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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

Michael J. Ryan [a], Janna R. Jackson [a,b,c], Yanlei Hao [a,b], Stephen S. Leonard [d], Stephen E. Alway [a,b,c,*]

[a] Laboratory of Muscle Biology and Sarcopenia, Division of Exercise Physiology, West Virginia University School of Medicine, Morgantown, WV 26506, USA
[b] Center for Cardiovascular and Respiratory Sciences, West Virginia University School of Medicine, Morgantown, WV 26506, USA
[c] Department of Physiology and Pharmacology, West Virginia University School of Medicine, Morgantown, WV 26506, USA
[d] Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV 26506, USA

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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.

* Corresponding author. Fax: +1 304 293 7105.
*E-mail address:* salway@hsc.wvu.edu (S.E. Alway).

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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