ENHANCED DEGRADATION OF OXIDATIVELY MODIFIED MYOFIBRILLAR PROTEINS BY CALPAIN AND CASPASE-3

By

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To my family and friends for their constant support and to the people who have played a significant role in my education
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>7</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>8</td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>9</td>
</tr>
<tr>
<td>2 LITERATURE REVIEW</td>
<td>11</td>
</tr>
<tr>
<td>Overview of Skeletal Muscle Disuse Atrophy</td>
<td>11</td>
</tr>
<tr>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td>Skeletal Muscle Characteristics</td>
<td>12</td>
</tr>
<tr>
<td>Mechanisms of Skeletal Muscle Dysfunction</td>
<td>12</td>
</tr>
<tr>
<td>Protein Synthesis and Degradation</td>
<td>12</td>
</tr>
<tr>
<td>Proteolytic Systems</td>
<td>13</td>
</tr>
<tr>
<td>Oxidative Stress and Disuse Muscle Atrophy</td>
<td>16</td>
</tr>
<tr>
<td>Summary</td>
<td>18</td>
</tr>
<tr>
<td>3 MATERIALS AND METHODS</td>
<td>21</td>
</tr>
<tr>
<td>Experiment 1: Animals</td>
<td>21</td>
</tr>
<tr>
<td>Animal Model Justification</td>
<td>21</td>
</tr>
<tr>
<td>Animal Housing and Diet</td>
<td>21</td>
</tr>
<tr>
<td>Experimental Design</td>
<td>21</td>
</tr>
<tr>
<td>Animal Protocol</td>
<td>22</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>22</td>
</tr>
<tr>
<td>General Methods</td>
<td>22</td>
</tr>
<tr>
<td>Biochemical Measurements</td>
<td>22</td>
</tr>
<tr>
<td>4 RESULTS</td>
<td>26</td>
</tr>
<tr>
<td>Redox Balance</td>
<td>26</td>
</tr>
<tr>
<td>Myofibril Digestion</td>
<td>26</td>
</tr>
<tr>
<td>Identification of Specific Protein Substrates</td>
<td>28</td>
</tr>
<tr>
<td>5 DISCUSSION</td>
<td>40</td>
</tr>
<tr>
<td>Overview of Principal Findings</td>
<td>40</td>
</tr>
<tr>
<td>Calpains and Caspase-3 Release Myofilaments</td>
<td>40</td>
</tr>
<tr>
<td>Induction of Oxidative Stress during Disuse Atrophy</td>
<td>41</td>
</tr>
<tr>
<td>Oxidation of Myofibrils Increases Protein Degradation by Calpain and Caspase-3</td>
<td>41</td>
</tr>
<tr>
<td>Conclusions and Future Directions</td>
<td>42</td>
</tr>
</tbody>
</table>
LIST OF REFERENCES ...............................................................................................................44

BIOGRAPHICAL SKETCH .........................................................................................................52
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Pathway of protein degradation by calpain followed by degradation by the proteasome.</td>
</tr>
<tr>
<td>2-2</td>
<td>Potential pathways for the activation of caspase-3 during MV.</td>
</tr>
<tr>
<td>3-1</td>
<td>Experimental model for examining the effects of various levels of oxidation on isolated diaphragm myofibrillar proteins on the activation of caspase-3 and calpain.</td>
</tr>
<tr>
<td>4-1</td>
<td>Determination of oxidative damage.</td>
</tr>
<tr>
<td>4-2</td>
<td>Isolated myofilaments from diaphragm muscle.</td>
</tr>
<tr>
<td>4-3</td>
<td>250 kDa band intensity (percent different versus control).</td>
</tr>
<tr>
<td>4-4</td>
<td>100 kDa band intensity (percent different versus control).</td>
</tr>
<tr>
<td>4-5</td>
<td>37 kDa band intensity (percent different versus control).</td>
</tr>
<tr>
<td>4-6</td>
<td>30 kDa band intensity (percent different versus control).</td>
</tr>
<tr>
<td>4-7</td>
<td>Myosin protein (percent different versus control).</td>
</tr>
<tr>
<td>4-8</td>
<td>Actin protein (percent different versus control).</td>
</tr>
<tr>
<td>4-9</td>
<td>α-actinin protein (percent different versus control).</td>
</tr>
<tr>
<td>4-10</td>
<td>Troponin protein (percent different versus control).</td>
</tr>
</tbody>
</table>
Prolonged periods of skeletal muscle inactivity results in fiber atrophy. The physiological consequences of disuse-induced muscle atrophy is a diminished muscle force generating capacity which could negatively impact activities of daily living. Importantly, oxidative stress has been linked to the signaling events responsible for protein degradation. However, the mechanisms by which oxidative stress increases the rate of proteolysis have not been fully elucidated. In theory, oxidation of proteins can alter their structure to affect susceptibility to proteolytic processing. Several proteolytic systems are capable of degrading muscle proteins. Calpain (I and II) and caspase-3 are proteases that are capable of degrading intact myofilament proteins and are also activated during disuse atrophy. Therefore, we hypothesized that oxidative modification of skeletal muscle proteins increases their susceptibility to degradation by calpain and/or caspase-3. To test this postulate we introduced isolated myofibrillar proteins from the diaphragm to four distinct levels of oxidation and subsequently treated each group independently with active calpain I, calpain II, and caspase-3. Protein degradation was measured using protein mapping. Our findings reveal, as oxidation of myofibrillar proteins increases, their degradation by calpain (I and II) and caspase-3 increases as well. Therefore, we conclude that oxidative modification of myofibrillar proteins accelerates protein degradation by both calpains and caspase-3.
Skeletal muscle inactivity produces a profound atrophy of muscle fibers. This atrophy is characterized by a decrease in fiber size and therefore a decrease in muscle mass (29). Bed rest, immobilization, spaceflight, muscle denervation, and mechanical ventilation are all conditions that promote skeletal muscle atrophy. Locomotor muscles affected in these conditions are normally involved in maintaining posture (15). Loss of functional capacity in these muscles can greatly impact daily life therefore understanding the mechanisms that contribute to disuse atrophy is imperative.

Skeletal muscle disuse results in an imbalance in muscle protein synthesis and degradation. During this catabolic condition there is a decrease in muscle protein synthesis followed by an increase in protein degradation. The proteolytic pathways involved in protein degradation are well known. Major proteolytic systems in skeletal muscle include the ubiquitin-proteasome system, calpains, and caspases. Importantly, these pathways appear to be markedly influenced by oxidative stress.

To date, all forms of disuse muscle atrophy are associated with an increase in protein oxidation. This is significant because oxidative modification of muscle proteins during disuse could increase their susceptibility to degradation. However, the mechanisms by which oxidative stress influences the rate of proteolysis are lacking (1, 46, 69, 71, 76, 82). In theory, oxidation of proteins can alter their structure to affect susceptibility to proteolytic processing (21, 22). Evidence indicates that protein oxidation enhances substrate recognition for the proteasome; however, this system is unable to degrade intact sarcomeric proteins.

Calpain (I and II) and caspase-3 are proteases that are capable of degrading intact myofilament proteins and are also activated during disuse atrophy. Therefore, these experiments
determined whether oxidative modification of myofibrillar proteins increases their susceptibility to degradation via calpain (I and II) or caspase-3. Specifically, our experiments were designed to achieve the following specific aim.

Specific Aim: To determine if oxidation influences myofibrillar protein breakdown when muscle proteins are independently exposed to active calpain I, calpain II, and caspase-3.

Rationale: Our previous work has demonstrated that inactivity-induced muscle atrophy is associated with oxidative stress and that the induction of oxidative stress accelerates proteolytic activity. Currently, the influence of muscle protein oxidation on substrate recognition for calpain and caspase-3 is unknown.

Hypothesis: Oxidative modification of myofibrillar proteins will increase their vulnerability to degradation by both calpain (I and II) and caspase-3.
CHAPTER 2
LITERATURE REVIEW

Periods of skeletal muscle disuse result in contractile dysfunction due to both a loss of muscle mass and a decrease in specific force generating capacity. Evidence suggests that oxidative stress is an important regulator in the pathways leading to muscle atrophy during periods of disuse. Models of disuse include: immobilization, chronic bed rest, physical inactivity, spaceflight, hindlimb unloading, and mechanical ventilation. These models are studied in order to better understand the mechanisms that contribute to muscle atrophy during disuse so that protective countermeasures can be developed.

Skeletal muscle weakness due to atrophy and contractile dysfunction can be attributed to changes in the rate of proteolysis (64, 76). Therefore, the first segment of this chapter will discuss the mechanisms of skeletal muscle atrophy and outline the different proteolytic systems involved in disuse-induced atrophy. The second segment of this chapter will discuss the induction of oxidative stress during inactivity. More specifically, oxidative damage occurs in skeletal muscle during periods of disuse and the second segment of this chapter will address both the mechanisms responsible for oxidative stress and the contribution of oxidative modification of proteins to skeletal muscle weakness.

Overview of Skeletal Muscle Disuse Atrophy

Introduction

Skeletal muscle tissue consists of ~40% of total body mass and provides basic functions such as locomotion, metabolism and respiration. Skeletal muscle exhibits a very high level of plasticity. Skeletal muscle hypertrophy is characterized by increased muscle size, protein content and strength (31, 32). Conversely, prolonged inactivity results in the loss of skeletal muscle mass. Skeletal muscle disuse atrophy occurs as a result of altered protein metabolism which
leads to a decrease in muscle contractile protein content and function (26, 80, 81, 88, 90). Due to the severity of complications arising from skeletal muscle disuse atrophy identification of the mechanism(s) responsible for inactivity-induced weakness is important.

**Skeletal Muscle Characteristics**

Skeletal muscle proteins can be divided into three classes based upon their solubility and location in the muscle. Sarcoplasmic proteins constitute ~30-35% of skeletal muscle and are the cytoplasmic proteins soluble in low salt solutions. These proteins comprise all of the glycolytic enzymes (19, 20). Stroma proteins constitute ~10-15% of total protein in muscle and are those proteins that are insoluble in aqueous solvent at neutral pH (19, 20). The third and largest class of skeletal muscle proteins are the myofibrillar proteins which constitute ~55-60% of total muscle protein (19, 20). Myofibrillar proteins consist of the myofibril or contractile structure in skeletal muscle. They are responsible for the contractile properties of muscle and for most of the functional properties (19, 20).

**Mechanisms of Skeletal Muscle Dysfunction**

**Protein Synthesis and Degradation**

Skeletal muscle mass is maintained through the balance of the rate of protein synthesis and protein degradation. Periods of prolonged muscle disuse can result in muscle wasting and alter the muscle’s physiological function. These conditions have been demonstrated during periods of immobilization, micro-gravity, muscle denervation, and mechanical ventilation (31, 32, 62, 87). Skeletal muscle atrophy is caused by a decrease in muscle protein synthesis and an increase in muscle protein proteolysis (31, 32). The decrease in protein synthesis is characterized by alterations in translational initiation and elongation and/or a decrease in cellular RNA (28, 41, 58, 59). Our laboratory has observed a decrease in diaphragmatic protein synthesis in as few as 6 hours following mechanical ventilation (75). Nevertheless, although inactivity results in
decreased protein synthesis our laboratory has also demonstrated that the development of atrophy during periods of inactivity is primarily due to an increase in protein degradation (12, 52, 53, 76). During skeletal muscle proteolysis myofibrillar protein is lost at a rate faster than other muscle proteins (31). These proteins must first be disassembled before they can be degraded and therefore, release of myofilaments is a required first step in contractile protein breakdown (19).

**Proteolytic Systems**

Disassembly and degradation of skeletal muscle proteins occurs via activation of several proteolytic systems (75, 76). There are at least four different proteolytic systems involved in skeletal muscle disuse atrophy. These systems include: 1) Lysosomal proteases 2) Ubiquitin-proteasome system 3) Ca^{2+}- dependent calpain system 4) Caspases. These systems all work together during proteolysis (24, 31, 32, 62, 66).

**Lysosomal proteases.** Lysosomal proteases include the family of proteases called cathepsins. These proteases are found ubiquitously in all tissue, but with greater levels in tissues having higher protein turnover (63). The major role of the cathepsins is to degrade membrane proteins, including receptors, ligands, channels, and transporters. Lysosomal proteases are activated during skeletal muscle atrophy and it has been argued that these proteases do not appear to significantly affect the rates of myofibrillar protein degradation or total protein degradation (17, 25, 68). Nonetheless, new evidence questions this concept and suggests lysosomal cathepsins may contribute to muscle protein breakdown via autophagy (47, 96). Hence, determining the precise role that lysosomal proteases play in muscle wasting remains an important area for future research.

**Ubiquitin-proteasome system.** The proteasome is comprised of a 20S ‘core’ proteasome subunit with a 19S regulatory proteasome subunit attached to each side. The combination of these three subunits makes up the 26S proteasome complex (21-23, 89). The 26S proteasome
degrades proteins that have been ubiquitinated, whereas the 20S is able to degrade proteins that have not been ubiquitinated. The pathway of degradation by the 26S proteasome complex begins with the protein E1, the ubiquitin-activating enzyme. After ubiquitin is activated it is then transferred to E2, the ubiquitin carrier protein. E2 then interacts with E3, an ubiquitin ligase responsible for catalyzing the transfer of ubiquitin to a protein substrate, marking the substrate for proteasomal degradation (46, 70, 85). The 19S regulatory complex is required for degradation of ubiquitinated proteins. Once a protein is ubiquitinated it is subsequently recognized by the 19S regulatory complex. Energy from ATP hydrolysis removes the polyubiquitin chain and unfolds the substrate protein. The unfolded protein then enters the 20S proteasome and is degraded (11, 22, 70).

Along with being a part of the 26S proteasome, the 20S proteasome has also been reported to be located intracellularly without any regulatory proteins attached (22). This ‘free’ 20S proteasome appears to be the predominant intracellular form. The 20S proteasome is capable of acting separately from ubiquitin and ATP (21, 22).

During muscle atrophy, the proteasome system is responsible for the degradation of actin and myosin. However, this system is unable to degrade intact sarcomeres (20, 89) and therefore, these complexes must be broken down by a separate proteolytic system prior to degradation by the proteasome (89, 93).

**Ca²⁺-dependent calpain system.** Calpains are calcium-dependent cysteine proteases that are activated in skeletal muscle during conditions that promote muscle wasting (e.g. inactivity, sepsis, cachexia) (6, 20, 24, 25). When calpains are activated they can cleave myofibrillar proteins and produce large peptides that can later be degraded by the proteasome (17, 30, 68). The calpains refer to calpain I and calpain II which are also referred to as μ- and m-
calpain respectively. These names are based upon the amount of calcium each requires to be catalytically active. Skeletal muscle contains approximately the same amount of both calpain I and II (20). Calpain (I and II) is located exclusively intracellularly and in the cell most is located on or next to the z-disk of the sarcomere with the I-band and the A-band also containing small amounts (18, 32). Due to the location of calpain the substrates that it primarily acts on are those involved in linking contractile elements together, and in general calpain I and II tend to act on the same substrates but at different rates (20). It is unknown if calpains can directly degrade actomyosin complexes but it is clear that calpain can release sarcomeric proteins by cleaving cytoskeletal proteins that anchor the contractile elements (19, 20). Some of the protein substrates known to be cleaved by calpain activation include α-spectrin, troponin I, desmin, and titin (34, 47, 68, 89). Due to the damaging effects calpain can have on the integrity of myofibers calpain activity is highly regulated and remains inactive most of the time. Calpain activity is regulated by several factors including cytosolic calcium levels, phosphorylation, and calpastatin concentration (20). Calpastatin is the only known endogenous inhibitor of calpain. In brief, high cytosolic levels of calpastatin inhibit calpain activation whereas a decrease in calpastatin levels favors calpain activation. Interestingly, calpastatin is a substrate of the protease caspase-3 and therefore, increased caspase-3 activation can reduce calpastatin levels and promote calpain activation (20, 61, 62).

**Caspases.** Caspases are cysteine-dependent proteases that are capable of degrading proteins and of promoting apoptosis (14, 84). Apoptotic pathways are activated in skeletal muscle during disuse atrophy. Apoptotic proteases have been reported to cleave actin in vitro which suggests that apoptotic proteases contribute to protein breakdown (33, 49). In particular, caspase-3 is reported to not only cleave actin but also degrade actomyosin complexes (55). In the
cell, caspases are expressed as inactive precursors called procaspases. Cleavage of the procaspases results in their activation, and once a caspase is activated it is able to cleave and activate other caspases (67). This cascade-like activation of caspases can be seen during the activation of caspase-3. Caspase-3 is a protease that has been found to act similarly to calpain in its ability to promote degradation of cytoskeletal proteins and the release of actin and myosin monomers to be subsequently degraded by the proteasome. Caspase-3 can be activated by a calcium-releasing pathway, a mitochondrial pathway, and calpain is also capable of activating caspase-3 (9, 67).

**Oxidative Stress and Disuse Muscle Atrophy**

The relationship between oxidative stress and disuse muscle atrophy was first reported by Kondo (36-39). These studies reported that immobilization of skeletal muscles was associated with oxidative injury to muscle. Oxidative stress is important during muscle wasting situations because oxidants play an important role in many signaling pathways that promote contractile dysfunction, disturbances in calcium homeostasis, and protease activation in skeletal muscle (1, 46, 69, 71, 76, 82, 83). Oxidative stress occurs when there is an imbalance in the production of reactive oxidant species (ROS) and their ability to be scavenged by antioxidants. Oxidants are able to modify proteins causing them to have a loss of function or a change in function. These changes lead to an enhanced degradation of the oxidized proteins (22). Physiologically, the degradation of oxidatively modified proteins is important in order to avoid excessive accumulation of damaged proteins. Therefore degradation of oxidized and oxidatively modified proteins is an important part of oxidant defense (22). Following oxidation, modifications to amino acids, fragmentation, and aggregation can all be induced. These changes in proteins can change the proteolytic susceptibility of protein substrates (22). Oxidation of amino acids can alter protein structure to affect susceptibility to
proteolytic processing. Many proteins change their secondary and tertiary structure upon oxidation (4, 22). In order to maintain cellular homeostasis and prevent accumulation of highly oxidized and cross-linked proteins, degradation of oxidized proteins is essential. Intracellular oxidation products need to either be repaired or removed. Increasing evidence indicates that cells are able to selectively degrade the oxidized forms of proteins (4). For example, evidence of changes in degradation susceptibility has been reported for glutamine synthetase. Specifically, it has been reported that oxidative modification of this enzyme increases its susceptibility for degradation by several proteases (45, 73). Similar results have been reported for other oxidized proteins (22). Oxidized proteins in cell free extracts were found to have no ATP dependency during proteolysis. Along with this, oxidized proteins have been found to be poor substrates for ubiquitination. This means that the 26S proteasome is not involved in the degradation of oxidatively modified proteins, which then increases their susceptibility to degradation by the 20S proteasome which does not require ubiquitination or ATP in order to recognize and degrade proteins (21, 22).

Since oxidative stress occurs in skeletal muscle during periods of inactivity and the proteasome system is unable to degrade intact sarcomeres, the role of calpains and caspase-3 may be important in the disassembly of the actomyosin complexes prior to degradation by the proteasome. Both proteolytic systems have the possibility of being activated by oxidative stress, and may also selectively degrade oxidized proteins (40, 44, 62, 67).

During oxidative stress, oxidants generate the formation of reactive aldehydes (i.e. 4-HNE). These reactive aldehydes have been shown to reduce plasma membrane Ca\(^{2+}\) ATPase activity (58). This decrease would delay Ca\(^{2+}\) removal from the cell and therefore contribute to cellular Ca\(^{2+}\) accumulation. This overload of Ca\(^{2+}\) in the cell could lead to the activation of
calpain resulting in increased proteolysis of diaphragmatic cytoskeletal proteins and the release of myofilaments for subsequent degradation by the proteasome system (Figure 2-1). The oxidative increase in Ca\(^{2+}\) overload and the activation of calpain are also the means by which caspase-3 can be activated. Increases in intracellular Ca\(^{2+}\) concentrations cleave and activate caspase-12 which in turn activates caspase-3. The activation of calpain by increased intracellular Ca\(^{2+}\) concentration also activates caspase-3. Oxidative stress can also activate caspase-3 via a mitochondrial pathway. Specifically, oxidative stress leads to an increase in the release of cytochrome c from the mitochondria, activating caspase-9. Activation of caspase-9 leads to the subsequent activation of caspase-3 enabling the protease to cleave skeletal muscle sarcomeres and release actin and myosin for degradation by the proteasome (Figure 2-2) (ref 9, 67).

Summary

Inactivity results in skeletal muscle atrophy; this atrophic response to disuse is caused by a decrease in protein synthesis and an increase in degradation. Our work reveals that inactivity-induced oxidative stress in respiratory skeletal muscle is a requirement for proteolytic activation, atrophy, and contractile dysfunction. In this regard, our lab has also demonstrated that prolonged inactivity results in increased activation of the proteases, calpain and caspase-3. Calpain (I and II) and caspase-3 can function as the rate-limiting step in muscle protein proteolysis by releasing sarcomeres for further degradation by the proteasome. Growing evidence indicates that protein oxidation enhances substrate recognition for the proteasome. In contrast, the influence of protein oxidation on substrate recognition for calpains or caspase-3 remains unknown and this forms the basis for the current experiments.
Figure 2-1. Pathway of myofilament release by calpain followed by actin and myosin degradation by the proteasome. Note that increases in intracellular Ca$^{2+}$ concentration activate calpain which degrades myofibrillar protein substrates releasing the myofilaments.
Figure 2-2. Pathways leading to the activation of caspase-3 during MV: 1) Mitochondrial pathway occurs through the release of cytochrome c and activation of caspase-9. 2) Calcium releasing pathway results in increases in intracellular Ca\(^{2+}\) concentration activating calpain and caspase-12.
CHAPTER 3
MATERIALS AND METHODS

This chapter will be divided into two sections. Section one includes the experimental design used in each of our experiments that are intended to determine if oxidative modification of myofibrillar proteins increases their susceptibility to degradation by calpain and/or caspase-3. In the subsequent section, we will provide the methodological details associated with each experimental protocol and biochemical technique.

Experiment 1: Animals

Animal Model Justification

To address our specific aim and determine if oxidatively modified myofibrillar proteins are more vulnerable to degradation by caspase-3 and calpain (I and II), adult (4-6 month old) female Sprague-Dawley (SD) rats were used. The animals were 4-6 months of age (young adult) at the time of sacrifice. The SD rat was chosen due to the similarities between the rat and human diaphragm in both anatomical and physiological parameters (2, 3, 54, 56, 60, 61, 95).

Animal Housing and Diet

All animals were housed at the University of Florida Animal Care Services Center according to guidelines set forth by the Institutional Animal Care and Use Committee. The Animal Care and Use Committee of the University of Florida approved these experiments. Animals were maintained on a 12:12 hour reverse light-dark cycle and provided food (AIN93 diet) and water ad libitum throughout the experimental period.

Experimental Design

Adult rats were randomly assigned to two primary experimental groups: 1) control (CON; n=8)), 2) oxidation (H$_2$O$_2$ and Fe$^{2+}$; n=8). The costal diaphragm was removed from each animal the myofibrillar proteins were isolated for subsequent biochemical assays. Group 1 was divided
further into four additional groups; these groups included a control group, a group treated with active caspase-3, active calpain I, and active calpain II respectively. Group 2 contained 3 groups. Each myofibrillar protein sample was exposed to different oxidizing treatments resulting in a low, moderate, and high level of protein oxidation. The three levels of oxidation were chosen in order to simulate the level of protein oxidation observed following 6, 12, and 18 hours of MV. The oxidation levels for the three groups were: 1) Low (25 \mu M H_2O_2 and 10 \mu M Fe^{2+}) 2) Moderate (25\mu M H_2O_2 and 25 \mu M Fe^{2+}) 3) High (25\mu M H_2O_2 and 50 \mu M Fe^{2+}). Each of these three groups was then independently treated with either active caspase-3, active calpain I, or active calpain II (Figure 3-1). Each group had a sample size n=8 chosen based upon a power analysis from preliminary data.

Animal Protocol

Animals in each group were acutely anesthetized with sodium pentobarbital (60 mg/kg IP). After reaching a surgical plane of anesthesia, the animals were sacrificed and the diaphragm was immediately frozen in liquid nitrogen and stored at -80°C for subsequent analyses.

Statistical Analysis

Comparisons between groups were made by a one-way ANOVA and when appropriate simple main effects tests and Tukey HSD tests were performed. Significance was established at P < 0.05.

General Methods

Biochemical Measurements

Myofibrillar isolation. Samples were prepared based on the method of Reid et al. 1994 (70). Diaphragm samples were first homogenized in a buffer containing 0.039 M sodium borate (pH 7.1), 0.025 M KCl, 5mM ethelyne glycol-bis(\beta-amoioethyl 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: 100mM 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 then determined (Bradford).

**Reactive carbonyl derivatives.** Reactive carbonyl derivatives were assessed using the Oxyblot Oxidized Protein Detection Kit from Chemicon International (Temecula, Ca) as described by the manufacturer. Myofibrillar protein samples were treated with one of four differing levels (Control, Low, Moderate and High) of the oxidants H₂O₂ and Fe²⁺ according to the experimental design and incubated at 37°C for 15 minutes. Samples were then immediately cooled to 4°C in order for the oxidation to be terminated. Samples were separated via polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with primary antibody in order to confirm four distinct levels of oxidation.

**Peptide mapping via gel electrophoresis.** Peptide mapping was used to investigate the fragmentation pattern generated by digestion of myofibrillar proteins by each protease. Briefly, 20μl of each of the myofibrillar protein samples was treated independently with 2μl of either active calpain I, calpain II, or caspase-3 and 2μl of 50μM Ca²⁺. Samples were then incubated at 37°C for 30 minutes. Samples were then immediately cooled to 4°C in order to terminate the reaction. Samples were separated via polyacrylamide gel electrophoresis and then stained with Coomassie Blue. The gels were then analyzed using Image J software in order to determine percentage change from control. The protein bands chosen for analysis were at the molecular weights of 250, 100, 37 and 30 kDa.
**Western blot analysis.** Proteins were separated via polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, non-specific sites were blocked for 2 h at room temperature in a PBS solution containing 0.05% Tween-20 and 5% non-fat milk. Membranes were then incubated overnight at 4°C with primary antibodies directed against the protein of interest. The myofibrillar proteins myosin, actin, troponin I, and α-actinin were all probed as a measurement of specific protein degradation. 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 extensively with PBS-Tween and either sheep anti-mouse (myosin) or donkey anti-rabbit (actin, troponin I and α-actinin) IgG horseradish peroxidase (amersham biosciences) diluted 1:2000. After washing, a chemiluminescent system was used to detect labeled proteins (GE healthcare) and membranes were developed using autoradiography film and a developer (Kodak).
Figure 3-1. Experimental design for investigating the effects of various levels of myofibrillar protein oxidation on protein degradation by caspase-3 and calpains.
CHAPTER 4
RESULTS

Redox Balance

Our experimental plan required the production of different levels of oxidized myofibrillar proteins. To achieve this goal, we exposed isolated myofibrillar proteins to three different concentrations of H₂O₂ and Fe²⁺. Protein carbonyl formation (i.e. reactive carbonyl derivatives; RCD) is an excellent biomarker of protein oxidation and was used in our experiments to document the level oxidative damage to myofibrillar proteins.

Oxyblot. Our results revealed that our lowest level of protein oxidation resulted in a significant increase in RCD formation when compared with control (P<0.001) (Figure 4-1). Moreover, the moderate oxidation treatment produced slightly higher levels of RCD formation when compared to both control and low oxidation (P<0.001 and P<0.01), respectively. Finally, our high oxidation treatment resulted in further elevated levels of RCDs when compared to control, low and moderate oxidation (P<0.001, P<0.001, P<0.05), thus showing distinct levels of oxidation.

Myofibril Digestion

Peptide mapping provides a method to quantify the controlled cleavage of myofibrillar proteins by individual proteases. Therefore, peptide mapping was used to identify protein degradation based upon a comparison between control samples (no exposure to proteases) and myofibrillar samples that were incubated with individual proteases. Polyacrylamide SDS-page gels were used in order to obtain molecular weight information about the peptides produced (Figure 4-2). By visual inspection, we identified numerous protein bands that were degraded by both calpain (I and II) and capase-3. From these bands, we selected four prominent bands for
quantitative analysis. A summary of the impact of myofibrillar protein oxidation on proteolytic degradation in these selected myofibrillar proteins follows.

**250-kDa protein band.** Compared to control, the 250 kDa molecular weight protein was decreased in band size and intensity when independently exposed to calpain I, calpain II, and caspase-3 (Figure 4-3). Indeed, all three proteases significantly degraded the 250 kDa band. Importantly, as the protein oxidation level increased, the degradation of the 250 kDa band increased in all proteases. Specifically, compared to control, independent exposure of the highest oxidized proteins to calpain I, calpain II, and caspase-3 resulted in degradation of more than 90% of the 250 kDa band.

**100-kDa protein band.** Protease degradation of a 100 kDa protein band was also significantly affected by increased oxidative modification (Figure 4-4). In the protein samples treated with caspase-3, each treatment resulted in significant increases in protein degradation. Both the moderate and high oxidation groups were significantly different from the no oxidation group. Caspase-3 degradation of the 100-kDa band ranged from 19% (no oxidation treatment) to 73% degradation (high oxidation treatment). The protein samples exposed to calpain I ranged in degradation from 23% to 71% as the level of oxidation increased from no oxidation to high oxidation. Similarly, exposure of protein samples to calpain II resulted in significant protein degradation ranging from 12% to 73% (i.e., low oxidation to high oxidation).

**37-kDa protein band.** The protein band at a molecular weight of ~37-40 kDa was degraded by all three proteases in a similar manner to the 250 and 100 kDa bands (Figure 4-5). Specifically, increased protein oxidation increased protein breakdown by calpain I, calpain II, and caspase-3.
**30-kDa protein band.** At the molecular weight of ~30 kDa there was a pair of protein bands that were substrates for both calpain and caspase-3 (Figures 4-2 and 4-6). Similar to the three previous proteins, increased oxidation resulted in a general increase in breakdown of these ~30 kDa proteins by calpains and caspase-3.

**Identification of Specific Protein Substrates**

To identify the specific proteins that calpain and caspase-3 were degrading we employed a Western blotting technique with antibodies directed toward four known substrates of calpain and caspase-3. These blots were then analyzed to determine the impact of protein oxidation on proteolytic degradation by calpain and caspase-3.

**Myosin heavy chain.** Myosin heavy chain was identified via monoclonal antibodies as the dominant protein band located at ~250 kDa. Myosin was degraded by both calpains and caspase-3 and oxidation increased the magnitude of degradation (Figure 4-7). Specifically, control (non-oxidized) myosin was 29% depleted with the addition of active caspase-3 and this protein continued to be degraded to a greater extent as the protein oxidation level increased (i.e., 85% degradation with high oxidation). Similar to the caspase-3 mediated degradation of myosin, both calpain I and calpain II degradation of myosin was also increased as a function of the level of protein oxidation.

**Actin.** Actin was identified as the major protein located at ~37-40kDa. Similar to myosin, actin was degraded by all three proteases and the magnitude of degradation increased as a function of the level of protein oxidation (Figure 4-8).

**α-actinin.** α-actinin was recognized as the prominent protein band located at ~100 kDa. This protein was degraded by both calpains and caspase-3, and oxidation increased the level of degradation via all three proteases. More specifically, each level of protein oxidation significantly increased the susceptibility of α-actinin to be degraded by caspase-3. In contrast,
moderate-to-high levels of oxidation were required to increase the susceptibility of $\alpha$-actinin to degradation by calpain I and II (Figure 4-9).

**Troponin I.** 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 4-10). Troponin I was also a substrate for caspase-3 and the level of degradation was accelerated by oxidation. Note, however, that compared to the calpains, the magnitude of caspase-3-mediated troponin I degradation was significantly less.
Figure 4-1. 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 (left) was not exposed to oxidants. The samples in lanes 2, 3, 4 were exposed to three levels of H₂O₂ and Fe²⁺ (i.e. 1) low, 2) moderate, 3) high). These results clearly indicate that the oxidizing treatment resulted in increased levels of protein oxidation in myofibrillar protein. Low = 25µm H₂O₂ and 10 µm Fe²⁺, moderate = 25µm H₂O₂ and 25µm Fe²⁺, and high =25µm H₂O₂ and 50 µm Fe²⁺. 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).
Figure 4-2. Isolated myofilaments from diaphragm muscle were either not oxidized or exposed to three levels of oxidation (low, moderate, high) via H$_2$O$_2$ and Fe$^{2+}$ prior to exposure to caspase-3 and calpain (I and II). Samples were then separated via SDS-PAGE and stained with Coomassie Blue. The figure depicts an increase in degradation of specific proteins with an increase in the level of oxidation.
Figure 4-3. 250 kDa band intensity (percent difference versus control). Values are mean percentage change ±SE. A) *Low oxidation significantly increased versus no oxidation (P<0.001). ¥Moderate oxidation significantly increased versus no oxidation (P<0.001). ∞High oxidation significantly increased from no oxidation and low oxidation (P<0.001, P<0.01). B) *Low significantly increased versus no oxidation (P<0.05). ¥Moderate significantly increased versus no oxidation (P<0.05). ∞High significantly increased from no oxidation and low oxidation (P<0.001, P<0.05). C) ¥Moderate significantly increased versus no oxidation (P<0.001). ∞High significantly increased from no oxidation and low oxidation (P<0.001, P<0.01).
Figure 4-4. 100 kDa band intensity (percent difference versus control). Values are mean percentage change ±SE. A) ¥Moderate oxidation significantly increased versus no oxidation (P<0.001). §High oxidation significantly increased from no oxidation, low oxidation and moderate oxidation (P<0.001, P<0.001, P<0.05). B) #Moderate significantly increased versus no oxidation and low oxidation (P<0.01, P<0.05). ∞High significantly increased from no oxidation and low oxidation (P<0.001, P<0.001). C) #Moderate significantly increased versus no oxidation and low oxidation (P<0.001, P<0.01). §High significantly increased from no oxidation low oxidation and moderate oxidation (P<0.001, P<0.001, P<0.05).
Figure 4-5. 37 kDa band intensity (percent difference versus control). Values are mean percentage change ±SE. A) *Low oxidation significantly increased versus no oxidation (P<0.05). #Moderate oxidation significantly increased versus no oxidation and low oxidation (P<0.001, P<0.05). ∞High oxidation significantly increased from no oxidation and low oxidation (P<0.001, P<0.001). B) #Moderate significantly increased versus no oxidation and low oxidation (P<0.01, P<0.05). ∞High significantly increased from no oxidation and low oxidation (P<0.001, P<0.001). C) ¥Moderate significantly increased versus no oxidation (P<0.01). ∞High significantly increased from no oxidation and low oxidation (P<0.001, P<0.01).
Figure 4-6. 30 kDa band intensity (percent difference versus control). Values are mean percentage change ±SE. A) *Low oxidation significantly increased from no oxidation (P<0.05). ¥Moderate oxidation significantly increased versus no oxidation (P<0.001). §High significantly increased from no, low and moderate oxidation (P<0.001, P<0.001, P<0.01). B) *Low significantly increased from no oxidation (P<0.01). ¥Moderate significantly increased versus no oxidation (P<0.001). §High significantly increased from no and low oxidation (P<0.001, P<0.001).
Figure 4-7. Myosin protein (percent difference versus control). Values are mean percentage change ±SE. A) *Low oxidation significantly increased from no oxidation (P<0.01). #Moderate significantly increased versus no and low oxidation (P<0.001, P<0.05). §High significantly increased versus no and low oxidation (P<0.001, P<0.001, P<0.05). B) *Low significantly increased from no oxidation (P<0.01). #Moderate significantly increased versus no and low oxidation (P<0.001, P<0.05). §High significantly increased from no, low and moderate oxidation (P<0.001, P<0.001, P<0.05). C) *Low significantly increased from no oxidation (P<0.001). ¥Moderate significantly increased versus no oxidation (P<0.001). ∞High significantly increased from no and low oxidation (P<0.001, P<0.001).
Figure 4-8. Actin protein (percent difference versus control). Values are mean percentage change ±SE. A) *Low oxidation significantly increased from no oxidation (P<0.05). ¥Moderate oxidation significantly increased versus no oxidation (P<0.001). ‡High oxidation significantly increased from no and low oxidation (P<0.001, P<0.05). B) ¥Moderate significantly increased versus no oxidation (P<0.01). ‡High significantly increased from no oxidation (P<0.001). C) ¥Moderate significantly increased versus no oxidation (P<0.05). ‡High significantly increased from no oxidation and low oxidation (P<0.001, P<0.05).
Figure 4-9. α-actinin protein (percent difference versus control). Values are mean percentage change ±SE. A) *Low oxidation significantly increased from no oxidation (P<0.001). ¥Moderate oxidation significantly increased versus no oxidation (P<0.01). ∞High oxidation significantly increased from no oxidation and low oxidation (P<0.001, P<0.05). B) ¥Moderate significantly increased versus no oxidation (P<0.01). ∞High significantly increased from no oxidation and low oxidation (P<0.001, P<0.001, P<0.05). C) ‡High significantly increased from no oxidation (P<0.01).
Figure 4-10. Troponin protein (percent difference versus control). Values are mean percentage change ±SE. A) ¥Moderate significantly increased versus no oxidation (P<0.05). ‡High significantly increased from no oxidation (P<0.01). B) ¥Moderate significantly increased versus no oxidation (P<0.01). ‡High significantly increased from no oxidation (P<0.01). C) ¥Moderate significantly increased versus no oxidation (P<0.05). ‡High significantly increased from no oxidation (P<0.01).
Overview of Principal Findings

These experiments provide new and important information regarding the effects of myofibrillar protein oxidation on the susceptibility to degradation by the proteases calpain (I and II) and caspase-3. We tested the hypothesis that protein oxidation-induced modification of myofibrillar proteins would increase their susceptibility to degradation by calpain and caspase-3. Our findings support this postulate as increased levels of protein oxidation augmented myofibrillar protein cleavage and degradation. A detailed discussion providing an interpretation of our experiments follows.

Calpains and Caspase-3 Release Myofilaments

The ubiquitin-proteasome system has been shown to be a major proteolytic system activated in skeletal muscle during periods of disuse. Evidence to support this statement comes from two key observations: 1) atrophying muscles contain an accumulation of ubiquitin conjugated proteins and increased proteasome activity; and 2) pharmacological inhibition of proteasome activity retards disuse muscle atrophy (5, 43, 65, 79, 86). However, the proteasome is incapable of breaking down intact actomyosin complexes, which constitute the bulk of muscle protein (42, 55, 78). Therefore, disassociation of actomyosin complexes appears to be the rate-limiting step in muscle protein degradation (78).

Growing evidence indicates that both calpain (I and II) and caspase-3 are capable of degrading intact actomyosin complexes. In this regard, Mitch and colleagues were the first to report that caspase-3 is capable of cleaving actomyosin complexes and myosin (14). These results suggest that caspase-3 may play a critical role in the initiation of muscle protein degradation because release of actomyosin complexes is required for subsequent protein
degradation by the ubiquitin proteasome system (55). Moreover, evidence also indicates that increased calpain activity promotes degradation of many structural and regulatory myofibrillar proteins (8, 20). This is significant because calpain activity is elevated in skeletal muscles during disuse and calpain activation lowers the levels of the cytoskeletal protein desmin in muscles exposed to prolonged periods of unloading (15).

To examine whether calpain (I and II) and caspase-3 are capable of cleaving intact actomyosin we performed preliminary experiments by treating intact actomyosin with active forms of each protease, and measuring protein degradation via peptide mapping. Our findings revealed that intact actomyosin is degraded by both calpains and caspase-3 (data not shown).

**Induction of Oxidative Stress during Disuse Atrophy**

Previous work from our lab has revealed that MV promotes diaphragmatic oxidative injury (7, 10, 16, 52, 53, 64, 74, 76, 91, 94). Therefore we postulated that oxidative modification of myofibrillar proteins during MV and other models of disuse atrophy caused these proteins to be more readily recognized and degraded by calpain (I and II) and caspase-3. To test this postulate, we oxidized myofibrillar proteins to varying degrees to replicate the three different levels of oxidative damage observed following 6, 12, and 18 hours of MV. Our results confirm that our experimental protocol was successful in oxidizing myofibrillar proteins to achieve three significantly different levels and that as protein oxidation increased, so did protein degradation by both calpain and caspase-3. This important finding will be discussed in detail in the next segment.

**Oxidation of Myofibrils Increases Protein Degradation by Calpain and Caspase-3**

Muscle proteins are differentially susceptible to oxidative modification during exposure to oxidants (27, 94). Our lab has previously shown that prolonged MV promotes oxidation of numerous diaphragmatic proteins including actin and myosin (94). Both actin and myosin are
degraded by calpains at a slow rate, however when these substrates become chemically modified they become more rapidly degraded by calpain I and II (19-22).

Our peptide maps of the proteolytic digests supported our hypothesis that oxidative modification of myofibrillar proteins increases the susceptibility of numerous proteins to degradation by calpain (I and II) and caspase-3. Compared to control, oxidized myofibrils exhibited an enhanced rate of protein breakdown at many molecular weights (e.g., 250, 100, 37, 30 kDa). In general, the magnitude of protein degradation increased as a function of the level of oxidation.

To identify the specific proteins that were undergoing degradation, we used monoclonal antibodies against four different proteins that matched the molecular weights of the protein bands that were degraded by calpains and caspase-3. This analysis revealed that the 250 kDa band was myosin heavy chain, the 100 kDa band was α-actinin, and the 37 kDa band was actin. Troponin I was also probed because it is a known substrate of each protease. Our results show that increased oxidative modification to each of these proteins resulted in increased degradation by both calpain (I and II) and caspase-3. Although some variation existed among these proteins, all of these substrates showed that increasing levels of oxidation from low to high resulted in a progressive rise in protein degradation.

Conclusions and Future Directions

In summary, our data demonstrate that calpain (I and II) and caspase-3 rapidly degrade numerous myofibrillar proteins. It also reveals that the protein oxidation resulting from prolonged periods of disuse is capable of enhancing the vulnerability of numerous proteins to degradation by calpain and caspase-3. These results also highlight the importance in attenuating oxidative stress during skeletal muscle disuse in order to decrease the enhanced cleavage of sarcomeric proteins and subsequently decrease the rate of disuse-induced atrophy.
Although it is now clear that oxidative stress promotes disuse muscle atrophy, numerous unresolved issues exist. For example, are disuse-induced redox disturbances responsible for the activation of both calpain and caspase-3 in skeletal muscles? Moreover, future studies should address the possibility that cross-talk may exist between calpain and caspase-3. That is, theoretically, active caspase-3 can promote calpain activation and vice-versa. Therefore, determining if there is potential for synergistic activity between these two proteases would assist in developing a genetic or pharmacological approach to inhibiting one or both of these proteases during muscle wasting conditions. Another important area for future studies would be to determine the site of oxidant production in skeletal muscles during prolonged disuse. Identifying the pathway(s) responsible for oxidant production in inactive skeletal muscle would provide the insight needed for the development of optimal therapeutic strategies to prevent or retard disuse-induced oxidant damage in skeletal muscle. Indeed, improving our understanding of the cell signaling pathways that regulate disuse muscle atrophy remains an exciting area for future research.


45. **Levine RL, Oliver CN, Fulks RM, and Stadtman ER.** *Proc Natl Acad Sci USA* 78: 2120-2124, 1981.


BIOGRAPHICAL SKETCH

Ashley Joslin Smuder was born in Naples, Florida. She earned a Bachelor of Science degree in exercise physiology from the University of Florida. After graduation, she pursued a master’s degree in exercise physiology and began her graduate work at the University of Florida in 2007 under the direction of Scott K. Powers. Ashley focused her studies on oxidative stress and proteolysis of the diaphragm during prolonged mechanical ventilation. She received her Master of Science degree in 2008.