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Oxidation enhances myofibrillar protein degradation via calpain and caspase-3

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Abstract

Oxidative stress has been linked to accelerated rates of proteolysis and muscle fiber atrophy during periods of prolonged skeletal muscle inactivity. However, the mechanism(s) that link oxidative stress to muscle protein degradation remain unclear. A potential connection between oxidants and accelerated proteolysis in muscle fibers is that oxidative modification of myofibrillar proteins may enhance their susceptibility to proteolytic processing. In this regard, it is established that protein oxidation promotes protein recognition and degradation by the 20S proteasome. However, it is unknown if oxidation of myofibrillar proteins increases their recognition and degradation by calpain (s) and/or caspase-3. Therefore, we tested the hypothesis that oxidative modification of myofibrillar proteins increases their susceptibility to degradation by both calpain and caspase-3. To test this postulate, myofibrillar proteins were isolated from rat skeletal muscle and exposed to *in vitro* oxidation to produce varying levels of protein modification. Modified proteins were then independently incubated with active calpain I, calpain II, or caspase-3 and the rates of protein degradation were assessed via peptide mapping. Our results reveal that increased protein oxidation results in a stepwise escalation in the degradation of myofibrillar proteins by calpain I, calpain II, and caspase-3. These findings provide a mechanistic link to connect oxidative stress with accelerated myofibrillar proteolysis during disuse muscle atrophy.

Keywords

disuse muscle atrophy; calpain; caspase-3; protein oxidation; proteolysis; oxidative stress; reactive oxygen species

INTRODUCTION

Prolonged periods of skeletal muscle disuse result in muscle fiber atrophy. Indeed, extended bed rest, limb immobilization, spaceflight, and mechanical ventilation are all conditions that promote skeletal muscle wasting (reviewed in [1]). Disuse muscle atrophy results in functional, morphological, and biochemical changes in muscles that can impair activities of daily living and therefore, understanding the mechanisms that contribute to disuse muscle atrophy is important.

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Abundant evidence indicates that disuse skeletal muscle atrophy occurs due to both a decrease in protein synthesis and an increase in protein degradation (reviewed in [1]). However, proteolysis is the predominant factor responsible for disuse skeletal muscle atrophy and myofibrillar proteins are lost at a rate faster than other muscle proteins [2]. The degradation of myofibrillar protein is a multistep process that requires the cooperation of several proteolytic components including the calpain, caspase-3, and ubiquitin-proteasome proteolytic systems [1–4].

All forms of disuse muscle atrophy are associated with increased oxidant production and oxidative stress accelerates the rate of skeletal muscle protein degradation [5]. Theoretically, inactivity-induced oxidative damage in diaphragm muscle can occur due to the interaction of several major oxidant-producing pathways. For example, xanthine oxidase production of superoxide, NADPH oxidase-mediated production of superoxide, and mitochondrial production of superoxide can all contribute to oxidative damage in muscle during prolonged periods of inactivity [5–8]. At present, it is unclear if the source of oxidant production in inactive skeletal muscle differs between the different models of disuse muscle atrophy (e.g., prolonged bed rest vs. immobilization).

Although it is clear that oxidative stress contributes to disuse muscle atrophy [5], the mechanism(s) by which oxidative stress promotes proteolysis remains uncertain. A potential means by which oxidative stress increases proteolysis is that oxidative modification of muscle proteins increases their susceptibility to proteolytic degradation. In this regard, prior research reveals that oxidation can enhance substrate recognition for several cellular proteases [9,10]. Nonetheless, the impact of skeletal muscle protein oxidation on substrate recognition and degradation by calpain and caspase-3 remains unknown. Therefore, these experiments were undertaken to determine if oxidatively modified myofibrillar proteins are more susceptible to degradation by calpains and caspase-3. Guided by our preliminary experiments, we hypothesized that oxidative modification of myofibrillar proteins will increase their vulnerability to degradation by both calpains and caspase-3.

METHODS

Animals

Tissues from young adult (six months old) female Sprague-Dawley (SD) rats were used in these experiments. All animals were housed at the University of Florida Animal Care Services Center and the Animal Care and Use Committee of the University of Florida approved these experiments.

Experimental Design

These experiments tested the hypothesis that oxidative modification of myofibrillar proteins increases their susceptibility to degradation by both calpain and caspase-3. To test this postulate, we isolated myofibrillar proteins from diaphragm muscle of rats and exposed these proteins to *in vitro* oxidation to produce three distinct levels of protein modification. Oxidized proteins were then independently incubated with active calpain I, calpain II, or caspase-3 (calbiochem) and the rates of protein degradation were assessed via peptide mapping. Specifically, isolated myofibrillar protein samples were divided into five treatment groups: 1) control (CON) with no protease treatment, 2) CON group with protease treatment, 3) Low oxidation with protease treatment, 4) Moderate oxidation with protease treatment, and 5) High oxidation with protease treatment (Figure 1).

Finally, to determine if our *in vitro* oxidation of myofibrillar proteins results in similar levels of *in vivo* protein oxidation that occurs in myofibrillar proteins from skeletal muscle undergoing

disuse atrophy, we performed the following experiment using mechanical ventilation-induced atrophy of the diaphragm which is a clinically relevant form of disuse muscle atrophy. Briefly, although mechanical ventilation is a life-saving intervention in patients suffering from respiratory failure, controlled mechanical ventilation results in diaphragmatic inactivity and a rapid onset of diaphragmatic oxidative stress and atrophy. Therefore, using a rat model of mechanical ventilation, we isolated myofibrillar proteins from diaphragm muscle of control animals, animals ventilated for 18 hours, and animals ventilated for 18 hours treated with the antioxidant Trolox. We then isolated myofibrillar proteins from the diaphragm and determined the levels of protein carbonyl formation using a western blot approach. Note that the experimental design describing our experimental model of mechanical ventilation and the antioxidant treatment of these animals has been previously described (22).

Biochemical Analyses

Isolation of myofibrillar protein—Insoluble (i.e., myofibrillar proteins) from rat diaphragm muscle were used in these experiments. Diaphragm muscle was chosen for these experiments for several reasons. First, the diaphragm is a mixed fiber skeletal muscle that contains all four of the muscle fiber types found in the adult rat [11]. Second, it is well established that diaphragmatic inactivity, due to prolonged mechanical ventilation, results in oxidative modification of diaphragmatic proteins and accelerated proteolysis [12,13]. Therefore, it is of interest to determine if oxidative modification of diaphragm muscle proteins accelerates substrate recognition and increases protein degradation via calpains and caspase-3.

Animals (n=8) were acutely anesthetized with sodium pentobarbital (60 mg/kg IP). After reaching a surgical plane of anesthesia, the costal diaphragm was removed and immediately frozen in liquid nitrogen and stored at -80°C for subsequent isolation of myofibrillar protein. Myofibrillar protein samples were then prepared based on the method of Reid et al. [14]. Briefly, muscle samples were first homogenized in a buffer containing 0.039 M sodium borate (pH 7.1), 0.025 M KCl, 5 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and a protease inhibitor cocktail (Sigma). The homogenate was then centrifuged at 4°C for 12 minutes at 1500g. After centrifugation, the supernatant was discarded and the pellet was resuspended and homogenized again. The second homogenization buffer contained: 100 mM KCl and 1.0% Triton X-100. This process was repeated twice. After the final centrifugation, the final pellet was obtained and resuspended in 0.4 M KCl, 50 mM Tris (hydroxymethyl)-aminomethane (Tris) (pH 7.4) and 1.0 mM dithiothreitol (DTT). Protein concentration was determined using the Bradford technique [15].

In vitro oxidation of myofibrillar protein—Following myofibrillar isolation, protein samples (n=8) were randomly divided into four groups. Proteins in group one were not exposed to oxidizing conditions and served as the control group (i.e., basal level of protein oxidation). The remaining three samples were divided into separate groups and were exposed to varying levels of hydrogen peroxide (H_2O_2) and iron (Fe^{2+}) to generate three differing levels of hydroxyl radical ($\cdot\text{OH}$) production and therefore, three distinct levels of protein modification: 1) Low oxidation (25 μM H_2O_2 and 10 μM Fe^{2+}); 2) Moderate oxidation (25 μM H_2O_2 and 25 μM Fe^{2+}); and 3) High oxidation (25 μM H_2O_2 and 50 μM Fe^{2+}). These concentrations of H_2O_2 and Fe^{2+} were chosen to generate differing levels of protein oxidation and to mimic the levels of *in vivo* myofibrillar protein oxidation observed in the diaphragm of animals exposed to prolonged mechanical ventilation [12]. Each protein oxidation treatment was performed at 37°C for 15 minutes. At the completion of this oxidation period, proteins were quickly removed from the oxidizing medium and placed on ice to inhibit further oxidation.

Assessment of protein oxidation via reactive carbonyl derivatives—The levels of reactive carbonyl derivatives in the myofibrillar protein samples were assessed as an index of

the magnitude of protein modification. This was accomplished using the Oxyblot Oxidized Protein Detection Kit from Chemicon International (Temecula, Ca) as described by the manufacturer. Briefly, the carbonyl groups in the protein side chains of myofibrillar proteins were derivatized to 2, 4-dinitrophenylhydrazine (DNP). These DNP derivatized proteins were then separated via polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with primary antibody against DNP to detect the presence of myofibrillar oxidation in the samples as described previously [12]. The levels of reactive carbonyl derivatives of different molecular weight proteins were determined via computerized image analysis by comparing the signal intensity of each lane.

In vitro proteolysis—To evaluate the impact of protein oxidation on the rates of myofibrillar protein breakdown via calpains and caspase-3, we incubated both control and oxidized myofibrillar protein samples with known quantities of purified and active calpains and caspase-3. Specifically, 20 µg myofibrillar protein was placed in a reaction medium containing 2 µl of either active calpain I, calpain II, or caspase-3. Two µl of 50 µM Ca^{2+} was then added to the medium and samples were incubated at 37°C for 30 minutes. At the completion of this incubation period, proteolytic activity was immediately stopped by placing the samples on ice, followed by the addition of laemmli buffer.

Peptide-mass mapping via gel electrophoresis—Peptide-mass mapping was used to investigate the rate of proteolysis and the protein fragmentation pattern generated by digestion of myofibrillar proteins by calpain I, calpain II, and caspase-3. Samples were separated via polyacrylamide gel electrophoresis (4–20% gradient gel containing 0.1% SDS) and then stained with Coomassie Blue. The protein band size and cleavage products identified on the gels were analyzed using computerized image analysis to determine the percent degradation compared to control. Following visual inspection of all gels, four prominent protein bands exhibited marked degradation by all three proteases and these bands were chosen for analysis; the molecular weights of these proteins were approximately 200, 100, 40 and 25 kDa.

Western blot analysis—To determine the primary identity of the myofibrillar proteins (i.e., 200, 100, 40 and 25 kDa) selected for study, membranes containing the insoluble proteins were probed with monoclonal antibodies specific for rat skeletal muscle myosin heavy chains, actin, troponin I, and α -actinin. The myofibrillar proteins myosin heavy chain, actin, troponin I, and α -actinin were selected for study because these proteins correspond to the approximate molecular weights of the four prominent protein bands that were degraded by these proteases. Myosin heavy chain was probed using 1:1000 dilution of a monoclonal antibody obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa Department of Biological Sciences. Actin was incubated with a 1:400 dilution of primary polyclonal antibody (Santa Cruz Biotechnology). Troponin I was incubated with a 1:1000 dilution of polyclonal antibody (Santa Cruz Biotechnology), and α -actinin was incubated with a 1:500 dilution of primary polyclonal antibody (Santa Cruz Biotechnology). Following incubation, membranes were washed with PBS-Tween and either sheep anti-mouse (myosin) or donkey anti-rabbit (actin, troponin I and α -actinin) IgG horseradish peroxidase secondary antibody (Amersham Biosciences) diluted 1:2000 was used. After washing, 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 percentage change from control.

Statistical analysis

Comparisons between groups for each dependent variable were made by a one-way analysis of variance (ANOVA) and when appropriate Tukey HSD (honestly significantly different)

tests were performed *post-hoc*. Significance was established at $P < 0.05$. Data are presented as means $\pm$ SE.

RESULTS

In vitro protein oxidation resulted in three levels of reactive carbonyl derivatives

To determine whether oxidative modification of myofibrillar proteins increases their susceptibility to degradation by both calpain and caspase-3, we exposed myofibrillar proteins to oxidizing conditions that were designed to create three diverse levels (i.e. low, moderate, and high) of protein oxidation. We then measured the levels of reactive carbonyl derivatives (RCD) in these protein samples as an index of protein oxidation. Our results show that a low level of protein oxidation resulted in an ~3-fold increase ($p < 0.05$) in RCD formation in myofibrillar proteins when compared with “control” proteins that were not exposed to *in vitro* oxidation ($P < 0.001$) (Figure 2A and 2B). Moreover, the moderate oxidation treatment produced a significantly higher level of RCD formation when compared to both control ($P < 0.001$) and low oxidation ($P < 0.01$). Finally, our high oxidation treatment resulted in further elevated levels of RCDs when compared to control, low and moderate oxidation ($P < 0.05$). Hence, our *in vitro* oxidation treatments were successful in creating three distinct levels of myofibrillar protein oxidation.

We then asked if exposure of myofibrillar proteins to oxidizing conditions resulted in a spontaneous degradation of proteins that occurs independent of protease activity. To address this question we separated both control and oxidized myofibrillar proteins using 2-D electrophoresis and stained the gels with coomassie blue to identify the protein bands. Our results revealed that even the highest level of protein oxidation did not result in a spontaneous breakdown of myofibrillar proteins (Figure 2C).

Finally, to determine that our oxidation protocol was physiologically relevant to *in vivo* protein oxidation observed in the diaphragm of animals undergoing prolonged mechanical ventilation, we isolated myofibrillar proteins from the diaphragm of control animals, animals ventilated for 18 hours and animals ventilated for 18 hours treated with the antioxidant Trolox. We then measured RCD formation and our results suggest that our *in vitro* protein oxidation protocol results in a physiological relevant level of protein oxidation (Figure 2D).

Impact of protein oxidation on rates of myofibrillar protein degradation

To determine if protein oxidation accelerates myofibrillar protein breakdown via calpain or caspase-3, we independently incubated oxidized myofibrillar proteins with active calpain I, calpain II, or caspase-3. Peptide mapping was then used to identify the magnitude and pattern of protein degradation based upon a comparison between control samples (i.e., without protease exposure) and myofibrillar samples that were incubated with individual proteases (Figure 3). By visual inspection, we identified 19 separate protein bands that were degraded by either calpains (I and II) and/or caspase-3. Using SDS-PAGE to identify molecular weights, we selected four prominent protein bands (~25, 40, 100, and 200 kDa) for quantitative analysis (Figure 3). Details of this analysis are highlighted in the following sections.

200-kDa protein band

Compared to control, the 200 kDa molecular weight protein was decreased in band size and intensity when independently exposed to calpain I, calpain II, or caspase-3 (Figure 4A–C). Indeed, all three proteases significantly degraded the 200 kDa band. Importantly, as the protein oxidation level increased, the degradation of the 200 kDa band increased in all proteases.

To identify the specific protein that calpains and caspase-3 were degrading at ~200 kDa we used a Western blotting approach with a monoclonal antibody directed toward myosin heavy chain protein. These blots confirmed that myosin heavy chain protein was the dominant protein band located at ~200 kDa. Using computerized image analysis we confirmed that myosin heavy chain proteins were degraded by both calpains and caspase-3 and that oxidation increased the magnitude of degradation in a similar fashion to the analysis of the 200 kDa protein band located on the gels stained with coomassie blue dye (Figure 4D–F).

100-kDa protein band

Protease degradation of a 100 kDa protein band was also significantly affected by increased oxidative modification (Figure 5). In the myofibrillar protein samples treated with caspase-3, each treatment resulted in increases in protein degradation. Similarly, exposure of protein samples to calpain I and II resulted in significant protein degradation. (Figure 5A–C).

Using monoclonal antibodies against α -actinin, we identified α -actinin as the prominent protein band located at ~100 kDa. Further analysis revealed that this protein was degraded by both calpains and caspase-3, and oxidation increased the level of degradation via all three proteases (Figure 5D–F). The pattern of α -actinin degradation followed a similar pattern to the analysis of the 100 kDa protein band located on the gels stained with coomassie blue dye.

40-kDa protein band

The protein band at a molecular weight of ~40 kDa was degraded by all three proteases in a similar manner to the 200 and 100 kDa bands (Figure 6). Specifically, an increase in myofibrillar protein oxidation increased the breakdown of the 40 kDa protein by calpain I, calpain II, and caspase-3. Using a monoclonal antibody, actin was identified as the major protein located at ~40kDa. Further analysis revealed that actin was degraded by all three proteases and the magnitude of degradation increased as a function of the level of protein oxidation (Figure 6).

25-kDa protein band

A molecular weight protein of ~25 kDa was also a substrate for both calpains and caspase-3 (Figure 3 and 7). Similar to the three previous proteins, increased oxidation resulted in a general increase in breakdown of this ~25 kDa protein by calpains and caspase-3.

Using a Western blot approach, the ~25 kDa protein band was identified as troponin I. Troponin I showed great susceptibility to degradation when exposed to both calpain I and II. Moreover, oxidation increased the vulnerability of this protein to calpain-mediated proteolysis (Figure 7). Troponin I was also a substrate for caspase-3 and the level of degradation was accelerated by oxidation. Nonetheless, compared to the calpains, the magnitude of caspase-3-mediated troponin I degradation was significantly lower.

DISCUSSION

Overview of Principal Findings

These experiments provide new and important information regarding the effects of oxidation on the vulnerability of myofibrillar proteins to degradation by calpains (I and II) and caspase-3. Specifically, our results clearly reveal that oxygen radicals increase the proteolytic susceptibility of numerous myofibrillar muscle proteins and support the hypothesis that oxidative modification of myofibrillar proteins increases their propensity to degradation by calpains and caspase-3. A detailed discussion of these and other important findings follows.

Myofibrillar protein damage promotes calpain-mediated proteolysis

Ground-breaking work by Davies and colleagues first demonstrated that oxygen radicals increased the breakdown of cellular proteins exposed to several purified proteases [10,16–18]. This landmark work has been expanded by several investigators and it is now clear that oxidized proteins are readily degraded by other proteases including the 20S proteasome [9, 19]. Importantly, the current study expands these earlier findings by demonstrating that oxidation increases the susceptibility of skeletal muscle myofibrillar proteins to degradation by the calcium-activated proteases, calpain I and calpain II. Indeed, the current study provides the first evidence that oxidation increases calpain-mediated myofibrillar protein breakdown in a dose-dependent manner and that following oxidation, myosin heavy chain, α -actinin, actin, and troponin I are all rapidly degraded by calpain I and II.

Although calpain has been shown to degrade over 100 different cellular proteins during *in vitro* assays, myosin heavy chain, α -actinin, and actin have been reported to be weak substrates for calpains [20]. Our results support this supposition but our findings reveal that oxidative modification of these proteins markedly accelerates their rate of degradation by both calpain I and II. Our data also reveal that many other myofibrillar proteins (i.e., molecular weights ranging from 200 kDa to ~10 kDa) are degraded by calpains and that oxidative modification of these proteins accelerates their susceptibility to proteolytic breakdown.

Oxidation promotes breakdown of myofibrillar proteins via caspase-3

Similar to our results with calpain I and II, this investigation provides the first evidence that oxidation of myofibrillar proteins increases their vulnerability to degradation by caspase-3. Specifically, our findings document that non-oxidized myosin heavy chain, α -actinin, actin, and troponin I proteins are weak substrates of caspase-3. However, our data clearly show that oxidation of these proteins increases their susceptibility to caspase-3 degradation in a dose-dependent manner.

Since the discovery that the major effectors of programmed cell death are the caspase family of proteases, significant efforts have been directed toward understanding which cellular proteins are substrates for caspases [21]. To date, almost 400 proteins have been shown to be substrates of one or more caspases [21]. Nonetheless, prior to 2004, limited effort had been directed toward identifying caspase-3 substrates in skeletal muscle because the role that caspase-3 plays in skeletal muscle wasting was relatively unknown. However, interest in caspase-3 substrates in skeletal muscle was greatly expanded when Du et al. demonstrated that caspase-3 plays a key role in several forms of skeletal muscle atrophy [22]. Importantly, our results reveal that the role caspase-3 plays in skeletal muscle atrophy can be amplified when myofibrillar proteins are oxidized.

Mechanistic link between protein oxidation and proteolytic degradation

Our data do not unveil the chemical mechanisms to explain why oxidation of myofibrillar proteins accelerates their rates of degradation by calpains and caspase-3. Nonetheless, based on earlier work, it seems likely that oxidative modification of muscle proteins increases their susceptibility to proteolysis, in part, due to unfolding of the molecule [10,16,17,23]. This oxidative damage to the protein molecule would lead to the modification of the secondary or tertiary structure such that previously shielded peptide bonds would be exposed to enzymatic hydrolysis [10,16,17]. Based on this model, Davies et al. has postulated that proteases should degrade .OH-modified proteins more efficiently than normal proteins and the current findings are consistent with this prediction [18].

Critique of experimental model

To determine if oxidatively modified myofibrillar proteins are more susceptible to degradation by both calpains and caspase-3, we isolated myofibrillar protein from rat skeletal muscle and performed a series of *in vitro* (i.e., cell free) experiments. This approach has several experimental advantages. First, this experimental platform offers direct access to complex biological processes where the chemical environment can be controlled and the rates of protein degradation accurately measured [24]. Moreover, the use of a cell-free system permits the independent study of specific classes of proteins (e.g., myofibrillar proteins). Furthermore, our *in vitro* experimental approach allows the exposure of isolated myofibrillar proteins to controlled and relatively well-defined levels of oxidation. This approach is essential to study the dose-response relationship between the levels of protein oxidation and proteolytic degradation. A final advantage of our experimental approach is that this *in vitro* model is one of the few experimental approaches that will permit the study of specific proteases. Indeed, it is not possible to investigate the effects of a single protease within living cells and therefore, a cell-free system is essential to achieve this experimental objective.

Although our experimental model has many advantages, we acknowledge that cell free systems also have limitations. A widely recognized restraint of cell free systems is their inability to perfectly duplicate the behavior of cells *in vivo*. For example, in the cell, the degree of protein damage caused by oxidants depends on many factors including the level and type of oxidant acting upon the protein. Therefore, it is unclear if our *in vitro* oxidation of myofibrillar proteins mimics the types of protein modifications that occur in myofibrillar proteins within skeletal muscle fibers during prolonged disuse. Nonetheless, our experiments attempted to circumvent this problem and were designed to mimic the levels of *in vivo* myofibrillar protein oxidation observed in a specific model of disuse muscle atrophy. Specifically, we endeavored to replicate the levels of protein oxidation in diaphragm muscle of animals exposed to varying durations of prolonged mechanical ventilation. In this regard, it is established that prolonged mechanical ventilation results in diaphragmatic inactivity, oxidative stress, and accelerated diaphragmatic proteolysis [12,13,25]. Further, we have demonstrated that prolonged mechanical ventilation results in a time-dependent increase in the levels of diaphragmatic protein oxidation [12]. Importantly, we have also shown that mechanical ventilation is sufficient to activate both calpain and caspase-3 in the diaphragm and that prevention of mechanical ventilation-induced oxidative stress via the antioxidant Trolox can prevent activation of both proteases [26]. Based on this, we used the levels of RCD's as a biomarker of protein oxidation in the diaphragm, and designed our *in vitro* oxidation protocol to mimic the levels of diaphragmatic protein oxidation observed during 6, 12 and 18 hours of mechanical ventilation. Importantly, our results indicate that we were successful in achieving this objective and our experiments provide proof of concept that *in vitro* oxidation of myofibrillar proteins increases their susceptibility to proteolytic degradation *in vitro*.

Conclusions and implications for disuse muscle atrophy

These experiments reveal that oxidation increases the proteolytic susceptibility of myofibrillar proteins to degradation by both calpains and caspase-3. These new and important findings provide a potential mechanistic link that connects oxidative stress with disuse muscle atrophy. Indeed, one of the hallmarks of disuse muscle atrophy is the observation that myofibrillar proteins undergo oxidation [5]. Moreover, antioxidant treatments have been shown to diminish the rate of disuse muscle atrophy [25,27]. This observation has been interpreted as evidence that oxidant stress accelerates proteolysis in muscle fibers during periods of prolonged disuse. In theory, oxidant stress could accelerate proteolysis by increasing protease activity and/or increasing the susceptibility of proteins to degradation via proteases. Our finding that oxidative modification of myofibrillar proteins accelerates their rate of degradation via calpains and

caspase-3 indicates that oxidation accelerates proteolysis and may provide an important cause and effect link between oxidative stress and accelerated disuse muscle atrophy.

As discussed earlier, several of the myofibrillar proteins investigated in our study are considered weak substrates for calpains and caspase-3. However, oxidative modification of these proteins greatly enhances their susceptibility to proteolytic degradation via these proteases. This is significant because myofibrillar proteins are the largest class of muscle proteins and constitute approximately 55–60% of the muscle by weight [28]. Further, compared to other classes of muscle proteins, myofibrillar proteins are degraded and lost at a faster rate during disuse muscle atrophy [28].

Finally, the proteasome is often considered to be the primary protease responsible for the degradation of myofibrillar proteins during muscle atrophy. However, our results clearly indicate that oxidation of myofibrillar proteins greatly enhances their susceptibility to degradation by both calpains and caspase-3. Given that myofibrillar proteins undergo oxidative modification during prolonged periods of skeletal muscle inactivity and, that both calpains and caspase-3 are activated in skeletal muscle during periods of disuse, our results suggest that these proteases may play an important role in the degradation of myofibrillar proteins during disuse muscle atrophy. This is an important possibility that warrants additional research.

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Figure 1.

Experimental design for investigating the effects of myofibrillar protein oxidation on protein degradation by caspase-3 and calpains.

Figure 2.

Assessment of the level of reactive carbonyl derivatives (RCD) in myofibrillar proteins exposed to varying levels of oxidizing treatments. A) Western blot to determine the level of RCD in myofibrillar protein isolated from the rat diaphragm. The control sample (lane 1) was not exposed to oxidants. The samples in lanes 2, 3, 4 were exposed to three levels of H_2O_2 and Fe^{2+} (i.e. low, moderate or high). These results clearly indicate that the oxidizing treatment resulted in increased levels of protein oxidation in myofibrillar protein. B) Fold difference (versus control) of the oxidation levels. *low oxidation significantly increased versus control ($P < 0.001$). #moderate oxidation significantly increased versus control and low oxidation ($P < 0.001$, $P < 0.01$). \$high oxidation significantly increased from control, low and moderate oxidation ($P < 0.001$, $P < 0.001$, $P < 0.05$). C) Representative gel of control and oxidized myofibrillar proteins separated via 2-D electrophoresis and stained the gels with coomassie. The observation that these gels appear identical in band number and size indicates that our three levels of protein oxidation does not result in a spontaneous breakdown of myofibrillar proteins. D) Western blot to determine the level of RCD in control animals (Con), animals ventilated for 18 hours (MV), and animals ventilated for 18 hours treated with Trolox (MVT).

Figure 3.

Representative SDS-PAGE gel (stained with coomassie blue) illustrating the separation of isolated myofilaments (control and three levels of oxidation) following exposure to active caspase-3 or calpain I and calpain II. This form of peptide mapping was used to identify the pattern of protein degradation based upon a comparison between control samples (i.e., without protease exposure) and myofibrillar samples that were incubated with purified proteases. Note the progressive increase in degradation of specific proteins (e.g., 200, 100, 40, and 25 kDa) with an increase in the level of protein oxidation.

FIGURE 4.

(A–C) Oxidation resulted in a step-wise increase in the degradation of the 200 kDa molecular weight protein when proteins were independently exposed to purified and active caspase-3, calpain I, and calpain II. (D–F) Using western blot analysis, this 200 kDa protein was identified as myosin heavy chain. Further analysis confirmed that non-oxidized myosin heavy chain proteins are degraded by both calpains and caspase-3 and that oxidation greatly increases the rate of proteolytic degradation. Values are mean percentage change $\pm$ SE. *low oxidation significantly increased versus control ($p < 0.05$). ¥moderate oxidation significantly increased from control ($p < 0.05$). #moderate significantly increased versus no and low oxidation ($p < 0.05$). \$high oxidation significantly increased versus control ($p < 0.05$).

∞high oxidation significantly increased from control and low oxidation ($p<0.05$). §high significantly increased from control, low and moderate oxidation ($p<0.05$).

Figure 5.

(A–C) Investigation of the breakdown of myofibrillar proteins by peptide mapping indicates that, compared to control, oxidation resulted in a step-wise increase in the degradation of the 100 kDa molecular weight protein when proteins were independently exposed to purified and active caspase-3 and two isoforms of calpain (i.e., I and II). (D–F) Using western blot analysis, the 100 kDa protein band was identified as α -actinin. Further analysis confirmed that α -actinin is modestly degraded by both calpains and caspase-3 but oxidation markedly increases the rate of proteolytic degradation. Values are mean percentage change $\pm$ SE.

*low oxidation significantly increased from control ($p < 0.05$). ¥moderate oxidation significantly increased from control ($p < 0.05$). ‡high significantly increased from control

($p < 0.05$). #moderate significantly increased from control and low oxidation ($p < 0.05$). ∞ high significantly increased from control and low oxidation ($p < 0.05$). §high oxidation significantly increased from control, low and moderate oxidation ($p < 0.05$).

FIGURE 6.

(A–C) Investigation of the proteolytic breakdown of myofibrillar proteins using peptide mapping shows that compared to control, protein oxidation promotes a step-wise increase in the degradation of the 40 kDa molecular weight protein when proteins were independently exposed to purified and active caspase-3, calpain I, and calpain II. (D–F) Using western blot analysis, this 40 kDa protein was identified as actin and subsequent analysis indicates that non-oxidized actin is mildly degraded by both calpains and caspase-3 but oxidation greatly increases the magnitude of proteolytic degradation. Values are mean percentage change $\pm$ SE.

*low oxidation significantly increased from control ($p < 0.05$). ¥moderate significantly increased from control ($p < 0.05$). ‡high significantly increased from control ($p < 0.05$). #moderate oxidation significantly increased from control and low oxidation ($p < 0.05$). ∞high oxidation significantly increased from control and low oxidation ($p < 0.05$).

FIGURE 7.

Analysis of the proteolytic breakdown of myofibrillar proteins via peptide mapping revealed that compared to control, oxidation resulted in a stepwise increase in the degradation of the 25 kDa molecular weight protein when proteins were independently exposed to purified and active caspase-3, calpain I, and calpain II. (**D–F**) Using western blot analysis, this 25 kDa protein was identified as troponin I. Subsequent analysis indicates that troponin I is degraded by both calpains and caspase-3 and that oxidation accelerates proteolytic degradation. Values are mean percentage change $\pm$ SE.

*low oxidation significantly increased from control ($p < 0.05$). ¥moderate oxidation significantly increased from control ($p < 0.05$). ‡high significantly increased from control ($p < 0.05$). ∞high significantly increased from control and low oxidation ($p < 0.5$). \$high significantly increased from control, low and moderate oxidation ($p < 0.05$).