Interplay Between Aging and Unloading on Oxidative Stress in Fast-Twitch Muscles

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This study evaluated the effect of aging on the adaptation potential of antioxidants and the accumulation of oxidative damage in fast-twitch muscles in response to non–weight-bearing conditions. Adult and old rats were randomized into 4 groups: normal weight bearing, hind-limb unloading for 3, 7, and 14 days. Activities of manganese superoxide dismutase, copper–zinc superoxide dismutase, catalase, and glutathione peroxidase and contents of glutathione, carboxylated proteins, and malondialdehyde were determined in tibialis anterior muscles. We found that the adaptability of most antioxidants in fast-twitch muscles with unloading is intact in aged rats except copper–zinc superoxide dismutase where its activity decreased by 14 days of unloading. Additionally, malondialdehyde accumulated in aged muscles with 14 days of unloading but not adult muscles. Collectively, the adaptation of copper–zinc superoxide dismutase in fast-twitch muscles with unloading is impaired with aging, which may be related to the greater accumulation of malondialdehyde.

Key Words: Antioxidants—Lipid peroxidation—Disuse.

Received August 18, 2012; Accepted November 2, 2012

Decision Editor: Rafael de Cabo, PhD

Individuals who are unable to ambulate secondary to illness, surgeries, or imposed bed rest experience periods of non–weight-bearing and their muscles are considered unloaded. During periods of non–weight-bearing, many changes occur including loss of muscle mass (atrophy) and muscle strength. For example, using magnetic resonance imaging as an assessment tool, muscle mass decreases by 6% in young men and strength decreases by 10%–15% following 7 and 14 days of muscle unloading (1,2). The decreased muscle mass and muscle strength with unloading impair muscle function (3). Interestingly, the unloading-induced muscle dysfunction is age dependent, and the age-dependent muscle dysfunction is significant in fast-twitch muscles. For example, muscle atrophy in fast-twitch muscles after unloading is found in aged rats but not in adult rats (25% decrease vs 10% decrease [4]). In humans, these age-related responses are also reported. In a study by Deschenes and colleagues (2), the force generation at high contractile speed is significantly decreased in the elderly population but not in young individuals following muscle unloading (6% decrease vs 0% decrease [2]). Collectively, these studies indicate that the age of individuals influences the degree or extent of alteration in muscle function of fast-twitch muscles with unloading.

One mechanism underlying the unloading-induced muscle dysfunction is oxidative stress (5–7), a condition where the generation of oxidants exceeds the scavenger capacity of antioxidants. Antioxidants are molecules that buffer the burden of oxidants and are upregulated in response to the increased generation of oxidants (8). During periods of unloading, muscles have a greater generation of oxidants (9–11) and the activities of antioxidants increase (12,13). The upregulation of antioxidants in response to the increased generation of oxidants helps maintain the redox balance of cells and decreases the accumulation of oxidative damage in muscles with unloading.

Although antioxidants adapt in response to the increased generation of oxidants, the adaptive capacity is affected by the age of animals (14,15). In general, the responses of antioxidants with physical perturbations are blunted in aged animals (13,16–18). For example, the adaptation of copper–zinc superoxide dismutase (Cu–ZnSOD) in muscles with 4.5 weeks of repetitive loading is found to be less in aged animals than that in young animals (0% increase vs 43% increase [16]). Consistent with Cu–ZnSOD, manganese superoxide dismutase (MnSOD) shows impaired adaptation potential in aged animals compared with young animals following 13 weeks of endurance exercise training (130% increase vs 40% decrease [17]). Similarly, the adaptive capacities of antioxidants glutathione (GSH) and catalase are attenuated in aged muscles with unloading (13,18). Notably, previous studies investigating the interplay between aging and muscle unloading focused on slow-twitch and mixed fiber–type muscles. Because fast-twitch muscles experience earlier and greater age-related changes compared with slow-twitch muscles (19,20), it is important...
to understand the influence of aging on muscle unloading in fast-twitch muscles.

The aim of this study was to understand the influence of aging on the adaptation of antioxidants and the accumulation of oxidative damage in fast-twitch muscles with non–weight-bearing or unloading. We hypothesized that (a) the adaptation of antioxidants in fast-twitch muscles with unloading would be compromised with aging and (b) the accumulation of oxidative damage would be greater in aged muscles compared with that in adult muscles with unloading. The tibialis anterior (TA) muscle was chosen to be investigated in this study because it is composed predominantly of fast-twitch muscle fibers and is a critical muscle that ensures a normal gait pattern (21,22).

**Methods**

**Animals and Hind-Limb Unloading**

Fischer 344 rats aged 13 months (the age at which more than 90% of rats in this strain are alive, 55–59 years old when matched to humans (23,24); n = 40) and 26 months (the age at which about 25% of rats in this strain are alive, 85–89 years old when matched to humans (23,24); n = 40) were purchased from the Minneapolis Veterans Administration Aged Rodent Colony. Rats in the same age group were randomized into four weight-bearing conditions: normal weight bearing (control; n = 10), hind-limb unloading (HU) for 3 (n = 10), 7 (n = 10), and 14 days (n = 10). The HU intervention was achieved as described previously (18,25). Briefly, the tail of the rat was mounted to a swivel at the top of the cage and the height of the suspension was adjusted to prevent hind limbs from touching the floor. Animals with HU were housed individually, whereas animals with normal weight bearing were group housed. All animals were housed in a research animal facility and were checked daily for any abnormal response to the HU. Rats were anesthetized with pentobarbital sodium (35 mg/kg body weight) after the intervention. TA muscles were collected, weighed, and immediately frozen in liquid nitrogen. The frozen muscles were stored in a –80°C freezer until later analysis. The protocol of this study was approved by Institutional Animal Care and Use Committees of University of Minnesota and Chang Gung University.

**Assays for Antioxidants**

**MnSOD and Cu–ZnSOD activities.**—Activities of MnSOD and Cu–ZnSOD were determined using spectrophotometric assay kits (Cayman, Ann Arbor, MI) according to the manufacturer’s instruction. Briefly, frozen TA muscles were homogenized in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.2) containing 1 mM ethylene glycol tetraacetic acid, 210 mM mannitol, and 70 mM sucrose. The homogenate was centrifuged at 1,500g for 5 minutes at 4°C, and the resulting supernatant was centrifuged again at 10,000g for 15 minutes at 4°C. The supernatant from the 10,000g of centrifugation was the cytosolic fraction and the pellet contained mitochondria. The mitochondrial pellet was homogenized in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer where 3 mM of potassium cyanide was added to inhibit both Cu–ZnSOD and extracellular superoxide dismutase (SOD), resulting in the detection of only MnSOD activity (mitochondrial fraction). Protein concentrations of both cytosolic and mitochondrial fractions were determined by bicinechonic acid (BCA) protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard (18).

When performing the assay, the radical detector tetrazolium salt was added into samples and SOD standards. Next, the enzymatic reaction was initiated by adding xanthine oxidase. After 20 minutes of incubation at room temperature, the generation of superoxide radicals was measured at 450 nm. The activities of MnSOD and Cu–ZnSOD were calculated according to the SOD standard curve generated under identical conditions. Values were expressed in U/mg protein, where 1 U was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

**Catalase activities.**—Catalase activities were determined using spectrophotometric assay kits (Cayman) according to the manufacturer’s instruction. This assay kit measured formaldelyde that was generated from the reaction of the catalase with methanol in the presence of an optimal concentration of H₂O₂. Briefly, frozen TA muscles were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. The homogenate was centrifuged at 10,000g for 15 minutes at 4°C. Protein concentrations of the supernatant were determined by BCA protein assay kit. When performing the assay, methanol was first added into the samples and then H₂O₂ was added to initiate the reaction. The reaction was terminated by adding potassium hydroxide after 20 minutes of incubation at room temperature. Next, 4-amino-3-hydrazino-5-mercaptop-1,2,4-triazole (Purpald), a chromogen, was added and incubated for 10 minutes at room temperature followed by the addition of potassium periodate and another 5 minutes of incubation at room temperature. The color change of Purpald was measured at 540 nm. Catalase activities were calculated based on the formaldehyde standard curve generated under identical conditions. Values were expressed in nmol/min/mg protein.

**Activities of glutathione peroxidase.**—Glutathione peroxidase (GPX) activities were determined using spectrophotometric assay kits (Cayman) according to the manufacturer’s instruction. This assay kit determined the GPX activity by a coupled reaction with GSH reductase. Briefly, frozen TA muscles were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM dithiothreitol (DTT). Protein concentrations of the supernatant were determined by BCA protein assay
kit. When performing the assay, provided assay buffer was added to samples and the enzymatic reactions were initiated by the addition of cumene hydroperoxide. NADPH levels were measured at 340 nm for 5 minutes. GPX activities were determined by the calculation of NADPH oxidation. Values were expressed in nmol/min/mg protein.

**Levels of GSH and glutathione disulfide.**—Levels of GSH and glutathione disulfide (GSSG) were determined using spectrophotometric assay kits (Cayman), according to the manufacturer’s instruction. This assay kit determined the GSH level by the reaction of GSH with 5,5′-dithiobis-(2-nitrobenzoic acid), which produced yellow-colored 5-thio-2-nitrobenzoic acid. The rate of 5-thio-2-nitrobenzoic acid production is proportional to the concentration of the GSH in the sample. Briefly, frozen TA muscles were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. The homogenate was centrifuged at 10,000g for 15 minutes at 4°C. In order to remove proteins from the sample to avoid interferences in the assay, 5% metaphosphoric acid was added to the supernatant followed by 5 minutes of centrifugation at 2,000g, 4°C. Finally, the resulting supernatant was added with 4M of triethanolamine. To measure GSSG, 2-vinylpyridine was added to the deproteinized sample and incubated at room temperature for 1 hour before performing the assay. This procedure conjugated GSH in the sample; thus, only GSSG could be measured. The assay reaction was initiated by adding the provided assay cocktail to standards and samples followed by the measurement at 410 nm for 25 minutes with 5 minute of intervals. GSH and GSSG levels were calculated according to the standard curve generated in the same setting. Values were expressed in µM/mg tissue.

**Markers of Oxidative Damage**

**Oxidative-modified proteins.**—OxiBlot (Oxyblot protein oxidation detection kit; Millipore, Billerica, MA), which detects the carbonyl groups of proteins, was used to determine the level of oxidative-modified proteins in muscles. Briefly, frozen TA muscles were homogenized in buffer containing 50 mM potassium phosphate, 1 mM EDTA, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, and 3 M urea (pH 7.0) followed by the centrifugation at 10,000g for 30 minutes at 4°C. Protein concentrations of the supernatant were determined by BCA protein assay kit. After the sample preparation, 2, 4-dinitrophenylhydrazine was added to the sample to react with the carbonyl groups in the protein side chains that formed 2,4-dinitrophenylhydrazone. Next, equal amounts (25 µg) of protein were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels and separated by electrophoresis using mini-vertical gel electrophoresis units (Bio-Rad, Hercules, CA). Proteins resolved on gels were transferred to polyvinylidene difluoride membranes by Mini Trans-Blot Electrophoretic Transfer Cells (Bio-Rad) at 110V for 90 minutes. The protein load was determined experimentally to be in the linear range of responses for the antibody. The protein-bound membranes were incubated with rabbit anti-dinitrophenyl antibody (1:300 dilution) overnight at 4°C, followed by the incubation with goat anti-rabbit antibody (horseradish peroxidase conjugated, 1:600 dilution) for 1 hour at room temperature. Membranes were then incubated with chemiluminescent reagent (Invitrogen, Carlsbad, CA) and images were captured immediately every 30 seconds until the reaction was saturated by BioSpectrum 500 (Level Biotechnology, Taipei, Taiwan). The optical density of each sample was quantified by densitometry using UN-SCAN-IT (Silk Scientific, Orem, UT). Note, an internal control (standard sample) was loaded and transferred on each blot, and the intensity of immune responses of all samples was normalized to the intensity of the standard sample. This method permitted the comparison of samples across multiple blots (26).

**Malondialdehyde assay.**—Malondialdehyde (MDA), the most abundant carbonyl compound decomposed from lipid peroxides, was determined using spectrophotometric assay kits (OxisResearch, Portland, OR) according to the manufacturer’s instruction. Briefly, frozen TA muscles were homogenized in phosphate-buffered saline buffer containing 5 mM butylated hydroxytoluene followed by 10 minutes of centrifugation at 3,000g, 4°C. Protein concentrations of the supernatant were determined by BCA protein assay kit. Samples and standards with known concentrations were incubated with chromogenic reagent, N-methyl-2-phenylindole, at 45°C for 60 minutes and then measured at 586 nm. Values were expressed in nmol/mg protein.

**Statistics**

Data were presented as mean ± standard error of the mean. Two-way analysis of variance was used to examine the main effect of aging and HU on dependent variables. Tukey’s honest significant difference test was used as a post hoc test when the two main effects have significant interaction. Differences were considered significant when p < .05.

**Results**

**Muscle Weight**

The changes of TA muscle wet weight with HU were age dependent (age × HU, p = .003). In adult rats, muscle wet weight did not change until 14 days of HU, which was 17% lower than that of control rats. In old rats, muscle wet weight started to show significant decline with 7 days of unloading. The muscle wet weights of old rats with 7 and 14 days of HU were 28% lower than that of control rats (Figure 1).


Activity of MnSOD

MnSOD is the predominant isoform of SOD in the mitochondria, where it converts superoxide anions to hydrogen peroxide. HU influenced MnSOD activities in TA muscles; however, the influence was dependent on the age of rats (age × HU, \( p = .03 \); Figure 2). In adult rats, the activity of MnSOD in TA muscles remained stable with HU. In old rats, the activity of MnSOD in TA muscles with 14 days of HU was 13.88 ± 0.92 U/mg protein, which was 1.5-fold of the activity in TA muscles of control weight-bearing rats (9.33 ± 0.84 U/mg protein).

Activity of Cu–ZnSOD

Cu–ZnSOD is an isoform of SOD that locates primarily in the cytosol with a small portion in the mitochondria intermembrane space. HU influenced Cu–ZnSOD activities in TA muscles, however, the influence was dependent on the age of rats (age × HU, \( p = .03 \); Figure 3). In adult rats, the activity of Cu–ZnSOD in TA muscles was unchanged with HU. In old rats, the activity of Cu–ZnSOD in TA muscles with 14 days of HU was 22.91 ± 3.34 U/mg protein, which was significantly lower than that in TA muscles with weight bearing and HU for 3 and 7 days (50.21 ± 6.65, 44.45 ± 6.19, and 55.62 ± 5.33 U/mg protein, respectively).

Activity of Catalase

Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen. Catalase activities in TA muscles of rats with 14 days of HU were 2.1-, 2.4-, and 1.5-fold compared with that in TA muscles of rats with control weight bearing and HU for 3 and 7 days, respectively. Regarding the age effect, catalase activities in TA muscles increased with aging (\( p = .02 \); Figure 4).

Activity of GPX

GPX catalyzes the reduction of hydrogen peroxide to water and the reduction of lipid hydroperoxides to their corresponding alcohols (27). Aging influenced the unloading-induced changes of GPX activities in TA muscles (age × HU, \( p = .01 \); Figure 5). In adult rats, the activity of...
GPX decreased with HU, where the activities of GPX of rats with 3, 7, and 14 days of HU were 29%, 25%, and 28% less, respectively, than that in muscles of rats with weight bearing (control group). In old rats, the activity of GPX did not show significant changes.

**GSH and GSSG Levels**

GSH, an important nonenzymatic antioxidant in cells, protects cells by being a cofactor in redox reactions as well as direct conjugation with oxidants. HU altered the GSH level in TA muscles, independent of age (age × HU, p = .30; Figure 6). The level of GSH in TA muscles of rats with 7 days of HU was 1.4-fold compared with that in TA muscles of rats with control weight bearing. GSSG, the oxidized form of GSH, did not show significant changes with HU or aging (Figure 7).

**Level of the Overall Oxidative-Modified Proteins**

Aging influenced the accumulation of the overall oxidized proteins in TA muscles with HU (age × HU, p = .03; Figure 8). In adult rats, the accumulation of oxidized proteins with HU was not significant. In old rats, the accumulation of oxidized proteins in muscles with 7 and 14 days of HU was lower than that in muscles with normal weight bearing and 3 days of HU.
MDA is the compound decomposed from lipid peroxides and is used as a marker of lipid oxidation of cells. HU affected the level of MDA in TA muscles; however, the effect was dependent on the age of rats (age × HU, \( p = .01 \); Figure 9). In adult rats, the MDA level in TA muscles did not change with HU. In old rats, the MDA level in TA muscles of rats with 14 days of HU was 1.65-, 1.85-, and 2-fold compared with that in TA muscles of rats with weight bearing and 3 and 7 days of HU.

**Discussion**

**Overview of Main Findings**

The aim of this study was to evaluate the effect of aging on the adaptation of antioxidants and the accumulation of oxidative damage in fast-twitch muscles with unloading. We found that the adaptability of most of the key antioxidants in fast-twitch muscles is intact in aged animals except Cu–ZnSOD under conditions of unloading. The activities of Cu–ZnSOD decreased in aged fast-twitch muscles with 14 days of unloading, whereas the activities of Cu–ZnSOD remained stable in adult muscles. Consistent with the impaired adaptation of Cu–ZnSOD in aged muscles, MDA accumulated significantly in aged muscles with 14 days of unloading. Contrary to our expectation, unloading did not induce a greater accumulation of carbonylated proteins in aged fast-twitch muscles, instead, the level of carbonylated proteins decreased in aged muscles.

**Adaptation of Antioxidants in Fast-Twitch Muscles With Aging and Unloading Is Varied**

We found the effect of aging on antioxidant adaptation in fast-twitch muscles (TA) with unloading is antioxidant specific. GSH and catalase adapted positively in muscles with unloading, regardless of the age of animals, whereas unloading-induced changes in MnSOD, Cu–ZnSOD, and GPX activities were age dependent. The finding of the antioxidant-specific responses in muscles with unloading and aging is similar to other studies. For example, Lawler and colleagues (28) found that 28 days of HU increased the activities of Cu–ZnSOD in slow-twitch muscles (soleus) of rats; however, the activities of MnSOD, GPX, and catalase were decreased. Servais and colleagues (12) found that while there was no change in MnSOD activities in slow-twitch muscles (soleus) of rats with 14 days of HU, activities of Cu–ZnSOD, GPX, and catalase had a compensatory increase. Similarly, Siu and colleagues (13) found that 14 days of HU increased catalase activities in mixed fiber–type muscles (gastrocnemious), but the activities of MnSOD and GPX were unchanged. Collectively, the antioxidants in skeletal muscles respond or adapt under conditions of unloading, however, the response is varied or antioxidant specific.

The varied responses of the antioxidants may be related to transcriptional and posttranscriptional regulations of each antioxidant. Specifically, each antioxidant has its unique genetic organization with some similarity between antioxidants. For example, although both Cu–ZnSOD and MnSOD have binding sites for nuclear factor-κB, activator protein-1, and activator protein-2, Cu–ZnSOD has the antioxidant-response elements in the promoter regions and MnSOD has the Forkhead box, class O (FOXO) binding site (29,30). Another mechanism that may cause the varied responses of antioxidant activity is posttranslational modification. For example, the catalytic activities of catalase
were found increased when it was posttranslationally modified by the activated c-Ab1-Arg complex in the environment with increased H$_2$O$_2$ concentration (31). The antioxidant-specific responses to the posttranslational modification were also seen in SOD. Posttranslational modifications by glycation and glutathionylation result in the decrease of enzymatic activity of Cu–ZnSOD but not MnSOD (32). Taken together, the antioxidant-specific transcriptional and posttranscriptional regulations may be responsible for the varied responses of antioxidants in muscles with unloading.

**Adaptations of GSH and Catalase in Muscles With Unloading are Age Independent**

We found that GSH and catalase in TA muscles adapted positively with HU, regardless of the age of animals. This finding suggests that (a) unloading causes a stress to fast-twitch muscles and (b) aged muscles have the capacity to respond to unloading. This finding is consistent with a previous report where levels of superoxide were found elevated in fast-twitch muscles with unloading (9). Similar to what was observed in muscles composed of primarily fast-twitch fibers, unloading induces greater levels of oxidants, altered antioxidant activities, and accumulation of oxidative damage in slow-twitch muscles and mixed fiber-type muscles with unloading (13,18,28). Taken together, unloading causes stress not only in slow-twitch muscles that are extensively investigated but also in fast-twitch muscles. Our results extend the current knowledge about oxidative stress in muscles with unloading by demonstrating an upregulation of antioxidants in fast-twitch muscles.

**Adaptations of MnSOD, Cu–ZnSOD, and GPX in Muscles With Unloading are Age Dependent**

The adaptations of MnSOD, Cu–ZnSOD, and GPX in fast-twitch muscles with unloading were found to be age dependent in this study. In our previous study, where a sensitive technique was used to determine superoxide production, we found the production of superoxide, both inside and outside of mitochondria, in fast-twitch muscles with 14 days of unloading was greater in old rats than that in adult rats (9). Based on this finding, we predicted the activities of MnSOD (the predominant isoform of SOD in the mitochondria, where it converts superoxide to hydrogen peroxide) and Cu–ZnSOD (the predominant isoform of SOD in cytosol) would have a compensatory increase in muscles with unloading. In addition, the extent of the positive adaptation of antioxidants would be greater in aged rats than that in adult rats. We found MnSOD activity increased in muscles of aged rats with 14 days of unloading, but the activity did not change in muscles of adult rats. This finding suggests that MnSOD in aged fast-twitch muscles have the capacity to adapt with unloading. The nonsignificant change of MnSOD activity in adult muscles with unloading is probably because the increase of the superoxide level in adult muscle with unloading is not high enough to induce significant adaptation of MnSOD.

The mechanism underlying the unloading-induced increase of MnSOD activity in fast-twitch muscles of old rats is unknown, however, transcriptional and posttranscriptional mechanisms may be involved. The expression of MnSOD gene is regulated at both the transcriptional level and the posttranscriptional level (29). Transcriptional factors that are involved in the regulation of MnSOD gene include nuclear factor-κB, activator protein-1, activator protein-2, and Forkhead box, class O3a. Through these transcriptional factors, stimuli such as proinflammatory cytokines and oxidative stress can rapidly modulate MnSOD gene. Posttranscriptional mechanisms regulate the expression of MnSOD gene by changing the stability of mRNA, affecting mRNA translation and posttranslational modification. For example, redox balance is one factor that regulates the expression of MnSOD gene posttranscriptionally (29).

Further studies are needed to understand the mechanism underlying the unloading-induced increase of MnSOD activity in fast-twitch muscles of old rats.

The second enzyme investigated in this study that showed age-dependent changes with unloading was Cu–ZnSOD. This enzyme catalyzes the conversion of superoxide outside of the mitochondrial inner membrane to hydrogen peroxide. In adult rats, the changes of Cu–ZnSOD activities were not significant. In contrast, the activities of Cu–ZnSOD decreased dramatically in muscles of old rats with 14 days of unloading. This finding did not follow our expectation where aged muscles were expected to have a greater positive adaptation in Cu–ZnSOD compared with adult muscles since previous research reported that unloading-induced increase of superoxide production outside the mitochondria in fast-twitch muscles was greater in old rats compared with adult rats (9). Thus, the decreased Cu–ZnSOD activity in aged muscles with 14 days of unloading implies a poor adaptation of Cu–ZnSOD in fast-twitch muscles with 14 days of unloading in old animals. The poor adaptation of Cu–ZnSOD may be deleterious because the high levels of superoxide can react with nitric oxide and form peroxynitrite, a highly cytotoxic compound.

The adaptation potential of Cu–ZnSOD in fast-twitch muscles of old animals was questioned before and was reported to be limited in animals following exercise training (16,33). The regulation of the Cu–ZnSOD gene is likely involved in the age-related adaptation of Cu–ZnSOD activity in muscles of old rats with additional stimuli. Transcriptional factors involved in the regulation of the Cu–ZnSOD gene include nuclear factor-κB, activator protein-1, activator protein-2, and NF-E2-related factor 2. The impaired activation, translocation, or the binding to DNA of these transcriptional factors would limit the adaptation of the Cu–ZnSOD gene with stimuli (29). Another potential mechanism to explain the age-dependent adaptation of Cu–ZnSOD with additional stimuli is posttranslational
modify the reactivity of Cu-ZnSOD. Posttranslational modifications such as nitration, glutathionylation, and glycation were found to decrease the activity of Cu-ZnSOD (32). Importantly, oxidative stress and aging are factors that promote these posttranslational modifications (32).

GPX was another antioxidant that showed age-dependent adaptation in fast-twitch muscles with unloading. The functions of GPX are to catalyze the reduction of hydrogen peroxide to water and lipid hydroperoxides to their corresponding alcohols. We found the activity of GPX decreased in adult muscles, but it remained unchanged in aged muscles with unloading. The results are difficult to interpret because GPX activities were unchanged in slow and mixed fiber-type muscles with unloading, regardless of the age of animals (13,18). Because GPX includes several isozymes that have their specific cellular locations and substrates (34), further studies are needed to understand which isoform plays the critical role in the decreased GPX activity in adult muscles with unloading.

Overall, fast-twitch muscles of old animals can adapt to unloading-induced greater generation of superoxide inside of mitochondria by increasing the activities of MnSOD, catalase, and GSH. Outside of mitochondria, the capacity to buffer the greater generation of superoxide in fast-twitch muscles of old animals with unloading may be limited by the impaired adaptation of Cu-ZnSOD. The unbuffered superoxide may lead to damage of cells.

**Effect of Aging on Oxidative Damage in Fast-Twitch Muscles With Unloading**

MDA, the major end product of lipid peroxidation, is a highly reactive compound that reacts with proteins to form protein adducts, leading to protein dysfunction (35). We found the level of MDA in aged muscles remained unchanged with 3 and 7 days of unloading but increased dramatically with 14 days of unloading. In contrast, in adult muscles, there was no significant change in the MDA level during the 14 days of unloading. The finding of the age-related accumulation of MDA in muscles with unloading is similar to a previous publication where mixed fiber-type gastrocnemius muscles with 14 days of unloading were investigated (13). These results suggest that 14 days is a critical period of muscle unloading for aged muscles where lipid peroxidation in the form of MDA starts to accumulate. Interestingly, we found the time point where MDA accumulated significantly in aged muscles with unloading matched the time point where the adaptation of Cu-ZnSOD became impaired (14 days of HU). Taken together, the cellular environment in aged muscles at 14 days of HU does not appear to be in a redox-balanced condition.

The finding of the dramatic decrease of Cu-ZnSOD and accumulation of MDA in aged muscles with 14 days of unloading corresponds with our previous study, where cytosolic superoxide in fast-twitch muscles was found increased at 7 days of HU and continued to increase at 14 days of HU (cytosolic superoxide level in aged muscles: 14 d > 7 d > control; [9]). Collectively, the findings of this study and our previous study suggest that the balance between oxidant (superoxide) and antioxidant (Cu-ZnSOD) is altered in aged fast-twitch muscles with 14 days of HU. The disrupted redox balance is likely contributing to the accumulation of oxidative damage (MDA).

Another potential mechanism for the MDA accumulation in aged muscles with 14 days of unloading is the impaired MDA removal in aged muscles in 14 days of unloading. The critical enzyme that detoxifies MDA is aldehyde dehydrogenase (36). It was reported that the activity of aldehyde dehydrogenase decreased significantly with the increase of oxidative stress (37). Further studies are needed to test this hypothesis.

Oxidative attacks on proteins introduce carbonyl groups into the side chains. The carbonylation of proteins may impair protein functions and make proteins more susceptible to degradation by the proteasomal system (38,39). On the other hand, protein carbonylation also plays a role in the redox signal transduction process (40,41). In this study, we found that the level of carbonylated proteins decreased in aged muscles with 7 and 14 days of unloading, whereas it remained unchanged in adult muscles during the 14 days of unloading. This finding is contrary to our prediction as we found that the adaptation of Cu-ZnSOD in muscles with unloading was impaired in aged rats. One explanation for the decreased level of carbonylated proteins in aged muscles with 7 and 14 days of unloading is the activation of the proteasomal system. The proteasomal system is the primary system that degrades carbonylated proteins, thus its activity influences the level of carbonylated proteins in the cells. Previous studies have reported that muscle unloading induces an upregulation of the proteasomal system in muscles from adult animals (12,42,43). In addition, the proteasome content was found two- to three-fold higher in aged muscles than that in adult muscles, which was thought to be a compensatory mechanism for the lower proteasome-specific activity in aged muscles (44,45). Thus, the compensatory increase in proteasome content with aging may contribute to the decreased level of oxidized proteins in aged muscles with unloading. Additional research is needed to test this statement.

**Effect of Aging on Muscle Weight in Fast-Twitch Muscles With Unloading**

We found that unloading-related muscle atrophy in fast-twitch muscles was more significant in aged rats. In old rats, TA muscles showed 28% of mass loss at 7 and 14 days of HU. In adult rats, unloading-related muscle loss was not significant until 14 days of HU (17% of mass loss). In our previous study where slow-twitch soleus muscles were investigated, the loss of muscle mass was significant in both aged and adult muscles with 7 and 14 days of HU with aged muscles having greater extent of muscle atrophy (46% and
29% of mass loss with 14 days of HU in aged and adult muscles, respectively). Thus, regarding the time point that muscle mass starts to show significant loss with unloading, aging affects fast-twitch muscles more than slow-twitch muscles. However, in terms of the extent of muscle mass loss with unloading, the effects of aging on fast-twitch muscles and slow-twitch muscles are similar (the extents of muscle mass loss in aged muscles with 14 days of HU were both 1.6-fold of that in adult muscles).

Limitations

One limitation of this study is the lack of information about muscle contractility such as maximal force and power. Thus, the relationship between the age-related changes of oxidative damages and muscle function in fast-twitch muscles with unloading cannot be identified. Second, muscle atrophy with aging and HU was characterized by changes of muscle weight, whereas cross-sectional areas of muscles and/or single fibers would allow for a more accurate reflection of atrophy. Another limitation of this study is that the carbonylated proteins that show age-related changes were not identified. Therefore, it is unknown whether the age-related changes in the accumulation of carbonylated proteins in muscles with unloading influence the redox signaling in muscles with unloading.

Conclusion

Muscle unloading causes a stress to fast-twitch muscles in both adult and old rats, characterized by the compensatory adaptation of several key antioxidants such as catalase and GSH. Although most antioxidants in aged muscles had the capacity to adapt to unloading, the activity of Cu–ZnSOD decreased significantly in aged muscles with 14 days of unloading. Consistently, lipid peroxidation accumulated significantly in aged muscles with 14 days of unloading, whereas it did not change in adult muscles with 14 days of HU.

Funding

This work was supported by National Science Council (NSC 99-2320-B-182-001) in Taiwan and National Institute on Aging (AG-17768) in the United States.

Acknowledgment

We thank Wang C-Y for technical assistance.

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