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Original Contribution

Inhibition of xanthine oxidase reduces oxidative stress and improves skeletal muscle function in response to electrically stimulated isometric contractions in aged mice

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ABSTRACT

Oxidative stress is a putative factor responsible for reducing function and increasing apoptotic signaling in skeletal muscle with aging. This study examined the contribution and functional significance of the xanthine oxidase enzyme as a potential source of oxidant production in aged skeletal muscle during repetitive in situ electrically stimulated isometric contractions. Xanthine oxidase activity was inhibited in young adult and aged mice via a subcutaneously placed time-release (2.5 mg/day) allopurinol pellet, 7 days before the start of in situ electrically stimulated isometric contractions. Gastrocnemius muscles were electrically activated with 20 maximal contractions for 3 consecutive days. Xanthine oxidase activity was 65% greater in the gastrocnemius muscle of aged mice compared to young mice. Xanthine oxidase activity also increased after in situ electrically stimulated isometric contractions in muscles from both young (33%) and aged (28%) mice, relative to contralateral noncontracted muscles. Allopurinol attenuated the exercise-induced increase in oxidative stress, but it did not affect the elevated basal level of oxidative stress that was associated with aging. In addition, inhibition of xanthine oxidase activity decreased caspase-3 activity, but it had no effect on other markers of mitochondrial-associated apoptosis. Our results show that compared to control conditions, suppression of xanthine oxidase activity by allopurinol reduced xanthine oxidase activity, H_2O_2 levels, lipid peroxidation, and caspase-3 activity; prevented the in situ electrically stimulated isometric contraction-induced loss of glutathione; prevented the increase in catalase and copper–zinc superoxide dismutase activities; and increased maximal isometric force in the plantar flexor muscles of aged mice after repetitive electrically evoked contractions.

The fundamental mechanisms contributing to aging-associated deterioration in muscle function and muscle mass are poorly understood, but a large body of evidence supports the hypothesis that oxidative stress contributes to aging in many tissues, including muscle [1–4]. Oxidative stress occurs when the cellular production of oxidants exceeds the capacity of the cell to inhibit or terminate oxidizing reactions. Increases in oxidative stress have been proposed as a principal component leading to skeletal muscle loss with aging (sarcopenia). Loss of myonuclei via apoptosis is another likely contributor to sarcopenia. However, oxidative stress and apoptosis may not be mutually exclusive events in aging. Rather, the elevation in oxidative stress that occurs with aging can regulate redox-sensitive signaling pathways [5,6], increase catabolic gene expression [7–9], and activate apoptotic pathways [10,11], thereby contributing to the progression of sarcopenia.

Mitochondria are a major source of oxidant production in skeletal muscle [12,13]. The consequence of prolonged exposure to relatively high levels of oxidants reduces mitochondrial membrane integrity and antioxidant enzyme activity [13]. In addition, oxidants can lead to increased mitochondria permeability and the release of mitochondria-specific proteins, including apoptosis-inducing factor (AIF) and cytochrome *c*, into the cytosol through the mitochondrial transition pore. Cytosolic AIF initiates a caspase-independent pathway, whereas cytosolic cytochrome *c* initiates the caspase cascade resulting in DNA fragmentation and myonuclear apoptosis. Thus, mitochondria may be important for regulating both oxidative stress and apoptotic signaling in aging skeletal muscle.

The functional implications of elevated oxidative stress in skeletal muscle include reduced specific force [14], altered myofilament function [15], and elevated muscle fatigue [16]. Although exercise is used as a strategy to attempt to reduce sarcopenia and improve muscle function,

Abbreviations: AIF, apoptosis-inducing factor; Apaf-1, apoptosis peptidase-activating factor 1; Bax, Bcl-2-associated X protein; Bcl-2, B cell leukemia/lymphoma-2; CuZnSOD, copper–zinc superoxide dismutase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; HAE, 4-hydroxyalkenal; MDA, malondialdehyde; MnSOD, mitochondrial manganese superoxide dismutase; NF-κB, nuclear factor κB; NFM, nonfat milk protein; RFU, relative fluorescence units; TBS-T, Tris-buffered saline with 0.05% Tween 20.

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acute exercise can also increase free radical generation in skeletal muscle [17]. This has important implications in a highly metabolic tissue such as skeletal muscle, in which basal oxidant production is already elevated with aging, and exercise has the potential to further increase oxidant production by as much as 80% [12].

There are three major sources of oxidant production with exercise. These include infiltrating immune cells, mitochondrial respiration, and xanthine oxidase activity [18]. The magnitude and the sources of oxidant production are dependent on the mode, duration, and intensity of exercise.

Under normal physiological conditions, xanthine dehydrogenase is the principal form of the enzyme that catalyzes the oxidation of both hypoxanthine and xanthine to form uric acid via the reduction of NAD^+ to NADH. Both reactions result in the generation of hydrogen peroxide. However, during repetitive muscle contractions, the increased ATP utilization and a brief localized period of ischemia will facilitate adenine nucleotide degradation, thus breaking down ATP to AMP and eventually to hypoxanthine. Xanthine dehydrogenase can be converted to xanthine oxidase by reversible sulfhydryl oxidation or by irreversible proteolytic modifications [19,20]. Xanthine oxidase catalyzes the oxidation of xanthine to uric acid using NAD as a substrate without the formation of hydrogen peroxide or other oxidants.

Increased xanthine oxidase activity within the vascular endothelium [21] is a contributing factor associated with oxidative stress and damage during exhaustive exercise [1,22–25]. Allopurinol, which is a structural isomer of hypoxanthine, acts as a competitive inhibitor of xanthine oxidase, protecting cells from oxidative damage associated with exhaustive exercise [24]. It has been hypothesized that the activation of the enzyme xanthine oxidase during exhaustive exercise is similar to the process observed during ischemia–reperfusion injury [24,26,27]. During repetitive muscle contractions, the combination of increased ATP utilization and intermittent localized periods of ischemia due to muscle contractions will facilitate adenine nucleotide degradation and accumulation of hypoxanthine.

Conversion of xanthine dehydrogenase to xanthine oxidase has been shown to be dependent on both calcium and oxidant concentrations [28]. During muscle contractions, intracellular calcium concentrations are elevated, which in turn activate proteases that cause the irreversible conversion of xanthine dehydrogenase to xanthine oxidase. Furthermore, increased oxidant production may lead to the oxidation of cysteine residues on xanthine dehydrogenase, forming disulfide bonds resulting in the reversible conversion to xanthine oxidase [20].

During muscle relaxation, the influx of oxygen-rich blood catalyzes the reactions of xanthine oxidase to form hypoxanthine and oxygen to form xanthine and superoxide. Within the muscle environment, H_2O_2 concentrations are expected to increase via the accumulation of superoxide formed by xanthine oxidase activity, mitochondrial sources, and NADPH oxidase activity, because the superoxide anion is quickly dismutated to H_2O_2 by superoxide dismutase (SOD). Decreases in antioxidant capacity with aging and exercise may lead to an increase in contractile protein and mitochondrial damage caused by an augmented duration and exposure to oxidants, thus potentially accelerating muscle loss [29,30].

Xanthine oxidase has been reported to make important contributions to oxidative stress in the heart [31] and gastrocnemius muscle [32–34] from aged rodents; however, this age-dependent elevation in xanthine oxidase activity is not observed universally [35]. Xanthine oxidase activity contributes, at least in part, to an increase in oxidant production during exhaustive exercise, but it is not known if xanthine oxidase is an important source of oxidant production with more moderate exercise in aged animals. Therefore, the purpose of this investigation was to determine the contribution of the xanthine oxidase enzyme as a source of oxidant production during repetitive isometric contractions and to determine if it further contributes to oxidative stress in aged skeletal muscle. A second aim of this study was to determine if increased xanthine oxidase levels play a role in regulating the decreased functional capacity and increased apoptotic signaling in aged muscles. We tested the hypothesis that the inhibition of xanthine oxidase will improve the redox environment within muscle by reducing oxidative stress and thus preserving functional capacity in aged animals after in situ electrically stimulated isometric contractions. The second hypothesis tested was that xanthine oxidase-associated oxidative stress will exacerbate the release of pro-apoptotic mitochondrial proteins into the cytosol, thereby increasing apoptotic signaling in aged skeletal muscle after in situ electrically evoked contractions, whereas reducing xanthine oxidase by allopurinol will prevent these negative changes in aging muscles. Our rationale was that if acute contractions induced detrimental changes in aged muscle (e.g., as a result of increased oxidant production), where oxidant levels are already high relative to muscle conditions in young animals [34,36], and if allopurinol could suppress xanthine oxidase-induced oxidative stress that occurs as a result of muscle contractions, then acutely, muscle redox and function (e.g., force) would be improved and oxidant damage in loaded muscles would be reduced.

Materials and methods

Suppression of xanthine oxidase during in situ electrically stimulated isometric contractions

All experimental procedures were carried out with approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care.

A subcutaneous 2.5-mg 21-day-release allopurinol pellet (Innovative Research of America, Sarasota, FL, USA) was implanted subcutaneously over the dorsal cervical column in anesthetized mice (Isotec 5; Ohmeda; 3% isoflurane/97% O_2) 7 days before the start of the in situ electrically stimulated isometric contraction protocol. The incision was closed with a 9-mm wound clip. A sham surgery was performed on control animals. Forty-eight young adult (3–5 months) and 48 aged (26–28 months) C57BL/6 mice were randomly separated into groups receiving the allopurinol pellet or only the sham surgery ($n=24$ per treatment group). In each treatment group, one-half of the gastrocnemius muscles were processed for whole-muscle homogenate and RNA isolation, and the gastrocnemius muscles from the other half of the treatment group were homogenized and separated into a mitochondrial fraction and a mitochondria-free cytosolic fraction. Mitochondria were isolated from gastrocnemius in other animals in each group.

In situ electrically stimulated isometric contractions were conducted on a custom-built mouse dynamometer that has been previously described [34]. Briefly, mice were anesthetized with a mixture of oxygen (97%) and isoflurane gas (3%) and placed on a plate that was heated to 37 °C. The left ankle was positioned at 90° of flexion and was aligned with the axis of rotation of the servomotor (Model 6350*350; Cambridge Technology, Cambridge, MA, USA). The foot was secured to the foot plate that was connected to the servomotor. Commercially available software (Dynamic Muscle Control; Aurora Scientific, Aurora, ON, Canada) was used to control the servomotor providing for the angular position of the foot. Muscle contractions of the plantar flexor muscles were stimulated via subcutaneous platinum electrodes that were placed on either side of the tibial nerve. Electrode placement was tested via a short stimulation of the nerve to cause plantar flexion. When stimulated, the foot plantar flexed without any visible appearance of eversion, or inversion, of the foot. Twenty electrically evoked (10-V, 100-Hz, 200-μs pulses) isometric contractions of the plantar flexor muscle group were obtained in one limb. Each contraction train lasted for 5 s, and a 25-s recovery period occurred between subsequent contractions. Isometric contractions were conducted over 3 consecutive days in the left limb, whereas the contralateral limb served as the intra-animal control. Muscle functional data were collected as a force × time

curve during isometric contractions for each session and values were normalized to each animal's body weight. The contractile data were analyzed offline (Dynamic Muscle Analysis software; Aurora Scientific).

Mitochondria isolation

Gastrocnemius muscles were dissected with the mice under deep anesthesia (5% isoflurane/95% oxygen). Careful precautions were made to keep the blood supply to the gastrocnemius intact until it was removed. Mitochondria and mitochondria-free cytosolic muscle fractions were obtained by protease digestion from the myofibrils, followed by centrifugation, using modifications of the manufacturer's recommendations (Mitoiso1-1KT; Sigma–Aldrich Co., St. Louis, MO, USA). Briefly, the gastrocnemius muscle was placed on ice and minced in a 1.5-ml Eppendorf tube. Samples were washed and resuspended in an extraction buffer containing 0.25 mg/ml trypsin. After a 20-min incubation period, albumin was added to a final concentration of 10 mg/ml to quench the proteolytic reaction. Samples were washed and resuspended in the extraction buffer and then homogenized with a Teflon pestle for two strokes of 5 s each. The homogenate was then centrifuged at 1100 *g* for 5 min. The supernatant was transferred to a new tube and centrifuged at 11,000 *g* for 10 min. The supernatant was collected as the mitochondria-free cytosolic fraction. The mitochondrial pellet was suspended in a storage buffer containing sucrose.

H_2O_2 concentration in whole-muscle homogenates and isolated mitochondria

Muscle hydrogen peroxide levels were determined with a fluorescent H_2O_2 assay (FLOH 100-3; Cell Technology, Mountain View, CA, USA). This assay utilizes a nonfluorescent detection reagent to detect H_2O_2. H_2O_2 oxidizes the detection reagent in a 1:1 stoichiometry to produce a fluorescent product, resorufin. This oxidation is catalyzed by peroxidase. The change in fluorescence signal is detected with an excitation of 530 nm and an emission of 590 nm and plotted against at standard curve of known concentrations of H_2O_2. Whole muscles were homogenized in phosphate-buffered saline (PBS) (pH 7.4). Isolated mitochondria were obtained as described above. Reagents and standards were prepared as recommended by the manufacturer with slight modifications and have been previously described [34]. H_2O_2 was normalized to the muscle protein concentration of each sample as determined by a DC protein concentration assay (Bio-Rad, Hercules, CA, USA). All analyses were done in duplicate.

Electron paramagnetic resonance spectroscopy (EPR) to measure reactive oxygen species (ROS) in isolated mitochondria

EPR spin trapping was used to detect short-lived free radicals, including hydroxyl radicals ($^{\bullet}OH$) and superoxide radicals ($O_2^{\bullet -}$) [37]. This method utilizes the reaction of a short-lived radical binding with a paramagnetic compound to form a spin adduct, which is a relatively long-lived free radical product. The spin adduct can be measured with conventional EPR. The intensity of the EPR signal is used to estimate the amount of short-lived radicals that were trapped. The hyperfine structure of the spin adducts represents characteristics of the original trapped radicals. The specificity and sensitivity of this assay make it optimal for detecting and identifying free radicals. All EPR measurements were conducted using a Bruker EMX spectrometer (Bruker Instruments, Billerica, MA, USA) and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl as reference standards [37]. Data acquisitions and analyses were conducted using the Acquisit data analysis program (Bruker Instruments). The assay was conducted as reported in detail previously [38]. Briefly, isolated mitochondria samples were suspended in PBS and then incubated with the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (200 mM) in the presence or absence of the excess complex I respiratory substrates glutamate and malate. The samples were incubated at 37 °C and measured at 20 °C in an EPR flat cell. Three scans were made of each cell. The reaction obtained with xanthine and xanthine oxidase was used as a reference. The relative radical concentration was estimated by measuring the peak-to-peak height (mm) of the observed spectra.

Concentration of reduced and oxidized glutathione

Glutathione (GSH) and oxidized glutathione (GSSG) were measured in muscle homogenates using commercially available substrates (21040; Percipio Biosciences, Burlingame, CA, USA). The data were expressed as the ratio of reduced to oxidized glutathione (GSH/GSSG). The assay was conducted as previously described [34]. Briefly, muscle tissue (~40 mg) was homogenized immediately after dissection in 530 μl of cold buffer (5% metaphosphoric acid for the GSH or 5% metaphosphoric acid and M2VO scavenger for the GSSG sample). The GSH/GSSG assay uses the thiol-scavenging reagent 1-methyl-2-vinyl-pyridinium trifluoromethanesulfonate to rapidly scavenge GSH but it does not interfere with the glutathione reductase assay. The method employs Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid), which reacts with GSH to form a spectrophotometrically detectable product at 412 nm. The appropriate amounts of sample chromogen (Ellman's reagent) and enzyme (glutathione reductase) were mixed and incubated at room temperature. NADPH was added and the absorbance (412 nm) of each sample was read for 3 consecutive minutes. GSSG was determined by the reduction of GSSG to GSH, which was then determined by the reaction with Ellman's reagent. This assay measures the change in color development during the reaction, and the reaction rate is proportional to the GSH and GSSG concentrations.

The concentration for each sample was determined via a DC protein concentration assay (Bio-Rad). The signals from each sample were normalized to the corresponding protein content of that sample.

Xanthine oxidase activity and hypoxanthine and xanthine concentrations in whole-muscle homogenate

Xanthine and hypoxanthine concentrations and xanthine oxidase activity were measured in muscle homogenates using commercially available reagents (A22182; Invitrogen, Eugene, OR, USA). In this assay, xanthine oxidase catalyzes the oxidation of purine bases, hypoxanthine or xanthine, to uric acid and superoxide. The superoxide spontaneously degrades to H_2O_2, and the H_2O_2, in the presence of horseradish peroxidase, reacts stoichiometrically with Amplex red to generate the red-fluorescent oxidation product, resorufin. The methods have been described previously by our laboratory [34]. 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 by subtracting the values derived from the non-xanthine-containing wells. Hypoxanthine and xanthine concentrations were determined by comparing sample values to values obtained from a standard curve. Relative concentrations of hypoxanthine and xanthine were determined by comparing sample values in relative fluorescence units (RFU). Each sample and standard was performed in duplicate and then normalized to protein concentrations (Bio-Rad) of the samples.

Lipid peroxidation in whole-muscle homogenate

Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were measured as indicators of lipid peroxidation (Bioxytech LPO-586; Percipio Biosciences). This assay is based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole, with MDA and HAE at 45 °C. One molecule of either MDA or HAE reacts with two molecules of *N*-methyl-2-phenylindole to yield a stable chromophore with a maximal absorbance at 586 nm. Methanesulfonic acid was used as the acid

solvent. The methods have been previously described [34]. Briefly, 75–100 mg of each muscle was homogenized in ice-cold PBS and 5 μl of 0.5 M butylated hydroxytoluene in acetonitrile. Absorbance of the supernatant was obtained at 586 nm. The samples were normalized for differences in the amount of protein in each sample as determined by a DC protein concentration assay (Bio-Rad).

Glutathione peroxidase (GPx) in whole-muscle homogenate

A commercially available cellular GPx assay (353919; EMD/Calbiochem, San Diego, CA, USA) was used to measure GPx activity in muscle homogenates. This assay measures GPx activity indirectly by a coupled reaction with glutathione reductase. GSSG, produced upon reduction of hydroperoxide by glutathione peroxidase, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ results in a decrease in absorbance at 340 nm. Under conditions in which the glutathione peroxidase activity is rate limiting, the rate of decrease in the A_{340} is directly proportional to the glutathione peroxidase activity in the muscle sample. We have previously reported the methods to measure GPx [34]. Briefly, a portion of each muscle was homogenized in PBS (pH 7.5) containing 5 mM EDTA and 1 mM dithiothreitol (DTT). The homogenate was centrifuged at 10,000 *g* and the supernatant was used for the assay. All reagents and samples were equilibrated to 25 °C. The absorbance was measured at 340 nm using a 96-well plate reader (Dynex Technologies, Chantilly, VA, USA). Each sample and standard was performed in duplicate.

Catalase activity in whole-muscle homogenate

Commercially available reagents (A22180; Invitrogen) were used to measure the activity of the catalase enzyme in whole-muscle homogenates. In this assay, catalase reacts with H_2O_2 to produce water and oxygen. The Amplex red reagent reacts with a 1:1 stoichiometry with any unreacted H_2O_2 in the presence of horseradish peroxidase to produce the highly fluorescent oxidation product resorufin. As catalase activity increases, the signal from resorufin decreases. The methods have been previously reported [36]. Briefly, 25 μl of muscle homogenate was mixed with 25 μl of 40 μM H_2O_2 solution and allowed to incubate in the dark for 30 min at room temperature. After 30 min, the sample containing solution was mixed with 50 μM Amplex red and 0.4 U/ml horseradish peroxidase 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. The change in fluorescence was determined by subtracting the sample value from that of the no-catalase control. The concentration of catalase was determined by comparing the sample to a standard curve. All analyses were measured in duplicate and the samples were normalized to the protein concentration in each sample as assessed using a DC protein concentration assay (Bio-Rad).

Mitochondrial manganese superoxide dismutase (MnSOD)

MnSOD activity was measured in the mitochondrial fraction of each muscle sample using commercially available reagents (574601; EMD/Calbiochem). The assay was performed with slight modifications to the manufacturer's directions and all samples and standards were measured in duplicate. The assay utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Mitochondrial MnSOD was measured with 1 mM potassium cyanide added to the assay to inhibit CuZnSOD and extracellular SOD, resulting in the detection of only MnSOD activity.

The assay was performed in a 96-well plate with each sample being treated with 10 μl of 12 mM potassium cyanide to inhibit any residual CuZnSOD and extracellular SOD activities. The absorbance was measured at 450 nm using a 96-well plate reader (Dynex Technologies). The samples were normalized to the protein concentration in each sample as assessed using a DC protein concentration assay (Bio-Rad).

Cytosolic copper–zinc superoxide dismutase activity

CuZnSOD activity was measured in the mitochondria-free fraction of muscle homogenates using reagents and methods (574601; EMD/Calbiochem) as described previously [34]. All samples and standards were measured in duplicate. This assay is based on the same principles as that used to measure MnSOD, except that potassium cyanide was not added to the assay buffer. This results in the detection of both CuZnSOD and MnSOD activity. MnSOD activity was then subtracted from total activity to obtain CuZnSOD activity. The assay was performed in a 96-well plate and the absorbance was measured at 450 nm using a 96-well plate reader (Dynex Technologies). The samples were normalized to the protein concentration in each sample as assessed using a DC protein concentration assay (Bio-Rad).

Measuring antioxidant enzyme mRNA concentrations

CuZnSOD, MnSOD, catalase, and GPx-1 mRNA were determined by means of reverse transcription–polymerase chain reaction (RT-PCR) according to previously published procedures from our laboratory [34,36,39]. Briefly, RNA was isolated from 60 μg of the gastrocnemius muscle homogenized in 1 ml of Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA). RNA purity was assessed using a minimum 260/280 ratio of 1.7. RNA was reverse transcribed using random primers, dNTP, and SuperScript II reverse transcriptase (Invitrogen/Life Technologies, Bethesda, MD, USA). The primers for CuZnSOD, MnSOD, GPx-1, and catalase have been previously published [36]. The signal from the gene was expressed as a ratio to the 18S signal from the same PCR product. The PCR product from each reaction was separated on a 1.5% agarose gel containing ethidium bromide via electrophoresis. The resulting signals were digitally captured (Kodak DC290; Eastman Kodak Co., Rochester, NY, USA) and quantified using 1D Kodak image analysis software (Eastman Kodak Co.).

Fluorimetric caspase-activity assay

The proteolytic activity of caspase-3 was determined using the fluorogenic substrate Ac-DEVD-AFC (Alexis Biochem/Enzo Life Sciences, PA, USA) as a substrate. Ac-LEHD-AFC (Alexis Biochem/Enzo Life Sciences) was used as the fluorogenic caspase-9 substrate. Briefly, 50 μl of caspase activity buffer (50 mM Pipes, 0.1 mM EDTA, 10% glycerol, 1 mM DTT), 50 μl of the cytosolic fraction of the muscle homogenate without protease inhibitor, and 10 μl of substrate (1 mM) were combined in a 96-well fluorescence microplate. Caspase activity was accessed at wavelengths of 400 nm for excitation and 505 nm for emission. The microplate was incubated for 2 h at 37 °C and caspase activity was determined by subtracting the 2-h reading from the initial reading. Caspase activity is expressed as the relative fluorescence units normalized to the protein concentration of each muscle sample (RFU/mg protein).

Western immunoblots

The protein contents of CuZnSOD, apoptosis peptidase-activating factor 1 (Apaf-1), AIF, and cytochrome *c* were measured in the cytosolic (mitochondria-free) protein fraction. MnSOD, Bax (Bcl-2-associated X protein), and Bcl-2 (B cell leukemia/lymphoma-2) protein abundance was measured in isolated mitochondrial proteins. Thirty micrograms of protein was loaded into each well of a 4–12% gradient polyacrylamide gel (Novex, Invitrogen) and separated by routine SDS–polyacrylamide gel electrophoresis for 1.5 h at 20 °C followed by transfer to a nitrocellulose membrane. All membranes were blocked in 5% nonfat milk protein (NFM) for 1 h at room temperature. The membranes were

incubated overnight at 4 °C, with primary antibodies directed against MnSOD (sc-11407; Santa Cruz Biotechnology, Santa Cruz, CA, USA), MnSOD (A300449A; Bethyl Labs, Montgomery, TX, USA), AIF (sc-13116; Santa Cruz), Apaf-1 (3018; BioVision, Mountain View, CA, USA), cytochrome *c* (sc-13561; Santa Cruz), Bcl-2 (2876; Cell Signaling Technology, Boston, MA, USA), or Bax (2772; Cell Signaling Technology). The antibodies were diluted in Tris-buffered saline with 0.05% Tween 20 (TBS-T) and 0.002% sodium azide. The membranes were washed in TBS-T followed by incubation in the appropriate dilutions of secondary antibodies (diluted in 5% NFM in TBS-T) conjugated to horseradish peroxidase. The signals were developed using a chemiluminescent substrate (ECL Advanced; Amersham Bioscience) and visualized by exposing the membranes to X-ray films (BioMax MS-1; Eastman Kodak). Digital records were obtained from each blot and the protein bands of interest were quantified using 1D analysis software (Eastman Kodak). The protein bands were quantified as optical density × band area and expressed in arbitrary units, normalized to GAPDH. The molecular sizes of the immunodetected proteins were verified using prestained standards (LC5925; Invitrogen/Life Technologies).

Results

Xanthine oxidase activity

Allopurinol blocked in situ electrically stimulated isometric contraction-induced and aging-induced increases in muscle xanthine oxidase activity. Xanthine oxidase activity was 65% greater ($P<0.05$) in control gastrocnemius muscles of aged mice that received the sham surgeries but no allopurinol, compared to young adult mice (Fig. 1A). Xanthine oxidase activity increased in muscles from young adult (33%, $P<0.05$) and aged animals (28%, $P<0.05$) after in situ electrically stimulated isometric contractions, compared to the contralateral control muscles. Allopurinol administration completely blunted both the aging-induced and the in situ electrically evoked increases in muscle xanthine oxidase activity. Allopurinol reduced xanthine oxidase levels in aged muscles so that there were no differences between any of the allopurinol-treated muscles (Fig. 1A).

Hypoxanthine and xanthine

Hypoxanthine, which is a product of purine degradation and a substrate for xanthine oxidase, was elevated by both aging and in situ electrically stimulated isometric contractions in gastrocnemius muscles, but it was not affected by allopurinol. The hypoxanthine concentration was greater in muscles of aged animals (62%, $P<0.05$) than in muscles of young adult animals (0.41 ± 0.07 μM/mg protein in young vs 0.667 ± 0.09 μM/mg protein in aged, $P<0.05$; Fig. 1B). Whereas in situ electrically stimulated isometric contractions increased hypoxanthine concentrations in both the young adult (31%, $P<0.05$) and the aged (54%, $P<0.05$) muscles, allopurinol did not change hypoxanthine concentrations in either control muscles or muscles after electrically stimulated isometric contractions in young or aged animals (Fig. 1B).

Xanthine oxidase catalyzes the reaction of hypoxanthine and oxygen, producing xanthine and superoxide. Xanthine abundance was similar in control muscles from young and aged mice. Electrically evoked isometric contractions increased xanthine oxidase in muscles of young adult and aged mice, but this electrically evoked increase was fully blunted by allopurinol treatment. In situ electrically stimulated isometric contractions elevated xanthine levels in the gastrocnemius muscle from young adult animals by 28% (0.364 ± 0.05 μM/mg protein in control muscles vs 0.465 ± 0.07 μM/mg protein in muscles after electrically evoked contractions, $P<0.05$) and by 86% in aged muscles (0.359 ± 0.06 μM/mg protein control muscles vs 0.668 ± 0.1 μM/mg protein in muscles after electrically evoked contractions, $P<0.05$). Allopurinol prevented any increase in xanthine associated with in situ electrically stimulated isometric contractions (Fig. 1C).

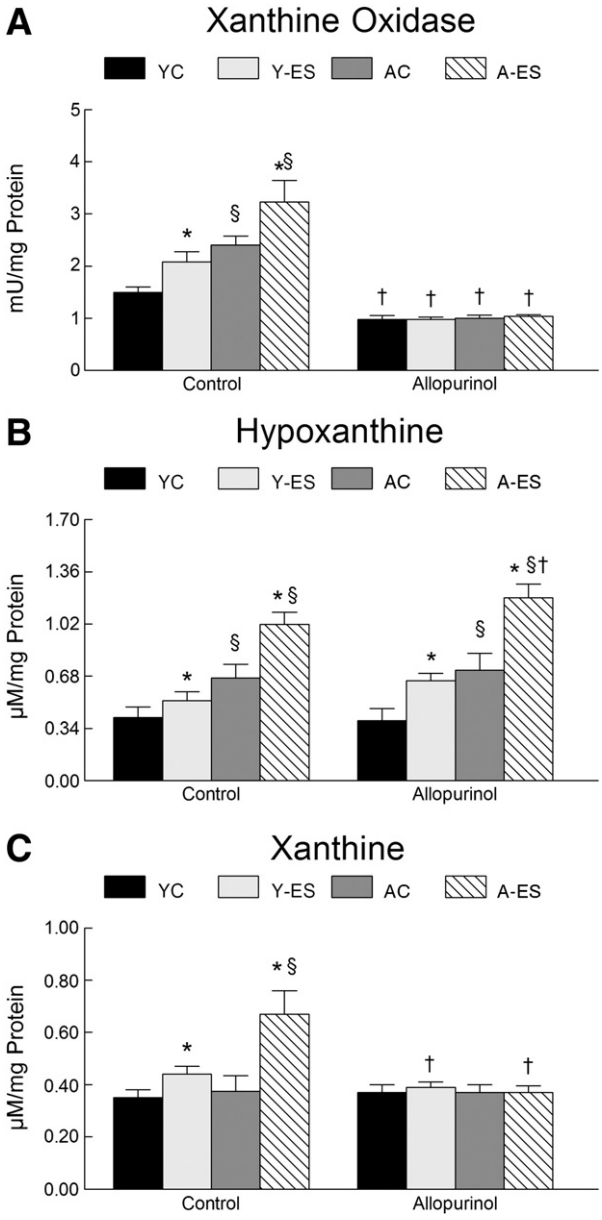

Fig. 1. Allopurinol attenuated the contraction-induced increase in xanthine oxidase activity and hypoxanthine and xanthine protein content. (A) Xanthine oxidase activity was determined fluorimetrically. Data are expressed as mU of activity/mg of total protein in gastrocnemius muscle homogenate. (B) Hypoxanthine and (C) xanthine levels were determined by a fluorimetric assay. Data are expressed as μM/mg of total protein in the gastrocnemius muscle homogenate. The normalized data for young control (YC), young electrically stimulated (Y-ES), aged control (AC), and aged electrically stimulated (A-ES) muscles are presented as means ± SEM. *$P<0.05$, significant difference between electrically stimulated muscles and contralateral control muscles; §$P<0.05$, significant effect of aging within the sham surgery or allopurinol-treatment groups; †$P<0.05$, significant effect of allopurinol treatment.

Hydrogen peroxide

H_2O_2 was measured as an indicator of oxidant production in muscle samples. Whole-muscle homogenate levels of H_2O_2 were elevated in gastrocnemius muscles after in situ electrically stimulated isometric contractions in both young adult (24%, $P<0.05$) and aged (44%, $P<0.05$) animals compared to the age-matched control limb. H_2O_2 was higher both in control muscles (21%, $P<0.05$) and after electrically evoked contractions (39%, $P<0.05$) in muscles of aged animals compared to muscles from young adult animals. Allopurinol attenuated the increase

in H_2O_2 associated with in situ electrically stimulated isometric contractions in both age groups (Fig. 2A).

Mitochondrial ROS production

Two approaches were used to determine if allopurinol affected ROS production in mitochondria. In the first method, H_2O_2 was measured in isolated mitochondria. H_2O_2 was similar in isolated mitochondria from control and electrically stimulated gastrocnemius muscles of young adult and aged mice. Furthermore, there was no decrease in H_2O_2 levels measured in mitochondria of animals that were treated with allopurinol (Fig. 2B). However, H_2O_2 diffuses readily from mitochondria, and therefore EPR was used as a second approach, to measure superoxide and hydroxyl radical oxidant production (i.e., ROS) in isolated mitochondria. EPR showed increased ROS radical production in mitochondria from electrically stimulated vs control muscles in aged mice and greater ROS radical production in mitochondria that were isolated from muscles of aged vs young adult animals. Allopurinol treatment significantly blunted the production of ROS radicals in isolated mitochondria from electrically stimulated muscles of old mice, but it did not change ROS radical production in mitochondria from electrically stimulated muscles of young adult mice (Fig. 2C).

Lipid peroxidation

The levels of MDA and HAE, both products of lipid peroxidation, were 56% ($P<0.05$) greater in control gastrocnemius muscles of aged vs young adult mice (4.8 ± 0.64 μM/mg protein in aged mice vs 3.06 ± 0.64 μM/mg protein in young animals; $P<0.05$). Lipid peroxidation was elevated by 29% (3.95 ± 0.74 μM/mg protein vs 3.06 ± 0.64 μM/mg protein; $P<0.05$) within the young adult muscles after in situ electrically stimulated isometric contractions and by 92% (9.2 ± 1.76 μM/mg protein vs 4.8 ± 0.64 μM/mg protein, $P<0.05$) in the aged electrically stimulated muscles. Allopurinol did not change MDA + HAE levels that were associated with electrically evoked contractions in the young adult (3.22 ± 0.61 μM/mg control muscles vs 3.27 ± 0.78 μM/mg in muscles after electrically evoked contractions, $P>0.05$) or aged muscles (5.17 ± 1.09 μM/mg protein in control muscles vs 5.68 ± 1.2 μM/mg protein in muscles after electrically evoked contractions, $P>0.05$). Allopurinol did not depress lipid peroxidation in control muscles of aged mice (4.80 ± 0.64 μM/mg protein after sham surgery vs 5.17 ± 1.09 μM/mg protein after allopurinol treatment, $P>0.05$; Fig. 2D).

Glutathione levels

Reduced GSH is a major tissue antioxidant that provides reducing equivalents for the reduction of hydrogen peroxide to water. In a

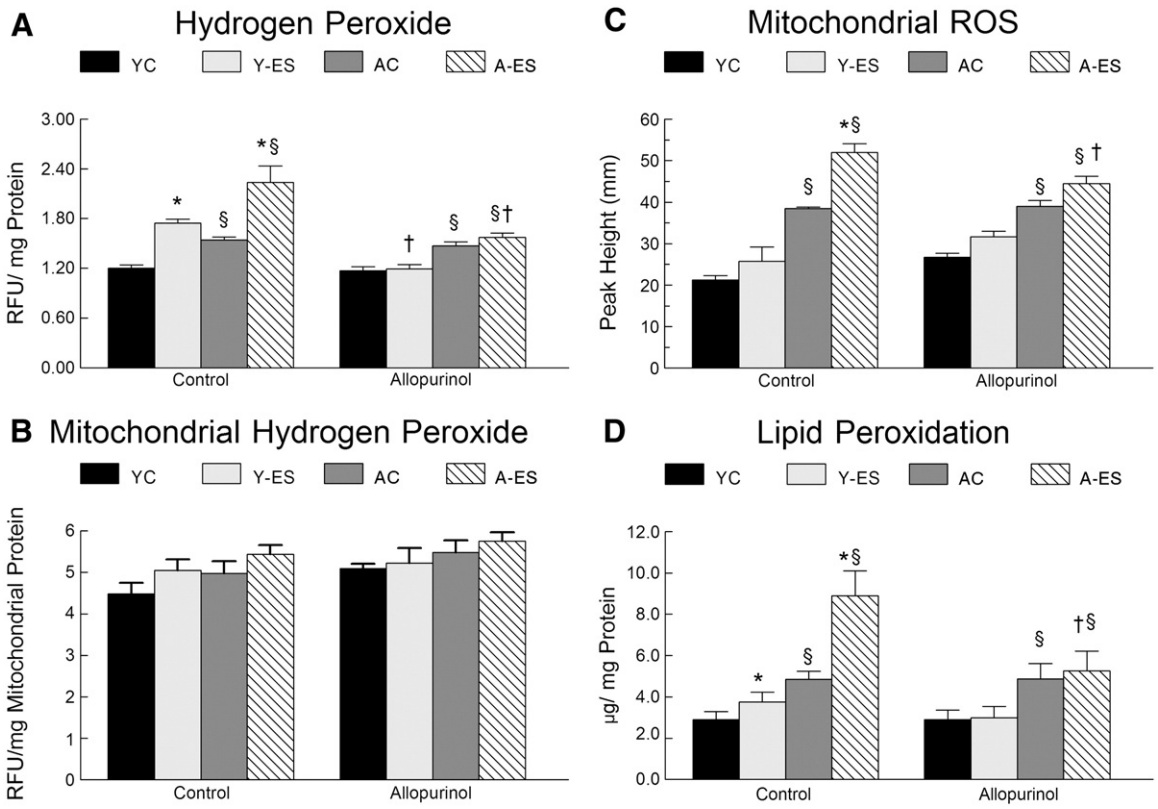

Fig. 2. Inhibition of xanthine oxidase activity attenuated the increase in H_2O_2 concentration, lipid peroxidation, and mitochondrial ROS radical production in response to in situ electrically stimulated contractions of aged mice. (A) The H_2O_2 concentrations were determined fluorimetrically in total muscle homogenates. Data are expressed as RFU/mg of total protein in gastrocnemius muscle homogenate. (B) The H_2O_2 concentrations were determined fluorimetrically in isolated mitochondrial homogenates. Data are expressed as RFU/mg of total mitochondrial protein. (C) Mitochondria were isolated from control and electrically stimulated gastrocnemius muscles of young adult and old mice that received either placebo or allopurinol treatment. EPR spectroscopy was performed to index ROS radical generation. Isolated mitochondria were incubated with 5,5-dimethyl-1-pyrroline-*N*-oxide in the presence or absence of excess complex I respiratory substrates glutamate and malate for 3 min at 37 °C. Radical ROS levels were measured at room temperature with instrument settings of 1000 mW, modulation amplitude 1.0 G, receiver gain 1.00×10^4, conversion time 40.960 ms, time constant 40.960 ms and sweep time 41.943 ms, microwave frequency 9.752 GHz, microwave power 126.90 mW. Representative spectra are shown and the arrows indicate ROS radical spikes. (D) Data represent combined malondialdehyde and 4-hydroxyalkenals and are normalized to the total protein concentration in the gastrocnemius muscle homogenate. The normalized data for young control (YC), young electrically stimulated (Y-ES), aged control (AC), and aged electrically stimulated (A-ES) muscles are presented as means ± SEM. *$P<0.05$, significant difference between in situ electrically stimulated muscle and contralateral control muscle; §$P<0.05$, significant effect of aging within the sham surgery or allopurinol-treatment groups; †$P<0.05$, significant difference of the allopurinol treatment.

reaction catalyzed by glutathione peroxidase, two GSH molecules form a disulfide bond resulting in the oxidized form of glutathione. The ratio GSH/GSSG is used as an indicator of oxidative stress. GSH was decreased in gastrocnemius muscles with aging and in muscles of old mice after electrically evoked contractions, but partially rescued by allopurinol treatment. As shown in Fig. 3A, the concentration of GSH was ~21% ($P<0.05$) lower in muscles of aged compared with young mice. Although it approached significance ($P=0.066$), the GSSG concentration was not statistically different in muscles obtained from young or aged mice (Fig. 3B). The ratio GSH/GSSG was 35% lower ($P<0.05$) in muscles of aged vs young adult animals (Fig. 3C). The GSH/GSSG ratio was reduced in gastrocnemius muscles after electrically evoked contractions in both young animals (15.0 ± 2.2 in control muscles vs 9.0 ± 1.4 in muscles after electrically evoked contractions, $P<0.05$) and aged animals (9.7 ± 0.98 in control muscles vs 3.2 ± 0.54 in muscles after electrically evoked contractions, $P<0.05$; Fig. 3C). Allopurinol treatment prevented the decrease in the GSH/GSSG ratio in muscles from young mice after electrically evoked contractions and partially attenuated the decrease in the GSH/GSSG ratio from control muscles from aged mice after electrically evoked contractions. These data suggest that aging increased oxidative stress and consequently lowered the GSH/GSSG ratio in these muscles, thus reducing the ability of the gastrocnemius muscle to tolerate increased oxidative production resulting from electrically evoked contractions.

GPx

Neither aging, in situ electrically stimulated isometric contractions, nor xanthine oxidase inhibition produced any significant changes in glutathione peroxidase enzyme activity or GPx-1 mRNA levels in mouse gastrocnemius muscles (Figs. 4A and B).

MnSOD

MnSOD activity was greater (37%, $P<0.05$) in the gastrocnemius muscles from aged animals compared to young adults (Fig. 4C). Neither in situ electrically stimulated isometric contractions nor allopurinol had any significant affect on MnSOD activity in either age group. Aging, in situ electrically stimulated isometric contractions, and xanthine oxidase inhibition all failed to affect muscle levels of MnSOD mRNA (Fig. 4D).

Catalase

Allopurinol blunted the increase in catalase activity that occurred in muscles after electrically evoked contractions, but it did not change the aging-associated increase in muscle catalase activity. The enzymatic activity of catalase was greater (66%, $P<0.05$) in the gastrocnemius muscles from aged animals compared to young adult animals (Fig. 5A), whereas catalase mRNA was ~180% greater ($P<0.05$) in muscles from old vs young adult mice (Fig. 5C). Neither catalase protein levels nor mRNA content was affected by allopurinol. In situ electrically stimulated isometric contractions increased the catalase activity by ~20% ($P<0.05$) in the muscles from aged animals, but they had no affect on catalase activity in muscles from the young adult animals. However, in aged gastrocnemius muscles, electrically evoked contractions did not elicit a significant increase in catalase protein abundance or mRNA content. The increase in catalase activity associated with electrically evoked contractions in the aged gastrocnemius muscles was completely attenuated by allopurinol administration (0.612 ± 0.11 nM/min/mg in control muscles vs 0.606 ± 0.1 nM/min/mg in muscles after electrically evoked contractions, $P>0.05$). In situ electrically stimulated isometric contractions did not alter catalase protein abundance or mRNA content in the gastrocnemius muscle from young adult or aged animals (Figs. 5B and C).

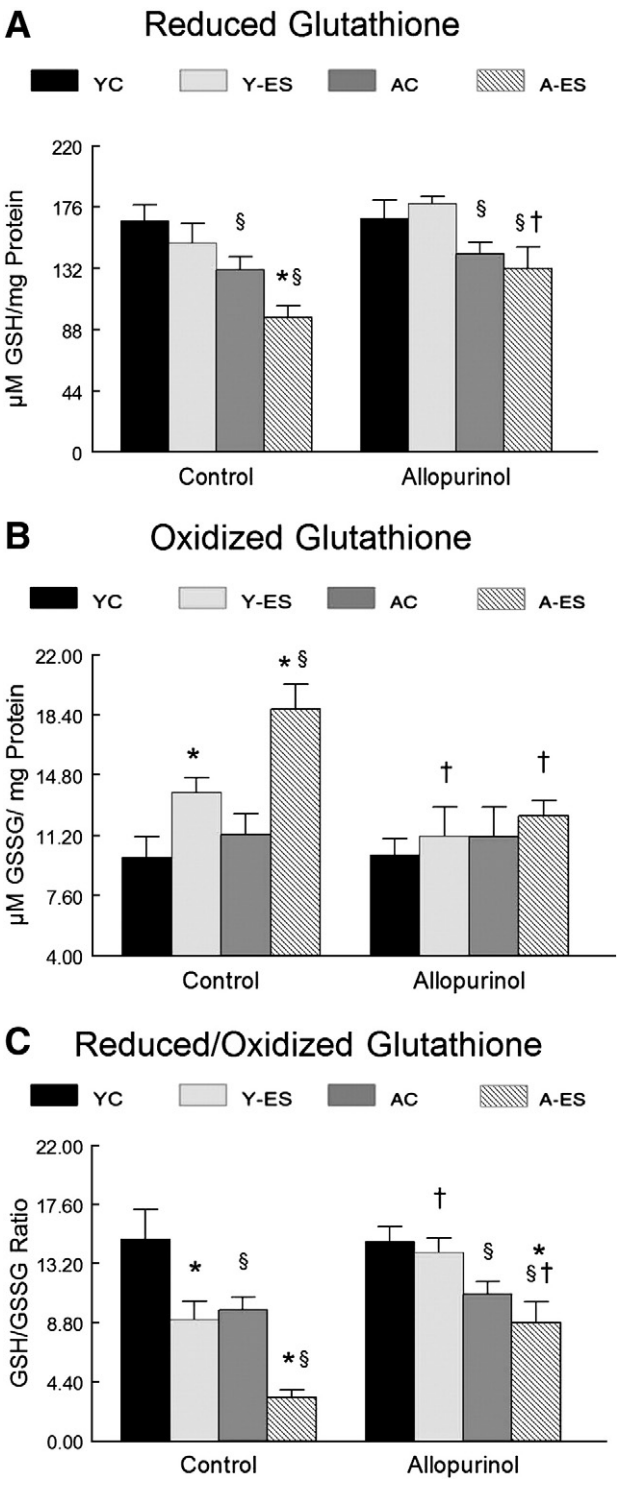

Fig. 3. Concentrations of reduced GSH and GSSG and the GSH/GSSG ratio. (A) The concentration of reduced glutathione is expressed as μM GSH normalized to total protein concentration (mg) in the gastrocnemius homogenate. (B) The concentration of oxidized glutathione is expressed as μM GSSG normalized to the total protein concentration (mg) of the gastrocnemius homogenate. (C) Data are depicted as the ratio of GSH to GSSG normalized to total protein content. Lower ratios are an indication of increased oxidative stress. The normalized data for young control (YC), young electrically stimulated (Y-ES), aged control (AC), and aged electrically stimulated (A-ES) muscles are presented as means ± SEM. *$P<0.05$, significant difference between muscles after repeated in situ electrically stimulated isometric contractions and the contralateral control muscles; §$P<0.05$, significant difference within either the sham surgery or the allopurinol-treatment groups due to aging; †$P<0.05$, significant difference due to the allopurinol treatment.

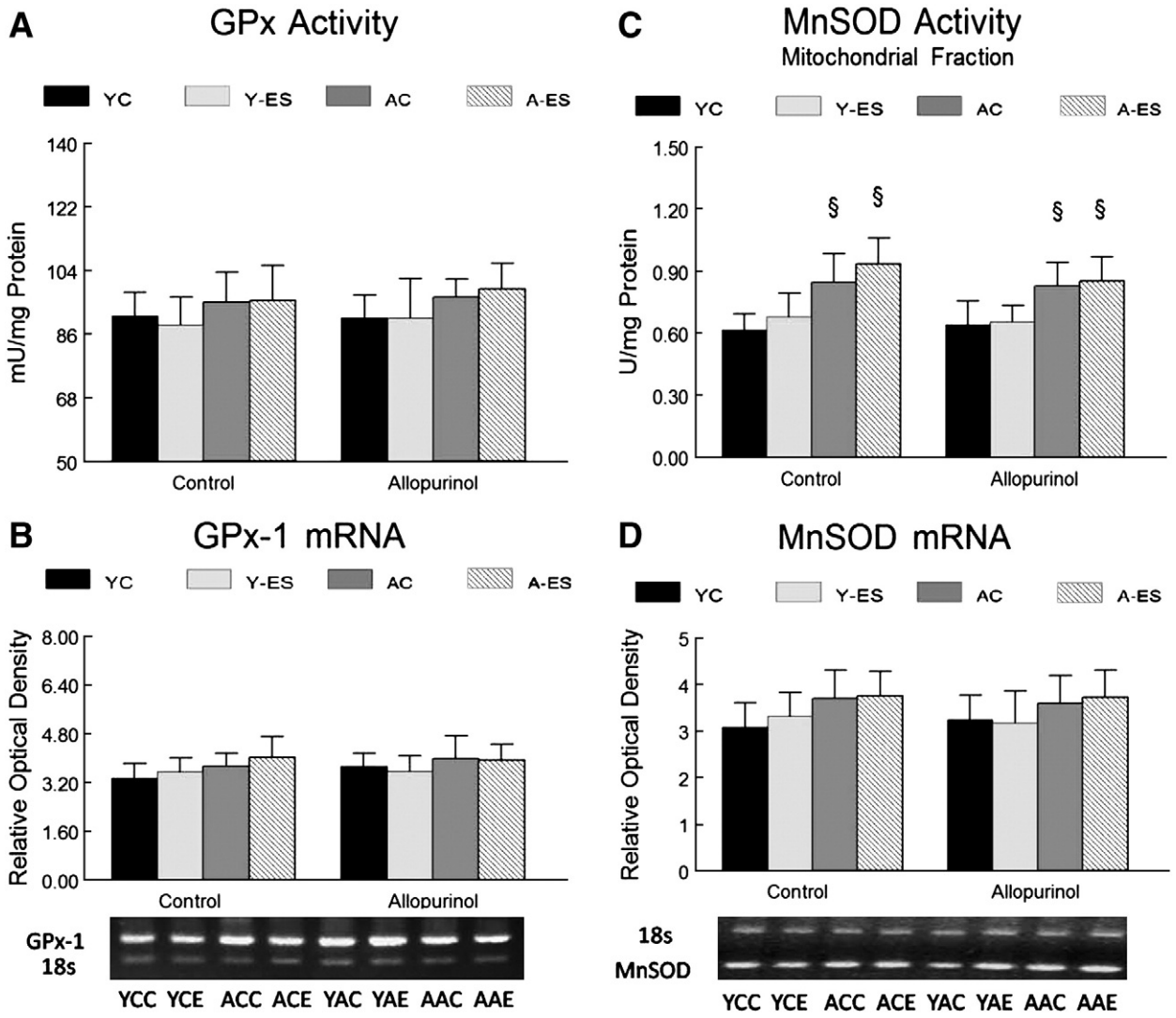

Fig. 4. GPx and MnSOD activity and mRNA regulation with in situ electrically stimulated isometric contractions and allopurinol treatment. The normalized data for young control (YC), young electrically stimulated (Y-ES), aged control (AC), and aged electrically stimulated (A-ES) muscles are presented as means ± SEM. §$P<0.05$, significant difference within the sham surgery or allopurinol treatment groups due to aging. (A) Total GPx activity is expressed as mU of GPx/ml of homogenate normalized to mg of total protein concentration in the gastrocnemius homogenate. (B) GPx-1 mRNA expression was determined from the total muscle homogenate by RT-PCR. The data are expressed as optical density × band area normalized to 18S rRNA (relative optical density). The inset shows representative gels for GPx-1 mRNA and 18S rRNA in young and aged (control and electrically stimulated) gastrocnemius muscle. (C) MnSOD activity was determined in the mitochondrial fraction that was obtained from the gastrocnemius muscle. MnSOD activity is expressed as mU of MnSOD/ml of homogenate normalized to mg of protein in the sample. (D) MnSOD mRNA expression was determined from the muscle by RT-PCR. The data are expressed as optical density × band area, normalized to 18S rRNA (relative optical density). The inset shows representative gels for MnSOD mRNA and 18S rRNA in young and aged (control and after in situ electrically stimulated isometric contractions) gastrocnemius muscle. YCC, young, control surgery, control non-electrically stimulated; YCE, young, control surgery, electrically stimulated; YAC, young, allopurinol, control non-electrically stimulated; YAE, young, allopurinol, electrically stimulated; ACC, aged, control surgery, control non-electrically stimulated; ACE, aged, control surgery, electrically evoked contractions; AAC, aged, allopurinol, control non-electrically stimulated; AAE, aged, allopurinol, electrically stimulated.

CuZnSOD

Allopurinol blunted the increase in CuZnSOD activity from muscles after electrically evoked contractions but not aging. CuZnSOD activity was greater (11%, $P<0.05$) in control gastrocnemius muscles of aged animals compared with young animals (1.06 ± 0.08 U/mg young vs 1.23 ± 0.08 U/mg aged, $P<0.05$; Fig. 5D). Allopurinol treatment did not affect CuZnSOD activity in control muscles. In situ electrically stimulated isometric contractions increased CuZnSOD activity in muscles of young adult (16%, $P<0.05$) and aged (11%, $P<0.05$) animals. Electrically evoked contractions also increased CuZnSOD protein abundance by 73% ($P<0.05$) in muscles from young animals and by 62% ($P<0.05$) in muscles from aged mice (Fig. 5E). Allopurinol suppressed the increase in CuZnSOD activity and protein content from gastrocnemius muscles after electrically evoked contractions in both young and aged mice. CuZnSOD mRNA levels were not altered by age, electrically evoked contractions, or allopurinol (Fig. 5F).

Proapoptotic mitochondrial signaling proteins

Cytosolic levels of gastrocnemius caspase-9 and caspase-3 activities were elevated with aging, but they were not affected by electrically evoked contractions or allopurinol treatment. Muscles from aged animals had greater levels of caspase-9 activity (122%, $P<0.05$) and caspase-3 activity (85%, $P<0.05$), compared to young adult mice. Although electrically stimulated contractions did not increase caspase-9 activity, caspase-3 activity was 30% greater ($P<0.05$) in muscles of aged mice after electrically evoked contractions compared to control muscles. Allopurinol suppressed the increase in caspase-3 activity that was induced by electrically evoked contractions. Neither electrically stimulated

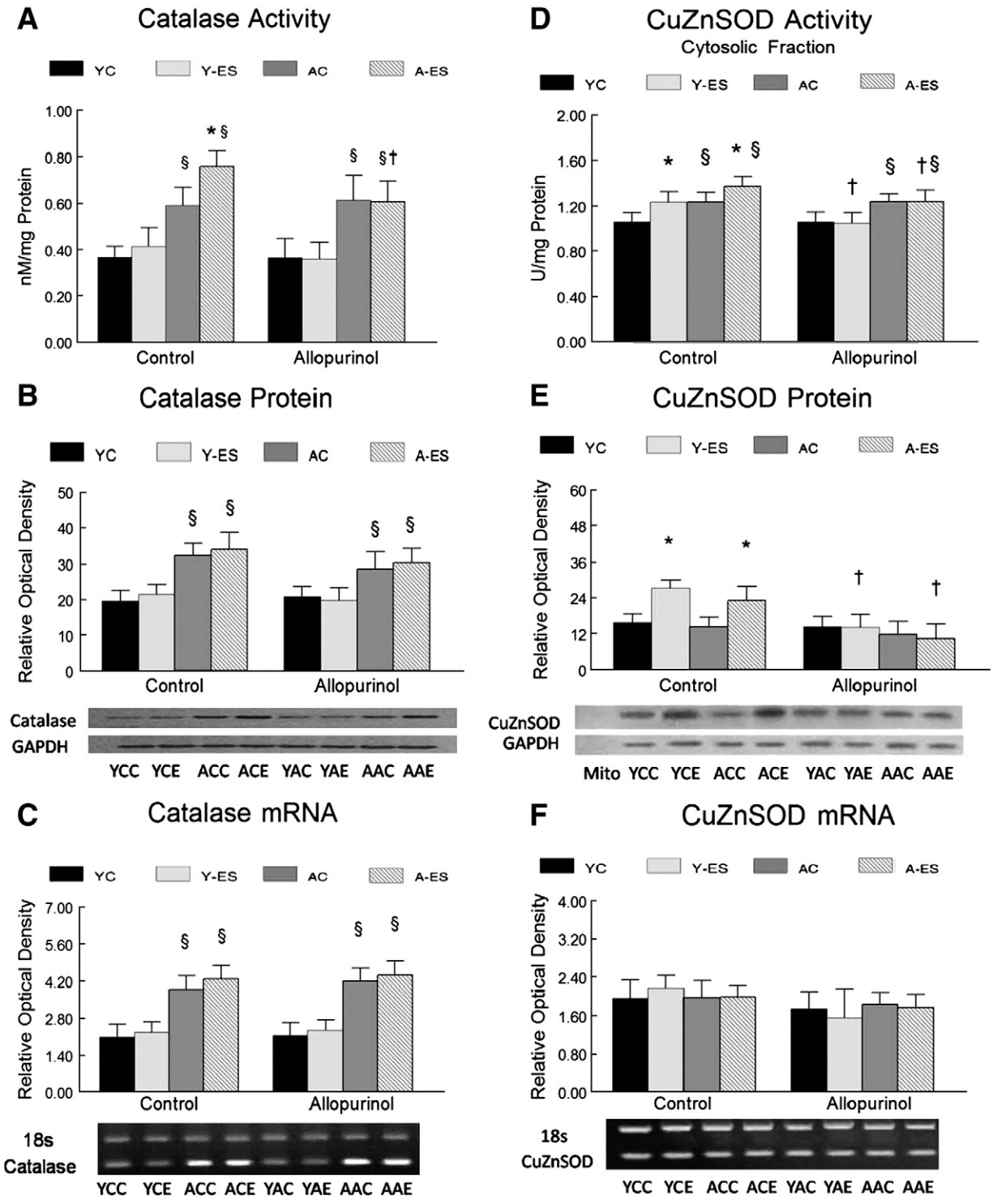

Fig. 5. Catalase and CuZnSOD activity, protein expression, and mRNA regulation with in situ electrically stimulated isometric contractions and allopurinol treatment. The normalized data for young control (YC), young electrically stimulated (Y-ES), aged control (AC), and aged electrically stimulated (A-ES) muscles are presented as means ± SEM. *$P<0.05$, significant difference between electrically stimulated muscles and contralateral control muscles; §$P<0.05$, significant difference within the sham surgery or allopurinol-treatment groups due to aging; †$P<0.05$, significant difference due to the allopurinol treatment. (A) Total catalase activity is expressed as nM activity/min normalized to mg of total protein in the gastrocnemius homogenate. (B) Catalase protein expression was determined in the mitochondria-free cytosolic fraction by Western immunoblotting. The data are expressed as optical density × band area, normalized to GAPDH (relative optical density). The inset shows representative blots for catalase and GAPDH content in young and aged gastrocnemius muscle. (C) Catalase mRNA expression was determined by RT-PCR from the total muscle homogenate. The data are expressed as optical density × band area, normalized to 18S rRNA (relative optical density). The inset shows representative gels for catalase mRNA and 18S rRNA in gastrocnemius muscles from young and aged (control muscles and muscles that received in situ electrically stimulated isometric contractions). (D) CuZnSOD activity was determined in the mitochondria-free cytosolic fraction of gastrocnemius muscles. CuZnSOD activity is expressed as mU of CuZnSOD/ml of homogenate normalized to mg of protein in homogenate. (E) CuZnSOD protein abundance was determined in the mitochondria-free cytosolic fraction by Western immunoblotting. The data are expressed as optical density × band area, normalized to GAPDH (relative optical density). The inset shows representative blots for CuZnSOD and GAPDH content in gastrocnemius muscles. (F) CuZnSOD mRNA expression was determined by RT-PCR from total muscle homogenates. The data are expressed as optical density × band area, normalized to 18S rRNA (relative optical density). The inset shows representative gels for CuZnSOD mRNA and 18S rRNA from gastrocnemius muscles under control conditions and muscles that received in situ electrically stimulated isometric contractions in young and aged mice. YCC, young, control surgery, control non-electrically stimulated; YCE, young, control surgery, electrically stimulated; YAC, young, allopurinol, control non-electrically stimulated; YAE, young, allopurinol, electrically stimulated; ACC, aged, control surgery, control non-electrically stimulated; ACE, aged, control surgery, electrically stimulated; AAC, aged, allopurinol, control non-electrically stimulated; AAE, aged, allopurinol, electrically stimulated.

contractions nor allopurinol altered apoptotic signaling in the muscles of young adult animals (Figs. 6A and B).

Mitochondrial proteins cytochrome *c* and AIF are released from the mitochondria to the cytosol in response to proapoptotic stimuli. Cytochrome *c* and AIF are apoptotic intermediates that when present in the cytosol suggest that mitochondria permeability has increased as a result of an increase in the formation of mitochondrial pore openings. Both cytochrome *c* (~237%; Fig. 6C) and AIF (~725%; Fig. 6D) were higher in the cytosolic fractions of gastrocnemius muscles from aged animals compared to muscles from young adult mice. Neither electrically evoked contractions nor allopurinol treatment altered cytochrome *c* or AIF accumulation in the cytosol of muscles of young or aged mice.

Bax and Bcl-2 protein abundance was estimated by Western blot analyses from isolated mitochondria that were obtained from control and electrically stimulated gastrocnemius muscles (Fig. 7). Bax protein abundance was greater in control mitochondria from aged animals than in young adult animals, but allopurinol removed any differences in mitochondrial Bax from young and aged animals. Electrically evoked contractile activity did not alter Bax abundance in mitochondria homogenates from either young adult or aged animals. Bcl-2 protein abundance was similar in mitochondria from control muscles of young adult and aged mice. However, repeated electrically evoked isometric contractions reduced Bcl-2 levels similarly in muscle mitochondrial homogenates from both young adult and aged animals.

Muscle function measurements

Maximal isometric plantar flexor muscle force was measured as an indicator of muscle function. The gastrocnemius muscle provides the greatest contribution to plantar flexion. The maximal isometric force recorded for the third day (which was the first contraction of that day)

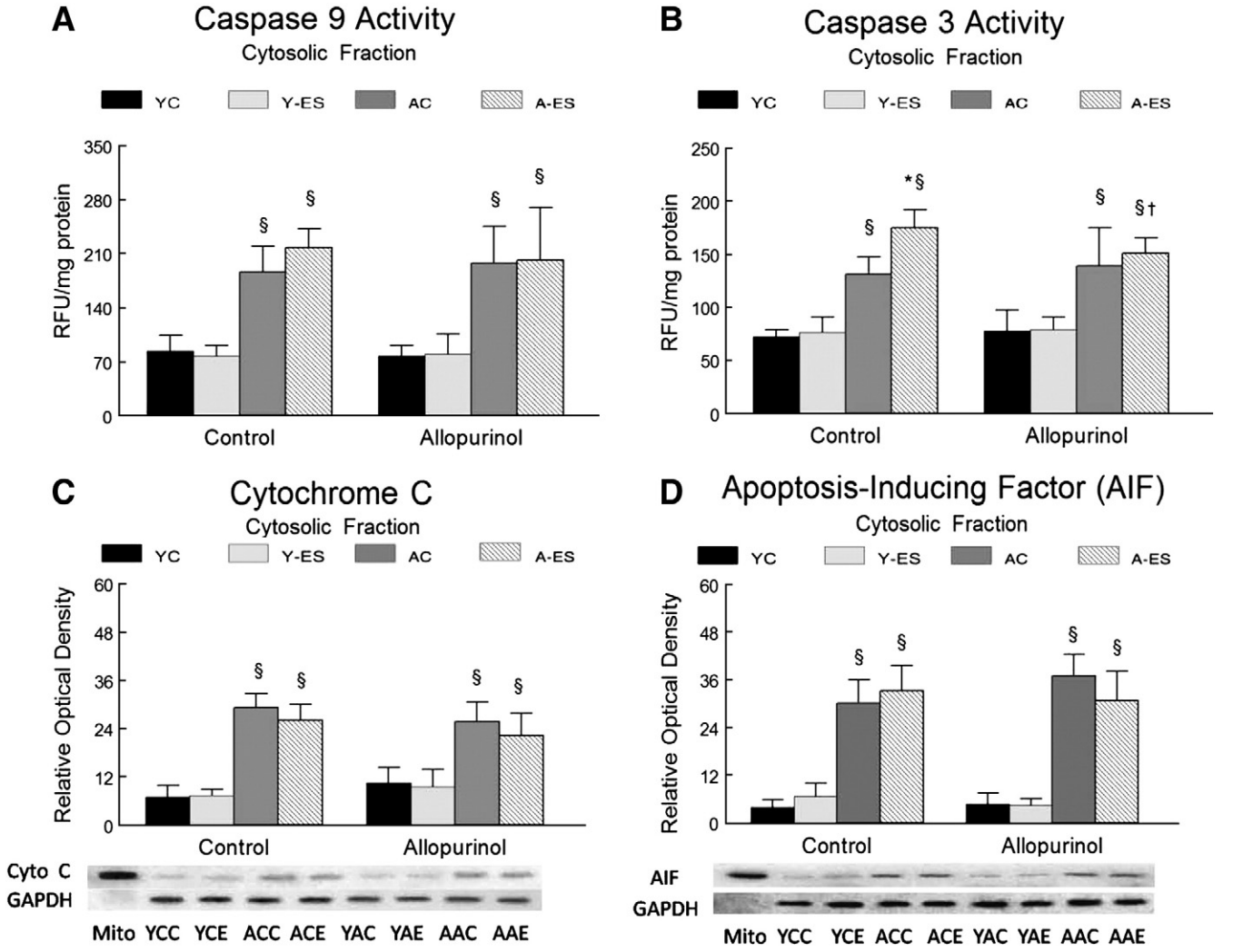

Fig. 6. Aging increases apoptotic signaling in skeletal muscle. The normalized data for young control (YC), young electrically stimulated (Y-ES), aged control (AC), and aged electrically stimulated (A-ES) muscles are presented as means ± SEM. *$P<0.05$, significant difference from muscles that received in situ electrically stimulated isometric contractions muscle and contralateral control muscle; §$P<0.05$, significant difference within the sham surgery or allopurinol treatment groups due to aging; †$P<0.05$, significant difference due to the allopurinol treatment. (A) Caspase-9 and (B) caspase-3 levels were determined in the mitochondria-free cytosolic fraction of the gastrocnemius muscle homogenate by a fluorimetric assay. Data are expressed as RFU/mg of protein in the homogenate. (C) Cytosolic cytochrome *c* protein expression was determined in the mitochondria-free fraction of the gastrocnemius homogenate by Western immunoblotting. The data are expressed as optical density × band area. The data are normalized to GAPDH (relative optical density). The inset shows representative blots for cytochrome *c* and GAPDH in gastrocnemius muscles from young and aged mice (control and repetitively electrically stimulated contractions). (D) Cytosolic AIF protein expression was determined in the mitochondria-free fraction of the gastrocnemius homogenate by Western immunoblotting. The data are expressed as optical density × band area, normalized to GAPDH (relative optical density). The inset shows representative blots for AIF and GAPDH in young and aged (control and repetitive loading) gastrocnemius muscle. YCC, young, control surgery, control non-electrically stimulated; YCE, young, control surgery, electrically stimulated; YAC, young, allopurinol, control non-electrically stimulated; YAE, young, allopurinol, electrically stimulated; ACC, aged, control surgery, control non-electrically stimulated; ACE, aged, control surgery, electrically stimulated; AAC, aged, allopurinol, control non-electrically stimulated; AAE, aged, allopurinol, electrically stimulated.

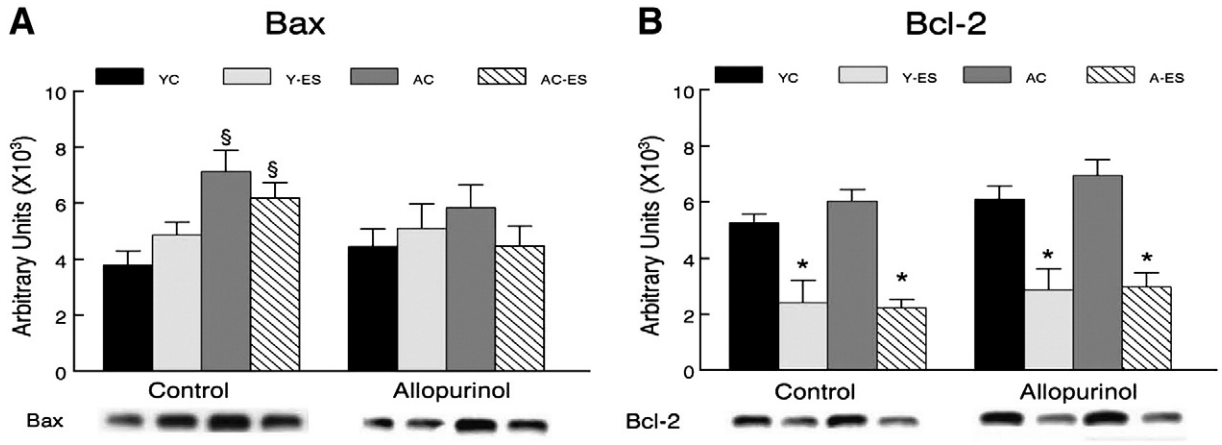

Fig. 7. Allopurinol blunts the increase in mitochondrial Bax that is elevated by electrically evoked contractions in skeletal muscle from aged mice. (A) Bax protein abundance was estimated by Western blot analyses in homogenates from isolated mitochondria. These data suggest that Bax is greater in mitochondria from aged animals, but allopurinol removed any differences in mitochondrial Bax from young and aged animals. Electrically evoked activity did not alter Bax abundance in mitochondria homogenates from young adult mice but it blunted the increase in mitochondrial Bax in muscles from aged animals. (B) Bcl-2 protein abundance was estimated in homogenates from gastrocnemius muscles of control muscles and muscles after 3 days of electrically evoked contractions. Bcl-2 levels were similar in control muscles from young adult and aged mice. Repeated electrically evoked isometric contractions reduced Bcl-2 levels similarly in muscle homogenates from young adult and aged animals. Data are presented for young control (YC), young electrically stimulated (Y-ES), aged control (AC), and aged electrically stimulated (A-ES) muscles, as means ± SEM. The insets show representative blots of mitochondrial Bax and Bcl-2. Ponceau S staining of the membranes confirmed equal loading and electrotransfer of the proteins from the gels to the membranes.

was normalized to the animal's body weight. Maximal isometric force was depressed with aging, but improved with allopurinol treatment. Maximal isometric force per gram of body weight was lower (−36.6%, $P<0.05$) in the plantar flexor muscles of aged animals compared to the young adult animals. Maximal isometric force in the plantar flexors from aged mice was greater (35%, $P<0.05$) in animals provided allopurinol compared to animals given the sham surgery, but allopurinol had no effect on force production in the young adult animals. Maximal isometric force did not differ between the first and the third session of electrically evoked contractions in control or allopurinol groups for either young adult or aged mice (Fig. 8A).

The rate of fatigue for the plantar flexors was assessed by calculating the net loss of force throughout the session of electrically evoked contractions relative to the first contraction in the session. Fatigue resistance was greater in muscles from the aged animals, but allopurinol treatment had no effect on isometric muscle fatigue in either young adult or aged animals (Fig. 8B).

Discussion

The main findings from this study are that in skeletal muscle: (1) acute in situ electrically stimulated isometric contractions and aging increased xanthine oxidase, hypoxanthine, xanthine, and markers of oxidative stress and damage; (2) reducing xanthine oxidase via allopurinol suppressed the in situ electrically stimulated isometric contraction-associated elevations in H_2O_2 and lipid peroxidation, prevented the loss of GSH that was induced by electrically evoked contractions, and prevented the increase in catalase and CuZnSOD activities, but had no effect on GPx and MnSOD activity or mRNA in electrically activated muscles of aged animals; and (3) allopurinol suppressed the in situ electrically stimulated isometric contraction-induced increase in caspase-3 activity in muscle homogenates and Bax protein abundance in isolated mitochondria of muscles from aged animals, but it did not reduce other markers of apoptotic signaling associated with aging or electrically evoked contractions.

Increased pro-oxidant production and damage have been shown to be associated with exercise [17,18,20,23,24,34,36,40–42] and aging [12,34,36,43–46]. Our data are consistent with these findings, because we found that both in situ electrically stimulated isometric contractions and aging lead to increases in skeletal muscle H_2O_2 concentrations, lipid peroxidation, and xanthine oxidase activity and a decrease in the GSH/GSSG ratio and greater mitochondrial ROS production. Nevertheless, maximal electrically evoked muscle contractions did not result in any obvious structural damage or inflammation to the fibers in the gastrocnemius muscle (Supplemental Fig. 1). H_2O_2 is highly permeative to membranes and therefore it can exit the mitochondria to the cytosol readily. Therefore, it was not surprising that H_2O_2 abundance in mitochondria fractions was not altered by electrical contractions or allopurinol (Fig. 2B). Nevertheless, our data clearly show that H_2O_2 abundance is elevated in muscle homogenates (Fig. 2A) and ROS radicals are increased in isolated muscle mitochondria (Fig. 2C) after electrically evoked muscle contractions in aged compared to young adult mice. Allopurinol suppresses mitochondrial ROS production in electrically contracting muscles (Fig. 2A) and mitochondria (Fig. 2C) from aged mice. The suppression of H_2O_2 that was measured in muscle homogenates after allopurinol treatment in electrically stimulated muscle of young adult mice (Fig. 2A) was probably from nonmitochondrial ROS sources, because ROS radical production was unaffected by allopurinol treatment in mitochondria of young adult mice after evoked contractions (Fig. 2C), yet muscle homogenates for H_2O_2 (Fig. 2A) and lipid peroxidation (Fig. 2D) were suppressed in muscles from young animals. In contrast, it is likely that both mitochondria and nonmitochondrial sources of ROS were suppressed after electrically evoked contractions in aged mice that received the allopurinol treatment.

Although allopurinol seemed to have a direct effect on mitochondria of aged mice, the mechanism for this is not known. One possibility is that this observation might represent an indirect effect on mitochondrial ROS production, by reducing oxidants (e.g., xanthine oxidase) in the mitochondrial niche and the cell's cytoplasm, which in turn reduces oxidation of mitochondrial proteins and reduces mitochondrial impairment and ROS production. Total muscle ROS accumulation could also be suppressed by lowering xanthine oxidase without changing other ROS sources, because exercise-induced accumulation of ROS can originate from other cellular sources, including NADPH oxidases located within the sarcoplasmic reticulum, transverse tubules, and the fiber sarcolemma (reviewed in [47]). It is also possible that allopurinol may have had a greater effect in the fast-contracting gastrocnemius compared to the responses that might have been found if a slow-contracting muscle had been examined. This is feasible, because mitochondria from fast type II muscle fibers such as in the gastrocnemius muscle, which was investigated in this study, have higher levels of ROS production than

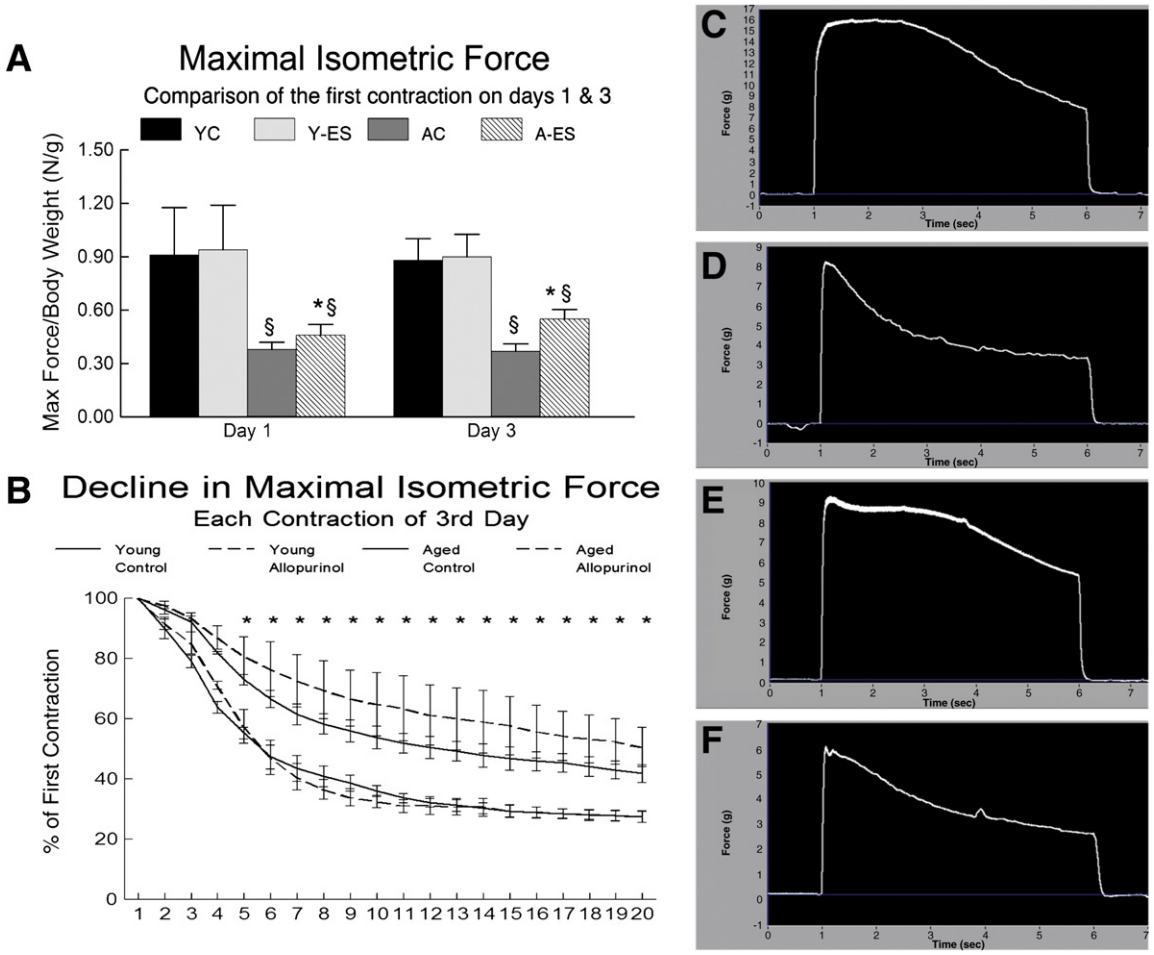

Fig. 8. Maximal isometric forces from the plantar flexor muscles. (A) Data are presented for young control (YC), young electrically stimulated (Y-ES), aged control (AC), and aged electrically stimulated (A-ES) muscles as means ± SEM of the maximal isometric force (N) recorded on the 3rd day of in situ electrically stimulated contractions by the left plantar flexor muscle group normalized to the body weight in grams of the animal. §$P<0.05$, significant effect of aging within the sham surgery or allopurinol-treatment groups. *$P<0.05$, significantly different from control muscle in aged animals. (B) Data are expressed as the means ± SEM of the relative difference between the maximal isometric forces produced on the each contraction and the maximal isometric force on the first contraction. All force measurements were normalized to body weight (g). *$P<0.05$, significant difference in both the aged sham surgery and the aged allopurinol-treatment animals versus the young adult sham surgery and allopurinol-treatment animals. (C–F) Representative force × time curves from the third session of electrically stimulated muscle contractions in the allopurinol-treated young adult and aged animals. (C) First contraction of the 3rd day in a young adult animal. (D) Twentieth contraction of the 3rd day in a young adult animal. (E) First contraction of the 3rd day in an aged animal. (F) Twentieth contraction of the 3rd day in an aged animal.

mitochondria from slow type I muscle fibers [48]. However, additional work is required to investigate this possibility.

Although either electrically evoked or voluntary isometric exercise that exceeds ~60% of maximal force production produces muscle ischemia [49], and ischemia can produce xanthine oxidase, we cannot rule out the possibility that ischemia-induced xanthine oxidase could differ between the repeated electrically evoked isometric muscle model used in this study and models that would use voluntary exercise.

Nondamaging isometric muscle contractions have been shown to elevate superoxide production in the extracellular space of the gastrocnemius muscle in mice [33]. We would anticipate that the additional superoxide from the isometric contractions in this study would be quickly converted to H_2O_2 by SOD, and this conversion could account for most of the observed increase H_2O_2 content. H_2O_2 has the potential to induce widespread oxidant damage simply because it easily crosses cellular membranes.

Xanthine oxidase is a source of oxidative stress in muscles after electrically stimulated isometric contractions

Xanthine oxidase produces superoxide in the cytosol of contracting rodent skeletal muscles [41]. In addition, xanthine oxidase has been shown to be present in endothelial cells from human skeletal muscle [50] and despite its location, it is relevant to muscle function, because it affects the responses of human muscle to exercise [51–53]. The present data suggest that muscle xanthine oxidase activity increased with aging, and it is elevated with in situ electrically stimulated isometric contractions in muscles of both aged and young adult mice. The inhibition of xanthine oxidase via allopurinol reduced the indices of oxidative stress associated with electrically evoked contractions (H_2O_2 concentration, lipid peroxidation, and the GSH/GSSG ratio). These data are consistent with the idea that xanthine oxidase makes an important contribution to oxidant production during exhaustive exercise [1,24,33,34,41]. Furthermore, increases in postexercise concentrations of hypoxanthine are accurate predictors of muscle energy exhaustion during exercise [54]. It is likely that the additional hypoxanthine was converted to xanthine and superoxide via xanthine oxidase. Although we did not measure superoxide formation in the cytosol, recent data show that superoxide is produced and released into the extracellular space via after isometric muscle [33].

In aged mice, hypoxanthine, which is upstream from xanthine oxidase, was increased in muscles of mice after electrically evoked contractions, when xanthine oxidase activity was inhibited by

allopurinol (Fig. 1). In contrast, xanthine, H_2O_2, and lipid peroxidation were all blunted by allopurinol in muscles of old mice after electrically evoked contractions. The increased hypoxanthine probably occurred as a result of reducing the conversion of hypoxanthine to xanthine, when xanthine oxidase was inhibited by allopurinol. Acute inhibition of xanthine oxidase by allopurinol reduced the indices of oxidative stress in muscles that were elevated by electrically evoked contractions but not in control muscles of aged mice.

To our knowledge, this is the first study to show that hypoxanthine concentration was greater in muscles of aged vs young adult mice; yet, the concentration of xanthine was similar in muscles of young and aged animals. It is possible that xanthine oxidase in muscles of aged mice was less efficient at converting hypoxanthine to xanthine and superoxide, and this resulted in the accumulation of hypoxanthine. However, additional investigations are needed to determine if this is the case. Another possibility is that xanthine oxidase was not limiting, but rather, xanthine was converted to uric acid at a rate similar to xanthine conversion, which yielded no net change in xanthine levels in muscles of aged animals. When xanthine oxidase activity was inhibited in muscles from aged control animals, there was no change in either hypoxanthine or xanthine concentrations.

The impact of allopurinol on endogenous antioxidant enzymes in aging in muscles after electrically evoked contractions

In general, the content of mRNA for antioxidant enzymes was not altered by allopurinol or aging. These data suggest that the observed differences in protein content and activity for antioxidant enzymes arise from posttranscriptional and/or posttranslational modifications [34,36]. In contrast with other antioxidant enzymes, catalase mRNA was greater in muscles of old vs young animals (Fig. 5C). The increased catalase mRNA, activity, and protein content may be an attempt to counterbalance the depletion of glutathione levels that has been observed within aging [13].

It is well known that strenuous muscle contraction promotes GSH oxidation and reduces the GSH/GSSG ratio [55]. Aging also decreases the GSH/GSSG ratio in skeletal muscle and this decrease is exacerbated by increased muscle activity [56]. Our data are consistent with these findings and show that aging increased oxidative stress and lowered the GSH/GSSG ratio in muscle. This presumably lowered the potential for the gastrocnemius muscle to tolerate the increased oxidative production that occurred in response to the repetitive electrically evoked contractions. In this study, allopurinol treatment prevented the decrease in the GSH/GSSG ratio in muscles from young mice after electrically evoked contractions, and it partially offset the decrease in the GSH/GSSG ratio that was observed in muscles from nonsupplemented aged mice after electrically evoked contractions.

The effects of electrically evoked contractions and xanthine oxidase inhibition on the endogenous antioxidant enzymes

Oxidant-sensitive transcription factors such as nuclear factor κB (NF-κB) have been shown to up-regulate antioxidant gene expression in response to chronic exercise [41,57]. However, the attenuation of oxidant production via inhibition of xanthine oxidase has been shown to prevent NF-κB activation and the subsequent up-regulation of MnSOD transcriptional activity after exhaustive aerobic treadmill running [41]. In contrast, we did not find evidence for transcriptional regulation of MnSOD, GPx, catalase, CuZnSOD, or MnSOD in skeletal muscle after 3 days of electrically evoked contractions. Nevertheless, the activities of the cytosolic-localized antioxidants CuZnSOD and catalase were greater in both young adult and aged gastrocnemius muscles in response to in situ electrically stimulated isometric contractions. This might be in part a result of the need to buffer cytosolic oxidants arising from anaerobic metabolic pathways, compared aerobic types of exhaustive exercise [41,57], which would be expected to have a greater need to buffer mitochondria-associated antioxidants arising from oxidative metabolism. In contrast to the lack of increase in antioxidant enzymes resulting from an acute series of muscle contractions over 3 days as in this study, it is likely that the antioxidant enzyme adaptations and especially mitochondrial antioxidant enzymes (e.g., MnSOD) would have increased in response to chronic loading types of exercise.

It has been postulated that low levels of oxidative stress can promote beneficial adaptive responses including an improved antioxidant defense capacity, because preventing oxidative stress associated with exercise prevents these positive adaptations [41]. For example, in this study, inhibition of xanthine oxidase-induced oxidative stress by allopurinol blunted the increase in the cytosolic protein content and activity of CuZnSOD in response to in situ electrically stimulated isometric contractions. Inhibition of xanthine oxidase by allopurinol in aged animals also attenuated the increase in catalase activity associated with electrically evoked contractions. Together, these data suggest that inhibition of xanthine oxidase reduces the need for an increase in antioxidant enzymes in response to electrically evoked contractions.

Allopurinol reduces apoptotic signaling in aged muscles

The current data are consistent with previous findings showing that aging is associated with increases in apoptotic signaling in skeletal muscle [39]. The abundance of mitochondrial proteins cytochrome *c,* AIF, and Bax was elevated in the cytosolic fraction of the muscle homogenates. Downstream from mitochondrial-released cytochrome *c,* the activity of both the initiator caspase-9 and the executioner caspase-3 was elevated in muscles of aged compared to young adult animals.

Aging has been associated with a depolarization of the mitochondrial membrane, decreased mitochondrial respiratory activity, and decreased antioxidant enzyme activity [13]. These decreases lead to a detrimental release and accumulation of oxidants within the cells [13]. Although most oxidants originate from the mitochondria in aging muscles [12,58], our data show that xanthine oxidase also contributes to oxidant production in aging and muscles after electrically evoked contractions.

Increased cellular stress can activate redox-sensitive pathways that initiate mitochondria apoptotic signaling [39,59,60]. Aged skeletal muscle has elevated basal oxidant production, and exhaustive aerobic exercise further increases oxidant production [12] and apoptotic signaling [61]. In this investigation, we show decreased mitochondrial Bcl-2 protein abundance and increased muscle levels of caspase-3 activity, without significant increases in caspase-9 activity or cytosolic cytochrome *c* in gastrocnemius muscle of aged mice after electrically evoked contractions compared to control muscles. These findings are consistent with other observations in gastrocnemius muscle in aged rats, in which exhaustive exercise increased caspase-3 activity without changes in caspase-9 activity [61]. One possibility is that the increased caspase-3 activity in the aged gastrocnemius muscle may be triggered via the extrinsic apoptotic pathway rather than through the mitochondria.

Allopurinol decreased mitochondrial Bax protein abundance and blunted the increase in caspase-3 activity in gastrocnemius muscle from aged animals after electrically evoked contractions. Although this suggests that xanthine oxidase activity has a role in regulating apoptotic signaling, this is probably not through mitochondria signaling pathways, because allopurinol had no effect on suppressing the elevated levels of cytochrome *c,* AIF, caspase-9, and caspase-3 associated with aging. Further research is needed to determine the upstream mechanisms that regulate the increase in caspase-3 activity after electrically evoked contractions to determine if allopurinol blunts extrinsic apoptotic signaling in response to aging and muscle contractions.

Allopurinol affects maximal isometric force in aged animals

Loss of muscle force with aging is typically greater than the loss in muscle mass per se. This occurs as a result of both a decrease in muscle mass and an altered myosin cross-bridge function, which is due at least in part to oxidative modifications of myosin and actin protein structures (reviewed in [62]). An important novel finding in this study is that allopurinol administration increased plantar flexor maximal isometric force by 35% ($P<0.05$), without having an effect on the force production in muscles from young adult animals. Our data in young adult animals differ from recent data from Gomez-Cabrera and colleagues [33], who reported a loss of in vitro maximal force production in extensor digitorum longus and soleus muscles from young (3 months of age) mice that were incubated with oxypurinol (the active metabolite of allopurinol). These differences may have been the result of different experimental approaches (in vitro vs in vivo), the compound used to suppress xanthine oxidase (allopurinol vs oxypurinol), and/or the muscle that was studied. Nevertheless, the data in the current study suggest that suppressing xanthine oxidase has the potential to improve maximal force production in aged muscles. Our in vivo data are not inconsistent with the idea that ROS generation during loading is important in various signaling pathways in muscle that lead to improved muscle force production [63]. However, excessive ROS production can also decrease force production (reviewed in [64]), and the higher basal ROS levels in aged muscles coupled with the contraction-induced increases in ROS may have contributed to force reduction in this study.

Recent data have shown that xanthine oxidase inhibits ryanodine receptor and/or dihydropyridine receptor function [65,66]. This results in dysregulation of calcium release from sarcoplasmic reticulum stores and lowers force production, whereas reducing or removing xanthine oxidase improves muscle function. Although speculative, the calcium leakage from the sarcoplasmic reticulum as a result of altered S-nitrosylation of the ryanodine receptor by xanthine oxidase [65] could also result in elevated intracellular calcium, which in turn could elevate xanthine oxidase levels and contribute to greater calcium dysregulation and loss of force. Our data are consistent with these data and support a model in which buffering of xanthine oxidase-induced oxidant stress in muscle of old animals may have lowered ROS levels to acceptable levels, so that force production was improved by the allopurinol treatment as a result of improved calcium handling.

Muscle fatigue during repetitive isometric contractions

In this study, we found that relative fatigue over each session of evoked contractions was less in muscles of aged compared to young adult animals. This observation is consistent with the literature that suggests that the rate of fatigue decreases with aging [67]. Several possibilities have been proposed to explain this finding, including recent observations that the metabolic cost of producing muscle contractions is decreased in aging [68]. Alternatively, the decreased fatigue may be due to the well-known shifting of fiber types toward a greater percentage of type I fibers [69]. Although allopurinol administration did increase maximal isometric force in the aged animals, it did not significantly influence the rate at which force declined in either age group. This is consistent with a recent study that found no improvement in fatigability of hindlimb muscles of young mice in response to an in vitro protocol of repeated electrical stimulation, in which xanthine oxidase was reduced by oxypurinol [33].

Conclusion

The data in this study indicate that xanthine oxidase-derived oxidant production has a wide range of effects on skeletal muscle physiology and function in aged mice. In this study, we sought to determine if xanthine oxidase played an important role in oxidant stress-induced regulation of aging after isometric contractions. We did not anticipate that the relatively short duration of xanthine oxidase inhibition used in this experimental protocol would be adequate to relieve the chronic basal elevations in oxidative stress that are associated with advanced aging. The novel findings in this study show that compared to control conditions, suppression of xanthine oxidase activity by allopurinol reduced xanthine oxidase activity, H_2O_2 levels, lipid peroxidation, and caspase-3 activity and reduced Bax accumulation in mitochondria of electrically stimulated muscles. In addition, allopurinol prevented the electrically evoked muscle contraction-associated loss of GSH, prevented the increase in catalase and CuZnSOD activities, and increased maximal isometric force in the plantar flexor muscles of aged mice after electrically evoked maximal repetitive isometric contractions.

The suppression of antioxidant enzymes by allopurinol in aged muscles after electrically evoked contractions might be viewed as a negative adaptation. However, another perspective is that antioxidant inhibition of xanthine oxidase activity reduced oxidative stress in aged muscles and removed the need for short-term adaptation of the endogenous antioxidant enzymes catalase and CuZnSOD to repetitive isometric contractions. Acute reduction of xanthine oxidase levels in aging muscles by allopurinol reduced caspase-3 activity and Bax accumulation in mitochondria, but it did not affect other indicators of mitochondria-associated apoptotic signaling. Additional studies are required to determine if long-term inhibition of xanthine oxidase will have an important role in reducing apoptotic signaling in extrinsic pathways. Finally, xanthine oxidase inhibition improved maximal isometric force in the plantar flexor muscles from the aged mice. From a clinical perspective, it is important to determine if allopurinol will provide an effective strategy for reducing oxidant stress and improving loss of muscle function with aging in exercising humans.

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