Free Radical Biology & Medicine 49 (2010) 1152–1160

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Oxidation enhances myofibrillar protein degradation via calpain and caspase-3

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ARTICLE INFO

Article history:
Received 13 November 2009
Revised 10 May 2010
Accepted 21 June 2010
Available online 30 June 2010

Keywords:
Disuse muscle atrophy
Calpain
Caspase-3
Protein oxidation
Proteolysis
Oxidative stress
Reactive oxygen species
Free radicals

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 links oxidative stress to muscle protein degradation remains 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 20 S proteasome. However, it is unknown whether oxidation of myofibrillar proteins increases their recognition and degradation by calpains and/or caspase-3. Therefore, we tested the hypothesis that oxidative modification of myofibrillar proteins increases their susceptibility to degradation by both calpains 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 connecting oxidative stress with accelerated myofibrillar proteolysis during disuse muscle atrophy.

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.

Abundant evidence indicates that disuse skeletal muscle atrophy occurs because of 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 because of 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 oxidative modification of muscle proteins that increases their susceptibility to proteolytic degradation. In this regard, prior research reveals that oxidation can enhance substrate recognition of 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 (6 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

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0891-5849/$ – see front matter © 2010 Published by Elsevier Inc.
doi:10.1016/j.freeradbiomed.2010.06.025

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 (Fig. 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 h, and animals ventilated for 18 h 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, and a protease inhibitor cocktail (Sigma). The homogenate was then centrifuged at 4 °C for 12 min at 1500 *g*. 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 (pH 7.4), and 1.0 mM dithiothreitol. Protein concentration was determined using the Bradford technique [15].

In vitro oxidation of myofibrillar protein

After myofibrillar isolation, protein samples ($n=8$) were randomly divided into four groups. Proteins in group 1 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 ($^{\bullet}OH$) 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 min. At the completion of this oxidation period, the proteins

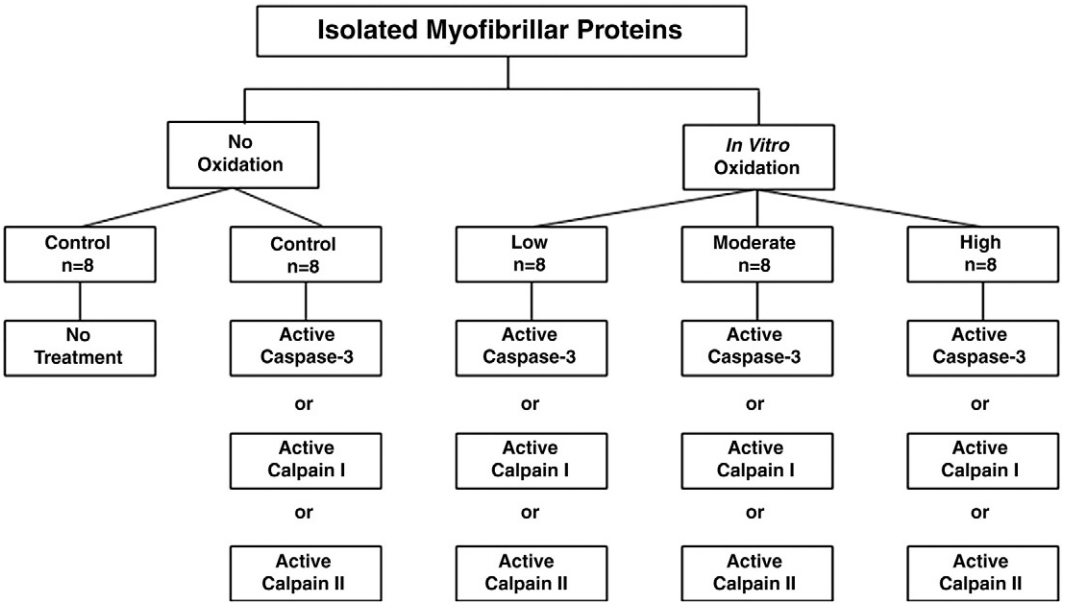

Fig. 1. Experimental design for investigating the effects of myofibrillar protein oxidation on protein degradation by caspase-3 and calpains.