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Suppression of Oxidative Stress by Resveratrol After Isometric Contractions in Gastrocnemius Muscles of Aged Mice

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This study tested the hypothesis that resveratrol supplementation would lower oxidative stress in exercised muscles of aged mice. Young (3 months) and aged (27 months) C57BL/6 mice received a control or a 0.05% *trans*-resveratrol-supplemented diet for 10 days. After 7 days of dietary intervention, 20 maximal electrically evoked isometric contractions were obtained from the plantar flexors of one limb in anesthetized mice. Exercise was conducted for three consecutive days. Resveratrol supplementation blunted the exercise-induced increase in xanthine oxidase activity in muscles from young (25%) and aged (53%) mice. Resveratrol lowered H_2O_2 levels in control (13%) and exercised (38%) muscles from aged animals, reduced Nox4 protein in both control and exercised muscles of young (30%) and aged mice (40%), and increased the ratio of reduced glutathione to oxidized glutathione in exercised muscles from young (38%) and aged (135%) mice. Resveratrol prevented the increase in lipid oxidation, increased catalase activity, and increased MnSOD activity in exercised muscles from aged mice. These data show that dietary resveratrol suppresses muscle indicators of oxidative stress in response to isometric contractions in aged mice.

Key Words: Exercise—Oxidative stress—Sarcopenia—Xanthine oxidase activity—NAD(P)H oxidase activity.

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THE causes of decreased muscle function associated with advanced aging are multifactorial and include muscle atrophy (sarcopenia), alterations in motor unit activity, and declines in metabolic efficiency. Exercise is a countermeasure that is partially effective for reversing the loss of muscle function. Although muscles in aged mammals adapt to chronic resistance exercise via improved muscular strength and muscle fiber hypertrophy, these adaptations are generally smaller than that reported in muscles from younger adult humans and animals (1,2). The mechanisms that regulate this attenuated adaptation to resistance exercise with advanced aging (3) are unknown, but it is possible that this may be mediated at least, in part, by the detrimental systemic effects associated with elevated oxidative stress (4).

The additive effects of an increase in oxidant production and an attenuated antioxidant buffering capacity leave aged skeletal muscles more vulnerable to oxidative stress and subsequently to oxidative damage. Age-related increases in oxidative stress have been associated with diminished muscular strength and physical performance (5). Specifically, elevated levels of oxidants have been shown to depress muscle force (6), alter myofilament function (6–8), and increase recovery time following injury (9,10).

Oxidative stress in skeletal muscles can arise from several sources (5,11). NAD(P)H oxidase is one potentially important contributor to oxidative stress in skeletal muscles because NAD(P)H oxidase activity increases in muscles of aging animals and also after long-duration exercise (5). The xanthine oxidase system is another important contributor to oxidative stress. Xanthine oxidase has been shown to be an important source of oxidant production in the vascular endothelium (12) and also a contributing factor to oxidative stress during strenuous exercise (13–15). A high demand on anaerobic metabolism, coupled with intermittent localized obstruction of blood flow and subsequent reperfusion within contracting muscles, raises the potential for xanthine oxidase to be an important source of oxidant production during intense resistance exercises. Previous studies have observed that after resistance training, type II fibers are preferentially hypertrophied in both young and aged muscle (2,16). Additionally, type II fibers tend to be more susceptible to oxidative damage than type I fibers (17); therefore, the increased oxidant production associated with aging could preferentially limit the ability of type II fibers to adapt to exercise training.

Resveratrol (3,4′,5-trihydroxystilbene) is a fat-soluble phytoalexin that has gained recognition as an effective antioxidant and antiaging nutraceutical (18–20). In vitro experiments with high doses of resveratrol have shown resveratrol to be effective at scavenging oxidants (21) and inhibiting

low-density lipoprotein oxidation (22). Whereas high concentrations of resveratrol appear to be an effective antioxidant (21,23), there is currently little evidence to show that a low concentration of resveratrol supplementation is an important oxidant scavenger in vivo (24). Resveratrol appears to improve muscle function in response to aerobic exercise; however, it is not clear if this is related to an antioxidant function of this compound. Furthermore, it is not known if resveratrol would reduce oxidative stress, including xanthine oxidase activity, oxidative damage, and/or muscle fatigue associated with acute resistance types of exercise. This information is important for designing optimal exercise programs for older people, because resistance types of exercise have been shown to develop considerable oxidative stress (1). Exercise-induced oxidative stress that is coupled with an age-related elevation in the basal levels of oxidative stress within skeletal muscle may increase the susceptibility of aged muscle to oxidative damage with exercise.

The objective of this investigation was to evaluate the efficacy of dietary resveratrol to attenuate oxidative stress that is induced via isometric contractions in muscles of aged rodents. Isometric exercise provides an approach that eliminates the potential for oxidant production to be the result of muscle damage related to inflammatory cell infiltration that has been observed in the muscles of aged animals in response to concentric and eccentric repetitive loading exercise (25). In the present investigation, it was hypothesized that resveratrol would reduce the indices of isometric exercise-induced oxidative stress in muscles of aged mice. Furthermore, we hypothesized that resveratrol supplementation would improve muscle function and attenuate the loss of force during acute repetitive isometric contractions from muscles of aged mice.

Methods

Animals

Experiments were conducted on 25 young adult (3–5 months) and 28 aged (26–28 months) C57BL/6 mice obtained from the National Institute on Aging colony (Harlan, Indianapolis, IN). The mice were housed in pathogen-free conditions at ~20ºC. All mice had free access to water and either a control diet (n = 12 young adult and n = 14 aged) (AIN-76A Rodent Diet; Research Diets Inc, New Brunswick, NJ) or an identical diet that contained 0.05% resveratrol (n = 13 young adult and n = 14 aged) (Research Diets Inc) for a total 10 days. The mice were given the experimental diet (control or resveratrol) for 7 days prior to the first exercise session and then kept on the same diet throughout the 3 days of exercise for a total of 10 days. Resveratrol was purchased from Orchid Pharmaceuticals (Tamil Nadu, India). All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards followed the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care and fully conformed to the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings."

Isometric Exercise

Mice were anesthetized with a mixture of oxygen (97%) and isoflurane gas (3%) using a small animal anesthetic system (Isotec 5, Ohmeda, Madison, WI). The left knee was secured in flexion by placing a metal rod on the lateral side of the knee. The left foot was secured to a footplate connected to a servomotor (Model 6350*350; Cambridge Technology Inc., Cambridge, MA). The ankle joint was aligned with the axis of rotation of the servomotor. Electrically evoked contractions of the plantar flexor muscles were accomplished by electrically stimulating (10 v, 100 Hz, 200-microsecond pulses) the tibial nerve via inserting platinum electrodes (Grass Medical Instruments, Astro-Med, Inc., West Warwick, RI) through the skin so that they were flanking either side of the nerve. The left plantar flexor muscle group from each animal was subjected to 20, electrically evoked, five-second isometric contractions (10 v, 100 Hz, 200microsecondmicrosecond pulses) with a 25-second recovery period between contractions, resulting in a daily 10-minute session for three consecutive days. The contralateral limb served as an intra-animal control.

Plantar flexion muscle functional data were collected as a force × time curve during isometric contractions for each session. The exercise sessions were performed on a custom-built mouse dynamometer. Briefly, the mouse was placed on a heated plate (37°C) with its right side down. Dynamic Muscle Control (DMC) software (Aurora Scientific Inc., Aurora, Ontario, Canada) was used to control the servomotor providing for the angular position of the foot. Muscle contractions were stimulated using a High-Power Bi-Phase Current Stimulator (Aurora Scientific Inc.). Data files from the DMC software were analyzed by the Dynamic Muscle Analysis software (Aurora Scientific Inc.).

Muscle Levels of Hydrogen Peroxide (H_2O_2)

H_2O_2 content in control and exercised mouse gastrocnemius muscles was measured in eight animals of each age and diet group by a fluorescent assay according to the manufacture's recommendations (Cell Technology, Mountain View, CA). The sample fluorescence was detected at an excitation of 530 nm and measured at 590 nm. All analyses were completed in duplicate. The data from the tissue samples were normalized to the muscle protein concentration of each sample, as measured according to Bradford (26). The reagents for the protein assay were purchased from Bio-Rad (Hercules, CA). Each sample and standard was obtained in duplicate.

Total Glutathione and Reduced Glutathione/Oxidized Glutathione Content of Aged and Exercised Muscles

A BIOXYTECH GSH/GSSG-412 (Percipio Biosciences, Inc., Burlingame, CA) assay kit was used to determine the total glutathione and the reduced glutathione to oxidized glutathione (GSH/GSSG) ratio in control and exercised gastrocnemius muscles of eight young and eight aged mice in each diet group. For total GSH measurements, ~40 mg of fresh muscle was homogenized immediately after dissection in 530 μL of cold 5% metaphosphoric acid (MPA). GSSG was obtained on tissue samples after homogenization in 500 μL cold 5% MPA and 30 μL of a M2VO scavenger. The tissue homogenates were flash-frozen and stored at −80°C until time of analysis.

The assay was conducted according to the manufacturer's recommendations and as described previously (1). The reaction was initiated by adding 50 μL of NAD(P)H, and the absorbance of each sample was read every 60 second at 412 nm for 3 minutes. The protein concentration of each sample was determined as described previously (26). The optical density (OD) from each sample was normalized to the protein content of the respective sample. Each sample and standard was performed in duplicate.

Muscle Levels of Xanthine Oxidase Activity

An Amplex Red, xanthine oxidase assay (#A22182; Invitrogen, Eugene, OR), was used to measure xanthine oxidase activity, xanthine, and hypoxanthine concentration in the gastrocnemius muscle homogenates from eight animals in each age group by following the manufacturer's suggestions. Briefly, tissue homogenates were mixed with 100 μM Amplex Red, 0.4 U/mL horseradish peroxidase, and 200 μM hypoxanthine and incubated at 37°C in the dark. Fluorescence was measured in a microplate reader using an excitation of 530 nm and emission detection at 590 nm. Each sample was corrected for background fluorescence and then normalized to protein concentrations (Bio-Rad) of the original samples. Hypoxanthine and xanthine were measured in the same manner; however, xanthine oxidase was used in the assay instead of hypoxanthine. Relative concentrations of hypoxanthine and xanthine concentrations were determined by comparing sample values relative fluorescent units (RFU). Each sample and standard was performed in duplicate.

Muscle Levels of Lipid Peroxidation

Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were measured using BIOXYTECH LPO-586 reagents (Percipio Biosciences, Inc., Burlingame, CA) as an indicator for the levels of lipid peroxidation in the gastrocnemius muscle samples from eight animals in each age and diet group, as described previously (1). Briefly, ~75mg of muscle was homogenized in ice-cold phosphate-buffered saline (PBS) containing 0.5 M butylated hydroxytoluene in acetonitrile. The tissue samples were homogenized, and the supernatant was used to determine lipid peroxidation. The resulting OD signals were measured with an absorbance at 586 nm (DYNEX Technologies, Chantilly VA). The protein content of the sample was determined as described previously (26) and used to normalize the OD of each sample. Each sample and standard was performed in duplicate.

NAD(P)H Oxidase Activity

Superoxide generation by NAD(P)H oxidase is a potential source of reactive oxygen species (ROS) in aging (5,27) and exercise (5). NAD(P)H oxidase activity was determined based on superoxide-induced lucigenin photoemission, as described by Cui and Douglas (28), with slight modifications. Enzyme assays were carried out in a final volume of 1 mL containing 50 mM phosphate buffer (pH 7.0), 1 mM ethylene glycol tetraacetic acid (EGTA), 150 mM sucrose, 0.5 mM lucigenin, 0.1 mM NADH, and 100 μg of muscle homogenate. Enzyme reactions were initiated with the addition of lucigenin. Photoemission was measured every minute for 15 minutes using a luminometer, and the integrated data were expressed as relative light units per minute. Assays were carried out in the dark, at 25°C, with appropriate controls. The protein content of the sample was determined as described previously (26) and used to normalize the data from each sample. Each sample and control was measured in duplicate.

NAD(P)H Oxidase Protein Levels

NAD(P)H oxidase has been shown to be regulated by resveratrol in some cells (29,30), although it is not known if this is also the case in skeletal muscle. We used western blot analyses to determine the protein levels of NAD(P)H oxidase subunit-4 (Nox4) and NAD(P)H oxidase subunit-1 (Nox1) in resveratrol-treated mouse muscles. Whole gastrocnemius muscle homogenates from six aged mice in each diet group and five young adult mice in each diet group were prepared according to the methods previously described in our laboratory (31,32). Briefly, 60 micrograms of protein was boiled for 5 minutes at 95°C in Laemmli buffer and was loaded on each lane of a 12% polyacrylamide gel and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with Ponceau S red (Sigma Chemical Co, St Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. The membranes were probed with Nox1 (1:500 dilution, ab55831) and Nox4 (1:500 dilution, ab81967), which were purchased from Abcam (Cambridge, MA). The primary antibody incubations were performed overnight at 4°C. Secondary antibodies were conjugated to horseradish peroxidase (Chemicon International, Temecula, CA), and signals were developed by an enhanced chemiluminescent assay (Amersham Biosciences, Piscataway, NJ). The signals were then visualized by exposing the membranes to x-ray films (BioMax MS-1; Eastman Kodak, Rochester, NY), and digital records of the films

were captured with a Kodak 290 camera. Resulting bands were quantified as OD × band area by a one-dimensional image analysis system (Eastman Kodak Company, Rochester, NY), and the signals were normalized to the corresponding GAPDH signal. The data are reported in arbitrary units. The molecular sizes of the immunodetected proteins were verified by using prestained standard (LC5925; Invitrogen/Life Technologies, Bethesda, MD).

Glutathione Peroxidase in Exercised and Control Muscles

Cellular glutathione peroxidase (GPx) was used to measure GPx activity in gastrocnemius muscle homogenates from eight animals in each age and diet group according to the manufacturer's recommendations (#35319; EMD/Calbiochem, San Diego, CA) and as previously described (1). The muscle samples were homogenized in PBS (pH 7.5) containing 5 mM EDTA and 1 mM dithiothreitol and then centrifuged. The supernatant was used for the GPx assay. The resulting absorbance was measured at 340 nm (DYNEX Technologies). Each sample and standard was performed in duplicate.

Catalase Activity

The activity of catalase was determined in gastrocnemius muscle homogenates (#219265; EMD/Calbiochem) as described previously (1). Eight animals were examined in each age and diet treatment group. The samples were read on a microplate reader (DYNEX Technologies) at an absorbance of 520 nm. All analyses were measured in duplicate, and the samples were normalized to the corresponding protein concentration of the sample (Bio-Rad).

Activity Levels of Manganese Superoxide Dismutase (MnSOD) and Copper–Zinc Superoxide Dismutase (CuZnSOD)

Superoxide dismutase was measured in gastrocnemius muscles (n = 16 young adult; n = 16 aged) using a commercially available SOD Assay Kit II (#574601; EMD/Calbiochem). SOD and MnSOD activities were measured in control and exercised gastrocnemius muscles. Eight animals were examined in each age and diet group. CuZnSOD was determined from assuming that CuZnSOD was the result of subtracting MnSOD activity from the total SOD activity. The assay was performed with slight modifications to the manufacturer's directions, and all samples and standards were measured in duplicate as described previously by our laboratory (1). Briefly, gastrocnemius muscle samples were homogenized in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid containing 1mM EGTA, 210 mM mannitol, and 70 mM sucrose, and the insoluble material was discarded. The supernatant was incubated either with or without 12 mM potassium cyanide to inhibit CuZnSOD and extracellular SOD activity. The sample absorbance was measured at 450 nm using a 96-well plate reader (DYNEX Technologies).

Messenger RNA Levels of Endogenous Antioxidant Enzymes

Messenger RNA (mRNA) for GPx-1, catalase, MnSOD, and CuZnSOD were measured in gastrocnemius muscle samples (n = 16 young adult; n = 16 aged) using reverse transcription–polymerase chain reaction (RT-PCR) according to the methods previously published by our laboratory (1,31). Eight animals were examined in each age and diet treatment group. Total RNA was reversed transcribed using random primers, dNTP, and SuperScript II reverse transcriptase (Invitrogen/Life Technologies). The primers for CuZnSOD, MnSOD, GPx-1, and catalase have been previously described (1). The signal from the gene of interest was expressed as a ratio to the 18S signal from the same PCR product. The PCR product from each reaction was separated by agarose gel electrophoresis. The gels were stained with ethidium bromide, and the resulting signals were digitally captured (Kodak 290). The signals were quantified using 1D Kodak image analysis software (Eastman Kodak Company).

Citrate Synthase Activity

Citrate synthase activity was measured in gastrocnemius muscle homogenates from 12 aged mice (n = 6 in each diet and age group) and 10 young adult mice (n = 5 in each diet and age group). Fifteen milligrams of gastrocnemius muscle was homogenized on ice in 0.1 M Tris buffer containing 0.1% Triton X-100, pH 8.35. Citrate synthase activity was determined spectrophotometrically according to the method of Srere (33) with slight modifications from that previously reported by our laboratory (34). Briefly, the homogenates were frozen under liquid nitrogen and thawed four times to disrupt the mitochondria. The assay system (Sigma–Aldrich, St. Louis, MO) contained in a total volume of 200 μL:100 mM Tris buffer (pH 8.35), 5 mM 5,5-dithiobis(2-nitrobenzoate), 22.5 mM acetyl-CoA, 25 mM oxaloacetate, and 4 μL of muscle homogenate. The color change was monitored at wavelength of 405 nm at 15-second intervals for a period of 3 minutes by using a plate reader. All measurements were performed in duplicate, in the same setting at 25°C. The solubilized protein extracts of the homogenates were quantified in duplicate by as described previously (26). The citrate synthase activity was normalized to the total protein content and was reported in as nanomoles per milligram protein per minute.

Mitochondrial Isolation

The procedure for the isolation of mitochondria was adapted from Cogswell et al. (35). The entire procedure was performed at 0°C–4°C. Gastrocnemius muscles from 12 aged mice (n = 6 in each diet and age group) and 10 young adult mice (n = 5 in each diet and age group) were immediately placed in ice-cold extraction buffer I (100 mM KCl, 5 mM $MgSO_4 \cdot 7H_2O$, 5 mM EDTA, 50 mM Tris–HCl,

pH 7.4) then blotted and weighed. Hundred milligrams of muscle was minced with scissors in 1 mL of extraction buffer II (extraction buffer I with 1 mM ATP, pH 7.4), homogenized in an ice-cold glass Potter-Elvehjem homogenizer, then homogenized with a loose-fitting Teflon pestle (seven strokes, 1200*g*). The homogenate was centrifuged at 800*g* for 10 minutes at 4°C. The supernate was centrifuged at 12,000*g* for 10 minutes at 4°C to pellet the mitochondria. The remaining myofibrillar pellet was rehomogenized and digested in 5 mg/kg trypsin on ice for 10 minutes. After diluting the sample twofold in extraction buffer II containing bovine serum albumin (BSA) (10 mg/mL), the resulting homogenate was centrifuged at 800*g* for 10 minutes at 4°C. The supernatant was centrifuged at 12,000*g* for 20 minutes at 4°C to pellet the mitochondria. The two mitochondria pellets were combined, resuspended in extraction buffer II, and centrifuged at 10,000*g* for 10 minutes at 4°C. The pellet containing isolated mitochondria was suspended in 100 μL of KME buffer (100 mM KCl, 50 mM 3-(N-morpholino)propanesulfonic acid, 0.5 mM EGTA, pH 7.0). The protein concentration of the mitochondria was quantified in duplicate by using a bicinchoninic acid assay (Pierce, Rockford, IL) and BSA standards. The mitochondria samples were frozen and stored at −80°C.

Electron Transport Chain Complex Activities

Electron transport chain (ETC) activities of complexes I, III, and IV were measured spectrophotometrically as previously described (36,37) in gastrocnemius muscles from 12 aged mice ($n = 6$ in each diet and age group) and 9 young adult mice ($n = 4$, control diet; $n = 5$, resveratrol diet). Complex I activity was determined by measuring the oxidation of NADH at 340 nm as previously described (37). The assay mixture for complex I contained 25 mM potassium phosphate buffer (pH 7.2), 5 mM $MgCl_2$, 2 mM KCN, 2.5 mg/mL BSA, 0.13 mM NADH, 65 decylubiquinone, and 2 μg/mL antimycin A. The reaction was initiated by adding purified mitochondria (45 μg), and the enzyme activity was measured for 3 minutes, with values recorded every 10 seconds after the initiation of the reaction. The complex I specific activity was inhibited by 2 μg/mL of rotenone. Complex III activity was determined as previously described (36,37) by following the reduction of cytochrome *c* at 550 nm in the presence of reduced decylubiquinone. Briefly, the assay buffer for complex III consisted of 500 mM sucrose, 2 mM EDTA, 100 mM Tris–HCl (pH 7.4), 1 mM cytochrome *c*, 200 mM KCN, 1mg/mL antimycin A and reduced decylubiquinone. Finally, complex IV activity was determined by measuring the oxidation of cytochrome *c* at 550 nm. Briefly, the assay mixture for complex IV consisted of 10 mM phosphate buffer (pH 7.4) and 20 μM reduced cytochrome *c*. Protein content was determined as described previously (26), and values expressed as activities in nanomoles of substrate consumed per minute per microgram of skeletal muscle protein.

Statistical Analysis

All statistical analyses were performed using SPSS software package (version 18; Chicago, IL). Comparison of means was determined using a multiple analyses of variance (MANOVA) to examine the main effect of aging, exercise, and resveratrol supplementation on the respective dependent variables. Pairwise comparisons were assessed post hoc using least significant difference tests. Statistical significance was established at $p < 0.05$. Data are reported as mean ± SEM.

Results

Body Weights and Food Intake

The average body weight of the aged animals was significantly ($p < .05$) more than the young animals (33.0 ± 0.8 g vs 25.5 ± 0.7 g). There was not a significant difference in body weight between nonsupplemented and resveratrol-supplemented animals, in either age group. Similarly, there was no difference in food intake between the control and resveratrol-supplemented diets. Although young adult animals had a similar food consumption (3.1 ± 0.3 g/d) as aged animals (3.8 ± 0.4 g/d) when normalized to body weight, the young animals received a greater amount of resveratrol per day (156.1 ± 18.1 g resveratrol/kg BW vs 113.5 ± 26.5 g resveratrol/kg BW).

Muscle Functional Measurements

Maximal isometric plantar flexion force was recorded for each evoked contraction. The greatest contributor to plantar flexion is the gastrocnemius muscle. The maximal isometric force recorded on the third day (which was the first contraction of that day) was normalized to the animal's body weight. Maximal plantar flexion isometric force normalized to body weight was 25% and 27% greater in young adult than aged animals that consumed the control or the resveratrol-supplemented diet, respectively. Resveratrol supplementation did not have a significant influence on maximal isometric force in either the young adult or the aged animals (Figure 1A).

The fatigability of the plantar flexors within each training session was assessed by comparing the net loss of force from the first contraction of the session to subsequent contractions. Neither aging nor resveratrol had any significant effect on the maximal force produced during the first five contractions of the exercise session. However, after the fifth contraction, on the third day, the aged animals on the control diet and the aged animals on the resveratrol diet showed a greater maintenance of force than young animals on the control diet ($p < .05$). No significant differences in fatigability were observed in the aged animals that received control or resveratrol-supplemented diets, whereas young animals who were supplemented with resveratrol showed an improved maintenance of force over the exercise session (Figure 1B). A representative plantar flexor isometric force tracing from

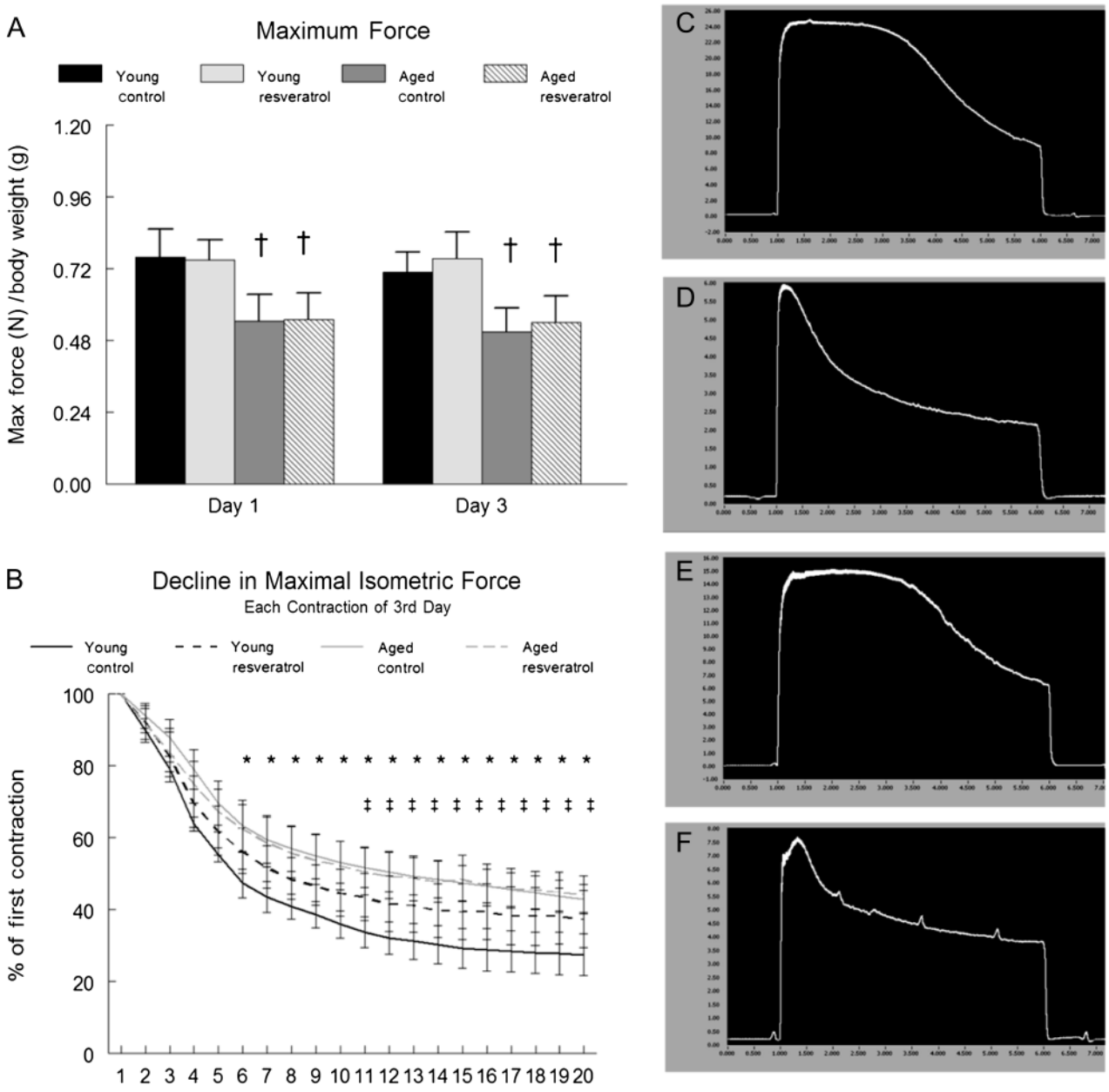

Figure 1. Maximal plantar flexor isometric force after 10 days of dietary supplementation with 0.05% resveratrol or a control diet and three consecutive days of isometric exercise. (**A**) Data are expressed as the mean ± SEM of the maximal isometric force recorded on the third day of exercise by the left plantar flexor muscles normalized to the body weight of the animal. Dagger signifies a difference ($p < .05$) from young adult diet-matched muscles. (**B**) Data are expressed as the mean ± SEM of the relative difference between the maximal isometric force on the first contraction and the force produced on subsequent contractions. All force measurements were normalized to body weight. Asterisk indicates a significant difference ($p < .05$) in the aged nonsupplemented control and resveratrol-supplemented diet from young nonsupplemented control diet. Double dagger indicates a significant difference ($p < .05$) in the young adult animals in the resveratrol-supplemented diet group from animals in the young adult nonsupplemented control diet group. (**C–F**) Representative force × time curves from the third consecutive exercise session in the resveratrol-treated young adult and aged animals. Force (in grams) is shown on the y-axis and time (in seconds) is shown on the x-axis. (**C**) First contraction of the third day in a young adult animal. (**D**) Twentieth contraction of the third day in a young adult animal. (**E**) First contraction of the third day in an aged animal. (**F**) Twentieth contraction of the third day in an aged animal.

the last exercise session is shown for a young animal in Figure 1C and D and for an aged mouse in Figure 1E and F.

Muscle Levels of H_2O_2

H_2O_2 was measured in gastrocnemius muscle homogenates as an indicator of oxidant production in basal and exercised conditions. Muscle homogenate levels of H_2O_2 were elevated in isometrically exercised muscles by 31% in young adult mice ($n = 8$, 1848 vs 2428 RFU/mg/protein) and 19% in aged in the animals ($n = 8$, 3689 vs 4401 RFU/mg/protein) on the control diet compared with the age-matched nonexercised control limb ($p < .05$; (Figure 2). This indicates that acute isometric exercise elevated muscle

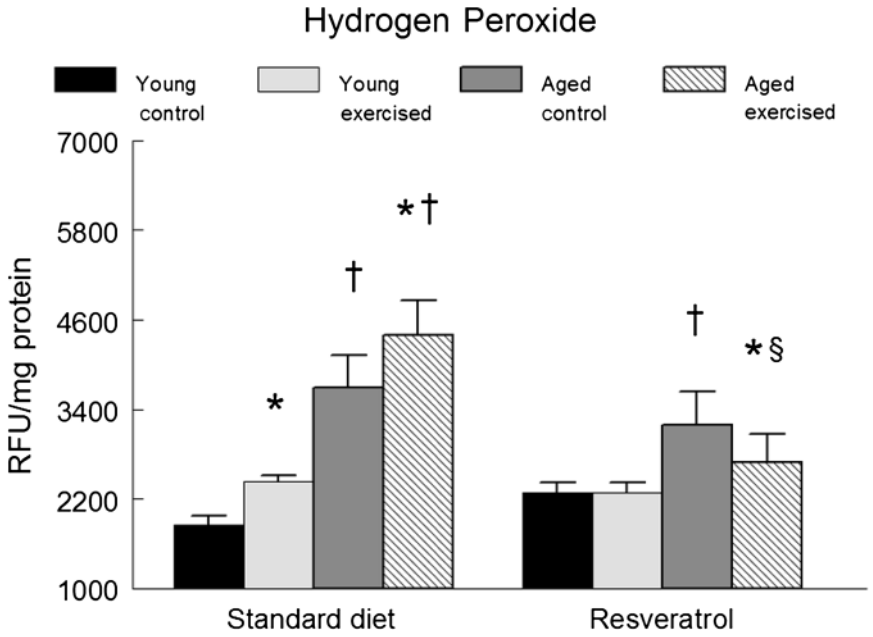

Figure 2. Resveratrol attenuated the increase in hydrogen peroxide (H_2O_2) concentration associated with exercise and aging. The H_2O_2 concentration was determined fluorometrically in muscles of mice after 3 days of isometric exercise. The animals were fed a control diet or a diet containing 0.05% resveratrol for 10 days. Data are expressed as relative fluorescent units (RFU) per milligram of total protein in the gastrocnemius homogenate. The normalized data are presented as mean ± SEM. *Significant difference ($p < .05$) of isometrically exercised muscle from contralateral control muscle; †significant difference ($p < .05$) from young exercised and diet-matched control muscles; §significant difference ($p < .05$) from age-matched animals on the nonsupplemented diet.

oxidant production. Control and repetitively loaded muscles from aged animals had 99% and 81% higher levels of H_2O_2, respectively, than their treatment-matched muscles from young adult mice ($p < .05$; Figure 2). Fortifying the standard diet with resveratrol lead to a 24% ($p < .05$) increase in H_2O_2 in the young exercised gastrocnemius muscles. Resveratrol significantly lowered H_2O_2 levels in both control and exercised muscles by 13% and 38%, respectively, from aged ($n = 8$) animals ($p < .05$; Figure 2). Isometric exercise did not affect muscle levels of H_2O_2 in the young adult mice ($n = 8$) that received a diet supplemented with resveratrol. However, resveratrol significantly reduced H_2O_2 by 15% in the exercised muscles of aged mice compared with nonexercised mice fed the control diet (3190 vs 2693 RFU/mg/protein; Figure 2).

NAD(P)H Oxidase Activity and Protein Content

NAD(P)H oxidase activity was not increased significantly by 3 days of isometric exercise in gastrocnemius muscles of young or aged mice (Figure 3A), although it did approach significance for the young control group ($p = .08$). Resveratrol did not affect NAD(P)H oxidase levels in control or exercised gastrocnemius muscles in either aged group. Nox4 protein abundance was similar in muscle samples from young adult and aged mice (Figure 3B). Exercise did not significantly increase Nox4 protein abundance in gastrocnemius samples from young adult or old muscles. However, 10 days of resveratrol treatment suppressed Nox4 protein abundance in exercised gastrocnemius muscles of both young and aged mice (Figure 3B). Nox 1 protein levels did not change with age, exercise, or resveratrol treatment (data not shown).

Hypoxanthine, Xanthine, and Xanthine Oxidase Activities

Hypoxanthine, a product of purine degradation and a substrate for xanthine oxidase, increased by 36% ($p < .05$) with aging. Furthermore, isometric exercise increased muscle levels of hypoxanthine by 21% ($p < .05$) in young adult mice ($n = 8$) and by 20% ($p < .05$) in aged mice ($n = 8$). Resveratrol supplementation blunted the increase in hypoxanthine in muscles from both young adult ($n = 8$) and aged ($n = 8$) mice, but it did not reduce the elevated levels of hypoxanthine associated with aging (Figure 4A).

Xanthine and superoxide are the products of hypoxanthine reacting with O_2 in the presence of xanthine oxidase, which acts as a catalyst for the reaction. Gastrocnemius muscle xanthine was elevated after isometric exercise in young adult ($n = 8$) and aged mice ($n = 8$) by 19% ($p < .05$) and 45% ($p < .05$), respectively. The increase in xanthine with exercise was prevented in the muscles from the mice fed the diet supplemented with resveratrol. No age-dependent changes in xanthine levels were observed (Figure 4B).

The activity of xanthine oxidase in gastrocnemius muscles from aged animals was elevated by 168% compared with muscles from young adult mice (258.9 vs 692.3 RFU/mg/protein, $p < .05$; Figure 4C). Resveratrol supplementation lowered

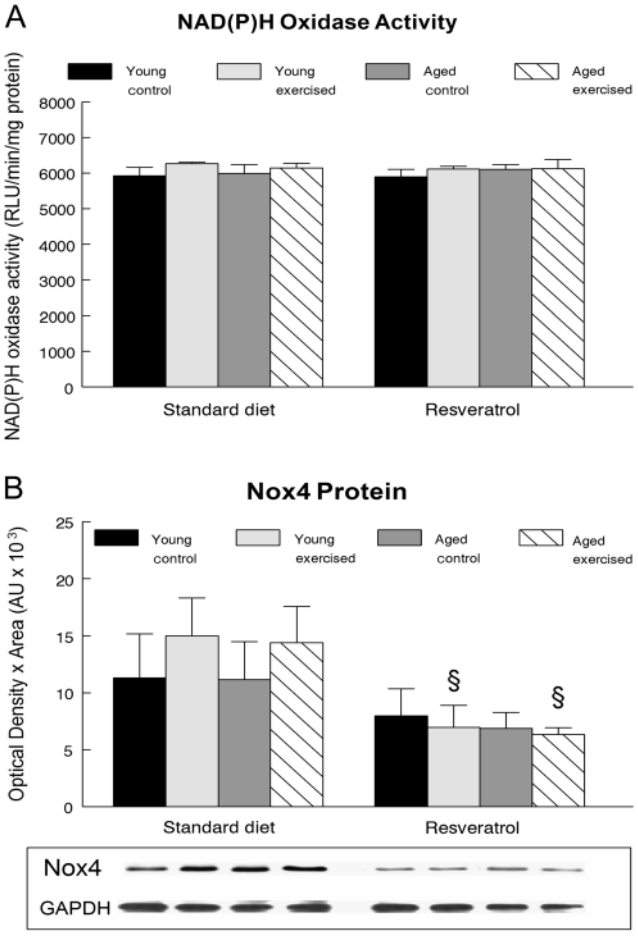

Figure 3. NAD(P)H oxidase activity. NAD(P)H oxidase activity was determined in control and exercised gastrocnemius muscle samples from 6 aged animals and 5 young adult animals in each diet and age group (A). The mice received a diet of 0.05% resveratrol or a control diet for 10 days, and exercise was for three consecutive days. Lucigenin photoemission was quantified for 15 minutes. The data are reported as mean ± SEM and expressed as relative light units (RLU) per minute per milligram of muscle protein. Nox4 protein abundance was determined by western blot analysis in control and exercised gastrocnemius muscle samples from 6 aged and 5 young adult animals in each diet group (B). A representative blot is shown. GAPDH was used as a loading control. Protein abundance was expressed relative to GAPDH.

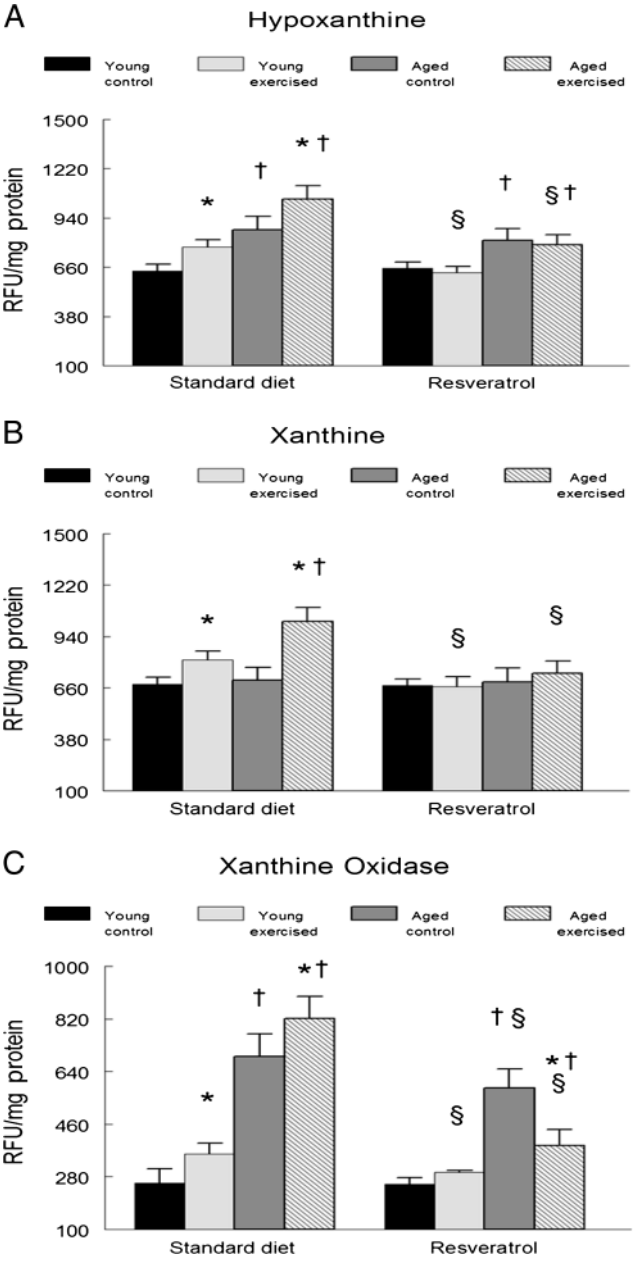

Figure 4. Resveratrol attenuated the increase in xanthine oxidase, hypoxanthine, and xanthine activity. Relative concentrations of hypoxanthine (**A**), xanthine (**B**), and enzymatic activity of xanthine oxidase (**C**) were determined fluorometrically. Data are expressed as relative fluorescent units (RFU) per milligram of total protein in gastrocnemius homogenates. The normalized data are presented as mean ± SEM. *Significant difference ($p < .05$) between isometrically exercised muscles from contralateral control muscles; †significant difference ($p < .05$) from young exercised and diet-matched control muscles; §significant difference ($p < .05$) from age-matched animals in the control (nonsupplemented) diet group.

xanthine oxidase activity in the nonexercised muscle from aged mice by 16% (692.3 vs 582.9 RFU/mg/protein, $p < .05$; Figure 4C), but it had no effect on the nonexercised control muscle from young adult mice. Compared with the contralateral control muscles, isometric exercise increased xanthine oxidase activity by 38% ($p < .05$) in gastrocnemius muscles from young adult mice and by 18% ($p < .05$) in muscles from aged animals on the control diet. Resveratrol supplementation blunted the increase in xanthine oxidase activity associated with exercise in the young adult muscle by 25% ($p < .05$) and reduced xanthine oxidase activity in the aged gastrocnemius by 53% ($p < .05$; Figure 4C). Resveratrol supplementation in the aged isometrically exercised muscles resulted in a 50% ($p < .05$) reduction in muscle xanthine oxidase activity when compared with the nonexercised muscle (Figure 4C).

The Concentration of Glutathione

Total glutathione decreased by 26% ($p < .05$) in the control diet nonexercised gastrocnemius muscle of aged mice ($n = 8$, 115 ± 18 μM GSH/mg protein) compared with young adult muscle ($n = 8$, 156 ± 26 μM GSH/mg protein). However, there was no significant difference between the young

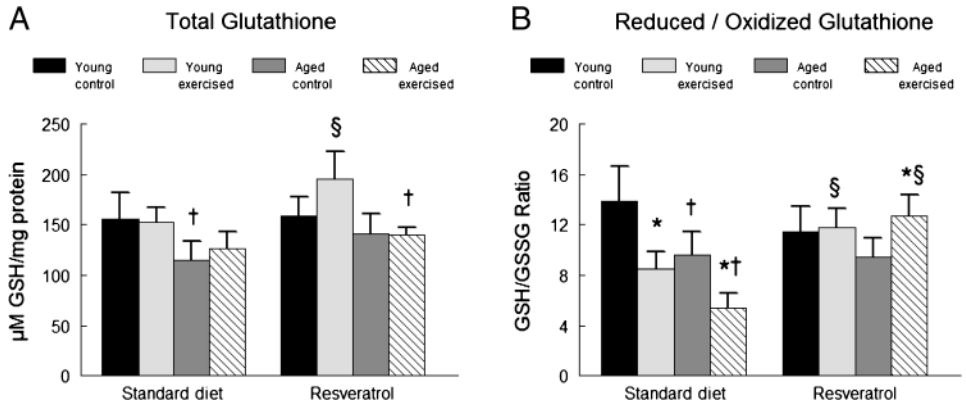

Figure 5. Total glutathione content and ratio of reduced glutathione to oxidized glutathione (GSH/GSSG). (**A**) Data indicate total glutathione concentration normalized to total protein content. (**B**) Data are reported as the ratio of GSH to GSSG normalized to total protein content. Lower GSH/GSSG ratios are an indication of increased oxidative stress. The normalized data are presented as mean ± SEM. *Significant difference ($p < .05$) of isometrically exercised muscle from contralateral control muscle; †significant difference ($p < .05$) from young exercised and diet-matched control muscles; §significant difference ($p < .05$) from age-matched animals on the nonsupplemented diet.

adult ($n = 8$, 153 ± 15 μM GSH/mg protein) and the aged exercised gastrocnemius muscles ($n = 8$, 126 ± 17 μM GSH/mg protein) and the age-matched mice receiving the control diet. Exercise alone failed to produce any changes in total glutathione within either age group receiving the control or the resveratrol fortified diet. A combination of resveratrol and exercise produced a 27% ($p < .05$) increase in total glutathione in the young adult animals (195 ± 27 μM GSH/mg protein) when compared with the young exercised animals that did not receive the fortified diet (153 ± 15 μM GSH/mg protein). No differences were observed in the aged animals. Resveratrol supplementation did not produce any significant differences in either the age group (Figure 5A).

The Ratio of GSH/GSSG

The GSH/GSSG ratio was 31% lower in control nonexercised muscles of aged mice (9.5 ± 1.3) compared with young mice (13.9 ± 2.5). This reduction in the GSH/GSSG ratio is indicative of an increase in oxidants as a result of both aging and exercise and may also be the result of impaired GSH metabolism and replenishment (38). The GSH/GSSG ratio was not altered by resveratrol in nonexercised control muscles from either young adult or aged mice (Figure 5B). Isometric exercise decreased the GSH/GSSG ratio by 39% ($p < .05$) and 43% ($p < .05$) in the muscles of young and aged mice, respectively, that were fed the control diet (Figure 5B). However, resveratrol supplementation prevented the exercise-induced decrease in the GSH/GSSG ratio in the gastrocnemius muscle because GSH/GSSG was 38% and 135% greater in muscles from resveratrol-treated exercised young and aged mice, respectively, as compared with muscles from age-matched mice on the control diets.

Muscle Lipid Peroxidation Levels

Aging increased the total amount of lipid peroxidation in nonexercised control gastrocnemius muscles by 57%, as shown by the greater MDA + HNE levels in control muscles from aged nonsupplemented rats compared with control muscles from young adult nonsupplemented animals ($n = 8$, 2.86 vs 4.49 μM/mg protein, $p < .05$; Figure 6). Three days of isometric exercise failed to alter lipid peroxidation levels within gastrocnemius muscles from the young adult mice, but it increased MDA + HAE levels by 63% ($n = 8$, 4.49 vs 7.31 μM/mg protein, $p < .05$; Figure 6) in the muscles from aged nonsupplemented mice. Resveratrol supplementation completely prevented the increase in MDA + HNE levels associated with exercise in the muscles from aged mice, but resveratrol did not blunt the increase in lipid peroxidation

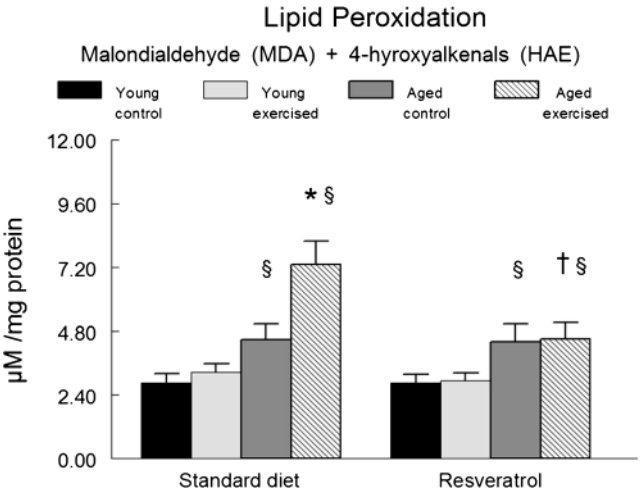

Figure 6. Resveratrol supplementation decreased lipid peroxidation associated with isometric exercise but not aging. The level of lipid peroxidation was estimated from malondialdehyde (MDA) plus 4-hydroxyalkenals (HAE) levels that were normalized to total protein content in the muscle sample. The normalized data are presented as mean ± SEM. *Significant difference ($p < .05$) of isometrically exercised muscles from contralateral control muscles; †significant difference ($p < .05$) from young exercised and diet-matched control muscles; §significant difference ($p < .05$) from age-matched animals on the nonsupplemented diet.

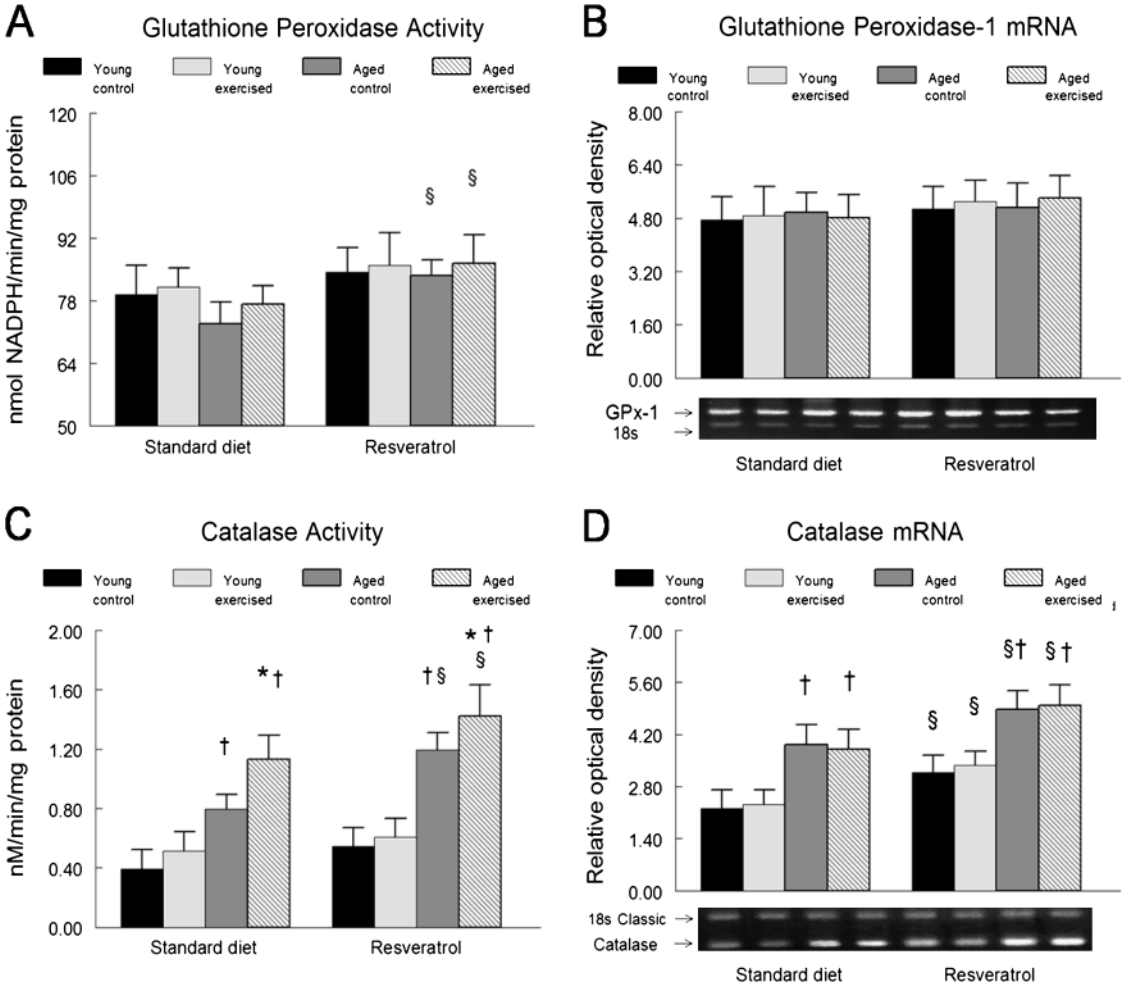

Figure 7. Glutathione peroxidase (GPx) activity, GPx-1 messenger RNA (mRNA), catalase activity, and catalase mRNA regulation with isometric exercise and resveratrol supplementation (**A**) Total GPx activity is expressed as nanomoles NADPH per minute per milligram of protein. (**B**) GPx-1 mRNA expression was determined by reverse transcription–polymerase chain reaction (RT-PCR). The data are expressed as optical density (OD) × band area and expressed as a relative OD. The inserts show representative gels for GPx-1 mRNA and 18S ribosomal RNA in young and aged (control and isometric exercised) gastrocnemius muscle. (**C**) Total catalase activity is expressed as nanomoles of activity per minute normalized per milligram of protein in the homogenate. (**D**) Catalase mRNA expression was determined by RT-PCR. The data are expressed as OD × band area and expressed as a relative OD. The inserts show representative gels for catalase mRNA and 18S mRNA in muscles from young and aged (control and isometrically exercised) mice. For all graphs, the normalized data are presented as mean ± SEM; *significant difference ($p < .05$) of isometrically exercised muscles from contralateral control muscles; †significant difference ($p < .05$) from young exercised and diet-matched control muscles; §significant difference ($p < .05$) from age-matched animals on the nonsupplemented diet.

associated with aging. Both control and isometrically exercised muscles from aged resveratrol-supplemented animals showed a ~53% ($p < .05$) increase in MDA + HAE levels when compared with their young adult counterparts. Resveratrol supplementation had no effect on lipid peroxidation in the muscles from young adult animals.

GPx and Catalase Enzyme Activity and mRNA Abundance

There was no apparent aging or exercise effect on GPx activity in the nonsupplemented gastrocnemius muscle. Furthermore, resveratrol supplementation did not lead to any changes in GPx activity in the control or exercised muscle from young adult animals. However, GPx activity increased 15% ($p < .05$) in control and 12% ($p < .05$) in exercised muscles from aged mice with resveratrol supplementation (Figure 7A). No significant changes were found among GPx-1 mRNA within any of the muscle samples (Figure 7B).

Catalase activity was higher in the gastrocnemius muscle from the aged animals when compared with their treatment-matched young adult counterparts. Neither isometric exercise nor resveratrol supplementation showed any significant changes in catalase activity within muscles from young adult animals. There was a 42% increase ($p < .05$) in catalase activity with isometric exercise in the aged animals on the standard diet and a 19% increase ($p < .05$) with the resveratrol-supplemented diet. Resveratrol supplementation increased catalase activity within the aged animals by 50% ($p < .05$) in the nonexercised control and 25% ($p < .05$) in the

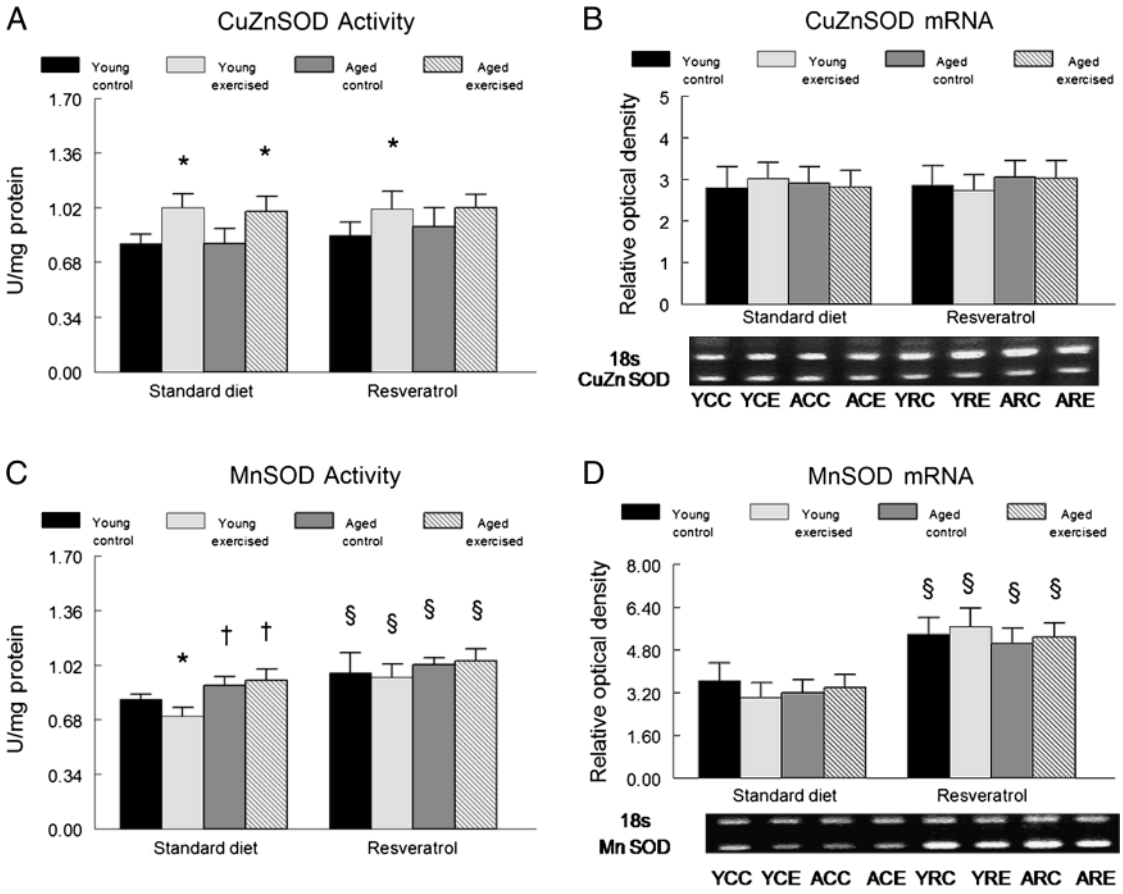

Figure 8. Superoxide dismutase activity and messenger RNA (mRNA) regulation with isometric exercise and resveratrol supplementation. (**A**) Copper–Zinc superoxide (CuZnSOD) activity was expressed as units of CuZnSOD per milliliter of homogenate normalized per milligram of protein in the homogenate. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. (**B**) CuZnSOD mRNA expression was determined by reverse transcription–polymerase chain reaction (RT-PCR). The data are expressed as optical density (OD) × band area and reported as relative OD. The inserts show representative gels for CuZnSOD mRNA and 18S ribosomal RNA (rRNA) in gastrocnemius muscles from young and aged (control and isometric exercised) mice. (**C**) Manganese superoxide dismutase (MnSOD) activity was determined expressed as units of MnSOD per milliliter of homogenate normalized per milligram of protein in homogenate. One unit is equal to the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical (D) MnSOD mRNA expression was determined by RT-PCR. The data are expressed as OD × band area and expressed as relative OD. The inserts show representative gels for MnSOD mRNA and 18S rRNA in gastrocnemius muscles from young and aged (control and isometric exercised) mice. For all graphs, the normalized data are presented as mean ± SEM; *significant difference ($p < .05$) of isometrically exercised muscles from contralateral control muscles; †significant difference ($p < .05$) from young exercised and diet-matched control muscles; §significant difference ($p < .05$) from age-matched animals on the nonsupplemented diet. ACC = aged-control diet-control nonexercise; ACE = aged-control diet-exercised; ARC = aged-resveratrol-control nonexercised; ARE = aged-resveratrol-exercised; YCC = young-control diet-control nonexercise; YCE = young-control diet-exercised; YRC = young-resveratrol-control nonexercise; YRE = young-resveratrol-exercised.

isometrically exercised gastrocnemius muscle (Figure 7C). Similar to the enzyme activity data, catalase mRNA content was greater in the gastrocnemius muscle from all groups of the aged animals when compared with their treatment-matched young adult counterparts. Supplementation with resveratrol increased catalase mRNA levels in the gastrocnemius muscle from both young adult and aged animals. However, exercise did not produce any significant changes in catalase mRNA levels within any of the groups (Figure 7D).

Superoxide Dismutase Enzyme Activity and mRNA Levels

Isometric exercise increased CuZnSOD enzyme activity ($p < .05$) by 27% in nonsupplemented ($n = 8$) and 19% in resveratrol-supplemented muscles ($n = 8$) from young adult animals compared with their contralateral control muscles. Exercise increased CuZnSOD activity by 25% ($p < .05$) in the gastrocnemius muscles from the aged animals fed the standard diet ($n = 8$), but no differences in CuZnSOD activity were observed between the control and the exercised muscles of resveratrol-supplemented animals ($n = 8$). No significant changes in enzyme activity were observed as a result of aging in any of the groups (Figure 8A). No significant differences were found among CuZnSOD mRNA within any of the muscle samples (Figure 8B).

MnSOD activity was 10% greater in the nonexercised control gastrocnemius muscle from aged animals compared with young animals (0.89 vs 0.80 U/mg protein). Surprisingly, isometric exercise lead to a decrease in MnSOD activity in the gastrocnemius muscle from young adult animals

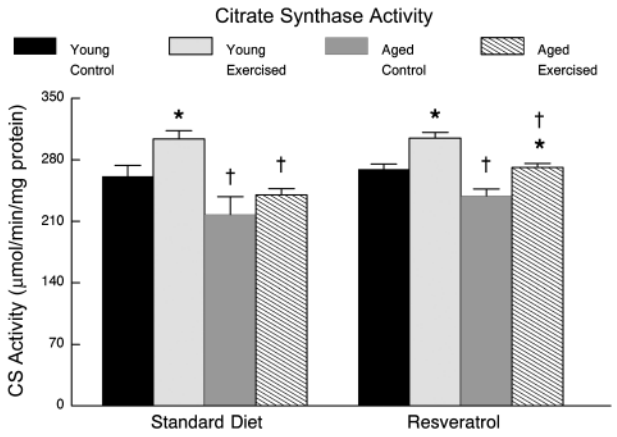

Figure 9. Citrate synthase (CS) activity. CS activity was obtained in gastrocnemius muscles from exercised and intra-animal young adult and aged mice that were fed either a control diet or 0.05% resveratrol. The data were normalized to the protein in the sample. The data are presented as mean ± SEM; *significant difference ($p < .05$) of isometrically exercised muscles from contralateral control muscles; †significant difference ($p < .05$) from young exercised and diet-matched control muscles.

feed the standard diet, whereas exercise had no effect on MnSOD activity in the muscles from aged animals. Resveratrol supplementation increased MnSOD activity by ~10%–15% in all groups and removed any differences between groups due to aging or exercise (Figure 8C). Although isometric exercise did not induce any changes in mRNA transcription for MnSOD, resveratrol feeding lead to a ~90% increase in MnSOD transcription in exercised and a ~50% increase in control muscles from both age groups (Figure 8D).

Citrate Synthase Activity

Citrate synthase activity was similar in control nonexercised gastrocnemius muscle samples from young animals on either control or resveratrol diets (Figure 9). Citrate synthase activity was 16% greater in the exercised muscles as compared with the intra-animal control muscle, but there was no difference between young adult mice fed resveratrol and control diets. Citrate synthase activity was not greater in exercised muscles than nonexercised muscles from animals on the control diet. In contrast, exercise improved citrate synthase activity in muscles from aged mice that were fed resveratrol, relative to the intra-animal control muscles.

Complexes I, III, and IV Activities

Complex I activity was 118% and 122% greater ($p < .05$) in the exercised muscle of young adult animals on control and resveratrol diets, respectively, as compared with the intra-animal control muscle (Figure 10). Complex I activity was not statistically different in gastrocnemius muscles of control and exercised muscles from aged animals on the control diet. In contrast, complex I activity was 95% greater

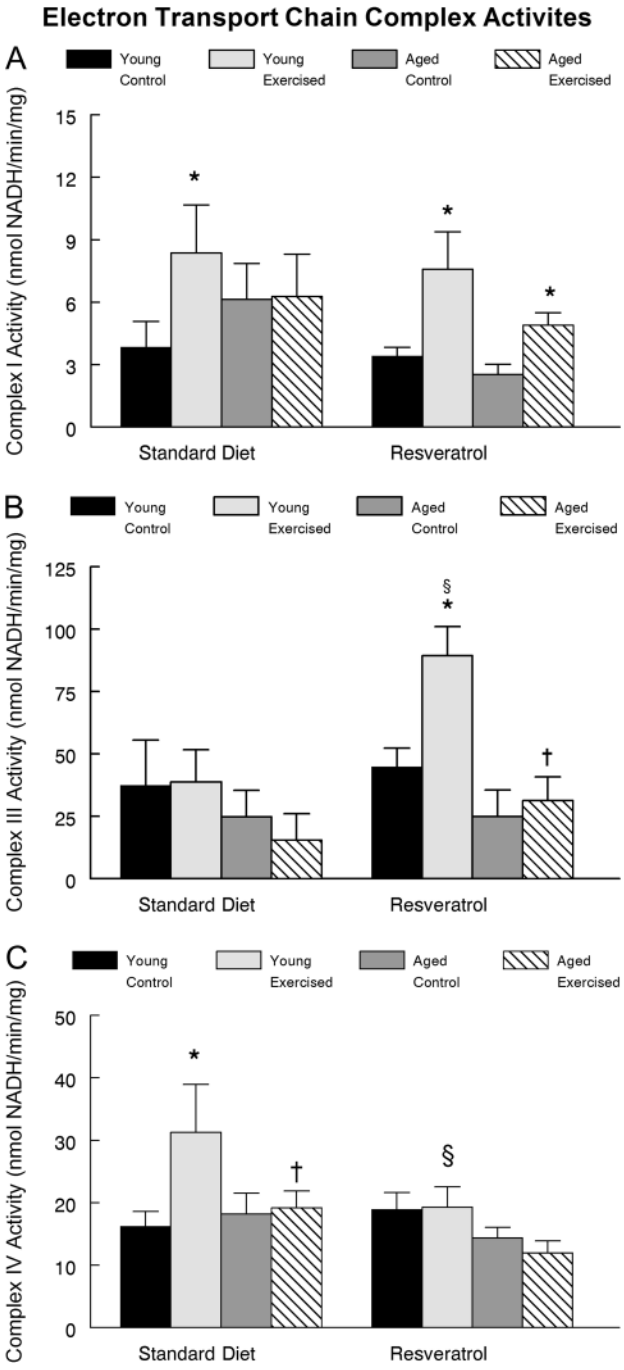

Figure 10. Electron transport chain (ETC) complex activities. ETC activities of complexes I (**A**), III (**B**), and IV (**C**) were measured spectrophotometrically in exercised and intra-animal gastrocnemius muscles from young adult ($n = 4$, control diet; $n = 5$, resveratrol diet) and aged mice ($n = 6$ control diet; $n = 6$, resveratrol diet). The data are presented as mean ± SEM of nanomoles of substrate consumed per minute per milligram of protein; *significant difference ($p < .05$) between isometrically exercised muscles from contralateral control muscles of the same animal group; †significant difference ($p < .05$) between muscles from old and young mice in the same exercise and diet group; §significant difference ($p < .05$) from age-matched animals on the nonsupplemented diet.

($p < .05$) in the exercised muscles versus the control muscles of aged animals that were supplemented with resveratrol. Although complex III activity also appeared to increase in the exercised versus control gastrocnemius muscles from

young animals, there was a high variability among the few samples that were investigated ($n = 4$ per group), and as a result, this failed to reach statistical significance in animals in the young control diet ($p = .21$) or the resveratrol diet ($p = .168$) group. However, complex III activity was greater in exercised muscles of resveratrol fed animals as compared with exercised muscles from animals on the control diet ($p < .05$).

MANOVA analysis indicated significant age ($F = 5.435$, $p = .026$) and diet ($F = 4.657$, $p = .038$) effects on complex IV activity. Post hoc analyses of the group means indicated that complex IV activity was not significantly affected by exercise or resveratrol in young either adult or aged animals (Figure 10). Although complex IV activity failed to increase with exercise in young adult animals, the increase as compared with the control muscle was close to significance ($p = .062$, $n = 4$). Complex IV activity was significantly greater ($p < .05$) in exercised muscles from young adult animals on the control diet as compared with the resveratrol diet (Figure 10).

Discussion

Oxidative stress is elevated with aging in most tissues, including skeletal muscle (1,39,40). Increased ROS production may contribute to aging-induced skeletal muscle wasting (ie, sarcopenia) (1,41). Although exercise is a useful approach to counter aging-induced sarcopenia, it also increases oxidative stress levels within the exercising muscles (40–42). The additive effects of an increase in oxidant production and an attenuated antioxidant buffering capacity potentially leave aged skeletal muscles vulnerable to oxidative damage. The novel data in this study show that dietary resveratrol reduces oxidative stress, including xanthine oxidase activity, in control and exercised muscles of aged mice and Nox4 protein abundance in exercised muscles of aged mice.

Xanthine Oxidase as One Source of Exercise-Induced Oxidant Stress

There are several intracellular sources producing oxidative stress in skeletal muscle. These include mitochondria, NAD(P)H oxidase, and xanthine oxidase (11). Although it is clear that oxidative stress is elevated in response to both acute exercise and aging, it has not been conclusively established that xanthine oxidase contributes to the increased oxidant production with advanced age in skeletal muscle. In the present study, we show that both xanthine oxidase activity and hypoxanthine levels are elevated in gastrocnemius muscles from aged mice compared with young adult mice. This is consistent with previous data showing that xanthine oxidase activity was higher in the gastrocnemius muscles from aged rats when compared with young animals (43) and in plasma from older versus young adult humans (44). Nevertheless, this contrasts with other data in humans that have shown an absence of age-associated increases in endothelial xanthine oxidase in antecubital venous cells from young and older subjects (45).

We anticipated that our model of repetitive maximal isometric contractions would increase oxidant stress in the exercised muscles. Our data show that repeated isometric exercise increased hypoxanthine levels, xanthine oxidase activity, and H_2O_2 production in the gastrocnemius muscles of both young and aged mice. These data are consistent with previous evidence that xanthine oxidase is, at least in part, responsible for oxidant production during exhaustive exercise.

Resveratrol Reduces Oxidative Stress

Resveratrol has been shown to exert a variety of health benefits that include the direct scavenging of ROS (21), the inhibition of xanthine oxidase (46,47), and the activation of intracellular pathways that improve metabolism and induce mitochondrial biogenesis (18,48). The current study suggests that resveratrol supplementation lowers muscle oxidative stress (H_2O_2, xanthine oxidase activity, GSH/GSSG ratio, and lipid peroxidation) associated with both normal aging and isometric exercise in muscles from aged mice. NAD(P)H oxidase activity and NOX1 protein abundance were not increased significantly by exercise in muscles of young or aged animals, although NAD(P)H oxidase activity did approach significance for the exercised muscles from young animals in the control diet group ($p = .08$). Resveratrol did not affect NAD(P)H oxidase levels in control or exercised gastrocnemius muscles of young or aged animals. Although resveratrol has been shown to reduce NAD(P)H oxidase activity (49), including regulation of Nox1 and Nox 4 (29,50), we were unable to find any effect of resveratrol on Nox 1 protein abundance in muscles from aged or young adult mice. This may have been due to the low numbers of animals, the low dose, and/or the short duration of resveratrol treatment (10 days) or a lower sensitivity of Nox1 to resveratrol, which together, prevented us from detecting any effect of resveratrol on NAD(P)H oxidase activity. In contrast, resveratrol appeared to suppress Nox4 protein abundance in the exercised muscles of young and aged mice. These data suggest that resveratrol may potentially suppress exercise-induced oxidative stress via a reduction of both xanthine oxidase activity and Nox4 protein levels, without altering overall NAD(P)H oxidase activity.

Muscle xanthine oxidase activity and H_2O_2 production were lower in exercised muscles of aged mice as compared with muscles of age-matched nonexercised nonsupplemented control muscles. In addition, resveratrol supplementation abolished the increase in xanthine oxidase activity and H_2O_2 production associated with isometric contractions in muscles from young mice. Together these data suggest that there may be an additive benefit to combining resveratrol supplementation with isometric exercise, especially in skeletal muscle from aged animals.

As expected, the short duration of resveratrol supplementation in the current study affected H_2O_2 concentrations, xanthine oxidase activity, and lipid peroxidation to a greater

degree with exercise than aging. It is therefore likely that aging and isometric exercise may regulate oxidant production via different mechanisms. For example, isometric exercise increased the activity of the cytosolic antioxidant enzymes, catalase, and CuZnSOD, along with an increase in xanthine oxidase activity. This observation suggests that at least part of the increase in H_2O_2 production with isometric exercise is not originating from the mitochondria. This possibility is in agreement with observations showing that aging increases oxidant production via mitochondrial sources, whereas exercise increases oxidants in muscles from multiple sources, including xanthine oxidase (5).

Resveratrol's putative role in reducing oxidative stress is likely a combination of many factors. When taken orally, *trans*-resveratrol is well absorbed by mammals, but its bioavailability is low due to its rapid first-pass metabolism (51). Therefore, its role as a direct scavenger of ROS (24) is likely to be limited. The most likely mechanism by which resveratrol can attenuate the increase in oxidative stress due to aging and exercise lies in its ability to induce transcriptional changes via the activation of silent mating type information regulation 2 homolog (Sirt1) (19). Sirt1 is a NAD^+-dependent histone deacetylase, that is, upstream of a wide variety of cellular pathways involved in energy homeostasis, longevity, cell survival, and apoptosis. Increases in SIRT1 transcription have been shown to occur after 3 days of resveratrol supplementation (52). Sirt1 activation sequentially leads to energetic adaptations within the muscle by activating the metabolic regulators nuclear receptor peroxisome proliferator-activated receptor *gamma* coactivator 1-alpha and AMP kinase and in turn enhancing components of the mitochondrial ETC, β-oxidation, and ATPases (18,48). Although speculative, one possibility is that resveratrol might reduce uncoupling of the mitochondrial ETC, leading to an increased availability of ATP and decreased superoxide formation. Previous findings have demonstrated that increases in post-exercise concentrations of hypoxanthine are accurate predictors of muscle energy depletion (53) and adenine nucleotide degradation during exercise (54). Our current data show increased hypoxanthine concentrations after exercise, which is indicative of elevated ATP utilization and depletion (54).

Mitochondria Function is Altered by Exercise and Resveratrol

Resveratrol supplementation reduced hypoxanthine levels following exercise, and this is consistent with the idea that resveratrol increased ATP availability to the exercising muscles. This may be the result of an improvement in mitochondria function, which could provide an important outcome for reducing oxidative stress after resveratrol supplementation. Mitochondria function appeared to be altered by exercise and resveratrol, in an age-specific manner. Muscle levels of citrate synthase increased with exercise in muscles of young animals in both diet groups but only increased in the muscles from aged animals that were supplemented with resveratrol. In addition, there appeared to be greater changes with proximal enzyme complexes of the ETC versus more distal complexes. For example, exercise showed greater improvements in complex I activity than complex III activity. Resveratrol increased the complex I activity in exercised muscles of aged mice, but it had no effects on downstream complex III or complex IV activities. Although resveratrol increased complex III activity in the exercise limb of young adult animals, it did not improve complex IV activity after exercise. These results suggest that both exercise and resveratrol influenced mitochondria function and enzyme activity, and this may have contributed directly or indirectly to reduced oxidative stress. Nevertheless, additional experiments are needed to determine if this is a result of increases in mitochondria biogenesis or some other mechanism that altered mitochondria function.

Antioxidant Enzymes and Resveratrol

To our knowledge, this is the first investigation to examine the effects of resveratrol supplementation on the regulation of the endogenous antioxidant system in response to isometric exercise in young and aged animals. Our study measured transcription and activity levels of the endogenous antioxidant enzymes catalase, GPx, MnSOD, and CuZnSOD. In general, the results do not support transcriptional control as a mechanism for altering the activity levels of the endogenous antioxidant enzymes within the muscles of nonsupplemented animals. Instead, these data are consistent with previous data that indicate that the activity of the endogenous antioxidant enzymes are regulated via various levels of posttranscriptional and/or posttranslational controls (1,55). Insufficient tissue was available in this study to determine if the protein levels of endogenous antioxidant enzymes had been altered by short-term resveratrol treatment.

Several studies have shown that there is no aging-induced change in MnSOD or CuZnSOD activity, although loss of CuZnSOD exacerbates muscle loss with aging (17). Generally, overexpression of antioxidant enzymes has not been shown to improve life span (56); however, this is not a universal finding (57). Our data indicate that aging did not alter the activity of GPx and CuZnSOD; however, aging increased the enzyme activity of MnSOD and catalase and catalase mRNA content. This increase in catalase transcription may be attributed to an attempt to compensate for the inability of the glutathione system to buffer H_2O_2. Age-related increases in catalase activity have been proposed as a potential means to counterbalance the depletion of glutathione levels in metabolically active tissues (58). Differences in age-dependent transcriptional control of the other endogenous antioxidant enzymes between our current study and that reported in other studies (1,55) may be due to differences in environmental conditions, animal models, or the muscles that were investigated.

Short-term adaptation of antioxidant enzymes to isometric exercise in both young adult and aged animals appeared to occur via an increase in catalase and CuZnSOD activity, two enzymes that are primarily located in the cytosol. Catalase activity increased only in the aged animals after isometric contraction, whereas CuZnSOD increased in muscles from both the young adult and the aged mice after isometric contractions. However, isometric contractions did not alter mRNA content for any of the endogenous antioxidant enzymes (GPx, catalase, CuZnSOD, or MnSOD) investigated in the current study. These observations are similar to previous data from our laboratory (1), where 4.5 weeks of repetitive loading exercise in the rat tibialis anterior muscle increased the activity of both catalase and CuZnSOD without changing mRNA content for these enzymes.

The absence of changes in mRNA suggests that posttranscriptional modifications might be responsible for either enhancing the capacity of the active site of the antioxidant enzymes or perhaps reducing protein degradation leading to increased enzyme content. Similarly, it has been shown that increases in CuZnSOD protein levels occur without changes in mRNA content after a single bout of endurance exercise (55).

Resveratrol supplementation increased the endogenous antioxidant enzymes, catalase and MnSOD (20), which are located in close proximity to the sites of electron transport production of ATP. For example, MnSOD is localized to the mitochondrial matrix, where it protects the mitochondria from oxidative damage. In addition, catalase is found in low concentrations in the cytosol, and it is thought to be contained mainly within peroxisomes, which are sites of fatty acid oxidation. This fits well with previous observations showing that resveratrol supplementation increases the transcription and the activity of both catalase (19,59) and MnSOD (19,20). Furthermore, our mitochondrial function data suggest that resveratrol may be able to improve mitochondrial components of aerobic metabolism (18,19).

No Effect of Resveratrol on Muscle Force

The data in the current study show that resveratrol supplementation did not improve the maximal isometric force output of the plantar flexors muscle group from either the young adult or the aged animals, at any point of the acute 3-day exercise regime. Furthermore, maximal isometric force did not change significantly from the first to the third exercise session (Figure 1A) in either young adult or aged mice. This is not surprising, given the relatively short duration of the supplementation and exercise intervention in this study. We did not anticipate that this short exercise period would result in hypertrophic adaptations, and therefore, they were not measured.

Resveratrol Does Not Improve Muscle Fatigability in Aged Mice

Muscle fatigability was measured as the relative decline in maximal isometric force by comparing the 1st and 20th contraction in the third exercise session. Muscles from aged animals in either the control or the resveratrol group had significantly greater fatigue resistance than muscles from young animals that where fed a control diet (Figure 1B). This cannot be explained by an age-associated improvement in mitochondria function or number because neither citrate synthase activity nor mitochondrial ETC complex activity was greater in muscles from aged as compared with young animals. Instead, the greater relative decrease in fatigue over the 20 contractions may be due to shift toward a greater percentage of fatigue-resistant type I fibers in the gastrocnemius muscle of aged mice (60,61) as compared with young mice. Another possibility is that because the larger-sized fibers from the young adult animals would have produced a greater maximal force and have a greater rate of ATP utilization (as a result of increased cross-bridge cycling, greater calcium release, and therefore greater ATP utilization by sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase [SERCA] pumps, greater heat production) than the fibers in muscles from aged mice, this would result in a greater relative decline in force over the exercise session. Nevertheless, the similar relative changes in hypoxanthine observed during exercise in the young adult and the aged animals would imply that if ATP utilization was greater in the young animals, then young muscles must possess a mechanism that limits purine degradation even though ATP levels were not fully resorted. It is clear from these data that additional research is needed to understand the mechanisms that underlie the age-dependent difference in muscle fatigue found in the current model of isometric exercise more fully.

The plantar flexor muscles from young adult animals supplemented with resveratrol had a significantly lower decline in muscle force over the 20 contractions (−27.5% ± 1.6) than animals that were fed the control diet (−37.4% ± 7.9). The decline in maximal isometric force was similar in resveratrol-supplemented young adult animals and aged animals from either control (−42.9% ± 4.1) or resveratrol diet (44.4% ± 5.1) groups.

Previous studies have reported that resveratrol supplementation improved mitochondria function and reduced fatigue associated with aerobic exercise (18,48). Our data show for the first time that resveratrol reduces muscle fatigue in response to repetitive anaerobic (isometric) contractions, and therefore, this effect is not activity specific. However, the effect of resveratrol with repetitive exercise occurs only in muscles of young animals. We hypothesize that resveratrol increased the availability of ATP, in part as a result of improved mitochondrial function, or perhaps by increasing β-oxidation and by increasing ATP content (18,19,48) in muscles of young mice. This possibility is plausible because increases in hypoxanthine have been shown to be predictors of muscle ATP exhaustion (53), and hypoxanthine concentrations were lower in muscles from resveratrol-supplemented mice in our current study. However, this investigation does not provide any direct evidence for this possibly because we

did not measure mitochondrial density or efficiency nor did we measure skeletal muscle ATP content.

If resveratrol has an age-specific effect on fatigue resistance, it might be argued to act in a muscle fiber type–specific manner. For example, there is a well-known increase in type I muscle fibers with aging, and muscles with a high percentage of type I fibers (eg, soleus) appear to be more resistant to resveratrol-induced increases in mitochondrial enzymatic activity and oxidative capacity than the gastrocnemius muscle (primarily composed of type II fibers) (48). Nevertheless, fiber type–specific responses cannot explain all the effects of resveratrol because even if there were some age-induced shift toward type I fibers, the gastrocnemius muscle, in the aged mouse still has a high percentage of type II fibers. Furthermore, mitochondria volume density is not a good predictor of fatigue resistance to isometric exercise (62), and therefore, improvements in mitochondria number or size would be anticipated to have minimal or no effects on isometric fatigability.

Another possibility to account for the improved fatigue resistance to maximal isometric exercise in muscles from young animals is that resveratrol could directly diminish exercise-induced ROS, which have been shown to be a mediator of muscle fatigue (10). However, we do not regard this as a strong putative mechanism because resveratrol decreased the exercise-induced elevation in H_2O_2 levels and other indices of oxidative stress in muscles from both young and aged mice yet, despite clear reductions in oxidative stress, resveratrol did not improve muscle fatigue resistance in aged animals. These findings indicate that acute increases in oxidative stress, including modulation of H_2O_2, and Nox4 protein abundance are not sufficient to moderate acute fatigue responses to maximal isometric exercise in skeletal muscle with aging.

Conclusions

The current data suggest that resveratrol supplementation reduces oxidant production and oxidative damage in gastrocnemius muscles from young adult and aged mice subjected to short-term isometric exercise. Ten days of resveratrol supplementation also diminishes the basal levels of oxidative stress associated with aging. Functional measurements of maximal isometric force and rate of fatigue were unaffected by resveratrol supplementation in the aged animals. Further work is required to understand the role that fortifying a normal diet with resveratrol may have on the adaptive response of mitochondria and skeletal muscle to long-term exercise with aging.

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References

1. Ryan MJ, Dudash HJ, Docherty M, et al. Aging-dependent regulation of antioxidant enzymes and redox status in chronically loaded rat dorsiflexor muscles. *J Gerontol A Biol Sci Med Sci*. 2008;63:1015–1026.
2. Kryger AI, Andersen JL. Resistance training in the oldest old: consequences for muscle strength, fiber types, fiber size, and MHC isoforms. *Scand J Med Sci Sports*. 2007;17:422–430.
3. Degens H, Alway SE. Control of muscle size during disuse, disease, and aging. *Int J Sports Med*. 2006;27:94–99.
4. Harman D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol*. 1956;11:298–300.
5. Bejma J, Ji LL. Aging and acute exercise enhance free radical generation in rat skeletal muscle. *J Appl Physiol*. 1999;87:465–470.
6. Andrade FH, Reid MB, Allen DG, Westerblad H. Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse. *J Physiol*. 1998;509(Pt 2):565–575.
7. Kondo H, Kodama J, Kishibe T, Itokawa Y. Oxidative stress during recovery from muscle atrophy. *FEBS Lett*. 1993;326:189–191.
8. Kondo H, Miura M, Itokawa Y. Antioxidant enzyme-systems in skeletal-muscle atrophied by immobilization. *Pflugers Arch*. 1993;422:404–406.
9. Kaneko T, Tahara S, Taguchi T, Kondo H. Accumulation of oxidative DNA damage, 8-oxo-2'-deoxyguanosine, and change of repair systems during in vitro cellular aging of cultured human skin fibroblasts. *Mutat Res*. 2001;487:19–30.
10. Reid MB. Free radicals and muscle fatigue: of ROS, canaries, and the IOC. *Free Radic Biol Med*. 2008;44:169–179.
11. Reid MB. Invited review: redox modulation of skeletal muscle contraction: what we know and what we don't. *J Appl Physiol*. 2001;90:724–731.
12. Houston M, Estevez A, Chumley P, et al. Binding of xanthine oxidase to vascular endothelium. Kinetic characterization and oxidative impairment of nitric oxide-dependent signaling. *J Biol Chem*. 1999;274:4985–4994.
13. Hellsten Y, Hansson HA, Johnson L, Frandsen U, Sjodin B. Increased expression of xanthine oxidase and insulin-like growth factor I (IGF-I) immunoreactivity in skeletal muscle after strenuous exercise in humans. *Acta Physiol Scand*. 1996;157:191–197.
14. Vina J, Gimeno A, Sastre J, et al. Mechanism of free radical production in exhaustive exercise in humans and rats; role of xanthine oxidase and protection by allopurinol. *IUBMB Life*. 2000;49:539–544.
15. Sachdev S, Davies KJA. Production, detection, and adaptive responses to free radicals in exercise. *Free Radic Biol Med*. 2008;44:215–223.
16. Roman WJ, Fleckenstein J, Straygundersen J, Alway SE, Peshock R, Gonyea WJ. Adaptations in the elbow flexors of elderly males after heavy-resistance training. *J Appl Physiol*. 1993;74:750–754.
17. Muller FL, Song W, Liu YH, et al. Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. *Free Radic Biol Med*. 2006;40:1993–2004.
18. Baur JA, Pearson KJ, Price NL, et al. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature*. 2006;444:337–342.
19. Pearson KJ, Baur JA, Lewis KN, et al. Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span. *Cell Metab*. 2008;8:157–168.
20. Robb EL, Winkelmolen L, Visanji N, Brotchie J, Stuart JA. Dietary resveratrol administration increases MnSOD expression and activity in mouse brain. *Biochem Biophys Res Commun*. 2008;372:254–259.
21. Stojanovic S, Sprinz H, Brede O. Efficiency and mechanism of the antioxidant action of trans-resveratrol and its analogues in the radical liposome oxidation. *Arch Biochem Biophys*. 2001;391:79–89.
22. Brito P, Almeida LM, Dinis TC. The interaction of resveratrol with ferrylmyoglobin and peroxynitrite; protection against LDL oxidation. *Free Radic Res*. 2002;36:621–631.
23. Bisht K, Wagner KH, Bulmer AC. Curcumin, resveratrol and flavonoids as anti-inflammatory, cyto- and DNA-protective dietary compounds. *Toxicology*. 2010; in press.
24. Bradamante S, Barenghi L, Villa A. Cardiovascular protective effects of resveratrol. *Cardiovasc Drug Rev*. 2004;22:169–188.
25. Baker BA, Mercer RR, Geronilla KB, Kashon ML, Miller GR, Cutlip RG. Stereological analysis of muscle morphology following exposure to repetitive stretch-shortening cycles in a rat model. *Appl Physiol Nutr Metab*. 2006;31:167–179.

26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248–254.
27. Adler A, Messina E, Sherman B, et al. NAD(P)H oxidase-generated superoxide anion accounts for reduced control of myocardial O2 consumption by NO in old Fischer 344 rats. *Am J Physiol Heart Circ Physiol*. 2003;285:H1015–H1022.
28. Cui XL, Douglas JG. Arachidonic acid activates c-jun N-terminal kinase through NADPH oxidase in rabbit proximal tubular epithelial cells. *Proc Natl Acad Sci U S A*. 1997;94:3771–3776.
29. Schilder YD, Heiss EH, Schachner D, et al. NADPH oxidases 1 and 4 mediate cellular senescence induced by resveratrol in human endothelial cells. *Free Radic Biol Med*. 2009;46:1598–1606.
30. Park DW, Baek K, Kim JR, et al. Resveratrol inhibits foam cell formation via NADPH oxidase 1- mediated reactive oxygen species and monocyte chemotactic protein-1. *Exp Mol Med*. 2009;41:171–179.
31. Siu PM, Alway SE. Mitochondria-associated apoptotic signalling in denervated rat skeletal muscle. *J Physiol*. 2005;565:309–323.
32. Siu PM, Pistilli EE, Butler DC, Alway SE. Aging influences cellular and molecular responses of apoptosis to skeletal muscle unloading. *Am J Physiol Cell Physiol*. 2005;288:C338–C349.
33. Srere PA. Citrate synthase. *Methods Enzymol*. 1969;13:3–5.
34. Siu PM, Donley DA, Bryner RW, Alway SE. Citrate synthase expression and enzyme activity after endurance training in cardiac and skeletal muscles. *J Appl Physiol*. 2003;94:555–560.
35. Cogswell AM, Stevens RJ, Hood DA. Properties of skeletal muscle mitochondria isolated from subsarcolemmal and intermyofibrillar regions. *Am J Physiol*. 1993;264:C383–C389.
36. Dabkowski ER, Williamson CL, Bukowski VC, et al. Diabetic cardiomyopathy-associated dysfunction in spatially distinct mitochondrial subpopulations. *Am J Physiol Heart Circ Physiol*. 2009;296:H359–H369.
37. Trounce IA, Kim YL, Jun AS, Wallace DC. Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines. *Methods Enzymol*. 1996; 264:484–509.
38. Chen CN, Brown-Borg HM, Rakoczy SG, Ferrington DA, Thompson LV. Aging impairs the expression of the catalytic subunit of glutamate cysteine ligase in soleus muscle under stress. *J Gerontol A Biol Sci Med Sci*. 2010;65:129–137.
39. Figueiredo PA, Powers SK, Ferreira RM, Appell HJ, Duarte JA. Aging impairs skeletal muscle mitochondrial bioenergetic function. *J Gerontol A Biol Sci Med Sci*. 2009;64:21–33.
40. Ji LL, Leeuwenburgh C, Leichtweis S, et al. Oxidative stress and aging. Role of exercise and its influences on antioxidant systems. *Ann N Y Acad Sci*. 1998;854:102–117.
41. Fulle S, Protasi F, Di Tano G, et al. The contribution of reactive oxygen species to sarcopenia and muscle ageing. *Exp Gerontol*. 2004;39:17–24.
42. McArdle A, Jackson MJ. Exercise, oxidative stress and ageing. *J Anat*. 2000;197 (Pt 4):539–541.
43. Hofer T, Marzetti E, Xu J, et al. Increased iron content and RNA oxidative damage in skeletal muscle with aging and disuse atrophy. *Exp Gerontol*. 2008;43:563–570.
44. Aranda R, Domenech E, Rus AD, et al. Age-related increase in xanthine oxidase activity in human plasma and rat tissues. *Free Radic Res*. 2007;41:1195–1200.
45. Eskurza I, Kahn ZD, Seals DR. Xanthine oxidase does not contribute to impaired peripheral conduit artery endothelium-dependent dilatation with ageing. *J Physiol*. 2006;571:661–668.
46. Huang XF, Li HQ, Shi L, Xue JY, Ruan BF, Zhu HL. Synthesis of resveratrol analogues, and evaluation of their cytotoxic and xanthine oxidase inhibitory activities. *Chem Biodivers*. 2008;5:636–642.
47. Jia Z, Zhu H, Misra BR, Mahaney JE, Li Y, Misra HP. EPR studies on the superoxide-scavenging capacity of the nutraceutical resveratrol. *Mol Cell Biochem*. 2008;313:187–194.
48. Lagouge M, Argmann C, Gerhart-Hines Z, et al. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell*. 2006;127:1109–1122.
49. Zhang H, Zhang J, Ungvari Z, Zhang C. Resveratrol improves endothelial function: role of TNF{alpha} and vascular oxidative stress. *Arterioscler Thromb Vasc Biol*. 2009;29:1164–1171.
50. Spanier G, Xu H, Xia N, et al. Resveratrol reduces endothelial oxidative stress by modulating the gene expression of superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1) and NADPH oxidase subunit (Nox4). *J Physiol Pharmacol*. 2009;60(suppl 4):111–116.
51. Wenzel E, Soldo T, Erbersdobler H, Somoza V. Bioactivity and metabolism of trans-resveratrol orally administered to Wistar rats. *Mol Nutr Food Res*. 2005;49:482–494.
52. Smith JJ, Kenney RD, Gagne DJ, et al. Small molecule activators of SIRT1 replicate signaling pathways triggered by calorie restriction in vivo. *BMC Syst Biol*. 2009;3:31.
53. Bianchi GP, Grossi G, Bargossi AM, Fiorella PL, Marchesini G. Can oxypurines plasma levels classify the type of physical exercise? *J Sports Med Phys Fitness*. 1999;39:123–127.
54. Sahlin K, Tonkonogi M, Soderlund K. Plasma hypoxanthine and ammonia in humans during prolonged exercise. *Eur J Appl Physiol Occup Physiol*. 1999;80:417–422.
55. Hollander J, Bejma J, Ookawara T, Ohno H, Ji LL. Superoxide dismutase gene expression in skeletal muscle: fiber-specific effect of age. *Mech Ageing Dev*. 2000;116:33–45.
56. Zhang Y, Ikeno Y, Qi W, et al. Mice deficient in both Mn superoxide dismutase and glutathione peroxidase-1 have increased oxidative damage and a greater incidence of pathology but no reduction in longevity. *J Gerontol A Biol Sci Med Sci*. 2009;64:1212–1220.
57. Schriner SE, Linford NJ, Martin GM, et al. Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science*. 2005;308:1909–1911.
58. Meng Q, Wong YT, Chen J, Ruan R. Age-related changes in mitochondrial function and antioxidative enzyme activity in fischer 344 rats. *Mech Ageing Dev*. 2007;128:286–292.
59. Kumar A, Kaundal RK, Iyer S, Sharma SS. Effects of resveratrol on nerve functions, oxidative stress and DNA fragmentation in experimental diabetic neuropathy. *Life Sci*. 2007;80:1236–1244.
60. Holloszy JO, Chen M, Cartee GD, Young JC. Skeletal muscle atrophy in old rats: differential changes in the three fiber types. *Mech Ageing Dev*. 1991;60:199–213.
61. Pansarasa O, Felzani G, Vecchiet J, Marzatico F. Antioxidant pathways in human aged skeletal muscle: relationship with the distribution of type II fibers. *Exp Gerontol*. 2002;37:1069–1075.
62. Alway SE. Is fiber mitochondrial volume density a good indicator of muscle fatigability to isometric exercise? *J Appl Physiol*. 1991;70: 2111–2119.