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Aging-Dependent Regulation of Antioxidant Enzymes and Redox Status in Chronically Loaded Rat Dorsiflexor Muscles

Michael J. Ryan,[1] Holly J. Dudash,[1] Megan Docherty,[1] Kenneth B. Geronilla,[1,2] Brent A. Baker,[2] G. Gregory Haff,[1] Robert G. Cutlip,[2] and Stephen E. Alway[1]

[1]Laboratory of Muscle Biology and Sarcopenia, Division of Exercise Physiology, West Virginia University School of Medicine, Morgantown.
[2]National Institute for Occupational Safety and Health, Morgantown, West Virginia.

This study compares changes in the pro-oxidant production and buffering capacity in young and aged skeletal muscle after exposure to chronic repetitive loading (RL). The dorsiflexors from one limb of young and aged rats were loaded 3 times/week for 4.5 weeks using 80 maximal stretch-shortening contractions per session. RL increased H_2O_2 in tibialis anterior muscles of young and aged rats and decreased the ratio of reduced/oxidized glutathione and lipid peroxidation in aged but not young adult animals. Glutathione peroxidase (GPx) activity decreased whereas catalase activity increased with RL in muscles from young and aged rats. RL increased CuZn superoxide disumutase (SOD) and Mn SOD protein concentration and CuZn SOD activity in muscles from young but not aged animals. There were no changes in protein content for GPx-1 and catalase or messenger RNA for any of the enzymes studied. These data show that aging reduces the adaptive capacity of muscles to buffer increased pro-oxidants imposed by chronic RL.

Key Words: Exercise—Oxidative stress—Sarcopenia—Superoxide dismutase.

THE consequence of oxidative stress is an important mechanism that may, at least in part, underlie the aging-induced attenuation of physiological adaptation to increased loading (1–4) in muscle. Increased pro-oxidant production is buffered in muscles of young animals and humans, but it is possible that aging reduces the ability for muscles to buffer oxidants. This reduced buffering capacity leads to oxidative stress and thus may lead to cellular damage. Oxidative stress as a result of increased muscle loading has been shown to depress muscle-specific force (5), modify myofilament function (6,7) and alter contraction-induced calcium release (8–10). Oxidative stress may also contribute to loss of muscle force by reducing recovery from injury (11,12). Increases in pro-oxidant production have also been shown to stimulate redox-sensitive signaling pathways (13–15), up-regulate catabolic gene expression in muscle (16–18) and activate apoptosis (19–21) in muscle.

It is clear that muscle atrophy is associated with increases in pro-oxidants and oxidative stress (6,7,22), as evidenced by increases in lipid peroxidation, glutathione oxidation, protein carbonyls, free iron content, and xanthine oxidase levels. Oxidative stress is also elevated with both aging and loading in chondrocytes (23), synovial cells (24), and muscle cells (25,26). The generation of pro-oxidants and oxidative stress have been implicated in mechanisms of muscle dysfunction and sarcopenia (27); however, there are no data that address the role of cumulative oxidative stress in repetitive loading in aging muscles. Furthermore, the underlying cause for increased pro-oxidant formation in aging muscle is unknown, yet this information is essential if we are to develop useful strategies for improving adaptations to loading in aging.

The mitochondrial theory of aging predicts that an increase in oxidative stress is responsible for cellular damage and ultimately apoptosis and cell death of various cell types (25,28) including skeletal muscle cells (29,30). Actin and myosin proteins do not appear to have increased oxidative damage with aging in rats (31). However, recent data from muscle biopsies obtained from young and older men showed that 8-hydroxy-2′-deoxyguanosine (8-OHdG), protein carbonyls, Mn superoxide dismutase (SOD) activity, and catalase activity were significantly higher in muscles from old as compared to young men (32). Full-length mitochondrial DNA was also lower, and mitochondrial deletions were prominent in muscles from old as compared to young men (32). These data support the hypothesis that even healthy aging is associated with oxidative damage to proteins and DNA in skeletal muscle and may be a primary reason for increased nuclear apoptosis that has been reported within aged muscles (33–37). Oxidative stress may reduce the muscle's ability to adapt to increased demands, as is the case in repetitive loading (RL). Although mitochondria may be the intrinsic initiators of oxidative stress, mitochondria, along with activated satellite cells, may also be the target of pro-oxidants in repetitively loaded muscle. If activated satellite cells are reduced or eliminated (e.g., by apoptosis), muscle adaptation to loading would be reduced or eliminated. Although it is clear that oxidative damage

accumulates with aging, the role of mitochondria in aging and oxidative damage still has several unanswered questions, including the involvement of mitochondria in apoptosis (38).

It is generally accepted that aging is associated with increases in oxidative stress. Given that skeletal muscle has a substantial potential for pro-oxidant production, due to its high level of oxygen consumption, increases in pro-oxidants may have a role in reducing muscle function in aging (29). We have recently shown that muscle force falls within 24 hours, and does not recover during the first 7 days of RL (39). We hypothesize that loss of muscular force is mediated, directly or indirectly, by the increase in oxidant activity during RL. This is known to occur in muscles exposed to exogenous reactive oxygen species (10,40–42). An aging-associated reduction in CuZn SOD may be a good candidate to explain the increased superoxide levels that lead to long-term oxidative damage and eventual loss of skeletal muscle, because CuZn SOD-deficient animals have a shorter life span and decreased muscle mass (27). Recent data (43) also suggest that CuZn SOD levels are lower in muscles and other tissues of old rodents, whereas long-lived animals do not show this decrease in CuZn SOD and other oxidative enzymes (44). CuZn SOD levels increase in cells of young animals after exercise (45). Although Vasilaki and colleagues (46) report an increased level of CuZn SOD in muscles of old animals after a single bout of electrical stimulation, it is possible that old muscles may fail to respond similarly with chronic RL. Furthermore, even if CuZn SOD does increase either comparably or greater in old versus young muscles with RL, it is not known if this will be sufficient to counteract the loading-induced pro-oxidant and intrinsic pro-oxidant produced by aging mitochondria (27) in old muscles.

It is widely recognized that glutathione peroxidase (GPx)-1, catalase, CuZn SOD, and Mn SOD enzymes provide a defense system, which is essential for the survival of aerobic organisms. Moderate nonfatiguing exercise has been shown to enhance the buffering capacity of these enzymes; therefore, exercise is thought to be a therapeutic tool for offsetting high levels of oxidative stress (47). However, the possibility exists that intensive exercise may exacerbate pro-oxidant production in muscles with aging, which may be detrimental to muscle function.

Aging rodents are frequently used as models for exercise training in humans, but there are few studies that have examined the effects of resistance training in aged rats. We have recently observed that RL results in a maladaptation in tibialis anterior (TA) muscles of old rats, whereas in the TA muscles from young adult rats the same stimulus results in an increase in force, muscle mass, and myofiber cross-sectional area (48). Although the mechanisms for these maladaptations to exercise in the aged muscle are unknown, we hypothesized that the increased level of oxidative stress in muscles of aged animals may in part reduce the ability of muscle to adapt to RL.

In this study, we tested the hypothesis that (a) TA muscles from aged rats would show evidence of oxidative stress; (b) chronic RL would increase antioxidant enzyme activity and decrease oxidant production in aged animals, thus decreasing the age associated oxidative stress generally observed in aged sedentary animals, compared with young adult animals; and (c) compared to exercised TA muscles from young adult rats, the exercised muscles from aged rats would have an attenuation in the increase of antioxidant enzyme activity, which is usually associated with an adaptation to exercise, and these increased enzyme activities would be closely correlated with the lower hypertrophic adaptations to RL with aging.

Methods

The left TA muscles of aged (30 months of age; $n = 8$) and young (12 weeks of age; $n = 8$) Fischer 344 Brown × Norway rats were subjected to RL exercise, which consisted of 3 sessions per week for 4.5 weeks of 80 eccentric/concentric contraction cycles per session (48). Muscle functional data were collected from a subset of four animals per group. The right TA was used as a contralateral control for each animal. The sessions were performed on a custom-built dynamometer by electrically stimulating the common peroneal nerve, causing contraction of the dorsiflexor muscles and moving the footplate from plantar flexion through dorsiflexion (49). Electrical stimulation of the TA was via supramaximal square wave pulses delivered to the common peroneal nerve. The voltage used in these experiments was 10% greater than the voltage required to obtain maximal force production. This method has been previously shown to produce a hypertrophic response in young adult rats (48). All animals had free access to rat chow and water. At the end of the training period, the rats were killed and the TA muscles were removed, cleaned of excess connective tissue, and weighed. A section of the muscle was obtained for the determination of the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). The remaining muscle was quickly frozen in liquid nitrogen and stored in a −80°C freezer until analysis.

RNA Isolation

Sixty micrograms of the TA muscle was homogenized in 1 mL of Tri-Reagent (Molecular Research Center, Cincinnati, OH). Muscle homogenates were centrifuged at 14,000 × *g* for 10 minutes at 4°C. Supernatants were transferred to a sterile tube, then 100 μL of 1-bromo-3-chloropropane (BCP; Molecular Research Center) was added to the supernatant. The sample was vortexed for 15 seconds and incubated at room temperature for 15 minutes. The samples were centrifuged (14,000 × *g* for 15 minutes at 4°C), and the top aqueous phase that contains RNA was transferred to a sterile 1.5 mL tube followed by addition of 500 μL of isopropanol and incubation at room temperature for 10 minutes. Samples were centrifuged at 14,000 × *g* for 8 minutes at 4°C, which collected the solubilized RNA into a small pellet. The RNA pellet was washed in 1 mL of 75% ethanol and centrifuged at 5500 × g for 5 minutes at 4°C. The supernatant was removed, and the RNA pellet was air dried in a fume hood and resuspended in 22 μL of sterile distilled H_2O. The RNA was treated with DNAse I using a DNA-free Kit (Ambion, Austin, TX) and quantified using a Bio-Rad SmartSpec 3000 (Bio-Rad, Hercules, CA). RNA

purity was determined using a minimum 260:280 ratio of 1.7. Samples with values less than this were re-treated for DNA contamination and quantified.

Reverse Transcription–Polymerase Chain Reaction

Two micrograms of total RNA were reverse transcribed using random primers (Invitrogen/Life Technologies, Bethesda, MD) via the following protocol: Random primers (1.0 μL) and 1.0 μL of 10 mM deoxyribonucleotide triphosphate (dNTP) mixture were added to 2 μg of RNA. Samples were heated to 65°C for 5 minutes then to 3°C for 5 minutes in a Biometra T3 thermocycler (Biometra, Göttingen, Germany). Seven microliters of a master mix containing 5X First Strand buffer, dithiothreitol (DTT), and RNase-Out were added to each sample. Tubes were returned to the thermocycler and incubated at 25°C for 10 minutes followed by 42°C for 2 minutes. One microliter of Super-Script II reverse transcriptase was added to each tube and lightly mixed by pipetting the solution up and down. Samples were returned to the thermocycler to be incubated at 42°C for 50 minutes, 70°C for 15 minutes, and then cooled to 3°C until removed for storage. This procedure yielded 20 μL of complimentary DNA (cDNA), which was stored at –80°C or used for polymerase chain reaction (PCR) analyses.

Primers for the genes of interest were constructed according to the following primer sequences: CuZn SOD sense, 5′-AGGCCGTGTGCGTGCTGA-3′; antisense, 5′-CCCAATCACACCACAAGCCA-3′; GPx-1 sense, 5′-CCT CGTGGCCTGGTGGTCCT-3′; antisense, 5′-AGGGGT TGCTAGGCTGCTTGGA-3′. The following primers were previously published from our laboratory: Mn SOD sense, 5′-GCGGGGGCCATATCAATCACAG-3′; antisense, 5′-GGCGGCAATCTGTAAGCGACCT-3′; Catalase sense, 5′-CGGGAACCCAATAGGAGATAAA-3′; antisense, 5′-CCACGAGGGTCACGAACTGT-3′ (35,50,51). To make certain that analyses were done in the linear range of amplification, preliminary tests were done to determine the proper number of PCR cycles. PCR products were verified by restriction digestion based on predicted PCR sequences. To control for any loading errors, the signal from the gene of interest was expressed as a ratio to the 18S signal from the same PCR product. Forty-nine microliters of a master mix containing 10X PCR buffer with $MgCl_2$, 5 mM dNTPs, 100 ng/mL of primer pairs, 18S primer pairs, and 1.0 μL of Taq DNA polymerase was combined with 1.0 μL of cDNA for PCR amplification. Amplification of PCR products was performed in a thermocycler using a denaturing step at 95°C for 45 seconds, an annealing step for 45 seconds, and an extension step at 72°C for 45 seconds. Following amplification, 20 μL of each reaction was subjected to electrophoresis on 1.5% agarose gels. Gels were stained with ethidium bromide to visualize the PCR products. The PCR signals were recorded via a digital camera (Kodak 290), and the signals were quantified in arbitrary units as optical density × band area, using 1D Kodak image analysis software (Eastman Kodak Company, Rochester, NY).

Protein Fractionation

Seventy-five micrograms of TA muscle samples were separated for cytoplasmic and nuclear protein fractions, adopted with slight modification from the methods of Rothermel and colleagues (52) as reported previously by our laboratory (35,53,54). However, a lower concentration of DTT was used in these experiments to prevent later interference with enzyme activity assays. Muscle samples were homogenized in 500 μL of ice-cold lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 20 mM HEPES at pH 7.4, 20% glycerol, 0.1% Triton X-100, and 10 μM DTT) with a mechanical homogenizer. Muscle homogenates were centrifuged at 100 × *g* for 5 minutes at 4°C. Supernatants were collected and centrifuged three more times at 3500 × *g* for 5 minutes at 4°C. The resulting supernatant was collected as a nucleus-free cytosolic fraction and divided into two equal portions: The first portion was frozen at –80°C until needed; in the second portion, protease inhibitor cocktail containing 104 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride hydrochloride, 0.8 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St. Louis, MO) was added before the sample was frozen at –80°C. Protein concentrations for each sample were determined in triplicate via a DC Protein Assay (Bio-Rad, Hercules, CA). The cytosolic fraction was used in the following assays: H_2O_2 concentration, catalase activity, GPx activity, CuZn and MnSOD activity, and western immunoblots.

Western Immunoblots

The protein content of GPx-1, catalase, CuZn SOD, and Mn SOD was measured in the cytosolic muscle fractions. Thirty micrograms of protein was loaded into each well of a 4%–12% gradient polyacrylamide gel (Novex; Invitrogen) and separated by routine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for 1.5 hours at 20°C followed by transfer to a nitrocellulose membrane. All membranes were blocked in 5% nonfat milk protein (NFM) for 1 hour at room temperature. In general, membranes were incubated in appropriate dilutions of primary antibodies (diluted in 1% NFM in Tris-buffered saline with 0.05% Tween-20 [TBS-T] overnight in a 4°C cold room). Membranes were washed in TBS-T followed by incubation in appropriate dilutions of secondary antibodies (diluted in 5% NFM in TBS-T) conjugated to horseradish peroxidase. Signals were developed using a chemiluminescent substrate (Lumigen TMA-6; Lumigen, Southfield, MI) and visualized by exposing the membranes to x-ray films (BioMax MS-1; Eastman Kodak). Digital records were captured by a Kodak 290 camera, and protein bands were quantified using 1D analysis software (Eastman Kodak). Bands were quantified as optical density × band area and expressed in arbitrary units.

H_2O_2 Levels

A fluorescent H_2O_2 detection kit (Cell Technology, Mountain View, CA) was used to determine the amount of H_2O_2 in the muscle tissue. Reagents and standards were prepared as recommended by the manufacturer. Briefly, 50 μL of controls, samples, or H_2O_2 dilutions were mixed with 50 μL of the reaction cocktail in each well to initiate the reaction. The plate was incubated in the dark for 10 minutes at 20°C, and fluorescence was detected with an excitation

at 530 nm and measured at 590 nm. All analyses were performed in duplicate, and samples were normalized to muscle protein concentration in each sample via a DC Protein Assay (Bio-Rad).

GSH/GSSG Ratio

A BIOXYTECH GSH/GSSG-412 assay (Oxis International, Inc., Foster City, CA) was performed to determine the GSH/GSSG ratio. Muscle tissue (~40 mg) was homogenized immediately after dissection in 530 μL of cold 5% metaphosphoric acid (MPA) for the GSH sample, and for the GSSG sample ~40 mg of muscle tissue was homogenized immediately after dissection in 500 μL of cold 5% MPA and 30 μL of M2VO scavenger. Homogenates were then frozen in liquid nitrogen and stored at –80°C until analyzed.

Samples were thawed, and cold 5% MPA was added to each sample (290 μL and 350 μL for GSSG and GSH, respectively). Samples were mixed and then centrifuged at 1000 × *g* for 10 minutes. For the GSSG sample, 25 μL of MPA extract and 350 μL of GSSG buffer were added to each tube then placed on ice until use. MPA extract (10 μL) and assay buffer (600 μL) were added to the GSH sample, then placed on ice. Sample (50 μL) and chromogen and enzyme (50 μL) were mixed in a cuvette followed by 5-minute incubation at room temperature. NADPH (50 μL) was added to each cuvette, and the absorbance of each sample was read every 60 seconds at 412 nm for 3 minutes. The concentration for each sample was determined via a DC Protein Assay (Bio-Rad). Signals from each sample were normalized to the corresponding protein content of that sample.

8-OHdG

Oxidized DNA was determined by a BIOXYTECH 8-OHdG ELISA (enzyme-linked immunosorbent assay; Oxis International Inc.). DNA was extracted from the muscle by using a DNeasy Tissue Kit (Qiagen, Chatsworth, CA). DNA was used if it had a minimum 260:280 ratio of 1.8. Fifty microliters of purified DNA was mixed with 50 μL of primary antibody. Samples were then incubated at 37°C for 1 hour. The wells were washed, then incubated in 100 μL of secondary antibody at 37°C for 1 hour. Chromogen (100 μL) was added to each well, shaken, then incubated at room temperature in the dark for 15 minutes. The reaction was terminated, and the samples were read at an absorbance of 450 nm. Samples were normalized to the DNA concentration measured via a plate reader (ND-1000; NanoDrop, Wilmington, DE). All analyses were done in duplicate.

Lipid Peroxidation

Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were measured using reagents from Oxis International (BIOXYTECH LPO-586). A 75 mg to 100 mg section of each muscle was homogenized in 500 μL of buffer containing ice-cold phosphate-buffered saline (20 mM, pH 7.4) and 5 μL of 0.5 M butylated hydroxytoluene in acetonitrile per 1 mL of tissue homogenate. Assay reagents were added following the manufacturer's recommendations. Briefly, the muscle homogenate was centrifuged at 3000 × *g* at 4°C for 10 minutes, and the supernatant was used for the assay and protein determination. The sample was incubated in the appropriate reagents at 45°C for 60 minutes, and then centrifuged at 15,000 × *g* for 10 minutes. An absorbance reading of the supernatant was obtained at 586 nm. Samples were normalized for differences in the amount of muscle protein in each sample as determined by a DC Protein Assay (Bio-Rad).

Catalase Activity

A catalase activity assay kit (#219265; EMD/Calbiochem, San Diego, CA) was used to determine the activity level of catalase in repetitively loaded and control muscles, according to the manufacturer's recommendations. After the appropriate incubations, the samples were read at an absorbance of 520 nm. All analyses were completed in duplicate, and samples were normalized to muscle protein in each sample via a DC Protein Assay (Bio-Rad).

Mn SOD and CuZn SOD

A commercially available SOD Assay Kit II (#574601; EMD/Calbiochem) was used to measure total and Mn SOD activity. CuZn SOD was determined by subtracting the value for Mn SOD activity from the total SOD activity. The assay was performed with modifications to the manufacturer's directions, and all samples and standards were measured in duplicate. Briefly, the muscle was homogenized in a buffer (20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) and centrifuged at 1000 × *g* for 10 minutes. The assay was performed in a 96-well plate with each sample being treated with and without 10 μL of 3 mM potassium cyanide. Potassium cyanide was used to inhibit CuZn SOD, resulting in the detection of only Mn SOD activity. The reagents and samples were protected from white light and incubated at 26°C for 20 minutes with periodic shaking. The absorbance was measured at 450 nm using a 96-well plate reader (DYNEX Technologies, Chantilly VA).

GPx

A commercially available cellular GPx Assay Kit (#35319; EMD/Calbiochem, San Diego, CA) was used to measure GPx activity in the cytosolic fractions of the muscle homogenates. The assay was preformed with slight modifications to the manufacturer's directions. Briefly, a portion of each muscle was homogenized in a buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM DTT. The homogenate was centrifuged at 10,000 g for 15 minutes at 4°C and the supernatant was used for the assay. All reagents and sample were equilibrated to 25°C and the remaining assay procedures followed manufacturer's specifications. The absorbance was measured at 340 nm using a 96-well plate reader (DYNEX Technologies). Experimental and control samples were measured in duplicate.

Statistical Analyses

Statistical analyses were performed using the SPSS 13.0 software package (SPSS, Chicago, IL). A multiple analysis of variance was used to examine differences between age and

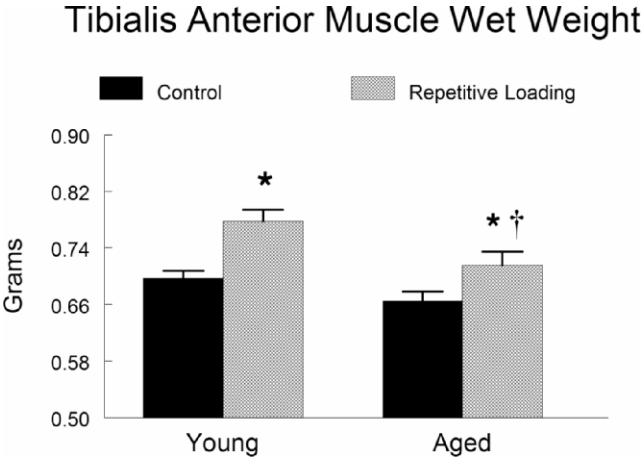

Figure 1. Repetitive loading (RL) induces muscle hypertrophy in the tibialis anterior (TA) muscle. Data are expressed as TA muscle wet weight in grams. *Significant difference between age-matched control and RL TA muscle, $p < .05$; †significant difference ($p < .05$) from young treatment-matched control muscles.

treatment (RL). Statistical significance was accepted at $p < .05$. Data are reported as mean ± standard error of the mean.

Results

Body Mass

The average body mass of the young animals was 326.5 ± 14.7 g before muscle loading and 317.2 ± 12.2 g after the 4.5 weeks of training, but this did not represent a significant change in body mass. However, the body mass of aged animals had a small but significant decrease from 597.3 ± 17.9 g before training to 558.3 ± 13.3 g after the 4.5 weeks of training.

Muscle Wet Weight

RL for 4.5 weeks resulted in a significant increase in TA muscle wet weight in the exercised limb of both the young adult (11.5 ± 1.6%, $p < .001$) and the aged adult (7.5 ± 1.9%, $p < .05$) rats as compared with the contralateral control muscle (Figure 1).

Muscle Functional Measurements

Maximal isometric muscle force, positive work, and negative work were used to measure the functional capacity of the dorsiflexors, which include TA muscle. There was no significant difference between the young adult and the aged animals for peak force, positive work, or negative work observed at the start of the study. However, 14 loading sessions increased peak force (51 ± 5.6%), positive work, (32 ± 3.9%), and negative work (37 ± 7.4%) as compared to the first session for young adult animals. In contrast, there was no significant change in any of the parameters used to assess functional changes in muscles of the aged animals over the training period (Figure 2).

H_2O_2

Muscle levels of H_2O_2 were elevated in the loaded muscles compared to the age-matched control limb ($p < .001$), suggesting a treatment effect and that chronic loading elevated oxidative stress. In aged rats, both control and experimental muscles had higher levels of H_2O_2 than did their treatment-matched muscles of young adult rats ($p < .001$). This finding suggests a systemic aging effect of oxidative stress on skeletal muscles (Figure 3).

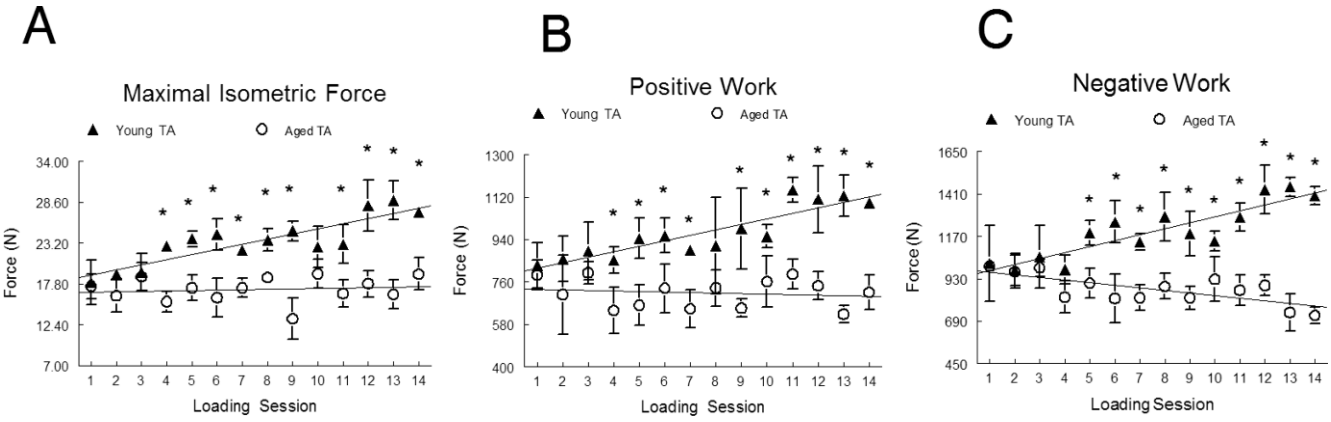

Figure 2. Repetitive loading increased muscle functional measurements in young adult dorsiflexor muscles while maintaining function in aged dorsiflexor muscles. **A,** Maximal force generated from the young and aged dorsiflexor muscles during each of the 14 training sessions. Data are expressed as the average maximum force for all animals in Newtons (N) produced during each exercise session ± standard error. *Solid line*: linear regression for all age-matched points. *Significant difference ($p < .05$) between young adult and aged dorsiflexor muscles. **B,** Positive work generated from a single eccentric/concentric movement preformed at the start of each training session from the young and aged dorsiflexor muscles during each of the 14 training sessions. Data are expressed as the mean ± standard error of the mean (*SEM*). *Solid line*: linear regression for all age-matched points. *Significant difference ($p < .05$) between young adult and aged dorsiflexor muscles. **C,** Negative work generated from a single eccentric/concentric movement performed at the start of each training session from the young and aged dorsiflexor muscles during each of the 14 training sessions. Data are expressed as the mean ± *SEM*. *Solid line*: linear regression for all age-matched points. *Significant difference ($p < .05$) between young adult and aged dorsiflexor muscles.

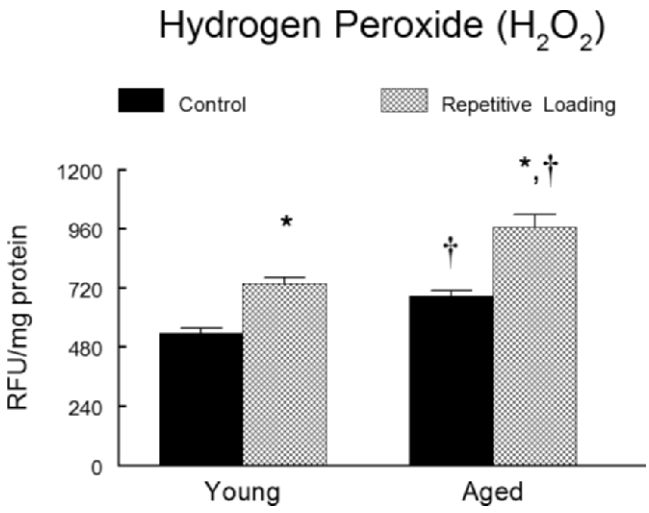

Figure 3. Concentration of hydrogen peroxide (H_2O_2) is elevated with repetitive loading (RL) and aging. H_2O_2 concentration was determined by a fluorometric assay. Data are expressed as relative fluorescent units (RFU) per milligram of total protein in tibialis anterior homogenate. Normalized data are presented as mean ± standard error of the mean. *Significant difference ($p < .05$) of RL muscle from contralateral control muscle. †Significant difference ($p < .05$) from young treatment-matched control.

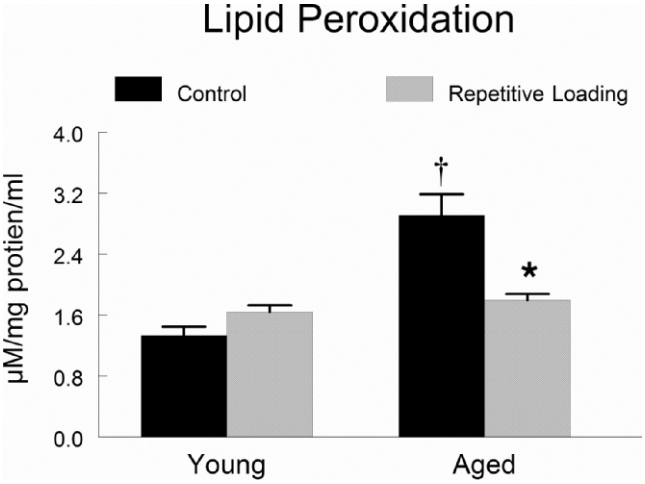

Figure 5. Repetitive loading (RL) decreases lipid peroxidation in tibialis anterior muscles of aged rats. Data are combined malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) normalized to total protein content. Data are presented as mean ± standard error of the mean. *Significant difference ($p < .05$) of RL muscle from contralateral control muscle. †Significant difference ($p < .05$) from young treatment-matched control.

GSH/GSSG Ratio

There was no training-induced difference in the GSH/GSSG ratio in muscles from young adult animals when comparisons were made to the control muscles. However, there was a significant reduction in the GSH/GSSG ratio of both control ($p < .05$) and loaded ($p < .01$) muscles of aged animals as compared to treatment-matched muscles in the young adult animals. The GSH/GSSG ratio was lower ($p < .05$) in the loaded muscles of the aged rats as compared to the young adult rats. These data suggest that aging reduces the basal ability to compensate for increased oxidative stress and therefore lowered the GSH/GSSG ratio when compared to muscles from young adult animals. Furthermore, aging reduced that ability to tolerate increased oxidative stress in chronically loaded skeletal muscles (Figure 4).

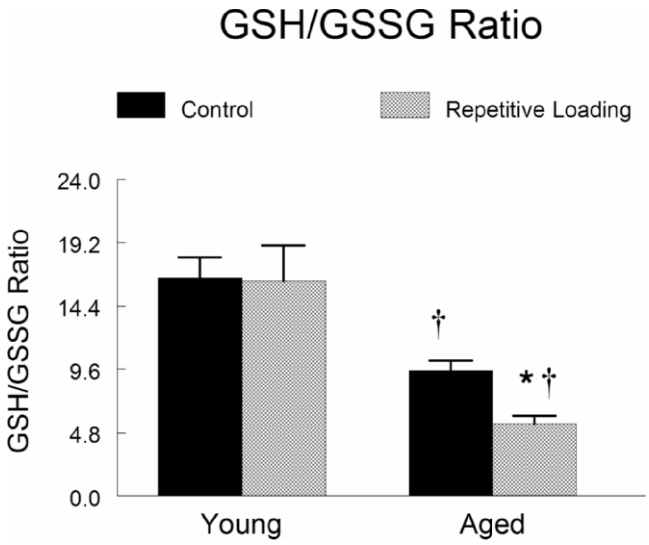

Figure 4. Ratio of reduced glutathione to oxidized glutathione (GSH/GSSG). Data are depicted as the ratio of GSH to GSSG normalized to total protein content. Lower ratios are an indication of increased oxidative stress. Normalized data are presented as mean ± standard error of the mean. *Significant difference ($p < .05$) of repetitively loaded muscle from contralateral control muscle. †Significant difference ($p < .05$) from young treatment-matched control.

Lipid Peroxidation

Aging increased the level of lipid oxidation as shown by greater MDA + HAE levels in control muscles of aged rats as compared to muscles from young adult animals ($p < .01$; Figure 5). RL appeared to activate adaptive responses in muscles of aged animals because MDA + HAE levels were lower in the loaded than control muscles of aged animals. No significant difference in MDA + HAE levels was observed among the control or chronically loaded muscles of young adult animals.

DNA Damage

Aging induced a significantly ($p < .05$) increased basal level of DNA damage as indicated by the amount of 8-OHdG detected in control muscles was significantly greater in aged versus young adult muscles. RL reduced the level of 8-OHdG in muscles of aged animals to that which was measured in muscles of young adult animals. Chronic loading did not change 8-OHdG in muscles of young adult animals. These data imply that chronic loading offsets the level of oxidative stress-induced DNA damage in aging rats (Figure 6).

GPx Activity

There was a loading effect but no age effect on GPx activity. Chronic RL decreased GPx activity in muscles

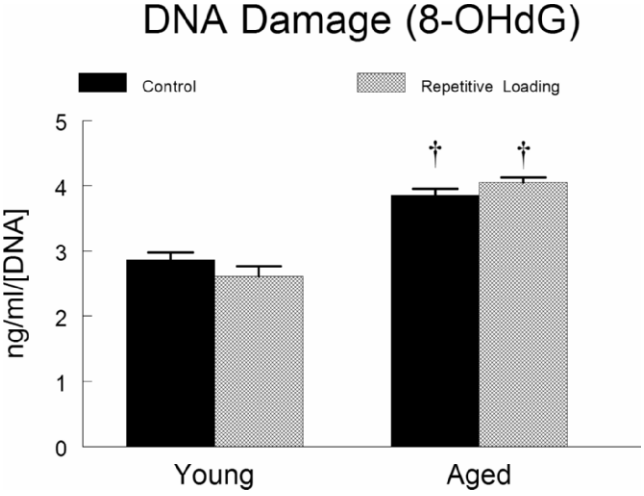

Figure 6. Aging increased the quantity of DNA damage whereas repetitive loading (RL) had no effect. Data are expressed as nanogram concentration of 8-hydroxy-2′-deoxyguanosine (8-OHdG) per milliliter of tibialis anterior (TA) homogenate per microgram of DNA. Normalized data are presented as mean ± standard error of the mean. †Significant difference ($p < .05$) from young treatment-matched control.

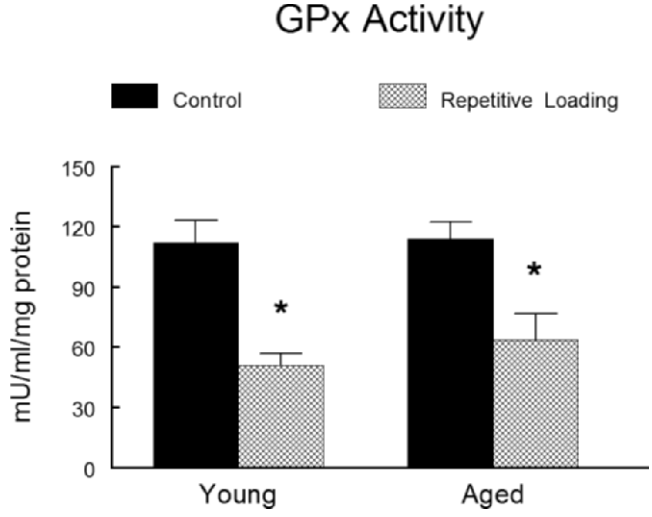

Figure 7. Glutathione peroxidase (GPx) activity decreased with repetitive loading (RL). Data are expressed as milliunits of GPx per milliliter of homogenate per milligram of protein. Normalized data are presented as mean ± standard error of the mean. *Significant difference ($p < .05$) of RL tibialis anterior muscle from contralateral control muscle.

from both young and aged animals ($p < .001$), but no differences were found between young and aged rodents (Figure 7). No significant differences were found among GPx-1 messenger RNA (mRNA) or protein levels within any of the muscle samples (data not shown).

Catalase Activity

Catalase activity increased with RL in muscles from the young adult rats ($p < .05$), but there was no significant change in catalase levels in muscles of the aged animals. Catalase activity was higher in control muscles of aged versus young adult animals, and it increased in loaded muscles of aged animals as compared to young adult animals ($p < .05$) (Figure 8). No differences were found in catalase mRNA or protein levels within any of the TA muscles (data not shown).

CuZn SOD Activity

CuZn SOD protein levels increased by 100% ($p < .05$) and CuZn SOD enzyme activity increased by 43% ($p < .05$) in repetitively loaded muscles from young animals as compared to the contralateral control muscles. However, these changes in CuZn SOD protein levels appeared to be posttranslationally regulated because no differences in CuZn SOD mRNA levels in control or loaded muscles from young animals were observed (data not shown). Aging suppressed any loading-induced changes in CuZn SOD, as there were no differences in mRNA, protein, or enzyme activity in the chronically loaded muscles compared to control muscles from aged animals (Figure 9).

Mn SOD

Mn SOD protein levels were increased by 75% in the loaded muscles of the young animals when compared to their contralateral control muscles ($p < .01$) as determined by western blot analyses. However, the increase in Mn SOD protein did not affect its activity levels nor was it driven by the alterations in Mn SOD mRNA, because no changes in any of the variables were seen in the aged animals (data not shown).

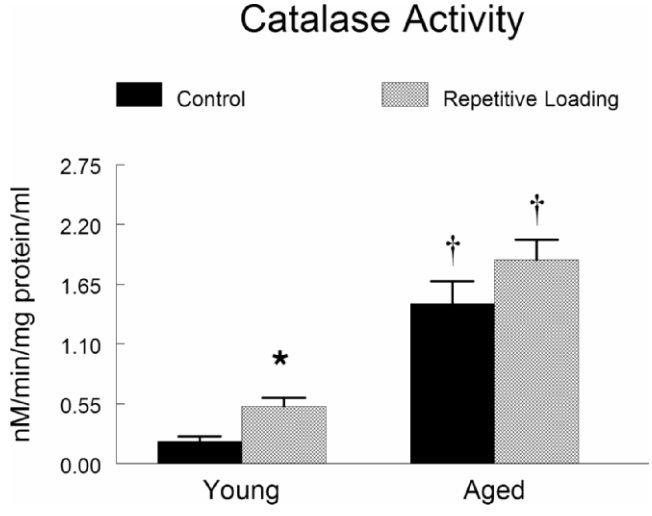

Figure 8. Catalase activity increased with repetitive loading (RL) and aging. Data are expressed as nanomolar of catalase activity per minute per milliliter of homogenate per milligram of protein. Normalized data are presented as mean ± standard error of the mean. *Significant difference ($p < .05$) of RL tibialis anterior muscle from contralateral control muscle. †Significant difference ($p < .05$) from young treatment-matched control.

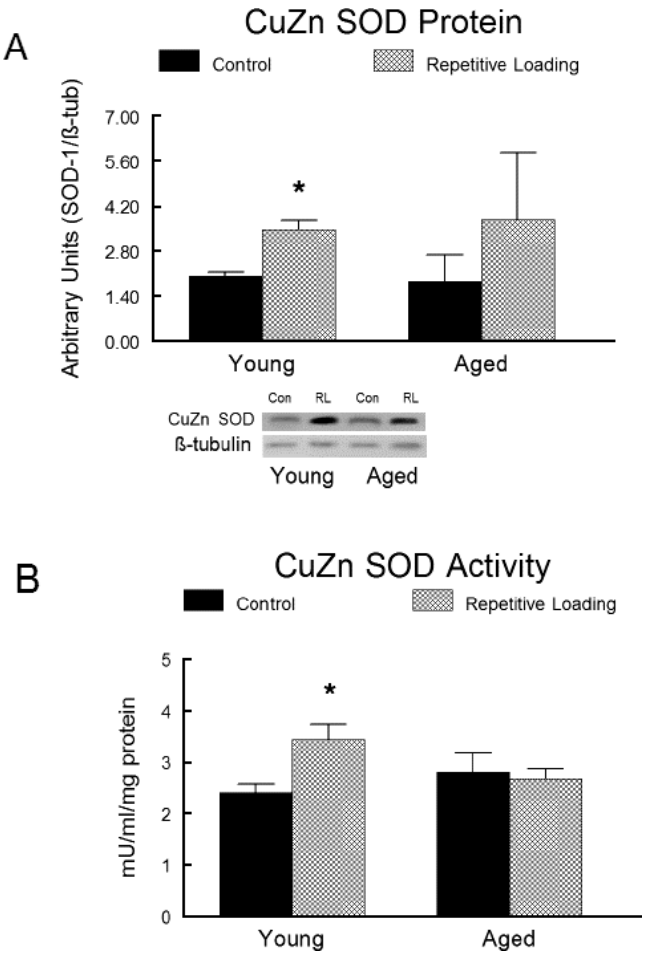

Figure 9. CuZn superoxide dismutase (CuZn SOD) protein levels and activity increased with repetitive loading (RL) in the tibialis anterior (TA) muscles of young but not old rats. **A,** CuZn SOD protein expression was determined in the total cytosolic fraction by western immunoblot analysis. Data are expressed as optical density × band area, and expressed in arbitrary units. *Inset:* Representative blots for CuZn SOD and β-tubulin in young and aged (control and RL) TA muscle. Normalized data are presented as mean ± standard error of the mean (*SEM*). *Significant difference ($p < .05$) of RL TA muscle from contralateral control muscle. **B,** CuZn SOD activity data are expressed as units of CuZn SOD per milliliter of homogenate per milligram of protein. Normalized data are presented as mean ± *SEM*. *Significant difference ($p < .05$) of RL TA muscle from contralateral control muscle.

Figure 10. Mn superoxide dismutase (Mn SOD) protein levels increase with repetitive loading (RL) in tibialis anterior (TA) muscles of young rats. Mn SOD protein expression was determined in the total cytosolic fraction by western immunoblots. Data are expressed as optical density × band area and expressed in arbitrary units. *Inset:* Representative blots for Mn SOD and β-tubulin in young and aged (control and RL) TA muscle. Normalized data are presented as mean ± standard error of the mean. *Significant difference ($p < .05$) of RL TA muscle from contralateral control muscle.

Discussion

Chronic adaptation to resistance exercise over many weeks in older women and men has been shown to improve muscular strength and also induce muscle fiber hypertrophy (55–58). However, it is not clear if the lesser extent of muscle hypertrophy that generally results from chronic loading in aged animals (59) is due, at least in part, to elevated levels of pro-oxidants. In this study, we found increases in muscle hypertrophy, muscle force, and work in chronically loaded muscles of young adult rats. Although there was some degree of muscle enlargement in aged animals with RL for 4.5 weeks, there was no improvement in muscle function, suggesting that muscle hypertrophy (based on muscle-wet weight) may not have been a result of an increase in contractile proteins in muscles of aged rats. Even though these findings vary slightly from those of a previous study using the same protocol, where a decrease in muscle mass and function was observed in old animals (48), it is likely that different cohorts of animals obtained from the National Institute on Aging (NIA) colony have slightly different responses and adaptive capabilities. Nevertheless, the current study and the previous study (48) are generally consistent in showing a reduced functional response to RL with aging compared with responses in young adult animals. Although we cannot rule out the possibility that part of the increase in muscle wet weight may have been the result of increases in collagen, or other contractile proteins, we did rule out the potential that inflammation (e.g., macrophages) contributed to enlargement of the loaded muscles of aged animals (B.A. Baker and R.G. Cutlip, 2005, unpublished observations).

Our data show that control muscles of aged rats had higher levels of oxidative stress as indicated by elevated H_2O_2, 8-OHdG, and MDA+HAE when compared to control muscles of young adult animals. These findings are consistent with observations from other laboratories showing that aging is associated with increased levels of oxidative stress in skeletal muscles and may be related, at least in part, to reduced muscle function with aging (60–62).

In the current study, 8-OHdG and MDA+HAE were measured to determine if the increases in pro-oxidant production were greater than the muscles' buffering capability, thus resulting in oxidative damage. Advanced

aging has been associated with increases in lipid peroxidation, DNA damage, and protein carbonyl formation. However, large age-associated increases in protein oxidation in muscle have not been consistently shown. For example, Mecocci and colleagues (63) reported large age-dependent increases in 8-OHdG and MDA but rather small increases in protein carbonyl formation in human skeletal muscle. Furthermore, Parise and colleagues (64) did not find any changes in muscle protein carbonyl content before and after acute resistance exercise or after 12 weeks of chronic resistance exercise training in humans, suggesting that protein oxidation does not increase with exercise in skeletal muscles with aging. It has also been reported that oxidative damage to the contractile proteins actin and myosin does not increase with aging in rats (31), so we did not directly assess oxidative damage of contractile proteins in loaded muscles of young or aged rats. Although we cannot rule out the possibility that oxidative damage to contractile proteins contributed to the lower muscle functional responses to RL in muscles of aged versus young adult rats, data from Thompson and colleagues (31) suggest that the decline in force production with age is not due to the accumulation of oxidation of myofibrillar proteins.

Chronic adaptation to loading that consisted of three exercise sessions per week for 4.5 weeks increased muscle levels of H_2O_2. Despite this increase, aging did not prevent improvement in several indices of oxidative stress in loaded skeletal muscles of aged rodents (e.g., lipid peroxidation and oxidative damage to DNA). Nevertheless, there was only a partial ability of muscles in aged animals to adapt to greater levels of oxidative stress. Of particular note, CuZn SOD and Mn SOD were not different in the chronically loaded muscles of aged rodents compared to the control muscles, despite increased oxidative loads (e.g., H_2O_2) and evidence of oxidative damage. We speculate that a failure to elevate CuZn SOD as part of an adaptive process to RL may be critically important in explaining the increased DNA and lipid oxidative damage with aging, because reduced levels of this antioxidant protein have been shown to coincide with reduced muscle mass and function (27).

In contrast, the adaptation of young adult animals to our RL protocol was more complete. For example, indices of oxidative stress (GSH/GSSG ratio) and oxidative damage (MDA+HAE and 8-OHdG) were similar in control and chronically loaded muscles from young animals. This finding suggests that the TA muscles were able to efficiently adapt and buffer the increase in pro-oxidant production in muscles of young adult animals. Furthermore, there was no significant difference in oxidative stress markers from the non-exercised control muscle. Adaptation in chronically loaded muscles from young adult animals appeared to occur via an increase in catalase and CuZn SOD activity, two enzymes that are primarily located in the cytosol. This result implies that the increase in pro-oxidant production may not primarily originate from the mitochondria, but may instead be generated in the cytosol, possibly through the xanthine oxidase pathway. We speculate that an increase in signaling of the xanthine oxidase pathway could increase the production of superoxide in the cytosol, thus increasing the localized stimuli for CuZn SOD production and activity, as well as an increase in H_2O_2 production. We have found that acute bouts of RL increased xanthine oxidase activity in TA muscle homogenate from mice (M.J. Ryan and S.E. Alway, 2008, unpublished data). This reasoning supports the previous suggestion from Parise and colleagues (27), who showed that, after 12 weeks of regular resistance training in aged humans, CuZn SOD and catalase activity were elevated, but no changes in Mn SOD were observed. The difference between the study by Parise and colleagues and our current work is that we found differences in CuZn SOD and catalase activity within only the young adult rats, not the aged rats. Two possible explanations for the difference are (a) the age of our rats (31 months at the end of the study, which would correlate to humans in their 80s; in the study by Parise and colleagues, the average participant age was 71 years); and (b) a different response between species.

In the present study, enzyme activity for CuZn SOD increased by 43%, which is similar to previous findings from our laboratory using the same electrically evoked RL model (65). The lack of an increase in CuZn SOD mRNA would suggest posttranscriptional modification that enhances protein synthesis. Similarly, Hollander and colleagues (66,67) found increases in CuZn SOD protein levels without changes in mRNA after a single bout of endurance exercise. Data from the current study differed from that reported by Hollander and colleagues (66,67) in that they showed no increase in CuZn SOD enzyme activity after an acute bout of exercise, whereas the present study shows increases in enzyme activity. These increases in CuZn SOD activity may be a result of a cumulative training effect resulting from chronic adaptation to repetitive stimulation over the 4.5-week period of the present study.

Xanthine oxidase-mediated oxidative stress has been shown to increase activation of nuclear factor-κ B (NF-κB) signaling resulting in an increase in the transcription of mRNA for Mn SOD (68). The lack of a detectable increase in Mn SOD mRNA in the current study does not exclude the possibility that it did not occur. There are two possible explanations; first, protein levels of Mn SOD are controlled by posttranslational modifications, similar to those found in CuZn SOD. Second, transcription of Mn SOD mRNA initially increased within the TA, which in turn led to an increase in Mn SOD protein levels, which then acted as a negative feedback regulatory mechanism, thus slowing down transcription of Mn SOD mRNA. Either possibility would explain our findings in the current study.

Various stages of posttranslational modification are required to make the Mn SOD enzyme catalytically active. We speculate that, without increased pro-oxidant production within the mitochondria, the Mn SOD protein would not have the stimulus needed for modification to its active form, thus we did not find any changes in Mn SOD activity levels. The lack of an increase in Mn SOD activity also lends support to our suggestion that the mode of exercise we implemented did not substantially increase pro-oxidant production within the mitochondria.

Chronic low intensity, high volume aerobic exercise has been shown to either maintain or increase levels of GPx activity (69,70). Therefore, the most surprising piece of data

was the loading-induced drop in GPx activity in muscles from both the young adult and aged animals. Furthermore, there were no significant changes in the mRNA or protein levels for GPx-1 between either young versus aged muscle or control versus exercise muscle. To the best of our knowledge, this is the first report of a posttranscriptionally determined decrease in GPx activity within the skeletal muscle of both young and aged animals with repetitive resistance loading. We expected that, similar to aerobic types of activity, resistance loading in the current study would increase H_2O_2 and decrease the GSH/GSSG ratio accompanied by an increase in GPx activity. GPx is an enzyme that is primarily thought to be responsible for reducing H_2O_2 and/or organic hydroperoxides to water or alcohol and is located in both the cytosol and the mitochondria. Catalase catalyzes the breakdown of H_2O_2 to form water and oxygen (71,72). Although there is some overlap between the function of catalase and GPx, GPx has a greater affinity for H_2O_2 than does catalase. Thus, when cellular levels of H_2O_2 are low, GPx is more active than catalase (73). It is possible that the high levels of loading-induced H_2O_2 in the current study may have created an unsuitable environment for improvements in GPx activity. In fact, GPx activity may have been suppressed as a result of chronically high levels of H_2O_2, which were exacerbated in the loaded muscles of aged animals. Nevertheless, this does not explain the reason why GPx activity also dropped with chronic loading in muscles of the young animals, unless the acute increase in H_2O_2 incurred during each loading session was sufficient to suppress GPx activity. Alternatively, catalase was responsive to the higher H_2O_2 induced by loading, and the increase in catalase may have been sufficient to buffer this peroxide in muscles of both young and old animals. However, Manna and colleagues (74) reported a decrease in GPx activity and other antioxidant enzymes in the testes and accessory sex organs of male rats that underwent 4 weeks of intense swim training; therefore, it is possible that intense resistance types of exercise may have effects similar to those of intense swimming on other tissues.

Conclusion

The data in this study show that muscles from aged rats have higher basal levels of oxidative stress and oxidative damage (e.g., to DNA and lipids) than muscles of young adult rats. Mechanical loading further exacerbates oxidative stress in muscles of aged rodents, but this appears to be well buffered in muscles of young adult animals. In response to high intensity chronic loading, there is a partial adaptation of oxidative enzymes to attempt to compensate for the increased oxidative insult in muscles of aged rats. However, this adaptation appears to be incomplete, because CuZn SOD and Mn SOD do not increase in chronically loaded muscles of aged rodents, but they do increase significantly after chronic adaptation to loading in muscles of young adult animals. The increase in catalase appears to be in response to loading-induced elevations in H_2O_2, but GPx is not increased. These data show that aging reduces the adaptive capacity of muscles to buffer the increased pro-oxidant production imposed by chronic RL. This reduced buffering capacity may compromise the muscles' abilities to hypertrophy and/or to improve muscle function in aged animals. Furthermore, it is possible that greater levels of oxidative stress in muscles of old animals may trigger increased levels of nuclear apoptosis that are associated with lower muscle mass and reduced hypertrophic adaptation with aging.

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Correspondence

Address correspondence to Stephen E. Alway, PhD, Division of Exercise Physiology, School of Medicine, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26506-9227. E-mail: salway@hsc.wvu.edu

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