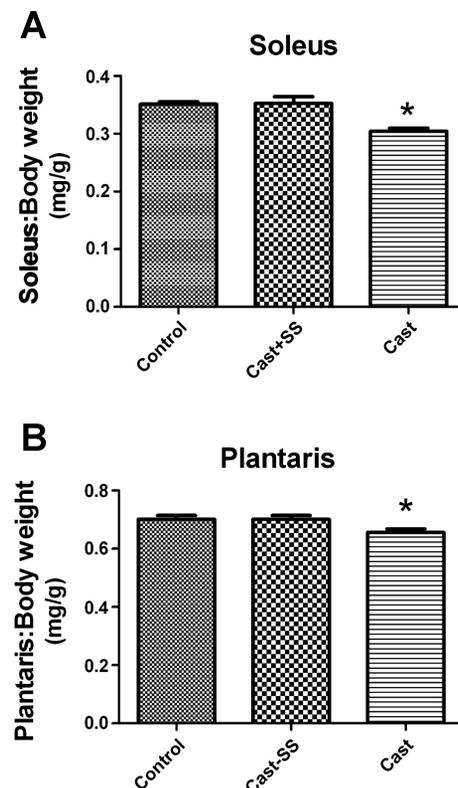

Fig. 1. Muscle-to-body weight ratio in soleus (*A*) and plantaris (*B*) muscle of control group, immobilization (cast) group, and hindlimb immobilization group treated with SS-31 (Cast + SS) after 14 days of immobilization. Values are means ± SE (*n* = 7/group). *Significantly different (*P* < 0.05) from control.
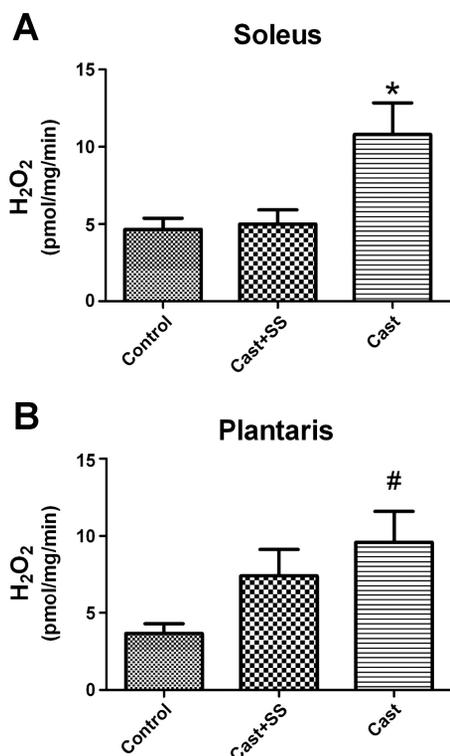
A Soleus

B Plantaris

Fig. 2. Rates of H_2O_2 release from mitochondria in permeabilized skeletal muscle fibers prepared from soleus (A) and plantaris (B) muscles from control, cast, and cast + SS groups. Values are means ± SE. *Significantly different ($P < 0.05$) from control and cast + SS. #Significantly different from control.

increase in mitochondrial ROS production prevented immobilization-induced fiber atrophy in the skeletal muscles.

Mitochondrial ROS Production Stimulates Protease Activation and Proteolysis in Immobilized Skeletal Muscles

Growing evidence shows that oxidative stress plays an important role in the activation of key proteases (e.g., calpain and caspase-3) during skeletal muscle atrophy (14, 28). Therefore, we determined whether prolonged immobilization-induced increases in mitochondrial ROS production are required to activate calpain and caspase-3 in skeletal muscle. Fourteen days of immobilization significantly elevated calpain-1 and caspase-3 activity, while treatment of animals with a mitochondrial-targeted antioxidant (SS-31) protected the skeletal muscles against the activation of calpain (Fig. 6) and caspase-3 (Fig. 7). These data reveal that mitochondrial ROS production is essential for prolonged immobilization-induced activation of calpain and caspase-3 in the skeletal muscles.

DISCUSSION

Overview of Major Findings

These experiments provide new and important information regarding the mechanism(s) responsible for immobilization-induced limb muscle atrophy. We hypothesized that mitochondrial ROS production plays an important role in immobilization-induced ROS production and oxidative stress in skeletal muscle. Our results support this postulate, as administration of a mitochondrial-targeted antioxidant protected the immobilized muscle against increased mitochondrial ROS production

and oxidative stress. Importantly, our findings also reveal that increased mitochondrial ROS production during periods of immobilization is an upstream signal to activate the key proteases calpain and caspase-3 in the inactive muscle. Finally, our data support the prediction that mitochondria are an important source of oxidant production in the skeletal muscles during prolonged immobilization. Importantly, our results also show that prevention of immobilization-induced increases in mitochondrial ROS production can protect skeletal muscle from disuse muscle atrophy.
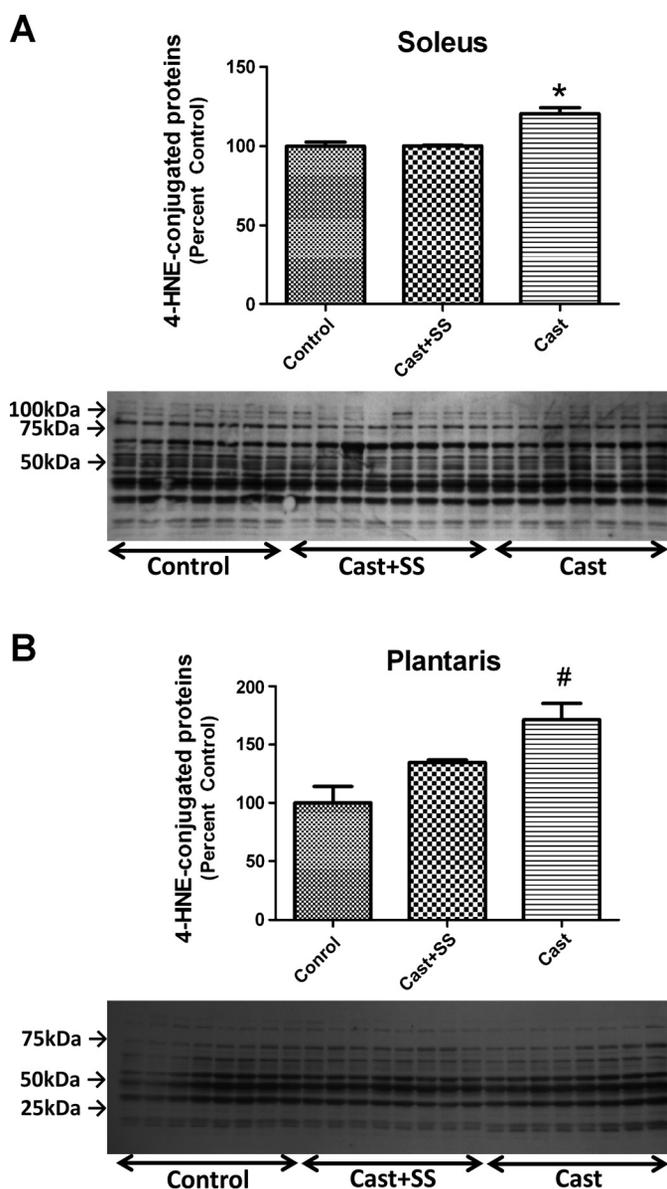
A Soleus

B Plantaris

Fig. 3. Levels of β-unsaturated 4-hydroxynonenal (4-HNE)-conjugated cytosolic proteins in soleus (A) and plantaris (B) muscles from control, cast, and cast + SS groups. Data were analyzed as an indicator of lipid peroxidation via Western blot from the 3 experimental groups. Values are means ± SE ($n = 7$/group). *Significantly different ($P < 0.05$) from control and cast + SS. #Significantly different from control.
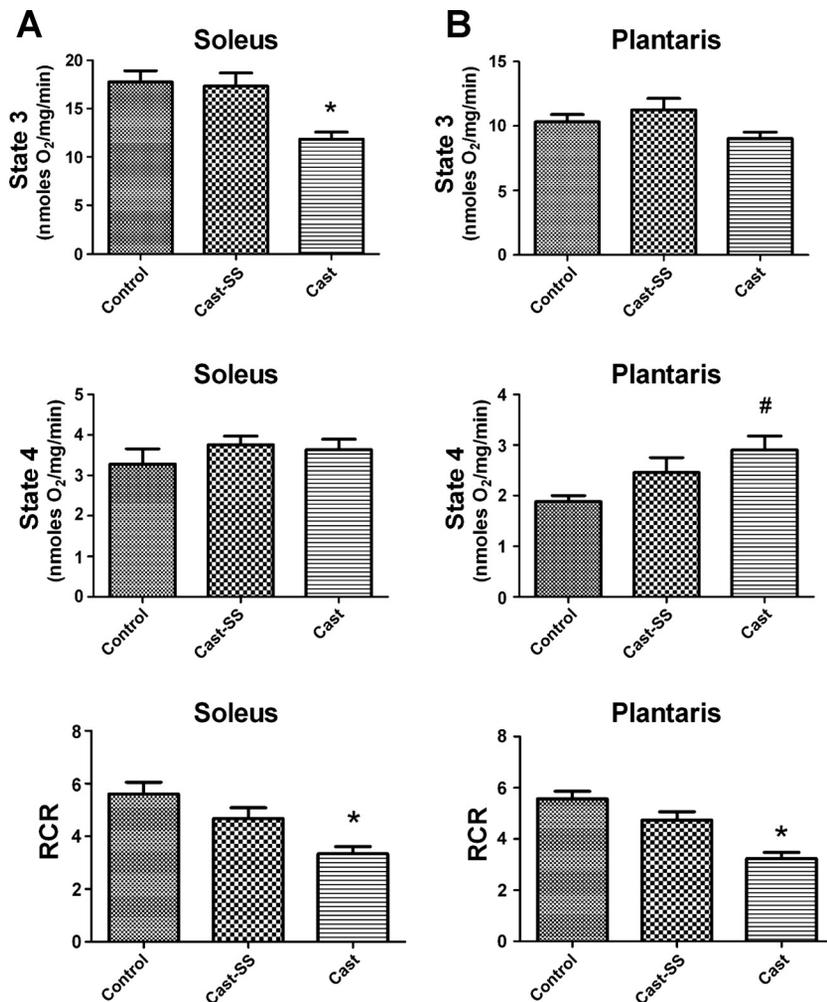
Fig. 4. State 3 respiration, state 4 respiration, and respiratory control ratio (RCR) of mitochondria in permeabilized fibers prepared from soleus (*A*) and plantaris (*B*) muscle from control, cast, and cast + SS groups. Data were obtained using pyruvate/malate as substrate. Values are means ± SE. *Significantly different ($P < 0.05$) from control and cast + SS. #Significantly different from control.

Use of a Mitochondrial-Targeted Antioxidant as an Experimental Probe

Although there are many sites for ROS production within skeletal muscle, the dominant source of ROS production in skeletal muscle during prolonged inactivity remains unknown. Based on our prior experiments in diaphragm muscle exposed to prolonged periods of inactivity (e.g., mechanical ventilation), we hypothesized that mitochondria are an important source of ROS production in hindlimb muscle during immobilization (8, 19). To determine whether mitochondria are an important source of ROS in skeletal muscles during prolonged immobilization, we utilized a new and innovative mitochondrial-targeted antioxidant designated SS-31. SS-31 belongs to a family of small cell-permeable peptides that target and concentrate >1,000-fold in the inner mitochondrial membrane, where it reduces mitochondrial ROS production without affecting membrane potential (29). This selective targeting of SS-31 provides extraordinary potency and selectivity for mitochondrial ROS. Indeed, previous studies reveal that SS-31 prevents increases in mitochondrial H_2O_2 production in skeletal muscle from rats fed a high-fat diet and development of insulin resistance (1). SS-31 provided similar protection against mitochondrial oxidative stress as the overexpression of mitochondrial catalase (1). Therefore, we used this highly selective mitochondrial-targeted antioxidant in our present experiments.

Prolonged Immobilization Increases Mitochondrial ROS Production in the Skeletal Muscle

It is well established that prolonged periods of skeletal muscle inactivity lead to increased ROS production, disturbed redox signaling, and oxidative damage (15, 18, 20, 28). However, the primary site(s) of immobilization-induced ROS production in skeletal muscle remain(s) unknown. In this regard, our previous work using mechanical ventilation as a model of respiratory muscle inactivity demonstrates that nitric oxide production is not increased in inactive skeletal muscles (24). In contrast, superoxide production in inactive skeletal muscles occurs via activation of NADPH oxidase and xanthine oxidase (14, 27). Nonetheless, NADPH oxidase and xanthine oxidase are not the dominant pathways of ROS production in inactive respiratory muscles (14, 27). Recently, our group reported that mitochondria are an important source of ROS production in diaphragm muscle during prolonged mechanical ventilation (8, 17). However, it remains unknown if mitochondria are an important source of ROS production in immobilized limb skeletal muscles. Hence, using a highly selective mitochondrial-targeted antioxidant (SS-31), we examined the role of mitochondria in ROS production in locomotor skeletal muscles exposed to prolonged immobilization. Our results clearly indicate that mitochondrial ROS production was significantly increased in soleus and plantaris muscles following prolonged
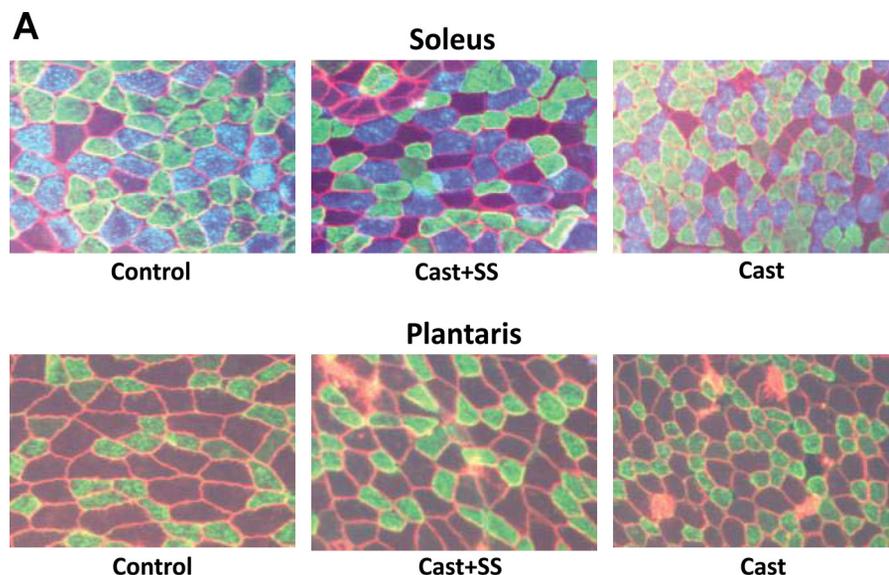
Fig. 5. Fiber cross-sectional area (CSA) of soleus and plantaris muscles from control, cast, and cast + SS groups. *A*: representative fluorescent staining of myosin heavy chain (MHC) I [4′,6-diamidino-2-phenylindole filter (blue)], MHC IIa [FITC filter (green)], and dystrophin [rhodamine filter (red)] proteins. *B*: type I, IIa, and IIx/IIb fiber CSA. Values are means ± SE (n = 7/group). *Significantly different (P < 0.05) from control and cast + SS.
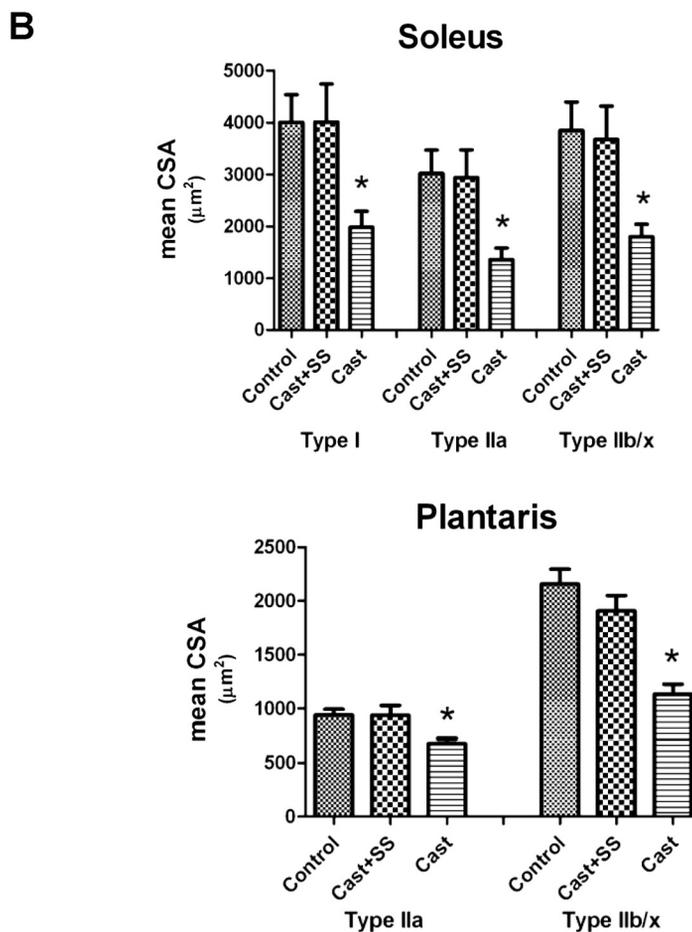
immobilization, and this increase was prevented by treatment with SS-31 (Fig. 2). Moreover, treatment with SS-31 protected skeletal muscle against inactivity-induced oxidative damage and also protected muscle mitochondria against uncoupling (Figs. 3 and 4). Collectively, these data support the hypothesis that mitochondria are an important source of ROS production in locomotor skeletal muscle during prolonged immobilization.

Mitochondrial ROS Production Contributes to Immobilization-Induced Atrophy

A key finding in this study is that administration of a mitochondrial-targeted antioxidant (SS-31) is sufficient to attenuate immobilization-induced skeletal muscle atrophy. Indeed, skeletal muscle atrophy was successfully prevented in
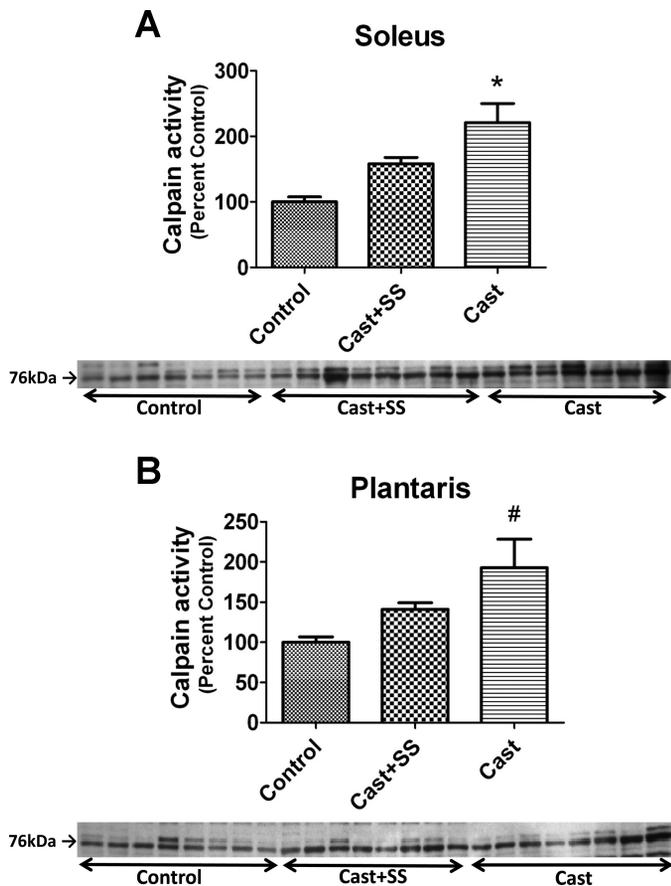
A
Soleus

B
Plantaris

Fig. 6. Calpain activity in soleus (A) and plantaris (B) muscles from control, cast, and cast + SS groups. Data were analyzed via Western blot from the 3 experimental groups. Values are means ± SE ($n = 7$/group). *Significantly different ($P < 0.05$) from control and cast + SS. #Significantly different from control.

type I, IIa, and IIx/b fibers during prolonged hindlimb immobilization in soleus and plantaris muscles (Fig. 5). Collectively, these novel results indicate that minimizing mitochondrial ROS production protected skeletal muscles from immobilization-induced muscle atrophy, and this protection occurs in slow- and fast-twitch muscle fibers.

Our laboratory and others have demonstrated that key proteases are a predominant factor responsible for disuse skeletal muscle atrophy (6, 12). Specifically, calpain and caspase-3 promote the degradation of myofibrillar proteins and are activated in skeletal muscle during a variety of conditions that promote muscle wasting (5, 12). Therefore, identification of the signal(s) responsible for activation of calpain and caspase-3 in inactive skeletal muscle is important. In reference to the "trigger" responsible for calpain and caspase-3 activation, we have shown that oxidative stress in inactive skeletal muscles is essential for the activation of proteases, including calpain and caspase-3 (2, 28). In addition, we have also shown that oxidatively modified proteins are more susceptible to degradation by calpain and caspase-3 (22). In this regard, the present experiments clearly provide evidence that mitochondrial ROS production is an upstream signal to activate calpain and caspase-3 in skeletal muscle during prolonged immobilization. In general, calpain activation requires elevated levels of cytosolic free calcium. Therefore, since it has been established that

oxidative stress can disturb calcium homeostasis by increasing cellular levels of free calcium (9), it is feasible that mitochondrial ROS production promotes calpain activation in inactive skeletal muscles by increasing cytosolic levels of calcium. Furthermore, it has been established that increased cellular ROS production can promote caspase-3 via a variety of signaling pathways (18, 28). Specifically, caspase-3 can be activated by one or more upstream pathways, including the activation of calpain-8, caspase-9, and/or caspase-12. In theory, oxidative stress could promote caspase-3 activation via one or more of these pathways (20). Future studies are needed to reveal the specific signaling pathways responsible for the activation of calpain and caspase-3 during disuse muscle atrophy.

Conclusions and Clinical Implications

The present experiments provide several new and important findings regarding the mechanisms responsible for immobilization-induced skeletal muscle atrophy. First, our data confirm that mitochondria are a source of ROS production in inactive skeletal muscles. Furthermore, our experiments reveal that increased mitochondrial ROS production contributes to limb muscle atrophy during prolonged immobilization. Our findings also indicate that mitochondrial ROS production is an upstream signal for inactivity-induced calpain and caspase-3 activation in locomotor skeletal muscles. Importantly, prevention of mitochondrial ROS production by the mitochondrial-targeted antioxidant SS-31 rescues locomotor skeletal muscle from

A
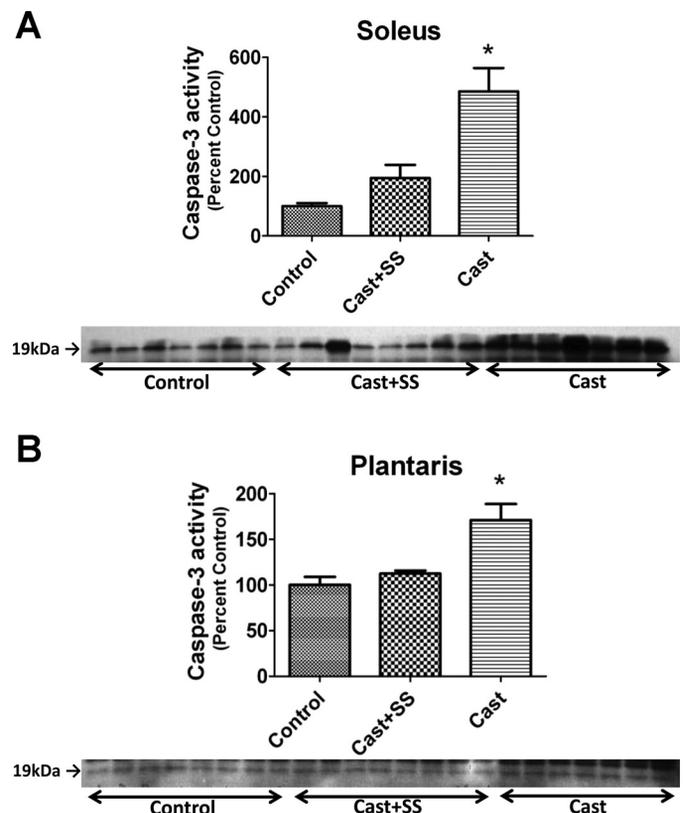### Soleus

B
Plantaris

Fig. 7. Caspase-3 activity in soleus (A) and plantaris (B) muscles from control, cast, and cast + SS groups. Data were analyzed via Western blot from the 3 experimental groups. Values are means ± SE ($n = 7$/group). *Significantly different ($P < 0.05$) from control and cast + SS.

disuse-induced atrophy. Collectively, our findings suggest that mitochondrial-targeted antioxidants may have therapeutic potential in protecting skeletal muscle against prolonged disuse-induced skeletal muscle atrophy.

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