Immobilization-induced activation of key proteolytic systems in skeletal muscles is prevented by a mitochondria-targeted antioxidant

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Talbert EE, Smuder AJ, Min K, Kwon OS, Szeto HH, Powers SK. Immobilization-induced activation of key proteolytic systems in skeletal muscles is prevented by a mitochondria-targeted antioxidant. J Appl Physiol 115: 529–538, 2013. First published June 13, 2013; doi:10.1152/japplphysiol.00471.2013.—Long periods of skeletal muscle disuse result in muscle fiber atrophy, and mitochondrial production of reactive oxygen species (ROS) appears to be a required signal for the increase in protein degradation that occurs during disuse muscle atrophy. The experiments detailed here demonstrate for the first time in limb muscle that the inactivity-induced increases in E3 ligase expression and autophagy biomarkers result from increases in mitochondrial ROS emission. Treatment of animals with a mitochondrial-targeted antioxidant also prevented the disuse-induced decrease in anabolic signaling (Akt/mammalian target of rapamycin signaling) that is normally associated with prolonged inactivity in skeletal muscles. Additionally, our results confirm previous findings that treatment with a mitochondrial-targeted antioxidant is sufficient to prevent casting-induced skeletal muscle atrophy, mitochondrial dysfunction, and activation of the proteases calpain and caspase-3. Collectively, these data reveal that inactivity-induced increases in mitochondrial ROS emission play a required role in activation of key proteolytic systems and the downregulation of important anabolic signaling molecules in muscle fibers exposed to prolonged inactivity.

Oxidative stress plays a major role in the disuse atrophy process and activates both calpain and caspase-3 in muscles exposed to prolonged inactivity (19, 23, 31). Mitochondrial oxidant production (e.g., superoxide and resulting hydrogen peroxide) appears to be a required upstream signal to promote calpain and caspase-3 activation in several models of muscle disuse, including hindlimb immobilization of mice (1, 12, 19, 31).

Presently, little is known about the redox sensitivity of the proteasome and the autophagy system during hindlimb disuse, although both systems are activated in diaphragm muscle in response to prolonged mechanical ventilation, another type of disuse muscle atrophy associated with oxidative stress (1, 23, 31). Therefore, we determined whether inactivity-induced mitochondrial oxidant production is required to activate both the proteasome system and autophagy during disuse muscle atrophy induced by hindlimb immobilization (i.e., casting). Furthermore, we also determined whether inactivity-induced oxidative stress in limb muscle plays a required role in the downregulation of anabolic signaling in the inactive muscles. Cause and effect were determined by treating immobilized animals with a mitochondrial-targeted antioxidant to scavenge reactive oxygen species (ROS) produced in mitochondria within the inactive skeletal muscles. We demonstrate for the first time in limb muscle that activation of both the proteasome system and autophagy system is sensitive to increased mitochondrial ROS emission. Additionally, our results provide the first evidence that increased mitochondrial ROS emission is responsible for the depressed anabolic signaling that occurs in skeletal muscle during prolonged disuse.

METHODS

Animals and Experimental Design

Adult female 300-g Sprague-Dawley rats (Charles River, Wilmington, MA) were assigned to one of four groups: 1) noncasted (ambulatory) receiving saline injections (Con, n = 10); 2) noncasted (ambulatory) receiving the mitochondria-targeted antioxidant SS-31 (Con-SS, n = 10); 3) hindlimbs casted receiving saline (Cast, n = 8); and 4) hindlimbs casted receiving the mitochondria-targeted antioxidant SS-31 (Cast-SS, n = 8). SS-31 was dissolved in saline and given via daily subcutaneous injection at a dose of 3 mg/kg. Saline animals received an equal volume of saline daily. Animals received their first injection of SS-31 immediately before casting while under anesthesia and received subsequent injections at the same time each day. All experiments were approved by the University of Florida Institutional Animal Care and Use Committee. Note that both muscle fiber size and mitochondrial functional data from the Con and Cast animals has been previously published (28).

Immobilization. Animals were casted under the influence of gaseous isoflurane (2% for induction of anesthesia, 0.5–1.5% to maintain a surgical plane of anesthesia). To prevent skin abrasions from the cast, a layer of Medipore Dress-it (3M Health Care, St. Paul, MN) was applied over each casted limb. All casts were developed (e.g., hindlimb cast immobilization of rodents used to mimic human limb immobilization) (25). Currently, details of the signaling pathways leading to disuse muscle atrophy remain largely unknown. Understanding the mechanisms responsible for inactivity-induced muscle atrophy is important, as currently no effective countermeasures exist to combat this problem (21).

Disuse muscle atrophy results from decreased protein synthesis and increased protein degradation, with degradation accounting for the majority of the atrophy (29). Four key proteolytic systems exist in skeletal muscle: autophagy, the ubiquitin proteasome system, the calpain system, and the caspase system. All four of these proteolytic systems are active during disuse muscle atrophy in humans and animals (25), but the relative contribution of each system to disuse atrophy remains unknown.

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used to cover the animal’s skin. Animals were casted with plaster-of-Paris (Gypsuma, Reynolds, Tamaulipas, Mexico) from the last rib down through both legs with the ankle plantar flexed as to induce maximum soleus and plantaris atrophy. A single layer of fiberglass (Scotchcast Plus, St. Paul, MN) was added to the top of the cast to decrease the animal’s ability to chew through the cast. Animals were monitored daily to ensure that animals did not experience swollen or abraded feet and did not damage the cast. Additionally, animals were checked to make sure they had free access to water and food.

**Tissue Harvesting**

Following 7 days of casting, animals were anesthetized to a surgical plane of anesthesia using isoflurane, and the cast was removed. The soleus and plantaris muscles were removed from both hindlimbs, and then the animals were killed. One soleus and one plantaris were split into two pieces. One piece of each muscle was dissected into two pieces and permeabilized to measure ROS emission and mitochondrial function, as described below. The other half of each plantaris and soleus was embedded in optimal cutting temperature (OCT) medium (Sakura Finetek USA, Torrance, CA) and frozen in liquid nitrogen-cooled isopentane, as previously described (18). The soleus and plantaris from the opposite leg were snap frozen in liquid nitrogen and stored at −80°C for Western blotting and real-time RT-PCR measurements.

**Biochemical Measures**

*Preparation of permeabilized muscle fibers.* Mitochondrial ROS release and mitochondrial oxygen consumption were determined in permeabilized muscle fibers, as previously described (19). Briefly, ~10 mg pieces of muscle were teased apart on ice in cold buffer X [60 mM K-2-(N-morpholino)ethanesulfonic acid (MES), 35 mM KCl, 7.23 mM K2EGTA, 2.77 mM CaK2EGTA, 20 mM imidazole, 0.5 mM DTT, 20 mM taurine, 5.7 mM ATP, 15 mM phosphocreatine, and 6.56 mM MgCl2, pH 7.1]. After dissection, muscle fiber membranes were permeabilized by rotation in buffer X containing 50 µM BSA, pH 7.1. Fiber bundles were incubated at 37°C in 96-well plates was used to determine the level of ROS release from permeabilized muscle fibers, as previously described (19). Briefly, ~10 mg pieces of muscle were teased apart on ice in cold buffer X [60 mM K-2-(N-morpholino)ethanesulfonic acid (MES), 35 mM KCl, 7.23 mM K2EGTA, 2.77 mM CaK2EGTA, 20 mM imidazole, 0.5 mM DTT, 20 mM taurine, 5.7 mM ATP, 15 mM phosphocreatine, and 6.56 mM MgCl2, pH 7.1]. After dissection, muscle fiber membranes were permeabilized by rotation in buffer X containing 50 µg/ml saponin at 3°C. After 30 min, the fiber bundles were rotated in buffer Z (110 mM K-MES, 35 mM KCl, 1 mM EGTA, 5 mM K2HPO4, 3 mM MgCl2, 0.05 mM glutamate, 0.02 mM malate, and 0.5 mg/ml BSA, pH 7.1) three times for 5 min to remove all saponin.

**Mitochondrial ROS production.** The Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies, Grand Island, NY) was used to determine the level of ROS release from permeabilized fiber bundles. Fiber bundles were incubated at 37°C in 96-well plates in reaction buffer (5 mM succinate, 10 mM MgCl2, 10 mM KH2PO4, 100 mM KCl, 50 mM MOPS, 1 mM EGTA, 0.4 µg/ml BSA, 0.02 units horseradish peroxidase, 2.5 µg Amplex Red, pH 7.0), as previously described (19). After 30 min of incubation, the amount of fluorescent resorufin produced was measured using an excitation wavelength of 545 nm and an emission wavelength of 590 nm in fluorometric multiwell-plate reader (SpectraMax, Molecular Devices, Sunnyvale, CA).

**Mitochondrial oxygen consumption.** Mitochondrial function was determined by measuring the rate of oxygen consumption of permeabilized soleus and plantaris muscle fibers using a respiration chamber (Hansatech Instruments), as previously described (19). Substrate (5 mM pyruvate and 2 mM malate) was added to 1 ml of buffer Z containing 20 mM creatine and the permeabilized fiber bundle. State 3 respiration was stimulated by 0.25 mM ADP. After 5 min, 10 µg/ml oligomycin was added to stimulate state 4 respiration. The respiratory control ratio (RCR) was determined by dividing maximal state 3 oxygen consumption by state 4 oxygen consumption.

**Myofiber cross-sectional area.** OCT-embedded pieces of muscle were sliced at 10 µm using a cryotome (Shandon, Pittsburgh, PA). Muscle sections were stained for dystrophin and myosin heavy chain isoforms, as described previously (18). Cross-sectional area (CSA) was determined by tracing using Scion Image software (Scion, Frederick, MD). A minimum of 100 fibers per muscle were analyzed for each animal.

**Western blotting.** Muscles were placed in 5 mM Tris/5 mM EDTA (pH 7.5) 1:10 (mg wt/µl buffer) and homogenized using a motorized glass-on-glass system. Protease inhibitor cocktail was used 1:20 (vol/vol) (Sigma-Aldrich). Samples were centrifuged at 4°C for 10 min at 1,500 g to pellet insoluble protein. Supernatant protein concentration was determined by the method of Bradford (3). Laemmli buffer (Bio-Rad, Hercules, CA) containing 5% β-mercaptoethanol was mixed 1:1 with equal amounts of muscle protein. Proteins were separated on 4–20% Tris-HCl gels (Bio-Rad). After blocking in Licor Blocking buffer, membranes were incubated with primary antibody against cleaved (active) calpain-1, cleaved (active) caspase-3, cleaved (active) caspase-9, light chain 3 (LC3), phosphorylated Akt (pAkt) (Ser473), Akt, phosphorylated mammalian target of rapamycin (pmTOR) (Ser2448), mTOR (Cell Signaling, Danvers, MA), and α-II spectrin (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were either visualized using the Odyssey system and fluorescent secondary antibodies (LICOR Biosciences, Lincoln, NE) or developed on photographic film using enhanced chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK). Films were analyzed using the Kodak Image Station 440 and Kodak 1D software. To control for protein loading and transfer differences, each protein was normalized to the level of α-tubulin detected on the same membrane.

**Real-time reverse-transcriptase PCR.** Our laboratory’s method for mRNA isolation and cDNA synthesis has been previously described (27). Briefly, total mRNA was isolated by homogenizing snap-frozen...
muscle tissue in TRIzol reagent (Life Technologies, Carlsbad, CA), according to the manufacturer’s directions. Spectrophotometry was used to determine RNA content, and 3 μg of mRNA were reverse-transcribed to cDNA using the Superscript III First Strand Synthesis System (Life Technologies). Taqman chemistry (Applied Biosystems, Foster City, CA) was used to determine the relative expression of our genes of interest using the computed tomography method (Applied Biosystems, User Bulletin no. 2, ABI PRISM 7700 Sequence Detection System) and the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Twenty-five microliter reactions containing 1 μl of cDNA were used to determine the expression of atrogin-1 (Entrez gene ID 171043), muscle RING-finger 1 (MuRF-1) (140939), peroxisome proliferator-activated receptor-α coactivator-1α (PGC-1α) (83516), and our housekeeping gene, β-glucuronidase (244434). Our laboratory has previously used β-glucuronidase as our reference gene based on work showing unchanged expression in mechanical ventilation, which is another type of disuse atrophy (6, 7).

Statistical Analysis

Comparisons between groups were made by a one-way analysis of variance, 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 ± SE.

RESULTS

SS-31 Treatment of Ambulatory Con Animals Does Not Alter Muscle Fiber Size or Mitochondrial ROS Emission

Treatment of ambulatory Con animals with SS-31 for 7 days did not alter mitochondrial ROS emission from soleus (Fig. 1A) or plantaris (Fig. 1B) muscle fiber bundles respiring on 5 mM succinate. Additionally, SS-31 treatment did not change muscle CSA of any fiber type in either the soleus (Fig. 2A) or the plantaris muscles (Fig. 2B). Thus statistical comparisons were made between only the Con, Cast, and Cast-SS groups.

Table 1. Mitochondrial function of permeabilized soleus fiber bundles

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Con</th>
<th>Cast</th>
<th>Cast-SS</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3 VO₂</td>
<td>5.40 ± 1.4</td>
<td>3.73 ± 1.1†</td>
<td>5.70 ± 1.5</td>
</tr>
<tr>
<td>State 4 VO₂</td>
<td>1.24 ± 0.3</td>
<td>1.88 ± 0.5*</td>
<td>1.17 ± 0.2</td>
</tr>
<tr>
<td>RCR</td>
<td>4.45 ± 1.2</td>
<td>2.17 ± 1.1*</td>
<td>4.76 ± 1.5</td>
</tr>
</tbody>
</table>

Values are means ± SE of state 3 and state 4 mean oxygen consumption (VO₂), normalized to fiber bundle dry weight. Respiratory control ratio (RCR) is the ratio of state 3 divided by state 4. Con, noncasted (ambulatory) receiving saline injections; Cast, hindlimbs casted receiving saline; Cast-SS, hindlimbs casted receiving the mitochondria-targeted antioxidant SS-31. Significantly different from †Cast-SS and *all other groups (P < 0.05).

Table 2. Mitochondrial function of permeabilized plantaris fiber bundles

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Con</th>
<th>Cast</th>
<th>Cast-SS</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3 VO₂</td>
<td>7.64 ± 0.60</td>
<td>7.01 ± 1.53*</td>
<td>8.78 ± 1.81</td>
</tr>
<tr>
<td>State 4 VO₂</td>
<td>1.20 ± 0.29</td>
<td>1.64 ± 0.59</td>
<td>1.67 ± 0.30</td>
</tr>
<tr>
<td>RCR</td>
<td>7.08 ± 2.37</td>
<td>3.71 ± 1.34†</td>
<td>5.51 ± 1.03</td>
</tr>
</tbody>
</table>

Values are means ± SE of state 3 and state 4 mean VO₂, normalized to fiber bundle dry weight. Significantly different from *Cast-SS and †Con (P < 0.05).
SS-31 Attenuates Casting-induced Increases in Mitochondrial ROS Emission

To assess the effectiveness of SS-31 in prevention of mitochondrial ROS emission, we measured hydrogen peroxide release from permeabilized soleus and plantaris fiber bundles. Compared with ambulatory Con animals, immobilization resulted in a significant increase in mitochondrial ROS release from both the soleus and plantaris muscles. Treatment with SS-31 attenuated this inactivity-induced increase in mitochondrial ROS emission from the soleus (Fig. 1A) and completely prevented the increased ROS emission from the plantaris (Fig. 1B).

SS-31 Prevents Casting-Induced Atrophy

Seven days of casting induced a significant decrease in the CSA of type I fibers of the soleus, and SS-31 treatment during casting completely prevented this decrease (Fig. 2A). No significant differences existed between groups in the CSA of type IIA muscle fibers in the soleus. In the plantaris muscle, casting significantly decreased the size of type IIA, type IIB/IIX, and hybrid IIA/IIB/IIX fibers (Fig. 2B). SS-31 treatment prevented the casting-induced decrease in each of these fiber types. No significant differences were observed in the CSA of type I fibers in the plantaris.

SS-31 Treatment Prevents the Mitochondrial Dysfunction Induced by Casting

Prolonged casting of the hindlimb muscles decreased the mitochondrial RCR in both the soleus and the plantaris muscles (Tables 1 and 2). RCR is the ratio of state 3 (ADP-stimulated) oxygen consumption divided by state 4 (oligomycin-inhibited) oxygen consumption, and decreases in RCR are indicative of impaired mitochondrial coupling. Daily SS-31 treatment attenuated the casting-induced decrease in RCR in both the soleus and plantaris muscle. Additionally, casting induced a significant increase in state 4 respiration in the soleus, which was completely prevented by SS-31 treatment. Finally, state 3 respiration in both plantaris and soleus muscle fibers was higher in Cast-SS animals compared with Cast animals. Our laboratory has previously demonstrated that treating ambulatory animals with SS-31 has no effect on skeletal muscle mitochondrial function (19).
SS-31 Prevents Casting-Induced Calpain Activation

Our laboratory has previously reported that SS-31 prevents the casting-induced increases in calpain activation in both the plantaris and soleus muscles in the mouse (19). We confirm these findings in a rat model of hindlimb muscle disuse (Fig. 3, A and B) and additionally demonstrate that SS-31 prevents immobilization-induced calpain activity measured by α-II-spectrin degradation in the soleus and plantaris. When calpain cleaves the cytoskeletal protein α-II-spectrin, a specific fragment is detectable at 145 kDa via Western blot. The presence of this fragment is an index of in vivo calpain activity over the last several hours of the experiment (30). Casting induced a significant increase in the calpain-specific fragment of α-II-spectrin in both the soleus and plantaris; this increase was completely prevented by SS-31 treatment (Fig. 3, C and D).

SS-31 Prevents Casting-Induced Caspase-3 Activation

Our group has also previously reported that SS-31 prevents the casting-induced activation of caspase-3 in a mouse model (19). We confirm this finding in a rat model of muscle atrophy, with SS-31 completely preventing the increase in active caspase-3 in the soleus muscle (Fig. 4A) and attenuating the

![Graphs showing the results of caspase-3 and α-II-spectrin cleavage](Image)

Fig. 4. Caspase-3 activation. Levels of active caspase-3 [soleus (A) and plantaris (B)] were assessed via Western blot. Caspase-3 activity [soleus (C) and plantaris (D)] was assessed by measuring levels of the specific α-II-spectrin cleavage product of caspase-3 (120 kDa). Caspase-9 activation, an upstream activator of caspase-3, was also assessed [soleus (E) and plantaris (F)]. Representative Western blots are shown below the graphs. Values are presented as fold control of mean arbitrary units ± SE. Blots were normalized to α-tubulin, which is shown under the representative blot. *Significantly different vs. all other groups (P < 0.05). †Significantly different vs. Con (P < 0.05).
casting-induced increase in active caspase-3 in the plantaris muscle (Fig. 4B). Additionally, our results indicate that SS-31 treatment attenuated the increase in the caspase-3-specific (120 kDa) cleavage fragment of α-II-spectrin in the soleus muscle (Fig. 4C). Similar to the 145-kDa fragment specific to calpain, the presence of this breakdown product is an index of caspase-3 activity over the last several hours of the experiment (30). Note that no group differences exist in the 120-kDa fragment of α-II-spectrin in the plantaris muscle (Fig. 4D). Finally, although no significant differences existed in caspase-9 activity in the soleus muscle (Fig. 4E), casting increased caspase-9 activation in the plantaris muscle (Fig. 4F), and SS-31 treatment attenuated this increase. This data suggests that caspase-9 may play a role in caspase-3 activation in the plantaris muscle during prolonged inactivity.

Activation of the Proteasome System Requires Mitochondrial ROS Production

Seven days of hindlimb casting resulted in a significant increase in the mRNA expression of both atrogin-1 (Fig. 5A) and MuRF-1 (Fig. 5B) in the soleus, and atrogin-1 (Fig. 5C) in the plantaris. In the soleus, these increases in E3 ligase expression were completely prevented by SS-31 treatment. Additionally, the increase in atrogin-1 expression in the plantaris was partially attenuated by daily SS-31 treatment, while no differences in MuRF-1 expression existed between groups in the plantaris (Fig. 5D). Increased expression of atrogin-1 and MuRF-1 are associated with increased signaling through Forkhead homolog FKHRL1 (FoxO3a) and NF-κB (11).

Mitochondrial ROS Stimulates Autophagy During Hindlimb Casting

The lysosomal protein microtubule-associated protein LC3 is conjugated with phosphatidylethanolamine during autophagy (20). LC3 without phosphatidylethanolamine is termed LC3 I, while lipidated LC3 is referred to as LC3 II. An increase in the ratio of LC3 II to LC3 I is representative of an increased presence of autophagic vesicles, which is indicative of accelerated autophagy. Casting significantly increased the ratio of LC3 II to LC3 I in both the soleus (Fig. 6A) and the plantaris muscles (Fig. 6B). Daily treatment of animals with SS-31 prevented this increase in LC3 II-to-LC3 I ratio in both muscles, demonstrating that mitochondrial ROS promotes an increase in the number of autophagic vesicles during prolonged inactivity. An increase in inactivity-induced autophagy in both the soleus and plantaris muscles is also supported by the finding of increased mRNA expression of cathepsin L, a protease involved in autophagic protein degradation (Fig. 6, C and D). Mitochondrial ROS is required for the increase in cathepsin L, as SS-31 treatment prevented the casting-induced increase in this protease in both the soleus and plantaris muscles.

SS-31 Does Not Protect Muscle Size by Promoting PGC-1α Expression

Recent evidence reveals that PGC-1α is decreased following denervation, and that overexpression of PGC-1α is sufficient to protect muscle against denervation and fasting-induced atrophy (26). Therefore, we determined whether increased mitochondrial ROS emission is responsible for the decrease in muscle PGC-1α mRNA induced by casting. SS-31 treatment did not
prevent the casting-induced decrease in PGC-1α in either the soleus or the plantaris muscles (Fig. 7, A and B, respectively). These findings suggest that the inactivity-induced decrease in PGC-1α mRNA in limb muscles is independent of mitochondrial ROS production. Finally, note that PGC-1α mRNA expression was greater in the soleus muscle compared with the plantaris, which agrees with previous work (16) (data not shown).

SS-31 Treatment Protects Markers of Anabolic Signaling During Casting

Although increased protein degradation is the predominant mechanism responsible for muscle fiber atrophy during prolonged disuse, decreases in muscle protein synthesis also occur and contribute to muscle wasting during prolonged disuse. The proteins Akt and mTOR both play important signaling roles in promoting protein synthesis by regulation of translation. Both proteins are activated by phosphorylation at specific sites (Akt at serine 473, mTOR at serine 2448), and the ratio of phosphorylated protein to total protein is an index of Akt/mTOR signaling (2). The ratio of pAkt to Akt was decreased by casting in both the soleus and the plantaris, and SS-31 treatment partially attenuated the decrease in pAkt/Akt in both muscles (Fig. 8, A and B). In the soleus muscle, the ratio of pmTOR to mTOR decreased in response to 7 days of casting, and this decrease was completely prevented by SS-31 treatment (Fig. 8C). In the plantaris muscle, casting did not decrease pmTOR-to-mTOR ratio, but treatment with SS-31 dur-
ing casting significantly increased the ratio of pmTOR to mTOR compared with Con animals (Fig. 8D). Finally, no differences existed between experimental groups in the protein levels of pAkt, Akt, pmTOR, or mTOR in either the plantaris or the soleus muscles (data not shown).

**DISCUSSION**

These experiments confirm the important role that increased mitochondrial ROS emission plays in promoting disuse muscle atrophy and demonstrate the ability of a mitochondrial-targeted antioxidant (SS-31) to prevent casting-induced atrophy of hindlimb muscles in the rat. Importantly, these are the first experiments to demonstrate that increased mitochondrial ROS emission plays a required role in inactivity-induced increases in key proteins involved in both the autophagy and the proteasome proteolytic systems in locomotor skeletal muscles. A discussion of these and other important findings follows.

It is well established that disuse muscle atrophy is associated with oxidative stress in the inactive skeletal muscles (12). Moreover, recent work has demonstrated that preventing inactivity-induced oxidative stress in muscle is sufficient to prevent disuse atrophy (1, 8, 31). Although ROS can be produced at several locations in skeletal muscles (24, 25), recent evidence indicates that the mitochondria are the primary site for ROS production in skeletal muscle during prolonged inactivity (19, 23). In the present study, we confirmed that treatment of animals with a mitochondrial-targeted antioxidant (SS-31) is sufficient to prevent immobilization-induced atrophy of the soleus and plantaris muscles.

Similar to previous results (19), we also observed that inactivity-induced activation of both calpain-1 and caspase-3 in the soleus and plantaris muscles require increased mitochondrial ROS emission. Indeed, treatment of animals with the mitochondrial-targeted antioxidant SS-31 prevents the casting-
induced activity of both proteases in the soleus muscle, and prevented calpain activation in the plantaris muscle. This is significant because previous work indicates that the inhibition of calpain or caspase-3 is sufficient to prohibit casting-induced atrophy, demonstrating that these proteases play important roles in disuse atrophy (28). Finally, we report that caspase-9, a caspase that can lead to the activation of caspase-3 via a mitochondria-mediated pathway (15), is also activated in the inactive plantaris muscle by increased mitochondrial ROS emission.

Much emphasis has been placed on the role that the proteasome system plays in disuse muscle atrophy. Importantly, these experiments reveal for the first time that increases in mitochondrial ROS emission are required for casting-induced increases in the muscle-specific E3 ligases atrogin-1 and MuRF-1. Increases in these E3 ligases are commonly associated with increased FoxO3a and NF-κB signaling (11).

Recent evidence suggests that increased autophagy contributes to different forms of skeletal muscle atrophy, including atrophy induced by denervation (5, 9, 10, 17, 22). Furthermore, accumulating evidence suggests that oxidative stress plays an important role in the induction of autophagy (reviewed in Ref. 14). In this regard, our results reveal that inactivity-induced increases in autophagy signaling in the plantaris and soleus muscles can be prevented by treatment of animals with the mitochondrial-targeted antioxidant SS-31. Note that, although all four key proteolytic systems are activated by prolonged disuse, it remains unclear which proteases play a dominant role during disuse-induced limb muscle atrophy.

Work by Sandri et al. (26) demonstrated that PGC-1α overexpression is sufficient to prevent denervation-induced muscle atrophy and increases in atrogin-1, MuRF-1, and cathepsin L. Additionally, work by Brault et al. (4) has demonstrated that PGC-1α overexpression is sufficient to attenuate myotube proteolysis during starvation. Based on these observations, we hypothesized that treatment of animals with SS-31 may prevent disuse muscle atrophy by protecting the expression of PGC-1α. However, SS-31 treatment did not prevent the casting-induced decreases in PGC-1α mRNA expression, suggesting that SS-31 prevents muscle atrophy through a mechanism not involving PGC-1α.

Finally, we hypothesized that the increase in mitochondrial ROS emission from limb muscles during casting promotes a decrease in anabolic signaling (i.e., Akt/mTOR signaling), which can decrease protein synthesis and accelerate protein degradation by activation of both the proteasome and autophagy systems. Our data support this prediction and reveal that increased mitochondrial ROS plays an important role in the inactivity-induced decrease in Akt and mTOR phosphorylation. Future experiments will be required to determine whether prevention of disuse-induced decreases in anabolic signaling can prevent inactivity-mediated decreases in muscle protein synthesis.

In these experiments, we sought to discern differences in the role of mitochondrial ROS production during disuse atrophy between a highly oxidative muscle (soleus) and a glycolytic muscle (plantaris). Our results reveal numerous similarities in the inactivity-induced activation of proteases and depression of anabolic signaling markers in these two muscles. However, the explanations for muscle-specific differences in caspase activation and MuRF-1 expression are unclear, but could be linked to the fiber-type differences between these two muscles or the fact that the time course of inactivity-induced protease activation differs between these two muscles. The lack of a casting-induced decrease in pmTOR-to-mTOR ratio in the plantaris may also be due to the time course of our experiments, and is consistent with pmTOR-to-mTOR ratios following 5 days of casting in the rat gastrocnemius, a mixed muscle similar to the plantaris (13). These results represent the first comprehensive work investigating the role that increased mitochondrial ROS plays in promoting inactivity-induced skeletal muscle atrophy. By measuring important molecules involved in the regulation of both protein synthesis and protein degradation, this work complements recent findings from our laboratory (19, 23) and demonstrates many similarities in the mechanisms of protein degradation both between rat and mouse limb muscle and between limb muscle and diaphragm muscle exposed to prolonged inactivity.

Conclusions

Our findings clearly demonstrate that increased mitochondrial emission of ROS is a crucial contributor to disuse muscle atrophy. Indeed, inactivity-induced activation of all four key proteolytic systems in both the soleus and plantaris muscles was prevented by treatment with a mitochondria-targeted antioxidant (i.e., SS-31). Finally, treatment with a mitochondrial-targeted antioxidant also prevented the disuse-induced decrease in anabolic signaling (i.e., Akt/mTOR) in the casted skeletal muscles, suggesting that increased mitochondrial ROS emission acts to depress the Akt/mTOR pathway in skeletal muscles undergoing disuse-induced atrophy.

DISCLOSURES

Patent applications have been filed by Cornell Research Foundation (CRF) for the technology (SS-31) described in this article, with H. H. Szeto and S. K. Powers as inventors. CRF, on behalf of Cornell University, has licensed the technology for further research and development to a commercial enterprise (Stealth Peptides International) in which CRF and H. H. Szeto have financial interests.

In an effort to comply with the US Animal Welfare Act and Public Health Service Policy to use the absolute minimum number of animals required to meet our scientific objectives, some data from the soleus muscles of animals in the “control” and “cast” groups have previously been accepted for publication in the Journal of Applied Physiology. We believe that the use of the data did not exceed the requirements for in vivo research at the University of Florida (protocol 01-12226A).

AUTHOR CONTRIBUTIONS


REFERENCES


