Myofibrillar Proteolysis in Chick Myotubes during Oxidative Stress

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Summary Changes in protein conformation and proteolysis in chick myotubes in response to the induction of oxidative stress by H₂O₂ treatment were studied. Myotubes were treated for 1 h with H₂O₂. After this treatment, the H₂O₂ was removed and the cells were cultured in serum-free medium for 6 and 24 h. Protein carbonyl content, as an index of protein modification, was increased at 6 and 24 h after H₂O₂ treatment. N⁷-methylhistidine release, as an index of myofibrillar proteolysis, was also increased at 6 and 24 h after H₂O₂ treatment. Calpain and cathepsin (B+L and D) activities were increased at 24 but not 6 h after H₂O₂ treatment. Proteasome activity was increased at 6 and 24 h after H₂O₂ treatment. These results indicate that oxidative stress increased proteasome activity and caused an increase in myofibrillar proteolysis during short-term incubation, whereas it increased calpain, proteasome and cathepsin activities during long-term incubation, finally resulting in an increase of myofibrillar proteolysis in chick myotubes.

Key Words myofibrillar proteolysis, proteasome, oxidative stress, chick myotubes, H₂O₂

Reactive oxygen species or free radicals produced either as a natural consequence of cellular metabolism or as a result of pathological events are known to attack lipids and protein, resulting in cellular injury. Oxidative damage of proteins can result in reduced biological functions and enhanced susceptibility to proteolysis (1–3). Proteins modified by reactive oxygen species or free radical attack are easily degraded by proteasome (3, 4) and some proteins with unusually long half lives, such as crystalline in lens, are particularly susceptible targets for free radical attack (4, 5). Skeletal muscle comprises about 40% of body weight and has very important roles in organ and whole body protein and amino acid metabolism. The turnover rate of skeletal muscle protein is comparatively slower than that of other tissue proteins (6) and therefore skeletal muscle protein is potentially highly susceptible to protein fragmentation or conformational changes induced by free radicals. Lipid peroxidation is intimately involved in the pathogenesis of cellular injury by oxygen radicals (7), and evidence for the involvement of free radicals in muscular dystrophy has been reported (8). Skeletal muscle proteins could be damaged (9) or modified (10) by oxidative stress. It is known that, in most cells, intracellular proteolytic enzymes selectively degrade oxidatively modified proteins (11–13). Proteolytic capabilities are therefore considered to be part of a secondary defense system that can avert or delay the accumulation of altered proteins (14, 15). However, the biochemical pathway for the degradation of muscle protein and its mode of activation under oxidative stress are still

obscure.

Like other tissues, skeletal muscle contains multiple proteolytic systems, each of which could play important roles in muscle atrophy. Intracellular proteolysis is carried out by lysosomal and non-lysosomal pathways in which intracellular proteases are directly responsible for the degradation of proteins. Calpains, cysteine proteases in the cytosol, are thought to be the main agents of non-lysosomal Ca²⁺-dependent proteolysis which occurs within the myofibril, and are capable of carrying out the initial step in myofibrillar proteolysis (16, 17). Proteasome, the multicatalytic protease complexes in the cytosol, is also thought to be responsible for non-lysosomal ATP-dependent proteolysis (18). Although proteasome has been implicated in the regulation of myofibrillar protein degradation (19, 20), its substrates and the control of its activity has not been fully studied in muscles. Proteasome may degrade muscle proteins released due to the action of the other proteases. Cathepsins, the main agents of lysosomal degradation, have been well established to contribute to muscle protein breakdown (21). Lysosomal proteases degraded sarco-plasmic proteins and released myofibrillar proteins (22). However, the precise roles of all these degradation systems in the breakdown of skeletal muscle proteins have yet to be determined. Thus, in the present study, the effect of oxidative stress on protein modification, myofibrillar proteolysis and protease (calpain, proteasome and cathepsins) activities in chick myotubes were investigated.

N⁷-methylhistidine is an amino acid formed by the post-translational methylation of specific histidine residues in myofibrillar proteins, actin and myosin. N⁷-methylhistidine cannot charge tRNA and therefore cannot be reutilized for protein synthesis. Because of this,

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Abbreviations: H₂O₂, hydrogen peroxide; TBARS, 2-thiobarbituric acid-reactive substrate.

and also because it does not undergo catabolism, the output of *N*⁷-methylhistidine has been used as an index of myofibrillar proteolysis (23, 24).

MATERIALS AND METHODS

Cells were isolated from the thigh muscles of 13-d-old chick embryos (25). Briefly, the muscle tissue obtained from the embryos was digested with dispase. The cell suspension was transferred to a 35-mm uncoated culture dish to allow fibroblast attachment. Cell numbers were counted and plated onto gelatin-coated 6-well plates at a density of 2.0×10^5 cells/well. The cells were cultured in M-199 containing 15% calf serum and 2.5% chicken embryo extract (basal medium) and were grown at 37°C in a 5% CO₂-enriched atmosphere of humidified air. The medium was replaced every other day during a 7-d incubation period, at the end of which 90% of the cells had formed myotubes. The myotubes were treated for 1 h at 37°C with H₂O₂ (0.5 and 1 mM) in serum-free M-199 medium. After this treatment the H₂O₂-containing medium was removed and the cells were cultured in fresh serum-free M-199 medium for 6 and 24 h.

At the end of the incubation, the medium was collected, the cell monolayer was washed three times with ice-cold PBS and the cells were detached by scraping with a rubber policeman using homogenization solution (20 mM Tris-HCl, 5 mM EDTA, 10 mM dithiothreitol, pH 7.5). The homogenate was centrifuged at $30,000 \times g$ for 30 min at 4°C. The supernatant was used for the measurement of total calpain (μ - plus m-calpain) and total proteasome (20S plus 26S proteasome) activities. Calpain activity was measured by the method of Sasaki et al. (26), using 0.2 mM succinyl-Leu-Leu-Val-Tyr-MCA, a fluorogenic synthetic peptide, as a substrate at pH 7.3. Proteasome activity was analyzed by the method of Tanaka et al. (27), determined with succinyl-Leu-Leu-Val-Tyr-MCA, a fluorogenic synthetic peptide, as a substrate at pH 8.0.

Activities of cathepsin B+L were measured by the method of Barrett and Kirschke (28) using the fluorogenic peptide, 10 μ M Z-Phe-Arg-MCA as a substrate at pH 5.5. Since this synthetic substrate is hydrolyzed by cathepsin L and also by cathepsin B, the activity represents cathepsin B+L activity. The activity of cathepsin D was measured by the method of Barrett and Kirschke (29) using 2% hemoglobin as a substrate. The cells were washed three times with the homogenization solution (20 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 2 mM EDTA, and 2 mM EGTA). It was then homogenized with 1 mL of the homogenization solution containing 0.2% Triton X-100, and lysed by sonication. The homogenate was centrifuged at $18,000 \times g$ for 15 min. The supernatant was dialyzed against the same amount of glycerol and stored at -80°C until analysis.

*N*⁷-Methylhistidine released into the medium was measured by the HPLC method after derivatization of fluorescamine with a treatment of perchloric acid and heating (30). 2-Thiobarbituric acid-reactive substrate (TBARS) was determined according to the method of

Ohkawa et al. (31). The protein carbonyl content of both the homogenate was measured using 2,4-dinitrophenylhydrazine (DNPH) (32). The carbonyl content was calculated using the molar absorbance, $21,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Proteins were measured by Lowry's method using bovine serum albumin as a standard (33).

Data were analyzed by one-way analysis of variance and Duncan's multiple comparison test. A *p* value <0.05 was considered to be statistically significant. Each result is the mean $\pm$ SD of the values obtained from six replicates.

RESULTS AND DISCUSSION

In the present study, the effect of oxidative stress on protein modification, myofibrillar proteolysis and protease (calpain, proteasome and cathepsins) activities in myotubes was investigated. The results of TBARS, protein carbonyl content and *N*⁷-methylhistidine release are shown in Fig. 1. TBARS (A), an index of lipid peroxidation, was significantly (*p*<0.05) increased at 6 and 24 h after H₂O₂ (0.5 and 1 mM) treatment. This indi-

Fig. 1. Effect of oxidative stress on TBARS (A), protein carbonyl content (B) and *N*⁷-methylhistidine release (C) in chick myotubes. Myotubes were treated for 1 h with H₂O₂ (0.5 and 1 mM). After this treatment the H₂O₂ was removed and the cells were cultured in serum-free medium for 6 and 24 h. Values with different letters are significantly different (*p*<0.05). Values are means $\pm$ SD of six replicates.

cates that H_2O_2 induces lipid oxidation in chick myotubes, which suggests that peroxy-radicals in addition to oxygen free radicals may have been involved in H_2O_2 -induced protein oxidation. Lipid radicals have been reported to damage proteins (34), and in particular can cause protein fragmentation (35). Protein carbonyl content (B), an index of oxidatively modified proteins, was significantly ($p < 0.05$) increased at 6 and 24 h after H_2O_2 (0.5 and 1 mM) treatment. This indicates that H_2O_2 induces modifications of proteins in chick myotubes. The change in TBARS paralleled that in protein carbonyl. It has been shown that oxidative stress causes an increase in protein carbonyl content in muscle (10, 36). Several studies examining the modification of proteins induced by oxidative stress have identified changes in amino acids that resulted in fragmentation and conformational modifications of proteins and subsequent changes in protein functionality (37, 38). N^7 -Methylhistidine is an amino acid formed by the post-translational methylation of specific histidine residues in the myofibrillar proteins, actin and myosin. N^7 -Methylhistidine cannot charge tRNA and therefore cannot be reutilized for protein synthesis. Because of this, and also because it does not undergo catabolism, the output of N^7 -methylhistidine has been used as an index of myofibrillar proteolysis (23, 24). N^7 -Methylhistidine release (C) as an index of myofibrillar proteolysis was significantly ($p < 0.05$) increased at 6 and 24 h after H_2O_2 (0.5 and 1 mM) treatment. This indicates that oxidative stress induces myofibrillar proteolysis in chick myotubes. These results are consistent with previous reports of increased protein degradation using [3H]-phenylalanine release in the medium of cultured muscle cells (39) subjected to oxidative stress as an index. However, the effect of oxidative stress on myofibrillar protein degradation in myotubes has not been reported previously. Our present results show that oxidative stress induces protein modification and myofibrillar proteolysis in chick myotubes.

The main purpose of the present experiments was to determine the proteolytic mechanism(s) involved in the oxidative stress-dependent increase in muscle protein degradation. The total activities of calpain (μ - plus m-calpain) and proteasome (20S plus 26S proteasome) are shown in Fig. 2. Calpain activity (A) was significantly ($p < 0.05$) increased at 24 but not 6 h after H_2O_2 (0.5 and 1 mM) treatment. Calpain has been implicated in the degradation of myofibrillar elements of muscle (16, 17). Proteasome (B) activity was significantly ($p < 0.05$) increased at 6 and 24 h after H_2O_2 (0.5 and 1 mM) treatment. Proteasome is an ATP-dependent multicatalytic proteolytic system (18). The multiple proteolytic pathways are essential for complete proteolysis. An increase in surface hydrophobicity has been used to demonstrate intracellular protein degradation using the hemoglobin breakdown by proteasome during exposure to H_2O_2 (40). Another study demonstrated an increase in ubiquitin-protein conjugate during the recovery of bovine lens epithelial cells following the induction of oxidative stress by H_2O_2 (41). Gomes-Mar-

Fig. 2. Effect of oxidative stress on calpain (A) and proteasome (B) activities in chick myotubes. Myotubes were treated for 1 h with H_2O_2 (0.5 and 1 mM). After this treatment the H_2O_2 was removed and the cells were cultured in serum-free medium for 6 and 24 h. Values with different letters are significantly different ($p < 0.05$). Values are means $\pm$ SD of six replicates.

condes and Tisdale (39) measured expression levels of proteasome subunits (20S α -subunit and 19 S regulator p42) and ubiquitin-conjugating enzyme (E2_{14k}) by Western blotting, concluding that ubiquitin-proteasome pathways are affected by oxidative stress-induced proteolysis in C₂C₁₂ myotubes. However, increased levels of expression for various enzymes do not necessarily reflect increased proteolytic activity, indicating that there is not a necessary relationship between expression levels and protein degradation (42).

The results of cathepsin B+L and D activities are shown in Fig. 3. Cathepsin B+L and D activities were measured by using separately cultured cells. Cathepsin B+L (A) activity was significantly ($p < 0.05$) increased at 24 but not 6 h after H_2O_2 (0.5 and 1 mM) treatment. Cathepsin D (B) activity was significantly ($p < 0.05$) increased for 24 but not 6 h after H_2O_2 (0.5 and 1 mM) treatment. Lysosomal cathepsin B and L are endopeptidases that are thought to play major roles in intracellular protein degradation (43). Lysosomal proteases may degrade released myofibrillar proteins (22). Several investigations have suggested another possibility, namely that oxidative stress or Ca^{2+} -dependent phosphorylation may induce structural alteration of proteins, subsequently leading to ubiquitination (44). Furthermore, we observed that oxidative stress, as indicated by indices of lipid peroxidation and protein carbonyl, is increased at 6 and 24 h after H_2O_2 treatment. This suggests that

Fig. 3. Effect of oxidative stress on cathepsin B+L (A) and D (B) activities in chick myotubes. Myotubes were treated for 1 h with H₂O₂ (0.5 and 1 mM). After this treatment the H₂O₂ was removed and the cells were cultured in serum-free medium for 6 and 24 h. Values with different letters are significantly different ($p < 0.05$). Values are means $\pm$ SD of six replicates.

the modification of protein in response to oxidative stress may be related to their proteolysis.

Like other tissues, skeletal muscle contains multiple proteolytic systems, each of which could play an important role in muscle atrophy. Calpains (non-lysosomal Ca²⁺-dependent proteolysis), proteasome (non-lysosomal ATP-dependent proteolysis), and cathepsins (the main agents of lysosomal degradation) have been well established to contribute to muscle protein breakdown. In the present experiment, we measured calpain, proteasome and lysosomal protease (cathepsin B+L and D) activities, and showed that oxidative stress induces calpain, proteasome and cathepsins activities in chick myotubes. This finding confirms that oxidative stress induces multiple proteolytic pathways in chick myotubes. In conclusion, the present study showed that oxidative stress increased proteasome activity and caused an increase in myofibrillar proteolysis during short-term incubation, whereas it increased calpain, proteasome and cathepsin activities during long-term incubation, finally resulting in an increase of myofibrillar proteolysis in chick myotubes.

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