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Aging-dependent effects of repetitive loading exercise and antioxidant supplementation on oxidative stress in skeletal muscle

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Aging-Dependent Effects of Repetitive Loading Exercise and Antioxidant Supplementation on Oxidative Stress in Skeletal Muscle

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Abstract

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Aging is associated with a reduced ability to buffer oxidants along with an increase in oxidant production, resulting in chronic oxidative stress. Oxidative stress is a putative factor responsible for reducing function ability of skeletal muscle and increasing oxidative damage. The objective of this set of investigations was to evaluate the efficacy of reducing oxidative stress on improving muscle function the adaptive response of skeletal muscle to repetitive loading exercise in aging rodents. To achieve this objective, three methods of reducing oxidative stress were utilized; the antioxidant vitamins E&C were used to buffer oxidants, the nutraceutical resveratrol was used to inhibit oxidant production and the pharmacological agent allopurinol was used to attenuate oxidant production specifically through the inhibition of xanthine oxidase activity. This set of investigations show evidence that muscles from aged animals have high basal levels of xanthine oxidase, and this is further exacerbated by resistance exercise. Antioxidant treatment in aged rodents will reduce oxidative stress associated with both aging and exercise. Furthermore, an increase in xanthine oxidase activity is a major contributor to the oxidative stress associated with resistance exercise (i.e. repetitive loading). Modulation of exercise-induced oxidative stress will effect adaptation of the endogenous antioxidant system and different therapeutic methods of reducing oxidative stress in aged muscle produce slightly different results in muscle function. The results suggest that resistance training increases xanthine oxidase activity, which contributes to exercise-induced oxidative stress in muscles of aged mice. Furthermore, resistance exercise invokes a distinctive response in the endogenous antioxidant enzymes that differ from that typically observed with aerobic exercise.

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List of Abbreviations

8-OHdG	8-hydroxy-2'-deoxyguanosine
AAALAC	American Association for Accreditation of Laboratory Animal Care
AAC	Aged, Allopurinol, Control non-exercised
AAE	Aged, Allopurinol, Exercised
ACC	Aged, Control (non-antioxidant treatment), Control non-exercise
ACE	Aged, Control (non-antioxidant treatment), Exercised
ADP	adenosine diphosphate
AIF	apoptosis inducing factor
Akt-mTOR-S6K	Akt/protein kinase B-mammalian target of rapamycin-p70 S6 kinase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	analyses of variance
AO	antioxidant
AP-1	activator protein 1
ARC	Aged-resveratrol-control non-exercised
ARE	Aged-resveratrol-exercised
ATC	Aged, Treatment (antioxidant), Control non-exercised
ATE	Aged, Treatment (antioxidant), Exercised
ATP	adenosine-5'-triphosphate
BHT	butylated hydroxytoluene
BW	body weight
C57BL/6	C57 black 6 mice
cDNA	complimentary DNA
CuZnSOD	copper-zinc superoxide dismutase
DMC	Dynamic Muscle Control
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EC-SOD	extra-cellular superoxide dismutase
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ESR	electron spin resonance
FADD	Fas-associated death domain protein
FoxO1	forkhead transcription factor-1
GPx	Glutathione Peroxidase
GSH	reduced glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
HAE	4-hydroxyalkenals
IGF-1	insulin-like growth factor-1
IL-6	interleukin-6
LDL	low density lipoprotein
LSD	least significant difference
MANOVA	multiple analyses of variance
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MnSOD	manganese superoxide dismutase
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate

NF-κB	nuclear factor kappaB
NFM	non-fat milk protein
NF-Y	nuclear factor-Y
NIA	National Institute on Aging
NO	nitric oxide
NOS	nitric oxide synthase
NS	non-supplemented
OD	optical density
PBS	phosphate-buffered saline
RFU	relative fluorescent units
RL	repetitive loading (exercise)
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT-PCR	reverse transcription-polymerase chain reaction
PAGE	polyacrylamide gel electrophoresis
PGC-1α	PPARγ co-activator
PPAR	peroxisome proliferator-activated receptor
SEM	standard error of the mean
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
Sirt1	silent mating type information regulation 2 homolog
SOD	superoxide dismutase
TA	tibialis anterior muscle
TBS-T	tris-buffered saline with 0.05% Tween-20
tGSH	total glutathione
TNF-α	tumor necrosis factor alpha
RDA	recommended daily allowances
USDA	United States Department of Agriculture
VO_{2max}	maximum oxygen uptake
YAC	Young, Allopurinol, Control non-exercise
YAE	Young, Allopurinol, Exercised
YCC	Young, Control (non-antioxidant treatment), Control non-exercise
YCE	Young, Control surgery, Exercised
YRC	Young-resveratrol-control non-exercise
YRE	Young-resveratrol-exercised

Chapter 1

Introduction & Specific Aims

Michael J. Ryan

1. Introduction

Aging is a natural process that is regulated by both genetic and environmental factors. It is associated with the degenerative modifications of all cell types. Although, the rate of cellular degeneration associated with aging differs among tissues and species, it is a process that is evolutionarily conserved. One of the major sites of age-related degeneration is in skeletal muscle. Loss of skeletal muscle mass and strength during senescence is termed sarcopenia. Sarcopenia has detrimental consequences in the elderly, as it may limit their ability to perform the necessary daily physical activities needed to maintain their independence. Simple daily tasks, such as rising from a seated position, become increasingly more difficult as muscle function declines. Additionally, sarcopenia is strongly associated with increased morbidity and mortality (4) costing families and taxpayers in excess of $18.5 billion per year (2). Given that the elderly is the fastest growing proportion of our population, these cost are expected to exponentially increase. Reducing the prevalence of sarcopenia by a mere 10% could result in savings of over $1.1 billion per year to the U.S. healthcare system. Understanding the mechanisms involved in sarcopenia is a critical step in helping clinicians develop safe and effective therapies designed to delay the onset of sarcopenia. Such therapies have the potential to assist the elderly in maintaining their independence and improve their quality of life along with reducing the finical burden to the American taxpayer.

There are many factors that contribute to sarcopenia; reduced physical activity, altered hormonal status, denervation of muscle fibers, inflammation and damage caused by a lifetime of exposure to reactive oxygen species (ROS) (1; 3), all of which are associated with oxidative stress. Exercise training cannot prevent sarcopenia, but may reduce the prevalence of sarcopenia. Oxidants generated during exercise may play key roles (both positive and negatively) in the muscles' adaptive responses to chronic exercise. Advanced age is associated with an increase in oxidant production and a decreased capacity to buffer oxidants, resulting in a chronic state of oxidative stress. Oxidative stress damages DNA, lipids, proteins, and leads to elevated apoptotic signaling, thereby limiting the ability of muscle from aged animals to adapt appropriately to increased stresses, including exercise. Xanthine oxidase is one such source of oxidative stress in exercised muscles, but this oxidant activator has not been extensively studied in aged animals and/or humans. It is possible that buffering oxidant production may improve the muscle's ability to adapt to exercise, thereby reducing the affects of sarcopenia.

The overall goals of this project are to: (a) characterize the endogenous antioxidant pathways in aged skeletal muscle and their adaptive response to repetitive loading (repetitive stimulation of the muscle while movement is restricted), (b) to determine the effectiveness of dietary antioxidant supplementation to enhance the adaptive response to repetitive loading in aged skeletal muscle and (c) to determine the contribution of xanthine oxidase in the production of oxidants during repetitive isometric contractions in young adult and aged skeletal muscle.

1.1 Central Hypothesis

The central hypothesis of this project is that skeletal muscles from aged animals will show increased evidence of oxidative stress following acute bouts of resistance exercise (repetitive loading) that sequentially influence adaptation to chronic exercise. However, increased basal levels of oxidative stress associated with aging will negatively influence the normal adaptive response the exercise that has been established in young adult animals. Antioxidant supplementation will increase the aged muscles' oxidative buffering capacity, thus attenuating the increase in oxidative stress associated with aging and improving adaptation to resistance exercise. Increased xanthine oxidase activity will be a contributing factor to the increase in oxidative stress in response to resistance exercise (i.e. repetitive loading) and aging.

1.2 Specific Aim 1: To determine if aging negatively regulates the endogenous anti-oxidant pathways during adaptation to repetitive loading and if antioxidant supplementation will improve these adaptive responses.

The dorsiflexors muscles of the left limb in young and aged rats will be loaded 3 times a week for 4.5 wks using 80 maximal stretch-shortening contractions per session while the contralateral (right) limb will serve as the intra-animal control. The animals will receive a diet consisting of a vitamin complex (vitamin E & vitamin C) or a control diet. Markers of oxidative stress will be measured in tibialis anterior muscles.

Hypothesis 1: Dietary supplementation with Vitamin E&C would attenuate the increase in basal levels of oxidative stress associated with aging allowing for a more complete adaptation in oxidative enzymes and improvements in muscle function after 4.5 weeks repetitive loading in the aged rats.

Sub-hypothesis 1.1: The tibialis anterior muscles from aged rats will experience greater levels of oxidative stress than the tibialis anterior from young adult rats.

Sub-hypothesis 1.2: Chronic repetitive loading will increase the oxidative buffering capacity and decrease the oxidant production of the tibialis anterior muscle, thus reducing the oxidative stress associated with aging.

Sub-hypothesis 1.3: Aging will attenuate the adaptive responses in the endogenous antioxidant pathways and this will be closely associated with attenuated functional adaptations to repetitive loading in skeletal muscle.

Sub-hypothesis 1.4: Dietary supplementation with a vitamin complex will lessen the oxidant activity in tibialis anterior muscles subjected to repetitive loading.

Sub-hypothesis 1.5: In muscles from aged rats, a combination of the antioxidant supplementation and repetitive loading exercise will reduce oxidative stress to a greater extent than either treatment independently.

Sub-hypothesis 1.6: In repetitively loaded muscles from both young adults and aged rats, antioxidant supplementation will improve muscle function as represented by increases in force production.

1.3 Specific Aim 2: To determine the efficacy of resveratrol supplementation as a possible countermeasure for the oxidative stress associated with aging exercise in skeletal muscle.

The plantar flexors of the left limb from young adult and aged mice will be activated by 20, five-second isometric contractions (10v, 100 Hz, 200 μs pulses) for 3 consecutive days. This protocol was selected to induce oxidative stress without causing damage to the muscle. The contralateral limb will serve as the intra-animal control. Mice will be randomly assigned to either a control (non-supplemented) diet or a diet supplemented with 0.05% *trans*-resveratrol.

Hypothesis 2: Fortification of normal mice chow with resveratrol will reduce the indices of isometric exercise-induced oxidative stress and attenuate the loss of force during acute repetitive isometric contractions from muscles of aged mice.

Sub-hypothesis 2.1: Resveratrol supplementation will lower indices of oxidative stress associated with aging and acute exercise in muscle from young adults and aged mice.

Sub-hypothesis 2.2: Resveratrol supplementation will improve muscle function and attenuate the loss of force during acute repetitive isometric contractions from muscles of both young adults and aged mice.

1.4 Specific Aim 3: To determine: (a) the contribution of xanthine oxidase as a source of oxidant production during repetitive isometric contractions in young adult and aged skeletal muscle, and (b) the effect of xanthine oxidase inhibition on the decreased functional capacity and increased apoptotic signaling associated with repetitively loaded skeletal muscle from aged animals.

Electrically-stimulated isometric contractions (10v, 100 Hz, 200 μs pulses) of the plantar flexors of the left limb will be conducted for 3 consecutive days in *y*oung and aged mice. The animals will receive a time released xanthine oxidase inhibitor tablet (Allopurinol) prior to repetitive loading and its' effects on markers of oxidative stress and apoptosis will be measured.

Hypothesis 3: The inhibition of xanthine oxidase will eliminate the majority of isometric exercise-induced oxidative stress thus preserving the muscles functional capacity while reducing mitochondrial apoptotic signaling in aged animals after exercise.

Sub-hypothesis 3.1: Repetitive isometric contractions will increase xanthine oxidase activity resulting in an increase in indices of oxidative stress. Allopurinol administration will abolish xanthine oxidase activity thus decreasing indices of oxidative stress.

Sub-hypothesis 3.2: Inhibition of xanthine oxidase (via allopurinol) will improve the redox environment within muscle attenuating the decrease in the functional capacity in aged animals after three days of isometric exercise.

Sub-hypothesis 3.3: Xanthine oxidase activity is elevated in aged muscles which in part, lead to increased apoptotic signaling; moreover reducing xanthine oxidase will decrease apoptotic signaling in aged muscle after exercise allowing for increased adaptation and improved force production.

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Chapter 2

Background and Significance

Michael J. Ryan

2. Background and Significance

2.1 Theory of oxidative stress and aging

Aging is a naturally occurring process that causes deleterious modifications at a genetic, cellular, tissue, and system level in all organisms. Presently, the fundamental mechanisms of aging are poorly understood, but a growing body of evidence supports an increase in oxidative stress as one of the principal components. In 1956, Harman suggested that aging occurs because of the accumulation of irreversible damage from free radicals to biologically important macromolecules (61). Advanced aging is associated with increases in lipid peroxidation, protein carbonyl formation and DNA damage. These forms of oxidative damage lead to altered receptor function, ion transport systems, enzyme activation/deactivation, and altered gene expression. Over the past two decades it has been shown that the accumulation of oxidative damage is dependent on more than just free radicals, but also all oxidizing agents or oxidants.

2.2 Oxidative stress in aging muscle

Oxidative stress is defined as a state in which the cellular production of oxidants exceeds the cells' physiological buffering capacity. Concomitantly with oxidative stress, advanced aging is associated with a loss of muscle mass and strength, known as sarcopenia. Reductions in muscle strength with aging are directly correlated with decreases in muscle mass (3, 39, 96). Muscle strength has been reported to decline an average of 20-40% in healthy men and women during the seventh and eighth decades of life (45). Muscle fiber size, determined by cross sectional area, is also reduced an average of 40% between the ages of 20 and 60 (45). Sarcopenia is a contributing factor to the loss of independence and frailty often observed in older adults (157).

Oxidative stress is associated with sarcopenia, however whether oxidative stress is a cause or a result of sarcopenia is unknown. Aging-induced oxidative stress can be an important mechanism hindering muscular adaptation to loading (18, 24, 79, 80). In aged animals, the ability to buffer increased oxidant production is reduced in comparison to muscle from young animals (53). Within skeletal muscle, oxidative stress has been shown to depress muscle specific force (30), alter myofilament function (5, 87, 88),and /or alter contraction-induced calcium release (7, 43, 48, 127). Chronic oxidative stress may also reduce muscle force by increasing recovery time associated with injury (86, 163). Furthermore, increases in oxidant production regulate redox-sensitive signaling pathways (76, 78, 92) which can lead to the up-regulation of catabolic gene expression (41, 101, 104, 159) and activate apoptotic pathways (84, 102, 137) in muscle.

Oxidative stress associated with aging has been reported in most tissue types, (106, 146, 164) including skeletal muscle (52, 55, 128, 145). Bejma and Ji (18) demonstrated that skeletal muscle from aged rats had a dramatic increase (~80%) in oxidant production. Elevations in oxidants are associated with cellular damage and ultimately apoptosis in various cell types, (44, 145) including skeletal muscle cells (53, 85). Recent data from muscle biopsies obtained from young and aged men showed that 8-hydroxy-2'-deoxyguanosine (8-OHdG), protein carbonyls, the manganese isoform of superoxide dismutase (MnSOD) and catalase activity are significantly higher in muscles taken from elderly men (55); similar results have been seen in rodents (128). The contractile proteins, actin and myosin, do not show evidence of increased oxidative damage (148), leading to speculation that increased oxidant production in muscle may have more important roles in regulating genes (i.e. transcription/translation) and metabolic mechanisms (i.e. electron transport chain). Muscle from aged animals has been shown to have a decreased quantity of full-length mitochondrial DNA and increased

mitochondrial deletions compared to young animals (55). Additionally, aging has been associated with a decrease in mitochondrial membrane integrity due to the depolarization of the mitochondrial membrane and decreased mitochondrial respiratory activity (112). A decrease in mitochondrial membrane integrity combined with a reduction in antioxidant enzyme activity leads to an unfavorable release and accumulation of oxidants within the cells (112). This is supported by evidence that shows that the majority of oxidants produced in skeletal muscle from aged animals originates within the mitochondria (18).

There is conflicting data on whether xanthine oxidase contributes to the increased oxidant production associated with advanced age. Both the heart (133) and gastrocnemius muscles (65) from aged rodents exhibit increased xanthine oxidase activity when compared to young, however this has not been observed by all (47). The contribution of increased xanthine oxidase activity leading to oxidative stress and apoptotic signaling associated with aging is insufficiently characterized.

2.3 Effects of aging on the endogenous antioxidant defenses

It has been widely recognized that glutathione peroxidase (GPx), catalase, Copper-Zinc containing isoform of superoxide dismutase (CuZnSOD) and MnSOD enzymes make up part of the endogenous antioxidant defense system that is essential for aerobic organisms to survive. These enzymes form the front line in the defense against oxidant attack and work in unison with several other non-enzymatic molecules (i.e. cellular vitamin E&C concentrations) to avoid oxidative damage. Superoxide dismutase catalyzes the reduction of superoxide anion into hydrogen peroxide (H_2O_2), which is subsequently detoxified by catalase and GPx. Three different isoforms of superoxide dismutase have been identified; they include the previously mentioned CuZnSOD and MnSOD along with extra-cellular superoxide dismutase (EC-SOD). CuZnSOD contains Cu and Zn atoms at its catalytic site and is primarily located in cytoplasm of cells, though small amounts of the enzyme have been found in the mitochondrial inter-membrane space (120). Conversely, MnSOD is localized to the inner mitochondrial matrix and contains a manganese atom in its catalytically active center. Like CuZnSOD, EC-SOD contains Cu and Zn atoms at its catalytic site. EC-SOD is primarily located on the extracellular side of cell surface membranes and throughout the extracellular matrix as well as, although to a lesser extent, circulating within the blood plasma (49). It has been reported that within mouse skeletal muscle, the concentration of CuZnSOD is 27 times greater than that of MnSOD and 166 times greater than EC-SOD (105).

Advanced aging is associated with a reduced capability of the endogenous antioxidant defenses to convert oxidants into more inert species (74, 121). It appears that the age-dependent increase in oxidant production overwhelms the endogenous antioxidant defense system resulting in oxidative stress. As a result there is a general elevation in the activity of the antioxidant enzymes within aged skeletal muscle (77, 121) which is regulated primarily at the post-translational level (67).

The most identifiable age- associated change in the endogenous antioxidant defenses is an increase in activity of MnSOD (55, 67, 74, 95, 98, 121). Theoretically this aging induced adaptation in MnSOD would be a result of the increased oxidant production from within the mitochondria from aged muscle. By and large there is thought to be an increase in GPx activity in aged muscle (67, 74, 98), however not all have reported age-dependent changes in GPx activity (67, 68, 121). Similar to GPx activity, catalase activity has generally been reported to increase within muscle from aged animals (55, 67, 77, 95, 98). However, this response may slightly differ depending on the muscle being investigated (67, 98).

An age-associated reduction in CuZnSOD efficiency appears to be a good candidate for explaining increased superoxide levels leading to long-term oxidative damage and eventual loss of skeletal muscle. CuZnSOD deficient mice demonstrate a 30% reduction in lifespan and decreased muscle mass in predominantly type II muscle (gastrocnemius and plantaris) but no changes were observed in the soleus which is composed of primarily type I fibers (117). Recent data (160) also suggest that CuZnSOD levels are lower in muscle tissue of aged pigs. Pansarasa et al. (121) have found that humans 66-75 years of age have significantly lower total SOD activity, but greater MnSOD activity than younger humans, suggesting that there is a decrease in CuZnSOD activity. Regrettably, there is not an agreement within the scientific literature that there is a decrease in CuZnSOD activity with age. It has been observed that long lived animals (i.e. humans and naked mole rats) do not show this decrease in CuZnSOD and other oxidative enzymes (34, 55). In Fisher 344 rats, it has been shown that CuZnSOD activity increases during ageing and this increase may be due to both translational and post-translational control (67). CuZnSOD levels increase in cells of young animals after exercise (66), and although Vasilaki et al. (150) report an increased level of CuZnSOD activity in muscles of old animals after a single bout of electrically activated muscle contractions, it is possible that old muscles may fail to respond in this manner when undergoing chronic repetitive loading. Furthermore, even if CuZnSOD does increase either similarly or greater in old vs. young muscles with repetitive loading, it is unknown if this will be sufficient to counteract the loading-induced and intrinsic (age-associated) increase in oxidant production in (116) aged muscles.

2.4 Oxidative stress in muscle wasting

A consensus of the available scientific data supports the conclusion that even healthy aging is associated with an escalation in oxidative damage and increased oxidant production. This could be the primary reason for augmented myonuclear apoptosis in aged skeletal muscles (91, 99, 125, 126, 136). However, the exact process of how increased oxidant production may influence and /or regulate apoptotic signaling is unknown. Both oxidative stress and increased myonuclear apoptosis have been implicated in the pathogenesis of sarcopenia. Muscle wasting, leading to atrophy, is accompanied by a reduction in protein synthesis, and/or an activation of proteolysis. These events may be initiated and/or mediated by oxidative stress (114). Furthermore, oxidative stress may also contribute to the activation of myonuclear apoptotic cascades in skeletal muscle (136, 137). It has been suggested that a number of small, random, deleterious effects of increased oxidants could explain the degenerative process in skeletal muscle resulting in sarcopenia. Muscle atrophy has been shown to be associated with many different pathways of oxidant production; generation of oxidants by xanthine oxidase (90), production of nitric oxide (NO) via nitric oxide synthase (NOS)(72), formation of oxidants by increased cellular levels of reactive iron(65, 89, 161), increases in NADPH oxidase (18), and increased oxidant production via the mitochondrial (18, 26, 33, 99). Additional evidence implicating that oxidative stress is linked with muscle atrophy is the observed increase in lipid peroxidation, glutathione oxidation and protein carbonyl formation from atrophic muscle (56, 87, 88, 90, 97). Furthermore, increased oxidant generation has been implicated as a reason for muscle dysfunction associated with sarcopenia (117). Fulle et al. (53) have suggested the following mechanisms for how reactive oxygen species may contribute to muscle atrophy: (a) biochemical alterations of cell structures (i.e. peroxidation of cell membranes that increase oxidant production during physical activity), (b) the increased fragility of muscle that accompanies mechanical injury and subsequent inflammation, (c) reduced satellite cell/muscle stem cell proliferation in conjunction with reduced protein synthesis thus limiting the antioxidant defense and repair capacity, and (d) reduced dietary antioxidant intake. However, there are no data that address the role of oxidative stress produced by repetitive loading exercise in aged

muscle. The underlying cause for increased oxidant formation associated with exercise in aged muscle is unknown; this information is essential to develop useful strategies for improving muscle adaptations to loading in elderly individuals.

2.5 Mechanisms of oxidant production during exercise

It is widely accepted that exercise increases oxidant production even though there are only a few studies that supply direct *in vivo* evidence of this. Measuring free radicals within biological systems is problematic because they are highly reactive and maintain low steady-state concentrations. Electron paramagnetic resonance spectroscopy, also referred to as electron spin resonance (ESR) spectroscopy, coupled with the use of spin traps, is perhaps the most specific and direct method of measuring free radical species (12). Electron paramagnetic resonance spectroscopy has been employed to show that the concentration of free radical species increase after exercise in both rat muscle (38) and human blood (10). Further studies using 2′,7′-dichlorodihydrofluorescein as an intracellular probe for H_2O_2 show strong evidence that after exercise oxidant production is heightened (18, 72, 108). Oxidants in muscle are primarily derived in the form of two molecules; superoxide and nitric oxide. Superoxide anions will rapidly dismutate to H_2O_2 (and water) but can form hydroxyl radicals and other small-molecular-weight oxidants or reactive oxygen species. In addition, NO originating within exercising muscle may act as a precursor for the formation of peroxynitrite, peroxynitrous acid, and other nitrogen-derived oxidizing molecules or reactive nitrogen species (72).

Skeletal muscle generates reactive oxygen species and reactive nitrogen species at low basal levels under resting conditions but during exercise the metabolic demand to sustain muscle contractions increase, resulting in an increase in oxidant production. It is generally accepted that there are three major sources of oxidant production with exercise; neutrophils and other infiltrating immune cells, mitochondrial respiration and xanthine oxidase activity (31, 72, 100, 131). Oxidant production from each source is dependent on the mode, duration, and intensity of exercise. During strenuous exercise there will be two phases of increased oxidant production. The first increase in intramuscular oxidant generation will occur during and immediately following a bout of exercise. This will be characterized by increases in oxidants within the working muscle and systemic oxidative stress is found in other tissues (144, 151). The second increase in oxidant generation is delayed and tends to be more localized to the working muscle, specifically damaged fibers. The delayed response is a product of phagocytic cell invasion (103, 164) resulting from injury and is not associated with an increase in the metabolic demand during exercise.

2.5.1 Oxidant production by neutrophils and other phagocytes

Strenuous exercise involving eccentric muscle contractions has been shown to result in substantial injury potentiating the release of cytokines. Cytokines can activate neutrophils and other phagocytes promoting their release into the circulation. The activated neutrophils produce oxidants to aid in the removal of damaged tissue and assist in the repair process after exercise. Neutrophil-derived oxidant production is directed at damaged tissue and can overwhelm the muscles endogenous antioxidant defense mechanisms (124). This process appears to be essential for removal of damaged tissue and muscle fiber regeneration. It is theorized that phagocytic oxidant production could damage neighboring healthy fibers, but there is no direct evidence supporting this idea. Neutrophil activation during exercise does not appear to increase lipid markers of oxidative stress in the circulation to any significant degree (124). Furthermore, stereological analysis of muscle 72 hours after repetitive loading showed there was a large increase in phagocytic cells within the interstitial space surrounding damaged myofibers, but the phagocytic infiltrates were not associated with healthy non-degenerative fibers (14). Phagocytic

cells cause an increase in intramuscular oxidants hours after a bout of exercise, but evidence of oxidative stress in non-injured myofibers may be overestimated when assessed by means of muscle homogenate. Antioxidant supplementation has been suggested as a possible countermeasure for the increased production of oxidants via phagocytic cells. When neutrophils are stimulated *in vitro,* antioxidant supplements such as N-acetylcysteine have been shown to be effective at buffering the increase in oxidants (124) , whereas vitamin E supplementation reduced the infiltration of neutrophils into muscle cells (124).

Although there is a lack of data showing evidence that oxidants produced via inflammatory infiltrates cause oxidative damage to neighboring healthy cells, a more oxidizing redox environment may alter muscle function (5, 30). Myofibrillar Ca^{2+} sensitivity appears to be especially susceptible to changes in the redox environment (5). The use of antioxidant supplements may prevent, or at least minimize, the formation of an unfavorable redox environment generated from neutrophils and other phagocytes.

2.5.2 Oxidant production by mitochondria during exercise

It is widely assumed that mitochondria are the major source of oxidant production during exercise (42, 53). Mitochondria have been shown to produce superoxide, hydrogen peroxide, and possibly hydroxyl radicals. There is considerable data showing a correlation between increased oxidative metabolism and increased oxidant production and oxidative damage (15). It has been accepted for over thirty years that superoxide generation in the mitochondria is produced via a membrane-bound multi-enzyme redox system (27) referred to as the mitochondrial electron transport chain. Surprisingly, there is little direct evidence from intact cells that the production of oxidants via the mitochondrial electron transport chain causes oxidative damage (79). Although the mitochondrial electron transport chain is very efficient, its own make up is based on controlling a series of alternating one-electron oxidation-reduction reactions that can predispose each electron carrier to side reactions with molecular oxygen. As an example, ubiquinone, which excepts electrons from complex I and II of the mitochondrial electron transport chain, cycles between the quinone (fully oxidized form) to semiquinone (one-electron reduction product) to quinol (fully reduced by two electrons), there is a tendency for an electron to pass or “leak” to oxygen generating superoxide. Nevertheless, acute bouts of increased oxidant production within the mitochondria (such as during exercise) are unlikely to cause oxidative damage because of high levels of superoxide dismutase and GPx.

Early work on isolated mitochondria estimated that 2-5 % of the total O_2 consumed was converted to oxidants via the leakage of electrons from the mitochondrial electron transport chain (22). Leakage of electrons is most common at complex I and complex III of the electron transport chain (22, 64, 115, 131). Muller et al. (115), published data that indicates that complex III can release superoxide to both sides of the inner mitochondrial membrane, providing a potential source for cytosolic superoxide generation. Additionally, complex I-dependent superoxide formation is exclusively released into the matrix and no detectable levels of superoxide escape from intact mitochondria (115).

The early estimations of oxidant production from the mitochondrial electron transport chain were obtained using isolated mitochondria in state IV respiration (22). Furthermore, oxidant production during exercise was predicted by multiplying state IV respiration by the increase in VO_2 during exercise (which in humans is ~20 times higher during exercise than during rest). State IV (resting) respiration is defined as oxygen consumption within isolated mitochondria in the presence of substrate without any ADP or inhibitors. State III (active) respiration is achieved by the addition of large amounts of ADP. Experiments using isolated

mitochondria have shown oxidant production is dramatically reduced by the addition of ADP (a State IV to State III conversation) (63).

During exercise, muscle contractions produce an increased demand for ATP which is initially met by the ATP-PC system, glycolysis and glycogenolysis. Electron transport is stimulated as the concentration of ADP from anaerobic metabolism increases. ADP and an inorganic phosphate bind to ATP synthase, thus permitting protons to travel down their chemiosmotic gradient into the mitochondrial matrix. The energy released as protons pass through the channel into the inner mitochondrial matrix is utilized to bind the inorganic phosphate to ADP producing ATP; this process is known as oxidative phosphorylation. The additional ADP formed during exercise is transported into the matrix which increases the rate that ADP and inorganic phosphate bind to ATP synthase, thus leading to more protons traveling through the channel into the inner mitochondrial matrix. As energy from the chemiosmotic gradient is consumed, the mitochondrial electron transport chain accelerates, pumping protons back out of the inner mitochondrial membrane in an attempt to maintain the gradient.

As more high-energy electrons pass through the mitochondrial electron transport chain there is a greater demand for molecular oxygen which acts as the final electron accepter. In this way oxygen consumption is coupled to ADP phosphorylation by ATP synthase through the protons chemiosmotic gradient.

The respiratory states of isolated mitochondria have been studied to gain a better knowledge of how mitochondria work, but one must remember that by definition *in vivo* there is no such thing as state IV respiration (15, 100). Even during rest when the energy demand is low, mitochondria are constantly carrying out oxidative phosphorylation at rates that are proportional to the availability of ADP. Since ADP is always present, *in vivo* mitochondria are somewhere in between state III & IV (79, 100). It has been suggested that oxidant production from the mitochondria during exercise (when metabolic activity is high and ADP abundant) has been over estimated (100).

In 2002, J. St-Pierre et al. (140) re-examined the rate at which mitochondria produced oxidants. This data indicated that the maximum estimation of electrons flowing through the mitochondrial electron transport chain resulting in oxidant production was ~ 0.15%, or less than 10% of the original minimum estimate. Additional evidence indicating that mitochondrial production of oxidants might be overestimated has recently been found using 2′,7′-dichlorodihydrofluorescein to probe for oxidant production in contracting C2C12 myotubes(108). 2′,7′-dichlorodihydrofluorescein is a specific method for measuring H_2O_2 in cells. Results showed the 2′,7′-dichlorodihydrofluorescein was distributed evenly throughout the cells with no evidence of accumulation at any specific intracellular site, or localization to mitochondria. In a similar set of experiments myotubes differentiated from isolated skeletal muscle satellite cells from wild-type, heterozygous MnSOD knockout mice (Sod2(+/-)), and MnSOD over-expressers (Sod2-Tg) to show oxidant production in non-contracting myotubes increased in the Sod2(+/-), whereas in the Sod2-Tg oxidant production decreased (149). These results suggest that in quiescent myotubes mitochondrial production of oxidants is largely influenced by the amount of antioxidant enzymes present in the system. In contrast, when the myotubes were electrically stimulated, oxidant production was unaltered by reducing or increasing MnSOD, signifying that oxidants in contracting myotubes are primarily generated by methods outside of the mitochondria (149).

In the previously mentioned studies, simply because 2′,7′-dichlorodihydrofluorescein activity was not localized to the mitochondria does not mean that oxidant production is not occur there. Mitochondria will produce superoxide that is quickly converted into H_2O_2 which has the capability to pass through lipid membranes. Therefore, it is impossible to rule out that the increase in 2′,7′-dichlorodihydrofluorescein activity was caused by increased superoxide production within the mitochondria, which was converted to H_2O_2, then diffused throughout the cell. Although this explanation is possible, the previously mentioned studies do not show data supporting increased mitochondrial oxidant production during exercise. Furthermore, it seems unlikely that H_2O_2 would diffuse so evenly throughout the cell. A more likely scenario is that the fluorescent intensity would be greatest at the source of H_2O_2 production and evenly dissipate outward from that point. Furthermore, if H_2O_2 was produced in the mitochondria during exercise and evenly diffused throughout the cell at a concentration above control levels, then it would be expected that mitochondria isolated from exercised muscle would show evidence of higher H_2O_2 concentrations. However, Bejma and Ji (18) failed to find any change in 2′,7′-dichlorodihydrofluorescein oxidation in isolated mitochondria from post-exercised muscle when compared to pre-exercised mitochondria, although post- exercised muscle homogenate demonstrated a 38% (young) to 50% (aged) increase in 2′,7′-dichlorodihydrofluorescein oxidation post-exercise.

Bejma and Ji's (18) data support the likelihood that sources outside the mitochondria are, at least in part, responsible for oxidant production during exercise; nevertheless they suggest that mitochondria are responsible for the majority of the increased oxidant production associated with aging. In all likelihood, if mitochondrial oxidant production during exercise is not substantial enough to overwhelm the antioxidant defenses, thus failing to cause oxidative damage, then at least slight increases in mitochondrial derived oxidants may be involved in the adaptive response to exercise. Furthermore, this is not an attempt to minimize the important role that mitochondria play in the increased oxidant production associated with aging and disease states. However, the lack of evidence showing mitochondria are the primary source of oxidant production associated with exhaustive exercise is sufficient to warrant the investigation of alternative sources of oxidant production.

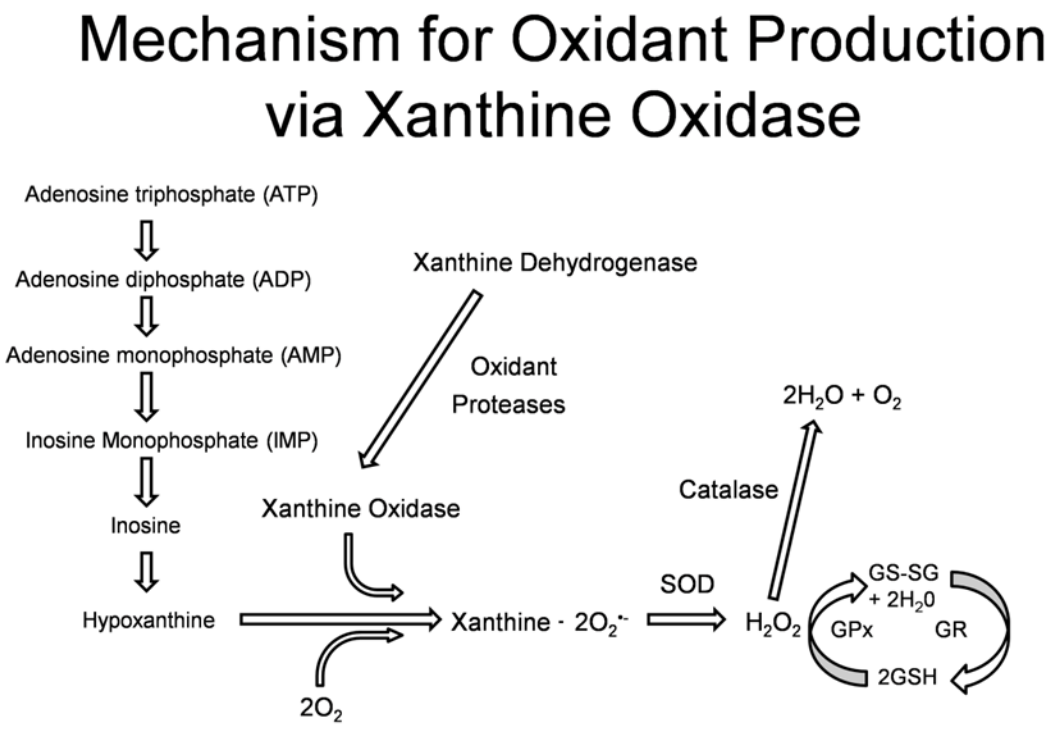
2.5.3 Xanthine oxidase activity in exercising muscle

Increased activity of the xanthine oxidase enzyme within the vascular endothelium (69) is an important source of extracellular oxidant production in the vascular endothelium (57, 69) and has been shown to be a contributing factor associated with oxidative stress during exercise (9, 46, 57, 62, 131, 152). Vina et al. (152, 153) have shown that exhaustive exercise leads to an increase in blood xanthine oxidase activity in rats and humans. Furthermore, the inhibition of xanthine oxidase with allopurinol administration prevented exercise-induced glutathione oxidation (GSH/GSSG ratio) and lower indices of lipid peroxidation (MDA) after exercise. It has been hypothesized that the activation of xanthine oxidase enzyme during exhaustive exercise is similar to the process observe during ischemia–reperfusion injury (110, 123, 152). Under normal physiological conditions, xanthine dehydrogenase is the principal form of the enzyme, which oxidizes both hypoxanthine and xanthine to form uric acid via the reduction of NAD^+ to NADH. However, during repetitive muscle contractions, the increased ATP utilization and a brief localized period of ischemia will facilitate adenine nucleotide degradation, thus breaking down of ATP to AMP and eventually hypoxanthine. Simultaneously to the increase in hypoxanthine xanthine dehydrogenase will be converted to xanthine oxidase either reversibly by oxidation, or irreversibly via proteolysis (32, 119). Conversion of xanthine dehydrogenase to xanthine oxidase has been shown to be dependent on both calcium and oxidant concentrations (111). During muscle contractions, intracellular calcium concentrations are elevated, which in turn may

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 that forms disulfide bonds resulting in the reversible conversion to xanthine oxidase.

During muscle relaxation the influx of oxygen rich blood would allow xanthine oxidase to catalyze the reaction of hypoxanthine and oxygen to form xanthine and superoxide. Within the muscle homogenate, at least part of the increased H_2O_2 concentration associated with exercise is expected to be the result of an increased accumulation of superoxide formed by xanthine oxidase activity, which has been dismutated into H_2O_2 via a reaction catalyzed by superoxide dismutase. See Figure 2.1.

Figure 2.1



2.6 Loading in aging muscle

Resistance training involves progressively increasing the load or resistance applied to a contracting muscle or groups of muscles. This form of exercise can provide several healthy benefits in older adults that include; increases in muscular strength, improved range of motion, decreased likelihood of falling, improved body composition and increases in bone density. Presently, resistance training is the most effective and safe intervention to attenuate or recover some of the loss of muscle mass and strength that is associated with aging (81). Within skeletal muscle the normal adaptive response to progressive overload results in muscle satellite cell activation, an increase in gene transcription and synthesis of muscle-specific proteins resulting in muscle hypertrophy (4). Chronic adaptation to resistance exercise over many weeks in older women and men has been shown to improve muscular strength and also induce muscle fiber hypertrophy (29, 51, 59, 93, 130, 135); however, these adaptations are generally smaller than that reported in young adults. The mechanisms that regulate this attenuated adaptation to resistance exercise in the aged (40) is not known, but one possibility is due, at least in part, to

elevated oxidative stress. Previous studies have observed that after resistance training type II fibers are preferentially hypertrophied in both young and aged adults (29, 59, 93, 130, 135). Type II fibers tend to be more susceptible to oxidative damage than type I fibers (117), therefore the increased oxidant production associated with aging could hinder the type II fibers' ability to adapt to training.

Aged rodents are frequently used as models for exercise training in humans, but there are few studies that have examined the effects of resistance training in aged rodents. One reason is the lack of sufficient and accepted training models. Although some rodent species will perform endurance exercise voluntarily, resistance exercise is difficult to accomplish in rodents. Successful models of progressive resistance training for rats have been developed (162), which have included securing progressively heavier loads to their tails, while the rats ascended a mesh incline to receive a piece of food. Other approaches include voluntary plantar flexion to receive a food reward (158). Although these methods accomplish some degree of muscle hypertrophy and elicited a training response, the rest interval between repetitions could not be controlled, nor could older animals be conditioned to comply

It has recently been observed in rats that aging results in an impaired ability of the tibialis anterior muscles to adapt chronic repetitive loading exercise (13, 35, 36). In young adult rats the same stimulus results in an increase in force, muscle mass, and myofiber cross-sectional area (36). The aged rats have been shown to display increases in pro-apoptotic signaling in activated muscle satellite cells (91) along with a diminished regenerative capacity and/or limited local muscle remodeling (13) that limited skeletal muscle adaptation to chronic repetitive loading exercise. Furthermore, this age-dependent divergence in the adaptive responses to repetitive loading exercise is exaggerated with increased velocity of movement (35). While the complete mechanisms for limiting the adaptive response to exercise in the aged muscle are unknown, it is hypothesized that the increased level of oxidative stress in muscles of aged animals may in part reduce the ability of muscle to adapt to repetitive loading.

2.7 Oxidative Stress in Muscle Loading

Resistance training has been identified as an effective means to delay the onset of sarcopenia, but there is still a paucity of studies that have evaluated oxidative stress during resistance training in humans. Studies that have evaluated high intensity or heavy resistance exercise have shown evidence of increased oxidant production (2, 11, 70, 109). However, few studies have examined oxidative stress and the long term benefits and/or consequences of resistance training within the increasingly oxidative environment that is associated with advanced age. Studies that investigating resistance training and oxidative stress in the elderly have found that chronic training will reduce the occurrence of oxidative damage (122, 154); however theses studies did not investigate the source of oxidant production or adaptation in the endogenous antioxidant system. Increased oxidative stress may reduce the muscles' ability to adapt to increased demands, as is the case with repetitive loading exercise. Mitochondria and activated satellite cells are the most common targets for oxidative damage in exercising muscle (17, 138). When activated, the normally quiescent satellite cells become more metabolically active and began to undergo mitosis, leaving them vulnerable to oxidant attack and thus can be subsequently eliminated (e.g., by myonuclear apoptosis). The inability to properly incorporate satellite cells as new myonuclei, may contribute to the maladaptation of skeletal muscle to repetitive loading seen in aged animals. Although it is clear that oxidative damage accumulates with aging, the role of mitochondria in aging and oxidative damage has several unanswered questions (17).

Since aging is associated with increased oxidative stress and diminished muscular strength, it is likely that increases in oxidants take part in reducing muscle function during repetitive loading. It has recently been shown that muscle force falls within 24 hrs after repetitive loading, and does not recover during the first 7 days (37). It is hypothesized that the loss of muscular force is mediated, directly or indirectly, by the increase in oxidant production during repetitive loading. This hypothesis is supported by the fact that force is diminished in muscles that are exposed to exogenous oxidants (5-7, 118).

Recent data have suggested that an increased production of free radicals, particularly during isometric or shortening contractions, may have beneficial adaptive effects (107). Isometric exercise could prove to be a useful tool in examining oxidative stress during exercise because the magnitude of damage following isometric exercise is relatively low (107).

Two interventions that show promise in reducing age-related oxidative stress are exercise training and antioxidant supplementation. Moderate non-fatiguing exercise has been shown to enhance the buffering capacity of these enzymes and therefore exercise can be thought of as a therapeutic means for offsetting high levels of oxidative stress (75, 128, 144). However, the possibility exists that intensive exercise may exacerbate oxidant production in muscles with aging, which could be detrimental to muscle function. These experiments will help to shed light on the topic of resistance/anaerobic training and its effects on oxidative stress in aged animals.

2.8 Antioxidant supplementation and oxidative stress

The effect of dietary antioxidant supplementation on reducing exercise and age-induced increases in oxidative stress has been given a good deal of attention throughout the years. In 1952, Staton (141) reported that 30 days of supplementation with 100 mg vitamin C resulted in lower accounts of muscle soreness after exercise when compared to a placebo. Harman (61) was one of first to suggest that nutritional supplements that are hydrogen donors could be beneficial in combating increases in oxidative stress and free radicals associated with aging. Many of the early studies that examined antioxidant supplementation and exercise centered on athletic performance. The rationale is simple; exercise is associated with increased energy expenditure and, as a consequence, oxidant production increases with metabolism; dietary antioxidants may help alleviate this process.

2.8.1 Vitamin E

Vitamin E (α-tocopherol) is an antioxidant and potent free radical scavenger that is suggested to have a protective effect in reducing or preventing oxidative injury to tissue. The antioxidant activity of vitamin E is based on the ease with which the hydrogen on the hydroxyl group of the chromogen ring can be donated to neutralize a free radical. Vitamin E is lipid-soluble and considered the predominant antioxidant protecting cellular membranes. Dietary supplementation of vitamin E has been shown to increase tissue resistance to exercise-induced oxidative damage, specifically lipid peroxidation (71, 113, 143). Furthermore, rodents (28, 38) and humans (113) that are deficient in vitamin E show massive increases in oxidant production and lipid peroxidation after exercise. McBride et al. (109) examined the effects of resistance training and vitamin E supplementation within humans. They found indicators of oxidative stress (lipid peroxidation measured by MDA) to increase immediately after heavy resistance training in both the supplemented group and the non-supplemented groups. However, six and 24 hours post exercise the supplemented groups MDA levels were back to baseline, whereas MDA levels were still elevated in the non-supplemented group. Although, there is opposing data that suggests supplementing with vitamin E could not completely protect elderly men from oxidative

damage caused by exercise (2). One possible reason was that exercise decreased levels of vitamin C in the elderly which could have reduced the effectiveness of vitamin E (2).

2.8.2 Vitamin C

Vitamin C (ascorbic acid) is a highly effective water-soluble antioxidant primarily found in the cytosol and extracellular fluid. Even in small amounts, vitamin C can protect proteins, lipids, carbohydrates, and nucleic acids from damage by oxidants generated during normal metabolism. What makes vitamin C such an effective antioxidant is its ability to interact directly with reactive oxygen and nitrogen species; superoxide, hydroperoxyl radicals, aqueous peroxyl radicals, singlet oxygen, ozone, peroxynitrite, nitrogen dioxide, nitroxide radicals, and hypochlorous acid, thereby preventing additional substrates from oxidation (20, 60). Jakeman and Maxwell (73) demonstrated that vitamin C, but not vitamin E, supplementation prior to eccentric exercise exerted a protective effect against muscle damage. Opposing results showed consumption of 200 mg vitamin C twice daily for three days after a 90 minute shuttle-run increased plasma concentrations of vitamin C, but the supplemented group failed to show improvements over the placebo group in muscle soreness, inflammatory response, or recovery of muscle function (147). Unfortunately neither of the preceding studies presents data on specific markers of oxidative stress.

2.8.3 Vitamin E & C combined.

A combination of Vitamin E & C has been shown to have a better antioxidant effect than either of the two vitamins alone (129). When vitamin E is oxidized, it forms a slightly more stable intermediate tocopheroxyl radical. vitamin C can reduce the tocopheroxyl radical, regenerating vitamin E (20, 60). In animal models of exercise, oxidants can lead to increased formation of protein carbonyls (2), plasma levels of MDA (109) and neutrophil chemotaxis (14); thus vitamin E and vitamin C supplementation may prevent these increases in cellular damage, neutrophil infiltration, and edema following an acute bout of exercise. Additional data suggest that prior supplementation with vitamin E & C ameliorates muscle functional decrements (decease in maximal force during isometric contractions) following eccentric muscle contractions (134). During heavy resistance training or maximal isometric contraction, there are brief periods of ischemia followed by reperfusion; an extreme example of this is exercise-induced claudication, common in patients with peripheral vascular disease. Recent findings suggest that, neutrophil chemotaxis is caused by increased xanthine oxidase-derived oxidants (82). Furthermore, vitamin E & C supplementation can inhibit oxidative damage, neutrophil infiltration and edema following an acute bout of contractile-induced claudication (83). Although, studies of exercise-induced claudication are extreme, they provide evidence that support the hypothesis of increased xanthine oxidase activity during exercise and the use of vitamin E & C supplementation to buffer oxidant production within the proposed model.

In a study of professional soccer players, dietary supplementation of vitamin E & C was shown to reduce lipid peroxidation and muscle damage after high intensity workouts, but failed to enhance athletic performance (166). Additionally, Rokitzki et al. (129) demonstrated in marathon runners that oral consumption of vitamin E & C for four and half weeks prior to competition lessened the increase in creatine kinase compared to a placebo group, which is indicative of reduced muscle damage. Supplementation schemes should take into a count the mode of exercise and where oxidants are being produced. Alessio et al. (2) has shown that aerobic training will produce greater protein carbonyl formation than isometric contraction, whereas isometric contractions produced greater evidence of lipid peroxidation. During a resistance exercise session increased oxidant production originating from outside the muscle fiber (i.e. xanthine oxidase from endothelial cells or eccentric contraction-induced damage from

phagocytic cells) may overwhelm natural cellular antioxidant defenses including membrane bound vitamin E, leading to lipid peroxidation. The combined supplementation of vitamin E & C could serve as a potent pro-oxidant scavenger, and chain-breaking antioxidant following intense exercise (58).

2.9 Resveratrol

Recent research has suggested that resveratrol has several beneficial health effects that include its use as an anti-cancer, anti-viral, anti-inflammatory, and anti-aging nutraceutical. Resveratrol (3,4',5-trihydroxystilbene), a phytoalexin, is produced naturally by numerous plants as a defense mechanism against infection by pathogens. Resveratrol works as an antibacterial and anti-fungal chemical, and is part of the hypersensitive response mechanism during the short-term immune response. During the hypersensitive response, resveratrol is employed to increase production of oxidants that assist in killing invading cells and initiate apoptosis in the area bordering the infection, essentially creating a physical barrier restricting the growth and spread of pathogens to other parts of the plant. When induced by stress, injury, infection or UV-irradiation, plants synthesize resveratrol from p-coumaroyl CoA and malonyl CoA (1, 132, 139). Resveratrol is a small fat-soluble molecule that occurs in two isoforms, a *trans*- (*E*) and a *cis*- (*Z*) configuration. The *cis*-isoform is easily oxidized and degraded when exposed to light, heat, and oxygen, while the *trans*- isoform is more stable under normal atmospheric conditions at room temperature (19).

When taken orally, *trans*-resveratrol is well-absorbed by mammals (i.e. humans, rats and mice), but its bioavailability is low due to its rapid metabolite limitation (8, 155, 156). When young adult men and women were given a 25 mg oral dose of *trans*-resveratrol, only traces of the unchanged resveratrol were detected in circulating blood plasma (155). The bioavailability of resveratrol in mammals is imperative to understanding how resveratrol supplementation achieves its beneficial effects. There has been a great deal of cell culture research that has exposed the cells to non-metabolized resveratrol at concentrations that are often 10-100 times greater than peak concentrations observed in human plasma after oral consumption. For that reason, we must be cautious in attempting to translate data attained through cell culture work to biological function *in vivo*. *In vitro* experiments have shown resveratrol to be effective at scavenging other oxidants (142) and inhibiting low density lipoprotein (LDL) oxidation (25, 50). However, there is little evidence supporting the role of resveratrol as an important oxidant scavenger *in vivo* (23). Based on the low bioavailability of resveratrol, there are other dietary (i.e. vitamin E & C) and endogenous antioxidants (i.e. glutathione, catalase, superoxide dismutase) that are present in much higher circulating and intracellular concentrations than resveratrol and are more likely to make a greater contribution to the oxidant defense. Given that after oral consumption the concentration of resveratrol is low, it has been hypothesized that biologically active resveratrol metabolites, which peak at concentrations around 2 mM/L in the plasma 30-60 minutes after consumption, could elicit much of the beneficial effects of resveratrol seen in mammals (155).

Even though the bioavailability of resveratrol at the tissue level is low, supplementation studies have shown beneficial results (16, 94). Oral resveratrol supplementation has been associated with longer lifespan, reduced insulin-like growth factor-1 (IGF-I) levels, increased AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-c coactivator 1α(PPAR-α) activity, increased mitochondrial number, and improved motor function (16). Treatment of mice with resveratrol has also been shown to significantly increase running time to exhaustion and consumption of oxygen in muscle, suggesting an improved aerobic capacity (94). In mammals, resveratrol supplementation modulates lifespan and metabolism

through the inhibition of insulin signaling pathways, AMPK, PGC-1a and activation of Sirt1 (16, 21, 94, 165). Recent work in skeletal muscle has also shown SIRT1 deacetylation of PGC-1α, will activate mitochondrial fatty acid oxidation genes (54) as well as enhance components of the electron transport chain, oxidative enzymes, and ATPases (94). Increases in these enzymes could contribute to an increased supply of ATP, which would explain the improved muscle endurance linked to resveratrol supplementation.

2.10 Summary & Conclusion

Advanced aging is accompanied by sarcopenia as a consequence of muscle fiber loss and atrophy of the individual muscle fibers. Advanced aging is also associated with an increase in oxidant production and a decrease ability to buffer oxidants. The resulting oxidative stress has been shown to contribute to the activation of myonuclear apoptosis(136, 137), a decrease in protein synthesis and activation of proteolysis, (114) all of which play a role in muscle atrophy. Sarcopenia will inevitably lead toward circumstances in which an elderly person is unable to accomplish everyday tasks. Resistance training has been identified as an effective means to delay sarcopenia, but there is still a paucity of studies that have evaluated oxidative stress during resistance training in humans. Even fewer studies have examined oxidative stress and the long term benefits and/or consequences of resistance training within the increasingly oxidative environment that is associated with advanced age. The majority of human studies are only able to report on a few markers of oxidative stress from blood samples', leaving one with only assumptions of what is happening within muscle tissue. Thus, animal models are important to collect larger amounts of tissue for extracting data. Unfortunately, there are even fewer studies that have investigated oxidative stress and resistance training in rodents than has been reported in humans. The proposed studies will be amongst a limited few that have exclusively examined oxidative stress with resistance exercise within an aging model. Furthermore, nutritional supplement companies have invested large sums of money into promoting antioxidant supplementation as an ergogenic aid to resistance training and aging, with only a minimal amount of research supporting their claims. A better understanding of how antioxidant supplements can be best employed to combat oxidative stress associated with aging and exercise would be beneficial to all. A further understanding of potential sources of oxidant production associated with resistance training will also allow for better identification of potential supplements and/or course of supplement therapy.

The major goal of this research is to better understand the role of oxidative stress in the adaptive hypertrophic response to repetitive loading in muscle and how it changes with advance aging. It is hypothesized that skeletal muscles from aged animals will show increased evidence of oxidative stress, while exercise (repetitive loading) and antioxidant supplementation would increase the aged muscles' oxidative buffering capacity and decrease the muscles oxidant production, thus attenuating the increase in oxidative stress associated with aging. Furthermore, resistance exercise (i.e. repetitive loading) and aging will be associated with an increase in xanthine oxidase activity which could be a contributing factor to oxidative stress. Hopefully the results from the proposed investigations will lead to further translational studies that promote a safe and more effective combination of resistance training and antioxidant treatment in the elderly population. The long term goal is developing an effective treatment for sarcopenia, which, in turn, will have a great benefit to the individual and their quality of life during their later years. Furthermore, such a treatment may potentially have a great economical value to society, given the expanding costs associated with caring for the increasing elderly population.

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Chapter 3

Aging-dependent regulation of antioxidant enzymes and redox status in chronically loaded rat dorsiflexor muscles

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Abstract

We have examined whether aging attenuates oxidant buffering capacity and decreases oxidant production after chronic repetitive loading (RL). The dorsiflexors from one limb of young adult and aged rats were loaded 3 times per week for 4.5 weeks using 80 maximal stretch-shortening contractions per session. RL increased H_2O_2 in the tibialis anterior muscle of young and aged rats and decreased the ratio of reduced/oxidized glutathione and lipid peroxidation in aged but not young adult animals. Glutathione peroxidase (GPx) activity and catalase activity increased with RL in muscles from both young and aged rats. RL increased CuZnSOD and MnSOD protein concentration and CuZnSOD activity in muscles from young animals but not old animals. There were no changes in protein content for GPx-1 and catalase or mRNA for any of the enzymes studied. These data show that aging reduces the adaptive capacity of muscles to buffer increased oxidants imposed by chronic repetitive loading.

Introduction

Oxidative stress is an important mechanism that may at least in part underlie the aging-induced attenuation of muscle physiological adaptation to increased loading (5; 6; 35; 36). Increased oxidant production is buffered in muscles of young animals and humans, but it is possible that aging reduces the ability for muscles to buffer oxidants. This is important because when the buffering of oxidants is compromised, oxidant stress will arise which can lead to cellular damage. Oxidative stress depresses muscle specific force (7), and alters myofilament function as a result of muscle loading (41; 42) or contraction-induced calcium release (2; 19; 61). Oxidative stress may also contribute to loss of muscle force by reducing recovery from injury (40). Increases in oxidant production have also been shown to stimulate redox-sensitive signaling pathways (32; 33; 46), up-regulate catabolic gene expression in muscle (17; 49; 51) and activate apoptosis (38; 50) in muscle.

It is clear that muscle atrophy is associated with increases in oxidants and oxidative stress (43; 47) evident by increases in lipid peroxidation, glutathione oxidation, protein carbonyls, free iron content, and xanthine oxidase levels. Oxidative stress is also elevated with both aging and loading in chondrocytes (52), synovial cells (74) and muscle cells (21). The generation of oxidants and oxidative stress has been implicated in mechanisms of muscle dysfunction and sarcopenia (55). However, there are no data that address the role of cumulative oxidative stress in repetitive loading in aging muscles. Furthermore, the underlying cause for increased oxidant formation in aging muscle is unknown, but this information is essential if we are to develop useful strategies for improving adaptations to loading in aging.

The mitochondrial theory of aging predicts that an increase in oxidative stress is responsible for cellular damage and ultimately apoptosis and cell death of various cell types (18) including skeletal muscle cells (22; 39). Although actin and myosin proteins do not appear to have increased oxidative damage with aging in rats (71), recent data showed that 8-hydroxy-2'-deoxyguanosine (8-OHdG), protein carbonyls, MnSOD activity and catalase activity were significantly higher in muscle biopsies obtained from older men as compared to young men (24). Full-length mitochondrial DNA was also lower and mitochondrial DNA deletions were prominent in muscles from old as compared to young men (23). These data support the conclusion that even healthy aging is associated with oxidative damage to proteins and DNA in skeletal muscle. This may be a primary reason for increased nuclear apoptosis that has been reported in aged muscles (45; 58; 59; 65). Oxidative stress may reduce the muscle's ability to adapt to increased demands, as is the case in repetitive loading. Although mitochondria may be the intrinsic initiators of oxidative stress, mitochondria, along with activated satellite cells may be the target for oxidative stress in loaded muscle. If activated satellite cells are reduced or eliminated (e.g., by apoptosis) muscle adaptation to loading would be reduced or eliminated. Although it is clear that oxidative damage accumulates with aging, the role of mitochondria in aging and oxidative damage still has several unanswered questions, including the involvement of mitochondria in apoptosis (4).

Because aging is associated with increases in oxidative stress, it is likely that oxidants have a role in reducing muscle function in aging and repetitive loading. Cutlip and colleagues have recently shown that muscle force falls within 24 hrs, and does not recover during the first 7 days of repetitive loading (14). We hypothesize that loss of

muscular force is mediated, directly or indirectly, by the increase in oxidant activity during repetitively loading. This is known to occur in muscles exposed to exogenous ROS (1; 2; 57). An aging-associated reduction in CuZnSOD appears to be a good candidate for explaining increased superoxide levels leading to long-term oxidative damage and eventual loss of skeletal muscle, because CuZnSOD deficient animals have a lower life span and decreased muscle mass (55). Recent data (73) also suggest that CuZnSOD levels are lower in muscles and other tissues of old rodents, whereas, long lived animals do not show this decrease in CuZnSOD and other oxidative enzymes (8). CuZnSOD levels increase in cells of young animals after exercise (26), and although Vasilaki et al. (72) report an increased level of CuZnSOD in muscles of old animals after a single bout of electrical stimulation, it is possible that old muscles may fail to respond in this manner with chronic repetitive loading. Furthermore, even if CuZnSOD does increase either similarly or greater in old vs. young muscles with repetitive loading, it is not known if this will be sufficient to counteract the loading-induced oxidant and intrinsic oxidant produced by aging mitochondria (54) in old muscles.

It has been widely recognized that GPx-1, catalase, CuZnSOD and MnSOD enzymes provide a defense system, which are essential for the survival of aerobic organisms. Moderate non-fatiguing exercise has been shown to enhance the buffering capacity by increasing the specific activity of these enzymes and therefore exercise is thought to be a therapeutic tool for offsetting high levels of oxidative stress (31). However, the possibility exists that intensive exercise may exacerbate oxidant production in muscles with aging, which may be detrimental to muscle function.

Aging rodents are frequently used as models for exercise training in humans, but there are few studies that have examined the effects of resistance training in aged rats. We have recently observed that repetitive loading results in a mal-adaptation in tibialis anterior muscles of old rats, whereas the same stimulus in the tibialis anterior muscle from young adult rats results in an increase in force, muscle mass, and myofiber cross-sectional area (13). While the mechanisms for these mal-adaptations to exercise in the aged muscle are unknown, we hypothesized that the increased level of oxidative stress in muscles of aged animals may in part reduce the ability of muscle to adapt to repetitive loading.

In this study we tested the hypothesis that: (I) tibialis anterior (TA) muscles from aged rats would show greater evidence of oxidative stress compared to muscles from young adult animals, (ii) chronic repetitive loading would increase the TA muscles' oxidative buffering capacity and decrease the muscles oxidant production, thus attenuating the increase in oxidative stress associated with aging, and (III) aging would attenuate adaptive responses in antioxidant pathways and this would be closely associated with attenuated hypertrophic adaptations to repetitive loading in aging.

Methods

The left TA muscles of old (30 months of age; n=8) and young (12 weeks of age; n=8) Fischer 344 Brown x Norway rats were subjected to repetitive loading exercise, which consisted of 3 sessions per week for 4.5 weeks, of 80 super-physiological eccentric / concentric contraction cycles per session (12). Muscle functional data were collected from a subset of four animals per group. The right TA was used as a contra-lateral control for each animal. The sessions were performed on a custom-built dynamometer by

electrically stimulating the common peroneal nerve, causing contraction of the dorsi flexor muscles and moving the footplate through plantar flexion (15). This method has been previously shown to produce a hypertrophic response in young adult rats (11). All animals had free access to rat chow and water. At the end of the 4.5 week loading period the rats were anesthetized with 2% isoflurane and the chronically loaded and control muscles were quickly removed, cleaned of excess connective tissue and weighed. The animals were euthanized by an overdose of pentobarbital. A section of each muscle was obtained for the determination of the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). The remaining muscle was quickly frozen in liquid nitrogen and stored in a -80°C freezer until analysis.

RNA Isolation. Sixty micrograms of the TA muscle was homogenized in 1ml of Tri-Reagent (Molecular Research Center, Cincinnati, OH) with a motorized blade homogenizer. Muscle homogenates were transferred to sterile 1.5ml Eppendorf tubes and centrifuged at 12,000 rpm for 10-minutes at 4˚C. Supernatants were transferred to a sterile 1.5ml tube then 100µl of 1-bromo-3-chloropropane (BCP, Molecular Research Center, Cincinnati, OH) was added to the supernatant. The sample was vortexed for 15s and incubated at room temperature for 15-minutes. The samples were centrifuged (12,000 rpm for 15-minutes at 4˚C) and the top aqueous phase that contains RNA was transferred to a sterile 1.5ml tube followed by addition of 500µl isopropanol and incubation at room temperature for 10-minutes. Samples were centrifuged at 12,000 rpm for 8-minutes at 4˚C, which collected the solubilized RNA into a small pellet. The RNA pellet was washed in 1ml of 75% ethanol and centrifuged at 7500 rpm for 5-minutes at 4˚C. The supernatant was removed and the RNA pellet was air dried in a fume hood and re-suspended in 22µl of sterile distilled H_2O. The RNA was treated with DNAse I using a DNA-free kit (Ambion, Austin, TX) and quantified using a BioRad SmartSpec 3000. RNA purity was accessed using a mimimum 260:280 ratio of 1.7. Samples with values less than this were re-treated for DNA contamination and quantified.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Two micrograms of total RNA were reversed transcribed using random primers (Invitrogen/Life Technologies, Bethesda MD) via the following protocol. 1.0µl of random primers and 1.0µl of 10mM dNTP mixture were added to 2µg of RNA. Samples were heated to 65˚C for 5-minutes followed by 3˚C for 5-minutes in a Biometra T3 thermocycler. 7.0µl of a master mix containing 5x First Strand buffer, DTT, and RNase-Out, were added to each sample. Tubes were returned to the thermocycler and incubated at 25˚C for 10-minutes followed by 42˚C for 2-minutes. 1.0µl of SuperScript II reverse transcriptase was added to each tube and lightly mixed by pipetting the solution up and down. Samples were returned to the thermocycler to be incubated at 42˚C for 50-minutes, 70˚C for 15-minutes, and then cooled to 3˚C until removed for storage. This procedure yielded 20µl of complimentary DNA (cDNA) which was stored at -80˚C or used for PCR analyses.

Primers for the genes of interest were constructed according to the following primer sequences: CuZnSOD sense-5’-AGGCCGTGTGCGTGCTGA-3’; anti-sense-5’-CCCAATCACACCACAAGCCA-3’; GPx-1 sense-5’-CCTCGTGGCCTGGTGGTCCT-3’; anti-sense-5’-AGGGGTTGCTAGGCTGCTTGGA-3’. The following primers were previously published from our lab: MnSOD sense-5'-GCGGGGGCCATATCAATCAC AG-3’; anti-sense-5'-GGCGGCAATCTGTAAGCGACCT-3'; Catalase sense- 5'-CGGGAACCCAATAGGAGATAAA-3'; anti-sense-5'-CCACGAGGGTCACGAACTGT-3'

(67; 69). To make certain analyzes were done in the linear range of amplification, preliminary tests were done to determine the proper number of PCR cycles. PCR products were verified by restriction digestion based on predicted PCR sequences. To control for any loading errors, the signal from the gene of interest was expressed as a ratio to the 18S RNA signal from the same PCR product. 49µl of a master mix containing, 10X PCR buffer with MgCl2, 5mM dNTPs, 100ng/ml of primer pairs, 18S primer pairs, and 1.0µl of Taq DNA polymerase, were combined with 1.0 µl of cDNA for PCR amplification. Amplification of PCR products were performed in a thermocycler using: a denaturing step at 95˚C for 45s, an annealing step for 45s, and an extension step at 72˚C for 45s. Following amplification, 20µl of each reaction were electrophoresed on 1.5% agarose gels. Gels were stained with ethidium bromide to visualize the PCR products. The PCR signals were recorded via a digital camera (Kodak 290) and the signals were quantified in arbitrary units as optical density x band area, using 1D Kodak image analysis software (Eastman Kodak Company, Rochester, NY).

Protein Fractionation. Seventy-five mg of TA muscle samples were separated for cytoplasmic and nuclear protein fractions, using methods reported previously by our lab (66; 70). However, a lower concentration of dithioreitol (DTT) was used in these experiments to prevent later interference with enzyme activity assays. Muscle samples were homogenized in 500 µl of ice-cold lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 20mM HEPES at pH 7.4, 20% glycerol, 0.1% Triton X-100, and 10µM dithioreitol) with a mechanical homogenizer. Muscle homogenates transferred to 1.5ml Eppendorf tubes and centrifuged at 800 rpm for 5-minutes at 4°C. Supernatants were collected and centrifuged three more times at 3500g for 5-minutes at 4°C. The resulting supernatant was collected as nuclei-free cytosolic fraction and divided into two equal portions; the first portion was frozen at -80°C until needed, in the second portion protease inhibitor cocktail containing 104mM 4-[2-aminoethyl]-benzenesulfonylflouride hydrochloride (AEBSF), 0.8mM aprotinin, 2mM leupeptin, 4mM bestatin, 1.5 mM pepstatin A and 1.4 mME-64 (Sigma-Aldrich, St. Louis, Mo, USA) was added before the sample was frozen at -80°C. Protein concentrations for each sample were determined in triplicate via a DC protein concentration assay (Bio Rad, Hercules, CA). The cytosolic fraction was used in the following assays: H_2O_2 concentration, Catalase activity, GPx activity, CuZn & MnSOD activity and western immunoblots.

Western Immunoblots. The protein content of glutathione peroxidase-1 (GPx-1), catalase, copper-zinc superoxide dismutase, (CuZnSOD) and manganese superoxide dismutase (MnSOD) was measured in the cytosolic muscle fractions. 30µg of protein was loaded into each well of a 4-12% gradient polyacrylamide gel (Novex, Invitrogen) and separated by routine SDS-polyacrylamide gel electrophoresis (PAGE) for 1.5 hours at 20°C followed by transfer to a nitrocellulose membrane. All membranes were blocked in 5% non-fat milk protein (NFM) for 1-hour at room temperature. In general, membranes were incubated in appropriate dilutions of primary antibodies (diluted in 1% NFM in tris-buffered saline with 0.05% Tween-20 (TBS-T) overnight in a 4°C cold room. Membranes were washed in TBS-T followed by incubation in appropriate dilutions of secondary antibodies (diluted in 5% NFM in TBS-T) conjugated to horseradish peroxidase. Signals were developed using a chemi-luminescent substrate (ECL Advanced, Amersham Bioscience) and visualized by exposing the membranes to X-ray films (BioMax MS-1; Eastman Kodak). Digital records were captured by a Kodak 290 camera and protein

bands quantified using 1-D analysis software (Eastman Kodak, USA). Bands were quantified as optical density (OD) x band area and expressed in arbitrary units.

H_2O_2 levels. A fluorescent hydrogen peroxide (H_2O_2) (Cell Technology, Mountain View, CA) detection kit was used to determine the amount of H_2O_2 in the muscle tissue. Reagents and standards were prepared as recommended by the manufacturer. Briefly, 50µL of controls, samples or H_2O_2 dilutions were mixed with 50µL of the reaction cocktail in each well to initiate the reaction. The plate was incubated in the dark for 10 minutes, at 20ºC and fluorescence was detected with an excitation at 530nm and measured at 590nm. All analyses were done in duplicate and samples were normalized to muscle protein concentration in each sample via a DC protein concentration assay (Bio-Rad, Hercules, CA).

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

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

8-hydroxy-2'-deoxyguanosine (8-OHdG). Oxidized DNA was determined by a BIOXYTECH 8-OhdG ELISA (enzyme linked immunoassay) (Oxis Research). DNA was extracted from the muscle via DNeasy Tissue Kit (Qiagen). DNA was used if it had a minimum 260:280 ratio of 1.8. 50µl of purified DNA was mixed with 50µl of primary antibody. Samples were then incubated at 37° C for one hour. The wells were washed then incubated in 100µl of secondary antibody at 37°C for one hour. 100µl of chromogen was added to each well, shaken then incubated at room temperature in the dark for 15 minutes. The reaction was terminated and the samples were read at an absorbance of 450 nm. Samples were normalized to the DNA concentration measured via a plate reader (ND-1000, NanoDrop, Wilmington, DE). All analyses were done in duplicate.

Lipid peroxidation. Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were measured using reagents from Oxis International, CA (Bioxytech LPO-586). A 75-100 mg section of each muscle was homogenized in 500µl of buffer containing ice-cold phosphate-buffered saline (PBS, 20 mM, pH 7.4) and 5 µL 0.5 M butylated hydroxytoluene (BHT) in acetonitrile per 1 ml of tissue homogenate. Assay reagents were added following the manufacturer's recommendations. Briefly, the muscle homogenate was centrifuged at

3000 g at 4°C for 10 minutes and the supernatant was used for the assay and protein determination. After incubation in the appropriate reagents, the sample was incubated at 45°C for 60 minutes, and then centrifuged at 15,000g for 10 minutes. An absorbance reading of the supernatant was obtained at 586nm. Samples were normalized for differences in the amount of muscle protein in each sample as determined by a DC protein concentration assay (Bio-Rad, Hercules, CA).

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

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

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

Statistical analyses. Statistical analyses were performed using an SPSS 13.0 software package. A multiple analyses of variance (MANOVA) were used to examine differences between age and treatment (RL). Statistical significance was accepted at $P < 0.05$. Data are reported as mean ± standard error mean (SEM).

Results

Body Mass. The average body mass of the young animals was 326.5 ± 14.7 g before muscle loading, and 317.2 ± 12.2 g after the 4.5 weeks of training, but this did not represent a significant change in body mass. However, the body mass of aged animals

had a small but significant decrease from 597.3 ± 17.9 g before training to 558.3 ± 13.3 g after the 4.5 weeks of training.

Muscle Wet Weight. Repetitive loading for 4.5 weeks resulted in a significant increase in tibialis anterior muscle wet weight in the exercised limb of both the young adult (11.5 ± 1.6%, $p<0.001$) and the aged adult (7.5 ± 1.9%, $p<0.05$) rats as compared with the contra-lateral control muscle. (Figure 3.1)

Insert Figure 3.1

Muscle Functional Measurements. Maximal isometric muscle force, positive work, and negative work were used to measure the functional capacity of the dorsiflexors, of which the greatest contributor is the tibialis anterior muscle. There was no significant difference between the young adult and the aged animals for maximal Isometric force (Figure 3.2A), positive work (Figure 3.2B) or negative work (Figure 3.2C) observed at the start of the study. However, 14 loading sessions increased maximal force (51± 5.6%), positive work, (32 ± 3.9%) and negative work (37± 7.4%) as compared to the first session for young adult animals. In contrast, there was no significant change in any of the parameters used to assess functional changes in muscles of the aged animals over the training period.

Insert Figure 3.2

H_2O_2. Muscle levels of H_2O_2 were elevated in the loaded muscles as compared to the age-matched control limb ($p<0.001$), suggesting a treatment effect and that chronic loading elevated oxidative stress. Both control and experimental muscles had higher levels of H_20_2 than their treatment matched muscles of young adult rats ($p<0.001$). This suggests a systemic aging effect of oxidative stress on muscles. (Figure 3.3) H_2O_2 concentration data expressed as a µmol/mg protein are discussed in the Limitation and Future Directions section within chapter 7. (Figure 7.2)

Insert Figure 3.3

GSH/GSSG ratio. There was no training-induced difference in the GSH/GSSG ratio in muscles from young adult animals when comparisons were made to the control muscles. However, there was a significant reduction in the GSH/GSSG ratio of both control ($p<0.05$) and loaded ($p<0.01$) muscles of aged muscles as compared to treatment-matched muscles in the young adult animals. The GSH/GSSG ratio was lower ($p<0.05$) in the loaded muscles of the aged rats as compared to the young adult rats. (Figure 3.4) These data suggest that aging increased oxidative stress and therefore lowered the GSH/GSSG ratio as compared to muscles in young adult animals and that aging reduced that ability to tolerate increased oxidative stress in chronically loaded skeletal muscles.

Insert Figure 3.4

Lipid Peroxidation. Aging increased the level of lipid oxidation as shown by greater MDA + HAE levels in control muscles of old rats as compared to muscles from young adult animals ($p<0.01$; Figure 3.5). Repetitive loading appeared to activate adaptive responses in muscles of old animals because MDA + HAE levels were lower in

the loaded than control muscles of aged animals. No significant difference in MDA + HAE levels was observed among the control or chronically loaded muscles of young adult animals.

Insert Figure 3.5

DNA Damage. Aging induced a significantly ($p<0.05$) increased basal level of DNA damage because the amount of 8-OHdG detected in control muscles was significantly greater in aged vs. young adult muscles. Repetitive loading reduced the level of 8-OHdG in muscles of old animals to that which was measured in muscles of young adult animals. Chronic loading did not change 8-OHdG in muscles of young adult animals. (Figure 3.6) These data imply that chronic loading offsets the level of oxidative stress-induced DNA damage in aging rats.

Insert Figure 6

GPx Activity. There was a loading effect but no age effect on GPx activity. Chronic repetitive loading increased GPx activity in muscles from both young and aged animals ($p<0.001$), but no differences were found between young and aged rodents. No significant differences were found among GPx-1 mRNA or protein levels within any of the muscle samples.

Insert Figure 3.7

Catalase activity. Catalase activity increased with repetitive loading in muscles from the young adult rats ($p<.05$), but there was no significant change in catalase levels in muscles of the aged animals. Catalase activity was higher in control muscles of aged vs. young adult animals, and it increased in loaded muscles of aged animals as compared to young adult animals ($p<.05$). (Figure 3.8) No differences were found between catalase mRNA or protein levels within any of the TA muscles.

Insert Figure 3.8

CuZn Superoxide dismutase activity. CuZnSOD protein levels increased by 100% ($p<0.05$) (Figure 3.9A) and CuZnSOD enzyme activity (Figure 3.9B) increased by 43% in ($p<0.05$) in repetitively loaded muscles from young animals as compared to the contra-lateral control muscles. However, these appeared to be post-translationally regulated because no differences in CuZnSOD mRNA levels in control or loaded muscles from young animals were observed. Aging suppressed any loading-induced changes in CuZnSOD, because there were no differences in mRNA, protein, or enzyme activity in the chronically loaded muscles compared to control muscles from aged animals.

Insert Figure 3.9

MnSOD. MnSOD protein levels were increased by 75% in the loaded muscles of the young animals as compared to their contra-lateral control muscles ($p<0.01$) as determined by western blot analyses (Figure 3.10); however, the increase in MnSOD protein did not affect the activity levels nor was it driven by the alterations in MnSOD mRNA. No changes in any of the variables were seen in the aged animals.

Insert Figure 3.10

Discussion

Chronic adaptation to resistance exercise over many weeks in older women and men has been shown to improve muscular strength and also induce muscle fiber hypertrophy (20; 63). However, it is not clear if the lower extent of muscle hypertrophy that generally results from chronic loading in aging (16) is due, at least in part, to elevated levels of oxidants. In this study, we found increases in muscle hypertrophy and muscle force and work in chronically loaded muscles of young adult rats. Although there was some degree of muscle enlargement in old animals with repetitive loading for 4.5 weeks, there was no improvement in muscle function, suggesting that muscle hypertrophy (based on muscle-wet weight) may not have been a result of increases in contractile proteins in muscles of aged rats. Although this varies slightly from a previous study using the same protocol, where a decrease in muscle mass and function was observed in old animals (10), it is likely that different cohorts of animals obtained from the NIA colony have slightly different responses and adaptive capabilities. Nevertheless, the current study and the previous study (9) are generally consistent in showing an attenuated functional response to repetitive loading with aging. While we cannot rule out the possibility that part of the increase in muscle wet weight may have been the result of increases in collagen or other contractile proteins, we did not detect evidence of inflammation (e.g., macrophages) or infiltrates in the loaded muscles of aged animals (Baker et al, unpublished observations).

Findings of the current study are that control muscles of aged rats had higher levels of oxidative stress as indicated by elevated H_2O_2, 8-OHdG and MDA+HAE as compared to control muscles of young adult animals. These findings are consistent with observations from other laboratories showing that aging is associated with increased levels of oxidative stress in skeletal muscles and may be related, at least in part to reduced muscle function with aging (3; 53; 64).

While chronic adaptation to loading, that was 3 times weekly for 4.5 weeks, increased oxidant levels of H_2O_2, aging did not prevent improvement in several indices of oxidative stress in loaded skeletal muscles of aged rodents (e.g., lipid peroxidation, and oxidative damage to DNA). Nevertheless, there was only a partial ability to adapt to greater levels of oxidative stress with aging. Of particular note, CuZnSOD and MnSOD were not different in the chronically loaded muscles of aged rodents compared to the control muscles, despite increased oxidative loads (e.g., H_2O_2) and evidence of oxidative damage. We speculate that a failure to elevate CuZnSOD as part of an adaptive process for repetitive loading may be critically important for explaining the increased DNA and lipid oxidative damage with aging, because reduced levels of this anti-oxidant protein has been shown to coincide with reduced muscle mass and function (55).

In contrast, the adaptation was more complete in muscles of young adult animals. For example, indices of oxidative stress (GSH/GSSG ratio) and oxidative damage (MDA +HAE and 8-OHdG) were similar in control and chronically loaded muscles from young animals. This suggests that the tibialis anterior muscles were able to efficiently adapt and buffer the increase in oxidant production in muscles of young adult animals. Furthermore, there was no significant difference in oxidative stress markers from the non-exercised control muscle. Adaptation in chronically loaded muscles from young adult animals appeared to occur via an increase in catalase and CuZnSOD activity, two enzymes that

are primarily located in the cytosol. This implies that the increase in oxidant production may not be primarily originating from the mitochondria, but may instead be generated in the cytosol, possibly through the xanthine oxidase pathway. We speculate that an increase in signaling of the xanthine oxidase pathway could increase the production of superoxide in the cytosol, thus increasing the localized stimuli for CuZnSOD production and activity as well as an increase in H_2O_2 production. We have found that acute bouts of repetitive loading increased xanthine oxidase activity in tibialis anterior muscle homogenate from mice (unpublished data).

In the present study, enzyme activity for CuZnSOD increased by about 43%, which is similar to previous findings from our lab using the same electrically evoked repetitive loading model (56). The lack of an increase in CuZnSOD mRNA would suggest post transcriptional modification that activates protein synthesis. Similarly, Hollander and colleagues (27; 29) found increases in CuZnSOD protein levels without changes in mRNA after a single bout of endurance exercise. The current study differed from data presented by Hollander et al (28; 30), in that previously they showed no increase in CuZnSOD enzyme activity after an acute bout of exercise, where the present study shows increases in enzyme activity. This may be a result of a cumulative effect resulting from chronic adaption to repetitive stimulation over the 4.5 week period of the present study.

Xanthine oxidase-mediated oxidative stress in the cytosol has been shown to increase activation of NF-κB signaling resulting in an increase in the transcription of mRNA for MnSOD. The lack of a detectable increase in MnSOD mRNA in the current study does not mean that it did not occur. There are two possible explanations; first protein levels for MnSOD are controlled by post translational modifications, similar to that found in CuZnSOD, and secondly, that transcription of MnSOD mRNA initially increased within the tibialis anterior, which in turn led to an increase in MnSOD protein levels which acted as a negative feedback regulatory mechanism to slow down transcription of MnSOD mRNA. Either possibility would explain the results we found in the current study.

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

Chronic exercise has been shown to either maintain or increase levels of GPx activity at least in aerobic high volume, low intensity types of exercise (25; 37). While generally, high-intensity exercise training has been shown to be more effective than low-intensity exercise in increasing of muscle GPx activity (62). Therefore, it was not surprising that repetitive muscle loading induced an increase in GPx activity in muscles from both the young adult and aged animals. However, there were no significant changes in the mRNA or protein levels for GPx-1 between either young vs. aged or control vs. exercise, this data suggests that repetitive loading induced a post-transcriptional increase in GPx activity within skeletal muscles of both young and aged animals. Similarly to GPx activity, catalase activity increased with repetitive loading exercise. GPx is an enzyme that is primarily thought to be responsible for reducing H_2O_2 and/or organic hydroperoxides to

water or alcohol and is located in both the cytosol and the mitochondria. Catalase catalyzes the breakdown of H_2O_2 to form water and O_2 (34; 48). The current data suggests that intense resistance training will lead to an increase in GPx and catalase activity in response to exercise induced H_2O_2 accumulation.

In conclusion, the data in this study show that muscles from aged rats have higher levels of oxidative stress and oxidative damage (e.g., to DNA and lipids) than muscles of young adult rats. Mechanical loading further exacerbates oxidative stress in muscles of aged rodents, but this appears to be well buffered in muscles of young adult animals. In response to high intensity chronic loading, there is a partial adaptation of oxidative enzymes to attempt to compensate for the increased oxidative insult in muscles of aged rats. However, this adaptation is incomplete, because CuZnSOD and MnSOD do not increase in chronically loaded muscles of aged rodents, but increase significantly after chronic adaptation to loading in muscles of young adult animals. The increases in GPx and catalase activity appear to be in response to loading induced elevations in H_2O_2. These data show that aging reduces the adaptive capacity of muscles to buffer the increased oxidant production imposed by chronic repetitive loading. This may compromise the muscles' abilities to hypertrophy or to improve muscle function in aged animals. Furthermore, it is possible that greater unbuffered levels of oxidative stress in muscles of old animals may trigger increased levels of apoptosis that are associated with lower muscle mass and attenuated hypertrophic adaptation in aging (44; 60; 68).

Figure Legends

Figure 3.1 **Repetitive loading induces muscle hypertrophy in the Tibialis Anterior Muscle**. Data are expressed as tibialis anterior muscle wet weight in grams. *, significance difference between age-matched control and RL TA muscle assigned at $p<0.05$; †, a significant difference ($p<0.05$) from young treatment-matched control muscles.

Figure 3.2 **Repetitive loading increased muscle functional measurements in young adult dorsiflexor muscles while maintaining function in aged dorsiflexor muscles. (A)** Maximal force generated from the young and aged dorsiflexor muscles during each of the 14 training sessions. Data are expressed as the average maximum force for all animals in Newtons (N) produced during each exercise session ± SE. Solid line represents the linear regression for all age-matched points. *, a significant difference ($p<0.05$) between young adult and aged dorsiflexor muscles. **(B)** Positive work generated from a single eccentric /concentric movement preformed at the start of each training session from the young and aged dorsiflexor muscles during each of the 14 training sessions. Data is expressed as the mean ± SEM. Solid line represents the linear regression for all age-matched points. *, a significant difference ($p<0.05$) between young adult and aged dorsiflexor muscles. **(C)** Negative work generated from a single eccentric /concentric movement preformed at the start of each training session from the young and aged dorsiflexor muscles during each of the 14 training sessions. Data is expressed as the mean ± SEM. Solid line represents the linear regression for all age-matched points. *, a significant difference ($p<0.05$) between young adult and aged dorsiflexor muscles.

Figure 3.3 **Concentration of hydrogen peroxide (H_2O_2) are elevated with RL & aging**. The H_2O_2 concentration was determined by a fluorometric assay. Data is expressed as Relative Fluorescent Unit (RFU) per mg of total protein in TA homogenate. The normalized data are presented as mean ± SEM. *, significant difference ($p<0.05$) of repetitively loaded (RL) muscle from contra-lateral control muscle. †, significant difference ($p<0.05$) from young treatment- matched control.

Figure 3.4 **Ratio of reduced glutathione to oxidized glutathione (GSH/GSSG)**. Data are depicted as the ratio of GSH to GSSG normalized to total protein content. Lower ratios are an indication of increased oxidative stress. The normalized data are presented as mean ± SEM. *, significant difference ($p<0.05$) of RL muscle from contra-lateral control muscle. †, significant difference ($p<0.05$) from young treatment- matched control.

Figure 3.5 **Repetitive loading decreases lipid peroxidation in tibialis anterior muscles of aged rats**. Data are combined malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) normalized to total protein content. The data are presented as mean ± SEM. *, significant difference ($p<0.05$) of RL muscle from contra-lateral control muscle. †, significant difference ($p<0.05$) from young treatment- matched control.

Figure 3.6 **Aging increased the quantity of DNA damage while RL had no effect**. Data is expressed as ng concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG) per ml of TA homogenate per µg of DNA. The normalized data are presented as mean ±

SEM. *, denotes significant difference ($p<0.05$) of RL TA muscle from contra-lateral control muscle. †, significant difference ($p<0.05$) from young treatment- matched control.

Figure 3.7 **Glutathione peroxidase (GPx) activity decreased with repetitive loading.** Data is expressed as mU of GPx per ml of homogenate per mg of protein. The normalized data are presented as mean ± SEM. *, significant difference ($p<0.05$) of RL TA muscle from contra-lateral control muscle; †, significant difference ($p<0.05$) from young treatment- matched control.

Figure 3.8 **Catalase activity increased with repetitive loading and aging.** Data is expressed as units (U) of catalase per ml of homogenate per mg of protein. The normalized data are presented as mean ± SEM. *, significant difference ($p<0.05$) of RL TA muscle from contra-lateral control muscle; †, significant difference ($p<0.05$) from young treatment- matched control.

Figure 3.9 **CuZn superoxide dismutase (CuZnSOD) protein levels and activity increased with repetitive loading in the tibialis anterior muscles of young but not old rats. (A)** CuZn Superoxide dismutase (CuZnSOD) protein expression was determined in the total cytosolic fraction by western immunoblot. The data is expressed as optical density (OD) x band area, and expressed in arbitrary units. The inserts show representative blots for CuZnSOD and β-tubulin in young and aged (control and RL) TA muscle. The normalized data are presented as mean ± SEM. *, significant difference ($p<0.05$) of RL TA muscle from contra-lateral control muscle. **(B)** CuZnSOD activity data is expressed as U of CuZnSOD per ml of homogenate per mg of protein. The normalized data are presented as mean ± SEM. *, significant difference ($p<0.05$) of RL TA muscle from contra-lateral control muscle.

Figure 3.10 **Mn superoxide dismutase (MnSOD) protein levels increased with repetitive loading in tibialis anterior muscles of young rats.** Mn Superoxide dismutase (MnSOD) protein expression was determined in the total cytosolic fraction by western immunoblot. The data is expressed as optical density (OD) x band area, and expressed in arbitrary units. The inserts show representative blots for MnSOD and β-tubulin in young and aged (control and RL) TA muscle. The normalized data are presented as mean ± SEM. *, denotes significant difference ($p<0.05$) of RL TA muscle from contra-lateral control muscle.

Figure 3.1

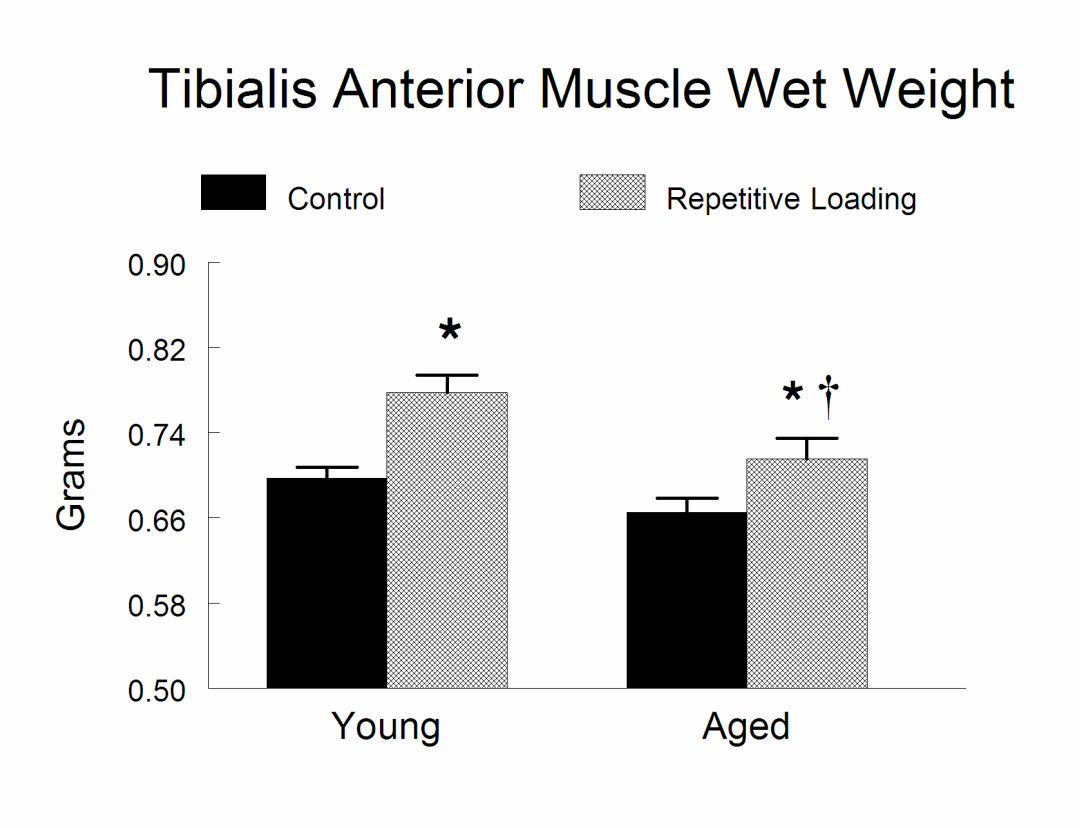

Tibialis Anterior Muscle Wet Weight
Control
Repetitive Loading
Grams
0.90
0.82
0.74
0.66
0.58
0.50
*
* †
Young
Aged

Figure 3.2

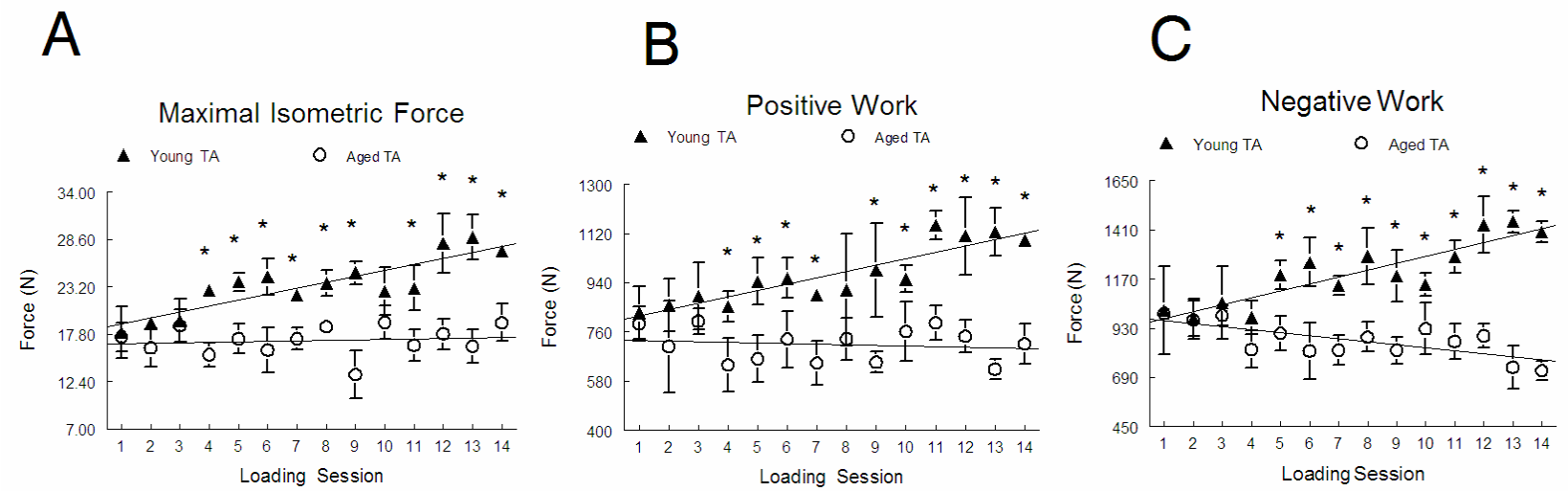

A
Maximal Isometric Force
Young TA
Aged TA
Force (N)
34.00
28.60
23.20
17.80
12.40
7.00
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Loading Session
B
Positive Work
Young TA
Aged TA
Force (N)
1300
1120
940
760
580
400
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Loading Session
C
Negative Work
Young TA
Aged TA
Force (N)
1650
1410
1170
930
690
450
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Loading Session

Figure 3.3

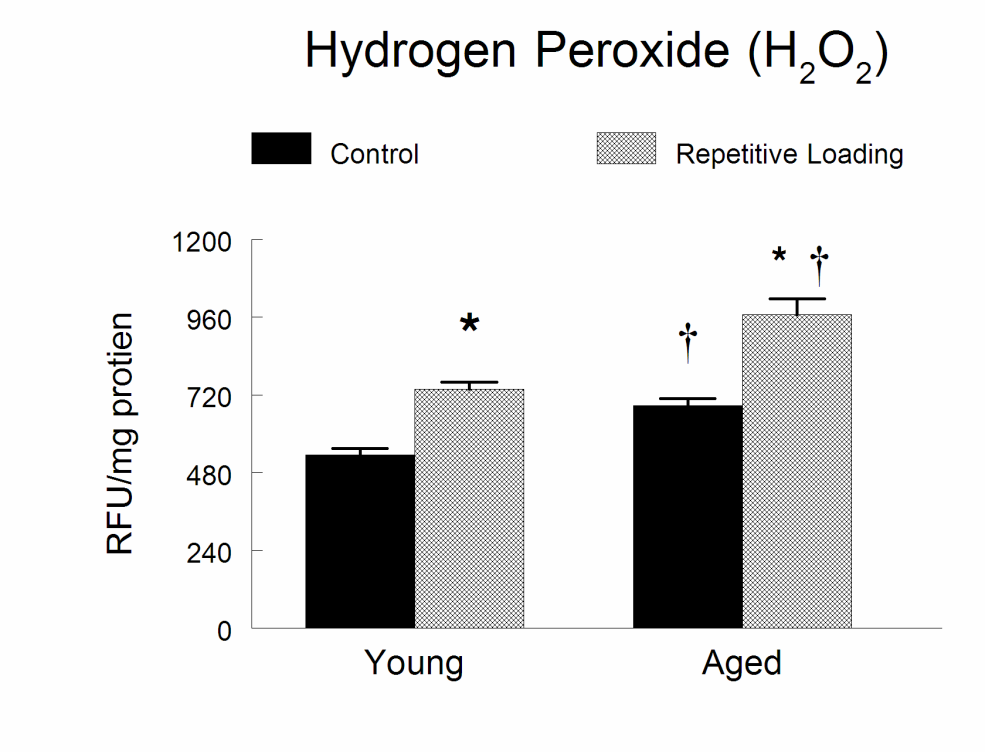

Hydrogen Peroxide (H_2O_2)
Control
Repetitive Loading
RFU/mg protien
1200
960
720
480
240
0
*
*
†
*
†
Young
Aged

Figure 3.4

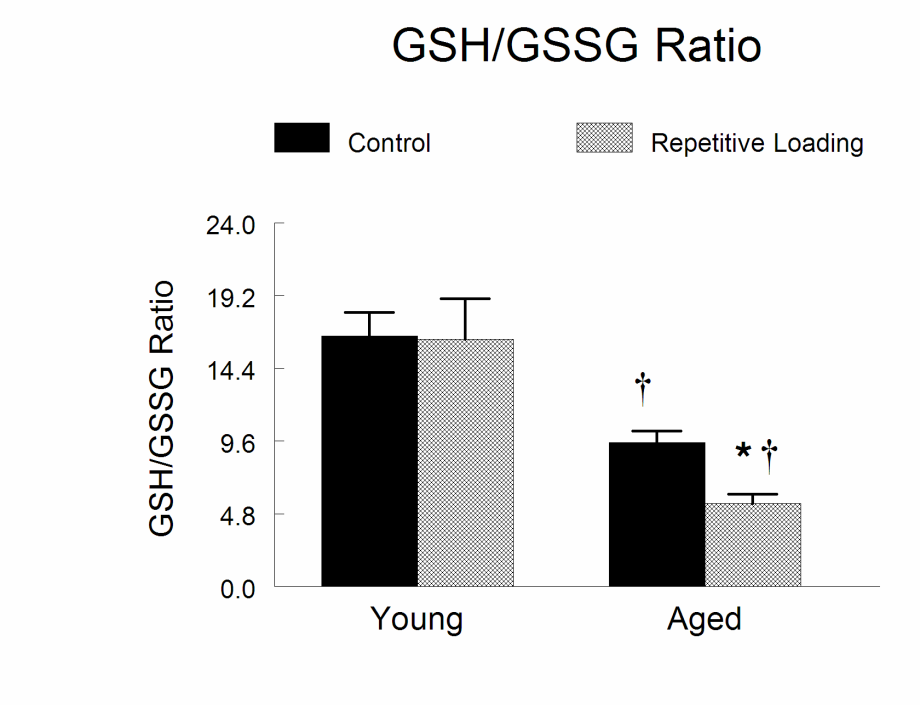

GSH/GSSG Ratio
Control
Repetitive Loading
GSH/GSSG Ratio
24.0
19.2
14.4
9.6
4.8
0.0
†
* †
Young
Aged

Figure 3.5

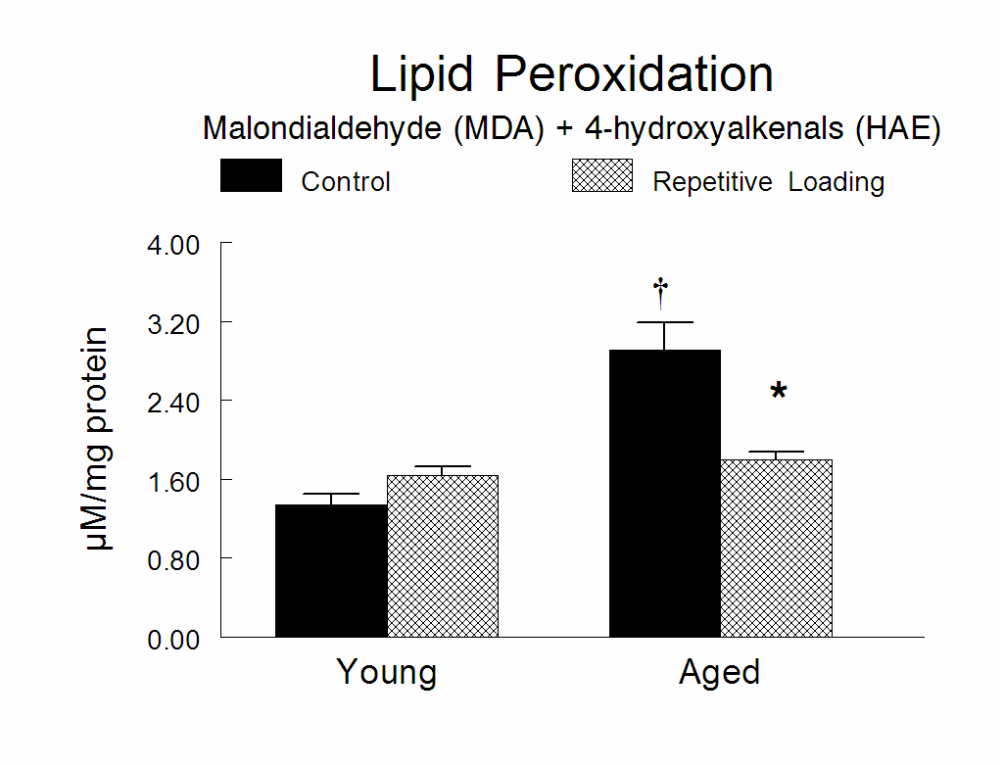

Lipid Peroxidation
Malondialdehyde (MDA) + 4-hydroxyalkenals (HAE)
Control
Repetitive Loading
µM/mg protein
4.00
3.20
2.40
1.60
0.80
0.00
†
*
Young
Aged

Figure 3.6

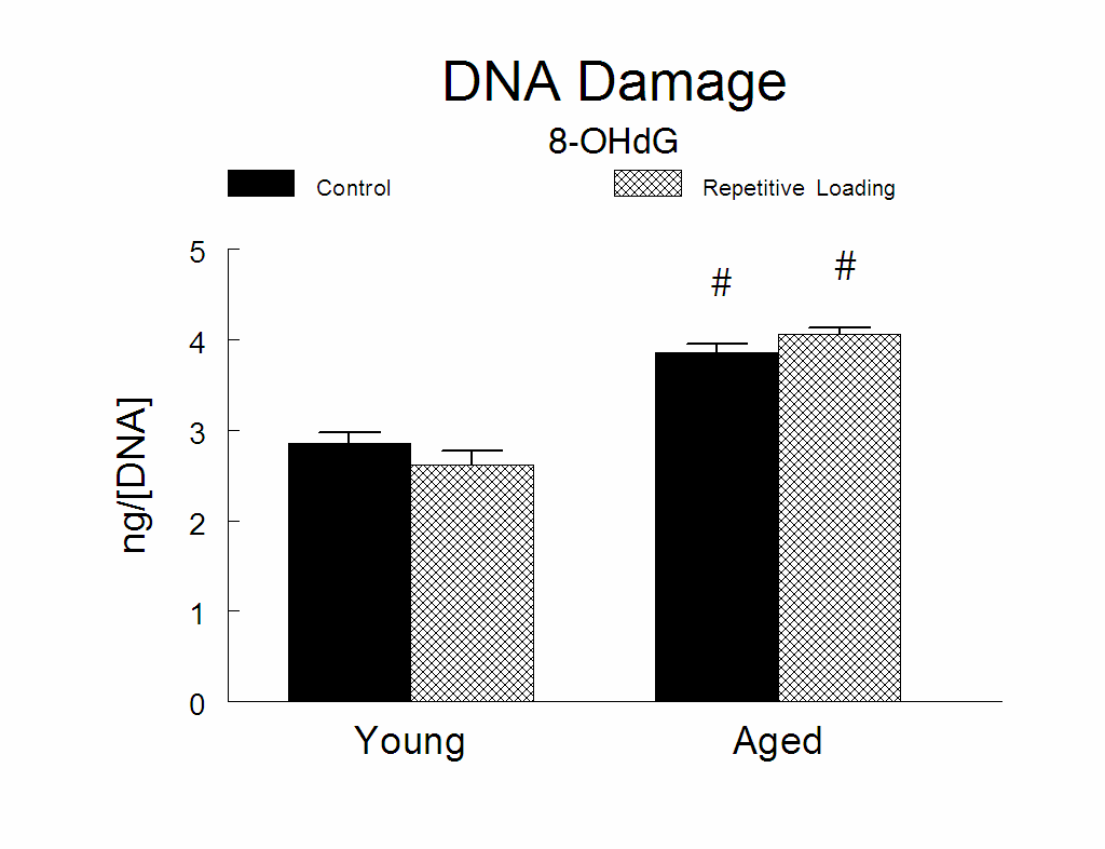

DNA Damage
8-OHdG
Control
Repetitive Loading
ng/[DNA]
0
1
2
3
4
5
#
#
Young
Aged

Figure 3.7

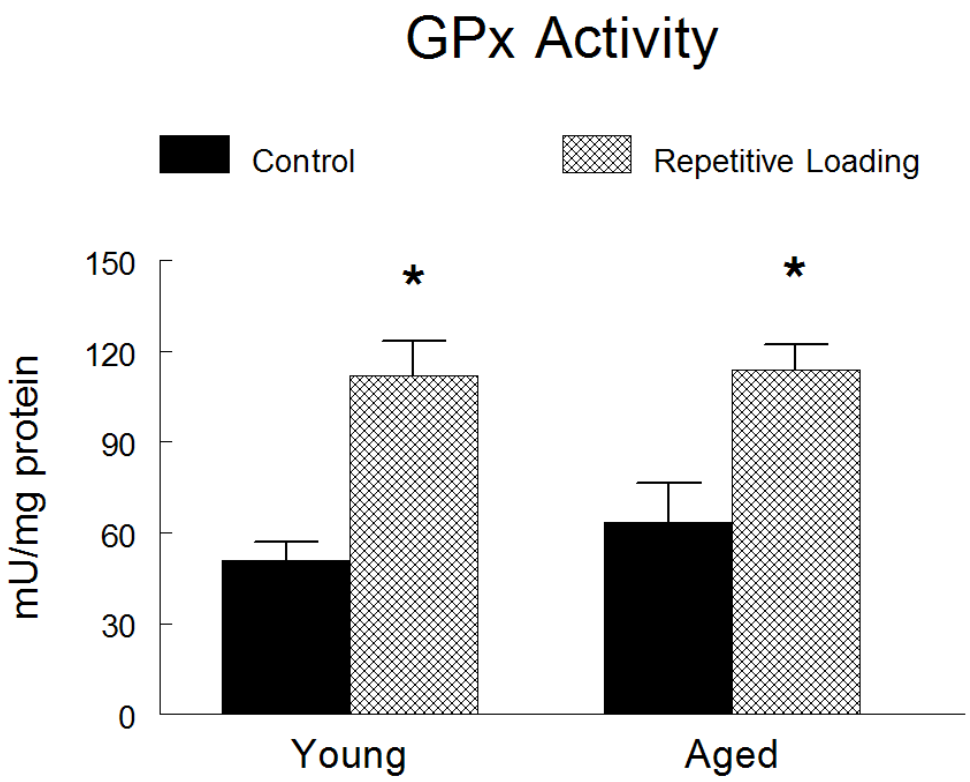

GPx Activity
Control
Repetitive Loading
mU/mg protein
150
120
90
60
30
0
*
*
Young
Aged

Figure 3.8

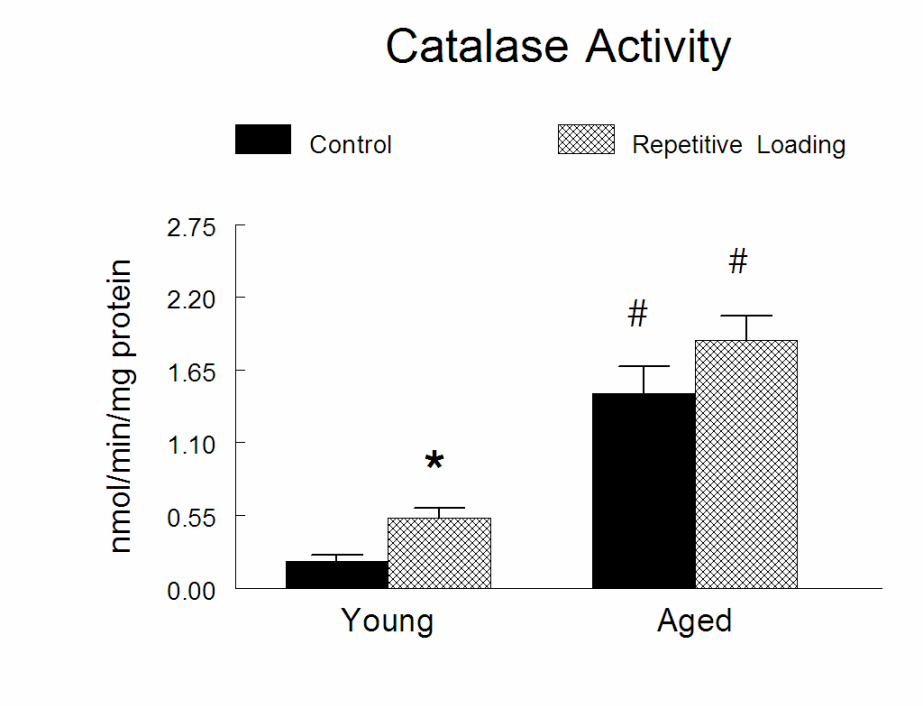

Catalase Activity
Control
Repetitive Loading
nmol/min/mg protein
2.75
2.20
1.65
1.10
0.55
0.00
*
#
#
Young
Aged

Figure 3.9

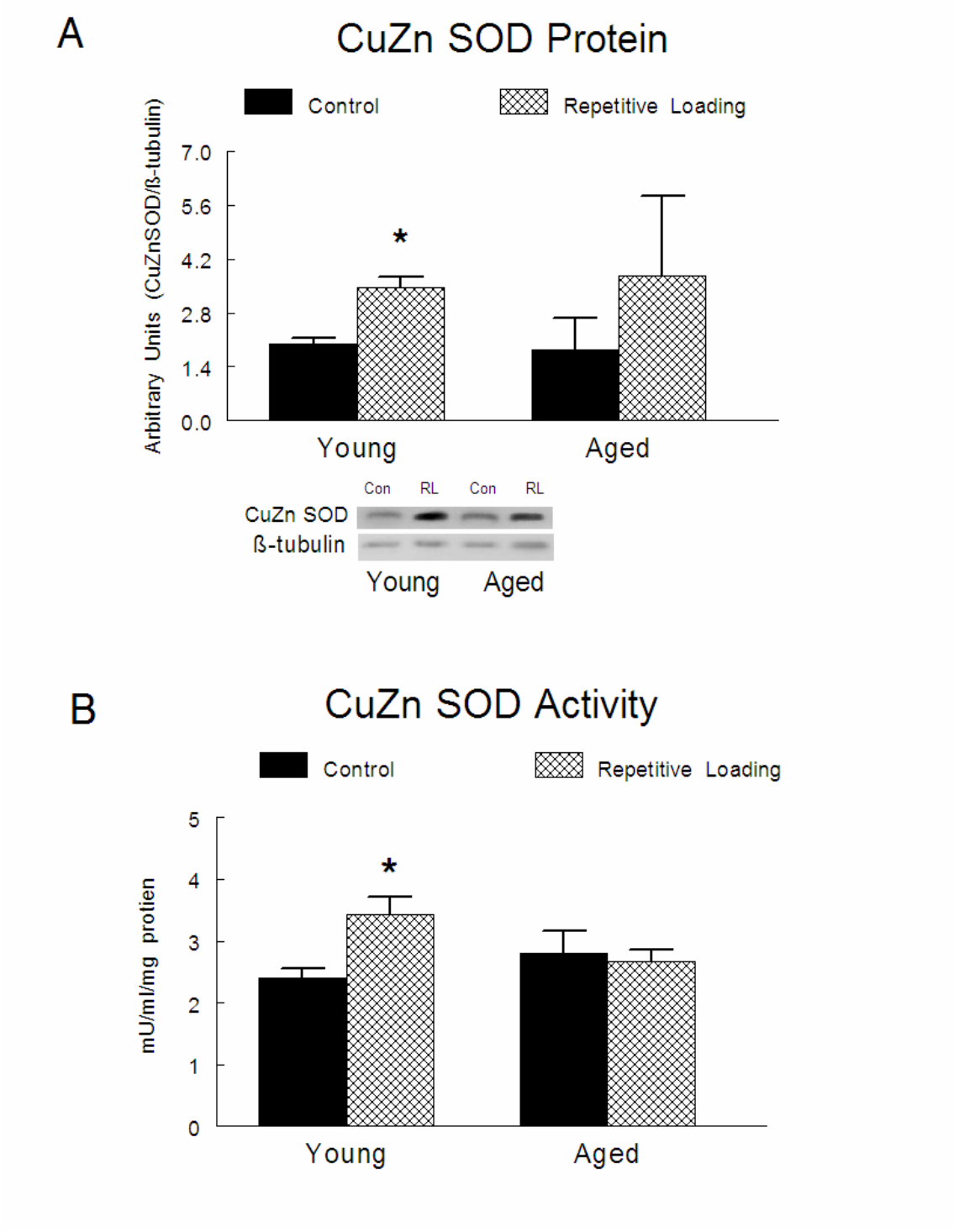
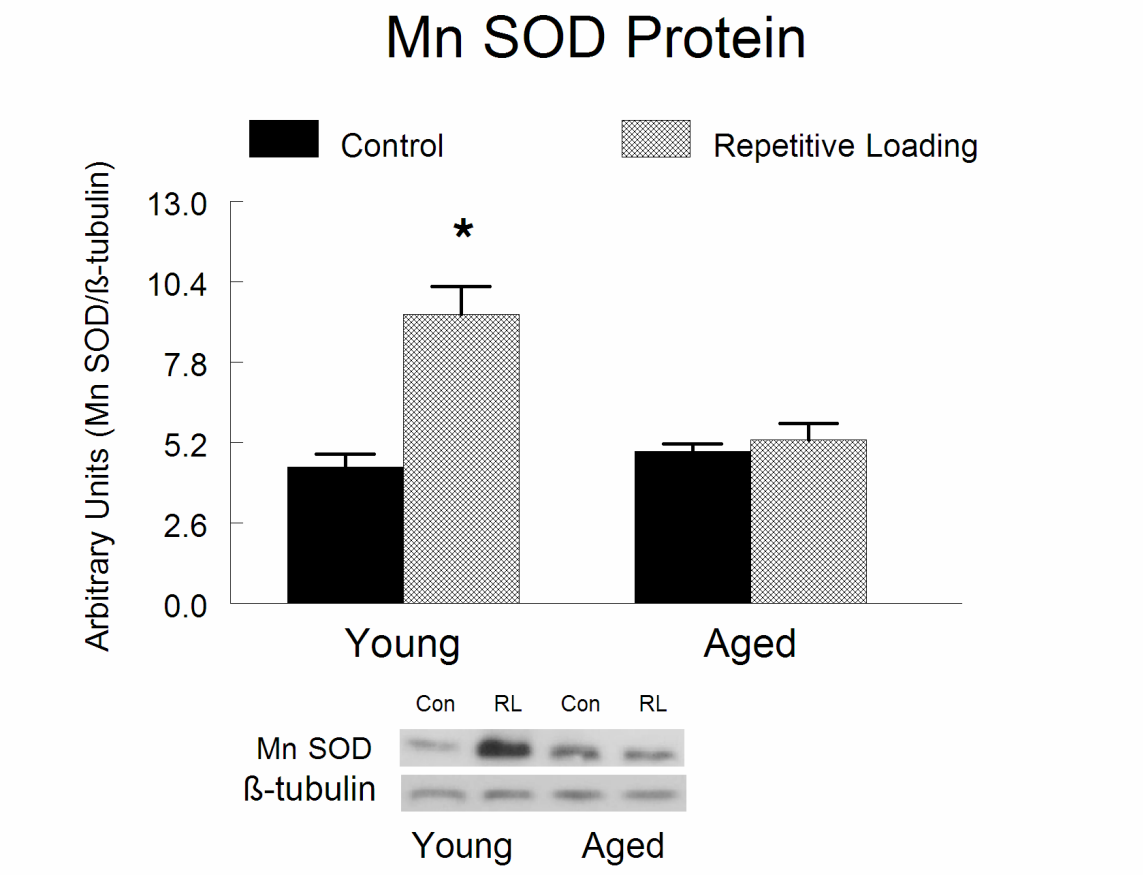

A
CuZn SOD Protein
Control
Repetitive Loading
Arbitrary Units (CuZnSOD/ß-tubulin)
7.0
5.6
4.2
2.8
1.4
0.0
*
Young
Aged
Con
RL
Con
RL
CuZn SOD
ß-tubulin
Young
Aged
B
CuZn SOD Activity
Control
Repetitive Loading
mU/ml/mg prolien
5
4
3
2
1
0
*
Young
Aged

Figure 3.10


Mn SOD Protein
Control
Repetitive Loading
Arbitrary Units (Mn SOD/ß-tubulin)
13.0
10.4
7.8
5.2
2.6
0.0
*
Young
Aged
Con
RL
Con
RL
Mn SOD
ß-tubulin
Young
Aged

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Chapter 4

Vitamin E and C supplementation reduces oxidative stress and improves antioxidant enzymes and positive muscle work in chronically loaded dorsiflexor muscles of aged rats

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Abstract

Aging is associated with increased oxidative stress that can be further elevated in skeletal muscle levels of oxidative stress are further elevated with exercise. The purpose of this study was to determine if dietary antioxidant supplementation would improve muscle function and cellular markers of oxidative stress in response to chronic repetitive loading in aging. The dorsiflexors of the left limb of aged and young adult Fischer 344 Brown x Norway rats were loaded 3 times a week for 4.5 weeks using 80 maximal stretch-shortening contractions per session. The contralateral limb served as the intra-animal control. The rats were randomly assigned to a diet supplemented with Vitamins E&C or normal non-supplemented rat chow. Biomarkers of oxidative stress were measured in the tibialis anterior muscle. Repetitive loading increased the muscle wet weight in all groups and maximal isometric force, negative and positive work in the young adult tibialis anterior muscle. Only positive work increased in the aged animals that were supplemented with Vitamin E&C. Markers of oxidative stress (H_2O_2, GSH/GSSG ratio, malondialdehyde and 8-OHdG) increased in the tibialis anterior muscles from aged and young adult animals with repetitive loading, but Vitamin E&C supplements attenuated this increase. MnSOD activity increased with supplementation in the young adult animals. CuZnSOD and catalase activity increased with supplementation in young adult and aged animals and GPx activity increased with exercise in the non-supplemented young adult and aged animals. The increased levels of endogenous antioxidant enzymes after Vitamin E&C supplementation appear to be regulated by post-transcriptional modifications that are affected differently by age, exercise and supplementation. These data suggest that antioxidant supplementation improves indices of oxidative stress associated with repetitive loading exercise and aging and improve the positive work output of muscles in aged rodents.

Introduction

Aging causes deleterious modifications at genetic, cellular, tissue, and system levels in all organisms. Presently, the fundamental mechanisms of aging are poorly understood, but a growing body of evidence supports the idea that oxidative stress is an important contributing factor to deterioration of organ and cell function that is associated with aging (2, 13, 17, 38, 43).

The age-associated loss of skeletal muscle mass and strength (i.e. sarcopenia), is an unavoidable part of aging. Sarcopenia is likely mediated, at least in part, by a lifetime of damage from oxidants. This is likely because aging is associated with an increase in oxidant production and a decrease in the capacity to buffer oxidants, resulting in a chronic state of oxidative stress. Oxidative stress can damage biomolecules (DNA, lipids and proteins), decrease muscle protein synthesis, elevate apoptotic signaling and protein degradation (13). Although exercise is one approach that may counterbalance sarcopenia, oxidative stress that is developed during muscle contractions may limit the ability of muscle from aged animals to hypertrophy in response to exercise (6, 28, 38).

Vitamin E (i.e., α-tocopherol) and Vitamin C (i.e., ascorbic acid) are antioxidants that are thought to have a protective effect by either reducing or preventing oxidative damage. Lipid soluble Vitamin E prevents lipid peroxidation chain reactions in cellular membranes by interfering with the propagation of lipid radicals. Vitamin C is a water-soluble antioxidant found in the cytosol and extracellular fluid that can interact directly with free radicals, thus preventing oxidative damage (5). Due to their different sub-cellular locations, a combination of Vitamins E and C has been shown to have a better antioxidant effect than either of the two vitamins alone (35, 37).

Oxidants generated near cellular membranes can oxidize Vitamin E forming a tocopheroxyl radical. Vitamin C may reduce the Vitamin E radical, thereby regenerating Vitamin E. This reaction forms the semi-dihydroascorbate (Vitamin C radical), which in turn is reduced by a glutathione (GSH)(37). Rodents (7) and humans (3) that are deficient in Vitamin E show massive increases in pro-oxidant production and lipid peroxidation after exercise. Furthermore, low plasma concentrations of Vitamin E, associated with nutritional deficiencies often seen in the elderly (16, 25), have been shown to contribute to a decline in physical function within these individuals (3). In contrast, dietary supplementation of Vitamin E has been shown to increase tissue resistance to exercise-induced oxidative damage (19, 23, 30). In addition, recent data suggests that antioxidant supplementation can stimulate muscle protein synthesis in aged rats, possibly through the protection of leucine metabolism (27). Furthermore, Vitamin E and C supplementation combined with resistance training has been shown to both increase fat free mass and muscle mass index in older adults more than resistance training alone (24).

Indicators of oxidative stress (lipid peroxidation measured by malondialdehyde (MDA)) have been shown to increase immediately after heavy resistance training in humans (30). MDA levels returned to baseline in subjects who consumed a diet that was supplemented with Vitamin E, whereas MDA levels continued to be elevated 24 hours after resistance exercise in the non-supplemented subjects (30). However, this is not a universal finding because dietary supplementation with Vitamin E does not completely protect elderly men from oxidative damage caused by exercise (1). This may be due to low levels of Vitamin C in the elderly, which could reduce the effectiveness of Vitamin E to protect against exercise-induced damage in the elderly (1). Thus, both Vitamins E and C may be important for effectively protecting muscles in aged people against oxidative damage.

Vitamin C is a highly effective water-soluble antioxidant primarily found in the cytosol and extracellular fluid. Even in small amounts, Vitamin C can protect proteins, lipids, carbohydrates, and nucleic acids from damage by pro-oxidants generated during normal metabolism. Vitamin E and glutathione also rely on Vitamin C for restoration back to their reduced isoforms. Vitamin C supplementation has been reported to have a protective effect against exercise-induced muscle damage (20). Though some studies have shown that taking antioxidants such as Vitamins E and C will prevent damage to tissues by reducing oxidant production, chronic use of these antioxidants could hinder the positive adaptive response that exercise has on the endogenous antioxidant defense system (36). It is not clear if Vitamin C has a direct role in muscle recovery from exercise, or if it has an indirect role in this process.

Resistance training has been shown to be an effective means of increasing muscular size and strength, although the extent of the increase is attenuated with aging. However, repetitive mechanical resistant-type loading exacerbates oxidative stress in muscles of aged rodents, whereas it appears to be well buffered in muscles of young adult animals (38). Oxidative stress increases in skeletal muscle after acute exercise; however, chronic exercise enhances the endogenous antioxidant defenses and decreases production of pro-oxidants resulting in lower indices of oxidative stress. Previous data have shown that aging reduces the adaptive capacity of muscle to buffer the increased oxidant production imposed by chronic repetitive loading. The reduced buffering capacity may compromise the muscles' abilities to hypertrophy and/or to improve muscle function in aged animals.

Although more work is needed in this area, the combined data suggest that antioxidant supplementation may be a potential strategy for reducing exercise-induced oxidative stress and reduce sarcopenia in the elderly. However, this is not a straight forward issue, because although Vitamin E and C supplementation will reduce oxidative stress post exercise, the reduction in oxidative stress may inhibit redox sensitive pathways that are associated with the positive adaptation to exercise.

Previous work suggests that there is only a partial ability for endogenous antioxidant enzymes to compensate for the increased oxidative insult in tibialis anterior muscles of aged rats in response to chronic repetitive loading as compared to young adult animals (38). The current study tested the hypothesis that dietary supplementation with Vitamins E and C would lessen oxidant activity and oxidative damage in tibialis anterior muscles of aged rats subjected to chronic repetitive loading. Furthermore, in this study we assessed whether dietary supplementation with Vitamins E and C would attenuate the increase in basal levels of oxidative stress associated with aging allowing for a more complete adaptation in oxidative enzymes and improvements in muscle function after 4.5 weeks repetitive loading in the aged rats.

Methods

Experimental design. The left tibialis anterior muscles of young (12 weeks of age; n=14) and old (30 months of age; n=14) Fischer 344 Brown x Norway rats were subjected to repetitive loading exercise. Seven animals from each age group were randomly assigned to a diet supplemented with Vitamin E (30,000 mg/kg) and Vitamin C (2% by weight), or normal non-supplemented (NS) rat chow containing 126 mg/kg of Vitamin E and 0% Vitamin C. All animals had free access to rat chow and water. The non-supplemented animals were a subset of animals described in another study (38). All experimental procedures carried 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 (AAALAC) and fully conformed to the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings."

Muscle function. Maximal isometric muscle force, positive work, and negative work were assessed in the left exercised and right control limbs on a custom-built dynamometer (9). The dorsi flexor muscle group was activated indirectly through electrical stimulation of the common peroneal nerve via platinum stimulating electrodes (Grass Medical Instruments, Quincy MA, USA). Muscle stimulation for all protocols was a 120 Hz square wave pulse at 200 μs pulse duration, and 4 volts. Dorsiflexor isometric force was measured at an ankle angle of 90 deg using a 300 ms stimulation duration. Positive and negative work was calculated from stretch-shortening contractions (9). The stretch-shortening contraction was performed by activating the dorsiflexor muscles for 300 ms then moving the load cell fixture from 70° to 140° at an angular velocity of 500°/s. The load cell fixture was immediately returned to 70°. Activation was continued for 300 ms after cessation of the movement. The change in force output over a training session was assessed by averaging the first three sessions as a pre value and the last three sessions as a post value and calculating the percent difference between the two.

Unilateral repetitive loading exercise. Repetitive loading consisted of 3 sessions per week for 4.5 weeks, of 80 stretch/shortening (i.e., eccentric / concentric) contraction cycles per session (9). This method has been previously shown to produce a hypertrophic response in young adult rats (9) although aging attenuates the hypertrophic response to these loading conditions (9, 38).

Muscle preparation. Forty-eight hours after the last exercise session, the tibialis anterior of both loaded and control limbs were removed with the animal under anesthesia (2% isoflurane). The rats were then euthanized, via an overdose of ketamine/xylazine (30%/70%, v/v). The tibialis anterior muscles were washed in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic and a pH of 7.4), blotted dry then weighed. A section of the muscle was obtained for the determination of the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). The remaining muscle was snap frozen in liquid nitrogen and stored at -80°C.

RNA Isolation. Sixty micrograms of frozen muscle was homogenized in 1ml of Tri-Reagent (Molecular Research Center, Cincinnati, OH) with a motorized blade homogenizer. The RNA was isolated according to our standard procedures (31, 38). The RNA was treated with DNAse I using a DNA-free kit (Ambion, Austin, TX) and quantified using a BioRad SmartSpec 3000. The RNA samples were quantified if their 260:280 ratio was 1.7 or greater.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Two micrograms of total RNA were reversed transcribed using 1.0μl of random primers, 1.0μl of 10mM dNTP, and 1.0μl of SuperScript II reverse transcriptase (Invitrogen/Life Technologies, Bethesda MD) as previously described (38). The resulting complimentary DNA (cDNA) was stored at -80˚C or used for PCR analyses.

Primers for the genes of interest were designed as follows: CuZnSOD sense-5'-AGGCCGTGTGCGTGCTGA-3'; anti-sense-5'-CCCAATCACACCACAAGCCA-3'; GPx-1 sense-5'-CCTCGTGGCCTGGTGGTCCT-3'; anti-sense-5'-AGGGGTTGCTAGGCTGCTTGGA-3'. The primers for MnSOD and catalase were the same as previously published by our lab (38). Preliminary experiments were conducted to ensure that the number of PCR cycles were completed in the linear range of amplification for each gene of interest. PCR products were

verified by restriction digestion based on predicted PCR sequences. Routine PCR amplification was conducted using PCR buffer, MgCl2, 5mM dNTPs, 100ng/ml of primer pairs, 18S primer pairs, 1.0µl of Taq DNA polymerase, and1.0 µl of cDNA (38). Amplification of PCR products were performed in a thermocycler using: a denaturing step at 95˚C for 45s, an annealing step for 45s, and an extension step at 72˚C for 45s. 20µl of each PCR product was separated by electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide to visualize the PCR products. The signal from each PCR gene product was expressed as a ratio to the 18S signal from the same PCR product. The PCR signals were recorded via a digital camera (Kodak 290) and the signals were quantified in arbitrary units as optical density x band area, using 1D Kodak image analysis software (Eastman Kodak Company, Rochester, NY).

Muscle Protein Fractionation. Cytoplasmic and nuclear protein fractions were obtained from 75 mg of frozen tibialis anterior using methods as reported previously by our lab (38, 42). Muscle samples were homogenized in 500 µl of ice-cold lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 20mM HEPES at pH 7.4, 20% glycerol, 0.1% Triton X-100, and 10µM dithioreitol) with a mechanical homogenizer. A lower concentration of dithioreitol was used than in previous studies (38, 42), to prevent interference with subsequent enzyme activity assays. Muscle homogenates were centrifuged at 800 rpm for 5-minutes at 4°C. The supernatants were collected and centrifuged three times at 3500g for 5-minutes at 4°C. The resulting supernatant was collected as the nuclei-free cytosolic fraction and divided into two equal portions; the first portion was frozen at -80°C until needed, and a protease inhibitor cocktail containing 104mM 4-[2-aminoethyl]-benzenesulfonylflouride hydrochloride (AEBSF), 0.8mM aprotinin, 2mM leupeptin, 4mM bestatin, 1.5 mM pepstatin A and 1.4 mME-64 (Sigma-Aldrich, St. Louis, Mo, USA) was added to the second portion before it was frozen at -80°C. Protein concentrations for each sample were determined in triplicate via a DC protein concentration assay (Bio-Rad, Hercules, CA). The cytosolic fraction was used in the following assays: H_2O_2 concentration, catalase activity, GPx activity, CuZnSOD and MnSOD activity and western immunoblots.

Western immunoblots. The protein content of glutathione peroxidase-1 (GPx-1), catalase, copper-zinc superoxide dismutase, (CuZnSOD) and manganese superoxide dismutase (MnSOD) was measured in the cytosolic protein fractions. Thirty µg of protein was loaded into each well of a 4-12% gradient polyacrylamide gel (Novex, Invitrogen) and separated by routine SDS-polyacrylamide gel electrophoresis (PAGE) for 1.5 hours at 20°C and transferred to a nitrocellulose membrane. The membranes were blocked in 5% non-fat milk protein (NFM) for 1-hour at room temperature then incubated in appropriate dilutions of primary antibodies (diluted in 1% NFM in Tris-buffered saline with 0.05% Tween-20 (TBS-T) overnight at 4°C. The membranes were washed in TBS-T followed by incubation in appropriate dilutions of secondary antibodies (diluted in 5% NFM in TBS-T) that were conjugated to horseradish peroxidase. The protein 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 captured by a Kodak 290 camera and protein bands quantified using 1-D analysis software (Eastman Kodak, USA). The bands were quantified as optical density (OD) x band area and expressed in arbitrary units.

Hydrogen peroxide (H_2O_2) levels. A fluorescent H_2O_2 detection kit (Cell Technology, Mountain View, CA) was used to determine the amount of H_2O_2 in the muscle tissue. Reagents and standards were prepared as recommended by the manufacturer. Briefly, 50µL of controls, samples, or H_2O_2 dilutions were mixed with 50µL of the reaction cocktail in each well to initiate the reaction. The plate was incubated in the dark for 10 minutes, at 20°C and fluorescence was detected with an excitation at 530nm and measured at 590nm. All analyses were done in

duplicate and the samples were normalized to muscle protein concentration in each sample via a DC protein concentration assay (Bio-Rad, Hercules, CA).

GSH and GSH/GSSG Ratio. Glutathione (GSH), oxidized glutathione (GSSG) and the GSH/GSSG ratio were determined by a BIOXYTECH GSH/GSSG-412 (Oxis International, Beverly Hills, CA) assay. Muscle tissue (~40 mg) was homogenized immediately after dissection in 530 µl cold 5% metaphosphoric acid (MPA) for the GSH sample and for the GSSG sample ~40 mg of muscle tissue was homogenized immediately after dissection in 500 µl cold 5% metaphosphoric acid (MPA) and 30µl of M2VO scavenger. Homogenates were then frozen in liquid nitrogen and stored at -80°C until analyzed.

The assay was preformed as described by the manufacturer. Briefly, cold 5% MPA was added to each sample mixed, and centrifuged at 1000 x g for 10 minutes. 50µl of sample and the appropriate buffer and 50µl of chromogen and enzyme were mixed and incubated at room temperature. 50µl of NADPH was added and the absorbance of each sample was read every 60 sec at 412 nm for three minutes. The protein concentration for each sample was determined via a DC protein concentration assay (BIO RAD). Signals from each sample were normalized to the corresponding protein content of that sample.

Oxidative DNA damage as measured by 8-hydroxy-2'-deoxyguanosine (8-OHdG). DNA was extracted from the muscle via DNeasy Tissue Kit (Qiagen, Valencia, CA). DNA was used if it had a minimum 260:280 ratio of 1.8. Oxidized DNA was determined on 50 µl of DNA by a BIOXYTECH 8-OhdG ELISA (enzyme linked immunoassay) according to the manufacturer's recommendations (Oxis International, Beverly Hills, CA). Briefly, DNA was incubated with the primary antibody, washed, and then incubated in secondary antibody. The chromogen was added to each well, and incubated at room temperature in the dark for 15 minutes. The reaction was terminated and the samples were read at an absorbance of 450 nm. Samples were normalized to the DNA concentration measured via a spectrophotmeter (ND-1000, NanoDrop, Wilmington, DE). All analyses were done in duplicate.

Lipid peroxidation. Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were measured as an indication of lipid peroxidation using the method and reagents from Oxis International, CA (BIOXYTECH LPO-586). Briefly, ~100 mg of muscle was homogenized in ice-cold PBS, containing 5 µL 0.5 M butylated hydroxytoluene (BHT) in acetonitrile per 1 ml of tissue homogenate. The muscle homogenate was centrifuged at 3000 g at 4°C and the supernatant was used for the assay and protein determination. The muscle sample was incubated in the appropriate reagents according to the manufacturer's instructions, and centrifuged at 15,000 g. An absorbance reading of the supernatant was obtained at 586nm. Samples were normalized for differences in the amount of muscle protein in each sample as determined by a DC protein concentration assay (Bio-Rad, Hercules, CA).

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

Manganese Superoxide Dismutase (MnSOD) and Copper-Zinc Superoxide Dismutase (CuZnSOD). A commercially available assay (#574601, EMD/Calbiochem, San Diego, CA) was used to measure total SOD and MnSOD activity. CuZnSOD was calculated by

subtracting the value for MnSOD activity from the total SOD activity. The assay was performed with modifications to the manufacturer's directions and all samples and standards were measured in duplicate. Briefly, the muscle was homogenized in 20mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210mM mannitol, and 70 mM sucrose and centrifuged at 1000g for 10 minutes. The assay was performed in a 96-well plate with each sample being treated with and without 10µL of 12 mM potassium cyanide. Potassium cyanide was used to inhibit CuZnSOD, resulting in the detection of only MnSOD activity. The reagents and samples were protected from white light and incubated at 26°C for 20 minutes with periodic shaking. The absorbance was measured at 450 nm using a 96-well plate reader (Dynex Tech., Chantilly VA., USA).

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

Statistical analyses. Statistical analyses were performed using an SPSS 18.0 software package. Statistical significance of the data was calculated by a multiple analyses of variance (MANOVA). When significant F scores were indentified from the MANOVA, subsequent protected one-way analysis of variance followed by Tukey post-hoc tests were used to identify differences between means. Statistical significance was accepted at $p < 0.05$. Data are reported as mean ± standard error mean (SEM).

Results

Body Weight. The average body weight of the aged animals was significantly ($p<0.05$) more than the young animals. A subset of the control non-supplemented animals used in this study was included in data reported previously (38). The body weight characteristics of all of the animals examined in this study are shown in Table 4.1. Bodyweight did not change over the course of the study in either the control or Vitamin E&C supplemented animals.

Insert Table 4.1

Muscle Wet Weight. Repetitive loading for 4.5 weeks resulted in a significant increase in tibialis anterior muscle wet weight in the exercised limb of both the young adult (690 ± 20 mg vs. 780 ± 40 mg, $p<0.05$) and the aged adult (670 ± 30 mg vs. 720 ± 30 mg, $p<0.05$) non-supplemented rats as compared with the contra-lateral control muscle. Similar results were observed in the exercised limb from Vitamin E&C supplemented rats, where the muscle wet weight of the tibialis anterior muscle from young adult rats increased 17.8% (720 ± 30 mg vs. 850 ± 40 mg, $p<0.05$) and 7.7% (670 ± 30 vs. 720 ± 20 mg, $p<0.05$) in the aged adults (Figure 4.1).

Insert Figure 4.1

Muscle Function. Maximal force, positive work and negative work were greater in control non-exercised muscles from young adult compared to aged rats. Maximal isometric force increased ($p<0.05$) by 48% in the young adult non-supplemented (0.053 ± 0.003 N/g body

weight vs. 0.082 ± 0.03 N/g body weight) and 40% Vitamin E&C supplemented (0.055± 0.007 N/g body weight vs. 0.079 ± 0.006 N/g body weight) animals (Figure 2A) after the 14 repetitive loading sessions. Negative work was also similarly increased ($p<0.05$) by 35.7% in the young adult non-supplemented (2.93 ± 0.29 J/g body weight vs. 4.11 ± 0.22 J/g body weight) and 31.9% Vitamin E&C supplemented (2.92 ± 0.21 J/g body weight vs. 4.03 ± 0.36 J/g body weight) animals at the end of the training period. Positive work increased ($p<0.05$) in the trained vs. control muscles of both the young adult non-supplemented (35.9%)(2.42 ± 0.13 J/g body weight vs. 3.45 ± 0.14 J/g body weight) and Vitamin E&C supplemented (30.6%) (2.51 ± 0.37 J/g body weight vs. 3.39 ± 0.2 J/g body weight 9) rats. Furthermore, positive work increased by 37.7% in the control non-exercised muscles of aged rats that consumed the Vitamin E&C supplemented diet (1.54 ± 0.3 J/g body weight vs. 2.34 ± 0.24 J/g body weight) compared to non-supplemented animals.

Maximal isometric force and negative work were unchanged by exercise training in aged rats in either dietary group (Figure 4.2A and 4.2B). Positive work improved in the aged animals that consumed the Vitamin E&C diet, but it did not change in the muscles of the aged non-supplemented animals during the training period (Figure 4.2C).

Insert Figure 4.2

H_2O_2. Muscle levels of H_2O_2 were elevated in the loaded muscles by 37.7% in young adult (543 ± 35 RFU/mg protein vs. 748 ± 45 RFU/mg protein) and 44.8% in aged muscles of the non-supplemented animals (696 ± 46 RFU/mg protein vs. 1008 ± 75 RFU/mg protein), suggesting a treatment effect and that chronic loading elevated oxidative stress. Vitamin E&C supplementation lowered H_2O_2 in both control and repetitively loaded tibialis anterior muscle from young adult and aged rats (Figure 4.3). H_2O_2 concentration data expressed as a µmol/mg protein are discussed in the Limitation and Future Directions section within chapter 7. (Figure 7.2)

Insert Figure 3

Total Glutathione. Total glutathione in the tibialis anterior muscle was 33% lower in the non-exercised muscles of aged as compared with young adult rats ($p<0.05$). Vitamin E&C lowered the total glutathione concentration by 41% in the non-exercised control muscles from young adult animals (243 ± 46 µM/mg protein vs. 141 ± 18 µM/mg protein), but it did not alter total glutathione levels in non-exercised muscles of aged animals (Figure 4.4A). In the young adult non-supplemented animals, muscle levels of total glutathione were not improved with exercise training, however, exercise training increased total glutathione by 43% as compared to control muscles in young adult animals that were fed the vitamin E&C supplemented diet (141 ± 18 vs. 203 ± 10 µM/mg protein) (Figure 4.4A). In contrast, Vitamin E&C supplementation did not alter total glutathione in exercised muscles from aged rats (Figure 4.4A).

GSH/GSSG ratio. The GSH/GSSG ratio was not increased by training in muscles from young adult non-supplemented animals when comparisons were made to the control muscles (Figure 4B). However, the tibialis anterior muscles from the aged non-supplemented animals had a significant reduction in the GSH/GSSG ratio in the loaded ($p<0.05$) muscles as compared to exercise-matched muscles in the young adult animals. The GSH/GSSG ratio was lower ($p<0.05$) in the loaded and control muscles of the aged non-supplemented rats as compared to the young adult non-supplemented rats. The combination of Vitamin E&C supplementation and

repetitive loading significantly increased ($p<0.05$) the GSH/GSSG ratio by 36.1% in exercised tibialis anterior muscles from the young adult (16.9 ± 1.4 vs. 23 ± 2) animals as compared to the intra-animal control muscles. Vitamin E&C supplementation did not improve GSH/GSSG in the unexercised control muscles from young adult or aged animals (Figure 4.4B).

Insert Figure 4.4

Lipid Peroxidation. Aging increased the level of lipid oxidation by 79.6% as shown by greater MDA + HAE levels in control muscles from aged non-supplemented rats as compared to muscles from young adult non-supplemented animals (1.67 ± 0.2 µM/mg protein vs. 2.99 ± 1.7 µM/mg protein, $p<0.01$; Figure 4.5A). Repetitive loading appeared to activate adaptive responses in muscles of aged non-supplemented animals because MDA + HAE levels were lower in the loaded than control muscles of aged animals. Furthermore, Vitamin E&C supplementation suppressed the increase in lipid peroxidation associated with aging, but a combination of exercise and supplementation showed no greater decline in lipid peroxidation than either treatment did individually. No significant difference in MDA + HAE levels was observed among the control or chronically loaded muscles of either the non-supplemented or Vitamin E&C supplemented young adult animals, or the aged Vitamin E&C supplemented animals.

DNA Damage. The aged muscle showed a 33.4% increase ($p<0.05$) in the basal level of oxidative DNA damage (2.86 ± 0.28 ng/µg DNA vs. 3.82 ± 0.2 ng/µg DNA) as indicated by the increase in 8-OHdG detected in control muscles from aged non-supplemented animals as compared to the control muscles from young adult non-supplemented animals. Chronic repetitive loading did not change 8-OHdG in muscles of young adult (non-supplemented or Vitamin E&C) or aged non-supplemented animals. In young adult animals, Vitamin E&C supplementation lowered 8-OHdG levels in control muscles by 38.4% (2.86 ± 0.28 ng/µg DNA vs. 1.76 ± 0.2 ng/µg DNA) and by 19.9% in exercised muscles (2.59 ± 0.33 ng/µg DNA vs. 2.08 ± 0.28 ng/µg DNA). In aged rats, Vitamin E&C reduced 8-OHdG levels in control muscles by 40.1% (3.82 ± 0.2 ng/µg DNA vs. 2.29 ± 0.28) and by 20.4% in exercised muscles (4.13 ± 0.16 ng/µg DNA vs. 3.29 ± 0.29 ng/µg DNA) when compared to animals that consumed the non-supplemented control diet (Figure 4.5B).

Insert Figure 4.5

Glutathione Peroxidase (GPx).

***Enzyme Activity*.** There was a loading effect but no age effect on GPx activity in the rat non-supplemented tibialis anterior muscle. Chronic repetitive loading significantly increased GPx activity in muscles from both young adult (123%; 52.1 ± 13.1 mU/mg protein vs. 116.2 ± 18.3 mU/mg protein) and aged non-supplemented animals (71.8%; 67.5 ± 21 mU/mg protein vs. 116 ± 18.5 mU/mg protein). GPx activity was similar in muscles from non-supplemented young and aged rodents. Vitamin E&C supplementation lowered total GPx activity in the tibialis anterior muscle from both control (60%; 52.1 ± 13.1 mU/mg protein vs. 23 ± 6.3 mU/mg protein) and exercised muscles (194%; 116.2 ± 18.3 mU/mg protein vs. 45.6 ± 13 mU/mg protein) from young adult rats. GPx activity was also reduced in control (48%; 67.5 ± 21 mU/mg protein vs. 34.7 ± 8 mU/mg protein) and exercised muscles (100%; 116 ± 18.5 mU/mg protein vs. 33.4 ± 13 mU/mg protein) from aged animals. Repetitive loading exercise increased GPx activity in the muscles from young rats in the Vitamin E&C supplementation group, but had it no additive effect in the muscles from aged supplemented animals (Figure 4.6A).

GPx protein abundance. Protein levels for GPx-1 increased with Vitamin E&C supplementation in both young adult and aged muscle. Exercise did not have a significant effect on GPx-1 protein concentrations (Figure 4.6B).

Changes in GPx mRNA. No significant differences were found among GPx-1 mRNA within any of the non-supplemented muscle samples. There was a 41.8% reduction in GPx-1 mRNA expression in the Vitamin E&C supplemented tibialis anterior muscle from young control muscle, which was reversed in the exercised muscle from young Vitamin E&C supplemented animals. Vitamin E&C supplementation reduced GPx-1 mRNA expression in both control and exercised muscles from aged animals (Figure 4.6C).

Insert Figure 4.6

Catalase.

***Enzyme Activity*.** Catalase activity was significantly greater in the tibialis anterior muscle from all groups of the aged animals when compared to their treatment matched young adult counterparts. Repetitive loading did not significantly alter catalase activity in muscles from the non-supplemented young adult rats or aged rats, nor did it alter catalase activity in the muscle from young adult animals that were feed the Vitamin E&C supplemented diet. Catalase activity was significantly higher in control muscles of aged vs. young adult animals, and it increased in loaded muscles of aged animals as compared to young adult animals ($p<0.05$) (Figure 4.7A).

***Catalase protein abundance*.** Repetitive loading exercise did not alter catalase protein abundance in the tibialis anterior muscles of any group. Similarly, catalase protein abundance was not altered by aging within the non-supplemented animals. Vitamin E&C supplementation increased catalase protein content by ~60% in the young adult control muscles as well as the aged control (~292%) and exercised (~246%) muscles when compared to their age-matched non-supplemented counterparts as determined by western blot analyses (Figure 4.7B).

Changes in catalase mRNA. Catalase mRNA was significantly greater in muscles of young vs. aged rats. Exercise did not produce any significant changes in muscle catalase mRNA levels within the any of the animal groups. Supplementation with Vitamin E&C reduced catalase mRNA levels from both control and exercised tibialis anterior muscle in the young adult by 24.7% and 22.6%, respectively. In a similar fashion to young animals, catalase mRNA expression was 44.4% and 43.9%, lower in control and exercised muscles, respectively, of Vitamin E&C treated aged animals (Figure 4.7C).

Insert Figure 4.7

Copper-Zinc Superoxide Dismutase (CuZnSOD)

Enzyme Activity. Repetitive loading increased CuZnSOD enzyme activity ($p<0.05$) by 64.1% (2.34 ± 0.33 mU/mg protein vs. 3.84 ± 0.5 mU/mg protein in non-supplemented and 35.7% (8.33 ± 1.2 mU/mg protein vs. 11.31 ± 2.18 mU/mg protein) in Vitamin E&C supplemented muscles from young adult animals as compared to the contra-lateral control muscles. No significant changes in enzyme activity were observed as a result of repetitive loading in the tibialis anterior muscle from aged animals. Surprisingly, Vitamin E&C supplementation increased CuZnSOD enzyme activity in control (255%; 2.34 ± 0.33 mU/mg protein vs. 8.33 ± 1.2 mU/mg protein) and repetitive loaded tibialis anterior muscles (195%; 3.84

± 0.5 mU/mg protein vs. 11.31 ± 2.18 mU/mg protein) of young animals. CuZnSOD enzyme activity was increased (20%; 4.32 ± 1.1 mU/mg protein vs. 5.2 ± 0.75 mU/mg protein) in repetitively loaded muscles of aged rats as compared to muscles that were obtained from non-supplemented old animals (Figure 4.8A).

CuZnSOD protein abundance. CuZnSOD protein abundance was not different between muscles obtained from non-supplemented or Vitamin E&C supplemented young adult rats. CuZnSOD protein abundance was 118% greater ($p<0.05$) in non-exercised Vitamin E&C supplemented compared to non-supplemented control muscles from aged animals (1.88 ± 0.85 vs. 4.12 ± 0.89) (Figure 4.8B).

Changes in CuZnSOD mRNA. CuZnSOD mRNA content was lower in both non-exercised and exercised muscles of aged rats in the Vitamin E&C supplemented group compared to aged rats in the non-supplemented group or in young adult animals of either diet group (Figure 4.8C).

Insert Figure 4.8

Manganese Superoxide Dismutase (MnSOD)

Enzyme Activity. Vitamin E&C supplementation significantly increased MnSOD activity levels within both the control and repetitive loaded tibialis anterior muscle from the young adult animals. No changes in MnSOD activity were found in muscles from the aged animals (Figure 4.9A).

MnSOD protein abundance. MnSOD protein levels were 113% greater ($p<0.05$) in the repetitive loaded muscles of the young adult non-supplemented animals as compared to their contra-lateral control muscles ($p<0.05$). Nevertheless, aging prevented any loading-induced increase in MnSOD protein abundance in the tibialis anterior muscle. Young and aged animals that were given the Vitamin E&C diet had greater MnSOD protein abundance in the non-exercised control muscle as compared to non-exercised muscles from non-supplemented animals. Repetitive loading decreased MnSOD protein abundance in the supplemented animals from both age groups (Figure 4.9B).

Changes in MnSOD mRNA. The changes that were observed in MnSOD protein levels did not seem to be driven by the alterations in MnSOD mRNA. The only significant changes in MnSOD mRNA levels were found in the aged Vitamin E&C supplemented animals, where supplementation lowered mRNA levels in the tibialis anterior muscles from exercise control and repetitively loaded limbs (Figure 4.9C).

Insert Figure 4.9

Discussion

Oxidative damage has long been implicated as a factor that progresses the aging process (17). Oxidative damage occurs in both type I and type II fiber types of aged animals (12), and is thought to underlie at least part of the deterioration in skeletal muscle with aging (4, 38). The aim of this study was to investigate the efficacy of dietary antioxidant supplementation to improve oxidative stress in skeletal muscle in aged rodents in response to repetitive loading. We chose to examine the tibialis anterior muscle, because it is composed of predominantly type II fibers, which are thought to be more susceptible to oxidative stress than type I fibers because their antioxidant defenses are less extensive. It was hypothesized that dietary supplementation with Vitamin E&C would lessen the oxidant activity and oxidative damage in tibialis anterior muscles in an age-dependent manner. The sub-hypothesis was that Vitamins E&C supplementation would attenuate the increase in basal levels of oxidative stress associated with aging, allowing for improved adaptation in oxidative enzymes and muscle function after 4.5 weeks of repetitive loading in the aged rats. The novel results of this investigation show that dietary supplementation with Vitamins E&C reduced oxidant levels in repetitively loaded muscles of aged rats, but there was no improvement in accumulation of muscle mass in the tibialis anterior muscle over 4.5 weeks of loading. Specifically, fortifying normal rat chow with Vitamin E&C, lowered the concentrations of H_2O_2, increased the ratio of GSH/GSSG, reduced indices of oxidative damage to DNA (8-OHdG) and cellular lipids (malondialdehyde & 4-hydroxyalkenals) in repetitively loaded tibialis anterior muscles from young adult and aged rats. Vitamin E&C supplementation did not improve maximal force production after more than four weeks of repetitive loading in muscles of aged animals. However, importantly, positive work output improved after exercise training in the dorisflexors of aged animals that received the Vitamin E&C supplemented diet.

Age-Related Adaptations of Muscle Weight to Repetitive Loading. Tibialis anterior muscle mass increased in response to 4.5 weeks of repetitive loading in aged rats (38), but the exercise-trained muscle in the aged animals only improved to a point that was similar to control untrained levels in the young adult animals. These results are consistent with previous observations in rodents using the same loading approach as the current study (38), or other models of loading in rodents (11) and humans (22) all showing that aging attenuates, but does not prevent muscle adaptation to loading until very old ages (6, 33). To our knowledge, this is the first report that has evaluated the aging-specific effects of repetitive resistance training in combination with Vitamin E&C supplementation on changes in muscle weight. Contrary to our expectations, fortifying the diet with Vitamins E&C in an attempt to reduce oxidative stress during repetitive loading exercise did not provide any additive effect to improvements in muscle weight in either young adult or aged rats. This implies that oxidative stress associated with aging may not be a limiting factor for protein accumulation in repetitively loaded muscles of aged animals. An alternative explanation is that the level of Vitamin E&C supplementation was too low to fully overcome any limitations to protein accumulation that occur in response to repetitive loading in muscles of aged rodents. Likely the antioxidant capacity of muscles in young adult animals was already sufficient to balance loading-induced increases in oxidative stress, so that elevating antioxidants via Vitamins E&C did not provide any greater improvement in muscle hypertrophy.

Age-Dependent Adaptations to Antioxidant Supplementation on Muscle function After Repetitive Loading. Repetitive loading increased maximal isometric dorsiflexor force production by >40%, positive work (work performed during the concentric portion of each contraction) by >31%, and negative work (work performed during the eccentric portion of each contraction) by >30% in young adult rats. The repetitively loaded tibialis anterior muscle weight

was not improved by the antioxidant supplementation, and since this muscle provides the greatest contribution to dorsiflexion, it is not surprising that Vitamin E&C supplementation did not further improve maximal dorsiflexion isometric force, or positive and negative work in the dorsiflexors of young adult rats. In contrast to the young adult rats, non-supplemented aged rats were unable to improve maximal force, positive or negative work over the training period. While Vitamin E&C supplementation did not improve either maximal force or negative work, the positive work was improved by ~ 38% in the aged rats that received the Vitamin E&C fortified diet. These findings indicate that although muscle size was not enhanced (and therefore maximal isometric force was not improved) by the antioxidant diet in the aged animals, the high oxidant environment of the aged muscle likely contributed to a rapid loss of force during each contraction in the non-supplemented animals. Vitamin E&C supplementation likely buffered (at least in part) the additional oxidant production imposed by repetitive loading, such that force was better maintained during each shortening contraction. As reactive oxygen species are mediators of muscle fatigue (34), it is therefore possible that Vitamin E&C acts to lower oxidative stress and thereby reducing fatigue within each contraction, and in doing so, improves positive work in muscles of aged animals.

Aging Increases Oxidative Stress but Vitamin E&C Reduces Oxidative Stress in Loaded Muscles. Several studies report an age-related increase of lipid peroxidation, oxidative modification to proteins, and DNA damage (4, 8, 13, 38, 41). Similarly, the results of the current study suggest that the tibialis anterior muscles from aged rats are under greater oxidative stress than muscles from young adult rats. In addition to the age-associated increase in oxidative stress, repetitive loading also elevated the oxidative load in skeletal muscles.

H_2O_2. The increase that we observed in cytosolic H_2O_2 content in the exercised tibialis anterior muscle is consistent with previous data showing that muscle contractions increase oxidant production (4, 10, 23, 29, 30, 38). Hydrogen peroxide is a relatively stable pro-oxidant that in biological systems is most commonly produced from the dismutation of superoxide. It is commonly assumed that during exercise most of the increases in H_2O_2 are the result of superoxide production.

In general, this study supports the idea that Vitamin E&C supplementation increases the ability of the muscle to buffer oxidant production associated with aging and exercise. To the authors knowledge the current data are the first to show that Vitamin E&C supplementation directly lowers cytosolic H_2O_2 concentrations in both control and exercised tibialis anterior muscle from young adult and aged animals. It has been previously shown via electron spin resonance spectroscopy that Vitamin E supplementation lowers the concentrations of free radicals produced during 30 min of exhaustive swimming exercise, however this study did not further distinguish the type or location of radicals being produced (23). Furthermore, no difference was reported in the muscles of non-exercised control animals (23). The majority of the data that report a decrease in oxidant production after exercise with Vitamin E&C supplementation have measured oxidative damage to lipids, protein and DNA, but they generally have failed to measure oxidant production (7, 19, 20, 24, 30, 35). In the current study, Vitamin E&C supplementation lowered cytosolic H_2O_2 concentrations in the aged muscle to the same level as the young adult, and both the young adult and aged muscle from the supplemented animals were lower than the control limb of the young adult non-supplemented tibialis anterior muscle. These data suggest that fortification of the rat's diet with Vitamins E&C was effective at increasing the oxidant buffering capacity, but combining exercise with supplementation did not further increase the buffering capacity of the tibialis anterior muscle to oxidative stress.

Glutathione. Total glutathione, and especially reduced glutathione (GSH), have important roles in protecting cells from oxidant damage. This protective function is achieved by direct conjugation with radicals as well as functioning as an electron donor in redox reactions. The redox reaction oxidizes GSH to GSSG while H_2O_2 and other peroxides are reduced (26). The ratio of reduced to oxidized glutathione (GSH/GSSG) is a good indicator of the redox status of the muscle. The results of this study show that aging decreased both the total GSH abundance and the GSH/GSSG ratio in skeletal muscle. This suggests a reduced potential for buffering oxidative stress in aged muscles. In addition, the GSH/GSSG ratio was further lowered in repetitively loaded muscles of non-supplemented aged rats as compared to muscles in young adult animals. This indicates that aging reduced the ability to tolerate increased oxidative stress in chronically loaded skeletal muscles.

Interestingly, total GSH was lower in muscles from Vitamin E&C supplemented young adult rats as compared to non-supplemented animals. In contrast, Vitamin E&C supplemented aged rats did not further reduce skeletal muscle GSH abundance. Nevertheless, Vitamin E&C supplementation increased the GSH/GSSG ratio in the both the young adult and aged tibialis anterior muscle after chronic repetitive loading exercise. These data are consistent with the H_2O_2 data, and together this is indicative of increases in oxidant production during exercise. These data suggest that aging increased oxidative stress and therefore lowered the GSH/GSSG ratio as compared to muscles in young adult animals, and that aging reduced that ability to tolerate increased oxidative stress in chronically loaded skeletal muscles. Furthermore, Vitamin E&C supplementation provided an effective buffer against oxidant stress in response to loading. It is not clear why the decrease in cytosolic H_2O_2 concentrations did not increase the GSH/GSSG ratio of non-exercised tibialis anterior muscle from young adult or aged animals receiving Vitamin E&C supplementation.

Oxidative Damage to DNA. Oxidative DNA damage (8-OHdG) increased with aging but not with repetitive loading exercise. This is consistent with previous data which indicate that aging is associated with increases in oxidative DNA damage (32, 38, 39). Furthermore, long-term exercise does not appear to elevate oxidative damage to DNA (38, 39), although the mode, duration and intensity of the exercise along with sampling procedures, may play a significant role in determining the effect that chronic exercise has on increased oxidative DNA damage in skeletal muscle. For example, in contrast to our current study using repetitive loading as a model of resistance exercise, 8 weeks of treadmill running resulted in an attenuation of the age-associated increase in 8-OHdG levels, and increased the activity of DNA repair in aged rats (32). It is likely that the differences in these studies are the result of the difference in the mode of exercise (high intensity, low duration vs. low intensity, high duration). Furthermore, chronic aerobic exercise has been shown to increase the mitochondria's oxidant buffering capacity and reduce oxidant production via the mitochondrial electron transport chain, whereas chronic resistance training has not been shown to elicit the same degree of adaptation within the mitochondria. It is also important to note that mitochondrial DNA is more susceptible to oxidative damage than nuclear DNA, so aerobic exercise-induced changes of oxidative stress may provide significant protection to mitochondria DNA, whereas this would likely not occur with resistance types of exercise. In the current study, Vitamin E&C supplementation decreased the 8-OHdG content in both young adult and aged muscle. This suggests that the antioxidant diet had a profound effect on buffering and reducing oxidative stress, resulting in lower DNA damage in muscles of both young adult and aged animals. Previous work has shown that Vitamin E supplementation decreased 8-OHdG in muscle of young men but supplementation failed to show a similar effect in elderly men (39). The variability of the subjects' heath/dietary

status might explain some of the variability in the different responses of subjects to antioxidant supplementation.

Lipids. In contrast to DNA, the current data suggests that the increase in cellular damage to lipids associated with aging can be attenuated after chronic repetitive loading. The beneficial effects of exercise on lowering levels of lipid peroxidation has been observed in previous investigations (1, 30, 38). The current study shows evidence that Vitamin E&C supplementation is as effective at reducing the elevated concentrations of MDA + HAE associated with aging as chronic repetitive loading alone. The current data is in agreement with previous work showing that aging increases oxidative stress (2, 4, 21) and that Vitamin supplementation (especially Vitamin E) protects lipids from oxidative damage (23) observed during exercise and aging. However, there was not an additive effect of combining exercise with Vitamin E&C supplementation.

Adaptation of Antioxidant Systems to Repetitive Loading and Antioxidant Supplementation. The current investigation measured transcription, protein levels and enzyme activity levels of endogenous antioxidant enzymes catalase, glutathione peroxidase, MnSOD and CuZnSOD . Overall the results do not support the likelihood that transcriptional control is a mechanism leading to increases in activity of the endogenous antioxidant enzymes. Instead, these data are consistent with the previously suggested notion that the endogenous antioxidant enzymes activities are regulated via various levels of post-transcriptional and/or post-translational controls (18, 38). However, the current data do not support the view that Vitamin E&C supplementation inhibits the positive adaptations to exercise within all of the endogenous antioxidant enzymes (36). The data in the current study suggest that there is an age-dependent effect of repetitive loading and Vitamin E&C supplementation within the tibialis anterior muscle. Vitamin E&C supplementation increased activity levels of catalase, MnSOD and CuZnSOD in both the exercise and control limbs from the young adult animals. There was no change in MnSOD activity but CuZnSOD and catalase also increased in the muscles of aged rats supplemented with Vitamins E&C. Although this not a universal finding (15, 36) our data are consistent with reports that antioxidant vitamin supplementation increases the activities of the enzymatic antioxidants in both healthy (40) as well as diseased animals with chronic elevations in oxidative stress (14).

Antioxidant supplementation has been previously reported to have a detrimental effect on producing expected antioxidant adaptations to chronic training (15, 36). However, any detrimental effect may be due in part to the method of administering the supplement. For example, antioxidant supplementation after chronic training that is provided in a concentrated form (oral gavage or pill) likely induces a bolus effect and may reduce intestinal absorption. Whereas, in this study, rather than one mega dose, we provided the fortification of the animal's food which would more likely represent more constant systemic levels of Vitamin E&C. It is not currently known how the endogenous antioxidants enzyme levels would respond to the long-term effects of a diet fortified with Vitamin E&C taken throughout a period of chronic exercise, as compared to a single supplemented dose of Vitamin E&C that would be given after adaptation to chronic exercise.

Conclusion

The current study provides data to show that chronic exercise and Vitamin E&C supplementation lower indices of oxidative damage (i.e. 8-OHdG, MDA + HAE) associated with aging. However, repetitive loading and Vitamin E&C supplementation affected DNA damage and lipid peroxidation differently. This raises the possibility that aging and repetitive loading

exercise increase oxidant production via different mechanisms. Several potential mechanisms exist that include elevations of oxidative stress via neutrophils and other infiltrating immune cells, mitochondria respiration, NADPH oxidase or xanthine oxidase activity. This possibility warrants additional studies, because previous work has suggested that aging increases oxidant production via the mitochondrial electron transport chain whereas exercise induced increase in muscle oxidants originate from multiple sources (4).

Assessing effectiveness of Vitamin E&C supplementation in preventing exercise-induced oxidative stress has been difficult to determine from previous studies. This is the result of a wide variance in study designs and experimental conditions (i.e. subject/ animal species, experimental conditions, length of study, dose of supplement, means of supplement administration and mode of exercise). Nevertheless, the data in our current study clearly show that Vitamin E&C supplementation lessens the oxidant activity and oxidative damage in tibialis anterior muscles from young and aged rats subjected to chronic repetitive loading. Furthermore, Vitamin E&C supplementation attenuated basal levels of oxidative stress associated with aging. Muscle size and other functional measurements were unaffected by Vitamin E&C supplementation, in aged rats, but there were important improvements in positive work in the aged animals after 4.5 week of repetitive loading that received the dietary supplementation. Additional studies are required to determine if skeletal muscles in elderly humans who supplement their diet with Vitamin E&C during chronic resistance types of muscle loading, will respond in a similar fashion to the rodents in the current study.

Table Legend

Table 4.1 **Body Mass of Young and Aged Rats Pre and Post 14 sessions of repetitive loading.** Data are mean ± SEM and presented as the mass of the rats before the first exercise session and after the 14th training session in grams. †, indicates a significant difference ($p<0.05$) from young exercise and diet-matched control rats. NS, non-supplemented diet.

Figure Legends

Figure 4.1 **Repetitive loading induces muscle hypertrophy in the Tibialis Anterior Muscle**. Tibialis anterior muscle wet weight is reported for young and aged rats that received no dietary supplement (NS) or a diet supplemented with Vitamin E&C (Vit E&C). Data are expressed as mean ± SEM. *, significant difference between age-matched control and repetitive loaded TA muscle assigned at $p<0.05$; †, a significant difference ($p<0.05$) from young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) from age-matched animals on the non-supplemented diet.

Figure 4.2 **Repetitive loading increased muscle functional measurements in young adult dorsiflexor muscles while maintaining function in aged dorsiflexor muscles. (A)** Maximal isometric force generated in the dorsiflexors from young and aged rats during each of the 14 training sessions. Data are expressed as the average maximum isometric force for all animals in Newtons (N) normalized to body mass (BM) in grams (g) produced during each exercise session ± SEM. There was no significant difference in muscle wet weight of the animals in the two diet groups. **(B)** Negative work generated from a single eccentric/concentric movement performed at the start of each training session from the young and aged dorsiflexor muscles during each of the 14 training sessions. There was not a significant difference between diets. **(C)** Positive work generated from a single eccentric /concentric movement preformed at the start of each training session from the young and aged dorsiflexor muscles during each of the 14 training sessions. §, indicates that there was a significant difference ($p<0.05$) from age-matched animals on the non-supplemented (NS) diet. Data are expressed as the mean ± SEM. The solid line represents the linear regression for all age-matched points. Maximal isometric force, negative work and positive work generated from the young animals was significantly different ($p<0.05$) from aged dorsiflexor muscles at all time points.

Figure 4.3 **Vitamin E&C supplementation attenuated the increase in hydrogen peroxide (H_2O_2) concentration associated with exercise & aging**. The H_2O_2 concentration was determined fluorometrically. The data are expressed as mean ± SEM of relative fluorescent units (RFU) per mg of total protein homogenate. *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contra-lateral control muscle; †, a significant difference ($p<0.05$) between young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched muscles from animals on the non-supplemented (NS) diet.

Figure 4.4 **Ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) & total glutathione content**. **(A)** Data are depicted as the ratio of GSH to GSSG normalized to total protein content. Lower ratios are an indication of increased oxidative stress. **(B)** Data indicate total glutathione concentration normalized to total protein content. The normalized data are presented as mean ± SEM. *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contra-lateral control muscle; †, a significant difference ($p<0.05$) between young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched muscles from animals on the non-supplemented (NS) diet.

Figure 4.5 **Vitamin E&C supplementation decreased evidence of oxidative damage associated with repetitive loading exercise and aging. (A)** The data represent oxidative damage as indicated by malondialdehyde (MDA) plus 4-hydroxyalkenals (HAE) normalized to total protein content. **(B)** Data are provided as mean ± SEM and they are expressed as the nannogram concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG) per µg of DNA; *, significant difference ($p<0.05$) of repetitively loaded muscle from contra-lateral control muscle; †, a significant difference ($p<0.05$) from young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) from age-matched animals on the non-supplemented (NS) diet.

Figure 4.6 **Glutathione peroxidase (GPx) regulation with repetitive loading and Vitamin E&C supplementation. (A)** Total GPx activity was expressed as mU of GPx per ml of muscle homogenate normalized per mg of protein in the homogenate. **(B)** GPx-1 protein expression was determined in the total cytosolic fraction by western immunoblots. The data are expressed as optical density (OD) x band area, and presented as relative optical density. The inserts show representative blots for GPx-1 and β-tubulin in young and aged (control and repetitive loading) tibialis anterior muscle. **(C)** GPx-1 mRNA expression was determined by RT-PCR. The data are expressed as optical density (OD) x band area, and presented as relative optical density. The inserts show representative gels for GPx-1 mRNA and 18s rRNA in young and aged (control and repetitively loaded) muscle. All data are presented as mean ± SEM; *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contra-lateral control muscle; †, a significant difference ($p<0.05$) between young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched animals on the non-supplemented (NS) diet; C, control; RL, repetitively loaded; Y, young; A, aged; E&C, diet supplemented with Vitamin E&C.

Figure 4.7 **Catalase regulation with repetitive loading and Vitamin E&C supplementation. (A)** Catalase activity was determined at 520 nm and expressed as nM of catalase per ml of homogenate normalized per mg of protein in homogenate. **(B)** Catalase protein expression was determined in the total cytosolic fraction by western immunoblots. The data are expressed as optical density (OD) x band area, and presented as relative optical density. The inserts show representative blots for catalase and β-tubulin in young and aged (control and repetitively loaded) muscles. **(C)** Catalase mRNA expression was determined by RT-PCR. The data are expressed as optical density (OD) x band area, and presented as relative optical density. The inserts show representative gels for catalase mRNA and 18s rRNA in young and aged (control and repetitively loaded) muscles. All data are presented as mean ± SEM; *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contra-lateral control muscle; †, a significant difference ($p<0.05$) between young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched animals on the non-supplemented (NS) diet; E&C, diet supplemented with Vitamin E&C; C, control; RL, repetitively loaded; Y, young; A, aged.

Figure 4.8 **CuZn superoxide dismutase (CuZnSOD) regulation with repetitive loading and Vitamin E&C supplementation. (A)** CuZnSOD activity was expressed as mU of CuZnSOD per ml of homogenate normalized per mg of protein in homogenate. **(B)** CuZnSOD protein expression was determined in the total cytosolic fraction by western immunoblot. The data are expressed as optical density (OD) x band area, and presented as relative optical density. The inserts show representative blots for CuZnSOD and β-tubulin in young and aged (control and repetitive loading) TA muscle. **(C)** CuZnSOD mRNA expression was determined by RT-PCR. The data are expressed as optical density (OD) x band area, and presented as

relative optical density. The inserts show representative gels for CuZnSOD mRNA and 18s rRNA in young and aged (control and repetitively loaded) muscle. All data are presented as mean ± SEM; *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contra-lateral control muscle; †, a significant difference ($p<0.05$) between young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched animals on the non-supplemented (NS) diet; E&C, diet supplemented with Vitamin E&C; C, control; RL, repetitively loaded; Y, young; A, aged.

Figure 4.9 **Mn superoxide dismutase (MnSOD) regulation with repetitive loading and Vitamin E&C supplementation. (A)** MnSOD activity was determined after inhibiting CuZnSOD activity by potassium cyanide. MnSOD was expressed as mU of MnSOD per ml of homogenate normalized per mg of protein in homogenate. **(B)** MnSOD protein expression was determined in the total cytosolic fraction by western immunoblot. The data are expressed as optical density (OD) x band area, and presented as relative optical density. The inserts show representative blots for MnSOD and β-tubulin in young and aged (control and repetitively loaded) muscle. **(C)** MnSOD mRNA expression was determined by RT-PCR. The data are expressed as optical density (OD) x band area, and presented as relative optical density. The inserts show representative gels for MnSOD mRNA and 18s rRNA in young and aged (control and repetitive loaded) muscle. All data are presented as mean ± SEM; *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contra-lateral control muscle; †, a significant difference ($p<0.05$) between young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched animals on the non-supplemented (NS) diet; E&C, diet supplemented with Vitamin E&C; C, control; RL, repetitively loaded; Y, young; A, aged.

Table 4.1

Body Mass of Young and Aged Rats Pre and Post 14 sessions of Repetitive Loading

	Young NS	Young Vit. E&C	Aged NS	Age Vit. E&C
Pre-RL (g)	343.5 ± 10.9	350.1 ± 26.1	513.8 ± 88.4 †	491 ± 63.5 †
Post-RL (g)	328.1 ± 11.8	340.2 ± 27.8	467.2 ± 91 †	441 ± 55.5 †

Each value expressed in grams as a mean ± SEM
NS = non-supplemented

Figure 4.1

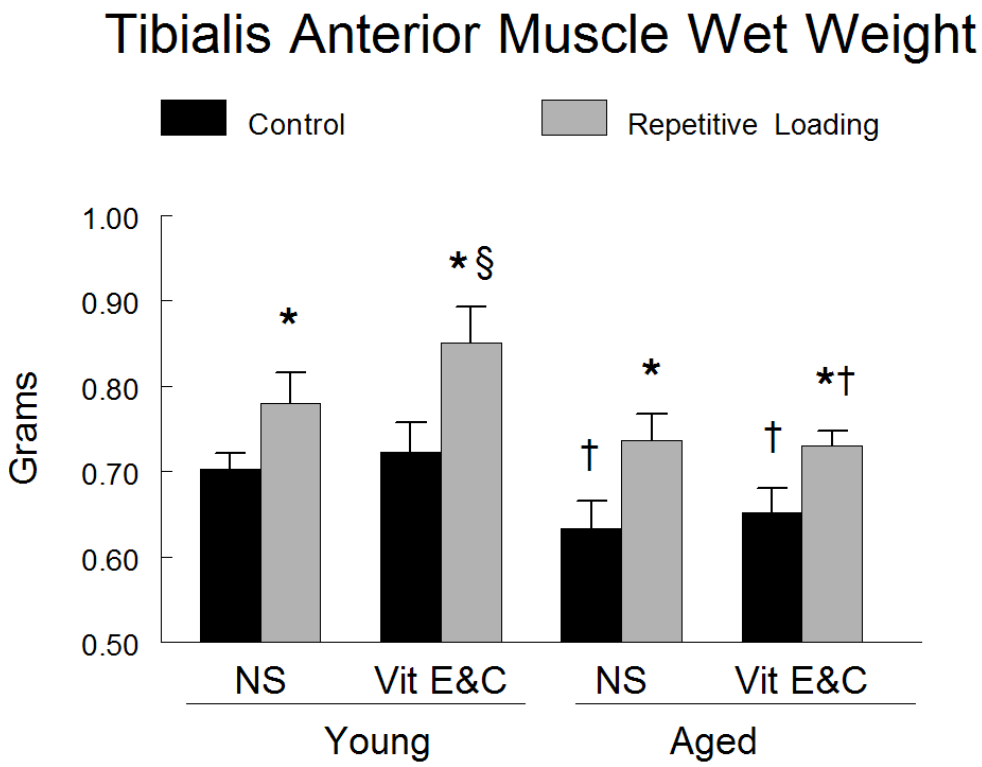

Tibialis Anterior Muscle Wet Weight
Control
Repetitive Loading
Grams
1.00
0.90
0.80
0.70
0.60
0.50
*
*§
*
*†
†
†
NS
Vit E&C
NS
Vit E&C
Young
Aged

Figure 4.2

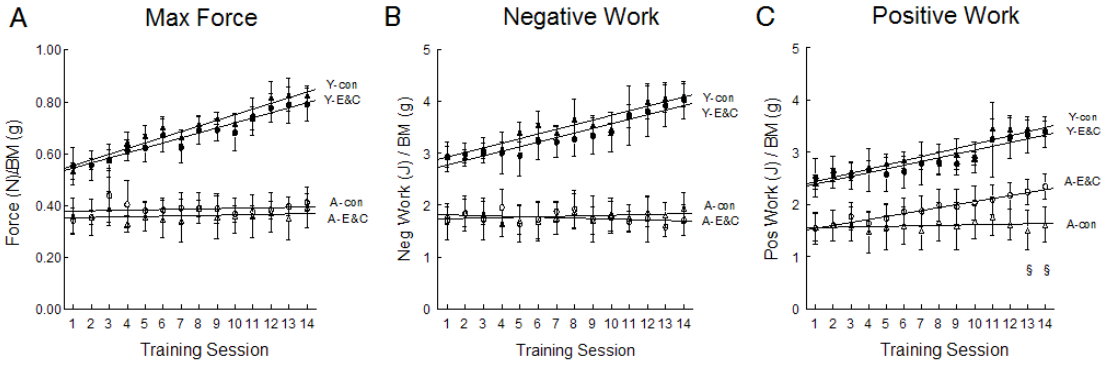

A
Max Force
Force (N)/BM (g)
Training Session
Y-con
Y-E&C
A-con
A-E&C
B
Negative Work
Neg Work (J) / BM (g)
Training Session
Y-con
Y-E&C
A-con
A-E&C
C
Positive Work
Pos Work (J) / BM (g)
Training Session
Y-con
Y-E&C
A-E&C
A-con
§ §

Figure 4.3

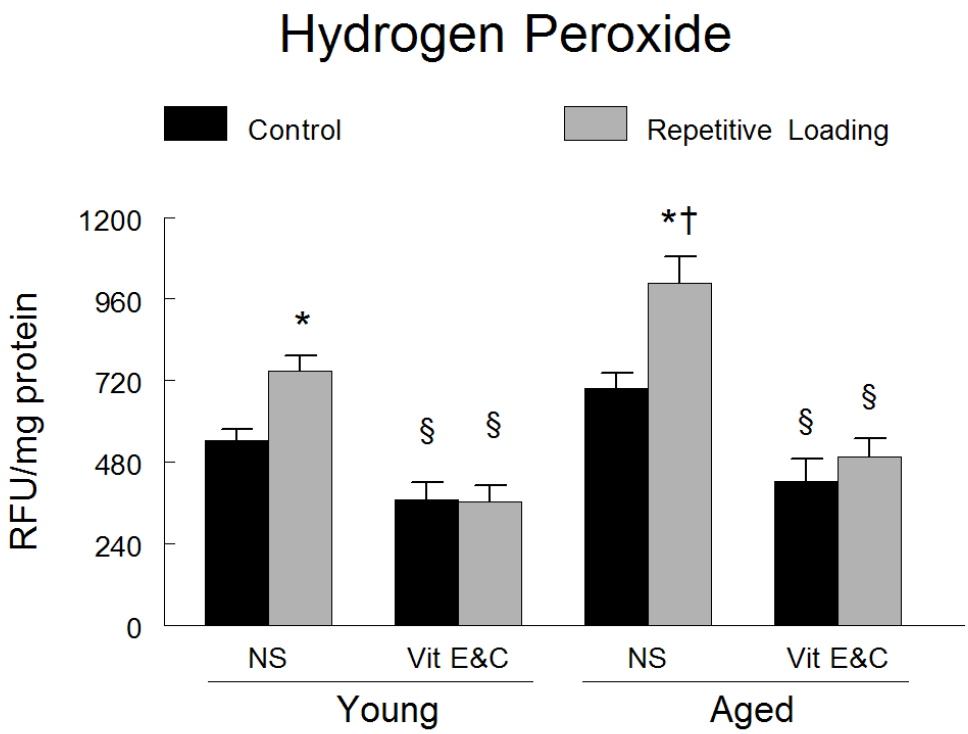

Hydrogen Peroxide
Control
Repetitive Loading
RFU/mg protein
1200
960
720
480
240
0
*
*†
§
§
§
§
NS
Vit E&C
NS
Vit E&C
Young
Aged

Figure 4.4

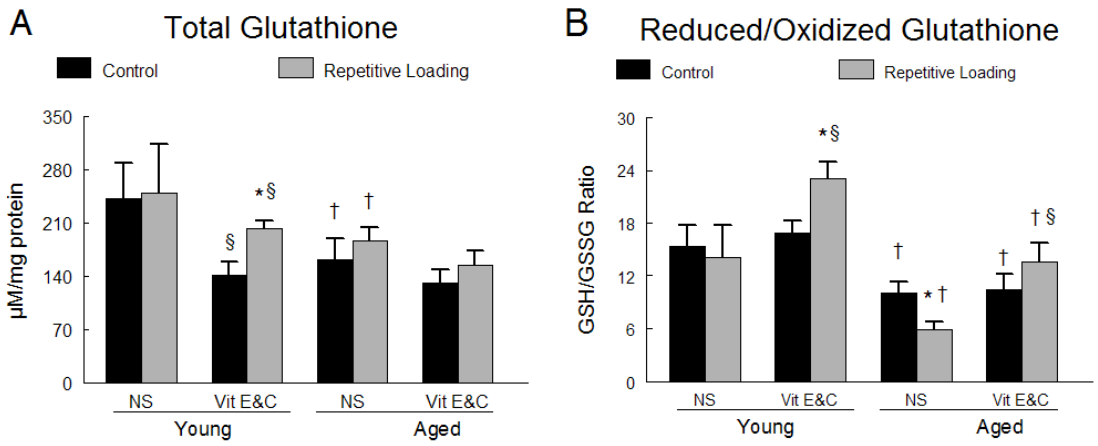

A
Total Glutathione
Control
Repetitive Loading
µM/mg protein
350
280
210
140
70
0
§
*§
†
†
NS
Vit E&C
NS
Vit E&C
Young
Aged
B
Reduced/Oxidized Glutathione
Control
Repetitive Loading
GSH/GSSG Ratio
30
24
18
12
6
0
*§
†
*†
†
† §
NS
Vit E&C
NS
Vit E&C
Young
Aged

Figure 4.5

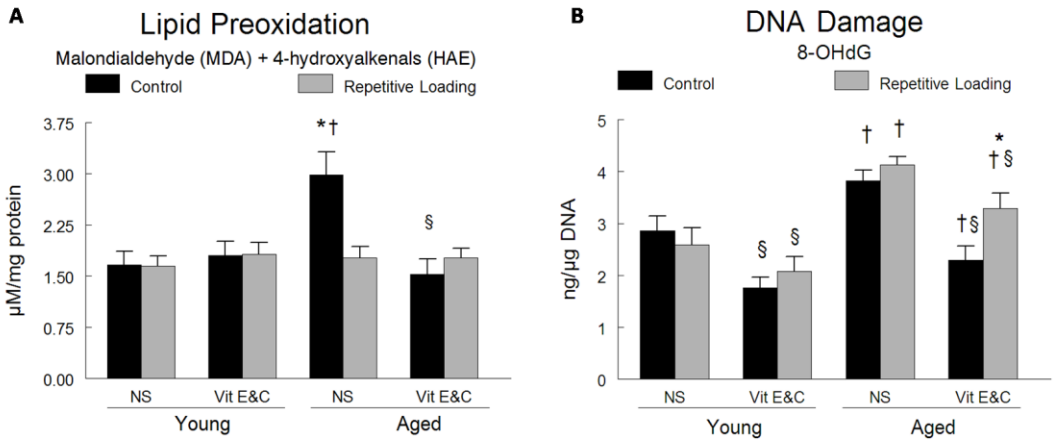

A
Lipid Preoxidation
Malondialdehyde (MDA) + 4-hydroxyalkenals (HAE)
Control
Repetitive Loading
μM/mg protein
3.75
3.00
2.25
1.50
0.75
0.00
*†
§
NS
Vit E&C
NS
Vit E&C
Young
Aged
B
DNA Damage
8-OHdG
Control
Repetitive Loading
ng/μg DNA
5
4
3
2
1
0
§
§
†
†
†§
*
†§
NS
Vit E&C
NS
Vit E&C
Young
Aged

Figure 4.6

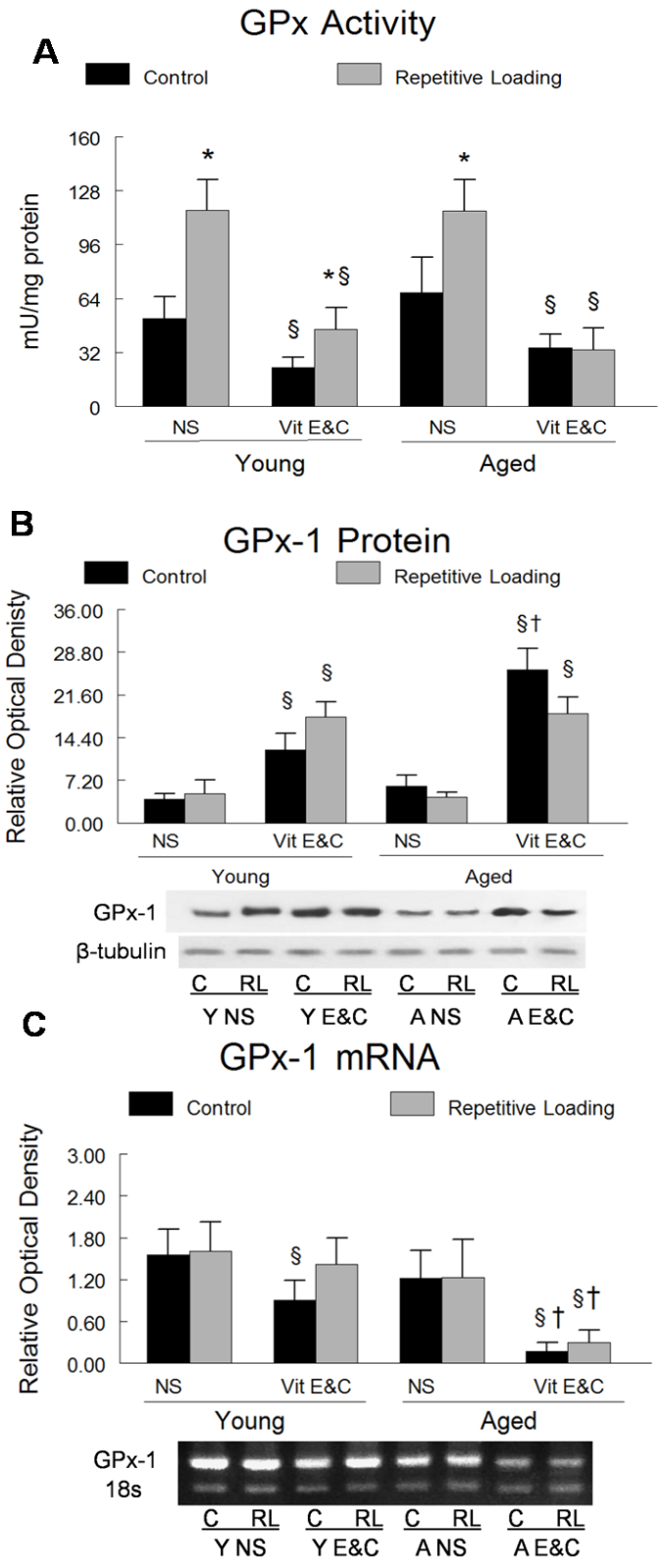

A
GPx Activity
Control
Repetitive Loading
mU/mg protein
160
128
96
64
32
0
*
*
*§
§
§
§
NS
Vit E&C
NS
Vit E&C
Young
Aged
B
GPx-1 Protein
Control
Repetitive Loading
Relative Optical Denisty
36.00
28.80
21.60
14.40
7.20
0.00
§
§
§†
§
NS
Vit E&C
NS
Vit E&C
Young
Aged
GPx-1
β-tubulin
C RL C RL C RL C RL
Y NS Y E&C A NS A E&C
C
GPx-1 mRNA
Control
Repetitive Loading
Relative Optical Density
3.00
2.40
1.80
1.20
0.60
0.00
§
§ †
§†
NS
Vit E&C
NS
Vit E&C
Young
Aged
GPx-1
18s
C RL C RL C RL C RL
Y NS Y E&C A NS A E&C

Figure 4.7

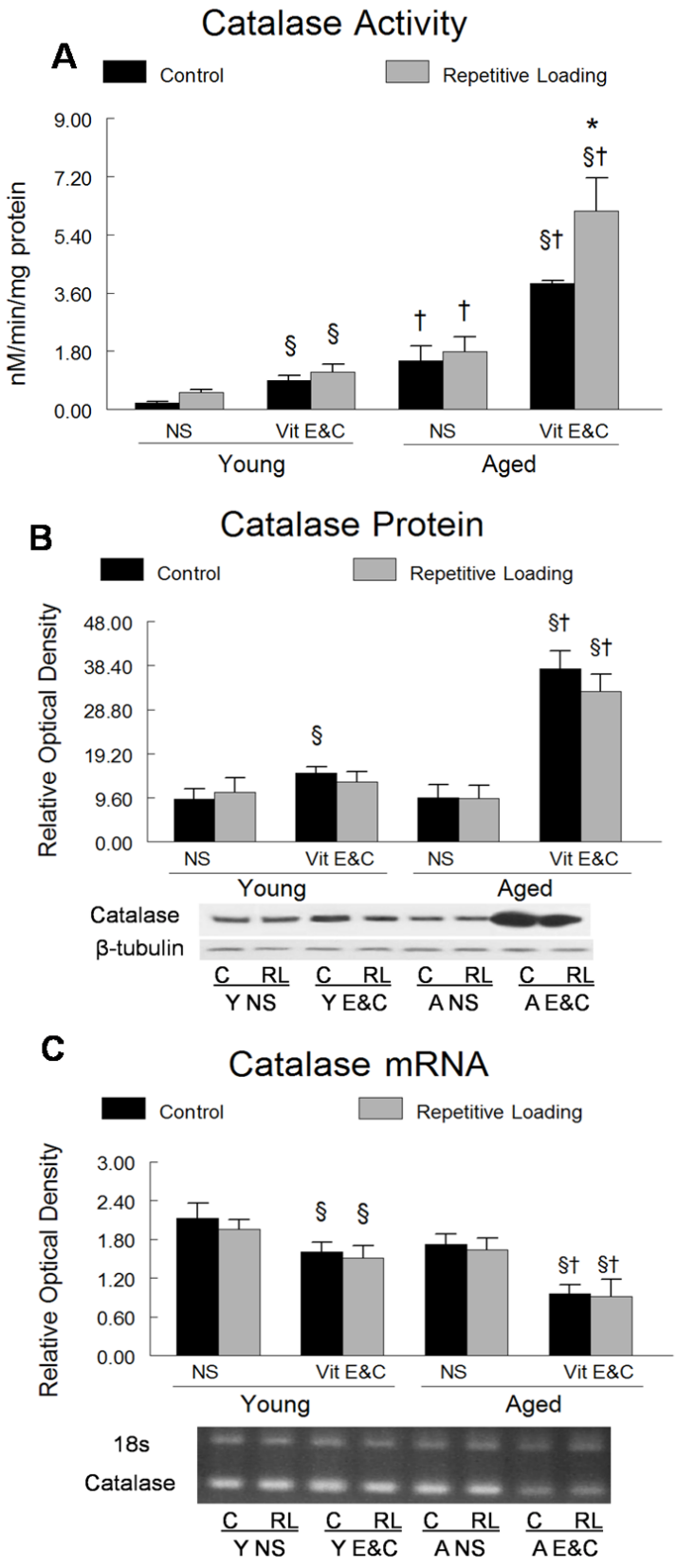

Catalase Activity
A
Control
Repetitive Loading
nM/min/mg protein
9.00
7.20
5.40
3.60
1.80
0.00
§
§
†
†
§†
§†
*
NS
Vit E&C
NS
Vit E&C
Young
Aged
Catalase Protein
B
Control
Repetitive Loading
Relative Optical Density
48.00
38.40
28.80
19.20
9.60
0.00
§
§†
§†
NS
Vit E&C
NS
Vit E&C
Young
Aged
Catalase
β-tubulin
C RL C RL C RL C RL
Y NS Y E&C A NS A E&C
C
Catalase mRNA
Control
Repetitive Loading
Relative Optical Density
3.00
2.40
1.80
1.20
0.60
0.00
§
§
§†
§†
NS
Vit E&C
NS
Vit E&C
Young
Aged
18s
Catalase
C RL C RL C RL C RL
Y NS Y E&C A NS A E&C

Figure 4.8

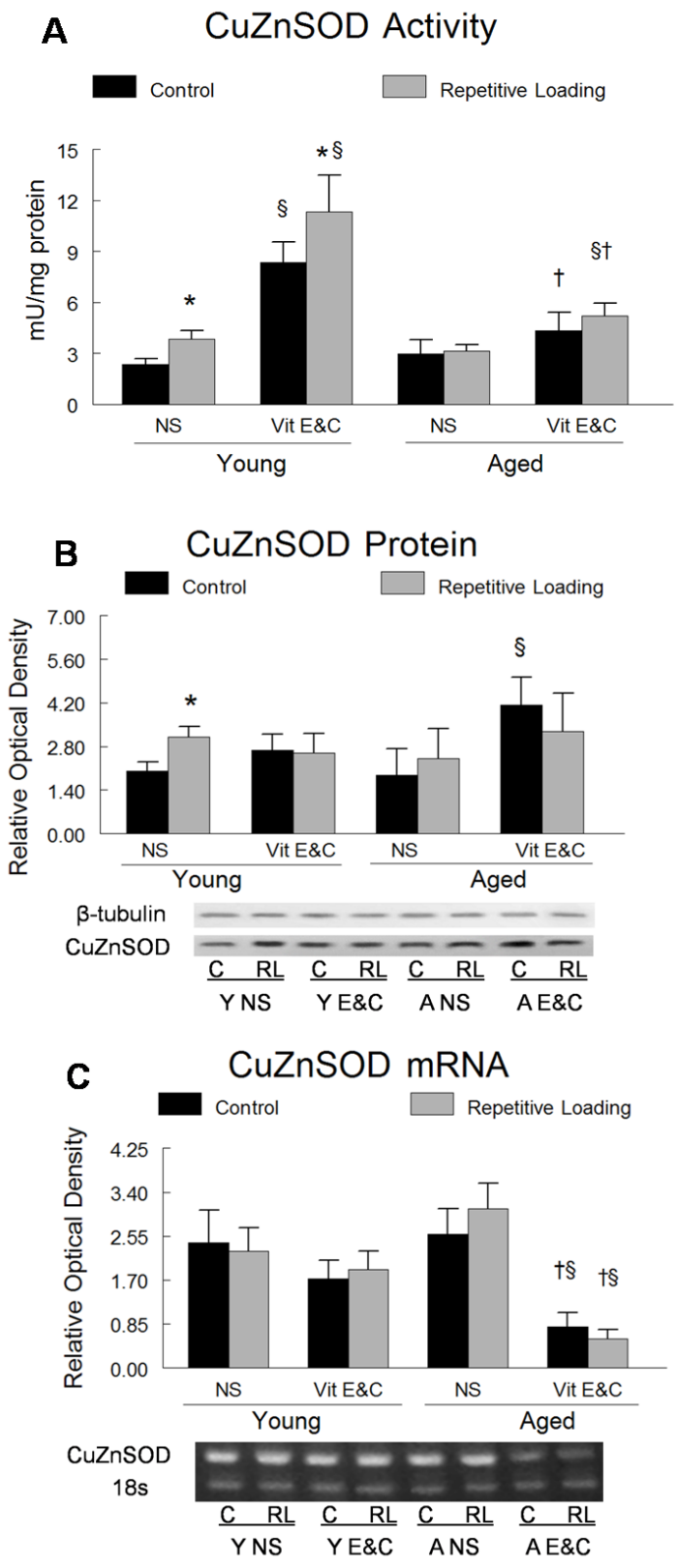
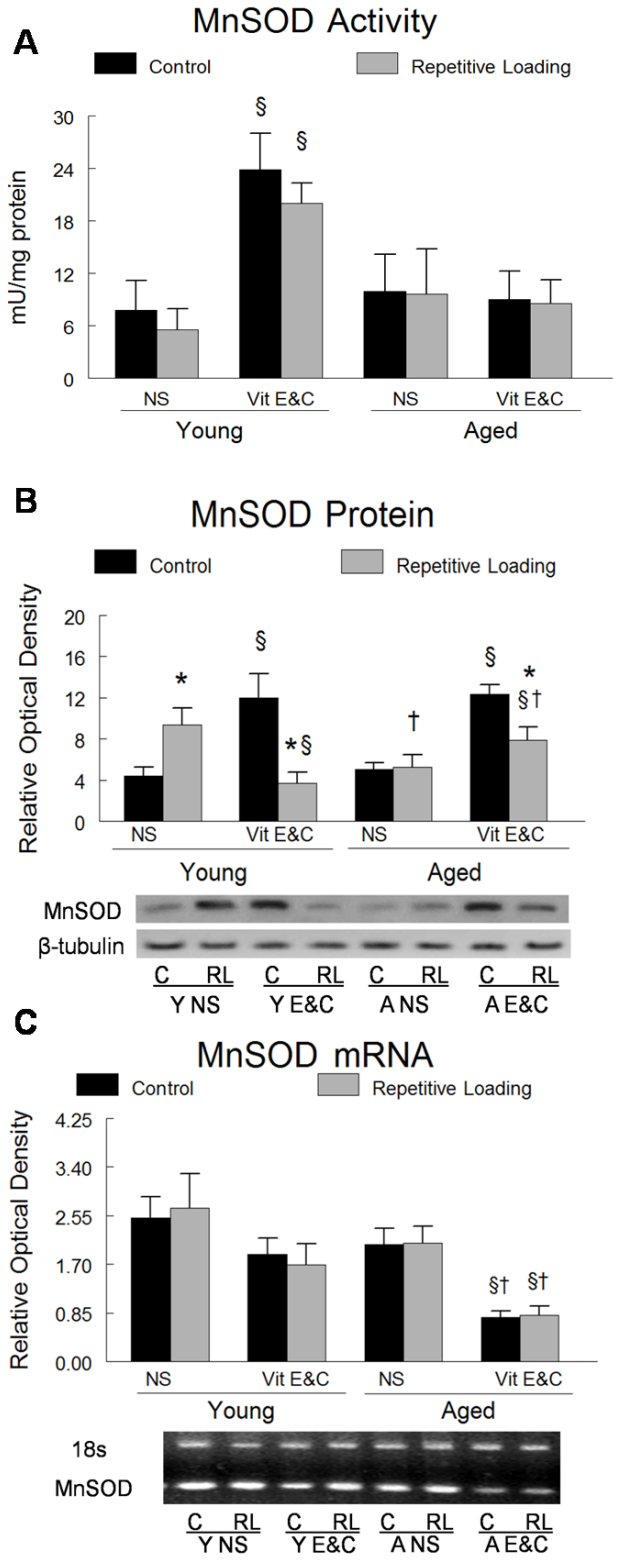

A
CuZnSOD Activity
Control
Repetitive Loading
mU/mg protein
15
12
9
6
3
0
*
*§
§
†
§†
NS
Vit E&C
NS
Vit E&C
Young
Aged
B
CuZnSOD Protein
Control
Repetitive Loading
Relative Optical Density
7.00
5.60
4.20
2.80
1.40
0.00
*
§
NS
Vit E&C
NS
Vit E&C
Young
Aged
β-tubulin
CuZnSOD
C RL C RL C RL C RL
Y NS Y E&C A NS A E&C
C
CuZnSOD mRNA
Control
Repetitive Loading
Relative Optical Density
4.25
3.40
2.55
1.70
0.85
0.00
†§
†§
NS
Vit E&C
NS
Vit E&C
Young
Aged
CuZnSOD
18s
C RL C RL C RL C RL
Y NS Y E&C A NS A E&C

Figure 4.9


A
MnSOD Activity
Control
Repetitive Loading
mU/mg protein
30
24
18
12
6
0
§
§
NS
Vit E&C
NS
Vit E&C
Young
Aged
B
MnSOD Protein
Control
Repetitive Loading
Relative Optical Density
20
16
12
8
4
0
*
§
*§
†
§
*
§†
NS
Vit E&C
NS
Vit E&C
Young
Aged
MnSOD
β-tubulin
C RL
C RL
C RL
C RL
Y NS
Y E&C
A NS
A E&C
C
MnSOD mRNA
Control
Repetitive Loading
Relative Optical Density
4.25
3.40
2.55
1.70
0.85
0.00
§†
§†
NS
Vit E&C
NS
Vit E&C
Young
Aged
18s
MnSOD
C RL
C RL
C RL
C RL
Y NS
Y E&C
A NS
A E&C

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Chapter 5

Regulation of oxidative stress and xanthine oxidase activity by resveratrol after isometric contractions in gastrocnemius muscles of aged mice.

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Abstract

This study tested the hypothesis that resveratrol supplementation would lower oxidative stress in exercised muscles of aged mice. Young (3 mo) and aged (27mo) C57BL/6 mice received a control, or a 0.05% trans-resveratrol supplemented diet. Twenty maximal electrically-evoked isometric contractions of the plantar flexors of one limb were obtained in anesthetized mice for 3 consecutive days. Resveratrol supplementation blunted the exercise-induced increase in xanthine oxidase activity in muscles from young (25%) and aged (53%) mice. Resveratrol lowered H_2O_2 levels in control (13%) and exercised (38%) muscles from aged animals, and increased the ratio of GSH/GSSG in exercised muscles from young (38%) and aged (135%) mice. Resveratrol prevented the increase in lipid oxidation, increased catalase activity, and increased MnSOD activity in exercised muscles from aged mice. These data show that dietary resveratrol reduces muscle indicators of oxidative stress in response to isometric contractions in aged mice.

Introduction

The causes of decreased muscle function associated with advanced aging are multifactoral and include, muscle atrophy (sarcopenia), alterations in motor unit activity and declines in metabolic efficiency. Exercise is a countermeasure that is partially effective in reversing the loss of muscle function. While muscles in aged mammals will effectively adapt to chronic resistance exercise, via improved muscular strength and muscle fiber hypertrophy, these adaptations are generally smaller than that reported in muscles from younger adult humans and animals (30, 41). The mechanisms that regulate this attenuated adaptation to resistance exercise with advanced aging (12) are unknown, but it is possible that this may be mediated, at least in part, by the detrimental systemic effects associated with elevated oxidative stress (16).

The additive effects of an increase in oxidant production and an attenuated antioxidant buffering capacity leaves aged skeletal muscles more vulnerable to oxidative stress and subsequently, oxidative damage. Age-related increases in oxidative stress have been associated with diminished muscular strength and physical performance (6). Specifically, elevated levels of oxidants have been shown to depress muscle force (2), alter myofilament function (2, 28, 29) and increase recovery time following injury (27, 38).

The xanthine oxidase system has been shown to be an important source of oxidant production in the vascular endothelium (23) and also a contributing factor to oxidative stress during strenuous exercise (17, 42, 47). A high demand on anaerobic metabolism, coupled with intermittent localized obstruction of blood flow and subsequent reperfusion within contracting muscles raises the question as to whether xanthine oxidase may also be an important source of oxidant production during intense resistance exercises. Previous studies have observed that after resistance training, type II fibers are preferentially hypertrophied in both young and aged muscle (30, 40). Additionally, type II fibers tend to be more susceptible to oxidative damage than type I fibers (35), therefore the increased oxidant production associated with aging could preferentially limit the ability of type II fibers to adapt to exercise training.

Resveratrol (3,4',5-trihydroxystilbene), is a fat-soluble phytoalexin that over the past few years has gained recognition as an effective antioxidant and anti-aging nutraceutical (5, 37, 39). *In vitro* experiments with high doses of resveratrol have shown resveratrol to be effective at scavenging oxidants (46) and inhibiting low density lipoprotein (LDL) oxidation (10). Whereas high concentrations of resveratrol appear to be an effective antioxidant (8, 46), there is currently little evidence to show that a low concentration of resveratrol supplementation is an effective oxidant scavenger *in vivo* (9). Resveratrol appears to improve muscle function in response to aerobic exercise. However, it is not clear if this is related to an antioxidant function of this compound. Furthermore, it is not known if resveratrol would reduce oxidative stress, including xanthine oxidase activity, oxidative damage, and/or muscle fatigue associated with acute resistance types of exercise. This is an important question to be considered in designing exercise programs for the elderly, because resistance types of exercise have been shown to develop considerable oxidative stress (41) and this coupled with an age-related elevation in the basal levels of oxidative stress within skeletal muscle may increase the susceptibility of aged muscle to oxidative damage with exercise.

The objective of this investigation was to evaluate the efficacy of dietary resveratrol to attenuate oxidative stress that is induced via isometric contractions in muscles of aged rodents. Isometric exercise provides an approach that eliminates the potential for oxidant production to be the result of muscle damage related to inflammatory cell infiltration that has been observed in the muscles of aged animals in response to concentric and eccentric repetitive loading exercise (4). In the present investigation, it was hypothesized that resveratrol would reduce the indices of

isometric exercise-induced oxidative stress in muscles of aged mice. Furthermore, we hypothesized that resveratrol supplementation would improve muscle function and attenuate the loss of force during acute repetitive isometric contractions from muscles of aged mice.

Methods

Animals. Experiments were conducted on 16 young adult (3-5 month) and 16 aged (26-28 month) C57BL/6 mice obtained from the National Institute on Aging colony (Harlan, Indianapolis, IN). The mice were housed in pathogen-free conditions at ~20^{o}C. All mice had free access to water and either a control diet (N=8 young adult & N=8 aged) (AIN-76A Rodent Diet, Research Diets Inc, New Brunswick, NJ), or an identical diet that contained 0.05% resveratrol (N=8 young adult & N=8 aged) (Research Diets Inc, New Brunswick, NJ) for a total 10 days. The mice were given the experimental diet (control or resveratrol) for seven (7) days prior to the first exercise session and then kept on the same diet throughout the three (3) days exercise. Resveratrol was purchased from Orchid Pharmaceuticals (Nungambakkam, India). All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards followed the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and fully conformed to the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings."

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

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

Muscle levels of hydrogen peroxide (H_2O_2). H_2O_2 content in control and exercised mouse gastrocnemius muscles was measured by a fluorescent assay according to the manufacture's recommendations (Cell Technology, Mountain View, CA). The sample fluorescence was detected at an excitation of 530nm and measured at 590nm. All analyses were completed in duplicate. The data from the tissue samples were normalized to the muscle protein concentration of each sample, as measured by a DC protein concentration assay (Bio-Rad Hercules, CA). Each sample and standard was performed in duplicate.

Total Glutathione & reduced glutathione/oxidized glutathione (GSH/GSSG) content of aged & exercised muscles. A BIOXYTECH GSH/GSSG-412 (Oxis International, CA) assay kit was used to determine the total glutathione and the GSH/GSSG ratio in control and exercised gastrocnemius muscles of young and aged mice. For total GSH measurements, approximately 40 mg of fresh muscle was homogenized immediately after dissection in 530 µl of cold 5% metaphosphoric acid (MPA). GSSG was obtained on tissue samples after homogenization in 500 µl cold 5% metaphosphoric acid (MPA) and 30µl of a M2VO scavenger. The tissue homogenates were flash frozen and stored at -80°C until time of analysis.

The assay was conducted according to the recommendations of the manufacturer and as described previously (41). The reaction was imitated by adding 50µl of NADPH and the absorbance of each sample was read every 60 sec at 412 nm for three minutes. The protein concentration of each sample was measured with a DC protein concentration assay (Bio-Rad Hercules, CA). The optical density from each sample was normalized to the protein content of the respective sample. Each sample and standard was performed in duplicate.

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

Muscle levels of lipid peroxidation. Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were measured using Bioxytech LPO-586 reagents (Oxis International, CA) as an indicator for the levels of lipid peroxidation in the gastrocnemius muscle samples as described previously (41). Briefly, ~75mg of muscle was homogenized in ice-cold PBS containing 0.5 M butylated hydroxytoluene in acetonitrile. The tissue samples were homogenized and the supernatant was used for the lipid peroxidation assay and also to determine the protein content of the sample. The resulting optical density signals were measured with an absorbance at 586nm (DYNEX technologies, Chantilly Va., USA). The protein content of the sample was determined (Bio-Rad, Hercules, CA) and used to normalize the optical density of each sample. Each sample and standard was performed in duplicate.

Glutathione Peroxidase in exercised and control muscles. Cellular glutathione peroxidase was used to measure glutathione peroxidase activity in gastrocnemius muscle homogenates according to the manufacture's recommendations (#35319, EMD/Calbiochem, San Diego, CA), and as previously described (41). Briefly, muscle samples were homogenized in PBS (pH 7.5) containing 5mM EDTA and 1mM DTT, and then centrifuged. The supernatant was used for the glutathione peroxidase assay. The resulting absorbance was measured at 340 nm (DYNEX technologies, Chantilly Va., USA). Each sample and standard was performed in duplicate.

Catalase activity. The activity of catalase was determined in gastrocnemius muscle homogenates (# 219265, EMD/Calbiochem, San Diego, CA) as described previously (41). The samples were read on a microplate reader (DYNEX technologies, Chantilly, VA) at an

absorbance of 520nm. All analyses were measured in duplicate and the samples were normalized to the corresponding protein concentration (Bio-Rad Hercules, CA).

Activity levels of Manganese Superoxide Dismutase (MnSOD) and Copper-Zinc Superoxide Dismutase (CuZnSOD). Superoxide dismutase was measured using a commercially available SOD Assay Kit II (#574601, EMD/Calbiochem, San Diego, CA). Both total SOD and MnSOD activity were obtained. CuZnSOD was determined from assuming that CuZnSOD was the result of subtracting MnSOD activity from the total SOD activity. The assay was performed with slight modifications to the manufacturer's directions and all samples and standards were measured in duplicate as described previously by our laboratory (41). Briefly, gastrocnemius muscle samples were homogenized in (20mM HEPES buffer, containing 1mM EGTA, 210mM mannitol, and 70 mM sucrose and the insoluble material was discarded. The supernatant was incubated either with, or, without, 12 mM potassium cyanide to inhibit CuZnSOD and extracellular SOD activity. The sample absorbance was measured at 450 nm using a 96-well plate reader (Dynex Tech., Chantilly VA., USA).

mRNA levels of endogenous antioxidant enzymes. mRNA for GPX-1, Catalase, MnSOD and CuZnSOD were measured in the gastrocnemius muscle using reverse transcription-polymerase chain reaction (RT-PCR) according to methods previously published by our laboratory (41, 44). Briefly, total RNA was reversed transcribed using random primers, dNTP, and SuperScript II reverse transcriptase (Invitrogen/Life Technologies, Bethesda, MD). The primers for CuZnSOD, MnSOD, GPx-1, and catalase have been previously described (41). 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 by agarose gel electrophoresis. The gels were stained with ethidium bromide and the resulting signals were digitally captured (Kodak 290) and the signals were quantified using 1D Kodak image analysis software (Eastman Kodak Company, Rochester, NY).

Statistical analysis. All statistical analyses were performed using SPSS software package (version 13, Chicago, IL). Comparison of means was determined using analyses of variance (ANOVA) to examine the main effect of aging, exercise and resveratrol supplementation. Pair-wise comparisons were assessed post hoc using least significant difference (LSD) tests. Statistical significance was established at $p < 0.05$. Data are reported as mean ± standard error mean (SEM).

Results

Body Weights and Food Intake. The average body weight of the aged animals was significantly ($p<0.05$) more than the young animals (33.0 ± 0.8g vs. 25.5 ± 0.7g). There was not a significant difference in body weight between non-supplemented and resveratrol supplemented animals, in either age group. Similarly, there was no observed difference in food intake between the control and resveratrol supplemented diets. While young adult animals had a similar food consumption (3.1 ± 0.3 g/day) as aged animals (3.8 ± 0.4 g/day), when normalized to body weight, the young animals received a greater amount of resveratrol per day (156.1 ± 18.1 g resveratrol/kg BW vs. 113.5 ± 26.5 g resveratrol/kg BW).

Muscle Functional Measurements. Maximal isometric plantar flexion force was recorded for each evoked contraction. Data was plotted as a force x time curve for each isometric contraction from each session. Representative data of the first and last contraction from the third day in the young adult (Figure 5.1C&D) and aged (Figure 5.1E&F) mice is shown in Figure 5.1. The greatest contributor to plantar flexion is the gastrocnemius muscle. The maximal isometric force recorded on the third day (which was the first contraction of that day) was normalized to the animal's body weight. Maximal plantar flexion isometric force normalized

to body weight was 25% and 27% greater in young adult than aged animals that consumed the control or the resveratrol supplemented diet, respectively. Resveratrol supplementation did not have a significant influence on maximal isometric force in either the young adult or the aged animals (Figure 5.1A).

The fatigability of the plantar flexors within each training session was assessed by comparing the net loss of force from the first contraction of the session to subsequent contractions. Neither aging, nor resveratrol, had any significant affect on the maximal force produced during the first five contractions of the exercise session. However, after the fifth contraction, on the third day, the aged animals on the control diet and the aged animals on the resveratrol diet showed a greater maintenance of force than young animals on the control diet ($p<0.05$). No significant differences in fatigability were observed in the aged animals that received control, or resveratrol supplemented diets, whereas, young animals who were supplemented with resveratrol showed an improved maintenance of force over the exercise session (Figure 5.1B).

Insert Figure 5.1

Muscle levels of H_2O_2. H_2O_2 was measured in gastrocnemius muscle homogenates as an indicator of oxidant production in basal and exercised conditions. Muscle homogenate levels of H_2O_2 were elevated in isometrically exercised muscles by 31% in young adult mice (1848 vs. 2428 RFU/mg protein) and 19% in aged in the animals (3689 vs. 4401 RFU/mg protein) on the control diet compared to the age-matched non-exercised control limb ($p<0.05$) (Figure 5.2). This indicates that acute isometric exercise elevated muscle oxidant production. Control and repetitively loaded muscles from aged animals had 99% and 81% higher levels of H_2O_2, respectively, than their treatment matched muscles from young adult mice ($p<0.05$)(Figure 5.2). Fortifying the standard diet with resveratrol lead to a 24% ($p<0.05$) increase in H_2O_2 in the young exercised gastrocnemius muscles. Resveratrol significantly lowered H_2O_2 levels in both control and exercised muscles by 13% and 38% respectively, from aged animals ($p<0.05$) (Figure 2). Isometric exercise did not affect muscle levels of H_2O_2 in the young adult mice that received a diet supplemented with resveratrol. However, resveratrol significantly reduced H_2O_2 by 15% in the exercised muscles of aged mice compared with non-exercised mice fed the control diet (3190 vs. 2693 RFU/mg/protein) (Figure 5.2).

Insert Figure 5.2

Hypoxanthine & Xanthine Oxidase activities. Hypoxanthine, a product of purine degradation and a substrate for xanthine oxidase was measured in young adult and aged gastrocnemius muscles. Hypoxanthine increased by 36% ($p<0.05$) with aging. Furthermore, isometric exercise increased muscle levels of hypoxanthine by 21% ($p<0.05$) in young adult mice and by 20% ($p<0.05$) in aged mice. Resveratrol supplementation blunted the increase in hypoxanthine in muscles from both young adult and aged mice, but it did not reduce the elevated levels of hypoxanthine associated with aging (Figure 5.3A).

The activity of xanthine oxidase in gastrocnemius muscles from aged animals was elevated by 168% compared to muscles from young adult mice (258.9 vs. 692.3 RFU/mg protein, $p<0.05$; Figure 5.3B). Resveratrol supplementation lowered xanthine oxidase activity in the non-exercised muscle from aged mice by 16% (692.3 vs. 582.9 RFU/mg protein, $p<0.05$; Figure 5.3B), but it had no effect on the non-exercised control muscle from young adult mice. Compared to the contralateral control muscles, isometric exercise increased xanthine oxidase activity by 38% ($p<0.05$) in gastrocnemius muscles from young adult mice and by 18% ($p<0.05$) in muscles from aged animals on the control diet. Resveratrol supplementation blunted the

increase in xanthine oxidase activity associated with exercise in the young adult muscle by 25% ($p<0.05$) and reduced xanthine oxidase activity in the aged gastrocnemius by 53% ($p<0.05$) (Figure 5.3B). Resveratrol supplementation in the aged isometrically-exercised animals resulted in a 50% ($p<0.05$) reduction in muscle xanthine oxidase activity when compared to the non-exercised muscle (Figure 5.3B).

Insert Figure 5.3

The concentration of glutathione. Total glutathione decreased by 26% ($p<0.05$) in the control diet, non-exercised gastrocnemius muscle of aged mice (115 ± 18 µM/mg protein) compared with young adult muscle (156 ± 26). However, in the exercised gastrocnemius muscle form the mice receiving the control diet there was no significant difference between the young adult (153 ± 15 µM/mg protein) and the aged muscles (126 ± 17 µM/mg protein). Exercise alone failed to produce any changes in total glutathione within either age group receiving the control or the resveratrol fortified diet. A combination of resveratrol and exercise produced a 27% ($p<0.05$) increase in total glutathione in the young adult animals (195 ± 27 µM/mg protein) when compared to the young exercised animals that did not receive the fortified diet (153 ± 15 µM/mg protein), but no difference were observed in the aged animals. Resveratrol supplementation in the non-exercised limb did not produce any significant differences in either age group.

The ratio of reduced to oxidized glutathione (GSH/GSSG). The GSH/GSSG ratio was 31% lower in control, non-exercised muscles of aged mice (9.5 ± 1.3) compared with young mice (13.9 ± 2.5). This reduction in the GSH/GSSG ratio is indicative of an increase in oxidants as a result of both aging and exercise and may also be the result of impaired GSH metabolism and replenishment (11). The GSH/GSSG ratio was not altered by resveratrol in non-exercised control muscles from either young adult, or aged mice (Figure 4B). Isometric exercise decreased the GSH/GSSG ratio by 39% ($p<0.05$) and 43% ($p<0.05$) in the muscles of young and aged mice that were fed the control diet (Figure 5.4B). However, resveratrol supplementation prevented the exercise-induced decrease in the GSH/GSSG ratio in the gastrocnemius muscle, such that GSH/GSSG was 38% and 135% greater in muscles from resveratrol treated exercised young and aged mice, respectively, as compared to muscles from age-matched mice on the control diets.

Insert Figure 5.4

Muscle lipid peroxidation levels. Aging increased total amounts of lipid peroxidation in non-exercised control gastrocnemius muscles by 57% as shown by the greater MDA + HNE levels from control muscles from aged non-supplemented rats compared to control muscles from young adult non-supplemented animals (2.86 vs. 4.49 µM/mg protein, $p<0.05$; Figure 5.5). Three days of isometric exercise failed to alter lipid peroxidation levels within muscles from the young adult mice but it increased MDA + HAE levels by 63% (4.49 vs. 7.31 µM/mg protein, $p<0.05$; Figure 5.5) in the muscles from aged non-supplemented mice. Resveratrol supplementation completely prevented the increase in MDA + HNE levels associated with exercise in the muscles from aged mice, but resveratrol did not blunt the increase in lipid peroxidation associated with aging. Both control and isometrically exercised muscles from aged resveratrol supplemented animals showed a ~53% ($p<0.05$) increase in MDA + HAE levels when compared to their young adult counterparts. Resveratrol supplementation had no effect on lipid peroxidation in the young adult animals.

Insert Figure 5.5

Glutathione peroxidase (GPx) and catalase enzyme activity and mRNA abundance. There was no apparent aging, or exercise effect on GPx activity in the non-supplemented gastrocnemius muscle. Furthermore, resveratrol supplementation did not lead to any changes in GPx activity in the control, or exercised muscle from young adult animals. However, GPx activity increased 15% ($p<0.05$) in control and 12% ($p<0.05$) in exercised muscles from aged mice with resveratrol supplementation (Figure 5.6A). There were no significant changes that were found among GPx-1 mRNA within any of the muscle samples (Figure 5.6B).

Catalase activity was higher in the gastrocnemius muscle from the aged animals when compared to their treatment matched young adult counterparts. Neither isometric exercise, nor resveratrol supplementation, showed any significant changes in catalase activity within muscles from young adult animals. There was a 42% increase ($p<0.05$) in catalase activity with isometric exercise in the aged animals on the standard diet and a 19% increase ($p<0.05$) with the resveratrol supplemented diet. Resveratrol supplementation increased catalase activity within the aged animals by 50% ($p<0.05$) in the non-exercised control and 25% ($p<0.05$) in the isometrically exercised gastrocnemius muscle (Figure 5.6C). Similar to the enzyme activity data, catalase mRNA content was greater in the gastrocnemius muscle from all groups of the aged animals when compared to their treatment matched young adult counterparts. Supplementation with resveratrol increased catalase mRNA levels in the gastrocnemius muscle from both young adult and aged animals. However, exercise did not produce any significant changes in catalase mRNA levels within any of the groups (Figure 5.6D).

Insert Figure 5.6

Superoxide dismutase enzyme activity and mRNA levels. Isometric exercise increased CuZnSOD enzyme activity ($p<0.05$) by 27% in non-supplemented and 19% in resveratrol supplemented muscles from young adult animals, compared to their contra-lateral control muscles. Within the gastrocnemius muscles from the aged animals, exercise increased CuZnSOD activity by 25% ($p<0.05$) in the animals fed the standard diet, but no differences were observed between the control and exercised muscles of resveratrol supplemented animals. No significant changes in enzyme activity were observed as a result of aging in any of the groups (Figure 5.7A). No significant differences were found among CuZnSOD mRNA within any of the muscle samples (Figure 5.7B).

MnSOD activity was 10% greater in the non-exercised control gastrocnemius muscle from aged animals compared to young animals (0.89 vs. 0.80 U/mg protein). Surprisingly, isometric exercise lead to a decrease in MnSOD activity in the gastrocnemius muscle from young adult animals feed the standard diet, whereas exercise had no affect on MnSOD activity in the muscles from aged animals. Resveratrol supplementation increased MnSOD activity by ~ 10-15% in all groups and removed any differences between groups due to aging, or exercise (Figure 5.7C). While isometric exercise did not induce any changes in mRNA transcription for MnSOD, resveratrol feeding lead to a ~90% increase in MnSOD transcription in exercised and a ~50% increase in control muscles from both age groups (Figure 5.7D).

Insert Figure 5.7

Discussion

Oxidative stress is elevated with aging in most tissues, including skeletal muscle (14, 25, 41). Increased reactive oxygen species (ROS) production may contribute to aging-induced skeletal muscle wasting (i.e., sarcopenia) (15, 41). Although exercise is a useful approach to counter aging-induced sarcopenia, it also increases oxidative stress levels within the exercising muscles (15, 25, 33). The additive effects of an increase in oxidant production and an

attenuated antioxidant buffering capacity potentially leaves aged skeletal muscles more vulnerable to oxidative damage. The novel data in this study show that dietary resveratrol reduces oxidative stress, including xanthine oxidase activity, in control and exercised muscles of aged mice.

Xanthine oxidase as one source of exercise-induced oxidant stress

Although it is clear that oxidative stress is elevated in response to both acute exercise and aging, it has not been conclusively established that xanthine oxidase contributes to the increased oxidant production with advanced age in skeletal muscle. In the present study, we show that both xanthine oxidase activity and hypoxanthine levels are elevated in gastrocnemius muscles from aged mice, compared to young adult mice. This is consistent with data showing that xanthine oxidase activity was higher in the gastrocnemius muscles from aged rats when compared to young animals (18), and in plasma from older vs. young adult humans (3). Nevertheless, this contrasts with other data in humans that have shown an absence of age-associated increases in endothelial xanthine oxidase in antecubital venous cells from the young and older subjects (13).

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

Resveratrol reduces oxidative stress

Resveratrol has been shown to exert a variety of health benefits that include the direct scavenging of ROS (46), the inhibition of xanthine oxidase (24, 26) and the activation of intracellular pathways that improve metabolism and induce mitochondrial biogenesis (5, 32). The current study suggests that resveratrol supplementation lowers muscle indices of oxidative stress (H_2O_2, xanthine oxidase activity, GSH/GSSG ratio and lipid peroxidation) associated with both normal aging and isometric exercise in aged mice. In fact, muscle xanthine oxidase activity and H_2O_2 production were lower in exercised muscles from aged mice, than in muscles of age-matched non-exercised, non-supplemented control muscles. In addition, resveratrol supplementation abolished the increase in xanthine oxidase activity and H_2O_2 production associated with isometric contractions in muscles from young mice. Together these data suggest that there may be an additive benefit to combining resveratrol supplementation with isometric exercise, especially in aged skeletal muscle.

As expected, the short duration of resveratrol supplementation in the current study affected H_2O_2 concentrations, xanthine oxidase activity, and lipid peroxidation to a greater degree with exercise than aging. It is therefore likely that aging and isometric exercise may regulate oxidant production via different mechanisms. For example, isometric exercise increased the activity of the cytosolic antioxidant enzymes, catalase and CuZnSOD, along with an increase in xanthine oxidase activity, implying that at least part of the increase in H_2O_2 production with isometric exercise is not originating from the mitochondria. This possibility is in agreement with observations showing that aging increases oxidant production via mitochondrial sources, whereas exercise increases oxidants in muscles from multiple sources, including xanthine oxidase (6).

Resveratrol's putative role in reducing oxidative stress is likely a combination of many factors. When taken orally, *trans*-resveratrol is well-absorbed by mammals, but its bioavailability is low due to its rapid first pass metabolism (48). Therefore, its role as a direct scavenger of

reactive oxygen species (9) is likely to be limited. The most likely mechanism by which resveratrol can attenuate the increase in oxidative stress due to aging and exercise lies in its ability to induce transcriptional changes via the activation of silent mating type information regulation 2 homolog (Sirt1) (37). Sirt1 is a NAD^+ dependent histone deacetylase that is upstream of a wide variety of cellular pathways involved in energy homeostasis, longevity, cell survival and apoptosis. Increases in Sirt1 transcription have been shown to occur after 3 days of resveratrol supplementation (45). Sirt1 activation sequentially leads to energetic adaptations within the muscle by activating the metabolic regulators PPARγ co-activator (PGC-1α) and AMP kinase (AMPK) and in turn enhancing components of the mitochondrial electron transport chain, β-oxidation and ATPases (5, 32). Although speculative, one possibility is that resveratrol might reduce uncoupling of the mitochondrial electron transport chain, leading to an increased availability of ATP and decreased superoxide formation. Previous findings have demonstrated that increases in post-exercise concentrations of hypoxanthine are accurate predictors of muscle energy depletion (7) and adenine nucleotide degradation during exercise (43). Our current data show increased hypoxanthine concentrations after exercise, which is indicative of elevated ATP utilization and depletion (43). Resveratrol supplementation decreased hypoxanthine levels following exercise, and this is consistent with the idea that resveratrol increased ATP availability to the exercising muscles. Nevertheless, the data in this study do not provide insight into whether greater ATP availability and/or improvement in mitochondria function are important outcomes for reducing oxidative stress after resveratrol supplementation.

Antioxidant enzymes and resveratrol

To our knowledge, this is the first investigation to examine the effects of resveratrol supplementation on the regulation of the endogenous antioxidant system in response to isometric exercise in young and aged animals. Our study measured transcription and activity levels of the endogenous antioxidant enzymes catalase, glutathione peroxidase, MnSOD and CuZnSOD. In general, the results do not support transcriptional control as a mechanism for altering the activity levels of the endogenous antioxidant enzymes within the muscles of non-supplemented animals. Instead, these data are consistent with previous data that indicate the activity of the endogenous antioxidant enzymes are regulated via various levels of post-transcriptional and/or post-translational controls (21, 41). Insufficient tissue was available in this study to determine if the protein levels of endogenous antioxidant enzymes had been altered by short-term resveratrol treatment.

Several studies have shown that there is no aging-induced change in MnSOD or CuZnSOD activity, although loss of CuZnSOD exacerbates muscle loss with aging (35). Furthermore, over-expression of antioxidant enzymes does not improve life span (49). Our data indicate that aging did not alter the activity of GPx and CuZnSOD; however, aging increased the enzyme activity of MnSOD and catalase and catalase mRNA content. This increase in catalase transcription may be attributed to an attempt to compensate for the inability of the glutathione system to buffer H_2O_2. Age-related increases in catalase activity have been proposed as a potential means to counterbalance the depletion of glutathione levels in metabolically active tissues (34). Differences in age-dependent transcriptional control of the other endogenous antioxidant enzymes between our current study and that reported in other studies (20, 41) may be due to differences in environmental conditions, animal models, or the muscles that were investigated.

Short-term adaptation of antioxidant enzymes to isometric exercise in both young adult and aged animals appeared to occur via an increase in catalase and CuZnSOD activity, two enzymes that are primarily located in the cytosol. Catalase activity increased only in the aged animals after isometric contraction whereas, CuZnSOD increased in muscles from both the young adult and aged mice after isometric contractions. However, isometric contractions did not

have an effect on mRNA content for any of the endogenous antioxidant enzymes (GPx, catalase, CuZnSOD or MnSOD) investigated in the current study. These observations are similar to previous data from our lab (41), where 4.5 weeks of repetitive loading exercise in the rat tibialis anterior muscle increased the activity of both catalase and CuZnSOD without changing mRNA content for these enzymes.

The absence of changes in mRNA suggests that post-transcriptional modifications might be responsible for either enhancing the capacity of the active site of the antioxidant enzymes, or perhaps reducing protein degradation leading to increased enzyme content. Similarly, it has been shown that increases in CuZnSOD protein levels occur without changes in mRNA content after a single bout of endurance exercise (19).

Resveratrol supplementation increased the endogenous antioxidant enzymes, catalase and MnSOD (39), which are located in close proximity to the sites of electron transport production of ATP. For example, MnSOD is localized to the mitochondrial matrix where it protects the mitochondria from oxidative damage. In addition, catalase is found in low concentrations in the cytosol and it is thought to be contained mainly within peroxisomes, which are sites of fatty acid oxidation. This fits well with previous observations showing that resveratrol supplementation increases the transcription and the activity of both catalase (31, 37) and MnSOD (37, 39). Furthermore, resveratrol can increase mitochondrial components of aerobic metabolism via its activation of Sirt1 (5, 37).

No effect of resveratrol on muscle force

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

Resveratrol does not improve muscle fatigability in aged mice

Muscle fatigability was measured as the relative decline in maximal isometric force by comparing the first and twentieth contraction in the third exercise session. Muscles from aged animals in either the control, or the resveratrol group had significantly greater fatigue resistance than muscles from young animals that where fed a control diet (Figure 1B). The greater relative decrease in fatigue over the 20 contractions may be due to an age dependent shift in muscle fiber type towards a greater percentage of type I fibers (22, 36). The larger sized fibers from the young adult animals would produce a greater maximal force and have a greater rate of ATP utilization (increased cross bridge cycling, greater calcium release and therefore greater ATP utilization by sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps, greater heat production) than the aged muscle, therefore having greater relative decline in force over time. However, the similar relative changes in hypoxanthine observed during exercise in the young adult and the aged animals would imply that if ATP utilization was greater in the young animals, then young muscle possess a mechanism that limited purine degradation even though ATP supplies were not fully restored. Further research is needed to fully understand the age-dependent difference in muscle fatigue found in the current model of isometric exercise.

The plantar flexor muscles from young adult animals supplemented with resveratrol had a significantly lower decline in muscle force over the 20 contractions (-27.5 ± 1.6%) than animals that were fed the control diet (-37.4 ± 7.9%). The decline in maximal isometric force

was similar in resveratrol supplemented young adult animals and aged animals from either control (-42.9 ± 4.1%), or resveratrol diet (44.4 ± 5.1%) groups.

Previous studies have reported that resveratrol supplementation improved mitochondria function and reduced fatigue associated with aerobic exercise (5, 32). Our data show for the first time that resveratrol reduces muscle fatigue in response to repetitive anaerobic (isometric) contractions, and therefore this effect is not activity specific. However, the effect of resveratrol with regard to repetitive exercise occurs only in muscles of young animals. It is possible that resveratrol increased the availability of ATP, by enhancing the density and efficiency of the mitochondrial electron transport chain, by increasing β-oxidation and by increasing ATPase content (5, 32, 37) in muscles of young mice. This possibility is plausible because increases in hypoxanthine have been shown to be predictors of muscle ATP exhaustion (7) and hypoxanthine concentrations were lower in muscles from resveratrol supplemented mice in our current study. However, this investigation does not provide any direct evidence for this possibly, because we did not measure mitochondrial density or efficiency, nor did we measure skeletal muscle ATP content.

If resveratrol has an age-specific effect on fatigue resistance, it might be argued to act in a muscle fiber-type specific manner. For example, there is a well known increase in type I muscle fibers with aging, and muscles with a high percentage of type I fibers (e.g. soleus) appear to be more resistant to resveratrol-induced increases in mitochondrial enzymatic activity and oxidative capacity than the gastrocnemius muscle (primarily composed of type II fibers) (32). Nevertheless, fiber type specific responses cannot explain all of the effects of resveratrol, because even if there were some age-induced shift towards type I fibers, the gastrocnemius muscle, in the aged mouse still has a high percentage of type II fibers. Furthermore, mitochondria volume density is not a good predictor of fatigue resistance to isometric exercise (1), and therefore improvements in mitochondria number, or size, would be anticipated to have minimal, or no effects on isometric fatigability.

Another possibility to account for the improved fatigue resistance to maximal isometric exercise in muscles from young animals is that resveratrol could directly diminish exercise-induced oxidant production which, have been shown to be a mediator of muscle fatigue (38). However, we do not regard this as a strong putative mechanism, because resveratrol decreased the exercise-induced elevation in H_2O_2 levels and other indices of oxidative stress in muscles from both young and aged mice yet, despite clear reductions in oxidative stress, resveratrol did not improve muscle fatigue resistance in aged animals. These findings indicate that acute increases in oxidative stress, including modulation of H_2O_2, are not sufficient to moderate acute fatigue responses to maximal isometric exercise in skeletal muscle with aging.

Conclusion

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

Figure Legends

Figure 5.1

Maximal plantar flexor isometric force after 3 consecutive days of isometric exercise. **(A)** Data are expressed as the mean ± SEM of the maximal isometric force recorded on the third day of exercise by the left plantar flexor muscles normalized to the body weight of the animal. † signifies a difference ($p<0.05$) from young adult diet-matched muscles. **(B)** Data are expressed as the mean ± SEM of the relative difference between the maximal isometric force on the first contraction and the force produced on subsequent contractions. All force measurements were normalized to body weight. * indicates a significant difference ($p<0.05$) in the aged non-supplemented control and resveratrol supplemented diet from young non-supplemented control diet. ‡ indicates a significant difference ($p<0.05$) in the young adult animals in the resveratrol supplemented diet group, from animals in the young adult non-supplemented control diet group. **(C-F)** Representative force x time curves from the third consecutive exercise session in the resveratrol treated young adult and aged animals**.** Force (in grams) is shown on the y-axis and time (in seconds) is shown on the x-axis. **(C)** First contraction of the 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 third day in an aged animal.

Figure 5.2

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

Figure 5.3

Resveratrol attenuated the increase in xanthine oxidase and hypoxanthine. Relative concentrations of hypoxanthine (**A**) and enzymatic activity of xanthine oxidase (**B**) were determined fluorometrically. Data are expressed as µ moles of hypoxanthine per mg protein or relative fluorescent units (RFU) per mg of total protein in gastrocnemius homogenates. The normalized data are presented as mean ± SEM. * significant difference ($p<0.05$) between isometrically exercised muscles from contra-lateral control muscles; † a significant difference ($p<0.05$) from young exercised and diet-matched control muscles; § significant difference ($p<0.05$) from age-matched animals in the control (non-supplemented) diet group.

Figure 5.4

Total glutathione content & ratio of reduced glutathione to oxidized glutathione (GSH/GSSG). **(A)** Data indicate total glutathione concentration normalized to total protein content. **(B)** Data are depicted as the ratio of GSH to GSSG normalized to total protein content. Lower GSH/GSSG ratios are an indication of increased oxidative stress. The normalized data are presented as mean ± SEM. * significant difference ($p<0.05$) of isometrically exercised muscle from contra-lateral control muscle; † a significant difference ($p<0.05$) from young exercise and diet-matched control muscles; § significant difference ($p<0.05$) from age-matched animals on the non-supplemented diet.

Figure 5.5

Resveratrol supplementation decreased lipid peroxidation associated with isometric exercise, but not aging. The level of lipid peroxidation was estimated from malondialdehyde (MDA) plus 4-hydroxyalkenals (HAE) levels that were normalized to total protein content in the muscle sample. The normalized data are presented as mean ± SEM. *, significant difference ($p<0.05$) of isometrically exercised muscles from contra-lateral control muscles; †, a significant difference ($p<0.05$) from young exercised and diet-matched control muscles; §, significant difference ($p<0.05$) from age-matched animals on the non-supplemented diet.

Figure 5.6

Glutathione peroxidase (GPx) activity, GPx-1 mRNA, catalase activity and catalase mRNA regulation with isometric exercise and resveratrol supplementation (A) Total GPx activity is expressed as mU of GPx per ml of homogenate normalized per mg of protein in homogenate. **(B)** GPx-1 mRNA expression was determined by RT-PCR. The data are expressed as optical density (OD) x band area, and expressed as a relative optical density. The inserts show representative gels for GPx-1 mRNA and 18s rRNA in young and aged (control and isometric exercised) gastrocnemius muscle. **(C)** Total catalase activity is expressed as nM of activity per min normalized per mg of protein in the homogenate. **(D)** Catalase mRNA expression was determined by RT-PCR. The data are expressed as optical density (OD) x band area, and expressed as a relative optical density. The inserts show representative gels for catalase mRNA and 18s mRNA in muscles from young and aged (control and isometrically exercised) mice. For all graphs the data are presented as mean ± SEM; *, significant difference ($p<0.05$) of isometrically exercised muscles from contra-lateral control muscles; †, a significant difference ($p<0.05$) from young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) from age-matched animals on the non-supplemented diet. **YCC**, Young-control diet-control non-exercise; **YCE**, Young- control diet-exercised; **YRC**, Young-resveratrol-control non-exercise; **YRE**, Young-resveratrol-exercised; **ACC**, Aged-control diet-control non-exercise; **ACE**, Aged- control diet-exercised; **ARC**, Aged-resveratrol-control non-exercised; **ARE**, Aged-resveratrol-exercised.

Figure 5.7

Superoxide dismutase activity and mRNA regulation with isometric exercise and resveratrol supplementation. (A) Copper-Zinc superoxide (CuZnSOD) activity was expressed as U of CuZnSOD per mg of protein in the homogenate. A unit was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. **(B)** CuZnSOD mRNA expression was determined by RT-PCR. The data are expressed as optical density (OD) x band area, and reported as relative optical density. The inserts show representative gels for CuZnSOD mRNA and 18s rRNA in gastrocnemius muscles from young and aged (control and isometric exercised) mice. **(C)** Manganese superoxide dismutase (MnSOD) activity was determined expressed as U of MnSOD per mg of protein in homogenate. A unit was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. **(D)** MnSOD mRNA expression was determined by RT-PCR. The data are expressed as optical density (OD) x band area, and expressed as relative optical density. The inserts show representative gels for MnSOD mRNA and 18s rRNA in gastrocnemius muscles from young and aged (control and isometric exercised) mice. For all graphs the normalized data are presented as mean ± SEM; *, significant difference ($p<0.05$) of isometrically exercised muscles from contra-lateral control muscles; †, a significant difference ($p<0.05$) from young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) from age-matched animals on the non-supplemented diet. **YCC**, Young-control diet-control non-exercise; **YCE**, Young- control diet-exercised; **YRC**, Young-resveratrol-control non-exercise; **YRE**, Young-resveratrol-exercised; **ACC**, Aged-control diet-control non-exercise; **ACE**, Aged- control diet-exercised; **ARC**, Aged-resveratrol-control non-exercised; **ARE**, Aged-resveratrol-exercised.

Figure 5.1

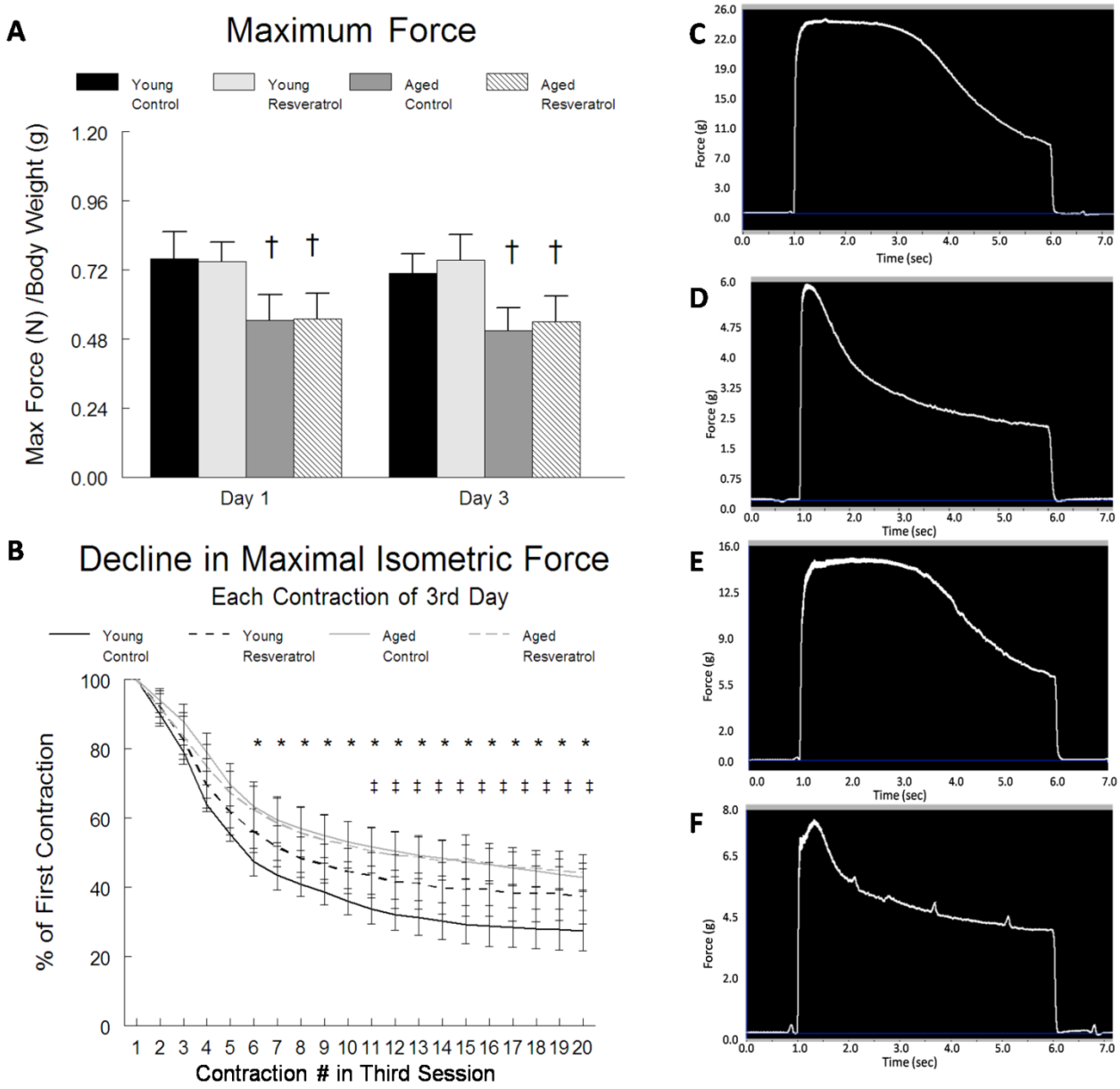

A
Maximum Force
Young Control
Young Resveratrol
Aged Control
Aged Resveratrol
Max Force (N) /Body Weight (g)
1.20
0.96
0.72
0.48
0.24
0.00
Day 1
Day 3
B
Decline in Maximal Isometric Force
Each Contraction of 3rd Day
Young Control
Young Resveratrol
Aged Control
Aged Resveratrol
% of First Contraction
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Contraction # in Third Session
C
D
E
F
Force (g)
Time (sec)

Figure 5.2

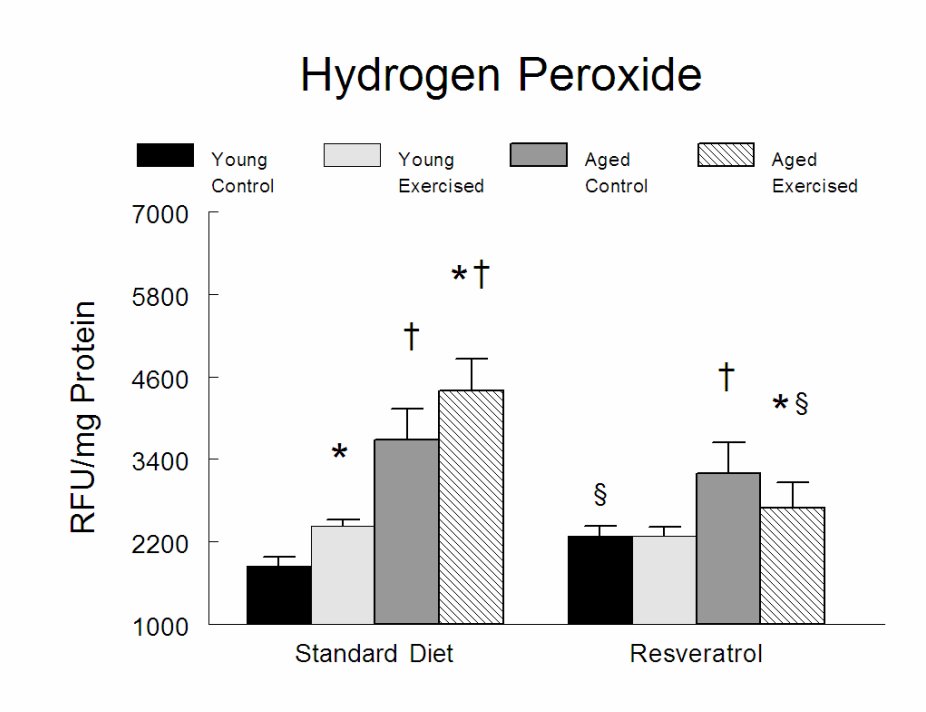

Hydrogen Peroxide
Young Control
Young Exercised
Aged Control
Aged Exercised
RFU/mg Protein
7000
5800
4600
3400
2200
1000
*
*†
†
†
§
*§
Standard Diet
Resveratrol

Figure 5.3

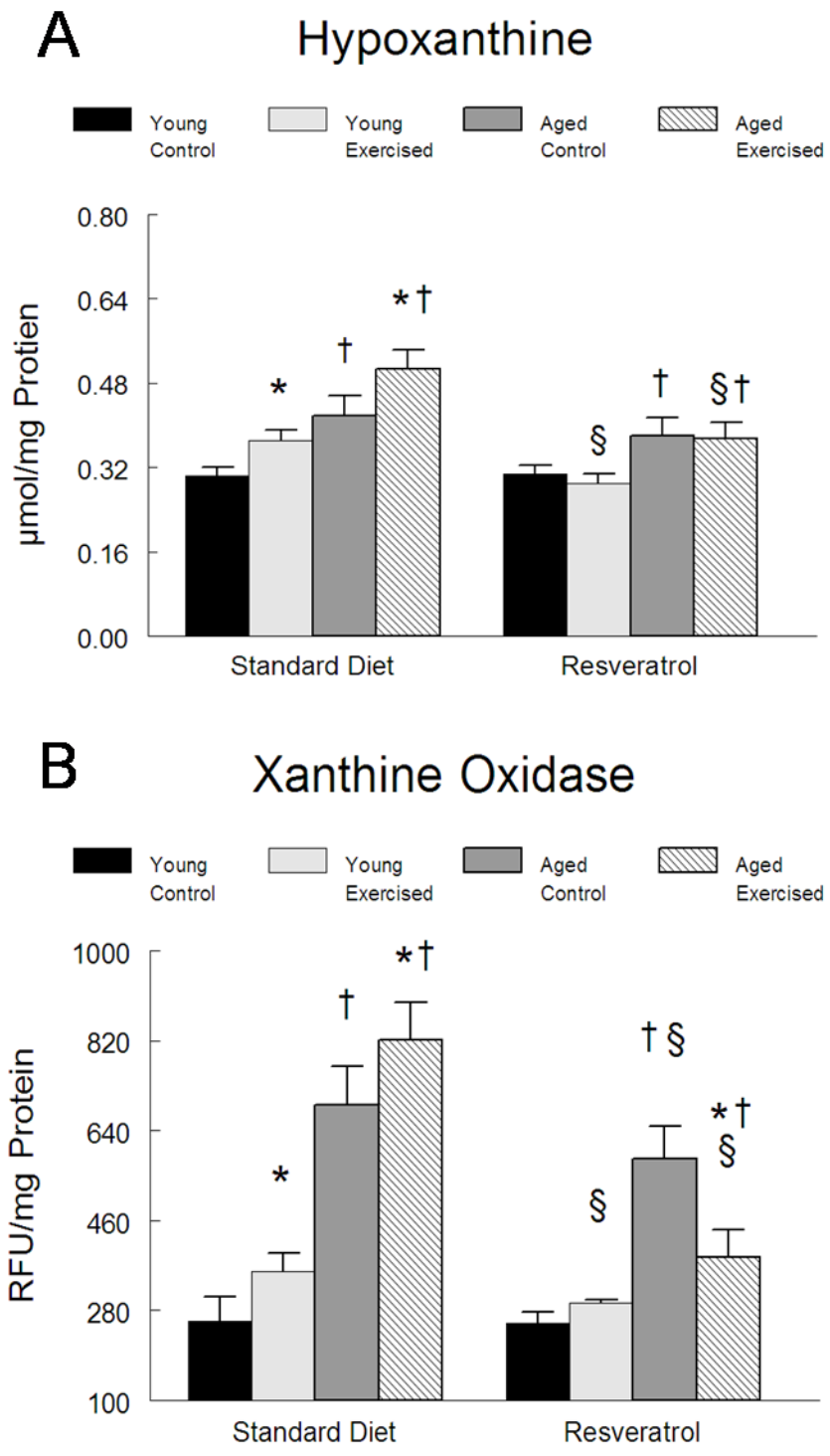

A
Hypoxanthine
Young Control
Young Exercised
Aged Control
Aged Exercised
µmol/mg Protien
0.80
0.64
0.48
0.32
0.16
0.00
*
†
*†
§
†
§†
Standard Diet
Resveratrol
B
Xanthine Oxidase
Young Control
Young Exercised
Aged Control
Aged Exercised
RFU/mg Protein
1000
820
640
460
280
100
*
†
*†
§
†§
*†
§
Standard Diet
Resveratrol

Figure 5.4

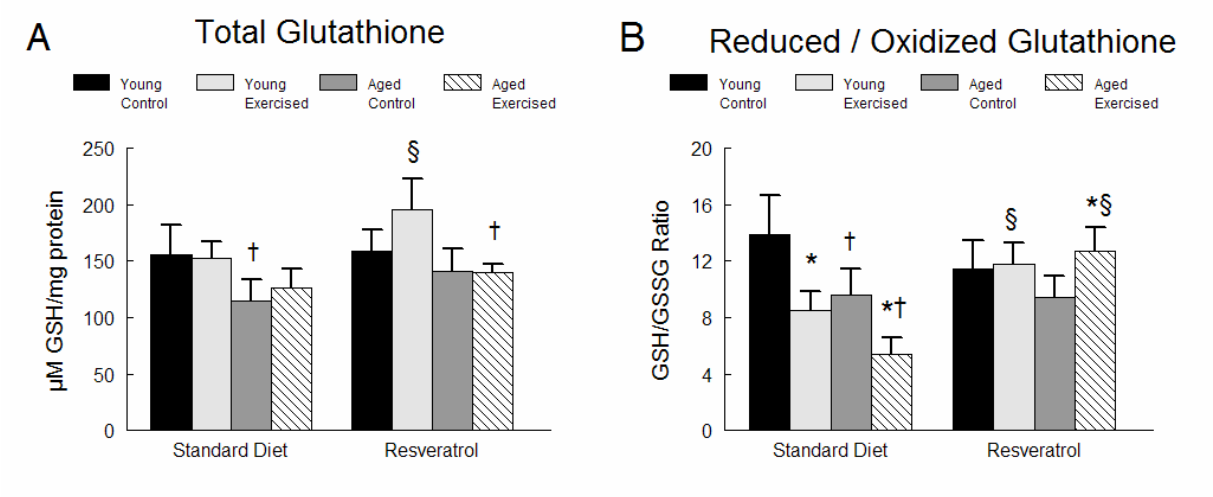


Figure 5.5

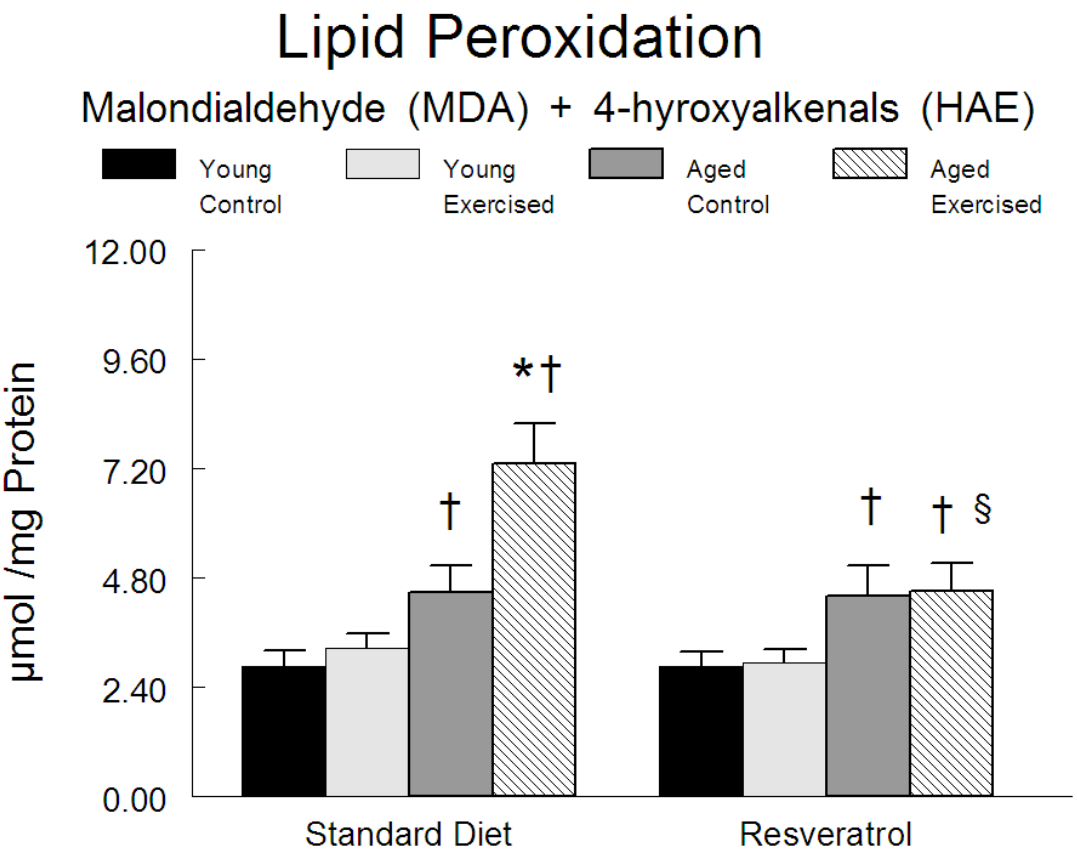


Figure 5.6

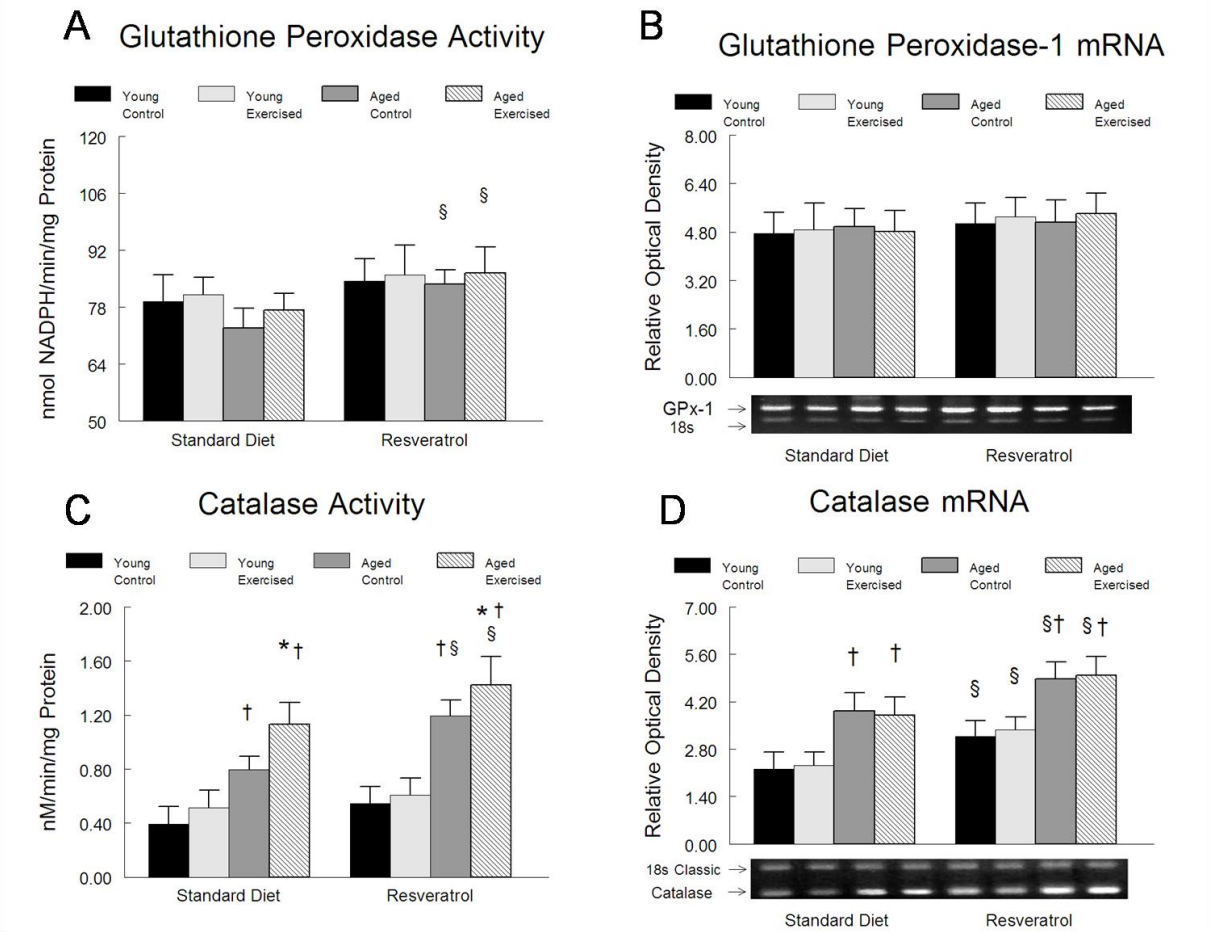
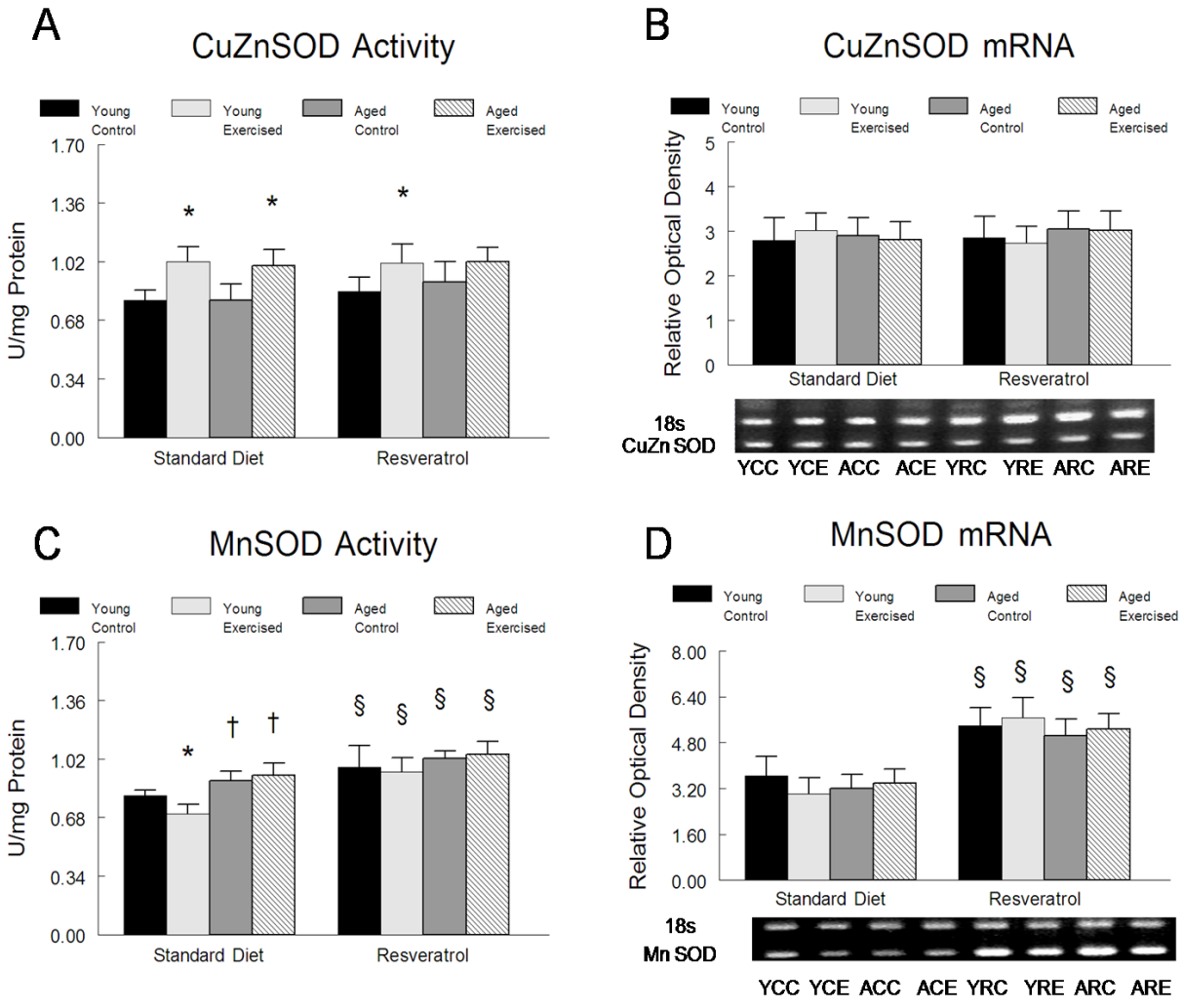

A
Glutathione Peroxidase Activity
Young Control
Young Exercised
Aged Control
Aged Exercised
nmol NADPH/min/mg Protein
120
106
92
78
64
50
§
§
Standard Diet
Resveratrol
B
Glutathione Peroxidase-1 mRNA
Relative Optical Density
8.00
6.40
4.80
3.20
1.60
0.00
GPx-1
18s
Standard Diet
Resveratrol
C
Catalase Activity
nM/min/mg Protein
2.00
1.60
1.20
0.80
0.40
0.00
*†
†
†§
*†
§
Standard Diet
Resveratrol
D
Catalase mRNA
Relative Optical Density
7.00
5.60
4.20
2.80
1.40
0.00
†
†
§
§
§†
§†
18s Classic
Catalase
Standard Diet
Resveratrol

Figure 5.7


A
CuZnSOD Activity
Young Control
Young Exercised
Aged Control
Aged Exercised
U/mg Protein
1.70
1.36
1.02
0.68
0.34
0.00
Standard Diet
Resveratrol
B
CuZnSOD mRNA
Relative Optical Density
5
4
3
2
1
0
18s
CuZn SOD
YCC YCE ACC ACE YRC YRE ARC ARE
C
MnSOD Activity
D
MnSOD mRNA
8.00
6.40
4.80
3.20
1.60
0.00
Mn SOD

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Chapter 6

Inhibition of xanthine oxidase reduces oxidative stress and improves muscle function in exercised skeletal muscle from 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 isometric exercise. 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 prior to the start of exercise. Mice were anesthetized, then the left posterior tibial nerve was subjected to 20, five second square wave pulses (10v, 100 Hz, 200 μs pulses), with 25 seconds between stimulus trains to produce maximal isometric contractions of the plantar flexor muscle group. The exercise was repeated for three consecutive days; the contralateral limb served as an intra-animal control. A force x time curve was obtained for each isometric contraction. Compared to young mice, xanthine oxidase activity was greater in the gastrocnemius muscle of aged mice (65%) and it increased after isometric exercise in muscles from both young (33%) and aged (28%) mice. Allopurinol treatment attenuated the exercise-induced increase in oxidative stress, but it did not affect elevated basal levels of oxidative stress associated with aging. Furthermore, inhibition of xanthine oxidase activity decreased caspase-3 activity, but had no effect on other markers of mitochondrial associated apoptosis. Additionally, the inhibition of xanthine oxidase increased maximal isometric force in the plantar flexor muscles from aged mice (35%). Our results suggest that repetitive isometric contractions increased xanthine oxidase activity, which contributes to exercise-induced oxidative stress in muscles of aged mice.

Introduction

The fundamental mechanisms contributing to aging are poorly understood, but a large body of evidence supports the hypothesis that oxidative stress (21) contributes to aging in many tissues. Oxidative stress occurs when the cellular production of oxidants exceeds the physiological buffering capacity of the tissue. 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 with aging. Rather, the elevation in oxidative stress that occurs with aging can regulate redox-sensitive signaling pathways (31, 33, 38), increase catabolic gene expression (11, 40, 42, 64), and activate apoptotic pathways (35, 41, 60), thereby contributing to the progression of sarcopenia.

Mitochondria are a major source of oxidant production in skeletal muscle (5, 46). The consequence of prolonged exposure to relatively high levels of oxidants reduces mitochondrial membrane integrity and antioxidant enzyme activity (46). 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. AIF release initiates a caspase independent pathway, while cytosolic cytochrome c initiates the caspase cascade resulting in DNA fragmentation and myonuclear apoptosis. Thus, mitochondria may be important for regulation of both oxidative stress and apoptotic signaling in aging skeletal muscle.

The functional implications of elevated oxidative stress in skeletal muscle include reduced muscle specific force (7), altered myofilament function (2, 37), and elevated muscle fatigue (54). Although exercise is used as a strategy to attempt to reduce sarcopenia and improve muscle function, acute exercise will also increase free radical generation in skeletal muscle (9). This has important implications in a highly metabolic tissue such as skeletal muscle, where basal oxidant production is already increased with aging and exercise has the potential to further increase oxidant production by as much as 80% (5).

There are three major sources of oxidant production with exercise. These include infiltrating immune cells, mitochondrial respiration and xanthine oxidase activity (39). The magnitude and the sources of oxidant production are dependent on the mode, duration and intensity of exercise. Increased xanthine oxidase activity within the vascular endothelium (28), is a contributing factor associated with oxidative stress and damage during exhaustive exercise (4, 14, 22, 58, 63). Allopurinol, which is a structural isomer of hypoxanthine, acts as a competitive inhibitor to xanthine oxidase protecting cells from oxidative damage associated with exhaustive exercise (63). 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 (44, 51, 63). 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 (See Figure 6.1).

Subsequent to the elevation in hypoxanthine, xanthine dehydrogenase is converted to xanthine oxidase either reversibly by oxidation, or irreversibly via proteolysis (8, 48). Conversion of xanthine dehydrogenase to xanthine oxidase has been shown to be dependent on both calcium and oxidant concentrations (45). During muscle contractions, intracellular calcium concentrations are elevated, which in turn, may 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 (48).

During muscle relaxation the influx of oxygen rich blood catalyzes the reaction of xanthine oxidase with hypoxanthine and oxygen to from 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, since the superoxide anion is quickly dismutated to H_2O_2 by 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 (30, 47, 66).

Xanthine oxidase has been reported to make important contributions to oxidative stress in the heart (59) and gastrocnemius muscles (3, 18, 57) from aged rodents; however, this age-dependent elevation in xanthine oxidase activity is not observed universally (15). 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 moderate exercise 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 exercise 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 plays 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 isometric exercise. The second hypothesis tested was that xanthine oxidase-associated oxidative stress will exacerbate the release of pro-apoptotic mitochondrial proteins into the cytosol resulting in increased apoptotic signaling in aged skeletal muscle after exercise but decreasing xanthine oxidase activity by allopurinol will prevent these negative changes in aging muscles.

Methods

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 (AAALAC) and fully conformed to the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings."

A subcutaneous 2.5 mg 21 day release allopurinol pellet (Innovative Research of America Inc., Sarasota, Fl) was implanted subcutaneously over the dorsal cervical column in anesthetized mice (Isotec 5, Ohmeda; 3% isoflurane/97% O_2), seven days prior to the start of the exercise protocol. The incision was closed with a 9mm wound clip. A sham surgery was performed on control animals. A total of 32 young adult (3-5 months) and 32 aged (26-28 months) C57BL/6 mice were randomly separated into groups receiving the allopurinol pellet, or only the sham surgery (n= 16 per treatment group). Each treatment group was randomly divided into groups of eight animals, where the gastrocnemius muscles from one group of eight were individually processed for whole muscle homogenate and RNA isolation while the gastrocnemius muscles from the other eight animals of the treatment group were individually homogenized and separated into a mitochondrial fraction and a mitochondrial free cytosolic fraction.

Isometric exercises were conducted on a custom-built mouse dynamometer that has been previously described (57). Briefly, mice were anesthetized with a mixture of oxygen (97%) and isoflurane gas (3%) and placed on their right side on a heated plate. The left ankle was positioned at 90° of flexion and was aligned with the axis of rotation of the servomotor (Cambridge Technology Inc. Model 6350*350, Cambridge, MA). The foot was secured to the foot plate connected to the servomotor. Dynamic Muscle Control software (Aurora Scientific Inc., Aurora, Ontario, Canada) controlled a servomotor providing for the angular position of the foot. Muscle contractions of the plantar flexor muscles were stimulated via subcutaneously placing platinum electrodes (Grass Medical Instruments) on either side of the tibial nerve. Electrode placement was tested via a short stimulation of the nerve to cause plantar flexion. Electrode placement was assumed to be correct when the foot would plantar flex without any visible appearance of eversion, or inversion, of the foot. Twenty electrically evoked (10v, 100 Hz, 200 μs pulses) isometric contractions of the plantar flexor muscle group were obtained in one limb. Each contraction train lasted for five seconds, and a 25 second recovery period occurred between subsequent contractions. Isometric contractions were conducted over three consecutive days in the left limb, while the contralateral limb served as the intra-animal control. Muscle functional data was collected as a force x time curve during isometric contractions for each session and values were normalized to each animal's body weight. The contractile data were analyzed by Dynamic Muscle Analysis software (Aurora Scientific Inc., Aurora, Ontario, Canada).

Mitochondrial isolation. The gastrocnemius muscle was 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 manufacture's recommendations (MITOISO1-1KT, Sigma-Aldrich Co., St Louis, MO). Briefly, the gastrocnemius muscle was placed on ice and minced in a 1.5ml Eppendorf tube. Samples were washed and re-suspended in an extraction buffer containing 0.25 mg/ml trypsin. After a 20 minute incubation period, albumin was added to a final concentration of 10 mg/ml to quench the proteolytic reaction. Samples were washed and re-suspended in the extraction buffer, then homogenized with a Teflon pestle for two strokes of five seconds each. The homogenate was then centrifuged at 1100g for 5 minutes. The supernatant was transferred to a new tube and centrifuged at 11,000 g for 10 minutes. The supernatant was collected as mitochondrial free, cytosolic fraction. The mitochondrial pellet was suspended in a storage buffer containing sucrose.

Whole gastrocnemius muscle homogenates concentration of H_2O_2. Hydrogen peroxide (H_2O_2) levels in the muscle tissue were determined by utilizing a fluorescent H_2O_2 detection kit (Cell Technology, Mountain View, CA). Whole muscles were homogenized in phosphate-buffered saline (PBS) (ph=7.4). Reagents and standards were prepared as recommended by the manufacturer with slight modifications and have been previously described (57). Samples were normalized to the muscle protein concentration of each sample as determined by a DC protein concentration assay (Bio-Rad, Hercules, CA). All analyses were done in duplicate.

Total concentration of Glutathione, oxidized glutathione and the reduced glutathione/oxidized glutathione (GSH/GSSG) ratio of aged & exercised gastrocnemius muscles. The concentration of total glutathione (tGSH) and oxidized (GSSG) glutathione was ascertained by the use of a Bioxytech GSH/GSSG-412 assay kit (Oxis International, CA). Furthermore the data were expressed as the ratio of reduced to oxidized glutathione

(GSH/GSSG). The assay was preformed according to the manufacturer's directions and have been previously described (57). Briefly, gastrocnemius muscle tissue (~40 mg) was homogenized immediately after dissection in 530 µl of cold buffer (5% metaphosphoric acid for the tGSH or 5% metaphosphoric acid and M2VO scavenger for the GSSG sample). The appropriate amounts of sample chromogen and enzyme were mixed and incubated at room temperature. NADPH was added and the absorbance (412 nm) of each sample was read for three consecutive minutes. The concentration for each sample was determined via a DC protein concentration assay (Bio-Rad, Hercules, CA). Signals from each sample were normalized to the corresponding protein content of that sample.

Xanthine Oxidase activity & hypoxanthine concentration in the gastrocnemius whole muscle homogenate. A commercially available Amplex Red® XO Assay Kit (#A22182, Invitrogen, Eugene, OR) was used to measure Hypoxanthine concentrations as well as xanthine oxidase activity in muscle homogenates. The methods have been described previously in our laboratory (57). 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. Values were normalized to protein concentrations for the original samples. Xanthine oxidase was substituted for hypoxanthine and added to the Amplex Red® reagent to obtain measurements for hypoxanthine concentrations. Hypoxanthine concentrations were determined by comparing sample values to values obtained from a standard curve.

Lipid peroxidation in the whole gastrocnemius muscle homogenate. Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were measured using the method and reagents from Oxis International, CA (BIOXYTECH LPO-586) and have been previously described (57). Briefly, 75-100 mg of each gastrocnemius muscle was homogenized in ice-cold PBS and 5 µL 0.5 M butylated hydroxytoluene (BHT) in acetonitrile. Absorbance of the supernatant was obtained at 586nm. The samples were normalized for differences in the amount of protein in each sample as determined by a DC protein concentration assay (Bio-Rad, Hercules, CA).

Glutathione Peroxidase (GPx) in the whole gastrocnemius muscle homogenate. A commercially available cellular GPx Assay Kit (#35319, EMD/Calbiochem, San Diego, CA) was used to measure GPx activity in gastrocnemius muscle homogenates (57). Briefly, a portion of each muscle was homogenized in PBS (pH 7.5) containing 5mM EDTA and 1mM DTT. The homogenate was centrifuged at 10,000g and the supernatant was used for the assay. All reagents and samples were equilibrated to 25°C and the assay was preformed according to the manufacturer's directions. 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 gastrocnemius muscle homogenate. A commercially available Amplex Red® Catalase Assay Kit (#A22180, Invitrogen, Eugene, OR) was used to measure the activity of the catalase enzyme in whole muscle homogenates. Briefly, 25µl of homogenates were mixed with 25µl of 40 µM H_2O_2 solution and allowed to incubate in the dark for 30mins at room temperature. After 30 mins the sample containing solution was mixed with 50 µM Amplex® Red, 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, Hercules, CA).

Manganese Superoxide Dismutase (MnSOD) activity in the mitochondrial fraction of the gastrocnemius muscle. A commercially available SOD Assay Kit II (#706002, Cayman Chemical Company, Ann Arbor, MI) was used to measure MnSOD activity in the mitochondrial fraction. The assay was performed with slight modifications to the manufacturer's directions and all samples and standards were measured in duplicate. The assay was performed in a 96-well plate with each sample being treated with 10µL of 12 mM potassium cyanide to inhibit any residual CuZn and extracellular SOD activities. The absorbance was measured at 450 nm using a 96-well plate reader (Dynex Tech., Chantilly VA., USA). The samples were normalized to the protein concentration in each sample as assessed using a DC protein concentration assay (Bio-Rad, Hercules, CA).

Copper-Zinc Superoxide Dismutase (CuZnSOD) activity in the cytosolic fraction of the gastrocnemius muscle. CuZnSOD activity was determined in the mitochondrial free fraction with slight modifications to the manufacturer's directions as described (57) and all samples and standards were measured in duplicate (#706002, Cayman Chemical Company, Ann Arbor, MI). The assay was performed in a 96-well plate and the absorbance was measured at 450 nm using a 96-well plate reader (Dynex Tech., Chantilly VA., USA). The samples were normalized to the protein concentration in each sample as assessed using a DC protein concentration assay (Bio-Rad, Hercules, CA).

Measuring mRNA concentrations of antioxidant enzymes in the gastrocnemius muscle. CuZnSOD, MnSOD, catalase and GPX-1 mRNA were determined by means of reverse transcription-polymerase chain reaction (RT-PCR) according previously published procedures from our laboratory (56, 60). Briefly, RNA was isolated from sixty micrograms of the gastrocnemius muscle homogenized in 1ml of Tri-Reagent (Molecular Research Center, Cincinnati, OH). RNA purity was accessed using a minimum 260:280 ratio of 1.7. RNA was reversed transcribed using random primers, dNTP, and SuperScript II reverse transcriptase (Invitrogen/Life Technologies, Bethesda MD). The primers for CuZnSOD, MnSOD, GPx-1, and catalase have been previously published (56). 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) and quantified using 1D Kodak image analysis software (Eastman Kodak Company, Rochester, NY).

Fluorometric Caspase-Activity Assay. The proteolytic activities of caspase-9 and caspase-3 were determined by using commercially available substrates (caspase-3 AC-DEVD-AFC & caspase-9Ac-LEHD, Alexis Biochemical, San Diego, CA). Briefly, 50µl of caspase activity buffer (50mM PIPES, 0.1 mM EDTA, 10% glycerol & 1mM DTT), 50µl of the cytosolic fraction of the gastrocnemius muscle homogenate without protease inhibitor, and 10µl of substrate (1mM) were combined in a 96-well fluorescent microplate. Caspase activity was accessed using a fluorescent microplate reader set at a wavelength of 400nm for excitation and 505nm for emission. The microplate was incubated for 2-hours at 37°C with caspase activity being determined by subtracting the time 2-hour reading from the initial reading. Caspase activity is expressed as the relative fluorescent units normalized to the protein concentration of each muscle sample (RFU / mg protein).

Western Immunoblots. The protein content of CuZnSOD, apoptosis inducing factor (AIF) and cytochrome C were measured in the cytosolic (mitochondrial free) fractions and MnSOD was measured in the mitochondrial fractions of the gastrocnemius muscle. Thirty µg of protein was loaded into each well of a 4-12% gradient polyacrylamide gel (Novex, Invitrogen, Eugene, OR) and separated by routine SDS-polyacrylamide gel electrophoresis (PAGE) for 1.5 hours at 20°C followed by transfer to a nitrocellulose membrane. All membranes were blocked in 5% non-fat milk protein (NFM) for 1-hour at room temperature. Membranes were incubated in the appropriate dilutions of primary antibodies (diluted in tris-buffered saline with 0.05% Tween-20 (TBS-T) and .002% sodium Azide overnight at 4°C). 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. Signals were developed using a chemi-luminescent substrate (ECL Advanced, Amersham Bioscience, Fairfield, CT) and visualized by exposing the membranes to X-ray films (BioMax MS-1; Eastman Kodak, Rochester, NY). Digital records were captured by a Kodak 290 camera and protein bands were quantified using 1-D analysis software (Eastman Kodak, Rochester, NY). Bands were quantified as optical density (OD) x band area and expressed in arbitrary units.

Statistical analysis. Statistical analyses carried out using the SPSS version 13 statistical software package (Chicago, IL). Analyses of variance (ANOVA) were implemented to observe the main effect of exercise, aging and allopurinol administration. Where the F value reached significance, least significant difference (LSD) post hoc analysis was performed to assess where the significant differences occurred. Statistical significance was recognized at $p < 0.05$. Data are reported as the mean ± standard error of the mean (SEM).

Results

Xanthine Oxidase activity. Xanthine oxidase activity was 65% and 59% greater in control non-exercised and isometrically exercised (2.08 ± .192 mU/mg young vs. 3.23 ± .416 mU/mg aged) gastrocnemius muscles compared to mice that received the sham surgeries (Figure 6.2A). Isometric exercise increased xanthine oxidase activity in muscle by 33% in young adult and 28% in aged animals compared to the contralateral control muscles. Allopurinol administration blunted the exercise-induced increase in xanthine oxidase activity on all treatment and control gastrocnemius muscles. Furthermore, allopurinol reduced xanthine oxidase levels of aged muscles so that there were no differences between any of the allopurinol treated muscles (Figure 6.2A).

Hypoxanthine. Hypoxanthine which is a product of purine degradation and a substrate for xanthine oxidase was measured in young adult and aged gastrocnemius muscle. The hypoxanthine concentration was 62% greater in muscles of aged animals (0.41 ± 0.07 µmol/mg in young vs. 0.667 ± 0.09 µmol/mg in aged) (Figure 6.2B). While isometric exercise increased hypoxanthine concentrations in both the young adult (31%) and aged (54%) muscles, allopurinol did not change hypoxanthine concentrations in either control or exercised muscles in young or aged animals (Figure 6.2B).

Insert Figure 6.2

Hydrogen peroxide (H_2O_2). H_2O_2 was measured as an indicator of oxidant production. Whole muscle homogenate levels of H_2O_2 were elevated with isometric exercise in young adult (24%) and in aged (44%) animals receiving the sham surgeries as compared to the age-matched control limb ($p<0.05$). H_2O_2 was higher in both control (21%) and isometrically exercised (39%) muscles ($p<0.05$) of aged animals as compared to young adult animals. Allopurinol attenuated the increase in H_2O_2 associated with isometric exercise in both age groups (Figure 6.3A).

Lipid Peroxidation. The levels of malondialdehyde (MDA) and 4-hydroxyalkenals (HAE), both products of lipid peroxidation, were 56% greater in non-exercised gastrocnemius muscles of aged vs. young adult mice (3.06 ± 0.64 µM/mg young vs. 4.8 ± 0.64 µM/mg aged). Isometric exercise elevated lipid peroxidation within the young adult muscles by 29% (3.06 ± 0.64 µM/mg vs. 3.95 ± 0.74 µM/mg) and by 92% in the aged muscles (4.8 ± 0.64 µM/mg vs. 9.2 ± 1.76 µM/mg). Allopurinol blunted the increase in MDA + HNE levels associated with exercise in the young adult (3.22 ± 0.61 µM/mg controls vs. 3.27 ± 0.78 µM/mg exercised) and aged muscles (5.17 ± 1.09 µM/mg vs. 5.68 ± 1.2 µM/mg). Allopurinol did not depress lipid peroxidation in control muscles of aged mice (4.80 ± 0.64 µM/mg sham surgery vs. 5.17 ± 1.09 µM/mg allopurinol) (Figure 6.3B).

Insert Figure 6.3

Glutathione. Glutathione (GSH) is a major tissue antioxidant that provides reducing equivalents for the reduction of hydrogen peroxide to water. In a reaction catalyzed by glutathione peroxidase, two GSH molecules form a disulfide bond resulting in the oxidized form of glutathione (GSSG). The ratio of reduced to oxidized glutathione (GSH/GSSG) is used an indicator of oxidative stress. As shown in Figure 6.4A, the concentration of GSH was ~21% lower in muscles of aged as compared with young mice. Although it approached significance ($p=0.066$), the GSSG concentration was not different in muscles obtained from young or aged

mice (Figure 6.4B). The ratio of reduced to oxidized glutathione (GSH/GSSG) was 35% lower ($p<0.05$) in muscles of aged vs. young adult animals (Figure 6.4C). The GSH/GSSG ratio was reduced in exercised gastrocnemius muscles of both young (15.02 ± 2.2 control vs. 9 ± 1.4 exercised) and aged animals (9.7 ± 0.98 control vs. 3.2 ± 0.54 exercised) (Figure 4C). Allopurinol treatment prevented the exercise-induced decrease in the GSH/GSSG ratio in muscles from the young mice and partially attenuated the decrease in muscles from aged mice. These data suggest that aging reduced the concentration of glutathione 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 exercise.

Insert Figure 6.4

Glutathione Peroxidase (GPx). Neither aging, isometric exercise, nor xanthine oxidase inhibition produced any significant changes in glutathione peroxidase enzyme activity or GPx-1 mRNA levels within gastrocnemius muscles (Figure 6.5A and Figure 6.5B).

Manganese Superoxide Dismutase (MnSOD). MnSOD activity was 37% greater in the gastrocnemius muscles from aged animals compared to young adults (Figure 6.5C). Neither isometric exercise nor allopurinol had any significantly affect on MnSOD activity in either age group. Aging, isometric exercise and xanthine oxidase inhibition failed to affect muscle levels of MnSOD mRNA (Figure 6.5D).

Insert Figure 6.5

Catalase. The enzymatic activity of catalase was 66% greater in the gastrocnemius muscles from aged animals compared to young adult animals (Figure 6.6A) whereas catalase mRNA was ~180% greater in muscles from old (3.87 ± 0.51 Relative Optical Density) vs. young adult (2.06 ± 0.49 Relative Optical Density) mice (Figure 6.6C). Neither catalase protein levels, nor mRNA content, were affected by allopurinol. Isometric exercise increase catalase activity by ~20% in the muscles from aged animal, but it had no affect on catalase activity in the young adult animals (0.366 ± 0.05 nM/min/mg non-exercised vs. 0.414 ± 0.08 nM/min/mg exercised). However, exercise in aged gastrocnemius muscles did not elicit a significant increase in catalase protein abundance or mRNA content. The increase in catalase activity associated with exercise in the aged gastrocnemius muscles was completely attenuated with allopurinol administration (0.612 ± 0.11 nM/min/mg non-exercised vs. 0.606 ± 0.1 nM/min/mg exercised). Isometric exercise did not alter catalase protein abundance, or mRNA content in the gastrocnemius muscle from young adult or aged animals (Figure 6.6B and Figure 6.6C).

Copper-Zinc Superoxide Dismutase (CuZnSOD). CuZnSOD activity was 11% greater in control gastrocnemius muscles of aged animals as compared with young animals (1.06 ± 0.08 U/mg young vs. 1.23 ± 0.08 U/mg aged, $P<0.05$)) (Figure 6.6D). Allopurinol treatment did not affect CuZnSOD activity in control muscles. Isometric exercise increased CuZnSOD activity by 16% in muscles from the young adult animals and by 11% in the aged animals (1.23 ± 0.08 U/mg aged non-exercised vs. 1.37 ± 0.07 aged exercised). Exercise also increased CuZnSOD protein abundance by 73% in muscles from young adult mice (15.67 ± 2.9 Relative Optical Density non-exercised vs. 27.17 ± 2.7 Relative Optical Density exercised) and 62% in the muscles from aged mice (14.14 ± 3.2 Relative Optical Density non-exercised vs. 23 ± 4.7 Relative Optical Density exercised) (Figure 6.6E). Allopurinol suppressed the exercise-induced increase in CuZnSOD activity and protein content in muscles from both young and aged mice. CuZnSOD mRNA levels were not altered by age, exercise or allopurinol (Figure 6.6F).

Insert Figure 6.6

Pro-apoptotic mitochondrial signaling proteins. Caspase-9 and -3 activities were measured in the cytosolic fraction of the gastrocnemius muscle. Caspase-9 activity and caspase-3 were 122% and 85% greater in muscles from aged compared to young adult mice. Although exercise did not increase caspase-9 activity, caspase-3 activity was 30% greater in exercised as compared to non-exercised muscles of aged mice. Neither caspase-9 nor caspase-3 was increased by exercise in young animals. Allopurinol did not alter either control or exercised muscles of young or aged animals (Figure 6.7A&B).

Mitochondrial proteins, cytochrome c and apoptosis inducing factor (AIF) are released from the mitochondria in response to pro-apoptotic stimuli. When cytochrome c and AIF are present in the cytosol this suggests that mitochondrial permeability has increased via opening of mitochondria permeability pores/channels. Both cytochrome c (~237%) (Figure 6.7C) and AIF (~725%) (Figure 6.7D) were higher in the cytosolic fractions of gastrocnemius muscles from aged animals compared to muscles from young adult mice. Neither exercise nor allopurinol altered cytochrome c or AIF accumulation in the cytosol of muscles of young or aged mice.

Insert Figure 6.7

Muscle Functional Measurements. Plantar flexor maximal isometric muscle force was measured as an indicator of muscle function. The gastrocnemius muscle provides the greatest contribution to plantar flexion. A representation of the raw data for the first and 20^{th} contraction from the last exercise session in the young adult and aged mice is shown in Figure 8C-F. The maximal isometric force recorded for the third day (which was the first contraction of that day) was normalized to the animal's body weight (BW) in grams (g). Maximal isometric force per gram of body weight was 36.6% lower in the muscles of aged animals compared to the young adult animals. Maximal isometric force in the plantar flexors from aged mice was 35% greater ($p<0.05$) in animals provided allopurinol as compared to animals given the sham surgery, but allopurinol had no effect on force production in the young adult animals. Maximal isometric force was similar in the first and third exercise session in control and allopurinol groups for either young adult or aged mice (Figure 6.8A).

The rate of fatigue for the plantar flexors was assessed by calculating the net loss of force throughout the exercise session relative to the first contraction. Fatigue resistance was greater in muscles from the aged animals, but allopurinol had no effect on isometric muscle fatigue in either young adult or aged animals (Figure 6.8B).

Insert Figure 6.8

Discussion

The main findings from this study are: 1) Acute isometric exercise and aging increased xanthine oxidase, hypoxanthine and markers of oxidative stress and damage. 2) Reducing xanthine oxidase via allopurinol suppressed the isometric exercise-associated elevations in H_2O_2, and lipid peroxidation, prevented the exercise-induced loss of GSH, and prevented the increase of catalase and CuZnSOD activities, but had no effect on GPx and MnSOD activity or mRNA in exercised muscles of aged animals. 3) Allopurinol suppressed the isometric exercise-induced increase in caspase-3 activities in exercised muscles of aged animals, but did not reduce other markers of apoptotic signaling associated with aging or exercise.

Increased oxidant production and damage have been shown to be associated with exercise (1, 9, 14, 17, 29, 39, 48, 56, 63) and aging (5, 13, 16, 21, 34, 43, 49, 56, 65). Our data are consistent with these findings, because we found that isometric exercise and aging both lead to increases in H_2O_2 concentrations, lipid peroxidation, xanthine oxidase activity and a decrease in the GSH/GSSG ratio in skeletal muscle. Furthermore, our data suggest that there is an additive effect of exercise and aging in gastrocnemius muscles of mice. Non-damaging isometric muscle contractions have been shown to elevate superoxide production in the extracellular space of the gastrocnemius muscle in mice (18). We would anticipate that the additional superoxide from the isometric contractions in the current 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 isometric exercise.

Xanthine oxidase has been shown to be present in endothelial cells from human skeletal muscle (23) and despite its location, it is relevant to muscle function because it affects the responses of human muscle to exercise (10, 19, 20). The present data suggest that xanthine oxidase activity increased with aging and also is elevated with isometric exercise in muscles of both aged and young adult mice. The inhibition of xanthine oxidase via allopurinol reduced the indices of oxidative stress associated with exercise (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 (17, 18, 22, 53, 57, 63). Furthermore, increases in post-exercise concentrations of hypoxanthine are accurate predictors of muscle energy exhaustion (6) during exercise. It is likely that the additional hypoxanthine was converted to xanthine and superoxide via xanthine oxidase. Although superoxide formation was not measured, recent data that show increased superoxide is produced and released into the extracellular space via after isometric muscle (18).

The impact of allopurinol on endogenous antioxidant enzymes in aging and exercised muscles

In general, mRNA content 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 post-transcriptional and/or post-translational modifications (25, 56, 57). In contrast with other antioxidant enzymes, catalase mRNA was greater in muscles of old vs. young animals (Figure 6C). The increased catalase mRNA, activity and protein content may be an attempt to counterbalance the depletion of glutathione levels observed within aging (46).

The affects of exercise and xanthine oxidase inhibition on the endogenous antioxidant enzymes

Oxidant sensitive transcription factors such as nuclear factor kappaB (NF-κB) have been shown to up-regulate antioxidant gene expression in response to exercise (17, 26, 32). However the attenuation of oxidant production via inhibition of xanthine oxidase has been shown to prevent NF-κB activation and the subsequent upregulation of MnSOD transcriptional activity after exhaustive aerobic treadmill running (17). In contrast, we did not find evidence for transcriptional regulation of GPx, catalase, CuZnSOD or MnSOD after acute exercise. Nevertheless, the activities of the cytosolic localized antioxidants CuZnSOD and catalase were greater in both young adult and aged gastrocnemius muscles in response to isometric exercise. This might be in part a result of the need to buffer cytosolic oxidants arising from anaerobic metabolic pathways, as compared aerobic types of exhaustive exercise (17, 26, 32) which,

would be expected to have greater need to buffer mitochondria associated antioxidants arising from oxidative metabolism.

It has been postulated that low levels of oxidative stress can promote beneficial adaptive response including an improved antioxidant defense capacity, because preventing oxidative stress associated with exercise prevents these positive adaptations (17, 55). For example, in the current study, inhibition of xanthine oxidase-induced oxidative stress by allopurinol blunted the increase in cytosolic protein content and activity of CuZnSOD in response to isometric. Xanthine oxidase inhibition also attenuated the increase in catalase activity associated with exercise in the aged animals. Together, inhibition of xanthine oxidase reduced the need for an increase in antioxidant enzymes in response to exercise.

Allopurinol reduces apoptotic signaling in aged muscles

The current data are consistent with previous findings from our lab and others, that aging is associated with increases in apoptotic signaling in skeletal muscle (12, 52, 60). Mitochondrial proteins, cytochrome *c* and AIF were both elevated in the cytosolic fraction of the muscle homogenates. Downstream from mitochondrial-release of cytochrome *c,* the activity of the initiator caspase-9 was elevated in conjunction with the executioner caspase-3.

Aging has been associated with a depolarization of the mitochondrial membrane, decreased mitochondrial respiratory activity and decreased antioxidant enzyme activity (46). This decrease leads to a detrimental release and accumulation of oxidants within the cells (46). While most antioxidants likely originate from the mitochondria in aging muscles (5, 62) our data show that xanthine oxidase also contributes to oxidant production in aging and exercised muscles.

Increased cellular stress can activate redox sensitive pathways that initiate mitochondria apoptotic signaling (12, 24, 60, 61). Aged skeletal muscle has elevated basal oxidant production, and exhaustive aerobic exercise further increases oxidant production (5) and apoptotic signaling (36). In this investigation we show increased caspase-3 activity without significant increases in caspase-9 activity or cytosolic cytochrome *c* in the exercised gastrocnemius muscle of aged mice. These findings are consistent with other observations in gastrocnemius muscle in aged rats, where exhaustive exercise increased caspase-3 activity without changes in caspase-9 activity (36). These data suggest that exercise-associated elevations in caspase-3 activity in the aged gastrocnemius muscle may be triggered via the extrinsic apoptotic pathway rather than through the mitochondria.

Allopurinol blunted the increase in caspase-3 activity in the exercised gastrocnemius muscle from aged animals. Although this suggests that xanthine oxidase activity has a role in regulating apoptotic signaling, this is likely 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 resulting from the increase in caspase-3 activity with exercise and if allopurinol blunts extrinsic apoptotic signaling in response to aging and exercise.

Allopurinol affects maximal isometric force in aged animal

An important novel finding in this study is that allopurinol administration increased plantar flexor maximal isometric force by 35%, without having an effect on the young adult animals. Our data in young adult animals differs from recent data from Gomez-Cabrera and colleagues (18) 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* and/or the muscle being study. Nevertheless, the data in the current study suggest that suppressing xanthine oxidase has the potential to improve *in vivo* maximal force production in aged muscles.

Muscle fatigue during repetitive isometric contractions

Similar to previous observations from our lab (57), in this study we found that relative muscle fatigue was less in muscles of aged as compared to young adult animals. This may be due, at least in part to, shifting towards a greater percentage of type I fibers (27, 50). 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 *in vivo* study that found no improvement in fatigability of hind limb muscles of young mice in response to an *in vitro* protocol of repeated electrical stimulation (18).

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 the current experimental protocol would be adequate to relieve the chronic basal elevations in oxidative stress that is associated with advanced aging. Our findings suggest that aging and repetitive *in vivo* isometric contractions increase xanthine oxidase activity in the gastrocnemius muscles from both young adult and aged mice. We recognize that additional studies are needed to determine if long-term inhibition of xanthine oxidase will provide positive improvements in skeletal muscle redox status, oxidative stress or function of aged animals.

The suppression of exercise-induced antioxidant enzymes by allopurinol in aged muscles 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 but not other indicators of mitochondria associated apoptosis. Additional studies are required to determine if long-term inhibition of xanthine oxidase will have an important role in reducing apoptotic signaling in mitochondria or 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.

Figure Legends

Figure 6.1**Mechanism of oxidant production via xanthine oxidase pathway.** GPx (Glutathione Peroxidase), GSH (Reduced Glutathione), GS-SG (Oxidized Glutathione), GR (Glutathione Reductase), H_2O_2 (Hydrogen Peroxide)

Figure 6.2**Allopurinol attenuated the increase in xanthine oxidase activity, hypoxanthine associated with exercise**. **(A)** Activity of xanthine oxidase was determined fluorometrically. Data are expressed as mU of activity per mg of total protein in gastrocnemius muscle homogenate. One unit of xanthine activity is defined as the amount of enzyme that will form 1mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C. **(B)** Hypoxanthine data are expressed as µmol concentration per mg of total protein in the gastrocnemius muscle homogenate. The normalized data are presented as mean ± SEM. * significant difference ($p<0.05$) between isometrically exercised muscles from contra-lateral control muscles; § signifies a significant effect of aging within the sham surgery or allopurinol treatment groups ($p<0.05$). † signifies a significant effect ($p<0.05$) of allopurinol treatment.

Figure 6.3**Inhibition of xanthine oxidase activity attenuated the increase in hydrogen peroxide (H_2O_2) concentration and lipid peroxidation associated with exercise.** (A) The H_2O_2 concentrations were determined a fluorometrically. Data are expressed as µmol per mg of total protein in gastrocnemius muscle homogenate. (B) Data are combined malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) and are normalized to the total protein concentration in the gastrocnemius muscle homogenate. The normalized data are presented as the mean ± SEM. * significant difference ($p<0.05$) of isometric exercised muscle from contra-lateral control muscle; § signifies a significant difference ($p<0.05$) due to aging. † signifies a significant difference ($p<0.05$) of the allopurinol treatment.

Figure 6.4**Concentration of total glutathione, oxidized glutathione (GSSG) and the ratio of reduced glutathione (GSH) / GSSG. (A)** The concentration of total 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 are presented as mean ± SEM. * significant difference ($p<0.05$) of isometric exercised muscle from contra-lateral control muscle; § signifies a significant difference ($p<0.05$) within either the sham surgery or allopurinol treatment groups due to aging. † signifies a significant difference ($p<0.05$) due to the allopurinol treatment.

Figure 6.5 **Glutathione peroxidase (GPx) and Manganese superoxide dismutase (MnSOD) activity & mRNA regulation with isometric exercise and allopurinol treatment.** For all graphs the normalized data are presented as mean ± SEM. § signifies a significant difference ($p<0.05$) within the sham surgery or allopurinol treatment groups due to aging. (A) Total GPx activity is expressed as nmol decrease in NADPH per minute normalized to mg of to 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 (OD) x band area normalized to 18s rRNA, and expressed in relative optical density. The inserts show representative gels for GPx-1 mRNA and 18s rRNA in young and aged (control and isometrically exercised) gastrocnemius muscle. (C) MnSOD activity was

determined in the mitochondrial fraction of the gastrocnemius muscle homogenate. MnSOD activity is expressed as U of MnSOD per ml of homogenate normalized to mg of protein in homogenate. One unit was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. (D) MnSOD mRNA expression was determined from the total muscle homogenate by RT-PCR. The data are expressed as optical density (OD) x band area, normalized to 18s rRNA and expressed in relative optical density. The inserts show representative gels for MnSOD mRNA and 18s rRNA in young and aged (control and isometric exercised) gastrocnemius muscle.

YCC= Young, Control surgery, Control non-exercise; **YCE**= Young, Control surgery, Exercised; **YAC**=Young, Allopurinol, Control non-exercise; **YAE**= Young, Allopurinol, Exercised; **ACC**= Aged, Control surgery, Control non-exercise; **ACE**= Aged, Control surgery, Exercised; **AAC**= Aged, Allopurinol, Control non-exercised; **AAE** Aged, Allopurinol, Exercised

Figure 6.6**Catalase and Copper zinc superoxide dismutase (CuZnSOD) activity, protein expression & mRNA regulation with isometric exercise and allopurinol treatment.** For all graphs the normalized data are presented as mean ± SEM. * significant difference ($p<0.05$) of isometrically exercised muscles from contra-lateral control muscles; § signifies a significant difference ($p<0.05$) within the sham surgery or allopurinol treatment groups due to aging. † signifies a significant difference ($p<0.05$) due to the allopurinol treatment. (A) Total catalase activity is expressed as nmol of activity per min normalized to mg of total protein in the gastrocnemius homogenate. (B) Catalase protein expression was determined in the mitochondrial free cytosolic fraction by western immunoblot. The data are expressed as optical density (OD) x band area, normalized to GAPDH and expressed in relative optical density. The inserts show representative blots for catalase and GAPDH content 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 (OD) x band area, normalized to 18s rRNA and expressed in relative optical density. The inserts show representative gels for catalase mRNA and 18s rRNA in young and aged (control and isometric exercised) gastrocnemius muscles. (D) CuZnSOD activity was determined in the mitochondrial free cytosolic fraction of gastrocnemius muscle homogenate. CuZnSOD activity is expressed as U of CuZnSOD per ml of homogenate normalized to mg of protein in homogenate. One unit was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. (E) CuZnSOD protein expression was determined in the mitochondrial free cytosolic fraction by western immunoblot. The data are expressed as optical density (OD) x band area, normalized to GAPDH and expressed in relative optical density. The inserts show representative blots for CuZnSOD and GAPDH content in gastrocnemius muscles. (F) CuZnSOD mRNA expression was determined by RT-PCR from total muscle homogenate. The data are expressed as optical density (OD) x band area, normalized to 18s rRNA and expressed in relative optical density. The inserts show representative gels for CuZnSOD mRNA and 18s rRNA in young and aged (control and isometric exercised) gastrocnemius muscle.

YCC= Young, Control surgery, Control non-exercise; **YCE**= Young, Control surgery, Exercised; **YAC**=Young, Allopurinol, Control non-exercise; **YAE**= Young, Allopurinol, Exercised; **ACC**= Aged, Control surgery, Control non-exercise; **ACE**= Aged, Control surgery, Exercised; **AAC**= Aged, Allopurinol, Control non-exercised; **AAE** Aged, Allopurinol, Exercised

Figure 6.7 **Aging increases apoptotic signaling.** For all graphs the normalized data are presented as mean ± SEM. * significant difference ($p<0.05$) of isometric exercised muscle from contra-lateral control muscle; § signifies a significant difference ($p<0.05$) within the sham

surgery or allopurinol treatment groups due to aging. † signifies a significant difference ($p<0.05$) due to the allopurinol treatment. Caspase-9 **(A)** and caspase-3 **(B)** levels were determined in the mitochondrial free cytosolic fraction of the gastrocnemius muscle homogenate by a fluorometric assay. Data are expressed as Relative Fluorescent Unit (RFU) per mg of protein in the homogenate. **(C)** Cytosolic Cytochrome C protein expression was determined in the mitochondrial free fraction of the gastrocnemius homogenate by western immunoblot. The data are expressed as optical density (OD) x band area, normalized to GAPDH and expressed in relative optical density. The inserts show representative blots for cytochrome c and GAPDH in young and aged (control and repetitive loading) gastrocnemius muscle. **(D)** Cytosolic AIF protein expression was determined in the mitochondrial free fraction of the gastrocnemius homogenate by western immunoblot. The data are expressed as optical density (OD) x band area, normalized to GAPDH and expressed in relative optical density. The inserts show representative blots for AIF and GAPDH in young and aged (control and repetitive loading) gastrocnemius muscle.

YCC= Young, Control surgery, Control non-exercise; **YCE**= Young, Control surgery, Exercised; **YAC**=Young, Allopurinol, Control non-exercise; **YAE**= Young, Allopurinol, Exercised; **ACC**= Aged, Control surgery, Control non-exercise; **ACE**= Aged, Control surgery, Exercised; **AAC**= Aged, Allopurinol, Control non-exercised; **AAE** Aged, Allopurinol, Exercised

Figure 6.8**Maximal isometric forces from the plantar flexor muscles**. **(A)** Data are expressed as the mean ± SEM of the maximal isometric force (N) recorded on the third day of exercise by the left plantar flexor muscle group normalized to the body weight in grams (g BW) of the animal. § signifies a difference ($p<0.05$) due to aging. **(B)** Data are expressed as the mean ± SEM of the relative difference between the maximal isometric forces produced on the each contraction to the maximal isometric force on the first contraction. All force measurements were normalized to body weight (g). * indicates a significant difference ($p<0.05$) in both the aged sham surgery and allopurinol treatment animals versus the young adult sham surgery and allopurinol treatment animals. **(C-F)** Representative force x time curves from the 3rd exercise session 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.

Figure 6.1

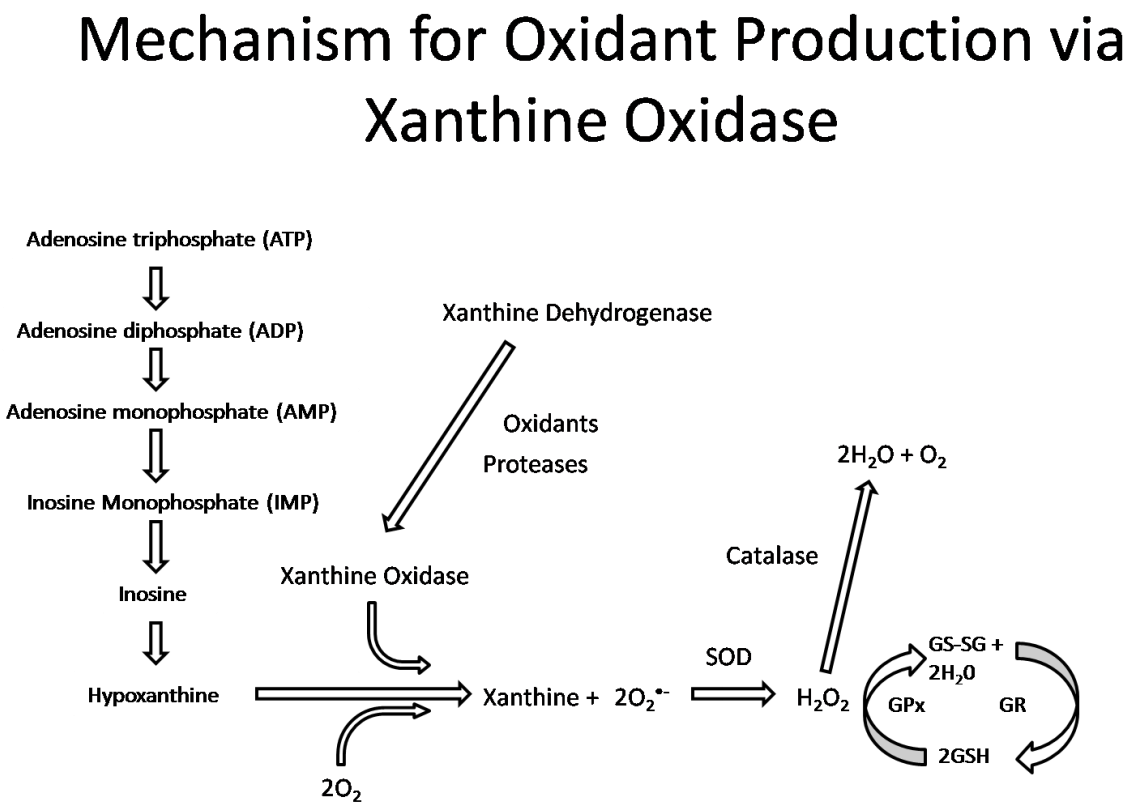

Mechanism for Oxidant Production via Xanthine Oxidase
Adenosine triphosphate (ATP)
Adenosine diphosphate (ADP)
Adenosine monophosphate (AMP)
Inosine Monophosphate (IMP)
Inosine
Hypoxanthine
Xanthine Dehydrogenase
Oxidants
Proteases
Xanthine Oxidase
$2O_2$
Xanthine + $2O_2^{\bullet-}$
SOD
H_2O_2
Catalase
$2H_2O + O_2$
GS-SG + $2H_2O$
GPx
GR
2GSH

Figure 6.2

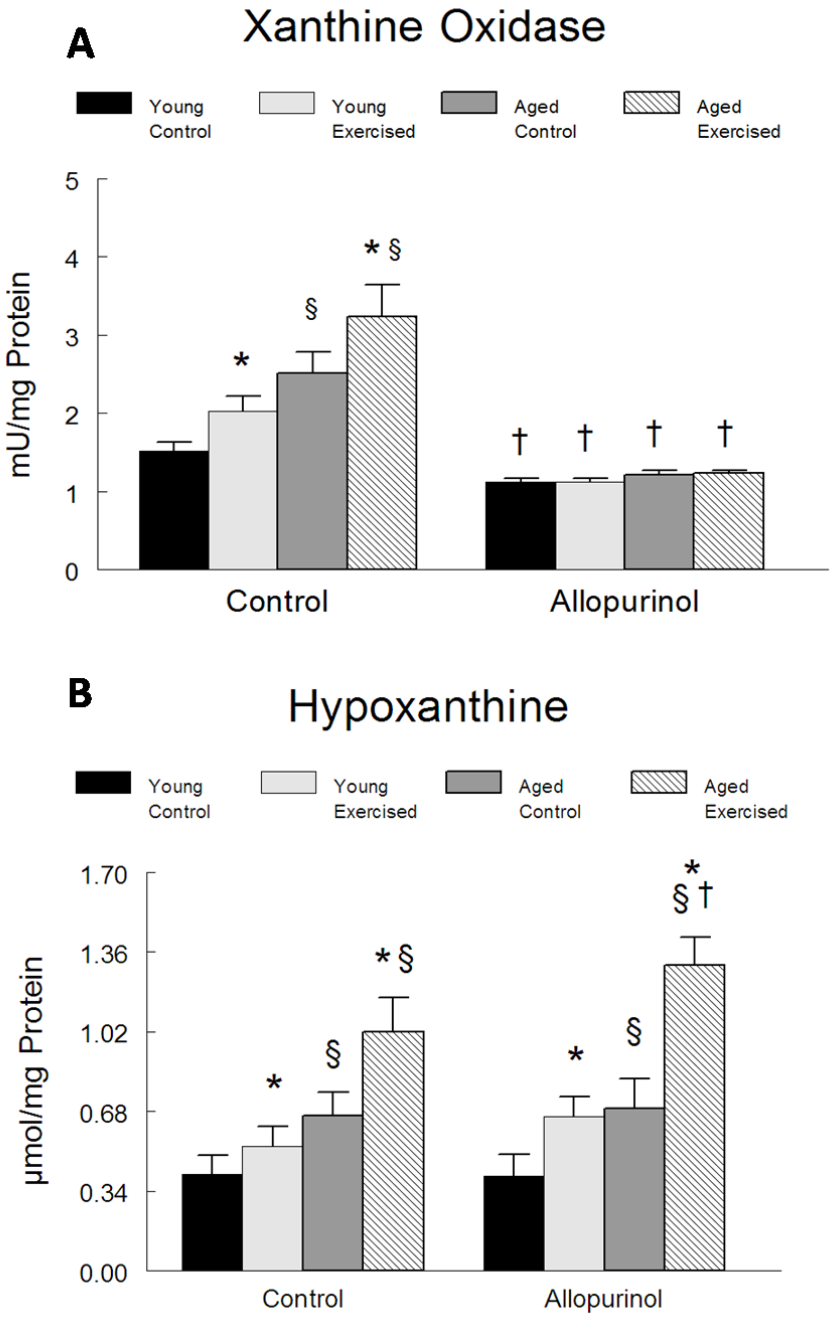

A
Xanthine Oxidase
Young Control
Young Exercised
Aged Control
Aged Exercised
mU/mg Protein
0
1
2
3
4
5
* §
§
*
† † † †
Control
Allopurinol
B
Hypoxanthine
Young Control
Young Exercised
Aged Control
Aged Exercised
µmol/mg Protein
0.00
0.34
0.68
1.02
1.36
1.70
* §
§
*
* § †
§
*
Control
Allopurinol

Figure 6.3

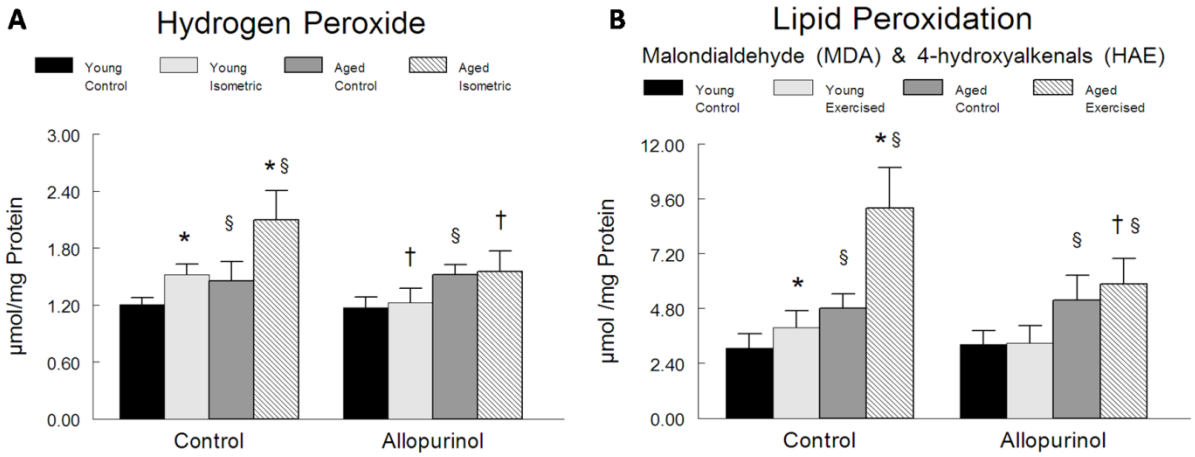

A
Hydrogen Peroxide
Young Control
Young Isometric
Aged Control
Aged Isometric
µmol/mg Protein
3.00
2.40
1.80
1.20
0.60
0.00
Control
Allopurinol
B
Lipid Peroxidation
Malondialdehyde (MDA) & 4-hydroxyalkenals (HAE)
Young Control
Young Exercised
Aged Control
Aged Exercised
µmol /mg Protein
12.00
9.60
7.20
4.80
2.40
0.00
Control
Allopurinol

Figure 6.4

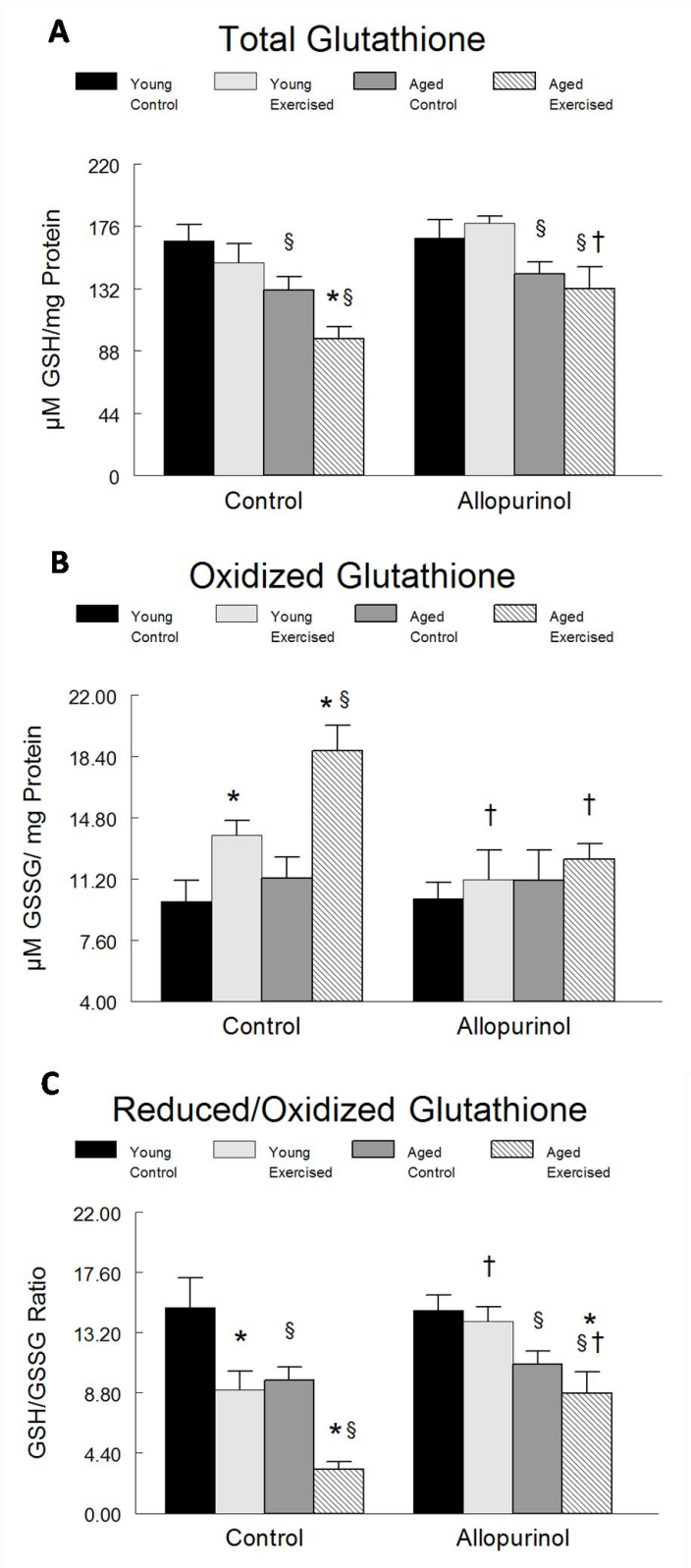

A
Total Glutathione
Young Control
Young Exercised
Aged Control
Aged Exercised
µM GSH/mg Protein
220
176
132
88
44
0
§
*§
§
§†
Control
Allopurinol
B
Oxidized Glutathione
Young Control
Young Exercised
Aged Control
Aged Exercised
µM GSSG/ mg Protein
22.00
18.40
14.80
11.20
7.60
4.00
*
*§
†
†
Control
Allopurinol
C
Reduced/Oxidized Glutathione
Young Control
Young Exercised
Aged Control
Aged Exercised
GSH/GSSG Ratio
22.00
17.60
13.20
8.80
4.40
0.00
*
§
*§
†
§
*
§†
Control
Allopurinol

Figure 6.5

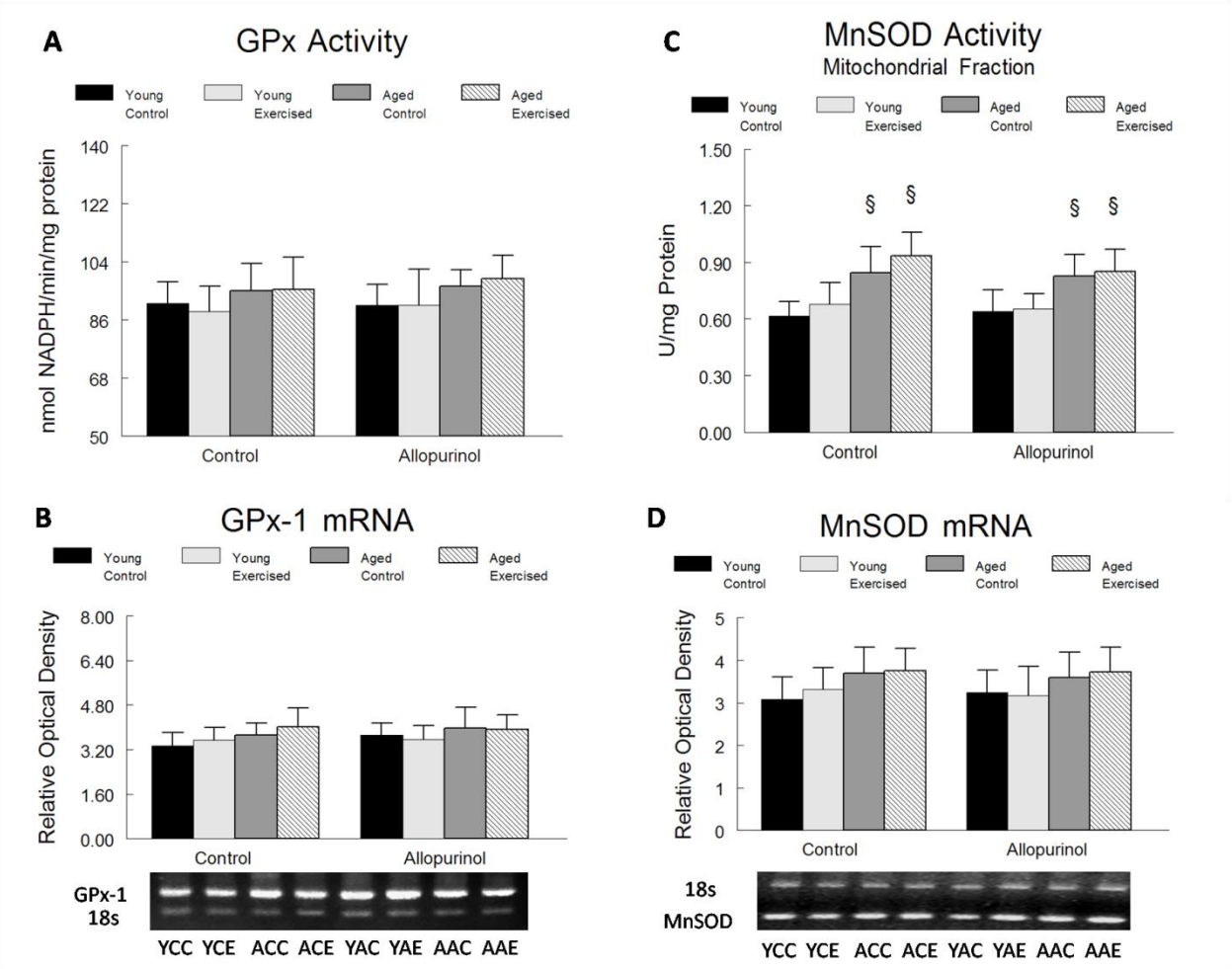

A
GPx Activity
Young Control
Young Exercised
Aged Control
Aged Exercised
nmol NADPH/min/mg protein
140
122
104
86
68
50
Control
Allopurinol
C
MnSOD Activity
Mitochondrial Fraction
U/mg Protein
1.50
1.20
0.90
0.60
0.30
0.00
§
B
GPx-1 mRNA
Relative Optical Density
8.00
6.40
4.80
3.20
1.60
0.00
GPx-1
18s
YCC YCE ACC ACE YAC YAE AAC AAE
D
MnSOD mRNA
5
4
3
2
1
0
18s
MnSOD

Figure 6.6

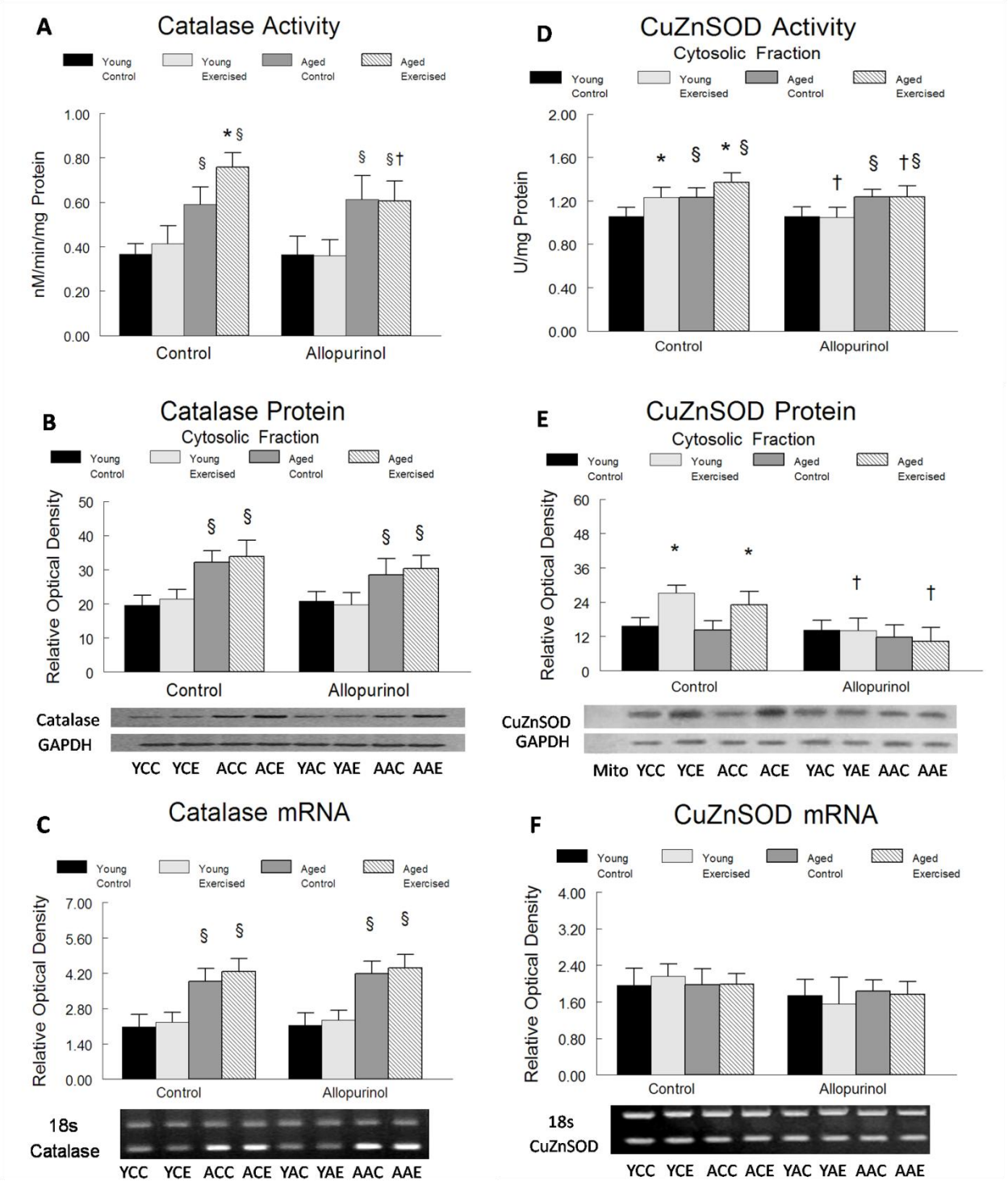

A
Catalase Activity
Young Control
Young Exercised
Aged Control
Aged Exercised
nM/min/mg Protein
1.00
0.80
0.60
0.40
0.20
0.00
Control
Allopurinol
D
CuZnSOD Activity
Cytosolic Fraction
U/mg Protein
2.00
1.60
1.20
0.80
0.40
0.00
B
Catalase Protein
Cytosolic Fraction
Relative Optical Density
50
40
30
20
10
0
Catalase
GAPDH
YCC YCE ACC ACE YAC YAE AAC AAE
E
CuZnSOD Protein
Cytosolic Fraction
60
48
36
24
12
0
CuZnSOD
GAPDH
Mito YCC YCE ACC ACE YAC YAE AAC AAE
C
Catalase mRNA
7.00
5.60
4.20
2.80
1.40
0.00
18s
Catalase
YCC YCE ACC ACE YAC YAE AAC AAE
F
CuZnSOD mRNA
4.00
3.20
2.40
1.60
0.80
0.00
18s
CuZnSOD
YCC YCE ACC ACE YAC YAE AAC AAE

Figure 6.7

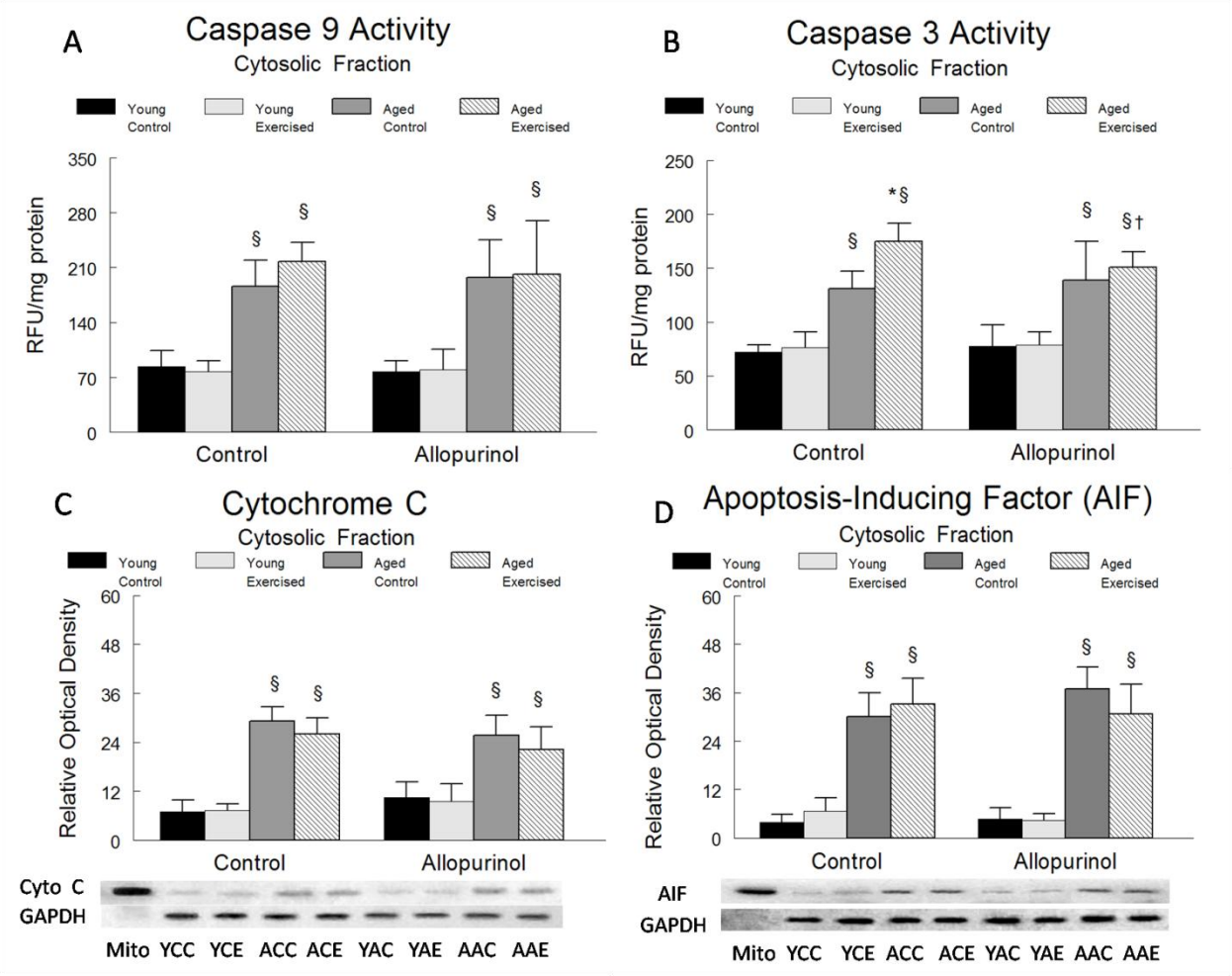
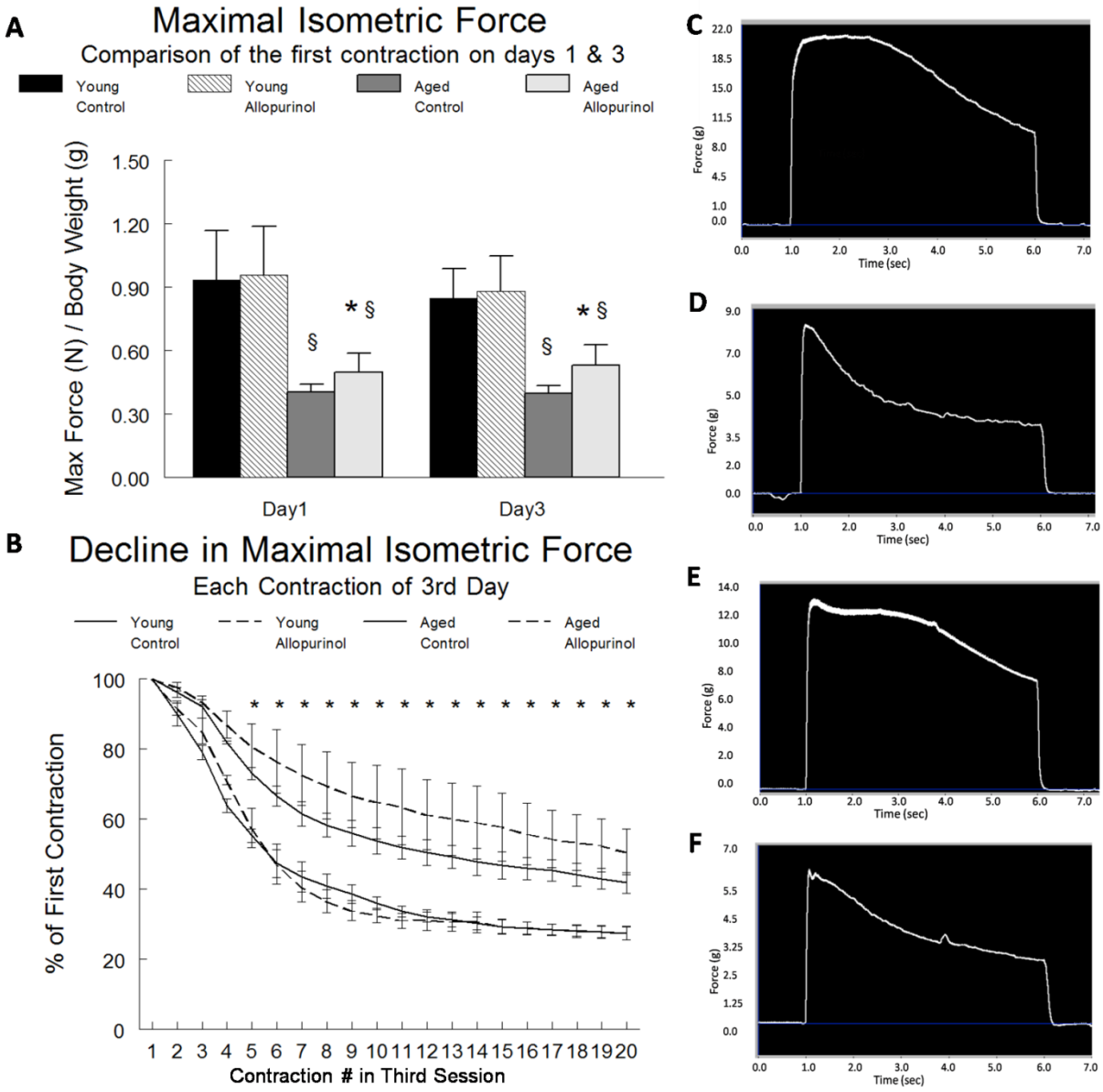

A
Caspase 9 Activity
Cytosolic Fraction
Young Control
Young Exercised
Aged Control
Aged Exercised
RFU/mg protein
350
280
210
140
70
0
§
Control
Allopurinol
B
Caspase 3 Activity
Cytosolic Fraction
250
200
150
100
50
0
*§
§†
C
Cytochrome C
Cytosolic Fraction
Relative Optical Density
60
48
36
24
12
Cyto C
GAPDH
Mito YCC YCE ACC ACE YAC YAE AAC AAE
D
Apoptosis-Inducing Factor (AIF)
Cytosolic Fraction
AIF
GAPDH
Mito YCC YCE ACC ACE YAC YAE AAC AAE

Figure 6.8


A
Maximal Isometric Force
Comparison of the first contraction on days 1 & 3
Young Control
Young Allopurinol
Aged Control
Aged Allopurinol
Max Force (N) / Body Weight (g)
1.50
1.20
0.90
0.60
0.30
0.00
§
* §
Day1
Day3
B
Decline in Maximal Isometric Force
Each Contraction of 3rd Day
Young Control
Young Allopurinol
Aged Control
Aged Allopurinol
% of First Contraction
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Contraction # in Third Session
C
D
E
F
Force (g)
Time (sec)

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Chapter 7

Summary and Conclusions

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Summary of research findings

The data presented in this project are consistent with previous studies that have shown an increase in oxidative stress within the skeletal muscle environment in response to exercise (1, 18, 21, 29, 65, 97) and advanced aging (7, 20, 25, 38, 53, 72, 77, 101). Specifically, in this study exercise and aging elevated lipid peroxidation, xanthine oxidase activity, the concentration of H_2O_2, and decreased the GSH/GSSG ratio in skeletal muscle. In addition, there was an age-related exacerbation of exercise-induced oxidative stress in the aged muscle. Together, these data provide support for a new hypothesis: *increases in xanthine oxidase activity are responsible for the majority of the oxidative stress associated with resistance training models of anaerobic exercise*.

The inhibition of xanthine oxidase by allopurinol attenuated the increase in oxidative stress associated with isometric exercise, but reduction of xanthine oxidase did not reduce oxidative stress that was associated with aging. These conclusions support the central hypothesis of this project that "**skeletal muscles from aged animals will show increased evidence of oxidative stress during resistance exercise (repetitive loading) and antioxidant supplementation will increase the aged muscles' oxidative buffering capacity, thus attenuating the increase in oxidative stress associated with aging. Increased xanthine oxidase activity will be a contributing factor to the increase in oxidative stress in response to resistance exercise (i.e. repetitive loading) and aging**". One difference from the original hypothesis that was not supported by the data were that the age related increase in xanthine oxidase activity did not appear to be a contributing factor to the oxidative stress associated with aging.

Another objective of this investigation was to evaluate the efficacy of reducing oxidative stress on the adaptive response of skeletal muscle to repetitive loading exercise in aging rodents. To achieve this objective, three methods of reducing oxidative stress were utilized; the antioxidants vitamins E&C were used to buffer oxidants (specific aim 1), the nutraceutical resveratrol was used to inhibit oxidant production (specific aim 2) and the pharmacological agent allopurinol was used to attenuate oxidant production specifically through the inhibition of xanthine oxidase activity (specific aim 3). While all three approaches effectively lowered oxidative stress (H_2O_2, the GSH/GSSG ratio, lipid peroxidation) associated with exercise, there was not a consistent pattern of response in endogenous antioxidant enzymes to the experimental interventions used in this study.

The affects of reducing oxidative stress on muscle function

Oxidative stress has been shown to depress muscular force (12), alter myofilament function (2, 58), mediate muscle fatigue (88) and/or modify contraction-induced calcium release (3, 19, 22, 82). Therefore, it was hypothesized in all three of the specific aims of this investigation that reducing aging related oxidative stress would improve muscle function in the aged animals. The animal models of *in vivo* repetitive loading that were implemented in these studies proved useful because the input parameters on the muscle were tightly controlled, thus any changes in performance parameters can be attributed to alterations within the muscle environment. Although methods using resistance training in conscious animals have been developed (98, 99), there are several problems with these approaches: a) it is difficult to motivate animals to exercise with resistance loads, b) using food rewards for exercise in aged animals is problematic and will result in loss of body weight, c) rests interval between repetitions in voluntary exercise are variable, and d) animal compliance to repeated voluntary

exercise is poor. Therefore, conscious animals models cannot rule out the possibility that changes in input parameters affect the results. Within the scope of the current studies the biomechanical loading signature (input parameters, i.e. frequency, intensity, duration along of the contraction along with the direction of movement and the speed of movement) for each contraction was identical. Because all motor units within the muscles were contracted simultaneously, the current models eliminated preferential fiber type recruit patterns. Although in the current models of repetitive loading the physiological recruitment patterns were altered, the force produced was within the physiological capabilities of the muscle. Within endurance training models of exercise; the ramp principle of fiber type recruitment would imply that at low to moderate intensities the slow oxidative and/or fast glycolytic-oxidative fibers would contract a greater number of times than the fast glycolytic fibers, which may result in “pockets” of greater oxidative stress based on input parameters.

In general, the current data supports the notion that muscle function declines with advanced aging, yet muscle is still able to adapt to exercise (10, 24, 37, 60, 92, 93). Nevertheless, these adaptations are generally less than that reported in muscles from young adults. While some elements of muscle function were improved, other aspects of function did not improve when oxidative stress was reduced. For example, vitamin E&C supplementation in the aged rats showed a ~ 38% improvement in positive work, but failed to improve either maximal force or negative work. Resveratrol supplementation did not improve maximal isometric force; however, somewhat surprisingly it did improve the young muscles ability to resist fatigue without affecting the fatigue in the aged muscle. Furthermore, allopurinol administration improved maximal isometric force in the aged animals, but failed to show any significant improvements in the fatigue resistance.

The improvements in positive work with vitamin E&C supplementation and the increase in maximal isometric force produced in the animals administered allopurinol can be directly related to the reduction in oxidative stress. However, with resveratrol supplementation it is unknown if the improved resistance to fatigue was a result of an increase in ATP availability or a diminished exercise-induced oxidant formation. Previous studies have reported that resveratrol supplementation reduced fatigue associated with aerobic exercise (6, 62). The increased resistance to fatigue has been attributed to increases in the density and efficiency of the proteins associated with the mitochondrial electron transport chain, by increasing β-oxidation and/or by increasing ATPase content (6, 62, 80, 95). These novel data represent the first evidence that resveratrol can reduce muscle fatigue in response to repetitive anaerobic (isometric) contractions.

Responses of endogenous antioxidant enzymes to exercise

Proper control of expression and activity of the endogenous antioxidant enzymes is the key component to the maintenance of redox homeostasis. Many of the endogenous antioxidant enzymes have been shown to be controlled via both transcriptional regulators and post-transcriptional and/or post-translational mechanisms. The antioxidant enzymes may be rapidly activated to manage acute oxidative stress due to a single bout of exercise or gradually up-regulated in response to chronic oxidative stress associated with aging or exercise training. However the overall response to either acute or chronic exercise may vary depending on the mode of exercise and the fiber type make up of the muscle (5, 39, 41, 46, 55, 64, 78, 79, 83, 84).

The majority of the research on the response of the antioxidant enzyme to exercise training has utilized moderate to exhaustive endurance activity as the mode of exercise. Some

of the general conclusions have shown that; (I) MnSOD activity has consistently been shown to increase with exercise training in an intensity-dependent manner(39, 46, 63, 64, 83), whereas CuZnSOD activity shows little change to chronic training(33, 39, 46, 55, 63). (II) GPx activity increases after endurance training (64, 84), and (III) exercise training effects on catalase activity has produced conflicting results (33, 39, 55, 63, 64, 83).

Mitogen-activated protein kinases (MAPKs) and nuclear factor kappaB (NF-κB) are two major redox-sensitive signal transduction pathways that have been shown to activate the gene expression of a number of enzymes and proteins that have an important role in the management of oxidative stress (52). Elevated oxidant production associated with exercise has been shown to activate MAPK , which in turn activates NF-κB in skeletal muscle resulting in increased gene expression of MnSOD and catalase (30, 50, 59). When antioxidants are used to diminish exercised induced oxidative stress it has been shown to attenuate the activation of the MAPK/ NF-κB signaling pathway resulting in a failure of exercise to elevate gene expression of endogenous antioxidant enzymes MnSOD and catalase.

MnSOD Regulation

The response of MnSOD to acute and chronic aerobic exercise has been widely studied. An acute bout of aerobic exercise has been shown to up-regulate NF-κB expression and activation leading to elevated MnSOD expression (30, 34, 44, 76). Tumor necrosis factor-alpha (TNFα), Interleukin-1(IL-1) and early growth-responsive-1 (Egr-1) have all been shown to up-regulate MnSOD gene expression through interactions with NF-κB binding to the promoter region of the MnSOD gene (17, 40, 56, 69).

Chronic endurance training has been shown to increase mRNA expression and increase both the protein content and activity of MnSOD (76). This suggests that in response to chronic endurance training MnSOD activity increased by transcriptional control. However, this is not a universal finding; Gore et al. (34) showed that endurance training in rats increased protein concentration of MnSOD by 66%, but had no affect on MnSOD mRNA abundance or enzyme activity. One difference between these two studies was the muscle in which MnSOD activity was measured from; the first being the soleus and the second the deep vastus lateralis. The soleus consists of primarily slow oxidative fibers while the deep vastus lateralis is composed of a large percentage of fast oxidative-glycolytic fibers. However, chronic exercise has been shown to change the enzymatic profile of the deep vastus lateralis to become similar to a slow oxidative fiber (35). Based on that evidence, it would be expected that the MnSOD expression and activity would increased as the fibers became more oxidative. There is a paucity of data on the regulation of MnSOD in response to either acute or chronic resistance training. Parise et al. (79) showed that neither acute or chronic resistance training in elderly men altered MnSOD activity and recently it has been shown that acute electrical stimulation did not affect MnSOD activity but did increase mRNA expression (81).

MnSOD also requires post-translational modification to become an active enzyme (68). MnSOD protein is produced as a large precursor enzyme (apoMnSOD) with several steps of modification required to become active. The superoxide radical is the most common activator of MnSOD, when apoMnSOD is produced it contains an Fe^{2+} in the active site, as the concentration of superoxide increases, Mn^{2+} is oxidized to Mn^{3+}, which replaces Fe^{2+} thereby activating the enzyme (85). Furthermore, there are several mechanisms that inactivate MnSOD, they include; metal mis-incorporation, glycation, S-glutathionylation, phosphorylation, nitration and high concentrations of H_2O_2 (68).

CuZnSOD Regulation

It is generally thought that acute aerobic exercise will activate the CuZnSOD enzyme and increase its activity without changes in its mRNA and protein levels (47, 54), however not all studies have shown changes in CuZnSOD activity in response to acute exercise (44, 81, 86). Increases in CuZnSOD activity associated with acute exercise have been suggested to be regulated via post-translational modifications to the protein (47). The most likely signal for increasing CuZnSOD activity is superoxide itself (9) and since CuZnSOD has a very short half-life, research suggests a transit regulation of the enzyme activity in response to superoxide concentrations (47). This suggestion is supported by training studies that have shown that chronic endurance exercise in rats can elevate CuZnSOD activity by 29%, without changes in enzyme protein content or mRNA expression (76). As with endurance training, progressive resistance training has resulted in a significant increase in CuZnSOD activity (79) however this may be due to increased mRNA expression and protein content (26).

Few studies have shown insight into the effect of exercise on the redox sensitive gene regulation of CuZnSOD expression in skeletal muscle. In contrast to the promoter region in MnSOD, the promoter region of the CuZnSOD gene (SOD1) is not known to contain as many regulatory sequences (47). Interestingly, the product of the dismutation of superoxide, H_2O_2, has been shown to increase activation of CuZnSOD gene expression, due to a H_2O_2-responsive element (HRE) on the promoter region of the SOD1 gene (100).

GPX Regulation

Like MnSOD, the promoter region on the GPx contains both NFκB and AP-1 binding sites (47). The gene for GPx-1 has also been shown to have two oxygen-responsive elements (15) that in response to depleted concentrations of oxygen, increase GPx activity proportional to the elevation of mRNA expression (14, 47). Exercise has commonly been shown to increase GPx activity (47, 48, 64) and similar to MnSOD the response of GPx activity to exercise training was muscle specific (63, 64, 83). Furthermore, it has been shown that the extent of the increase in GPx activity is associated with the duration of exercise however it is relatively independent of intensity. Even though the literature consistently shows that its GPx activity is increases after an acute bout of exercise and endurance training little is known in regards to GPx's response to resistance training and what data is available does not show increases in GPx activity with progressive resistance training (26, 79).

Catalase Regulation

Catalase is a heme containing antioxidant enzyme whose expression is largely transcriptionally regulated (67). Catalase is regulated by transcription factors binding one of two CCAAT boxes in the promoter region (67, 75). Nuclear factor-Y (NF-Y) a redox sensitive transcription factor (71) has been shown to increase catalase gene expression in mouse muscle cells in response to increases in H_2O_2 concentrations (67).

The response of catalase activity to either acute or chronic exercise has produced inconsistent and sometimes conflicting results (26, 33, 39, 55, 63, 64, 79, 81, 83). Catalase catalyses the conversion of H_2O_2 into water and oxygen and is found in high concentrations in the peroxisomes. The catalytic efficiency of the catalase is very high, which might explain the inconsistency in the response to exercise. Because of the high efficiency of the enzyme, increases in activity may not be needed to handle the increase in H_2O_2 associated with exercise. Catalase activity could also be linked to GPx activity, Powers et al (84), suggested

that, when cellular levels of H_2O_2 are low, GPx is more active and catalase activity might not increase until higher H_2O_2 concentrations are achieved.

Within the scope of the current set of experiments the regulation of antioxidant enzyme levels in aged and exercised muscles occur by translational or post- translational mechanisms. A summary of the changes in activity (Figure 7.1A), protein content (Figure 7.1B) and mRNA expression (Figure 7.1C) are presented in Figure 7.1. The results from the current studies do not support transcriptional control as a mechanism leading to increases in activity of the endogenous antioxidant enzymes in response to exercise and only in the mice (specific aims 2 & 3) did age result in an increase in catalase mRNA resulting in increased catalase activity. Across all three investigations exercise and antioxidant treatment did not result in consistent changes in the activity of the endogenous antioxidant enzymes. A possibility for this could be the different species, but also could be attributed to the different exercise protocols and different muscles that were investigated.

In sharp contrast to the observed increase in MnSOD with endurance training, both chronic and short term repetitive loading failed to alter MnSOD activity. Furthermore, consistent throughout all of the studies was an age-dependent increase in catalase activity. In the mice the elevation in catalase activity was accompanied by an increase mRNA expression, however the rats showed no increase in mRNA and/or protein levels in the rat vitamin E&C study. The regulation of GPx appeared to be dependent on the species of rodent used in the investigation. In the rats (specific aim 1), GPx activity was significantly elevated with exercise, independent of changes in transcriptional or translational activity and was unaffected by aging. However, GPx activity was unaltered by exercise or aging within the mouse studies (specific aim 2 & 3). Activity of the catalase enzyme showed a response opposite to GPx activity in the non-antioxidant treated animals. The short term exercise program in the mice studies increased catalase activity, but chronic exercise training in the rats did not alter catalase activity.

Even though these data support the suggestion by Powers et al (84) of a differential response in catalase and GPx activity, this is not a consistent response observed by all (64). A major limitation in comparing the responses of endogenous antioxidant enzymes between the rats in the vitamin E&C study and the mice in the resveratrol and the allopurinol investigations was the differences in the duration of the exercise protocols. The vitamin E&C study (specific aim 1) characterized the response of antioxidant enzymes to chronic exercise, whereas the resveratrol (specific aim 2) and the allopurinol (specific aim 3) studies examined the antioxidant and short-term adaptations to isometric exercise. It is unknown if the muscles from mice would have adapted in a similar manner as the rats, if their exercise protocol was the extended for the same duration in both species of rodents.

In general these data are consistent with the notion that the activities of the endogenous antioxidant enzymes are regulated via various levels of post-transcriptional and/or post-translational controls (42). Furthermore, the response of MnSOD to anaerobic forms of resistance exercise may differ from more commonly examined aerobic (28, 43, 49) types of exercise. It is speculated that this may be due in part to the utilization of ATP-phoshocreatine and glycolytic metabolic pathways in resistance exercise, versus a strong dependency on oxidative phosphorylation in aerobic exercise. The current data and that of others (26, 79, 81) support the formation of a new hypothesis stating that resistance types of exercise do not increase MnSOD activity in the same manner that has been observed with endurance exercise.

Responses of endogenous antioxidant enzymes to reducing oxidative stress

Analogous to the responses of endogenous antioxidant enzymes to exercise and aging, overall the activities of the endogenous antioxidant enzymes in response to the different methods of reducing oxidative stress appear to be regulated via various mechanisms of post-transcriptional and/or post-translational control. Furthermore, control via transcription, translation and activity of the individual enzymes appeared to vary depending on the method that was used to reduce oxidative stress, the age of the animal and if the muscle underwent exercise training. These data did not support the view that vitamins E&C supplementation inhibited the positive adaptations to exercise within all of the endogenous antioxidant enzymes (32, 89) and in the case of catalase and CuZnSOD, vitamin E&C supplementation enhanced the adaptive response to exercise. However, the data collected from the allopurinol treated mice would suggest that inhibiting oxidant production via the xanthine oxidase pathway removed the stimulus required to initiate an adaptive response in the endogenous antioxidant system. These differences may suggest that distinct mechanisms of reducing oxidative stress may lead to slightly diverse responses in the adaptation of the endogenous antioxidant enzymes.

It is likely that in the vitamin E&C study, oxidants were still being produced at a high rate during exercise, even after 4.5 weeks of repetitive exercise. If we assumed that the majority of those oxidants are coming from an increase in endothelial derived xanthine oxidase activity, then large concentrations of vitamin E&C within the muscle cells might only affect regulation of the endogenous antioxidant enzymes within those muscle cells. However, within the endothelial cells and/or the extracellular space, increased oxidant production may be activating redox sensitive pathways that in turn increase the activity of endogenous antioxidant enzyme specific to that sub-cellular location.

The assumptions of the allopurinol study were that there was never a large increase in oxidant production because the xanthine oxidase activity was inhibited by the drug. It is difficult to compare the resveratrol supplemented animals with the vitamin E&C and allopurinol studies in this regard because resveratrol has been shown to have direct control over gene regulation that is upstream of a wide variety of cellular pathways involved in energy homeostasis, longevity, cell survival, and apoptosis. For example, within the current investigation, it is unknown if resveratrol increased mitochondrial number which could have resulted in an increase MnSOD activity. Measuring mitochondrial morphology (16) and enzyme levels in resveratrol fed animals would have answered this question. It was assumed that Vitamin E&C supplementation along with allopurinol would regulate the endogenous antioxidant enzymes solely via reducing oxidative stress and activation of redox sensitive pathways.

Limitations and future directions

Increased cellular levels of H_2O_2 can result in oxidative stress and cause cellular damage. Oxidative damage to cells has been associated with the progression of the aging process and may lead to increased pathological damage associated with advanced aging. Nevertheless, low concentrations of H_2O_2 have been shown to be an important signaling molecule that regulates many cellular processes (96). A comprehensive review of the literature by Giorgio et al. (27) lead them propose that *in vivo* changes in H_2O_2 concentrations in the 10^{-8} molar range lead to proliferation and increased cellular signaling, whereas changes in the 10^{-6} molar range caused growth arrest and cellular damage whereas 10^{-4} molar changes induced apoptosis.

The original intent of this project was to determine if effectiveness of antioxidants in reducing oxidative stress associated with exercise and aging in skeletal muscles. Throughout the scope of this project H_2O_2 levels were determined to measure changes in the abundance of oxidants within the skeletal muscle environment. Determining changes in H_2O_2 by reporting the data as relative florescent units (RFU) /mg protein was effective in showing changes in H_2O_2 between groups within each individual study, however because actual concentrations of H_2O_2 were not calculated the interpretation of these data and the direct comparison, between studies within this project and that of others, is limited. In the rat vitamin E&C study (specific aim 1), going back and determining a standard curve for the H_2O_2 data proved to be difficult because the exact gain of the plate reader was not properly recorded. By comparing the readings from the blank wells an estimation of the gain setting was determined and a standard curve was ascertained (Figure 7.2a). This allowed for some interpretation of whether the changes in H_2O_2 were involved in cellular signaling or pathological in nature. Within the chronic exercise study in rats (specific aim 1) exercise and aging both increased H_2O_2 concentrations (1.5-2.9 x 10^{-7} mol/mg protein) in a range in-between promoting cellular growth and causing damage. Furthermore, because the muscle samples were ascertained 24 hours after the last bout of exercise and the relatively short half life of H_2O_2, it is postulated that the current elevations are the result of a signaling response. To further support this notion, the increase in lipid peroxidation associated with exercise in the aged animals was attenuated after 4.5 weeks of exercise training. Vitamin E&C supplementation attenuated the increases in the H_2O_2 concentrations associated with aging and exercise.

Within the resveratrol study (specific aim 2) the standard curve was not ascertained at the same time as the RFU data was collected, however the exact settings of the plate reader (i.e. gain) was duplicated to determine a standard curve for H_2O_2 concentrations (Figure 7.2b). Interpretation of the H_2O_2 concentration data suggests that immediately after exercise H_2O_2 concentrations were elevated by 1.7 x 10^{-6} mol/mg protein and was sufficient to increase oxidative damage (lipid peroxidation). In the aged animals exercise increased H_2O_2 concentrations by 7 x 10^{-7} mol/mg protein and elevated lipid peroxidation. This would suggest that the increase in H_2O_2 associated with aging was sufficient to increase localized oxidative damage within the muscle environment.

Within the allopurinol study (specific aim 3) a standard curve was established at the same time the RFU data was collected (Figure 6.3A). This allowed for more accurate determination of H_2O_2 concentrations. The elevation in H_2O_2 concentrations in the aged animals was not as great as in the resveratrol study (allopurinol = 3 x 10^{-7} mol/mg protein vs. resveratrol = 17 x 10^{-7} mol/mg protein); however, as in the resveratrol study there was increase in lipid peroxidation suggesting the increase in H_2O_2 was sufficient to cause oxidative damage. Similar results were found in the resveratrol and allopurinol studies in regard to the magnitude of the increase in the H_2O_2 concentrations (allopurinol = 6 x 10^{-7} mol/mg protein vs. resveratrol = 7 x 10^{-7} mol/mg protein) associated with exercise in the gastrocnemius muscle from aged mice. A brief interpretation of these data suggest that acute or short-term resistance exercise produced concentrations of H_2O_2 that resulted in cellular damage, however elevations in H_2O_2 concentrations associated with chronic resistance exercise may have acted as inter- and/or intra-cellular signals that resulted in the attenuation of oxidative damage associated with exercise in the muscles of the aged animals.

Other limitations of in the vitamin E&C study (Aim 1) were; (a) xanthine oxidase and NADPH oxidase activity were not measured, so the source of oxidant production (e.g., mitochondria, NADPH oxidase or xanthine oxidase) could not be determined, (b) the specific

concentration of vitamins E&C within the blood, the muscle and/or the various cell types within the muscle environment were not measured. Further studies to determine the tissue specific concentrations of vitamins E&C and the sub-cellular location of oxidant production are needed to obtain a clearer picture of how they affect the regulation of the endogenous antioxidant enzymes in response to anaerobic resistance types of exercise.

The second specific aim was to determine the efficacy of resveratrol supplementation as a possible countermeasure for oxidative stress associated with aging and acute exercise in skeletal muscle. The major limitations in the resveratrol study (specific aim 2) were that the duration of treatment might have been too short to identify all of the potential responses to resveratrol supplementation. It is possible that if the aged animals were given the resveratrol fortified diet for a longer time period they would have shown significant improvements in muscle fatigue as did the young animals. Since it is speculated that resveratrol is acting to reduce oxidant formation by means of Sirt1 activation and improved ATP availability, a longer pre-exercise time on the resveratrol diet might allow greater activation of the transcriptional mechanisms need to improve adaptation. However, this investigation does not provide any direct evidence of Sirt1 activation, increases in mitochondrial density and/or efficiency, nor did it measure skeletal muscle ATP content. Further research is needed to determine the significance of resveratrol to manipulate metabolic pathways that would increase ATP content and decrease the rate of fatigue.

Future directions that directly extend from the data in the second study include examining the capacity of resveratrol to increase the availability of ATP and decreased superoxide formation in skeletal muscle by reducing uncoupling of the mitochondrial electron transport chain. Possible targets for improving ATP availability should include the mitochondrial electron transport chain, β-oxidation, and gluconeogenesis. A future study could be designed to test if resveratrol increases ATP availability by increasing β-oxidation in skeletal muscle via decreasing lipogenesis and increasing lipolysis in adipocytes (95), thus allowing for greater substrate availability for ATP production within the contracting muscle. Moreover, Sirt1 activation via resveratrol has been shown to facilitate the nuclear translocation of forkhead transcription factor-1 (FoxO1) thus activating gluconeogenesis in hepatocytes (23). A future study could evaluate the role of Sirt1 in regulating gluconeogenesis within the liver, and leading to higher blood glucose levels. The ability to maintain blood glucose levels during exercise would lead to an increased glucose availability that the working muscle could use for ATP production via glycolysis.

Another important question that is extended from data in Aims 1 and 2 is whether long term resveratrol supplementation would lower the hypertrophic adaptation to high-intensity, short-duration resistance training. There exists the possibility that resveratrol would inhibit muscle growth in favor of improving aerobic function and endurance. It has been shown that concurrent training (combining diverse contractile activity i.e. high-intensity, short-duration and prolonged, low-intensity) may not be optimal for promoting specific adaptations that would simultaneously promote both anabolic and aerobic responses (13). Similar to resveratrol supplementation, the Sirt1 activated AMPK-PGC-1α pathway is up-regulated in response to endurance exercise. Additionally, endurance exercise has been shown to repress the Akt/protein kinase B-mammalian target of rapamycin-p70 S6 kinase (Akt-mTOR-S6K) pathway to which would normally increase protein synthesis and muscle growth in response to resistance exercise (4). It is unknown, if a similar response would occur in both animals and humans undergoing long–term resveratrol supplementation simultaneous with resistance training.

The third specific aim of this project examined how xanthine oxidase inhibition (via allopurinol) affected the increased apoptotic signaling in skeletal muscle from aged animals. It was hypothesized that increased xanthine oxidase activity due to exercise would contribute to decreasing the mitochondrial membrane integrity thus leading to increased apoptotic signaling in aged muscles after exercise. However, it was found that xanthine oxidase does not have a significant role in increasing mitochondrial derived apoptotic signaling. Nevertheless, the inhibition of xanthine oxidase diminished an increase in caspase 3 activity in the aged muscle after isometric contractions. Recent findings imply that increased caspase 3 activity after exhaustive exercise in aged rats is dependent on caspase 8 activation and the extrinsic apoptotic pathway.

The primary limitation of the allopurinol experiments were that direct measures of apoptosis were not examined nor were specific signaling pathways that initiate apoptosis. Future research stemming from these findings in the third aim should focus on determining if the increase in caspase 3 activity initiates an increase in apoptosis and which upstream mechanisms result in the increase of caspase 3 activity are activated by increase in xanthine oxidase activity. These investigations should focus on the extrinsic apoptotic pathway. Fifty minutes of strenuous treadmill running has been shown to increase plasma levels of TNF-α and interleukin-6 (IL-6) which was associated with an increase in apoptosis (57). However, TNF-α and IL-6 are pro-inflammatory cytokines that increase in response to cellular damage and the isometric contractions used in the current study are assumed to be a form of non-damaging contractile activity. It would be essential to determine if the isometric protocol used in the current study, induced increases in TNF-α and IL-6 and if so, did TNF-α and IL-6 increase because contractile damage or if perhaps if exercise-induced oxidative damage leads to inflammation.

TNF-α along with other ligands in the TNF superfamily such as FAS or TRAIL can activate the extrinsic apoptotic pathway when they bind to their receptors (sometimes referred to as death receptors) on the cytoplasmic side of the cellular membrane. This activation of the death receptors stimulates recruitment of Fas-associated death domain protein (FADD) leading to caspase 8 activation subsequent cleaving pro-caspase 3 to its active form. Future directions should characterize the response in death receptor ligands to non-phagocytic xanthine oxidase production and determine if increased extracellular oxidant production via xanthine oxidase increases the death receptor ligands and/or may modify the death receptors themselves. It has recently been shown that increases in intercellular H_2O_2 up-regulates the expression of death receptors (94). It is possible that the elevation in intercellular oxidative stress associated with increased mitochondrial dysfunction associated with aging leads to an increase in the death receptors enhancing the cells sensitivity to death receptor ligands.

Recommendations in human populations

Vitamin E&C

The current investigation shows evidence supporting the use of vitamin E&C as an antioxidant supplement, however the limited scale of these studies make it difficult to produce any practical recommendations in humans. Vitamin E&C supplementation will decrease evidence of oxidative damage caused by exercise and aging, which traditional has been thought to be a healthy benefit leading to the suggestion that antioxidant consumption needs increased in both athletes and the elderly. However, there is growing evidence that low concentrations of oxidants are needed to induce the expression of antioxidant enzymes in response to exercise. Indeed, recent evidence in humans (90) and rats (31) has suggested that antioxidant supplements like vitamin C and/or E can interfere with the benefits of exercise to increase muscle levels of antioxidant enzymes. This phenomenon is best characterized in the concept of

hormesis, which is a dose–response relationship in which low concentrations of a substance stimulate a response and a high concentrations inhibit a response (8). Hormesis has been applied to the oxidant generating effects of exercise as a means to explain why antioxidant supplementation inhibits muscular force production and blocks many of the beneficial effects of exercise on metabolism and the endogenous antioxidant (51, 87, 90). In this context oxidants are viewed as beneficial, rather than as deleterious.

It is important to note that the investigations that suggests antioxidant supplementation prevents the health-promoting effects of exercise have been done in young adult humans (90) or rats (31) performing mostly aerobic exercise. In the elderly, the elevation in basal concentrations of oxidants may offset the redox balance, thereby exposing cells to higher levels of oxidants that have the potential to result in oxidative damage to protein, DNA and lipids. In addition to aged-dependent oxidative stress, elderly people often have low plasma concentrations of the antioxidant vitamins, as a result of their nutritional deficiencies (36, 66). In contrast, dietary supplementation of vitamin E has been shown to significantly reduce systolic blood pressure in sedentary elderly individuals (45). A combination of aerobic exercise and antioxidant supplementation in the elderly doubled the drop in their systolic blood pressure and decreased their diastolic blood pressure, as well as enhanced weight loss and significantly improved maximal oxygen uptake (VO_{2max}) (45). Recent data have suggested that antioxidant supplementation can stimulate muscle protein synthesis in aged rats, possibly through the protection of leucine metabolism (70). In addition, Vitamin E&C supplementation combined with resistance training has been shown to increase fat free mass and the muscle mass index more than resistance training alone in older adults (61).

Based on the current knowledge of antioxidant supplementation, it should be suggested that a dietary analysis of vitamin intake be performed in elderly persons, as this would highlight possible nutritional deficiencies in their diet. The typical American diet for all age groups tends to be inadequate in supplying the recommended allowance of fresh fruits and vegetables. Europeans consume more fruits and vegetables in their diet than Americans, which has been associated with an overall higher dietary vitamin E&C intake in Europe countries compared with in the United States (11). The dietary analysis could be used to determine the proper dosages of supplements to be prescribed based on fulfilling the USDA recommended daily allowances (RDA). However, in the elderly, the RDA values may be inadequate to combat the increased oxidant production and the decreased buffering capacity associated with aging (25). Currently, no specific guidelines for vitamins E or C intake exist for elderly Americans. The data collected in this investigation and some of the research highlighted above suggests vitamin E&C supplementation may be beneficial for the elderly participating in vigorous physical activity. Further research is needed to determine if and to what extent the RDA values need to be adjusted for elderly individuals.

It has been suggested that antioxidant supplementation may be a beneficial countermeasure to the increased oxidant production associated with exercise in young adult athletes. In a study of professional soccer players, dietary supplementation of vitamin E & C was shown to reduce lipid peroxidation and muscle damage after high intensity workouts, however supplementation failed to enhance athletic performance (102). Additionally, it has been shown that oral consumption of vitamin E&C in marathon runners prior to competition reduced post-competition creatine kinase levels compared to a placebo group (91), which would be indicative of reduced muscle damage . However, for each study providing evidence for a positive effect there are other studies providing equally convincing evidence for either no effect or, occasionally, a negative effect of antioxidant supplementation (73).

Resveratrol

Resveratrol is naturally occurring in the skins and seeds of dark grapes, peanuts, and Japanese knotweed (which is an ingredient in many eastern herbal medicines), among other plants, resveratrol is produced by plants as a defense against molds, UV radiation and other pathogens. Oral resveratrol supplementation has been associated with longer lifespan, improve metabolism, decreased oxidative stress and increased aerobic performance (6). Recent research has suggested that dietary intake of resveratrol, together with habitual exercise, is beneficial for suppressing the decline in physical performance associated with aging (74). Theoretically, resveratrol supplementation sounds beneficial to ones overall health and well being. However, there is a paucity of convincing data from human clinical trials that supports resveratrol supplementation. To the best of my knowledge there are no controlled investigations that have examined resveratrol's effect on athletic performance and therefore it is difficult to make any conclusions regarding the efficacy of resveratrol use by athletes.

Within a quick search of the internet an individual will easily indentify many supplement companies that sell resveratrol, but the potency and purity of many of these supplements are not confirmed and/or regulated and in my opinion simply be a waste of time and money. Further research that establishes dose response and kinetic characteristics of the effects of long term resveratrol supplementation on both animals and humans is needed before any professional, scientifically based recommendation could be given. The existing data on the benefits of resveratrol supplementation surely warrant such investigations; however, caution should be advised to any notions of resveratrol being a "fountain of youth".

Allopurinol

Allopurinol is a commonly prescribed drug for the treatment of hyperuricemia (high levels of uric acid). Common complications of hyperuricemia include kidney stones and chronic gout. Allopurinol is in a class of medications called xanthine oxidase inhibitors. The distribution of allopurinol is regulated by the government and should only be given to humans when prescribed by a doctor for the treatment and/or prevention of hyperuricemia or as an inhibitor of xanthine oxidase activity in controlled scientific research projects that are monitored by a medical doctor. Therefore, it is premature to consider regular use by allopurinol as a therapeutic aid to reduce oxidative stress in muscles of the elderly who exercise. Nevertheless, other less toxic substances that reduce xanthine oxidase might eventually prove to be of some benefit for reducing oxidative stress in elderly humans.

Conclusion

The results of this set of investigations show evidence that advanced aging is associated with elevated levels of oxidative stress including oxidative stress from xanthine oxidase. Furthermore, an increase in xanthine oxidase activity provides an important contribution to the oxidative stress associated with resistance exercise (i.e. repetitive loading). Muscles from aged animals have high basal levels of xanthine oxidase, and this is further exacerbated by resistance exercise. Modulation of exercise-induced oxidative stress will effect adaptation of the endogenous antioxidant system and different therapeutic methods of reducing oxidative stress in aged muscle produce slightly different results in muscle function. These studies do not conclusively address the capacity that oxidative stress has on the regulating myonuclear apoptosis, decreasing protein synthesis and/or activating proteolysis pathways, which in turn may contribute to the functional decline in skeletal muscle associated with advanced aging. Further investigations are warranted to find a direct link between oxidative stress and muscle atrophy.

Further work is required to determine if reducing oxidative stress will improve muscle function, fatigue, or apoptotic signaling in muscles of elderly subjects. A greater awareness of the sub-cellular location of oxidant production related to resistance training may allow researchers to identify potential signaling targets that might be manipulated by supplements and/nutritional therapy in muscles of aging humans. Such treatment strategies may potentially have a great economical value to society, given the expanding costs associated with caring for the increasing elderly population.

Figure 7.1

A - Enzyme Activity		GPx activity			Catalase activity			MnSOD activity			CuZnSOD activity		
		Vitamin E&C	Resveratrol	Allopurinol	Vitamin E&C	Resveratrol	Allopurinol	Vitamin E&C	Resveratrol	Allopurinol	Vitamin E&C	Resveratrol	Allopurinol
Effect of Age in Non-Supl, Control Muscle	YCC vs. ACC	⟺	⟺	⟺	⬆	⬆	⬆	⟺	⬆	⬆	⟺	⟺	⬆
Effect of Exercise in Non-Supl from Aged mice	ACC vs. ACE	⬆	⟺	⟺	⟺	⬆	⬆	⟺	⟺	⟺	⟺	⬆	⬆
Effect of antioxidant on age related response	ACC vs. ATC	⇩	⬆	⟺	⬆	⬆	⟺	⟺	⬆	⟺	⟺	⟺	⟺
Effect of Antioxidant on exercise response in aged muscle	ACE vs. ATE	⇩	⬆	⟺	⬆	⬆	⇩	⟺	⬆	⟺	⟺	⟺	⇩
Effect of exercise in the antioxidant supplemented aged animals (Changes in antioxidant only)	ATC vs. ATE	⟺	⟺	⟺	⬆	⬆	⟺	⟺	⟺	⟺	⬆	⟺	⟺

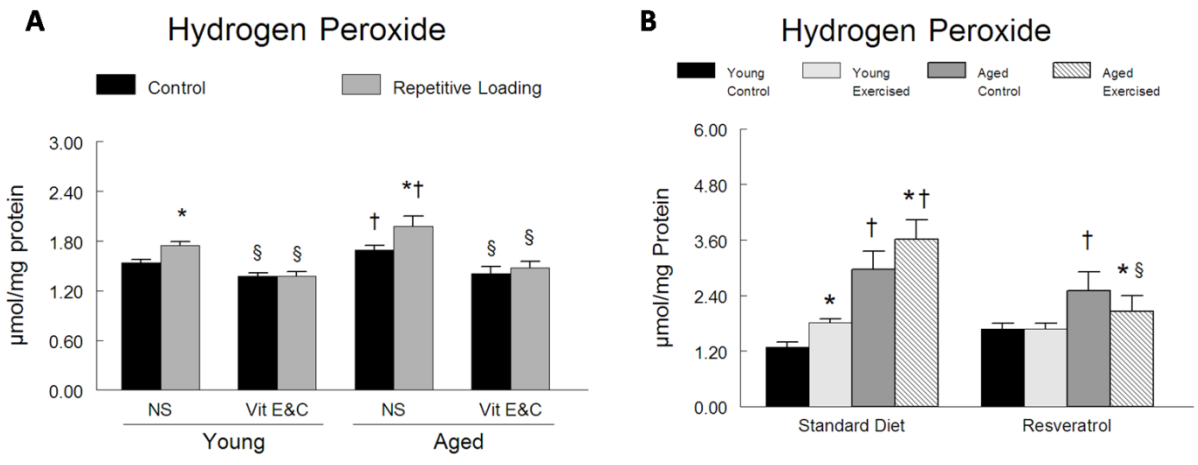
B - Protein Content		GPx-1 Protein			Catalase Protein			MnSOD Protein			CuZnSOD Protein		
		Vitamin E&C	Resveratrol	Allopurinol	Vitamin E&C	Resveratrol	Allopurinol	Vitamin E&C	Resveratrol	Allopurinol	Vitamin E&C	Resveratrol	Allopurinol
Effect of Age in Non-Supl, Control Muscle	YCC vs. ACC	⟺	N/A	N/A	⟺	N/A	⬆	⟺	N/A	N/A	⟺	N/A	⟺
Effect of Exercise in Non-Supl from Aged mice	ACC vs. ACE	⟺	N/A	N/A	⟺	N/A	⟺	⟺	N/A	N/A	⟺	N/A	⬆
Effect of antioxidant on age related response	ACC vs. ATC	⬆	N/A	N/A	⬆	N/A	⟺	⬆	N/A	N/A	⬆	N/A	⟺
Effect of Antioxidant on exercise response in aged muscle	ACE vs. ATE	⬆	N/A	N/A	⬆	N/A	⟺	⬆	N/A	N/A	⟺	N/A	⇩
Effect of exercise in the antioxidant supplemented aged animals (Changes in antioxidant only)	ATC vs. ATE	⟺	N/A	N/A	⟺	N/A	⟺	⇩	N/A	N/A	⟺	N/A	⟺

C - mRNA Expression		GPx-1 mRNA			Catalase mRNA			MnSOD mRNA			CuZnSOD mRNA		
		Vitamin E&C	Resveratrol	Allopurinol	Vitamin E&C	Resveratrol	Allopurinol	Vitamin E&C	Resveratrol	Allopurinol	Vitamin E&C	Resveratrol	Allopurinol
Effect of Age in Non-Supl, Control Muscle	YCC vs. ACC	⟺	⟺	⟺	⟺	⬆	⬆	⟺	⬆	⟺	⟺	⟺	⟺
Effect of Exercise in Non-Supl from Aged mice	ACC vs. ACE	⟺	⟺	⟺	⟺	⟺	⟺	⟺	⟺	⟺	⟺	⟺	⟺
Effect of antioxidant on age related response	ACC vs. ATC	⇩	⟺	⟺	⇩	⬆	⟺	⇩	⬆	⟺	⇩	⟺	⟺
Effect of Antioxidant on exercise response in aged muscle	ACE vs. ATE	⇩	⟺	⟺	⇩	⬆	⟺	⇩	⬆	⟺	⇩	⟺	⟺
Effect of exercise in the antioxidant supplemented aged animals (Changes in antioxidant only)	ATC vs. ATE	⟺	⟺	⟺	⟺	⟺	⟺	⟺	⟺	⟺	⟺	⟺	⟺

Across study comparison of regulation of the endogenous antioxidant enzymes
Comparison of the effect of aging, exercise and antioxidant treatment on activity of the endogenous antioxidant enzymes **(A)**, enzyme protein content **(B)** and enzyme mRNA expression **(C)**. ⬆ Indicates a significant ($p<0.05$) increase in activity. ⟺ Indicates no significant ($p<0.05$) change in enzymatic activity. ⇩ Indicates a significant ($p<0.05$) decrease in enzymatic activity. N/A (not applicable) Indicates data was not collected for that variable.

YCC= Young, Control (non-antioxidant treatment), Control non-exercise; ACC= Aged, Control (non-antioxidant treatment), Control non-exercise; ACE= Aged, Control (non-antioxidant treatment), Exercised; ATC= Aged, Treatment (antioxidant), Control non-exercised; ATE = Aged, Treatment (antioxidant), Exercised

Figure 7.2



Corrected concentrations for hydrogen peroxide (H_2O_2) The H_2O_2 concentration was determined fluorometrically. The data are expressed as mean ± SEM of µ moles per mg of total protein homogenate. **(A)** H_2O_2 concentrations from the vitamin E&C study (specific aim 1) *, significant difference ($p<0.05$) between age-matched repetitively loaded muscle and contra-lateral control muscle; †, a significant difference ($p<0.05$) between young exercise and diet-matched control muscles; §, significant difference ($p<0.05$) between age-matched muscles from animals on the non-supplemented (NS) diet. **(B)** H_2O_2 concentrations from the resveratrol study (specific aim 2) * significant difference ($p<0.05$) of isometrically exercised muscle from contra-lateral control muscle; † a significant difference ($p<0.05$) from young exercise and diet-matched control muscles; § significant difference ($p<0.05$) from age-matched animals on the non-supplemented diet.

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Appendix

Table 7.1
(Reprint of Table 4.1)

Body Mass of Young and Aged Rats Pre and Post 14 sessions of Repetitive Loading

	Young NS	Young Vit. E&C	Aged NS	Age Vit. E&C
Pre-RL (g)	343.5 ± 10.9	350.1 ± 26.1	513.8 ± 88.4 †	491 ± 63.5 †
Post-RL (g)	328.1 ± 11.8	340.2 ± 27.8	467.2 ± 91 †	441 ± 55.5 †

Each value expressed in grams as a mean ± SEM
NS = non-supplemented

Body Mass of Young and Aged Rats Pre and Post 14 sessions of repetitive loading

Data are mean ± SEM and presented as the mass of the rats before the first exercise session and after the 14th training session in grams. †, indicates a significant difference ($p<0.05$) from young exercise and diet-matched control muscles. NS, non-supplemented diet.

Table 7.2

Body Mass of Young and Aged Mice

Study	Young Non-AO	Young AO	Aged Non-AO	Aged AO
Resveratrol	25.4 ± 0.8	25.5 ± 0.6	33.1 ± 0.7*	32.9 ± 0.9*
Allopurinol	26.0 ± 0.6	25.8 ± 1.5	33.1 ± 1.0*	32.8.0 ± 0.8*

Each value expressed in grams as a mean ± SEM
*Indicates a significant difference ($p<0.05$) in the aged animals from the young adult treatment matched animals
Non-AO = Non-antioxidant treatment group
AO = Antioxidant treatment group

Body mass of young and aged mice in the resveratrol and allopurinol studies Data are mean ± SEM and presented as the mass of the mice in grams. *, indicates a significant difference ($p<0.05$) in the aged mice from the young treatment matched control mice.