Cysteine Suppresses Oxidative Stress-Induced Myofibrillar Proteolysis in Chick Myotubes

Kazuki Nakashima, Shigehiko Masaki, Makoto Yamazaki, and Hiroyuki Abe

Nutrient Function Laboratory, Department of Animal Physiology and Nutrition, National Institute of livestock and Grassland Science, Tsukuba, Ibaraki 305-0901, Japan

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The effects of cysteine as an antioxidant nutrient on change in protein modification and myofibrillar proteolysis in chick myotubes by induction of oxidative stress by H$_2$O$_2$ treatment were investigated. Myotubes were treated for 1 h with H$_2$O$_2$ (1 mM). After this treatment, the H$_2$O$_2$ was removed and the cells were cultured in cysteine (0.1 and 1 mM) containing serum-free medium for 24 h. Protein carbonyl content as an index of protein modification and N$^\text{ε}$-methylhistidine release as an index of myofibrillar proteolysis were increased at 24 h after H$_2$O$_2$ treatment, and the increment was reduced by cysteine. Calpain, proteasome and cathepsin (B + L and D) activities were increased at 24 h after H$_2$O$_2$ treatment, and the increment was also reduced by cysteine. These results indicate that cysteine suppresses protein modification by oxidative stress, resulting in a decrease of protease activities, finally resulting in a decrease in myofibrillar proteolysis in chick myotubes.

Key words: cysteine; antioxidant nutrient; myofibrillar proteolysis; proteasome; oxidative stress

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 function and enhanced susceptibility to proteolysis. Proteins modified by reactive oxygen species or free radical attack are easily degraded by proteasome, and some proteins with unusually long half lives, such as crystalline in lens, are particularly susceptible targets for free radical attack. Skeletal muscle comprises about 40% of body weight and has very important roles in muscle atrophy. Intracellular proteolytic enzymes selectively degrade oxidatively modified proteins, and proteolytic capabilities are therefore considered to be part of a secondary defense system that can avert or delay the accumulation of altered proteins. 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 operated by lysosomal and non-lysosomal pathways in which intracellular proteases are directly responsible for the degradation of proteins. Calpains, cathepsine proteases in the cytosol, are thought to be the main agents of non-lysosomal Ca$^{2+}$-dependent proteolysis which occurs within the myofibril, and are capable of carrying out the initial step in myofibrillar proteolysis. Proteasome, the multicatalytic proteinase complexes in the cytosol, is also thought to be responsible for non-lysosomal ATP-dependent proteolysis. Although proteasome has been implicated in the regulation of myofibrillar protein degradation, 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. Lysosomal proteases degrade sarcoplasmic proteins and released myofibrillar proteins. However, the precise roles of all these degradation systems in the breakdown of skeletal muscle proteins have yet to be determined. In our previous study, we have showed that oxidative stress stimulates proteases (calpain, proteaseme and cathepsins) activities, resulting in an increase of myofibrillar proteolysis in chick myotubes. This finding confirms that oxidative stress induces multiple proteolytic pathways in chick myotubes.
One of the antioxidant defenses in cells is endogenous thiols (sulfhydryl-containing compounds). Cysteine is a thiol, a most potent biological antioxidant, and also a redox status-regulating amino acid for intracellular glutathione. Droge et al. have reported that supplementation of cysteine or N-acetylcysteine could effectively prevent protein catabolism in cancer cachexia and human immunodeficiency virus infection. Ikemoto et al. have also reported that cysteine supplementation prevents unweighting-induced muscle proteolysis in association with redox regulation in rats. However, the effect of cysteine on oxidative stress-induced myofibrillar proteolysis has not been examined.

Thus, in the present study, the effect of cysteine as an antioxidant nutrient on change in protein modification, myofibrillar proteolysis and protease (calpain, proteasome and cathepsins) activities in chick myotubes by the induction of oxidative stress induced by H$_2$O$_2$ treatment was investigated.

Materials and Methods

Cell culture. Cells were isolated from the thigh muscles of 13-d-old chick embryos. 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 24-well plates at a density of 2.0 × 10$^5$ cells/well. The cells were cultured in minimum essential medium (MEM) containing 15% calf serum and 2.5% chicken embryo extract (basal medium) and were grown at 37°C in a 5% CO$_2$-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 H$_2$O$_2$ (1 mM) in serum-free MEM. After this treatment, the H$_2$O$_2$-containing medium was removed and the cells were cultured in fresh cysteine (0.1 and 1 mM) containing serum-free MEM for 24 h.

Proteases activities. At the end of 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 × g for 30 min at 4°C. The supernatant was used for the measurement of total calpain (m- plus μ-calpain) and total proteasome (20S plus 26S proteasome) activities. Calpain activity was measured by the method of Sasaki et al., 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., determined with succinyl–Leu–Leu–Val–Tyr–MCA, a fluorogenic synthetic peptide, as a substrate at pH 8.0.

The activity of cathepsin B + L was measured by the method of Barrett and Kirschke using the fluorogenic peptide 10 μM 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 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 × g for 15 min. The supernatant was dialyzed against the same amount of glycerol and stored at −80°C until analysis.

Chemical analysis. N’-methylhistidine released into the medium was measured by the HPLC method after derivatization of fluorescamine with a treatment of perchloric acid and heating. The protein carbonyl content of both the homogenate was measured using 2,4-dinitrophenylhydrazine (DNPH). The carbonyl content was calculated using the molar absorbance, 21,000 M$^{-1}$cm$^{-1}$. Proteins were measured by Lowry’s method using bovine serum albumin as a standard.

Statistical analysis. Data were analyzed by one-way analysis of variance and Duncan’s multiple comparison test. A p value of <0.05 was considered to be statistically significant. Each result is the mean ± standard deviation of the values obtained from six replicates.

Results and Discussion

In the present study, the effect of cysteine, an antioxidant nutrient, on protein modification, myofibrillar proteolysis and protease (calpain, proteasome and cathepsins) activities by the induction of oxidative stress induced by H$_2$O$_2$ treatment in chick myotubes was investigated. The results of protein carbonyl content and N’-methylhistidine release are shown in Fig. 1. Protein carbonyl content (A), an index of oxidatively modified proteins, was significantly (P < 0.05) increased at 24 h after H$_2$O$_2$ treatment. The increment of protein carbonyl content due to H$_2$O$_2$ was significantly (P < 0.05) reduced by cysteine (1 but not 0.1 mM). This indicates that cysteine suppresses modifications of proteins by H$_2$O$_2$ in chick myotubes. 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. We reported previously that oxidative stress induces protein modification in chick myotubes. Cysteine, a thiol antioxidant, has also been documented in vivo and in vitro to act as a pro-oxidant, although in most studies it is an effective antioxidant. It has been
reported that cysteine is a protective antioxidant that can prevent oxidative stress. Redox regulation by intrinsic reducing suppliers, including cysteine and thioredoxin, is essential to maintain intact cellular functions against oxidative stress in many mammalian cells. Ikemoto et al. have reported that cysteine supplementation prevents unweighting-induced oxidative stress in muscles of rats. This result is consistent with ours. The present study shows that cysteine suppresses oxidative stress in chick myotubes.

N'-methylhistidine is an amino acid formed by the post-translational methylation of specific histidine residues in the myofibrillar proteins, actin and myosin. N'-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'-methylhistidine has been used as an index of myofibrillar proteolysis. N'-methylhistidine release (B), as an index of myofibrillar proteolysis, significantly increased at 24 h after H₂O₂ treatment. This result is also consistent with previous reports of increased protein degradation using [³H]-phenylalanine release in the medium of cultured muscle cells subjected to oxidative stress as an index. Our previous study also showed that oxidative stress induces myofibrillar proteolysis in chick myotubes. The increment of N'-methylhistidine release due to H₂O₂ was significantly reduced (P < 0.05) by cysteine (1 but not 0.1 mM). This indicates that cysteine suppresses myofibrillar proteolysis by H₂O₂ in chick myotubes. Droge et al. have reported that supplementation of cysteine could effectively prevent protein catabolism in cancer cachexia and human immunodeficiency virus infection. Ikemoto et al. have also reported that cysteine supplementation prevents unweighting-induced muscle proteolysis in association with redox regulation in rats. However, the effect of antioxidant on oxidative stress-induced myofibrillar protein degradation in myotubes has not been reported previously. Our present study indicates that cysteine, an antioxidant nutrient, suppresses modifications of proteins and myofibrillar proteolysis by oxidative stress in chick myotubes.

The main purpose of the present experiments was to determine the proteolytic mechanism(s) involved in the suppression of oxidative stress-induced myofibrillar proteolysis by cysteine. The activities of total calpain (m- plus µ-calpain) and total proteasome (20S plus 26S proteasome) are shown in Fig. 2. Calpain activity (A) significantly increased at 24 h after H₂O₂ treatment.
treatment. The increment of calpain activity due to H$_2$O$_2$ was significantly ($P < 0.05$) reduced by cysteine (1 but not 0.1 mM). Calpain has been implicated in the degradation of myofibrillar elements of muscle.$^{16,17}$ We have previously showed that oxidative stress induced calpain activity in chick myotubes.$^{23}$ However, the effect of antioxidants on oxidative stress-induced calpain activation in myotubes has not been reported previously. Our present result indicates that cysteine suppresses calpain activity by the induction of oxidative stress in chick myotubes. Proteasome (B) activity significantly ($P < 0.05$) increased at 24 h after H$_2$O$_2$ treatment. The increment of proteasome activity due to H$_2$O$_2$ was also significantly ($P < 0.05$) reduced by cysteine (0.1 and 1 mM). 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$_2$O$_2$.$^{41}$ 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$_2$O$_2$. Gomes-Marcondes et al.$^{40}$ measured expression levels of proteasome subunits (20S $\alpha$-subunit and 19 S regulator p42) and ubiquitin-conjugating enzyme (E2$_{14k}$) by Western blotting, concluding that ubiquitin-proteasome pathway are affected by oxidative stress-induced proteolysis in C$_2$C$_{12}$ myotubes. Ikemoto et al. have showed that oxidative stress triggers ubiquitination of muscle protein caused by unweighting.$^{26,43}$ We have also reported that oxidative stress-induced myofibrillar proteolysis in chick myotubes is associated with an increase in proteasome activity.$^{23}$ In addition, several investigations have suggested that oxidative stress or Ca$^{2+}$-dependent phosphorylation may induce structural alteration of proteins, subsequently leading to ubiquitination.$^{44}$ This result is consistent with our result. Ikemoto et al. have reported that cysteine supplementation prevents unweighting-induced muscle proteolysis and ubiquitination in association with redox regulation in rats.$^{26}$ This result is also consistent with ours. Suppression of oxidative stress-induced myofibrillar proteolysis by cysteine in chick myotubes may be associated with a decrease in proteasome activity.

The results of cathepsin B + L and D activities are shown in Fig. 3. Cathepsin B + L and D activities were measured using separately cultured cells. Cathepsin B + L (A) activity significantly ($P < 0.05$) increased at 24 h after H$_2$O$_2$ treatment. The increment of cathepsin B + L due to H$_2$O$_2$ was significantly ($P < 0.05$) reduced by cysteine (1 but not 0.1 mM). Cathepsin D (B) activity was significantly ($P < 0.05$) increased for 24 h after H$_2$O$_2$ treatment. The increment of cathepsin D of due to H$_2$O$_2$ was significantly ($P < 0.05$) reduced by cysteine (1 but not 0.1 mM). Lysosomal cathepsin B and L are endopeptidases that are thought to play major roles in intracellular protein degradation.$^{45}$ Lysosomal proteases may degrade released myofibrillar proteins.$^{22}$ We have also reported that oxidative stress induces cathepsin B + L and D activities in chick myotubes.$^{23}$ However, the effect of antioxidants on oxidative stress-induced cathepsins activation in myotubes has not been reported previously. Our present result indicates that cysteine suppresses cathepsins activity by the induction of oxidative stress in chick myotubes.

In the present study, oxidative stress induces protein modification and resulting in an increase of myofibrillar proteolysis in chick myotubes. Our present results are consistent with previous reports of increased protein degradation using [$^3$H]-phenylalanine release in cultured muscle cells$^{40}$ subjected to oxidative stress. Gomes-Marcondes et al.$^{40}$ have also reported that oxidative stress (H$_2$O$_2$) induces protein degradation using [$^3$H]-phenylalanine release by the ubiquitin-proteasome pathway in C$_2$C$_{12}$ myotubes. We have also reported that oxidative stress-induced myofibrillar proteolysis in chick myotubes is associated with an increase in proteasome activity.$^{23}$ Therefore, it is suggested that protein modification by oxidative stress may relate with its proteolysis. The later is a possibility since oxidatively
damaged protein can be degraded by proteinases, especially by the ubiquitin-proteasome system.\textsuperscript{2,4,23,41,42}

Furthermore, we have observed that cysteine, an antioxidant nutrient, prevents oxidative stress-induced muscle proteolysis. Droge \textit{et al.} have reported that supplementation of cysteine could effectively prevent protein catabolism in cancer cachexia and human immunodeficiency virus infection.\textsuperscript{25} Ikemoto \textit{et al.} have also reported that cysteine supplementation prevents unweighting-induced muscle proteolysis in association with redox regulation in rats.\textsuperscript{26}

Like other tissues, skeletal muscle contains multiple proteolytic systems, each of which could play an important role in muscle atrophy. Calpains (non-lysosomal \textit{Ca}\textsuperscript{2+}-dependent proteolysis), proteasome (nons-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 cathepsin activities in chick myotubes. This finding confirms that oxidative stress induces multiple proteolytic pathways in chick myotubes. In addition, cysteine suppresses oxidative stress-induced myofibrillar proteolysis through the multiple proteolytic pathways in chick myotubes.

In conclusion, the present study showed that cysteine suppresses protein modification by oxidative stress, resulting in a decrease in protease activities, finally resulting in a decrease of myofibrillar proteolysis in chick myotubes.

References


