Calpain-1 is required for hydrogen peroxide-induced myotube atrophy

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Submitted 1 October 2008; accepted in final form 17 December 2008

McClung JM, Judge AR, Talbert EE, Powers SK. Calpain-1 is required for hydrogen peroxide-induced myotube atrophy. Am J Physiol Cell Physiol 296: C363–C371, 2009. First published December 24, 2008; doi:10.1152/ajpcell.00497.2008.—Recent reports suggest numerous roles for cysteine proteases in the progression of skeletal muscle atrophy due to disuse or disease. Nonetheless, a specific requirement for these proteases in the progression of skeletal muscle atrophy has not been demonstrated. Therefore, this investigation determined whether calpains or caspase-3 is required for oxidant-induced C2C12 myotube atrophy. We demonstrate that exposure to hydrogen peroxide (25 μM H2O2) induces myotube oxidative damage and atrophy, with no evidence of cell death. Twenty-four-hour exposure to H2O2 significantly reduced both myotube diameter and the abundance of numerous proteins, including myosin (–81%), α-actinin (–40%), desmin (–79%), talin (–37%), and troponin I (–80%). Myotube atrophy was also characterized by increased cleavage of the cysteine protease substrate αH-spectrin following 4 h and 24 h of H2O2 treatment. This degradation was blocked by administration of the protease inhibitor leupeptin (10 μM). Using small interfering RNA transfection of mature myotubes against the specific proteases calpain-1, calpain-2, and caspase-3, we demonstrated that calpain-1 is required for H2O2-induced myotube atrophy. Collectively, our data provide the first evidence for an absolute requirement for calpain-1 in the development of skeletal muscle myotube atrophy in response to oxidant-induced cellular stress.

skeletal muscle; protease; oxidative stress

SKELETAL MUSCLE ATROPHY OCCURS AS A CONSEQUENCE OF NUMEROUS PATHOLOGICAL CONDITIONS, including cachexia and disuse-related events (reviewed in Refs. 27 and 34). Numerous cellular events contribute to muscle atrophy, including those regulating protein synthesis, protease activation, ubiquitin conjugation, and autophagy (9, 17, 30, 31, 37, 39, 40). In recent years, progress has been made toward understanding the contributions of both ubiquitin ligase/proteasomal and lysosomal/autophagic-mediated skeletal muscle degradation processes. Although it has been argued that activation of the ubiquitin-proteasome system may play a dominant role in skeletal muscle atrophy (4, 21, 34), the role of cellular proteases in skeletal muscle atrophy is not well defined. In fact, new evidence suggests that a coordinated network of degradative processes is responsible for muscle atrophy (40).

Myofilament proteins make up the majority of protein in skeletal muscle cells, and the proteasome is important for the degradation of these proteins during disuse (reviewed in Ref. 4). Evidence reveals that the ubiquitin-proteasome system cannot degrade intact myofilibrill proteins, indicating that myofilament release is an initial and required step for proteasome-mediated degradation of myofilibrill proteins (4, 7, 20, 26, 27, 34). The inhibition of cysteine proteases (i.e., calpain and caspase-3) in skeletal muscle attenuates disuse muscle atrophy (16, 19, 38). We and others have hypothesized that disturbances in redox balance are critical regulatory mechanisms for the activation of both the Ca2+-dependent calpains I (μ- and II (m-) and caspase-3 during atrophy and these proteases are responsible for myofilament release during the initial stages of muscle atrophy (26, 27, 32).

Oxidative stress has been linked to skeletal muscle atrophy in numerous models of muscle wasting, including inactivity (27), diabetes (18), cancer (12), heart failure (13), chronic alcohol intake (25), human immunodeficiency virus infection (24), and sepsis (23). Although oxidative stress can activate cysteine proteases, the specific role that calpains and caspase-3 play in oxidant-induced muscle atrophy remains unclear. Therefore, this study investigated whether activation of cysteine proteases is a requirement for oxidant-induced skeletal muscle atrophy. Specifically, using both a pharmacological approach and RNA interference technology, we determined whether activation of calpain-1, calpain-2, or caspase-3 is required for hydrogen peroxide (H2O2)-induced skeletal muscle C2C12 myotube atrophy. We hypothesized that these cysteine proteases are required for H2O2-induced myotube atrophy in vitro. Our findings demonstrate a specific role for calpain-1 in H2O2-induced skeletal muscle atrophy in this model.

METHODS

Cultured myogenic cell line. Myoblasts derived from mouse skeletal muscle (C2C12 cells; American Type Culture Collection, Rockville, MD) were cultured on six-well dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% newborn calf serum, 1% penicillin/streptomycin, and 0.1% fungizone at 37°C in the presence of 5% CO2 until 80% confluence was reached as visualized by light microscopy. Myotube differentiation was then initiated by replacement of the growth medium with differentiation medium: DMEM supplemented with 2% heat-inactivated horse serum, 1% penicillin/streptomycin, and 0.1% fungizone at 37°C in the presence of 5% CO2, for 7 days. The muscle cells were examined for evidence of myotube formation and growth by light microscopy. H2O2 treatment of C2C12 cells involved dilution in differentiation media to final desired concentrations and standard incubation (37°C in the presence of 5% CO2). H2O2-treated media were replaced at 12-h intervals for incubation times greater than 12 h.

Cell viability. H2O2-induced alterations in C2C12 viability were analyzed via Trypan blue exclusion assay as previously described (36). Briefly, fully differentiated cells were treated with varying concentrations of H2O2 (0 μM control, 25 μM, 50 μM, 100 μM, and 200 μM) for predetermined durations (1 h, 2 h, 4 h, 24 h, 48 h, and 72 h). Media were removed, and the cells were incubated; 0.25% Trypan blue (Sigma) in PBS was applied to cultures for 5 min, and

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cultures were then rinsed twice with PBS. As a positive control, some untreated cultures were exposed to 80% ethanol in PBS for 30 min before application of Trypan blue. Images were obtained via an inverted microscope (Carl Zeiss Axiovert 200) camera system at ×10 magnification. Approximately 150 myotubes per treatment, a number chosen by determination of no additional change in standard deviation, were counted for the inclusion/exclusion of dye at each concentration and duration by a blinded investigator, and the percentage of viable cells (excluding dye) was determined.

**Transient transfections.** Differentiated myotubes were transiently transfected in six-well plates with small interfering RNA (siRNA) sequences for cysteine proteases. At least three validated siRNA sequences (Ambion) were evaluated for knockdown efficacy for each cysteine protease. Efficiency of knockdown was deemed adequate when mRNA abundance for each protease was reduced by ~70%. RNA oligos were transfected into differentiated myotubes using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Briefly, siRNA and Lipofectamine 2000 were separately diluted in Opti-MEM (Gibco). The diluted Lipofectamine 2000 reagent was incubated for 5 min and was then added to the siRNA mixture at a final volume of 500 μl/well. The lipid/siRNA mixtures were allowed to complex for 20 min during which the cells were rinsed twice with Opti-MEM and bathed in a final volume of 2 ml Opti-MEM/well. Lipid/siRNA complexes were then applied to the cells (final transfection volume of 2.5 ml). Separate experiments were performed using equivalent amounts of lipid reagent devoid of siRNA. Cultures were rocked every 2 h for the initial 6 h of incubation and were incubated with the transfection mixture for a total of 24 h. After 24 h, transfection medium was removed and replaced with differentiation medium. Parallel cultures were transfected with fluorescein (FAM)-labeled nonsilencing control and calpain-1 siRNA oligomers (Ambion) and were imaged on an inverted fluorescence microscope (Carl Zeiss Axiovert 200) camera system at ×10 magnification to determine transfection efficiency. Cultures were maintained for an additional 24 h before H2O2 treatment.

**Oxidative stress.** 4-Hydroxynonenal (trans-4-hydroxy-2-nonenal; 4-HNE, C9H16O2) is a α,β-unsaturated hydroxyalkenal that is produced by lipid peroxidation in cells (35). 4-HNE modification of proteins was analyzed as an indicator of oxidative damage in C2C12 cells via Western blotting as described (1). Values for 4-HNE were corrected for duration-matched untreated controls (0 μM H2O2) and are presented as fold changes.

![Fig. 1. Cell viability and oxidative stress in C2C12 myotubes treated with H2O2.](http://ajpcell.physiology.org/)
**Myotube atrophy.** Myotube diameter morphological analysis was performed as previously described (22) with the following modifications. Briefly, images of cultures were obtained via phase contrast at ×100 magnification after the appropriate treatment durations were obtained on an inverted microscope (Carl Zeiss Axiovert 200) camera system at ×10 magnification. The diameters were measured in a total of 100 myotubes from at least 10 random fields, a number chosen by determination of no additional change in standard deviation, using computerized image analysis (Scion Image, Frederick, MD). Each myotube analyzed was measured at three points along the length of the myotube in a blinded fashion, and results are expressed as percentage of the control treatment diameter.

**Western blot analysis.** On completion of the appropriate incubation time, cells were rinsed 2× in ice-cold 1× PBS and scraped for protein isolation in 130 μl nondenaturing lysis buffer (NDL buffer; 1% Triton X-100, 300 mM NaCl, 50 mM Tris-base, 3.1 mM sodium azide, 95 mM NaF, 22 μM Na3VO4), vortexed, incubated at 4°C for 25 min, and centrifuged at 1,000 g for 5 min. The supernatants were subsequently assayed for protein using the Bradford method (Sigma, St. Louis, MO), separated by polyacrylamide gel electrophoresis via 4–15% gradient, and transferred to nitrocellulose membranes (100 V for 3 h at 4°C) for Western blot analysis. As verification of equal loading and transfer, the resulting transfer membrane was stained with Pierce ECL reagents (Thermo Scientific, Rockford, IL), and exposed to the film for 1 min.

**RNA isolation and cDNA synthesis.** Cells harvested for RNA isolation were rinsed twice in ice-cold 1× PBS and scraped in TRIzol reagent (Life Technologies, Foster City, CA). Total RNA (5 μg) was then reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies) using oligo(dT)20 primers and the manufacturer’s instructions.

**Real-time polymerase chain reaction.** One microliter of cDNA was added to a 25-μl PCR reaction for real-time PCR using Taqman chemistry and the ABI Prism 7000 Sequence Detection System (ABI, Foster City, CA). Relative quantitation of gene expression in treated

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<tr>
<th>Duration of 25 μM H2O2 Treatment</th>
<th>Myotube Diameter (% Control)</th>
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<tbody>
<tr>
<td>Con</td>
<td>100</td>
</tr>
<tr>
<td>4 h</td>
<td>55 ± 0.05*</td>
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<tr>
<td>24 h</td>
<td>60 ± 0.05*</td>
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**Table 1. Sarcomeric protein abundance**

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<th>Myotube Diameter (% Control)</th>
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<tr>
<td>100</td>
</tr>
<tr>
<td>55 ± 0.05*</td>
</tr>
<tr>
<td>60 ± 0.05*</td>
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</table>

Values are means ± SE and are representative of 6 independent experiments. Sarcomeric protein abundance decreases in C2C12 myotubes with 25 μM H2O2 treatment. Protein expression was verified by Western blot analysis.

Significantly different (P < 0.05) from control (Con).

*Significantly different (P < 0.05) from 4-h treatment duration.
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and calpastatin (GenBank NM_009817.3) mRNA transcripts were assayed using presdesigned rat primer and probe sequences commercially available from Applied Biosystems (Assays-on-Demand).

**Statistical analysis.** A priori analysis was used to determine any differences between control treatments of varying durations. When no differences existed between groups, respective groups were pooled. Comparisons between treatments were made by a one-way analysis of variance and, when appropriate, a Tukey honestly significant difference test was performed post hoc. Significance was established at $P < 0.05$. Values are reported as means ± SE.

**RESULTS**

*Cell viability and oxidative stress.* The toxicity of $H_2O_2$ treatment to differentiated C2C12 myotubes was determined using the Trypan blue exclusion assay in an attempt to determine the appropriate concentration of peroxide and duration of administration for our experiments. Only $H_2O_2$ concentrations of 25 μM or 50 μM failed to induce myotube viability loss at all durations of exposure (1 h, 2 h, 4 h, 24 h, 48 h, or 72 h; Fig. 1A). We then tested these nontoxic $H_2O_2$ concentrations to determine whether they were sufficient to induce myotube oxidative damage. $H_2O_2$ concentration of 25 μM or 50 μM, regardless of the duration of administration (4 h, 24 h, 48 h, or 72 h), resulted in increased 4-HNE modification of myotube proteins (Fig. 1B and Supplemental Fig. S1A; supplemental material for this article is available online at the *American Journal of Physiology: Cell Physiology* website).

*Antioxidant enzyme expression.* We also analyzed the protein expression of catalase, CuZnSOD, and MnSOD to determine whether the administration of 25 μM $H_2O_2$ alters the baseline abundance of endogenous myotube antioxidants (Supplemental Fig. S1B). There were no alterations in the abundance of any antioxidant enzyme analyzed after 24 h of 25 μM $H_2O_2$ treatment. After extended periods of exposure, however, CuZnSOD optical density (OD) (48 h: 1.88 ± 0.11 OD; 72 h: 4.24 ± 0.59 OD) and MnSOD (72 h: 1.51 ± 0.10 OD) protein abundances increased from control values (1.0 ± 0.07 OD and 1.0 ± 0.05 OD, respectively).

*$H_2O_2$-induced myotube atrophy.* Myotube diameter was analyzed as an indicator of myotube atrophy in response to 25 μM $H_2O_2$ treatment. Myotube diameter was not altered from control values following 4 h of peroxide treatment (91.78 ± 7.9% of control). Myotube diameter decreased 31–37% with 25 μM $H_2O_2$ treatment during 24, 48, or 72 h. This finding demonstrated 2 important points: 1) that this lower dose of $H_2O_2$ treatment was sufficient to induce myotube atrophy and 2) that the 24-h duration of exposure resulted in roughly the same level of myotube atrophy as the 72 h.

*Sarcomeric protein expression.* Four hours of 25 μM $H_2O_2$ exposure resulted in decreases in myosin (−22%), α-actinin (−23%), and desmin (−80%) protein expressions (Table 1 and Supplemental Fig. S1C). Interestingly, 4 h of exposure also increased (101%) the protein expression of troponin I. Twenty-four hours of 25 μM $H_2O_2$ exposure resulted in further decreases in myosin and desmin, and decreases in talin (−37%), troponin I (−80%), and αII-spectrin (−29%) proteins. Actin protein expression was not altered by $H_2O_2$ exposure at any time-point analyzed. Overall, these data suggest that $H_2O_2$ exposure differentially alters the expression of numerous sarcomeric proteins in atrophying myotubes.

**Cysteine protease requirement for $H_2O_2$-induced myotube atrophy.** A key role for calpain and caspase cysteine proteases in the regulation of skeletal muscle size is their cleavage of sarcomeric protein substrates (including αII-spectrin), resulting in the disorganization of normal sarcomeric architecture (4, 29). The percentage of total αII-spectrin protein cleaved by proteases with 4 h or 24 h of 25 μM $H_2O_2$ treatment increased (Fig. 2A), indicating the amount of relative alterations in cleaved αII-spectrin and total αII-spectrin with $H_2O_2$ treatment. Furthermore, addition of the nonspecific protease inhibitor leupeptin to the differentiation medium attenuated myotube diameter loss associated with 24 h of 25 μM $H_2O_2$ treatment (Fig. 2B).

To determine the specific cysteine protease (i.e., calpain or caspase-3) required for the development of myotube atrophy, we tested the efficiency and specificity of calpain-1, calpain-2, and caspase-3 RNA interference (Table 2). After the establishment of specific and efficient knockdown of only the mRNA abundances and protein expressions of the targeted proteases, myotubes were transfected with each siRNA individually, subsequently treated with 25 μM $H_2O_2$ for 24 h, and diameter measurements were obtained (Fig. 3A). Calpain-1 knockdown protected C2C12 myotubes from diameter loss during peroxide treatment (Fig. 3B). Calpain-2 or caspase-3 interference proved ineffective in protecting the myotubes from atrophy (Fig. 3B).

The mRNA abundance for calpain-1 is transiently increased with 4 h of $H_2O_2$ treatment and returned to baseline with 12 h and 24 h of $H_2O_2$ treatment (Fig. 4A). Transfection with siRNA reduces mRNA abundance of calpain-1 independent of $H_2O_2$ administration. The mRNA abundance of the endogenous calpain inhibitor calpastatin increased only after 24 h of $H_2O_2$ treatment (Fig. 4B). Further analysis of the calpain-1 knockdown demonstrated a significant reduction in the early (4 h) autoproteolytic cleavage and activation of calpain protein (Fig. 5, A and B) and cleavage of αII-spectrin (Fig. 5, A and C). Neither calpain-2 nor caspase-3 interference reduced the $H_2O_2$ cleavage of αII-spectrin.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Calp-1 siRNA</th>
<th>Calp-2 siRNA</th>
<th>Casp-3 siRNA</th>
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<tbody>
<tr>
<td>Protein expression</td>
<td>0.31±0.04*</td>
<td>1.19±0.15</td>
<td>1.19±0.15</td>
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<tr>
<td>Calpain-1</td>
<td>0.93±0.05</td>
<td>0.41±0.05*</td>
<td>0.89±0.06</td>
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<tr>
<td>Calpain-2</td>
<td>0.78±0.10</td>
<td>0.84±0.11</td>
<td>0.38±0.05*</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>0.29±0.10*</td>
<td>0.83±0.26</td>
<td>1.23±0.13</td>
</tr>
<tr>
<td>mRNA abundance</td>
<td>1.07±0.10</td>
<td>0.31±0.07*</td>
<td>0.81±0.17</td>
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<tr>
<td>Calpain-1</td>
<td>1.33±0.16</td>
<td>1.21±0.29</td>
<td>0.28±0.04*</td>
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</table>

Values are means ± SE and are representative of 6 independent experiments. Efficiency and specificity of protease small interfering (si)RNA in C2C12 myotubes. C2C12 cells were allowed to differentiate for 4 days and were transfected with siRNA against calpain-1, calpain-2, or caspase-3. Cells were then treated and harvested for protein or RNA. Protein abundance was verified by Western blot analysis, and values are presented as fold induction from control siRNA-treated cells. mRNA expression was verified by real-time RT-PCR, and values are presented as corrected for hprt and are normalized to control siRNA-treated cells. *Significantly different (P < 0.05) from respective control cells.
DISCUSSION

Overview of principle findings. Several new and important findings emerged from these experiments. First, these results demonstrate that exposure of C2C12 myotubes to low levels of H$_2$O$_2$ is sufficient to induce myotube oxidative stress and atrophy within 24 h. This H$_2$O$_2$-induced atrophy is characterized by an early and sustained loss of numerous sarcomeric proteins and cleavage of the structural myofilament protein, αII-spectrin. Second, activation of the cysteine protease calpain-1 is a requirement for H$_2$O$_2$-induced atrophy. A brief discussion of these key results follows.

Requirement for calpain-1 in H$_2$O$_2$-induced myotube atrophy. The most important finding from the current study is that the knockdown of calpain-1 attenuated H$_2$O$_2$-induced myotube atrophy. This suggests a direct requirement for calpain-1 in the progression of H$_2$O$_2$-induced myotube atrophy. The magnitude of protease knockdown in the current study was not sufficient to completely abrogate calpain mRNA abundance or protein expression. It is possible that residual levels of protease may continue to mediate the normal response to physiologically relevant stimuli, resulting in the attenuation but not complete prevention of atrophy or substrate cleavage. Our data clearly demonstrate that this level of interference was sufficient to protect the myotubes from atrophy and the cleavage of protease substrate proteins. The transfection efficiency observed in the current study combined with the large number of myotubes and sample sizes used for the analysis of diameter and biochemistries, however, suggest that residual physiologically activated calpains are insufficient to overcome the magnitude of protective effects of the protease knockdown achieved. The C2C12 myotubes used in the present study were analyzed after a treatment period encompassing a total of 7 days of differentiation, allowing for sufficient time for mature myotube formation. However, residual calpain may also be localized to nondifferentiated myoblasts or in nontransfected myotubes in culture, providing another potential mechanism for continued protease activation, mRNA abundance, and protein expression by analysis.
To date, the mechanistic link between oxidants and activation of calpain-1 remains unclear. A potential mechanism for calpain activation by reactive oxygen species (ROS) in skeletal muscle involves the formation of reactive aldehydes that damage calcium handling proteins and increase cytosolic Ca\(^{2+}\) levels. Our finding that exposure of myotubes to H\(_2\)O\(_2\) increases cellular levels of the reactive aldehyde (4-HNE) is consistent with this notion. Regardless of the mechanism of how H\(_2\)O\(_2\) activates calpain in myotubes, our data suggest that calpain-1 activity participates in myofilament protein release in atrophying muscles. Finally, while active calpain-1 participates in myofilament release, it is also possible that calpains could contribute to skeletal muscle atrophy in other ways as well. For example, additional reported functions of calpain proteases in skeletal muscle include transcription factor degradation and modification of protein kinase B (PKB/Akt) activity (reviewed in Ref. 34).

H\(_2\)O\(_2\)-induced muscle atrophy. Oxidants such as H\(_2\)O\(_2\) are theorized to play important roles in the progression of skeletal muscle atrophy due to inactivity or various diseases. In fact, antioxidant administration attenuates myofiber atrophy in both disease and disuse models (20, 25, 32). Hydrogen peroxide is often used as an exogenous oxidant treatment to promote oxidative stress and proteolytic signaling pathways in both isolated skeletal muscles (6, 28) and in vitro myotubes (14, 15). Although the ability of H\(_2\)O\(_2\) to induce skeletal muscle myofiber atrophy has been inferred in several studies, the current study is the first to demonstrate a direct link between H\(_2\)O\(_2\) and myotube atrophy. Moreover, our results also extend previous work (15) by demonstrating that proteases involved in the ubiquitin-proteasome proteolytic cascade are activated and transcriptionally induced by the treatment of C2C12 myotubes with H\(_2\)O\(_2\). Note that we cannot attribute the effects of the H\(_2\)O\(_2\) treatment solely to the biochemical properties of this oxidant. Indeed, it is possible that H\(_2\)O\(_2\) could react with transition metals and/or superoxide in the cell to produce more reactive species such as the hydroxyl radical. Hence, the specific oxidant(s) directly responsible for our results remain unknown.

Recently, the classical dogma on oxidants as cellular disruptors of homeostasis by overwhelming endogenous antioxidant systems has undergone revision. A role for ROS in compartmentalized cellular signaling regulating cellular functions has been introduced (8, 10). Specifically, oxidants have been recognized as modifiers of cellular signal transduction, phosphatase and protease activity, chaperone proteins, and transcription factors (reviewed in Ref. 8). H\(_2\)O\(_2\) is receiving amplified attention as a cellular signaling molecule due to its ability to reversibly oxidize amino acids, specifically cysteines (8), resulting in posttranslational protein modifications that affect cellular function. A continuum of redox-based cellular modifications, proposed by Forrester and Stamler (3), suggests that reversible posttranslational modifications of cysteine thiol side chains (S-nitrosothiol, sulfenic acid, and disulfides) are demonstrative of physiological signaling modifications initiated by oxidants. In contrast, irreversible modifications (protein carbonyl and methionine sulfoxide formation) are maladaptive and represent oxidative stress-induced cellular injury. In this context, the low dose of H\(_2\)O\(_2\) used in the current study was insufficient to induce cell death but resulted in the induction of endogenous antioxidant (CuZnSOD and MnSOD) protein abundance, development of oxidative stress (4-HNE modification of proteins), and atrophy. It remains plausible, therefore, that H\(_2\)O\(_2\) functions in multiple stages along the redox contin-
Fig. 5. Calpain-1 interference inhibits H$_2$O$_2$-induced protease activity. Fully differentiated C2C12 myotubes were transfected with control (mismatched) or calpain-1 siRNA and subsequently treated with either 0 μM vehicle (control) or 25 μM H$_2$O$_2$ for 4 h. Cellular lysates were collected and analyzed via Western blot for the sarcomeric protease substrate αII-spectrin as well as cleaved (activated) calpain-1.

A: representative blot images of both total and cleaved calpain-1 and αII-spectrin. B: fold induction of autoproteolytically cleaved calpain-1 protein. Values are representative of 6 independent experiments, corrected for Ponceau-stained total protein within lane, normalized to control values, and presented as means ± SE. C: % αII-spectrin cleavage. Values are representative of 6 independent experiments, corrected for Ponceau-stained total protein within lane, and presented as the %cleaved αII-spectrin protein, means ± SE. *Significantly (P < 0.05) different from control. †Significantly (P < 0.05) different from 25 μM H$_2$O$_2$. 

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uum, first as a signaling intermediate to initiate protein modifications and cellular signaling and later as promoter of oxidative injury. At present, the relative contributions of the overlapping phases of this redox continuum to the progression of skeletal muscle atrophy remains unknown.

Cysteine protease-mediated muscle proteolysis. The involvement of cysteine proteases in skeletal muscle atrophy has been implicated in reports suggesting that inhibition of calpain or caspase-3 retards both disuse and sepsis-induced muscle atrophy (2, 16, 19, 38). We and others have postulated that myofilament protein release may be a rate-limiting step in myofilament protein degradation by the ubiquitin-proteasome system in atrophying skeletal muscle (7, 11, 21, 34). The current study further corroborates this idea by demonstrating that protease activation and myofilament substrate cleavage occur early during the development of atrophy in myotubes. In direct contrast to this theory, Zhao et al. (40) treated cells with both lysosomal and proteasomal inhibitors and demonstrated a 90% reduction in overall proteolysis. These investigators failed to inhibit overall muscle proteolysis with specific pharmacological inhibitors of caspases and concluded that caspases and/or calpains play a minor role in overall cellular proteolysis. The current findings corroborate a negligible role for caspase-3 in skeletal muscle atrophy due to H2O2 treatment. However, our results clearly demonstrate an important role for calpain-1 in oxidant-induced myotube atrophy. We appreciate that inhibition of the proteasome (4) would attenuate a portion of skeletal muscle proteolysis in response to atrophic stimuli. Nonetheless, since the proteasome cannot degrade intact myofilaments, it is feasible that proteolytic release of these proteins remains an initial and required step for the subsequent degrada-
tion of myofilament proteins via the proteasome proteolytic system. In support of this postulate, a recent report indicates that calpain inhibition can retard muscle proteolysis independent of key ubiquitin ligases deemed critical to the proteasome system (2). Therefore, we conclude that an important role for calpains in skeletal muscle atrophy remains.

Calpains are classically defined as calcium-activated proteases found in most cell types (5). Despite the fact that H2O2-induced atrophy and cellular oxidative stress result in calpain activation and myotube atrophy, a direct link between calcium release and oxidative stress has not been definitively proven in the current study. A possible link between cellular oxidative stress and cellular calcium overload involves the oxidant-induced production of reactive aldehydes (4-HNE) that inhibit plasma membrane Ca2+-ATPase activity and attenuate the ability of the cell to remove cytosolic Ca2+ (33). Our study demonstrates that the formation of reactive aldehydes is an initial event in response to the stress provided by H2O2 treatment of myotubes in vitro. This may provide insight into a possible mechanism for Ca2+-induced calpain activation in the current model. Future studies are needed to examine the role of oxidant stress in Ca2+-induced calpain activation and should investigate stress-induced alterations in Ca2+-ATPase membrane channel function or structure as a potential mechanism.

Conclusions. Our study revealed that the development of hydrogen peroxide-induced myotube atrophy in culture requires calpain-1, but not calpain-2 or caspase-3 cysteine proteases. The exact role for calpain-1 activity in atrophying myotubes is not entirely clear, but it may be due in part to its cleavage of structural myofilament proteins in response to hydrogen peroxide treatment. Although a requirement for calpain-1 for all types of muscle atrophy has not been established, our discovery that calpain-1 is required for H2O2-induced atrophy could lead to refined pharmacological or genetic therapies targeted at the maintenance of protein balance in skeletal muscle during wasting conditions involving oxidative stress.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant RO1-HL-072789 (to S. K. Powers).

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