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The Effects of Resveratrol on Oxidative Stress and Muscle Atrophy

Janna Renee Jackson
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The Effects of Resveratrol on Oxidative Stress and Muscle Atrophy

Janna Renee Jackson

Dissertation Submitted to the School of Medicine at West Virginia University
In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Physiology

Stephen E. Alway, Ph.D., Chair, Mentor
Robert G. Cutlip, Ph.D.
Stan M. Hileman, Ph.D.
John M. Hollander, Ph.D.
William T. Stauber, Ph.D.

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Abstract

The Effects of Resveratrol on Oxidative Stress and Muscle Atrophy

Janna Renee Jackson

Increases in oxidative stress and apoptosis are associated with skeletal muscle atrophy caused by prolonged periods of muscle disuse and also as part of the normal aging process. The loss of muscle size and strength with age is known as sarcopenia and has detrimental implications to the quality of life for elderly individuals. Although the causes of sarcopenia and disuse-mediated atrophy are largely unknown, an increased oxidant load has the potential to negatively impact both muscle mass and function by directly causing oxidative damage to tissue constituents and also via the initiation of the intrinsic mitochondrial apoptotic pathway. Due to its multi-nucleated cellular structure, skeletal muscle is an exception with regard to the linear relationship between apoptosis and cell death. Apoptosis in skeletal muscle results in the potential loss of both myonuclei and/or muscle precursor cells (i.e. satellite cells). This is of particular significance in skeletal muscle given that skeletal muscle is a post-mitotic tissue and thus it's only means of regeneration rests with its ability to activate and propagate satellite cells. Therefore, oxidative stress may potentially contribute to the process of muscle atrophy via a dual-fold mechanism, by both directly oxidizing the nucleic acids, lipids and proteins that make up the muscle tissue and by creating a more favorable apoptotic environment leading to an eventual loss of myonuclei.

Resveratrol (3,5,4,trihydroxystilbene) is a naturally occurring polyphenol found in over seventy plant species that has been shown to have anti-oxidant, anti-apoptotic and anti-aging properties. Resveratrols' capacity to enhance the endogenous anti-oxidant system, upregulate anti-apoptotic proteins and improve mitochondrial function, presumably through the activation of the NAD$^+$ dependent deacetylase Sirtuin1, suggests that supplementation with resveratrol may potentially protect skeletal muscle from the detrimental effects of increases in oxidative stress and the subsequent increase in apoptotic signaling that are present in many atrophic conditions. Therefore, the major goals of this dissertation are to further understand the interplay between oxidative stress and skeletal muscle atrophy and to evaluate the efficacy of resveratrol as a counter measure to both mitochondrial-induced oxidative stress and apoptosis.

The first study used hindlimb suspension (HLS) as a model of disuse atrophy as it is known to elicit muscle atrophy, oxidative stress and apoptosis in skeletal muscle. The aim of the investigation was to analyze the capacity of resveratrol administration at a moderate dose of 12.5mg/kg/day for 21 days to attenuate oxidative stress, apoptosis and muscle force loss following 14 days of HLS in young and aged rats. The study yielded mixed results. Resveratrol administration effectively reduced oxidative stress and subsequently oxidative damage in muscles from aged animals. Furthermore, resveratrol administration attenuated the relative loss of muscle mass as a ratio of animal body weight in gastrocnemius muscles from aged animals; however, despite reductions in apoptotic signaling in aged muscles, resveratrol administration was unable

to mitigate apoptosis as measured by DNA fragmentation. Moreover, resveratrol administration had no protective effect on gastrocnemius muscles from young animals with regard to oxidative or apoptotic indices. Based on these results resveratrol has the potential to be an effective therapeutic agent to treat muscle functional decrements associated with disuse in aged individuals, via improving the redox status associated with these conditions.

The second half of the dissertation focuses on the capacity of long-term dietary supplementation with resveratrol to protect against aging-induced oxidative stress and to enhance mitochondrial signaling, and thus stem the progression of sarcopenia in aged skeletal muscle. In this study middle-aged (18mo) C57BL/6 mice were supplemented with a diet containing .05% trans-resveratrol for 10 months until they reached senescence (28mo). Gastrocnemius, plantaris and vastus lateralis muscles from supplemented animals were compared for anti-oxidant enzyme content and activities, oxidant load, oxidative damage, mitochondrial integrity and mass, as well as muscle function indices with muscles from young and middle-aged animals receiving a control diet. Resveratrol specifically upregulated the capacity of the mitochondrial isoform of superoxide dismutase (MnSOD) and concomitantly decreased hydrogen peroxide concentrations. This was paralleled by reductions in lipid peroxidation, but not protein oxidation in muscles from supplemented animals. Despite reductions in oxidant load and lipid peroxidation resveratrol, supplementation was unable to confer protection against sarcopenia. Furthermore, plantaris muscles from supplemented aged animals did not show enhanced resistance to muscle fatigue, nor an increase in maximal force production.

Taken as a whole these results suggest that resveratrol may be most effective when used as a pharmacological pre-conditioner to help confer resistance to oxidative damage under perturbations that are known to increase oxidative stress. Specifically, resveratrol may be an effective therapeutic agent to improve the redox status of aged skeletal muscle and therefore allow for improved adaptation and recovery following chronic illness and/or injuries during which the capacity of the endogenous antioxidant system may be overwhelmed in aged individuals. Gaining clearer insight into the molecular signaling pathways involved in aging and disuse muscle atrophy is paramount in developing nutritional and/or pharmacological interventions to minimize protein loss and attenuate the functional decrements associated with atrophic conditions.

Dedications

This dissertation is dedicated to my friends and family. To my Mom for always being proud of me and for accepting the fact that her 34 year old daughter is still in school. To my wonderful husband Jason, whose faith in me far exceeds the faith that I have in myself; for that I am eternally grateful. To my daughter Evelyn who has unknowingly changed me in a way that only a child can. Her very presence motivates me to be the best person I can be; thank you for clarifying what's really important in life.

Last, but not least, I would also like to dedicate this dissertation to all of my friends that I've met during my time at WVU, they have truly made Morgantown a home away from home and will remain permanent fixtures in my life.

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Glossary of Terms

ASK-1	Apoptosis signaling kinase 1
ATP	Adenosine triphosphate
Bax	Bcl-2 associated x protein
Bcl-2	B-cell lymphoma 2
CuZnSOD	Copper-zinc superoxide dismutase
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FOXO	Forkhead family of transcription factors
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HAE	4-hydroxyalkenals (HAE)
HLS	Hindlimb suspension
H_2O_2	Hydrogen peroxide
Hz	Hertz
IHC	Immunohistochemistry
JNK	c-june kinase
kD	KiloDalton
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MnSOD	Manganese superoxide dismutase
mtPTP	Mitochondria transition pore
N	Newton
NFκB	nuclear factor-κB
NO	Nitric oxide
$OH^{\cdot-}$	Hydroxyl radical
ONOO-	Peroxynitrite
OS	Oxidative stress
O_2	Molecular oxygen
$O_2^{\cdot-}$	Superoxide
PBS	Phosphate buffered saline
PGC1	PPARγ co-activator
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
REDOX	Oxidation-reduction status
RFU	Relative fluorescent unit
ROS	Reactive oxygen species
SDS-Page	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
Sirt1	Silent mating type information regulation homolog1
TRX	Thioredoxin

Specific Aims

With advanced age there is a progressive loss of skeletal muscle mass, termed sarcopenia (32). The frailty and loss of function associated with sarcopenia puts the elderly at an increased risk of falls and subsequent injury, resulting in a marked decrease of their quality of life (6; 17). Furthermore, sarcopenia represents a tremendous financial burden; the estimated direct healthcare costs attributable to sarcopenia in the United States in the year 2000 was $18.5 billion (18). The causative factors of sarcopenia are multi-factorial and include a progressive denervation of muscle fibers (11), an altered hormonal milieu (35), and an increase in protein degradation and a concomitant decrease in protein synthesis, resulting in a net loss of contractile proteins (19; 29). Moreover, sarcopenia can be compounded by reductions in dietary intake (31) and activity levels (10) often associated with advanced age.

Both advanced age and disuse are associated with atrophy and an increased production of reactive oxygen species (ROS) in skeletal muscle (24; 25; 28), leading to an augmented oxidant-load. When an increase in pro-oxidant production exceeds an organisms' capacity to buffer them, via a complex coordination of the endogenous antioxidant defense system, oxidative stress occurs. Oxidative stress, left unchecked over time leads to the oxidation, and thus damage, of cellular macromolecules, including lipids (26), nucleic acids (15) and proteins (2; 9; 16). The progressive oxidative assault on cellular organelles is believed to be a main contributor to the aging process (13) and is thought to be responsible for many of the pathologies associated with aging, including genomic instability, mitochondrial dysfunction and chronic inflammation (13; 14).

Oxidative stress is believed to be a common underlying mechanism potentiating many of the factors leading to muscle loss with aging (12; 28), of particular interest is the redox sensitive apoptotic pathway (37). Oxidative stress is upstream of apoptotic signaling in muscle cells (34) and results in the initiation of the intrinsic mitochondrial apoptotic pathway. Due to its multi-nucleated cellular structure, skeletal muscle is an exception with regard to the linear relationship between programmed cell death and apoptotic signaling. Instead, apoptosis in skeletal muscle results in a loss of myonuclei and consequent fiber atrophy (1).

Antioxidant supplementation has been shown to be an effective counter measure to combat oxidative stress in a wide variety of tissue types and conditions (7; 28) and it is speculated that this might be an approach to reduce muscle wasting associated with disuse (33) and aging (7). Dietary supplementation with the polyphenol, resveratrol, exerts beneficial effects not only through its' ability to directly scavenge free radicals (3; 5), but also by its' capacity to modulate the signal transduction and gene expression of several pathways regulating cellular proliferation (30), mitochondrial biogenesis (20; 36), metabolism (12; 13; 28), and survival. The efficacy of acute resveratrol administration has been established for several pathological conditions (4; 20; 27); however, the use of chronic resveratrol supplementation as a counter-measure to combat oxidative stress-induced muscle loss and sarcopenia has not been established.

The _long-term goal_ of this study is to characterize the mechanisms responsible for the progression and pathogenesis of sarcopenia and disuse-induced muscle loss and to use this information to develop strategies for the prevention and treatment of muscle loss with aging. _The main objectives of the project were:_ (1) to characterize the

endogenous antioxidant defense system in young and old skeletal muscles exposed to hindlimb suspension (HLS); (2) to determine the ability of resveratrol to reduce oxidative stress associated with both aging and skeletal muscle disuse via an enhancement of the endogenous antioxidant defense system; (3) to evaluate the potential of long-term resveratrol supplementation to attenuate the detrimental effects of aging in skeletal muscle via reductions in pro-oxidant production by enhancing cellular mitochondrial content, integrity and function; (4) to characterize the relationship between oxidative stress and myonuclear apoptosis contributing to skeletal muscle atrophy.

The *central hypothesis* of the project is that enhancing the muscle's endogenous antioxidant defense system and promoting mitochondrial biogenesis will reduce oxidative stress, consequently reducing muscle cell apoptosis. Attenuation of myonuclear apoptosis will result in a reduction of skeletal muscle atrophy associated with both disuse and advanced age. Our central hypothesis is based on data from our laboratory and published data from other laboratories, suggesting that sarcopenia and muscle atrophy occurs concurrently with a reduction in mitochondria content and increases oxidative stress (12; 22; 23). Resveratrol may act to both reduce oxidative stress and improve mitochondria biogenesis (4; 8; 20; 21). The _rationale_ for this project is that a delineation of the potential importance of resveratrol to reduce oxidative stress and mitochondrial dysfunction potentially leading to attenuation of apoptosis and the improved regulation of muscle mass during aging would potentially provide opportunities for intervention, or prevention, of the development of sarcopenia in aged individuals.

Specific Aim 1: **To evaluate the efficacy of resveratrol administration to ameliorate muscle atrophy associated with muscle disuse by reducing oxidative stress and apoptotic signaling.**

To address aim 1, indices of oxidative stress and the content of mitochondria-associated apoptotic proteins were assessed in the gastrocnemius muscles of young adult and old rats subjected to resveratrol treatment during acute muscle disuse induced by HLS. Additionally, muscle function was assessed, pre and post-HLS, to determine the efficacy of resveratrol to preserve muscle mass and function following disuse.

- **Hypothesis 1.1:** Hindlimb suspension will increase oxidative stress in gastrocnemius muscles; Resveratrol administration will improve the redox status of these muscles by increasing their endogenous antioxidant defense systems.

- **Hypothesis 1.2:** The gastrocnemius muscles from old HLS animals will exhibit greater increases in oxidative stress than gastrocnemius muscles from young HLS animals; Resveratrol administration will attenuate this increase.

- **Hypothesis 1.3:** Hindlimb suspension will increase apoptotic signaling in gastrocnemius muscles from both young and old animals; Resveratrol administration will reduce apoptotic signaling in these muscles.

- **Hypothesis 1.4:** Resveratrol administration will improve muscle function in the gastrocnemius muscles from HLS animals by reducing oxidative stress and subsequently reducing myonuclear apoptosis-induced atrophy, leading to maintenance of muscle force following HLS.

Specific Aim 2: **To determine the ability of long-term resveratrol supplementation to counteract the detrimental effects of aging on skeletal muscle mass, function, and oxidant load.**

To address aim 2 select antioxidant enzyme capacities, oxidative stress indices, mitochondrial-associated signaling proteins and muscle mass and function (force and fatigability) were evaluated in the hindlimb muscles of old mice subjected to long-term resveratrol supplementation. The dietary supplementation with 0.05% trans-resveratrol began at middle-age (18 months old) mice and continued through old age (28 months old). Sirtuin1 (Sirt1) were measured because it is potentially activated by resveratrol. Therefore, Sirt1 may have a key role in regulating mitochondrial-associated changes in response to long-term supplementation.

- **Hypothesis 2.1:** Long-term resveratrol supplementation will reduce oxidative stress and consequently oxidative damage in aged skeletal muscle, there by slowing the progression of sarcopenia by helping to maintain the muscle mass of old mice.

- **Hypothesis 2.2:** Long-term resveratrol supplementation will prevent the age-associated decline in force production in hindlimb muscles of old mice by providing a more favorable muscle redox environment.

- **Hypothesis 2.3:** Long-term resveratrol supplementation will activate Sirt1 and in turn will promote mitochondrial biogenesis and improve mitochondrial membrane integrity, thus improving resistance to fatigue in skeletal muscles of old mice.

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Chapter 1: Background and Significance

1.1: Sarcopenia; An overview of the consequences and underlying mechanisms:

Loss of muscle strength and muscle wasting are characteristic of aging and are caused by both a loss of muscle fibers and atrophy of the remaining fibers (33; 113). Age associated loss of muscle mass and function is termed sarcopenia (113). Sarcopenia is a ubiquitous problem facing the elderly and is characterized by marked frailty, impaired mobility and an increased risk of injury (58; 113). The combination of these factors leads to a severe reduction in the quality of life for older individuals and presents an ever increasing burden on our already taxed healthcare system. In the year 2000, it was estimated that a 10% reduction in the prevalence of sarcopenia would result in savings of $1.1 billion per year in U.S. healthcare costs (58). The percentage of the U.S population in their seventh and eighth decade of life is increasing at a faster rate than at any other time in U.S. history (1), underscoring the importance of finding effective countermeasures to combat age-related pathologies, including sarcopenia. Although it is known that sarcopenia results in ~40% reduction in skeletal muscle mass by the age of 80 (77), the mechanisms underlying this severe muscle atrophy have not been completely elucidated. However, age-related increases in pro-oxidant production and the resultant oxidative stress associated with this increase are thought to be important mediators of the mechanisms triggering sarcopenia and the aging process as a whole (45). One pathway known to be activated by oxidative stress (137) and also implicated in the progression of sarcopenia (6) is the programmed cell death, or

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apoptotic pathway. Apoptosis results in cell death for mono-nucleated cells and a loss of nuclei in multi-nucleated cells, such as skeletal muscle (6). Since the number of nuclei within a muscle fiber is proportional to the size of the fiber, the loss of myonuclei is a key factor in skeletal muscle atrophy caused by a wide variety of muscle wasting conditions (5; 6), including denervation (2; 126), disuse (38; 128), immobilization (142) and advanced age (6). Furthermore, aged individuals must contend with the additive effect of the basal incidence of oxidative stress and apoptotic signaling that are inherent with advanced age, compounded by the fact that aging potentiates apoptosis during disuse, resulting in an enhanced atrophic environment (116; 130).

1.2 Oxidative stress and the process of aging:

Aging is an inevitable process that results in detrimental structural and functional changes starting at the subcellular level and eventually affecting the integrity and function of tissues, organs and whole systems within animals. Although the pathologies associated with aging are well documented (84), the exact mechanisms underlying these pathologies are a constant source of debate. One theory that has gained popularity over the past two decades, is "the mitochondrial theory of aging", originally presented by Dr. Denham Harmon in 1956. The theory is based on the idea that with advanced age there is an increase in free radical production from mitochondria due to reductions in the organelles integrity and function and that these free radicals oxidize and subsequently damage biomolecules (45; 46; 124). Thus, the accumulation of irreversible damage to lipids (94), proteins (52) and nucleic acids (49) drives the aging

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process. The accrual of damage induced by oxidative stress can ultimately lead to cell death in various tissues and cell types, including muscle stem cells (39; 62; 62).

Furthermore, oxidative stress is positively correlated with skeletal muscle atrophy induced by aging (39), denervation (90), disuse and immobilization (69; 71; 100). These atrophic stimuli are associated with concomitant increases in lipid peroxidation , glutathione oxidation, protein carbonyl formation (133), free iron content (50), xanthine oxidase levels (50) and nucleic acid damage (50; 133), which can lead to genomic instability and altered gene expression. Compounding the effect of increased pro-oxidant production and the associated macromolecule damage seen with advanced age (39) and muscle disuse (129) are the inherent alterations in the endogenous antioxidant defense systems that occur with aging (95; 103). Although there is an incongruence in the literature as to whether the endogenous antioxidant system activity increases or decreases with aging, there is an overwhelming amount of literature suggesting that despite increases in enzymatic activity and/or content of the antioxidant enzymes, the antioxidant defense system in aged individuals can become more easily overwhelmed in situations that potentiate oxidant production leading to oxidative stress and consequent damage (94; 116; 129).

1.3 The endogenous anti-oxidant system:

Antioxidant enzymes and compounds are present in organisms as simple as single-celled yeasts (57), to the most complex of organisms, humans (95). Their ubiquitous presence underscores the importance of protecting oneself from the harmful

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effects of pro-oxidants. The endogenous anti-oxidant system is composed of a complex coordination of enzymes and small molecules, whose sole responsibilities are to keep the levels of pro-oxidants in check. Antioxidant enzymes function by converting reactive, and thus toxic, pro-oxidants into less reactive forms, in a series of enzymatic reactions resulting in more inert biological molecules. An example of this type of chain reaction is exemplified by the coordination of two enzymes, superoxide dismutase (SOD) and catalase in the stepwise removal of the highly reactive superoxide anion ($O_2^{\bullet}$). The superoxide anion is a harmful free radical that is generated in the mitochondria when electrons escape the electron transport chain and react with molecular oxygen, to form $O_2^{\bullet -}$ (14). The protonated form ($HO_2^{\bullet -}$) can initiate lipid peroxidation (44; 79) and lead to the formation of radical species such as peroxynitrite (ONOO-) and the hydroxyl radical ($OH^{\bullet}$) (44). Therefore, limiting the production and enhancing the removal of $O_2^{\bullet -}$ via SOD is a key aspect in limiting the potential downstream oxidation of biomolecules (87). Superoxide dismutase has several isoforms that all catalyze the same reaction in different subcellular locations; the dismutation of $O_2^{\bullet -}$ into the less reactive pro-oxidant hydrogen peroxide (H_2O_2) (11; 87). Catalase,(located in both the cytosol and in micro-peroxisomes in skeletal muscle) (20; 108), then further converts H_2O_2 into O_2 and water. Alternatively, H_2O_2 can be reduced by other peroxidases, such as glutathione peroxidase, a very abundant enzyme found in the cytosol. The glutathione redox cycle reduces H_2O_2 by the continual interplay between glutathione reductase and glutathione peroxidase. Glutathione peroxidase catalyzes the reaction in which glutathione donates an electron to reduce H_2O_2 and glutathione reductase regenerates glutathione via a NADH- dependent reaction, thus this cycle is self-sustaining and extremely beneficial in

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the prevention of oxidative stress (11). Additionally, there are other non-enzymatic molecules present in biological systems that function as anti-oxidants, such as vitamin C and vitamin E (102). These small molecules exert their antioxidant capabilities by acting as electrons donors, and thus effectively reducing pro-oxidants before they can cause oxidative damage. They can also play a role in the repair of oxidized biomolecules. (102). Overall, the endogenous antioxidant defenses system is very effective in protecting cellular components against oxidation; however, under extenuating circumstances, in which pro-oxidant production increases dramatically, or is present for prolonged periods of time, the system can become overwhelmed, leading to oxidative stress and the associated downstream effects (11).

1.4 Mitochondrial dysfunction; A source of reactive oxygen species with aging:

Age-related mitochondrial alterations underlie a wide variety of diseases such as diabetes (81), neurodegeneration (122) and sarcopenia (17). The premise behind this relationship is that with advanced age there are more dysfunctional mitochondria present within a cell (80). These defective mitochondria contain "leaky" electron transport chains and thus more pro-oxidants are produced leading to oxidative stress (17; 49). The process is cyclic, with more mitochondrial uncoupling, there are more pro-oxidants present to further damage vulnerable membrane phospholipids (49; 94) and perhaps more importantly oxidatively damage mtDNA, which unlike nuclear DNA, is without the protection of chromatin condensation and histones (26). Thus, damaged mtDNA can lead to a further decrease in the quality and quantity of mitochondria within

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a cell, potentiating an oxidizing environment. Although mitochondria are not the sole

source of ROS within a cell, they are routinely viewed as the principle site of superoxide

generation, which is the primary source of damaging ROS within muscle cells (55; 56).
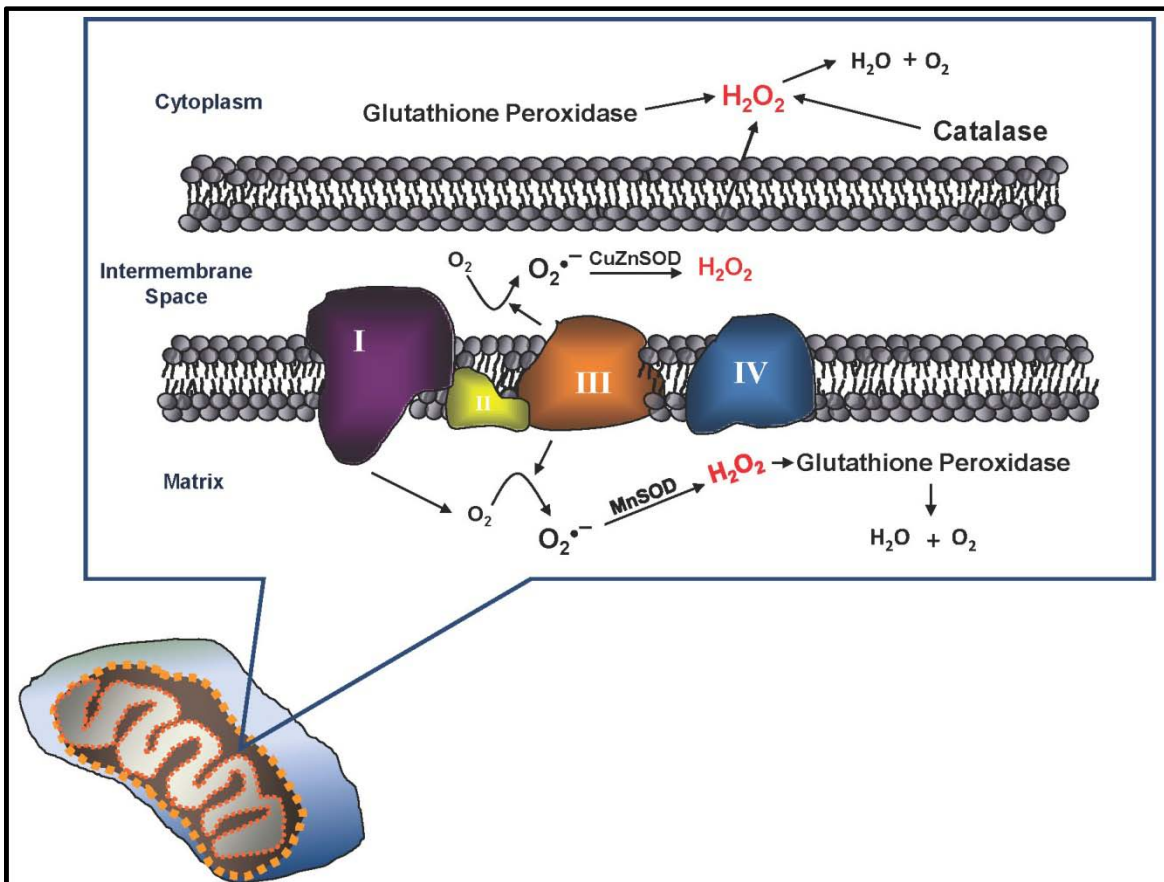
Figure 1.1- Mitochondria mediated ROS generation.

Mitochondrial derived superoxide generation increases with advanced age (91).

Additionally, mitochondria have recently been shown to be the primary source of ROS

within muscle precursor cells (143) (146). The potential for ROS to damage satellite

cells is of particular concern in a post-mitotic tissue like skeletal muscle, as satellite cells

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represent the only mechanism for muscle repair, adaptation and regeneration; thus the only mechanism available to combat sarcopenia (114; 123). Further evidence linking mitochondrial dysfunction and skeletal muscle atrophy is the negative association between muscle fiber size and the content of dysfunctional mitochondria (48; 88).

1.5 Oxidative stress and muscle atrophy:

Oxidative stress is associated with skeletal muscle atrophy caused by a variety of underlying conditions (89; 136). During extended periods of oxidative stress there is an eventual loss of cellular integrity mediated by the oxidation of lipids (94; 118), proteins (29; 89) and nucleic acids (50), promoting a cycle of increased pro-oxidant production, and thus increased oxidative damage, which limits both the cellular repair system and the enzymatic antioxidant defense system (39). The exact mechanisms by which oxidative stress acts as a potentiator of muscle atrophy are largely unknown, however, several relationships between oxidative stress and atrophy have been postulated (39; 101). Specifically, skeletal muscle atrophy is characterized by an overall reduction in protein synthesis and/or a concomitant increase in protein catabolism resulting in a net protein loss (10; 63). Oxidized proteins are preferentially targeted by the 20S proteasome (60) and are subsequently degraded via proteolysis (60). However, severely oxidized proteins are poorly recognized by the proteasome system and thus can aggregate and lead to an accumulation of dysfunctional proteins (42; 95; 132). Additionally, several key signaling pathways responsible for regulating muscle

precursor cell proliferation, differentiation and survival are redox sensitive, including the forkhead family of transcription factors (FOXOs) (141; 145) and nuclear factor-κB (NFκB) (76; 115; 136). Therefore, in an oxidizing environment these proteins have the potential to promote cell cycle arrest and decrease the proliferation of satellite cells, potentiating muscle atrophy. Furthermore, key mitogen-activated kinases (MAPKs), involved in the cellular stress response, are positively regulated by oxidative stress (31; 137) and when activated, act as upstream activators of the apoptotic pathway (118), which is associated with skeletal muscle atrophy. Clearly, the potential is there for oxidative stress to negatively impact the proliferation and differentiation capacity of satellite cells, the muscles only source of regenerative potential (24; 76; 140), has it therefore could influence muscle size and function.

1.6 Oxidative stress and muscle function:

Given that oxidative stress is positively correlated with muscle atrophy (32; 39; 129), it is likely negatively associated with muscle force production (21; 23; 53; 107). Furthermore, increased pro-oxidant production and/or an attenuated capacity to buffer these pro-oxidants, may result in reductions in muscle function beyond the linear relationship between cross-sectional area and muscle force generation (22; 53; 107) due to alterations of neural and/or metabolic factors (22).

This reduced functional capacity may be particularly true in aged skeletal muscle, where the increase in pro-oxidant production can overwhelm the endogenous anti-

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oxidant defense system, leading to cellular damage, increased muscle frailty and thus a decreased functional capacity (39; 49).

Specifically, increases in oxidative stress have the potential to depress muscle specific force (21; 99), alter myofilament function as a result of loading (70; 72) and perturb calcium handling induced by muscle contractions leading to calpain activation (35; 36; 97; 125). Additionally, it is possible that oxidative stress could alter muscle function by impeding recovery from injury (64) and other pathological conditions, given that muscle precursor cells are sensitive to oxidative stress (86; 104; 140) .

1.7 Oxidative stress and apoptosis; the mitochondrial link:
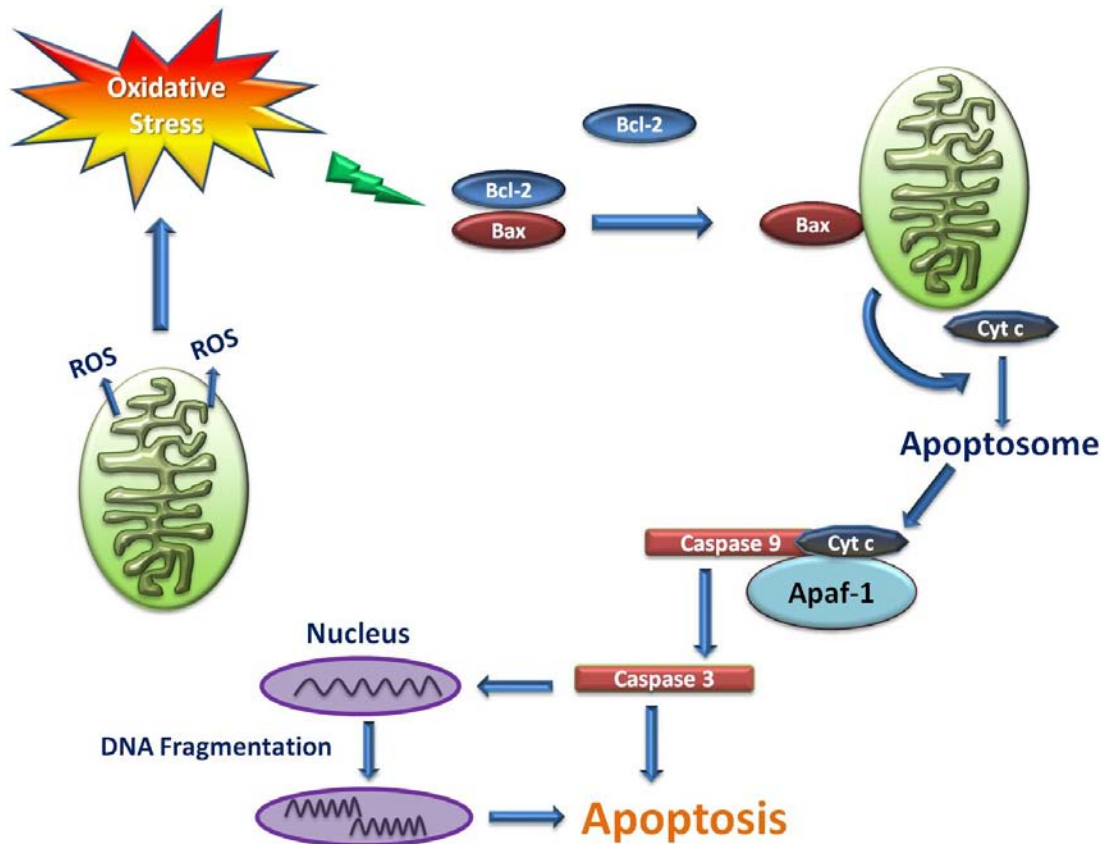
Figure 1.2- Mitochondria as mediators of oxidative stress and apoptosis.

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Myonuclei undergo apoptosis in many muscle wasting conditions including, but not limited to, advanced age (6), disuse (128) and denervation (92; 126). Likewise, these conditions are also associated with increases in oxidative stress (101), presumably generated through mitochondrial dysfunction, given the importance that the mitochondria play in maintaining cellular integrity via ROS production and the regulation of the apoptotic pathway (3; 8; 139). Mitochondria house several proteins, which in times of cellular stress, are released in to the cytosol through the mitochondrial transition pore (mtPTP) in the outer mitochondrial membrane (8; 66; 118). These proteins include several modulators of the apoptotic signaling pathway, including apoptosis inducing factor (AIF), Cytochrome c, endonuclease G, and smac/diablo which initiate a cascade of proteolytic events that converge on the nucleus leading to the fragmentation of DNA and ultimately cell death (8; 66). Regulation of the mtPTP opening is mediated via the B-cell lymphoma-2 (Bcl-2) family of proteins (47; 66), which contains both anti and pro-apoptotic members. Specifically, the pro-apoptotic member, Bax, is responsible for the mitochondrial transition pore channel formation via homo-oligermization and subsequent insertion into the outer mitochondrial membranes resulting in pore formation (see Figure 1.2) (47; 66). Conversely the channel is negatively regulated by the anti-apoptotic Bcl-2 protein, which acts to inhibit mtPTP formation by dimerizing with Bax (8; 66), thus preventing Bax from translocating and inserting into the membrane.

One pathway that directly links oxidative stress and apoptosis starts with the phosphorylation and subsequent activation of two members of the redox sensitive MAPK pathway; c-jun kinase (JNK) and p-38 (121). Both JNK and p38 are activated by

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apoptosis signaling kinase 1 (ASK-1) (18), a kinase upstream of the mitochondria (18). In a reducing environment ASK-1 remains inactive due to its dimerization with reduced thioredoxin (TRX) an anti-oxidant compound that functions in a very similar manner to the glutathione redox system and effectively reduces peroxides, such as H_2O_2 (27; 150). However, in an oxidizing environment TRX is oxidized and can no longer bind ASK-1, leaving the kinase free to phosphorylate its downstream targets, which include both JNK and p38 (27; 121; 150). Both Bax and Bcl-2 have multiple phosphorylation sites (30; 147; 148), which when phosphorylated confer conformational changes that can either promote, or inhibit their ability to regulate the mtPTP opening and consequently apoptosis (65). JNK and p-38 have been shown to each have the capability to phosphorylate both Bcl-2 and Bax (65). Bcl-2 phosphorylation causes the protein to lose its affinity for Bax, preventing Bcl-2 from sequestering Bax in the cytosol and thus abrogating its anti-apoptotic potential (43). Conversely, the phosphorylation of Bax causes a conformational change that exposes its N-terminus and promotes its ability to insert into the outer mitochondrial membrane and initiate apoptosis (65)

It should be noted that these pathways, although well characterized *in vitro*, have been very hard to elucidate *in vivo* and to date no study exists (to the author's knowledge), characterizing the actual, not theoretical, role that the ASK-1 pathway may play in myonuclear apoptosis in skeletal muscle. The lack of direct evidence is likely due, in part, to the lack of effective antibodies for use in rodent skeletal muscle given that ASK-1 gene expression is well characterized in skeletal muscle (13). ASK-1 protein levels do not appear to be present in high concentrations in skeletal muscle, making elucidating the role that ASK-1 may play in the oxidative stress apoptotic continuum

hard to clarify. Although direct manipulations of the signaling that occurs in skeletal muscle in response to oxidative stress is difficult to ascertain, taken as a whole the literature shows evidence that a causal relationship between an oxidant mediated stressor and the initiation of both the extrinsic (inflammatory mediated) and intrinsic (mitochondrial mediated) apoptotic pathways does exist.

1.8 Countermeasures to oxidative stress:

Gaining clearer insight into the molecular signaling pathways involved in aging and disuse-mediated muscle atrophy is paramount in developing nutritional and/or pharmacological interventions to minimize protein loss and attenuate the functional decrements associated with atrophic conditions. To date, numerous studies have been undertaken to evaluate the efficacy of various compounds, including antioxidants (vitamin E & C) (9; 124; 133), enzyme antagonists (allopurinol) (85) and other signaling modulators (NFk-B inhibitors) (59), in improving physiological and functional outcomes following atrophic stimuli. The results of these interventional studies vary from showing no beneficial effects of supplementation interventions (37) , to showing promising protective effects with regard to muscle mass (9; 124; 149) and function (9; 85). Although the potential seems clear for antioxidant and other signal modifying compounds to provide protection against oxidative stress by acting as free radical scavengers, antioxidant enzyme enhancers, or promoters of mitochondrial biogenesis, there are many variables to consider. The bioavailability of a compound, the chosen

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route of administration and its metabolism within an animal (82), may alter their efficacy as anti-atrophic countermeasures, potentially explaining the lack of congruency in the literature (51).

If in fact one believes that mitochondria are the primary source of ROS within a cell and that their integrity and content are directly related to the aging process (45; 49), then an ideal countermeasure to both aging and disuse-associated oxidative stress and subsequent muscle atrophy would be a compound with direct free radical scavenging capabilities, the capacity to upregulate the endogenous antioxidant defense system and directly improve mitochondrial content and function, leading to a reduction in pro-oxidant production. Fortunately, it seems such a compound exists; resveratrol.

1.9 Resveratrol, an antioxidant and activator of Sirtuin 1:

Resveratrol (3,5,4,trihydroxystilbene) is a naturally occurring polyphenol found in over seventy plant species, including grapes, peanuts and mulberries. It belongs to the stilbene family of phytoalexins, which are anti-microbial compounds produced in plants in response to bacterial, or fungal infections (15). Resveratrol has gained popularity over the past decade due to its potent anti-oxidant and anti-aging properties (83; 96; 109). Recent studies have shown resveratrol to improve the outcomes of pathological conditions ranging from cancer (16; 95; 112; 138; 149), chronic inflammation (34; 105; 111) and neurodegenerative diseases (111). Specifically, resveratrol has been shown to mediate cardiomyocyte survival following simulated hypoxia/reperfusion by up-regulating both the antioxidant thioredoxin and the anti-apoptotic protein Bcl-2 (27).

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Resveratrol also stimulated glutathione synthesis in alveolar epithelial cells exposed to cigarette smoke extract (68; 95). Additionally, several recent studies demonstrated the ability of resveratrol to specifically induce the transcription of two key antioxidant enzymes, catalase (68; 73; 95; 117) and MnSOD (68; 95; 109; 110; 117), both *in vitro* and *in vivo.*

With regard to skeletal muscle, resveratrol administration has effectively attenuated protein degradation in murine myotubes treated with proteolysis-inducing factor and attenuated muscle atrophy, *in vivo*, in a mouse model of cachexia (149). Furthermore, acute dietary resveratrol supplementation attenuated lipid peroxidation and improved the redox status, (quantified by the reduced to oxidized glutathione ratio), in aged murine skeletal muscle exposed to a repetitive loading protocol (117). Likewise, acute resveratrol administration was able to attenuate lipid peroxidation and reduce H_2O_2 concentrations in skeletal muscles from aged animals undergoing extended unloading (54). In an *in vitro* model of anti-viral therapy, the addition of resveratrol to primary myoblasts isolated from human quadriceps muscles which were treated with protease inhibitors, the hallmark treatment of those individuals infected with the Human Immunodeficiency Virus (HIV), resveratrol completely ablated the increase in ROS that is known to occur following protease inhibitor treatments. Moreover, supplementation with a combination of a low dose of resveratrol and coenzyme Q improved both the inflammatory status and morphological profile in quadriceps muscles from dystrophic mice (98). The protective effect of resveratrol with regard to the aforementioned parameters is significant because muscle regeneration is the one of the key deficiencies

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with muscular dystrophies and therefore it is possible that resveratrol may enhance the capacity of muscle to regenerate under pathological conditions.

Despite the fact that resveratrol has the biochemical capacity to directly scavenge free radicals due to its phenol ring structure and in fact, resveratrol has repeatedly been shown to be a potent scavenger of ROS in numerous cell types exposed to pro-oxidants (16; 61; 83; 110). However, its rapid first pass metabolism in mammals and thus relatively low bioavailability (28; 40; 82), may limit its role as a direct ROS scavenger *in vivo*. Consequently, *in vivo*, the various health benefits of resveratrol can more likely be attributed to its ability to activate the NAD dependent deacetylase, silent mating type information regulation homolog1, or sirtuin1 (Sirt1) (4; 74).

Sirt1 has been shown to play a role in a variety of important physiological functions including gluconeogenesis, energy expenditure, lipid oxidation, mitochondrial biogenesis and the regulation of oxidative stress and survival across a wide array of cell types (67; 74; 93; 144; 150). Sirt1 effectively protects certain mammalian cells from apoptosis by deaceytylating and thus inactivating p53 (41), furthermore, Sirt1 specifically protects against Bax-induced apoptosis through activation of the Bax transcriptional inhibitor Ku70 (7). Additionally*,* Sirt1 upregulates the transcription of both MnSOD and catalase (41), further showcasing its potential as a protective mechanism against oxidative stress. Sirt1 is also activated with exercise (134) and under conditions of nutrient deprivation (19; 145), underscoring its effectiveness as a caloric restriction mimetic. This is significant because both exercise (127; 131) and caloric restriction (12; 19; 75) are proven countermeasures to combat both oxidative stress and myonuclear apoptosis. Therefore, we would expect resveratrols' ability to activate Sirt1, to effectively

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reduce muscle loss during both disuse and aging. In a recent study, resveratrol stimulated muscle precursor cell proliferation in a Sirt1 dependent manner (106), further expanding the mechanisms by which resveratrol supplementation may improve muscle mass and function by enhancing muscles regenerative capacity, which has been shown to be augmented with aging (24; 25) and in certain pathological conditions. Furthermore, and perhaps most importantly, Sirt1 is a powerful regulator of PPARγ co-activator (PGC-1α) (135), which is considered to be the master regulator of mitochondrial function (78). PGC-1α is decreased during atrophic conditions (119; 120), but muscle specific overexpression of PGC-1α confers protection against both denervation and fasting-induced skeletal muscle atrophy (120). Therefore, the ability of Sirt1 to promote mitochondrial biogenesis, including structural components of the electron transport chain, ATPases, oxidative enzymes and the induction of MnSOD, provides compelling evidence with regard to resveratrols' potential ability to protect skeletal muscle from oxidative stress-induced damage and thus myonuclear apoptosis (see figure 1.3).
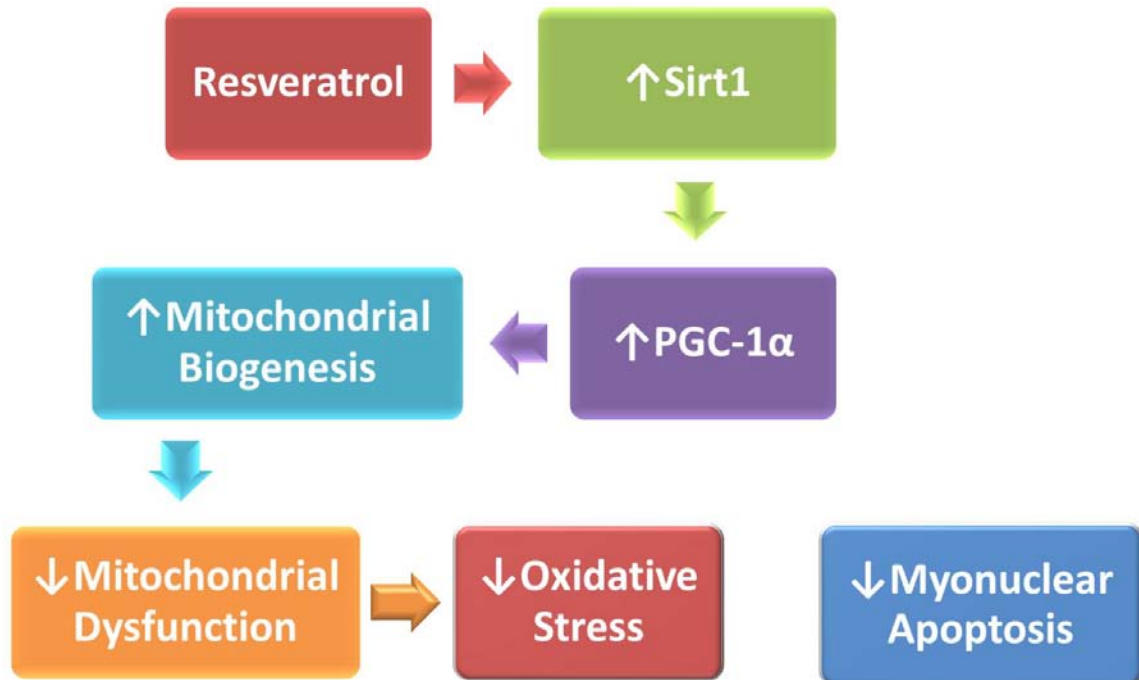
Figure 1.3- Potential mechanism(s) of resveratrol mediated protection.

1.10 Conclusion:

As of 2008 the United States Department of Health and Human Services projects that the number of individuals over the age of 65 will double in the next 20 years, making sarcopenia a debilitating and costly reality for an ever increasing percentage of our population. A relationship between increases in oxidative stress and decreases in muscle mass and function clearly exists, thus it is increasingly imperative that effective therapies are found to alleviate oxidative stress and thus improve the quantity and quality of skeletal muscle in elderly individuals. Overall, the reliance of skeletal muscle viability on proper muscle function skeletal muscle viability highlights the importance of

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promoting mitochondrial biogenesis and conserving mitochondrial membrane integrity as important countermeasures to reduce skeletal muscle atrophy associated with disuse and aging. Sirt1 activation is positively associated with an increased resistance to oxidative stress and apoptosis, likely due to its capacity to promote mitochondrial biogenesis. Resveratrol is a potent activator of Sirt1 and thus shows promise as an effective therapy to lessen mitochondrial-induced oxidative stress and apoptosis. There has been limited animal research conducted regarding the protective effects of resveratrol supplementation on skeletal muscle. Given the inherent differences between cell culture models and whole animals models, it is important to elucidate the specific signaling pathways of resveratrol, *in vivo*, to further our understanding of its' capacity to protect against skeletal muscle atrophy.

The major goals of this research are to further understand the interplay between oxidative stress and skeletal muscle atrophy and to evaluate the efficacy of resveratrol as a countermeasure to both mitochondrial-induced oxidative stress and apoptosis. It is hypothesized that with skeletal muscle disuse and advanced age there will be an increase in both oxidative stress and a concomitant decrease in muscle mass and function. Resveratrols' capacity to upregulate the endogenous anti-oxidant defense system, increase mitochondrial biogenesis and thus enhance cell viability, makes it an ideal candidate to combat oxidative stress and sarcopenia (see Figure 1.4). Thus it is hypothesized that resveratrol will improve muscle mass and function with both disuse and aging-induced muscle atrophy. It is expected that the results of the current study will help to further our understanding of the usefulness of dietary supplements as effective countermeasures to alleviate the mechanism(s) underlying sarcopenia.

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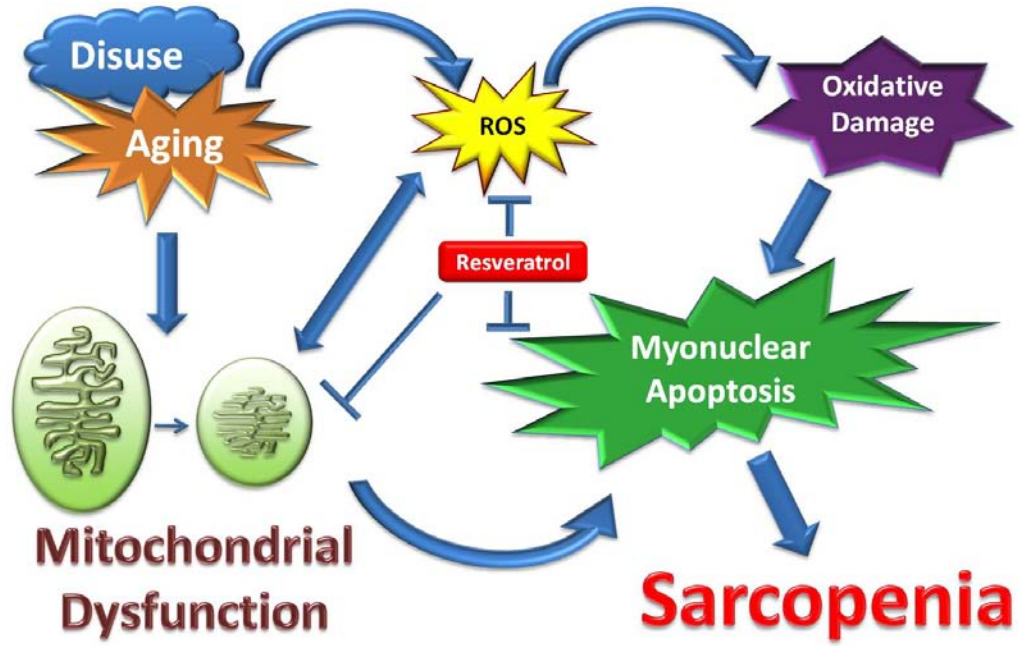
Figure 1.4- Working model of resveratrol mediated protection.

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Chapter 2:

Mediation of Endogenous Antioxidant Enzymes and Apoptotic Signaling by Resveratrol Following Muscle Disuse in the Gastrocnemius Muscles of Young and Old Rats

Janna R. Jackson[1], Michael J. Ryan[1], Yanlei Hao[1], and Stephen E. Alway[1]

[1]Laboratory of Muscle Biology and Sarcopenia, Division of Exercise Physiology, West Virginia University School of Medicine, Morgantown, West Virginia 26506

Send correspondence and reprint requests to:

Stephen E. Alway, Ph.D., Division of Exercise Physiology, School of Medicine,

Robert C. Byrd Health Sciences Center, West Virginia University,

Morgantown WV 26506-9227, USA

Phone: 304-293-0772

Fax: 304-293-7105

Email: salway@hsc.wvu.edu

Running Head: Resveratrol administration and skeletal muscle disuse

ABSTRACT

Hindlimb suspension (HLS) elicits muscle atrophy, oxidative stress, and apoptosis in skeletal muscle. Increases in oxidative stress can have detrimental effects on muscle mass, function and can potentially lead to myonuclear apoptosis. Resveratrol is a naturally occurring polyphenol possessing both anti-oxidant and anti-aging properties. To analyze the capacity of resveratrol to attenuate oxidative stress, apoptosis and muscle force loss were measured following 14 days of HLS. Young (6 mo) and old (34 mo) rats were administered either 12.5mg/kg/day of trans-resveratrol, or 0.1% carboxymethylcellulose for 21days, including 14 days of HLS.   HLS induced a significant decrease in plantarflexor isometric force, but resveratrol blunted this loss in old animals. Resveratrol increased gastrocnemius catalase activity, MnSOD activity, and MnSOD protein content following HLS. Resveratrol reduced hydrogen peroxide and lipid peroxidation levels in muscles from old animals after HLS. Caspase 9 abundance was reduced and Bcl-2 was increased, but other apoptotic markers were not affected by resveratrol in the gastrocnemius muscle after HLS. The data indicate that resveratrol has a protective effect against oxidative stress and muscle force loss in old HLS animals; however, resveratrol was unable to attenuate apoptosis following HLS. These results suggest that resveratrol has the potential to be an effective therapeutic agent to treat muscle functional decrements via improving the redox status associated with disuse.

Introduction

Both advanced age and skeletal muscle disuse are associated with atrophy and an increased production of reactive oxygen species (ROS) in skeletal muscle (27), leading to an augmented oxidant-load. Oxidative stress occurs when an increase in oxidant production exceeds an organisms' capacity to buffer them, via a complex coordination of the endogenous antioxidant defense system. During extended periods of oxidative stress there is an eventual loss of cellular integrity mediated by the oxidation of lipids (29), proteins (26) and nucleic acids (18), promoting a cycle of increased oxidant production. This results in elevated levels of oxidative damage, which limits both the cellular repair system and the enzymatic antioxidant defense system (14). The exact mechanisms by which oxidative stress acts as a potentiator of muscle atrophy are largely unknown, however, several links between oxidative stress and atrophy have been postulated (32).

Oxidative stress is upstream of apoptotic signaling in muscle cells *in vitro* (46), and results in the initiation of the intrinsic mitochondrial apoptotic pathway. Of particular interest in the current study was to determine if redox sensitive apoptotic signaling (49) could be suppressed by resveratrol administration during experimentally-induced muscle disuse. This is an important area of inquiry, because myonuclei undergo apoptosis during muscle disuse (42,44), and this is thought to contribute to fiber atrophy, especially in aging muscles (2,3). Likewise, muscle disuse is associated with increases in oxidative stress (1,27), presumably mediated through mitochondrial dysfunction via ROS production and the regulation of the apoptotic pathway (17,44).

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Antioxidant supplementation has been shown to be an effective counter measure to combat oxidative stress in a wide variety of tissue types and conditions (7,33), and it is speculated that this might be an approach to reduce muscle wasting associated with both disuse (40) and aging (7). Resveratrol (3,5,4,trihydroxystilbene) is a naturally occurring polyphenol found in over seventy plant species, including grapes, peanuts and mulberries (5). Resveratrol has gained popularity over the past decade due to its potent anti-oxidant and anti-aging properties (24,36). Recent studies have shown resveratrol to have positive effects on the outcomes of pathological conditions ranging from cancer (6) chronic inflammation (19) and neurodegenerative diseases (37). Specifically, resveratrol has been shown to mediate cardiomyocyte survival following simulated hypoxia/reperfusion by up regulating both the antioxidant thioredoxin and the anti-apoptotic protein Bcl-2 (4,10), thus underscoring its potential to act as both an antioxidant and anti-apoptotic compound. Likewise in a recent study in PC12 cells exposed to hydroxynonenal , an oxidizing byproduct of lipid peroxidation, pretreatment with resveratrol decreased the amount of the pro-apoptotic Bax protein, decreased caspase 3 activity and increased the amount of anti-apoptotic Bcl-2 protein, conferring complete protection against oxidative stress and apoptotic signaling (41). Additionally, recent data suggest that resveratrol induces the transcription of two key antioxidant enzymes, catalase (9,21) and MnSOD (36,39). Furthermore, a recent study in our laboratory found acute dietary supplementation with resveratrol to effectively reduce indices of oxidative stress in repetitively loaded skeletal muscles, presumably through the upregulation of antioxidant enzymes including MnSOD, an important mitochondrial antioxidant in muscles from both young and old animals (39). The efficacy of acute

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resveratrol administration has been established for several pathological conditions; however, the use of chronic resveratrol administration as a counter-measure to prevent muscle loss caused by disuse has not been established.

Therefore, the primary aim of this study was to evaluate the efficacy of a daily moderate dose of resveratrol to ameliorate oxidative stress and subsequent myonuclear apoptosis induced by skeletal muscle disuse in both young and old animals. In the present investigation, it was hypothesized that resveratrol would reduce the indices of hindlimb suspension (HLS)-induced oxidative stress in skeletal muscle and thus lessen the potential for downstream apoptotic signaling and subsequent muscle atrophy stemming from myonuclear loss. Furthermore, it was hypothesized that resveratrol administration would preserve muscle function following HLS both by preservation of muscle mass during the HLS protocol and by providing a more favorable redox environment.

METHODS

Animals. All experiments were conducted on young adult (6mo) and old (34mo) Fischer Brown Norway x 344 male rats. The animals were obtained from the NIA colony house at Harlan (Indianapolis, IN) and kept in pathogen-free conditions at ~20°C, on a reversed twelve-hour light cycle. 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."

Hindlimb Suspension. The hindlimb suspension technique employed in the current study is a modification of the technique originally described by Morey-Holton and Globus (25). This method allows for an even load distribution on the tail, permits the animals with 360° of movement around the cage, and assures that the forelimbs maintain contact with a grid floor, allowing the animals to move and access food and water freely as previously described (44,45). Briefly, orthopedic tape was applied along the proximal one-third of the tail then placed through a wire harness that is attached to a swivel placed at the top of a specially designed hindlimb suspension cage. Sterile gauze was then wrapped around the orthopedic tape and was subsequently covered with a thermoplastic material, which formed a hardened cast (Vet-Lite; Veterinary Specialty Products, Boca Raton, FL). The distal tip of the tail was examined daily to verify that the procedure did not occlude blood flow to the tail. The suspension height was

adjusted to prevent the animal's hindlimbs from touching any supportive surface, with care taken to maintain a suspension angle that did not exceed 30°. Weight bearing control animals were allowed to ambulate freely in their cages.

Experimental Protocol. Prior to commencement of the experimental protocol, all animals went through a 7-day acclimation period, which included the daily oral gavage of 1mL of distilled H_2O. Animals were hindlimb suspended (HLS) for 14 days. Seven days preceding suspension and continuing throughout the suspension protocol, all animals received either 1mL of 0.1% Carboxymethylcellulose dissolved in deionized water, or 1mL of trans-resveratrol suspended in 1mL of 0.1% Carboxymethylcellulose. Resveratrol was purchased from Orchid Pharmaceuticals (India). The resulting solutions were administered via oral gavage at a dose of 12.5mg/kg/day, for a total of 21 days. This dosage was chosen to provide a low to moderate daily dose of resveratrol that would have the potential to be therapeutic, but would not be high enough to be pro-apoptotic as can be seen with higher doses of resveratrol (13). Age-matched, non-suspended animals served as weight-bearing controls (Figure 1).

Refer to Figure 1

Force Analysis. Pre and post-HLS force measurements were assessed in anesthetized animals using a custom built rat dynamometer (8,38). The rat was placed supine on a heated X–Y positioning table of the rodent dynamometer, and anesthesia was induced with 5% isoflurane. Anesthesia was maintained in the animals over the duration of the

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experiment with 2% isoflurane. The left foot was secured to the footplate at an ankle angle of 90° and the knee was braced to ensure that forces were transmitted to the footplate. Vertical forces applied to the aluminum sleeve fitted over the dorsum of the foot were translated to a load cell transducer in the load cell fixture on the footplate. Platinum stimulating electrodes (Grass Medical Instruments, Quincy, MA) were inserted subcutaneously to span the tibial nerve in the popliteal fossa. The maximal isometric force of the plantar flexor muscle group was evaluated by stimulating the tibial nerve using supramaximal square wave pulses at 100Hz for a duration of 1000 ms using a SD9 stimulator (Grass Medical Instruments, Quincy, MA). The voltage used in these experiments was 10% greater than the voltage required to obtain maximal force production. Maximal force was determined off-line via custom Labview based software (8). The maximal forces for three isometric contractions were averaged for each data point. These *in vivo* force records were obtained before and after HLS. The percent in force loss with HLS was obtained by comparing the average pre-HLS for record with the post-HLS force record.

Muscle preparation. Immediately following the last contraction, the gastrocnemius muscle was dissected from both limbs muscle, weighed and snap frozen. To eliminate the possibility that the isometric muscle function tests could acutely affect measurements of oxidative stress, the gastrocnemius muscle from the limb that was not evaluated for muscle function (the right limb) was used for analysis of oxidative enzyme activities, oxidative stress, and apoptotic indices. The animals were then euthanized by removing the heart.

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Protein Isolation. Seventy-five micrograms of gastrocnemius muscle from each animal was homogenized in RIPA buffer (1% Triton x-100, 150mM NaCl, 5mMEDTA, 10mM Tris; pH: 7.4) for assessment of protein expression, caspase activity and cell death as measures by an ELISA. For all oxidative enzyme activity assays and/or redox status assessments muscle samples were homogenized in either PBS, or the kit specific buffer provide by the manufacturer. Muscle samples were homogenized in 500 µL of the appropriate ice-cold lysis buffer using a mechanical homogenizer. Muscle homogenates were centrifuged at 1000 x *g* for 5 minutes at 4°C. The resulting supernatant was collected and divided into two portions and frozen at –80°C either with, or without a protease inhibitor cocktail containing 104 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride hydrochloride, 0.8 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St. Louis, MO) added to it. Protein concentrations for each sample were determined in duplicate using the DC Protein Assay kit (Bio-Rad, Hercules, CA).

Catalase Activity. The activity of catalase was determined in gastrocnemius muscle homogenates using a commercially available kit (# 707002 Cayman Chemical Company, Ann Arbor, MI) as described previously in our laboratory (39). 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 µg of protein per µL of muscle homogenate.

Manganese Superoxide Dismutase (MnSOD) and Copper-Zinc Superoxide Dismutase (CuZnSOD) Activity Levels. Superoxide dismutase activity was measured in gastrocnemius muscle homogenate using a colorimetric enzyme activity kit (Cayman Chemical Company Ann Arbor, MI) following the manufacturer's guidelines. Both total SOD activity and MnSOD activity were obtained. CuZnSOD activity was determined by subtracting MnSOD activity from total SOD activity. The assay was performed with slight modifications to the manufacturer's directions. All analyses were measured in duplicate and the samples were normalized to µg of protein per µL of muscle homogenate as described previously by our laboratory (14). Briefly, muscle samples were homogenized in 20mM HEPES buffer, containing 1mM EGTA, 210mM mannitol, and 70 mM sucrose, and centrifuged at 1000 x g for 10 min. 50 µl of the resulting 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).

Immunoblots. The protein content of the oxidative enzymes Catalase, CuZnSOD, and MnSOD and the apoptotic markers Bax and Bcl-2 were assessed in gastrocnemius muscle homogenates. Either β-tubulin or GAPDH was used as loading controls. Although most blots were probed with β-tubulin, GAPDH was used as a loading control for blots in which we probed for Bax, because we had intended on also measuring apoptosis inducing factor (AIF) on the same blot, because AIF and Bax run at different molecular weights. However, AIF runs at a similar molecular weight as β-tubulin (data not shown) and therefore, we could not use β-tubulin as the loading control for these

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blots.   Thirty to forty micrograms of protein were loaded into each well of a 4%–12% gradient polyacrylamide gel (Invitrogen, Carlsbad, CA) and separated by routine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for 1.5hrs at 20°C, followed by transfer to a nitrocellulose membrane for 70min at 35V. All membranes were blocked in 5% nonfat milk (NFM) for 1 hour at room temperature. The membranes were then incubated overnight at 4°C in the appropriate primary antibodies. Primary antibodies were diluted in Tris-buffered saline, with 0.1% Tween-20 (TBST) and 10% sodium azide. Membranes were then washed in 0.05% TBST followed by incubation in the appropriate dilutions of secondary antibodies (diluted in 5% NFM in TBST) conjugated to horseradish peroxidase. The signals were developed using a chemiluminescent substrate (Lumigen TMA-6; Lumigen, Southfield, MI) and visualized by exposing the membranes to x-ray films (BioMax MS-1; Eastman Kodak). Digital records were captured by a Kodak 290 camera, and protein bands were quantified using 1D analysis software (Eastman Kodak). Bands were quantified as optical density x band area and expressed in arbitrary units relative to the loading control bands.

Hydrogen Peroxide (H_2O_2) Content. H_2O_2 content in gastrocnemius muscle homogenate was measured using a fluorescent assay according to the manufacturer's recommendations (Cell Technology, Mountain View, CA). Briefly, 50 μL of control, unknown muscle samples, or H_2O_2 standards were mixed with 50 μL of the reaction cocktail provided in the kit and added to each well to initiate the reaction. The plate was then incubated in the dark for 10 minutes at room temperature. The intensity of the fluorescent signal was detected using an excitation wavelength of 530 nm and an

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emission wavelength of 590 nm. A linear regression was performed by plotting the resultant florescent intensities from the known standards and subsequently the unknown samples were fit to the corresponding linear equation. All analyses were measured in duplicate and the samples were normalized to µg of protein per µL of muscle homogenate as determined by the DC Protein Assay Kit (Bio-Rad Hercules, CA).

Lipid Peroxidation. Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were assessed with reagents from Oxis International (BIOXYTECH LPO-586). Approximately 100 mg of gastrocnemius muscle was homogenized in 750 µL of ice cold buffer containing phosphate-buffered saline (20 mM, pH 7.4) and 5 µL of 0.5 M butylated hydroxytoluene in acetonitrile per 1 mL of tissue homogenate. Assay reagents were added following the manufacturer's recommendations. Briefly, the muscle homogenate was centrifuged at 3000 x g at 4°C for 10 minutes, and subsequently the supernatant was used to assess lipid peroxidation. The sample was incubated in the proper reagents at 45°C for 60 minutes, and then centrifuged at 15,000 x g for 10 minutes. The absorbance of the resulting supernatant was read at 586 nm. All analyses were measured in duplicate and the samples were normalized to µg of protein per µL of muscle homogenate as determined by the DC Protein Assay Kit (Bio-Rad Hercules, CA).

Caspase Activity. The proteolytic activity of caspase 9 and 3 were assessed with a fluorometric activity assay using the commercially available substrates; AC VDVAD-AFC and AC-DEVD-AFC (Alexis Biochemical, San Diego, CA) respectively. Briefly,

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50µL of caspase activity buffer (50 mM PIPES, 0.1 mM EDTA, 10% glycerol, 1 mM DTT) was added to each well of a 96-well fluorescent microplate (Thermo Fisher Scientific). Subsequently, 50µL of gastrocnemius muscle homogenate and 10µL of the appropriate caspase substrate (1mM) were added to each well. Caspase activity was determined by measuring fluorescent intensity using a fluorescent microplate reader at an excitation of 400nm and an emission of 505nm. The microplate was incubated at 37°C for 2-hours with caspase activity determined by subtracting the OD readings at time 2-hour from the initial reading, to eliminate any background fluorescence that was not mediated by caspase activity. All analyses were measured in duplicate and the samples were normalized to µg of protein per µL of muscle homogenate as determined by the DC Protein Assay Kit (Bio-Rad Hercules, CA).

Cell-Death ELISA Assay. A commercially available ELISA kit (Cell Death Detection ELISA, Roche Diagnostics, Indianapolis, IN) was used to quantify DNA fragmentation in gastrocnemius muscle homogenate. Briefly, the wells of a 96-well plate were coated with a primary anti-histone mouse monoclonal antibody. Following the addition of 100 µL of muscle homogenate, a secondary anti-DNA mouse monoclonal antibody coupled to peroxidase was added to each well. The substrate, 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) (ABST) was used to photometrically determine the amount of peroxidase retained in the immunocomplex. The colorimetric change of each well was determined at a wavelength of 405nm using a Dynex MRX plate reader and computer software (Revelation, Dynatech Laboratories, CA). All analyses were measured in duplicate and the samples were normalized to µg of protein per µL of

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muscle homogenate as determined by the DC Protein Assay Kit (Bio-Rad Hercules, CA). The resulting OD was recorded as the apoptotic index (OD_{405}/ mg protein). This assay measures DNA fragmentation in myonuclei, satellite cells, and non-muscle cell nuclei. However, because DNA fragmentation as determined by this ELISA is directly proportional to TUNEL identification of apoptotic muscle nuclei in old rat muscle after hindlimb suspension (30), the data obtained in the ELISA assay are a reasonable indicator of the apoptotic environment in skeletal muscle of the experimental animals.

Statistics. Statistical analyses were performed using the SPSS 13.0 software package (SPSS, Chicago, IL). A multiple analysis of variance was used to examine differences between age, suspension and supplementation. Tukey's post-hoc analyses were performed to protect against type 1 errors. Where appropriate, student t-tests were implemented to evaluate paired comparisons. Statistical significance was accepted at $p \leq 0.05$. Data are reported as mean ± standard error of the mean.

RESULTS

Body Weight. Resveratrol was unable to suppress the body weight loss associated with HLS. All animals were weighed prior to the experimental protocol and immediately before euthanasia (Table 1). Older animals were significantly heavier than young animals (460 ± 38g vs. 548 ± 39g; $p \leq 0.05$). Young vehicle control suspended (YVCS) animals lost ~15% of their body weight during the 14-day suspension protocol, while old vehicle control suspended (OVCS) animals lost ~ 13.5% of their body weight during the 14-day suspension protocol. There was no significant effect of resveratrol administration on the body weight of young vehicle control (YVC), YVCS, old vehicle control (OVC) or old OVCS animals. There was no significant difference between the percent of total body weight lost during the 21-day experimental protocol when comparing groups of HLS animals (Table 1).

Refer to Table 1

CuZnSOD Enzyme Activity and Protein Content. Both aging and HLS significantly increased the activity of CuZnSOD in gastrocnemius muscles, but this was not affected by resveratrol. CuZnSOD activity was significantly increased in gastrocnemius muscles from old animals compared to gastrocnemius muscles from young animals (0.083 ± .007 vs. 0.055 ± 0.002 U/ml/mg; $p \leq 0.05$), resveratrol administration did not alter CuZnSOD further with aging (Figure 2A). HLS increased CuZnSOD activity in gastrocnemius muscles from both young and old animals regardless of resveratrol administration. There was no change in CuZnSOD protein content in the gastrocnemius

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muscle from any treatment, or age group (Figure 2B). Resveratrol increased CuZnSOD

activity (Figure 2A) but not CuZnSOD content (Figure 2B) in control muscles from old

animals.

<u>Refer to Figure 2</u>

MnSOD Enzyme Activity and Protein Content. MnSOD activity was increased by

aging and resveratrol in control muscles of old animals. While HLS decreased MnSOD

activity, MnSOD activity in resveratrol-treated muscles after HLS was elevated over the

control muscles of old rats. Relative to young animals, MnSOD activity was 78% greater

($p ≤ 0.05$) in vehicle control old animals and resveratrol administration further increased

MnSOD activity in old non-suspended animals by an additional ~20% ($p ≤ 0.05$),

(Figure 2C). HLS had no effect on MnSOD activity in young animals; however, HLS

significantly reduced MnSOD activity in old vehicle control animals by approximately

23% (20.3 ± 1.7 vs. 14.6 ± 0.98 U/ml/mg, $p ≤ 0.05$), resveratrol administration was able

to completely abolish the decrease in activity seen with HLS. There was no aging effect

on MnSOD protein content in non-suspended animals, however, similar to activity

levels, MnSOD protein content was significantly reduced in old suspended animals

(Figure 2D). Furthermore, resveratrol administration mitigated the effects of HLS on

MnSOD protein content.

Catalase Activity and Protein Content. Resveratrol increased catalase activity in

control muscles of old rats. Catalase activity significantly increased with aging in

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gastrocnemius muscles from both vehicle control animals and animals that were administered resveratrol ($p \leq 0.05$) as compared to young adult animals (Figure 2E). Furthermore, gastrocnemius muscles from old non-HLS animals who received resveratrol had higher catalase activity than old non-HLS vehicle controls (6.14 ± 0.61 vs. 8.56 ± 0.81 mU activity /mg protein, $p \leq 0.05$). HLS had no significant effect on catalase activity in either age group, and resveratrol administration showed no further increase in catalase activity following HLS. Catalase protein content increased with HLS in both young and old animals (Figure 2F). Resveratrol further augmented catalase protein abundance in young HLS animals and old non-suspended animals.

Hydrogen Peroxide (H_2O_2) Concentrations. H_2O_2 was assessed in gastrocnemius muscle homogenates as an indicator of oxidant load during aging and HLS. Aging and HLS increased H_2O_2 levels in gastrocnemius muscles of old rats, but resveratrol suppressed this increase in H_2O_2 content. H_2O_2 levels were ~3 fold higher ($p \leq 0.05$) in gastrocnemius muscles from old animals when compared to gastrocnemius muscles from young animals (Figure 3A). There was a significant interaction of age and suspension, ($p \leq 0.05$). HLS had no significant effect on H_2O_2 content in young gastrocnemius muscles, regardless of resveratrol administration. Resveratrol administration significantly ($p \leq 0.05$) reduced H_2O_2 levels in gastrocnemius muscles from old control animals and old-HLS animals by 23% and 16% respectively, when compared to gastrocnemius muscles from old vehicle control and old vehicle control-HLS animals, ($p \leq 0.05$). There was still a significant increase in H_2O_2 content following HLS in gastrocnemius muscles from both vehicle control (2.29 ± 0.06 vs. 2.55 ± 0.05

µmols H_2O_2 /µg protein; $p \leq 0.05$) and resveratrol administered animals (1.77 ± 0.16 vs. 2.14 ± 0.06µmols H_2O_2 /µg protein; $p \leq 0.05$), however there was no difference in the H_2O_2 content between gastrocnemius muscles from old vehicle control and old resveratrol-HLS animals (2.29 ± 0.06 vs. 2.14 ± 0.05µmols H_2O_2 /µg protein).

Refer to Figure 3

Lipid Peroxidation. Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were assessed in whole muscle homogenates as indicators of oxidative damage, specifically as markers of muscle lipid peroxidation. Both aging and HLS increased lipid peroxidation in the gastrocnemius muscle, but this was suppressed by resveratrol. HLS significantly increased lipid peroxidation by ~55 % ($p \leq 0.05$) in young animals regardless of resveratrol administration (Figure 3B). Lipid peroxidation was increased in old animals compare to their young counterparts (0.171 ± .02 vs. 0.079 ± 0.01 µM [MDA/HAE]/mg protein; $p \leq 0.05$). There was no further significant increase in MDA and HAE levels in old animals that underwent HLS; however, gastrocnemius muscles from old animals that were administered resveratrol had significantly reduced levels of lipid peroxidation compare to old vehicle control suspended animals (0.199 ± 0.02 vs. 0.159 ± 0.01 µM [MDA/HAE]/mg protein; $p \leq 0.05$), this represented a 20% decrease in MDA and HAE levels in old resveratrol suspended animals.

Bax and Bcl-2 Protein Contents. Aging was generally associated with increased pro-apoptotic protein signaling in the gastrocnemius muscle. The pro-apoptotic Bax protein levels in gastrocnemius muscles from old animals increased ~1.8 fold ($p \leq 0.05$), regardless of resveratrol administration (Figure 4A). There was no further effect of HLS, or resveratrol administration, in either age group. Similarly, Bcl-2 protein levels increased 2.6 fold ($p \leq 0.05$) in gastrocnemius muscles from old vehicle control animals when compared to their young counterparts (Figure 2B). Resveratrol administration further increased Bcl-2 protein content in gastrocnemius muscles from old resveratrol non-HLS animals by ~20% ($p \leq 0.05$), when compared to gastrocnemius muscles from non-HLS vehicle control animals. HLS significantly increased Bcl-2 protein content in gastrocnemius muscles from old vehicle control-HLS animals by ~18.6% ($p \leq 0.05$), when compared to gastrocnemius muscles from non-HLS vehicle control rats. There was no effect of HLS on Bcl-2 content in gastrocnemius muscles from old animals. HLS caused significant increases in Bcl-2 protein content in gastrocnemius muscles from both young vehicle control (36%, $p \leq 0.05$) and young resveratrol administered (29%, $p \leq 0.05$) animals. There was no effect of resveratrol administration in gastrocnemius muscles from young animals.

Refer to Figure 4

Caspase 9 and Caspase 3 Activities. Caspase 9 and 3 activities were measured via fluorometric assays as an assessment of the contribution of mitochondrial mediated caspase dependent apoptotic signaling. Resveratrol blunted only the aging and HLS-

induced increase in caspase-9 activity in muscles of old rats. Both caspase 9 (Figure 4C) and caspase 3 (Figure 4D) activities were increased in gastrocnemius muscles from old animals, when compared to their young counterparts. Neither caspase 9 nor caspase 3 were altered by HLS, or resveratrol administration, in the muscles of young animals. There was no effect of resveratrol administration on caspase 9 activity in old-HLS animals, however, resveratrol administration significantly reduced caspase 9 activity in gastrocnemius muscles from old non-HLS animals ($p \leq 0.05$), eliminating the aging effect (Figure 4C). HLS significantly ($p \leq 0.05$) increased caspase 3 activity, in gastrocnemius muscles from vehicle control-HLS animals; this increase was significantly ($p \leq 0.05$) attenuated by resveratrol administration.

DNA fragmentation. A cell death ELISA was used as an indicator of DNA fragmentation and the data are presented as an apoptotic index. The apoptotic index was increased by aging and HLS in rat gastrocnemius muscles, but resveratrol failed to attenuate this increase. Old animals exhibited a 200% increase ($p \leq 0.05$) in DNA fragmentation regardless of resveratrol administration (Figure 4E). Similarly, gastrocnemius muscles from both young and old HLS animals treated with, or without, resveratrol, has significantly increased levels of fragmented DNA indicating greater levels of apoptosis. HLS increased the apoptotic index by ~5 fold ($p \leq 0.05$) in young animals and ~3 fold ($p \leq 0.05$) in old animals respectively. Resveratrol had no effect on DNA fragmentation in any treatment group (Figure 4E).

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Gastrocnemius Muscle Wet Weight. Resveratrol attenuated the HLS-induced atrophy of old rats. Gastrocnemius muscles from young animals were significantly heavier than their older counterparts (2.28 ± 0.15g vs.1.55 ± 0.06g; $p \leq 0.05$) (Figure 5A). HLS elicited significant atrophy in the gastrocnemius muscles from young and old rats. Gastrocnemius muscles from young HLS animals weighed on average 28.3% ($p \leq 0.05$) less than gastrocnemius muscles from young ambulatory control animals. Similarly, gastrocnemius muscles from old-HLS animals weighed 26.1% ($p \leq 0.05$) less than gastrocnemius from old non-HLS animals. Resveratrol administration had no effect on the muscle wet weight of gastrocnemius muscles from young animals following HLS (1.62 ± 0.12g vs.1.65 ± 0.11g) (Figure 5A). Gastrocnemius muscle wet weight from old vehicle control suspended animals (1.09 ±.09g) was 14.2% less than muscle wet weight in old resveratrol-treated suspended animals (1.28 ± 0.07g). Although, this difference did not quite reach statistical significance ($p = 0.062$), it reflects a potential for resveratrol to protect against muscle loss in old animals. There were losses of both body weight and muscle weight with HLS in old animals. To determine if resveratrol provided a muscle specific but not a body weight specific effect, muscle weight was normalized to the animal's body weight. When muscle wet weight was normalized to body weight, resveratrol administration significantly attenuated the relative proportion of gastrocnemius muscle lost during HLS in old animals (2.21 ± 0.11mg/g vs. 2.80 ± 0.21mg/g; $p \leq 0.05$) (Figure 5B). This preservation of relative muscle mass, but not body weight following HLS was not seen in young animals that were administered resveratrol.

Refer to Figure 5

Muscle Functional Measurements. The HLS-induced force loss was markedly attenuated by resveratrol in old animals. Three *in vivo* maximal isometric contractions were averaged to obtain the pre-HLS force record. This was repeated after HLS to obtain the post–HLS force data. The percent decrease of *in vivo* isometric force was determined by comparing the pre-HLS to post-HLS force records. Maximal isometric measurements were obtained on age-matched ambulatory rats at the two time points that corresponded to the pre-HLS and post-HLS measures in the experimental animals. Maximal plantar flexor force did not change over the duration of the study period for either young adult or old ambulatory rats (data not shown). Young animals lost an average of 33.7% of their maximal isometric force following HLS, and resveratrol administration did not attenuate the force loss in these animals ($32.09 \pm 9.79\%$ vs. $35.37 \pm 7.74\%$)(Figure 5C). However, resveratrol administration significantly preserved isometric force output following HLS in old animals, ($45.9 \pm 6.8\%$ vs. $31.6 \pm 7.4\%$; $p \leq 0.05$) (Figure 5C). To determine if muscle force was preferentially maintained during weight loss, we normalized isometric force to the animal's body weight (Figure 5D). When the percent decrease in isometric force following HLS was normalized to body weight, old vehicle control-HLS animals lost a significantly larger ($p \leq 0.05$) proportion of isometric force output than did their young counterparts, (Figure 5D). This aging effect was abolished with resveratrol administration.

DISCUSSION

Oxidative stress is positively correlated with skeletal muscle atrophy induced by aging (27), denervation (45), disuse (22) and immobilization (45). These atrophic stimuli are associated with concomitant increases in lipid peroxidation (47), glutathione oxidation (47), protein carbonyl formation (44) and myonuclear apoptosis (12). Furthermore, oxidative stress is linked to proteolytic processes that mediate muscle loss through caspase 3 activation (45). Although skeletal muscle disuse induces oxidative stress in both young and old animals (6,28), skeletal muscle from older animals possesses a higher basal level of oxidative stress placing more strain on the endogenous antioxidant system, and thus leaving skeletal muscle from these animals at greater risk for oxidative damage during periods of disuse, and subsequent diminished recovery following disuse.

The current investigation sought to shed light on the mechanisms underlying muscle atrophy with disuse and to ascertain if oxidative stress and/or apoptosis were possible contributing factors. Furthermore, the present study sought to elucidate the potential role of resveratrol administration to alleviate oxidative stress and apoptosis in skeletal muscle from old and young animals. Dietary supplementation with the polyphenol, resveratrol, has the potential to exert beneficial effects not only through its' ability to directly scavenge free radicals (36,39) and upregulate components of the endogenous antioxidant system (34), but also by its' capacity to modulate the signal transduction and gene expression of several pathways regulating cellular proliferation (20,53), mitochondrial biogenesis (50,53), metabolism (41), and apoptosis (20). The efficacy of acute resveratrol administration has been established for several

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pathological conditions (35,36,39); however, this study provides the first insight into the use of resveratrol supplementation as a counter-measure to combat muscle loss and function caused by disuse.

Hindlimb suspension resulted in a significant loss of gastrocnemius muscle mass as estimated from muscle wet weight (Figure 5A). Although there was no significant preservation of gastrocnemius muscle mass resulting from resveratrol administration, there was a significant preservation of the relative ratio of muscle mass preserved normalized to animal body weight in old HLS animals (Figure 5B). This protective effect may suggest a preferential preservation of skeletal muscle tissue with resveratrol administration in old animals following a prolonged period of disuse, since there was no difference in the total percentage of body mass lost between old resveratrol supplemented and old non-supplemented animals during the experimental period (Table1). Likewise, in old, but not young animals, there was a partial maintenance of maximal isometric force of the plantar flexor muscle group following HLS. The percent decrease in isometric force following HLS in old resveratrol administered animals was similar to the force decrements seen in the plantar flexors of young animals following HLS, thus eliminating the interaction of age and suspension. The findings showing a protective effect of resveratrol on force production in the current study, are consistent with data from Lagouge and colleagues (23), showing greater muscle strength in mice fed a high fat diet supplemented with resveratrol.

In general, the *in vivo* isometric force output that was measured in the current study, is the sum of forces that were generated by all of the individual muscles comprising the plantar flexor group. The plantar flexor muscle group consists of the

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soleus, plantaris and gastrocnemius muscles which, contribute approximately 6%, 16% and 78% respectively, to the mass of the rat plantar flexor muscles (11,51). As a result, the gastrocnemius muscle provides the greatest contribution to plantar flexion force output, and thus, the partial preservation of relative gastrocnemius muscle wet weight and plantar flexion force production are likely linearly related.

Resveratrol administration mediated endogenous antioxidant enzymes and oxidative stress indices in old animals. Specifically, resveratrol administration significantly increased MnSOD activity and protein content, but not CuZnSOD activity in old HLS animals (Figure 2). This is analogous with data showing that resveratrol protected against oxidative stress through the specific induction of MnSOD (39). Additionally, in the current study, resveratrol administration increased the content and activity of catalase in old non-suspended animals, but it did not further increase catalase activity, or expression following HLS. This may be in part because catalase activity is already increased with aging and HLS, so it may have reached a maximal point of induction. Perhaps most importantly, resveratrol administration reduced indices of oxidative stress in gastrocnemius muscles of old HLS animals, as estimated by H_2O_2 levels and the lipid peroxidation byproducts (MDA and HAE). This is congruent with recent data from our laboratory and others that have shown that resveratrol protects against H_2O_2 mitigated lipid peroxidation in *vivo* (39,41) and *in vitro* (4). The induction of catalase by resveratrol has previously been demonstrated (39), and along with increases in MnSOD, may represent important mechanisms by which resveratrol acts to reduce H_2O_2 and H_2O_2-mediated damage. However, these protective effects of increases in antioxidant enzyme activity and concomitant decreases in markers of

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oxidant load were not seen in young animals administered resveratrol, suggesting that there is a differential effect of resveratrol in young and old animals. The fact that resveratrol only seems to have an effect in old animals is interesting and is plausibly due to different underlying signaling mechanisms that may occur in old animals during disuse. It is also possible that younger animals are able to handle the stress of hindlimb suspension and the subsequent detrimental effects that are associated with skeletal muscle disuse and therefore the pre-conditioning effect of resveratrol administration helps to augment the stress response in old, but not young animals where it is not needed. This is congruent with our finding that H_2O_2 concentrations were not increased in young animals following suspension. Although, lipid peroxidation markers were increased in muscles from young suspended animals, despite no increases in H_2O_2 concentrations, this might indicate a temporal role of oxidative stress in young animals during muscle disuse.

Oxidative stress is upstream of apoptotic signaling in muscle cells *in vitro* (46) and results in the initiation of the intrinsic mitochondrial apoptotic pathway. Due to its multi-nucleated cellular structure, skeletal muscle is an exception to the linear relationship between apoptosis and cell death. Instead, apoptosis in skeletal muscle results in a loss of myonuclei and consequent fiber atrophy (3). Myonuclei undergo apoptosis in many muscle wasting conditions including, but not limited to, advanced age (31), disuse (42,44) and denervation (1,43). Likewise, these conditions are also associated with increases in oxidative stress (1,27) presumably mediated through mitochondrial dysfunction, given the importance that the mitochondria play in

maintaining cellular integrity via ROS production and the regulation of the apoptotic pathway (2,17,44).

Resveratrol administration altered apoptotic signaling (e.g., Bcl-2, caspase 9 activity) but it did not appear to blunt overall myonuclear apoptosis in muscles from old animals. Resveratrol administration increased the protein content of the anti-apoptotic protein Bcl-2 in old non-HLS animals suggesting a preconditioning effect by which resveratrol could potentially improve the apoptotic environment of skeletal muscle undergoing disuse. Our data are in agreement with a study by Brito and colleagues (12), suggesting that resveratrol was able to protect against oxidant-induced apoptotic signaling through the upregulation of Bcl-2, without any reductions of Bax protein content. Downstream of the mitochondrial mediated apoptotic signaling cascade are the proteolytic caspase enzymes. Resveratrol administration significantly decreased caspase 9 activity in old non-HLS animals and significantly decreased caspase 3 activity in old HLS animals. In parallel with the other findings from the current study, caspase activity was only attenuated in the gastrocnemius muscles from old animals, with no effect being seen in muscles from the young animals.

Interestingly, the alleviation of oxidative stress and damage in old HLS animals administered resveratrol did not translate into protection from apoptosis as estimated from the quantity of DNA fragmentation present following HLS. This suggests that the protective effect of resveratrol may be in part to its anti-inflammatory and/or anti-proteolytic capacities rather than anti-apoptotic capabilities. This would be in agreement with the fact that resveratrol was able to decrease caspase 3 following HLS in old animals, despite having no efficacy in ameliorating apoptosis following HLS. Caspase 3

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is known to be redox sensitive and to play an initiating role in muscle proteolysis via the ubiquitin-proteasome system (20). Although it is not known why a significant increase in Bcl-2 protein content did not confer resistance to apoptosis following HLS, it could be speculated that the relative contribution of apoptosis versus necrosis and other mechanisms of disuse mediated atrophy are temporal in nature. Since data in our study were only collected at one time point (14 days), it is possible that early protection against apoptosis was not detected, but it still may have contributed to the preservation of muscle mass and maintenance of isometric force seen in old animals administered resveratrol.

It is also probable, (although not evaluated in this study), that the protective effects of resveratrol observed in the current investigation stem from the ability of resveratrol to activate the silent mating type information regulation 2 homolog (Sirt1), a NAD+ dependent histone deacetylase (20). Sirt1 has been shown to play a role in a variety of important physiological functions including the regulation of oxidative stress. Congruent with our current observations regarding resveratrol administration, Sirt1 activation is reported to upregulate the transcription of both MnSOD (48) and catalase (15,16) and reduce reactive oxygen species-induced apoptosis (15). This highlights the potential of Sirt1 to act as a protective mechanism against oxidative stress. The wide range of Sirt1's cell signaling capacity underscores the potential ability of resveratrol to mediate a variety of cell signaling pathways and therefore provides many potential therapeutic targets that may be responsible for the attenuation of skeletal muscle atrophy and force preservation observed during disuse in old animals. Gaining clearer insight into the molecular signaling pathways involved in aging and disuse muscle

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atrophy is paramount in developing nutritional and/or pharmacological interventions to minimize protein loss and attenuate the functional decrements associated with atrophic conditions.

In summary, the results of the current study show that resveratrol treatment reduces the functional decrements and the oxidative stress levels in rat hindlimb muscles in response to disuse. One potential mechanism for improved muscle function in resveratrol treated animals is via a Sirt1 mediated improvement in the endogenous antioxidant enzyme activity and the redox status of the aging muscle during disuse. However, it is also possible that the protective effects of resveratrol are unrelated to Sirt1 or blunting oxidative stress. For example, administration of resveratrol has been shown to attenuate protein degradation in murine myotubes treated with proteolysis-inducing factor, enhanced the proliferation of muscle precursor cells (34), and blunted muscle weight loss, and protein degradation, *in vivo*, in a mouse model of cachexia (52). Additional studies are needed to determine if resveratrol activation of Sirt1 has the potential to be an effective therapeutic intervention, by reducing ROS, or if resveratrol functions via another mechanism, to lower the extent of loss of muscle mass or function in aged humans, in response to reduced activity or bed rest.

Acknowledgements

Table Legends

Table 2.1 **Animal Body Weight.** Animals were weighed pre and post-hindlimb suspension (HLS). Body weights (BW) are represented in grams (g) and as percent changes (%Δ) in body weights during the 21 day the experimental protocol. YVC = Young Vehicle Control, YVCS = Young Vehicle Control Suspended, YRC = Young Resveratrol Control, YRS = Young Resveratrol Suspended, OVC = Old Vehicle Control, OVCS = Old Vehicle Control Suspended, ORC = Old Resveratrol Control, ORS = Old Resveratrol Suspended. **Denotes significantly difference ($p \leq 0.05$) between non-suspended treatment matched control (Suspension effect). # denotes significantly different than young treatment matched (Aging effect).

Figure 2.1   **Experimental protocol**. Resveratrol was administered via oral gavage at a dose of 12.5 mg/kg/day for 21 days. One milliliter of 0.01% carboxymethylcellulose was used as a vehicle control. Following 7 days of resveratrol administration animals were randomly assigned to 14 days of hindlimb suspension (HLS), or continued ambulation. Following the experimental protocol, the gastrocnemius muscles were dissected and assessed for muscle wet weight, oxidative stress, and apoptotic indices. MWW, muscle wet weight.

Figure 2.2   **Enzymatic activities and protein contents of CuZnSOD, MnSOD and catalase in response to aging and HLS (A)** CuZnSOD activity was assessed using a spectrophotometric assay and is expressed as units of enzymatic activity (U)/mL/mg protein. **(B)** CuZnSOD protein content was determined via immunoblotting; a representative blot is shown. **(C)** MnSOD activity was assessed using a spectrophotometric assay and is expressed as units of enzymatic activity (U)/mL/mg protein. **(D)** MnSOD protein content was determined via immunoblotting; a representative blot is shown. **(E)** Catalase activity was assessed using a spectrophotometric assay and is expressed as milliunits of enzymatic activity (mU)/mg protein. **(F)** Catalase protein content was determined via immunoblotting; a representative blot is shown. YC= Young Control, YS = Young Suspended, OC = Old Control, OS = Old Suspended, YVC = Young

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Vehicle Control, YVCS = Young Vehicle Control Suspended, YRC =

Young Resveratrol Control, YRS = Young Resveratrol Suspended , OVC

= Old Vehicle Control, OVCS = Old Vehicle Control Suspended, ORC =

Old Resveratrol Control, ORS = Old Resveratrol Suspended.** $p \leq 0.05$,

non-suspended treatment vs. matched control (Suspension effect). # $p \leq$

0.05, young treatment matched (Aging effect). * $p \leq 0.05$, vehicle control

vs. age-matched and treatment matched group (Effect of resveratrol).

Figure 2.3   **Resveratrol attenuated increases in hydrogen peroxide (H_2O_2)**

concentration and lipid peroxidation associated with HLS in old

animals. (A) H_2O_2 concentrations were determined fluorometrically in

gastrocnemius muscle homogenate. Data are expressed as

μmols/H_2O_2/μg protein. Significance was set at ($p \leq 0.05$) and all data are

represented as mean ± standard error. **(B)** MDA and HAE levels were

evaluated as a combined marker of lipid peroxidation and expressed in

μM[MDA/HAE]/mg protein. YC= Young Control, YS = Young Suspended,

OC = Old Control, OS = Old Suspended, YVC = Young Vehicle Control,

YVCS = Young Vehicle Control Suspended, YRC = Young Resveratrol

Control, YRS = Young Resveratrol Suspended, OVC = Old Vehicle

Control, OVCS = Old Vehicle Control Suspended, ORC = Old Resveratrol

Control, ORS = Old Resveratrol Suspended. ** $p \leq 0.05$, non-suspended

control vs. suspended animals (Suspension effect). # $p \leq 0.05$, old vs.

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young treatment matched (Aging effect). * $p \leq 0.05$, vehicle control vs. age-matched and treatment matched groups (Effect of resveratrol).

Figure 2.4   **The effects of resveratrol administration and HLS on apoptotic signaling in young and old animals. (A)** Bax protein content was determined via immunoblotting; a representative blot is shown **(B)** Bcl-2 protein content was determined via immunoblotting; a representative blot is shown **(C)** Caspase 9 Activity was assessed using a fluorometric assay **(D)** Caspase 3 Activity was assessed using a fluorometric assay **(E)** Cell Death ELISA YC= Young Control, YS = Young Suspended, OC = Old Control, OS = Old Suspended, YVC = Young Vehicle Control, YVCS = Young Vehicle Control Suspended, YRC = Young Resveratrol Control, YRS = Young Resveratrol Suspended, OVC = Old Vehicle Control, OVCS = Old Vehicle Control Suspended, ORC = Old Resveratrol Control, ORS = Old Resveratrol Suspended. ** $p \leq 0.05$, non-suspended control vs. suspended animals (Suspension effect). # $p \leq 0.05$, old vs. young treatment matched (Aging effect). * $p \leq 0.05$, vehicle control vs. age-matched and treatment matched groups (Effect of resveratrol).

Figure 2.5   **Gastrocnemius muscle wet weight and plantar flexor maximal isometric force.** The gastrocnemius muscles were dissected, immediately blotted and weighed as a gross estimation of muscle size and represented in grams (g, A), or as ratio to the animal's body weight (mg/g,

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B). Pre and post-HLS force measurements were assessed. Three isometric contractions were recorded per animal for both pre and post–HLS assessments. The average of the three contractions at each time point was used to determine the decrease in isometric force output following HLS. The average force decrease with HLS (C) or the decrease in force normalized to body weight (D) demonstrates that resveratrol preserved loss of function in hindlimb muscles of the old rats. Significance was set at ($p \leq 0.05$) and all data are represented as mean ± standard error. YC= Young Control, YS = Young Suspended, OC = Old Control, OS = Old Suspended, YVC = Young Vehicle Control, YVCS = Young Vehicle Control Suspended, YRC = Young Resveratrol Control, YRS = Young Resveratrol Suspended, OVC = Old Vehicle Control, OVCS = Old Vehicle Control Suspended, ORC = Old Resveratrol Control, ORS = Old Resveratrol Suspended. **$p \leq 0.05$, non-suspended control vs. suspended animals (Suspension effect). [#] $p \leq 0.05$, old vs. young treatment matched (Aging effect). *$p \leq 0.05$, vehicle control vs. age-matched and treatment matched groups (Effect of resveratrol).

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Tables

Table 2.1: Animal Body Weights.

	Pre- HLS BW (g)	Post- HLS BW (g)	%ΔBW
YVC	470 ± 15.1	467± 23	>1%
YVCS	451 ± 41	382 ± 30**	-15%±4%
YRC	464± 55	465 ±45	>1%
YRS	455± 43	383 ± 34**	-16%±3%
OVC	516 ± 38	519± 33$^{\#}$	>1%
OVCS	541 ± 39	478 ± 32**	-12%±4%
ORC	567 ± 25	566 ± 24$^{\#}$	>1%
ORS	565 ± 55	471 ± 59**	-15%±3%

Figures
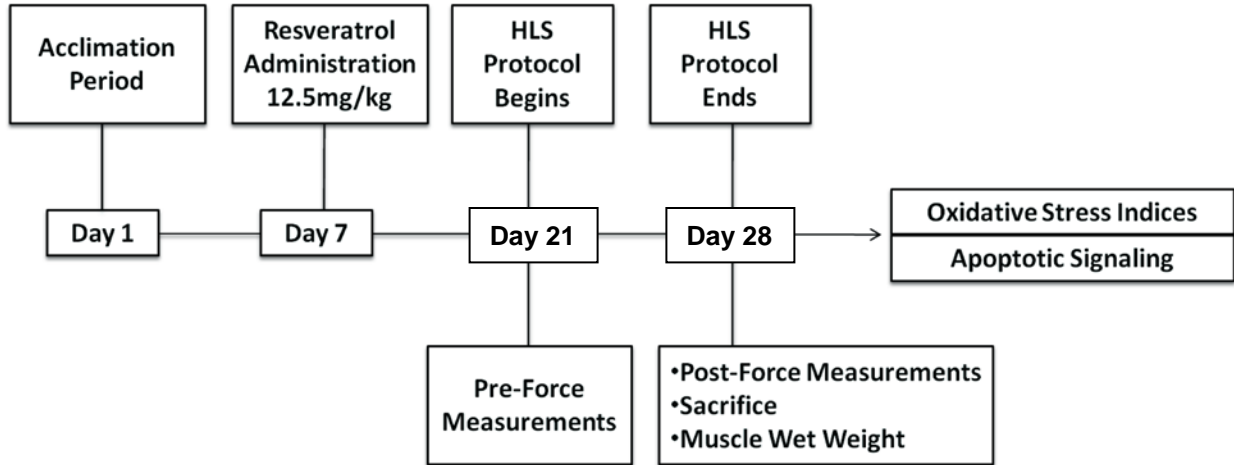
Figure 2.1: Experimental Protocol.

Figure 2.2: Enzymatic activities and protein contents of CuZnSOD, MnSOD and catalase in response to aging and HLS.
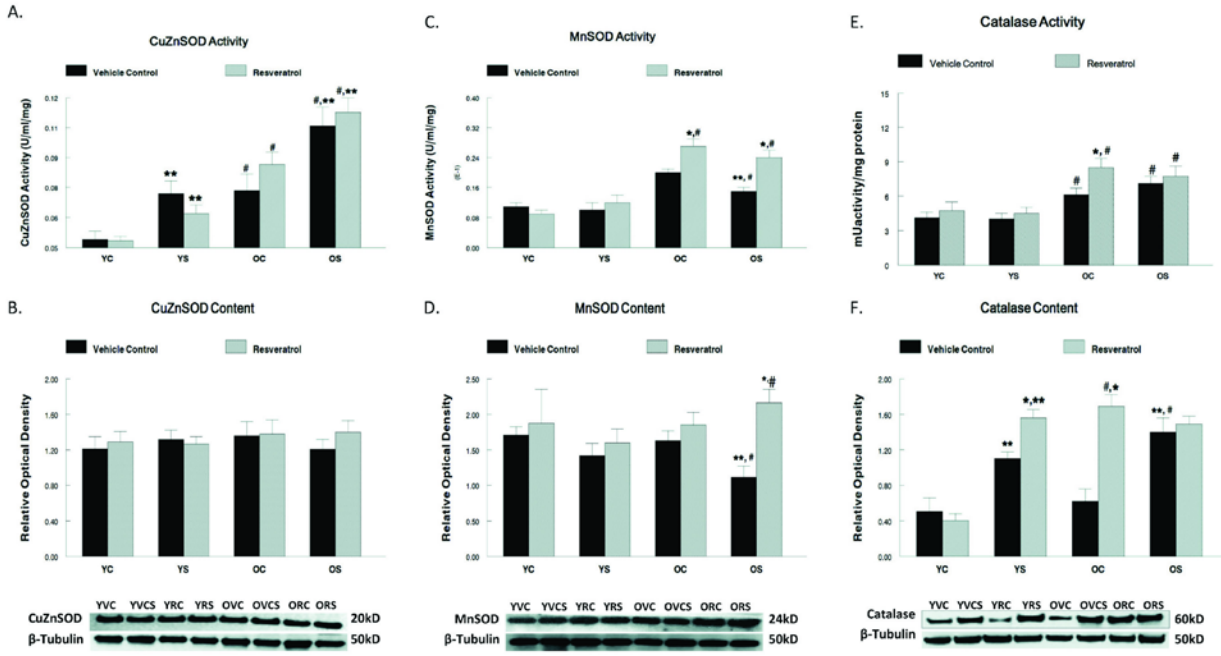
Figure 2.3: Resveratrol attenuated increases in hydrogen peroxide (H_2O_2) concentration and lipid peroxidation associated with HLS in old animals.
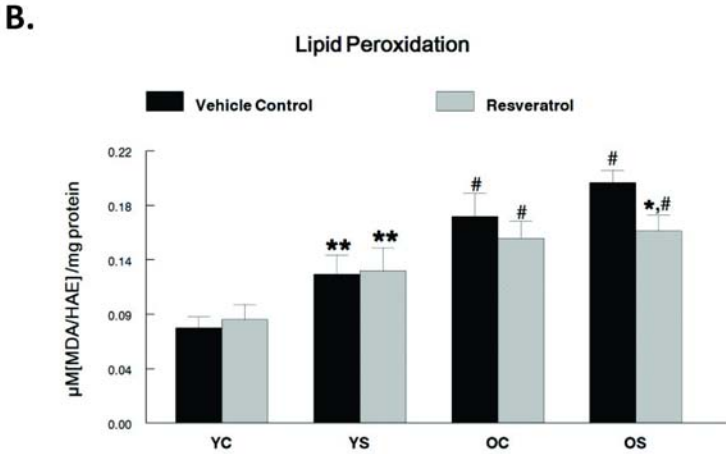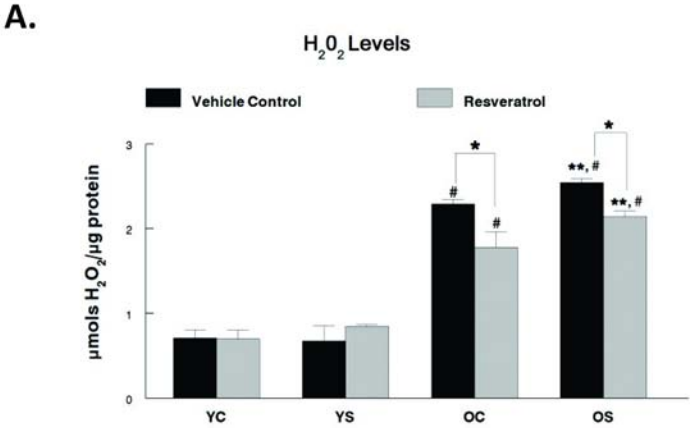
Figure 2.4: The effects of resveratrol administration and HLS on apoptotic signaling in young and old animals.
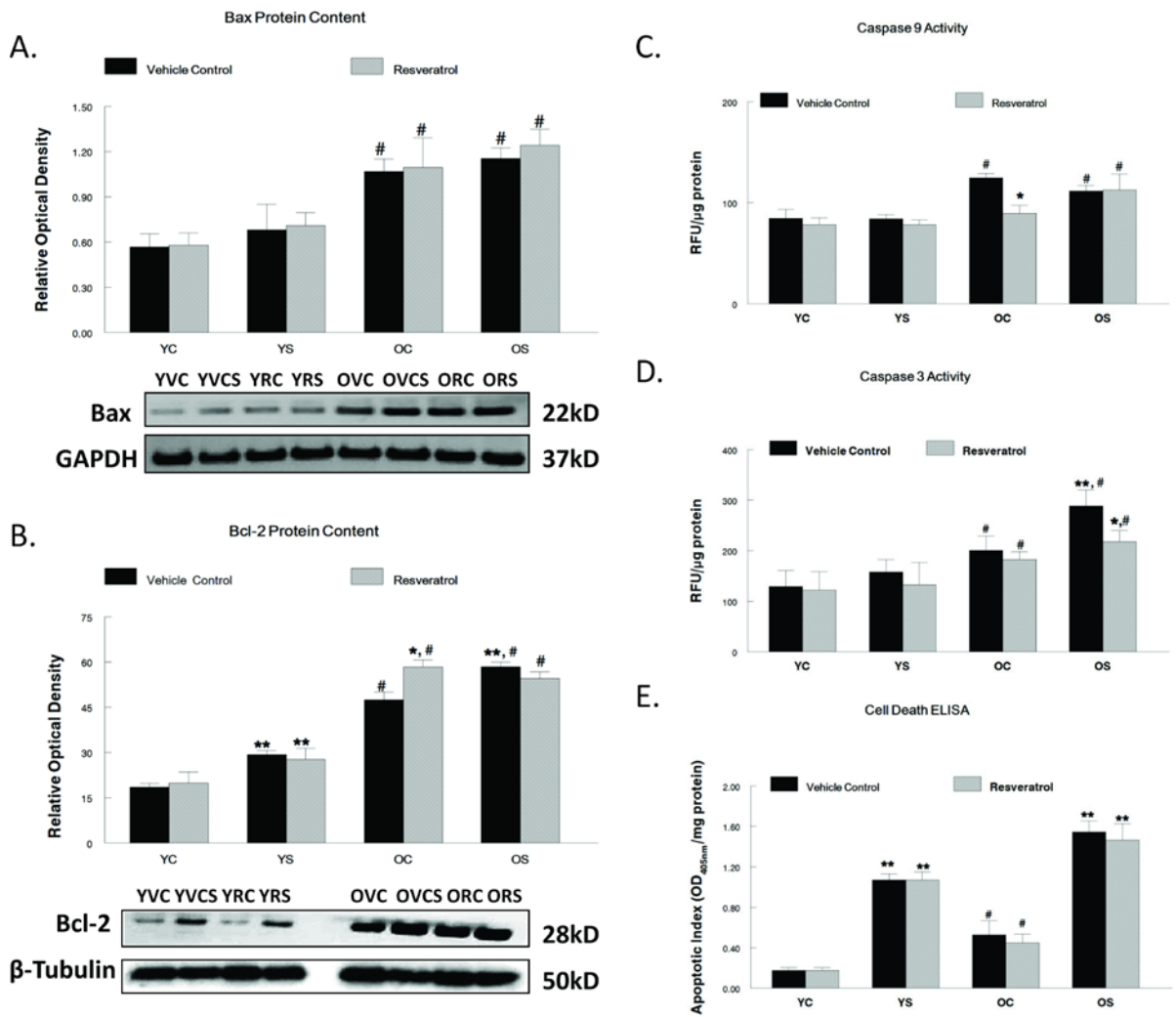
Figure 2.5: **Gastrocnemius muscle wet weight and plantar flexor maximal isometric force.**
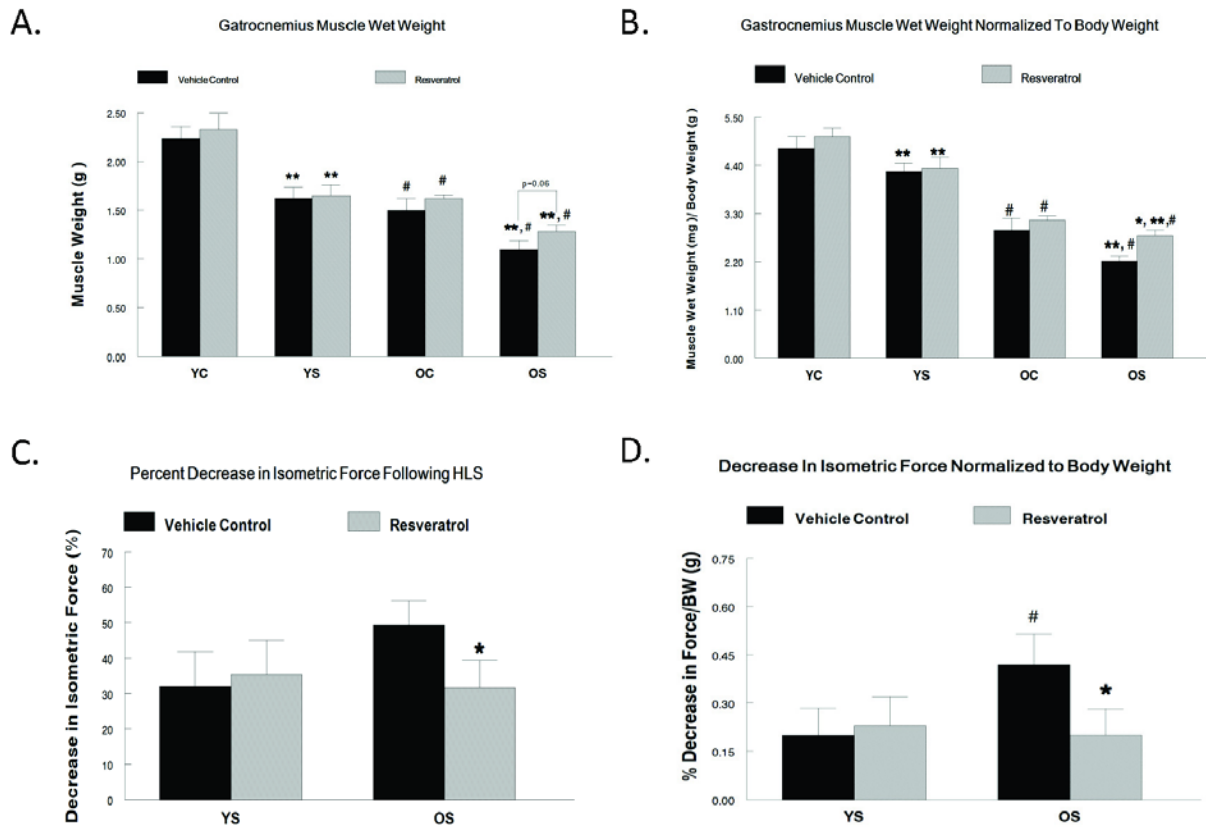
Long-Term Supplementation with Resveratrol Alleviates Oxidative Stress,

but Does not Attenuate Sarcopenia in Aged Mice.

Janna R. Jackson, Michael J. Ryan, and Stephen E. Alway

Laboratory of Muscle Biology and Sarcopenia, Division of Exercise Physiology,

West Virginia University School of Medicine, Morgantown, West Virginia 26506

Send correspondence and reprint requests to:

Stephen E. Alway, Ph.D., Division of Exercise Physiology, School of Medicine,

Robert C. Byrd Health Sciences Center, West Virginia University,

Morgantown WV 26506-9227, USA

Phone: 304-293-0772

Fax: 304-293-7105

Email: salway@hsc.wvu.edu

Running Head: Resveratrol and skeletal muscle aging

ABSTRACT

This study analyzed the capacity of resveratrol, a naturally occurring polyphenol, to reduce aging-induced oxidative stress, and protect against sarcopenia. Middle-aged (18mo) C57/Bl6 mice were randomly assigned to receive either a control diet or a diet supplemented with 0.05% trans-resveratrol for 10 months. Young (6mo) and middle-aged (18mo) mice were used as controls. Resveratrol supplementation did not reduce the aging-associated loss of muscle mass or improve maximal isometric force production, but it appeared to preserve fast-twitch fiber contractile function. Resveratrol supplementation did not improve mitochondrial content, the subcellular localization of cytochrome c protein content, or PGC1 protein content. Resveratrol increased MnSOD, reduced H_2O_2, and lipid peroxidation levels in muscle samples, but it was unable to significantly reduce protein carbonyl levels. The data suggest that resveratrol has a protective effect against aging-induced oxidative stress in skeletal muscle, likely through the upregulation of MnSOD activity, but sarcopenia was not attenuated by resveratrol.

Key Words: Muscle atrophy-Oxidative stress—Sarcopenia—Mitochondria

Introduction

Advanced age leads to a loss of muscle mass and function, termed sarcopenia (64). The causative factors of sarcopenia are multifactoral, and include a progressive denervation of muscle fibers (21), an altered hormonal milieu (75), and a net loss of contractile proteins, (37; 57). While no causal relationship has been established, skeletal muscle atrophy is often associated with an increased production of reactive oxygen species (ROS), (48; 49; 56), leading to an amplified oxidant-load. An increase in oxidant exposure, in conjunction with the less effective endogenous anti-oxidant systems often present in aged animals (54; 58) can lead to oxidative stress. Chronic oxidative stress left unchecked over time leads to the oxidation, and thus damage, of cellular macromolecules, including lipids (53), nucleic acids (29) and proteins (11; 20; 30). This progressive and cyclical oxidative assault on cellular macromolecules is believed to be an important contributor to the aging process (25) and is thought to be responsible for many of the pathologies associated with aging, including genomic instability, mitochondrial dysfunction and chronic inflammation (25; 26), all of which are associated with sarcopenia (13).

Supplementation with the naturally occurring phytoalexin, resveratrol (3,4',5-trihydroxystilbene), has recently been shown to protect against oxidative stress in rodent skeletal muscle (32; 67) and to act as a systemic anti-inflammatory agent *in vivo* (24). Specifically, in skeletal muscle, resveratrol has been shown to upregulate components of the endogenous antioxidant system (32; 67), reduced the oxidant load within the muscle environment and consequently attenuate oxidative damage associated with muscle loading (67), unloading (32) and aging (32; 67). Furthermore, resveratrol administration was able to significantly mitigate muscle atrophy in a mouse

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model of cachexia (80). Resveratrols' ability to directly scavenge free radicals resides in its phenol ring and resveratrol has repeatedly been shown to be a potent scavenger of reactive oxygen species (ROS) in numerous cell types exposed to oxidants (12; 36; 47; 62). However, its rapid first pass metabolism in mammals and thus relatively low bioavailability (19; 23; 46), may limit its role as a direct ROS scavenger *in vivo*. Consequently, *in vivo*, resveratrols' various health benefits can more likely be attributed to its ability to activate the NAD^+ dependent deacetylase, silent mating type information regulation homolog1, or sirtuin1 (Sirt1) (2; 40).

Sirt1 has been shown to play a role in a variety of important physiological functions including inflammation (24), mitochondrial biogenesis (40) and the regulation of oxidative stress and anti-oxidant enzymes (27; 39) in a wide array of cell types (38; 40; 51; 78). Sirt1 is also activated with exercise (74) and under conditions of nutrient deprivation (14; 79), underscoring resveratrols effectiveness as a caloric restriction mimetic. These conditions in which Sirt1 activation is elicited are significant because both exercise (71; 73) and caloric restriction (7; 14; 41) are proven countermeasures to combat both oxidative stress and sarcopenia. Furthermore, Sirt1 is a powerful positive mediator of PPARγ co-activator (PGC-1α) (76), which is considered to be the master regulator of mitochondrial function and biogenesis (43). PGC-1α is decreased during atrophic conditions (68; 69) and muscle specific over expression of PGC-1α confers protection against both denervation and fasting-induced skeletal muscle atrophy (69). Increases in PGC1 signaling have the potential to alleviate age-related mitochondrial alterations which underlie a wide variety of diseases such as diabetes (45), neurodegeneration (70) and sarcopenia (13).

Although mitochondria are not the sole source of ROS within a cell, they are routinely viewed as the principle site of superoxide generation, which is the primary source of damaging ROS within muscle cells (33; 34). Resveratrol's ability to increase PGC1 signaling, via Sirt1 activation, could potentially provide a two-fold mechanism with which to protect aged skeletal muscle against oxidative stress. Firstly, resveratrol has been shown to act as a direct and indirect anti-oxidant diminishing the likelihood of phospholipid oxidation and thus damage within the mitochondrial membrane preserving the integrity of the membranes and thus preventing "leaky" mitochondria. In addition, resveratrol has been shown to promote mitochondrial biogenesis in skeletal muscle in a Sirt1 dependent fashion (40) thus potentiating the possibility to replace damaged mitochondria in aged animals.

Acute resveratrol administration has been recently shown to reduce oxidative stress and improve functional outcomes in rodent skeletal muscle (32; 67); however, the efficacy of chronic resveratrol supplementation as a counter-measure to combat sarcopenia has not been established. Thus, the purpose of the current investigation was to determine if long-term dietary supplementation, from middle-age through senescence, with moderate doses of resveratrol would effectively attenuate the loss of muscle mass and function that occurs in aged animals. It was hypothesized that resveratrol's ability to activate Sirt1 would consequently enhance the endogenous anti-oxidant system and increase PGC1 signaling, thereby reducing the release of ROS from mitochondrion. Furthermore, it was hypothesized that resveratrol supplementation would maintain muscle function in aged animals both by preservation of muscle mass and by providing a more favorable redox environment

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Methods

Experimental Protocol. Experiments were conducted on young adult (3 mo), middle-aged (18 mo) and aged (28 mo) C57BL/6 mice obtained from the National Institute on Aging colony (Harlan, Indianapolis, IN). The mice were housed in pathogen-free conditions at ~20°C. All mice had free access to food and water. Middle-aged mice were put on either a control diet of normal mouse chow, (AIN-76A Rodent Diet, Research Diets Inc, New Brunswick, NJ), or an identical diet containing 0.05% resveratrol (Research Diets Inc, New Brunswick, NJ). Resveratrol was purchased from Orchid Pharmaceuticals (India). Mice of 3 mo and 18 mo of age were used as control animals only. Aged animals were sacrificed at 28 months after 10 months of being on either the control, or resveratrol-supplemented diet. Immediately following sacrifice, skeletal muscles were dissected for use in either biochemical analyses, mitochondrial isolation and/or physiological analyses. All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine.

Ex vitro **muscle physiological analysis.** Isometric muscle contractile properties were examined in the plantaris muscles of control and resveratrol-treated mice. The muscles were placed in an oxygenated ringers solution (in mM: 137 mM NaCl, 4.7mM KCl, 3.4mM $CaCl_2$, 1.2mM $MgSO_4$ 1 NaH_2PO_4, and 112 D-glucose). The Ringer solution was aerated with 95% O2 and 5% CO, (pH 7.4). The temperature of the solution was kept at 20°C. The distal end of the muscle was attached to a stationary plexiglass plate,

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and the proximal end was fixed to the lever arm of a 300C dynamometer (Aurora Scientific, Aurora Ontario, Canada). The muscles were stimulated by passing a constant current through platinum plates that were positioned on either side of the muscle. *Ex vivo* isometric twitch and tetanic contractions were obtained using a Constant Current/Constant Voltage Stimulator (Aurora Scientific) that provided DC-square wave signals at stimulation current of 12 Volts, with a 200 μs pulse width. Muscles were adjusted to the optimal muscle length (L_o) by a micromanipulator that controlled the base position of the electrode clamp. L_o was established as the muscle length that produced maximal isometric twitch tension. L_o was periodically checked by the same procedure throughout each experiment. Force-frequency isometric force records were obtained by stimulating the muscle at 10, 20, 40, 50, 75 and 100 Hz, with 3 minutes of rest between each contraction. Physiological contractile measures included peak isometric twitch force (PT), time to peak twitch contraction tension (CT), ½ relaxation time of twitch contraction (½ RT), and peak isometric tetanic force (Po), as previously described (3; 4). Following isometric contractions, the muscles remained in oxygenated Ringers for 5 minutes prior to the repeated stimulation fatigue protocol. Muscle fatigue was assessed by stimulating the muscle at 40Hz for 3 minutes with a duty cycle of 330 ms of stimulation followed by 660 ms of rest (15). The fatigue index was calculated as the difference in force from the average of the first three contractions to the average of the final three contractions. The contractile and fatigue measurements were analyzed off line using commercial software (DMI, Aurora Scientific).

Protein Isolation. Approximately 40 μg of gastrocnemius muscle from each animal was homogenized in 500 μL of RIPA buffer (1% Triton x-100, 150mM NaCl, 5mMEDTA,

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10mM Tris; pH: 7.4) using a mechanical homogenizer, for use in the assessment of protein expression via immunoblotting. Muscle homogenates were then centrifuged at 1000 x *g* for 5 minutes at 4°C. The resulting supernatant was collected and divided into two portions and frozen at –80°C either with, or without a protease inhibitor cocktail containing 104 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride hydrochloride, 0.8 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St. Louis, MO) added to it.

For all enzyme activity assays, and/or oxidative damage assessments muscle samples were homogenized in the appropriate volume of either PBS, or the kit specific buffer provide by the manufacturer. Protein concentrations for each sample were determined in duplicate using the DC Protein Assay kit (Bio-Rad, Hercules, CA).

Mitochondrial isolation. The vastus lateralis muscles were carefully removed while the mice remained under deep anesthesia (5% isoflurane / 95% oxygen). Precautions were made to assure that the blood supply to the muscles remained intact until it was removed to prevent the artificial accumulation of oxidants. Mitochondria and mitochondria free cytosolic muscle fractions were obtained using a commercially available mitochondrial isolation kit specifically designed for animal tissue (MITOISO1-1KT, Sigma-Aldrich Co., St Louis, MO). The fractions were obtained using sequential separation steps involving a protease digestion followed by separation of the fractions via centrifugation using slight modifications of the manufacturer's recommendations. 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 then washed and re-suspended in extraction buffer and then gently homogenized with a Teflon pestle. The homogenate was then centrifuged at 600 x g for 5 minutes. The supernatant was transferred to a new tube and centrifuged at 11,000 x g for 10 minutes and transferred to a new tube. The supernatant was centrifuged for 10min at 11,000 x g and transferred to a new tube to assure a clean mitochondrial free cytosolic fraction. The mitochondrial pellet was suspended in a sucrose storage buffer.

Manganese Superoxide Dismutase (MnSOD) and Copper-Zinc Superoxide Dismutase (CuZnSOD) Enzyme Activity Levels. Superoxide dismutase activity was measured in vastus lateralis muscle homogenate that was partitioned into a mitochondrial pellet and mitochondrial-free cytosolic fraction. Enzymatic activity was assessed using a colorimetric enzyme activity kit (Cayman Chemical Company Ann Arbor, MI) following the manufacturer's guidelines. CuZnSOD activity was obtained from the cytosolic fractions and MnSOD activity was measured using isolated mitochondria. All samples and standards were measured in duplicate. The assay was performed in a 96-well plate and mitochondrial samples were treated with 10µL of 12 mM potassium cyanide to inhibit any residual CuZnSOD activity. The absorbance of the resulting colorimetric changes were measured at a wavelength of 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).

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Immunoblots. The protein content of CuZnSOD and MnSOD were measured in vastus lateralis total muscle homogenate. Cytochrome C protein content was measured in the cytosolic (mitochondrial free) fraction of the vastus lateralis muscle, the corresponding mitochondrial fractions and total muscle homogenate. The protein content of Sirt1 and PGC-1 were measured in gastrocnemius total muscle homogenates. β-tubulin was used as a loading control for cytosolic fractions and total homogenate. A ponceau stain was used to validate equal loading of the mitochondrial fraction. Thirty to forty micrograms of protein were loaded into each well of a 4%–12% gradient polyacrylamide gel (Invitrogen, Carlsbad, CA) and separated by routine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for approximately 1.5hrs at 20°C, followed by transfer to a nitrocellulose membrane for 70-120 min at 35V. All membranes were blocked in 5% nonfat milk (NFM) for 1 hour at room temperature. Membranes were then incubated in the appropriate primary antibodies, overnight at 4°C. Primary antibodies were diluted in Tris-buffered saline, with 0.1% Tween-20 (TBST) and 10% sodium azide. Membranes were then washed in 0.05% TBST followed by incubation in the appropriate dilutions of secondary antibodies (diluted in 5% NFM in TBST) conjugated to horseradish peroxidase. Signals were developed using a chemiluminescent substrate (Lumigen TMA-6; Lumigen, Southfield, MI) and visualized by exposing the membranes to X-ray films (BioMax MS-1; Eastman Kodak). Digital records were captured by a Kodak 290 camera, and protein bands were quantified using 1D analysis software (Eastman Kodak). Bands were quantified as optical density x band area and expressed in arbitrary units.

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Sirt1 Activity. Sirt1 activity was measured using components of the Sirt1 Fluorimetric Drug Discovery Kit (BIOMOL, Plymouth Meeting, PA # AK -555). In short, endogenous Sirt1 activity was measured in total gastrocnemius muscle homogenates homogenized in ice-cold PBS (pH =8.0) using the fluorescent deacetylase substrate and developer provided in the kit. Following homogenization each sample was quantified using a DC protein concentration assay (Bio-Rad, Hercules, CA) and diluted to 2.5 µg/µL using the kit supplied assay buffer. The fluorescent substrate in conjunction with 100 µM of the co-substrate NAD$^+$ was incubated with 25 µL of each sample for 30 minutes at RT in a ½ volume 96 well white microplate. Following this incubation 2mM Nicotinamide (Sirt1 inhibitor) and the provided fluorescent developer were added to each well to stop the reaction and produce a fluorophore that is linearly related to Sirt1 activity. Resveratrol and Suramin were combined with the provided recombinant Sirt1 and were used as positive and negative controls respectively. Additionally, a mouse liver homogenate was used as an additional positive control. The intensity of the fluorescent signal was detected using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Data are presented as fluorescent units normalized to the milligrams of protein present in each homogenate.

Citrate Synthase Activity. Citrate synthase activity was measured in gastrocnemius whole tissue extracts using a commercially available kit (CS0720 Sigma-Aldrich, Saint Louis, MO). Citrate synthase activity has been extensively used as a marker of mitochondrial mass (40; 59) and is a suitable marker for use in whole muscle homogenate given that it's located in the mitochondrial matrix; the enzyme's activity

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would be minimally affected by the homogenization procedure. Muscle samples were homogenized in ice-cold CelLytic MT Cell Lysis Reagent (C3228) and the resultant homogenate was centrifuged at 12,000 x g for 10 minutes to remove cellular debris. The resulting supernatant was used for the kinetic assessment of citrate synthase activity. The assay was performed in a 96 well plate as per manufactures instructions. Briefly, 8μL of each muscle homogenate was added to a master mix containing the supplied assay buffer, 30mM Acetyl CoA solution and 10mM DTNB solution. The reaction was initiated with the addition of 10μL of oxaloacetic acid and the formation of citric acid was determined spectrophotometrically at a wavelength of 412nm at an interval of 90seoncds for 10minutes. Net citrate synthase activity was calculated as the endogenous citrate synthase activity subtracted from total activity. All analyses were measured in duplicate and the samples were normalized to μg of protein per μL of muscle homogenate as determined by the DC Protein Assay Kit (Bio-Rad Hercules, CA).

Hydrogen Peroxide (H_2O_2) Content. H_2O_2 content in gastrocnemius muscle homogenate was measured using a fluorescent assay according to the manufacturer's recommendations (Cell Technology, Mountain View, CA). Briefly, 50 μL of control, unknown muscle samples, or H_2O_2 standards were mixed with 50 μL of the reaction cocktail provided in the kit and added to each well to initiate the reaction. The plate was then incubated in the dark for 10 minutes at room temperature. The intensity of the fluorescent signal was detected using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. A linear regression was performed by plotting the

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resultant florescent intensities from the known standards and subsequently the unknown samples were fit to the corresponding linear equation. All analyses were measured in duplicate and the samples were normalized to µg of protein per µL of muscle homogenate as determined by the DC Protein Assay Kit (Bio-Rad Hercules, CA).

Lipid Peroxidation. Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were measured using the methods and reagents from Oxis International, CA (BIOXYTECH LPO-586). Approximately 50 mg of gastrocnemius muscle was homogenized in 750 µL of ice cold buffer containing phosphate-buffered saline (20 mM, pH 7.4) and 5 µL of 0.5 M butylated hydroxytoluene in acetonitrile per 1 mL of tissue homogenate. Assay reagents were added following the manufacturer's recommendations and have been previously described by our laboratory (66). Briefly, the muscle homogenate was centrifuged at 3000 x *g* at 4°C for 10 minutes, and the supernatant was carefully collected and used to quantify lipid peroxidation. The supernatant was subsequently incubated in the proper reagents at 45°C for 60 minutes, as per the manufactures instructions, and then centrifuged at 15,000 x *g* for 10 minutes. The absorbance of the resulting sample was read at 586 nm. All analyses were measured in duplicate and the samples were normalized to µg of protein per µL of total gastrocnemius muscle homogenate as determined by the DC Protein Assay Kit (Bio-Rad Hercules, CA).

Protein Carbonyls. A colorimetric protein carbonyl assay kit (#10005020 Cayman Chemical Company, Ann Arbor, MI) was used to determine the level of protein oxidation in gastrocnemius muscle homogenates. All procedures were carried out according to

the manufactures guidelines. In brief, ~50mg of gastrocnemius muscle was homogenized in ice-cold PBS (pH = 6.7) containing 1mM EDTA and subsequently centrifuged for 10,000 x g for 15min at 4°C to obtain a clear supernatant. 400 µL of the supernatant was subsequently transferred to two separate tubes, one to be used as a background control and one to be used to asses protein carbonyl formation. 2,4-dinitrophenylhydrazine was added to the sample tube and 2.5 M hydrochloric acid was added to each control tube. Both tubes were then incubated in the dark for 1 hour at room temperature. Following this incubation 20 % trichloroacetic acid was added to all tubes and then briefly placed on ice. The samples were then exposed to a series of sequential acid incubations, centrifugations and wash steps as per the manufactures directions. After the final wash the pellets were re-suspended in guanidine hydrochloride and transferred to the wells of a 96 well plate. The absorbance change was then measured at a wavelength of 360nm using a plate reader. Following the readings all samples were quantified using a DC protein concentration assay (Bio-Rad, Hercules, CA) and the results are presented as nmols of protein carbonyls normalized to the µg of protein present in each sample.

Statistics. Statistical analyses were performed using the SPSS 13.0 software package (SPSS, Chicago, IL). An analysis of variance was used to examine differences between the three age groups and additionally resveratrol supplementation. Bonferroni post hoc analyses were performed between significant means. A *p* value $\leq$ 0.05 was considered statistically significant. Data are reported as mean ± standard error of the mean.

RESULTS

Descriptive Data. All animals were weighed at the beginning and end of the experimental protocol. The data displayed in Table 1 are indicative of the final weight of the animals immediately prior to sacrifice. There was no significant difference between the body weights of aged control vs. aged resveratrol supplemented animals. Middle-aged and both aged animal groups were significantly heavier than young animals (34.3 ± 2.3g vs. 34.5 ± 3.9g vs. 26.5 ± 2.1g; $p \leq 0.05$). There was no significant effect of resveratrol administration on body weight within aged animals. Conversely, both gastrocnemius and plantaris muscle wet weights were significantly greater in young animals compared to aged and middle-aged animals. The difference was even more prominent when muscle wet weights were normalized to their body weights, (Table 1). These results are indicative of sarcopenia; however, resveratrol showed no protective effect with regard to maintaining muscle mass during aging in either the plantaris, or gastrocnemius muscles.

Refer to Table 1

Silent mating type information regulation homolog1, (Sirt1) Enzyme Activity and Protein Content. Surprisingly, Sirt1 Activity was significantly greater by 25% in gastrocnemius muscles from aged animals, compared to their young counterparts. Gastrocnemius muscles from resveratrol supplemented animals had a modest, but additional increase in Sirt1 activity (2867 ± 108 vs. 3243 ± 107 AFU/μg), (Figure 1A).

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Sirt1 protein content, as measured by immunoblotting, was similarly greater in muscles from aged animals, although to a much larger extent than the activity levels. Muscles from aged animals showed, more than a two-fold greater Sirt1 protein content compared to muscles from young animals. Resveratrol supplementation did not significantly increase Sirt1 protein content in muscles of middle-aged animals, although the results approached statistical significance ($p = .063$), (Figure 1B).

Refer to Figure 1

Peroxisome proliferator-activated receptor gamma coactivator 1 (**PGC1) Protein Content and Citrate Synthase Enzyme Activity.** PGC1 protein content and citrate synthase activity were measured as indirect markers of oxidative metabolism and mitochondrial content, respectively. PGC1 protein content measured in total gastrocnemius muscle homogenate remained unchanged regardless of age, or resveratrol supplementation, (Figure 2A). Citrate synthase activity was ~20% lower in muscles from aged animals, but resveratrol supplementation had no effect on the enzyme's activity (Figure 2B).

Refer to Figure 2

Subcellular Localization of Cytochrome c Protein. Cytochrome c protein content was measured, via immunoblotting, in the total, cytosolic (mitochondrial-free), and mitochondrial fractions of vastus lateralis muscles as an estimation of both

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mitochondrial content and mitochondrial membrane permeability. Cytochrome c protein content in both the total homogenate and the mitochondrial fractions, remained unchanged regardless of age or resveratrol supplementation (Figure 3A). Cytochrome c present in the mitochondrial free cytosolic fraction was ~ 10 fold greater in muscles from both middle-aged and aged animals regardless of resveratrol supplementation, suggesting increased mitochondrial membrane permeability and perhaps an increased likelihood of mitochondrial dysfunction present in middle-aged and aged animals (Figure 3A).

Refer to Figure 3

SOD Enzyme Activity and Protein Content. Superoxide dismutase activity was measured in vastus lateralis muscles fractioned into mitochondrial and cytosolic (mitochondrial-free) fractions. MnSOD activity was assessed in isolated mitochondria and CuZnSOD activity was measured in the corresponding mitochondrial free cytosolic faction. Total homogenates from the vastus lateralis muscle was used to determine the protein content of each SOD isoform. MnSOD activity was similar in mitochondria isolated from vastus lateralis muscles from middle-aged and aged animals relative to young muscles. Resveratrol supplementation increased MnSOD activity in muscles from aged animals by an additional ~40%, (Figure 4A). There was no aging, or supplementation effect on MnSOD protein content (Figure 4B). CuZnSOD activity was significantly increased in gastrocnemius muscles from old animals compared to

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gastrocnemius muscles from young animals (121 ± 6.3 vs. 73.8 ± 6.6 U/ml/mg; $p \leq$ 0.05). Resveratrol supplementation significantly decreased CuZnSOD activity in aged animals, by 33%, reducing the enzymes activity level to that of young animals (Figure 4C). There was no change in CuZnSOD protein content with resveratrol supplementation, however, aging significantly increased CuZnSOD protein content in both middle-aged and aged animals compared to their young counterparts, (Figure 4D).

<u>Refer to Figure 4</u>

Hydrogen Peroxide (H_2O_2) Concentrations. H_2O_2 was assessed in gastrocnemius muscle homogenates as an indicator of oxidant load during aging. H_2O_2 levels were ~80% higher in gastrocnemius muscles from aged and middle-aged animals when compared to gastrocnemius muscles from young animals, (1.88 ± 0.11 vs. 1.80 ± 0.25 vs. 1.05 ± 0.12 μmols H_2O_2 /μg protein; $p \leq 0.05$). Resveratrol supplementation significantly reduced H_2O_2 levels in gastrocnemius muscles from aged animals compared to gastrocnemius muscles from old control animals, (1.52 ± 0.10 vs. 1.88 ± 0.11 μmols H_2O_2 /μg protein; $p \leq 0.05$). This reduction represented a 24% decrease in H_2O_2 concentrations, but muscles from aged resveratrol animals still had significantly higher levels of H_2O_2 when compared to young muscles (Figure 5A).

Lipid Peroxidation. Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were assessed in whole muscle homogenates as indicators of oxidative damage, specifically as markers of muscle lipid peroxidation. Lipid peroxidation was increased in aged

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animals compare to their young counterparts (0.251 ± 0.04 vs. 0.442 ± 0.05 µM [MDA/HAE]/mg protein; $p ≤ 0.05$). Muscles from middle-aged animals did not show elevated markers of lipid peroxidation when compared to muscles from young animals. Resveratrol supplementation was able to partially attenuate the increase in lipid peroxidation that occurred due to aging. Gastrocnemius muscles from aged supplemented animals had a 20% reduction in MDA and HAE levels compared to aged control animals (0.354 ± 0.02 vs. 0.442 ± .05 µM [MDA/HAE]/mg protein; $p ≤ 0.05$), (Figure 5B).

Protein Oxidation. Protein carbonyl formation was measured in gastrocnemius muscle homogenates as an indicator of protein oxidation caused by oxidative stress. Muscles from both aged and middle-aged animals showed significantly greater concentrations of protein carbonyl formation compared to muscles from their young counter parts (7.43 ± 1.3 vs. 7.10 ± 1.2 vs. 4.63 ± .67 nmol/ml/µg; $p ≤ 0.05$). Resveratrol supplementation was unable to mitigate protein oxidation in aged animals as measured by carbonyl formation (Figure 5C).

Refer to Figure 5

Ex Vitro **Physiological Analyses.** Isometric muscle contractile properties were examined in the plantaris muscles of control and resveratrol-treated mice (Figure 6). The force frequency curve was shifted significantly rightward in resveratrol supplemented aged mice, compared to aged control mice (Figure 6A). This rightward

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shift is indicative of a faster muscle profile. These results were completely unexpected given that previous literature has shown that dietary resveratrol supplementation improves muscular endurance (40) and increases mitochondrial biogenesis in skeletal muscle (6; 40) which would likely shift the force frequency curve leftward to represent a slower muscle profile. Additionally, CT and ½ RT were analyzed to determine if resveratrol had any effect on the twitch properties of plantaris muscles, (Figure 6B). Plantaris muscles from middle-aged animals had a significantly shorter contraction times than did muscles from aged control animals, (32.8 ± 2.1 ms vs. 39.6 ± 1.4 ms; $p \leq 0.05$). Similarly, muscles from resveratrol supplemented animals also showed shortened contraction times compared to aged control animals (34.7 ± 0.8 ms vs. 39.6 ± 1.4 ms; $p \leq 0.05$), eliminating any aging effect between middle-aged and aged resveratrol supplemented animals. These data are consistent with the rightward shift in the force frequency curve of muscles from resveratrol supplemented animals (Figure 6.A**)**. No differences were found in the ½ RT (Figure 6.B), or the twitch to tetanus ratio (P_t/P_o) of plantaris muscles with respect to age or resveratrol supplementation, (Figure 6C). Lastly, a modified Burke protocol (10) was implemented to assess muscle fatigue in plantaris muscles. The fatigue protocol consisted of 3 minutes of 120 electrically evoked contractions at 40 Hz. Each contraction was for 1.0s with a 33.3% duty cycle. The pulse duration was 200 µs. There was no difference between the fatigue index of middle-aged and aged control animals and furthermore, resveratrol was unable to improve fatigue resistance in plantaris muscles (Figure 6D).

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DISCUSSION

Oxidative stress is believed to be a common underlying mechanism potentiating many of the factors leading to muscle loss with aging (22; 56). There appears to be inherent alterations in the endogenous antioxidant defense systems that occur with aging in skeletal muscle (54; 58). Although there is an incongruence in the literature as to whether antioxidant enzyme activities increase, or decrease, with aging; there is an abundance of evidence suggesting that the antioxidant defense system in aged individuals can be more easily overwhelmed by oxidants leading to oxidative stress and consequent oxidative damage (53; 65; 72). Increased oxidant production and/or an attenuated capacity to buffer oxidants may result in reductions in muscle function due to both muscle atrophy and also functional decrements that go beyond the linear relationship between muscle cross-sectional area and force generation (16; 31; 61). Given that the definition of sarcopenia is the loss of muscle mass and function with aging, (64) it stands to reason that there may be a possible link between increases in oxidative stress with aging and the progression of sarcopenia.

The source of increased ROS present in aged skeletal muscle most likely originates in the mitochondria. Age-related mitochondrial alterations underlie a wide variety of diseases such as diabetes (45), neurodegeneration (70) and sarcopenia (13). The premise behind this relationship is based on the theory that with advanced age there are more dysfunctional mitochondria present within a cell (44). These defective mitochondria contain "leaky" electron transport chains and thus more oxidants are produced leading to oxidative stress (13; 28). The process is cyclic; with more

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mitochondrial uncoupling, there are more pro-oxidants present to further damage vulnerable membrane phospholipids (28; 53). Consequently mitochondrial derived superoxide generation increases with advanced age (50).

Supplementation with the naturally occurring polyphenol, resveratrol, has the potential to alleviate oxidative stress in aged skeletal muscle via activation of Sirt1, by favorably augmenting endogenous anti-oxidant enzymes (27; 39) and enhancing mitochondrial function (40) and biogenesis (6). The present study sought to determine the efficacy of long-term resveratrol supplementation to alleviate oxidative stress from middle-age through senescence and to attenuate the progression of sarcopenia.

As expected, sarcopenia was present in both plantaris and gastrocnemius muscles from aged animals (Table1). Interestingly, although muscles from middle-aged animals were also significantly heavier than muscles from aged animals, when normalized to bodyweight, muscles from middle-aged animals already showed a relative decrease in muscle mass when compared to muscles from young animals. Counter to our hypothesis, resveratrol supplementation was not able to attenuate the loss of muscle mass or relative muscle mass in aged animals, nor did dietary resveratrol supplementation affect animal body weight (Table1).

Resveratrol supplementation moderately, but significantly, enhanced Sirt1 activity (Figure 1A) in gastrocnemius muscles from aged animals compared to muscles from animals on the control diet. There was a tendency for Sirt1 protein levels to be elevated by dietary resveratrol supplementation, however, these results did not reach statistical significance (p=0.063) (Figure 1B) and this is congruent with literature showing that supplementation with resveratrol primarily acts via increasing Sirt1 activation, not

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protein content (6). Somewhat unexpectedly, both Sirt1 activity and protein levels increased with aging (Figure 1A-B). We had originally theorized that Sirt1 activity would be decreased in aged animals in light of the fact that upregulation of Sirt1, with resveratrol, can protect against certain facets of aging (5; 55). However, Sirt1 has recently been found to be upregulated in heart muscle in response to both exogenous stress and aging (1), suggesting that Sirt1 is elevated in muscles of aged animal and likely represents an attempt to confer protection against endogenous and exogenous stressors. These results are in agreement the findings of the current study. Nevertheless, this elevation in Sirt1 protein and activity was insufficient to protect skeletal muscle against sarcopenia.

Sirt1 is a known positive regulator of PGC1 (63) and as such has the potential to influence pathways involved in mitochondrial biogenesis and oxidative metabolism. Long-term resveratrol supplementation did not increase total PGC1 protein content in aged gastrocnemius muscles, nor did total PGC1 protein content change with aging (Figure 2 A). Given that total PGC1 protein levels may not be sensitive to changes in the actual functional capacity of PGC1, the enzymatic activity of citrate synthase was also analyzed as a marker of oxidative metabolism and mitochondrial content. Citrate synthase activity was similarly unaffected by resveratrol supplementation, however, citrate synthase activity was sensitive to age-related alterations and was significantly decreased in aged animals, (Figure 2B). Congruent with the results of the citrate synthase assay, cytochrome c release from the mitochondria was increased in muscles from aged and middle-aged animals, but was not ameliorated with resveratrol supplementation, (Figure 3A). It should be noted that in the current investigation the

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subcellular localization of cytochrome c was used as an indicator of mitochondrial membrane integrity and content, although traditionally cytochrome c release is used as a hallmark of apoptosis (1) . Taken as a whole, these data seem to indicate that although Sirt1 activity was enhanced with resveratrol supplementation, it was not increased enough to enhance PGC1 signaling and thus had no measurable influence on mitochondrial membrane integrity, mass, or metabolism.

Long-term resveratrol supplementation was successful at mediating endogenous antioxidant enzymes and markers of oxidative stress and oxidative damage in muscles from aged animals. Specifically, resveratrol administration significantly increased MnSOD activity, while having an opposing effect on CuZnSOD activity by significantly decreasing it in vastus lateralis muscles, (Figure 4A & C). On the other hand, resveratrol supplementation did not alter either SOD isoform at the protein level (Figure 4 B & D). The ability of resveratrol to induce MnSOD is commonly accepted and it is believed that resveratrol augments Sirt1 activity which in turn upregulates MnSOD in a FOXO3a dependent manner (77). Acute resveratrol supplementation has previously been shown to enhance MnSOD at both a gene (67) and protein (32) level in addition to enhancing the enzyme's activity, suggesting that there may be differential signaling with regard to MnSOD regulation between acute and chronic exposure to resveratrol. CuZnSOD activity is known to be increased with aging and in other conditions eliciting elevated levels of oxidative stress (35), so it seems plausible that resveratrol's ability to reduce the muscle's oxidant load by reducing of H_2O_2 concentrations (Figure 5 A) may have prevented the aging-induced increase in CuZnSOD activity, resulting in a younger antioxidant profile.

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The reduction of H_2O_2 concentrations in gastrocnemius muscles from aged animals supplemented with resveratrol appears to have assuaged the accumulation of the lipid peroxidation byproducts Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE). This is consistent with recent data from our laboratory and others that have shown that resveratrol protects against H_2O_2 mitigated lipid peroxidation in *vivo* (32; 67) and *in vitro* (9). Although the muscle oxidant load (H_2O_2) was reduced and the level of lipid peroxidation was attenuated in aged gastrocnemius muscles, resveratrol supplementation was unable to protect skeletal muscle from aging-induced protein oxidation as measured by protein carbonyl formation (Figure 5C). This observation differs from recent rodent studies in both diabetic (52) and cancer (8) models showing that resveratrol was able to diminish protein oxidation in vivo. It is probable that the results of the current study are more representative of the effects that resveratrol can have under basal conditions in aged animals and it may prove to be more protective under perturbations that involve excessive stress such as disease states and/or injuries resulting in chronic inflammation, or under muscle wasting conditions such as cachexia or hindlimb suspension.

Given that resveratrol supplementation has been shown to improve muscular endurance (40) and increase mitochondrial biogenesis in skeletal muscle (6; 40), it was conjectured that the primarily fast twitch plantaris muscles from supplemented animals would show an improved resistance to fatigue. Presumably these muscles would show a shift in muscle characteristics that would be more in line with a slower muscle phenotype. This did not turn out to be the case, and in fact, resveratrol supplementation not only conferred no protection against muscle fatigue (Figure 6D), it in fact shifted the

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force-frequency curves of the plantaris muscles rightward (Figure 6A), indicating that the muscles had properties more consistent with a faster phenotype. One possible explanation for this phenomenon is that resveratrol supplementation, by retarding oxidative stress and damage, may have prevented the atrophy and/or loss of type II fibers that is known to occur in aged skeletal muscle (42) and thus was able to preserve a younger muscle profile. It is also possible, although not addressed in the current study, that resveratrol could improve the capacity of aged muscles to regenerate. In a recent study, resveratrol stimulated muscle precursor cell proliferation in a Sirt1-dependent manner (60), further expanding the mechanisms by which resveratrol supplementation may improve muscle mass and function by enhancing muscles regenerative capacity, which has been shown to be augmented with aging (17; 18) and in certain pathological conditions.

Taken as a whole, the results of the current study indicate that long-term dietary supplementation with moderate doses of resveratrol may prove to be beneficial by upregulating MnSOD and thus reducing the oxidant load present in the skeletal muscle environment and hence preventing some measures of oxidative damage. However, the current experimental protocol proved to be insufficient to enhance mitochondrial integrity or content. It is possible that a greater increase in Sirt1 protein and/or activity may be needed before a change in mitochondrial function will be observed. Further work is needed to determine if resveratrol has the potential to be an effective therapeutic agent to treat muscle functional decrements associated with elevated oxidative stress in aged individuals or in conditions of muscle loss such as prolonged

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bedrest, presumably through a pharmacological pre-conditioning effect resulting in an improved redox status associated with these conditions.

Acknowledgements

TABLE LEGENDS

Table 1   **Descriptive Data.** Animals were weighed prior to sacrifice, body weights (BW) are represented in grams (g). Gastrocnemius and plantaris muscles were dissected, immediately blotted and weighed as an estimate of muscle size. The data are presented in grams (g), or as a ratio to the animal's body weight (mg/g). Food consumption was assessed for each experimental group and is reported as g of food per day. Resveratrol was fed to aged mice for 10 months in a rodent chow that contained 0.05% trans-resveratrol. Resveratrol intake is reported as grams per day normalized to kg of bodyweight (g/kg/day). Significance was set at ($p \leq$ 0.05) and all data are represented as mean ± standard error. [#] denotes significantly different than young animals (Aging Effect).

Figure 1   **Silent mating type information regulation homolog1 (Sirt1) enzyme activity and protein content. (A)** Sirt1 protein content was measured via immunoblotting in total gastrocnemius muscle homogenate **(B)** Sirt1 enzyme activity was determined fluorometrically in gastrocnemius muscle homogenate. Data are expressed as arbitrary fluorescent units (AFU)/µg protein. **YC= Young Control, MAC = Middle-Aged Control, AC = Aged Control, AR = Aged Resveratrol**. Significance was set at ($p \leq 0.05$) and all data are presented as mean ± standard error. **#** $p \leq 0.05$, young control (Aging Effect). * $p \leq 0.05$, aged control vs. age resveratrol (Supplementation Effect).

Figure 2   **PGC1 protein content and citrate synthase enzyme activity (A)** PGC1 protein content was measured via immunoblotting in gastrocnemius muscle homogenate **(B)** Citrate synthase enzyme activity was measured kinetically in homogenates from gastrocnemius muscles. Data are expressed as µmol/min/mg protein. **YC= Young Control, MAC = Middle-Aged Control, AC = Aged Control, AR = Aged Resveratrol.** Significance was set at ($p \leq 0.05$) and all data are represented as mean ± standard error. **#** $p \leq 0.05$, young control (Aging Effect). * $p \leq 0.05$, aged control vs. age resveratrol (Supplementation Effect).

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Figure 3   **Subcellular cytochrome c protein content. (A)** Cytochrome c protein

content was measured, via immunoblotting, in the total, cytosolic

(mitochondrial-free), and mitochondrial fractions of vastus lateralis

muscles as an estimation of both mitochondrial content and mitochondrial

membrane integrity. **(B)** Immunoblots of the mitochondrial form of SOD

(MnSOD) and the cytosolic form of SOD (CuZnSOD) to illustrate the purity

of the tissue fractions. **YC= Young Control, MAC = Middle-Aged

Control, AC = Aged Control, AR = Aged Resveratrol**. Significance was

set at ($p \leq 0.05$) and all data are represented as mean ± standard error. **#**

p ≤ 0.05, young control (Aging Effect). * *p* ≤ 0.05, aged control vs. age

resveratrol (Supplementation Effect).

Figure 4   **Isoform specific superoxide dismutase activity and protein content.**

(A) MnSOD activity was assessed colorimetrically in mitochondria isolated

from vastus lateralis muscles. Data are expressed as U/mL/mg protein.

(B) MnSOD protein content was analyzed via immunoblotting in isolated

mitochondria of vastus lateralis muscles **(C)** CuZnSOD activity was

assessed colorimetrically in the mitochondria-free cytosolic fraction from

vastus lateralis muscles. Data are expressed as U/mL/mg protein. **(D)**

CuZnSOD protein content was analyzed via immunoblotting in the

mitochondria-free cytosolic fraction of vastus lateralis muscles. **YC=**

Young Control, MAC = Middle-Aged Control, AC = Aged Control, AR

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= Aged Resveratrol. Significance was set at ($p \leq 0.05$) and all data are represented as mean ± standard error. Significance was set at ($p \leq 0.05$) and all data are represented as mean ± standard error. **#** $p \leq 0.05$, young control (Aging Effect). * $p \leq 0.05$, aged control vs. age resveratrol (Supplementation Effect).

Figure 5   **Resveratrol attenuated increases in hydrogen peroxide (H_2O_2) concentration and lipid peroxidation associated with aging, but did not prevent protein carbonyl formation. (A)** H_2O_2 concentrations were determined fluorometrically in gastrocnemius muscle homogenate. Data are expressed as μmols/H_2O_2/μg protein. Significance was set at ($p \leq 0.05$) and all data are represented as mean ± standard error. **(B)** MDA & HAE levels were evaluated as a combined marker of lipid peroxidation and expressed in μM[MDA/HAE]/mg protein. **(C)** Protein carbonyl formation was analyzed as a marker of protein oxidation in gastrocnemius muscle homogenate. Data are expressed as nmol/mL. **YC= Young Control, MAC = Middle-Aged Control, AC = Aged Control, AR = Aged Resveratrol.** Significance was set at ($p \leq 0.05$) and all data are represented as mean ± standard error. **#** $p \leq 0.05$, young control (Aging Effect). * p ≤ 0.05, aged control vs. age resveratrol (Supplementation Effect).

Figure 6   *In vitro muscle physiological analyses.* Isometric muscle contractile

properties were examined in the plantaris muscles of control and

resveratrol-treated mice. (**A)** Graphical depiction of a force frequency

curve illustrating the maximal force produced in plantaris muscles at a

given frequency. Data are presented as the force production at a given

stimulation frequency relative to maximal force of that muscle. **(B)**

Contraction time (CT)and ½ relaxation time (½ RT) were analyzed to

determine twitch properties in plantaris muscles. Data are presented as a

unit of time (ms) **(C)** The twitch to tetanus ratio (P_t/P_o) was assessed in

plantaris muscles. Data are presented as a ratio of twitch force to maximal

force.**(D)** A modified Burke protocol was implemented to asses muscle

fatigue in plantaris muscles. Data are presented as a measure of fatigue

index, calculated as a percent change from the first to last contraction

(120th). **MAC = Middle-Aged Control, AC = Aged Control, AR = Aged**

Resveratrol. Significance was set at ($p \le 0.05$) and all data are

represented as mean ± standard error. * $p \le 0.05$, aged control vs. age

resveratrol (Supplementation Effect).

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Table 3.1:

Descriptive Data	Young Control	Middle Aged Control	Aged Control	Aged Resveratrol
Body Weight (BW)	26.5 ± 2.2	34.3 ± 2.3#	33.8 ± 3.9#	35.2 ± 2.3#
Gastrocnemius Muscle Wet Weight (g)	.141 ± .02	.150 ±.01	.126 ±.01#	.130 ±.01#
Gastrocnemius Muscle Wet Weight(g)/Body Weight(g)	5.34 ± .59	4.26 ± .28#	3.74 ± .33#	3.71 ± .25#
Plantaris Muscle Wet Weight (g)	.020 ± .001	.023 ± .004	.18 ± .002#	.019 ± .003
Plantaris Muscle Wet Weight/Body Weight(g)	.80 ± .03	.66 ± .04#	.54±.02#	.55 ± .02#
Food Intake (g/day)	3.4 ± .18	3.8 ± .39	3.1 ± .27	3.2 ± .14
Resveratrol (g/kg/day)	n/a	60.8 ± 4.0	n/a	45.9 ± 3.9#

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Figure 3.1:

Figure 3.2:

A. PGC1 Protein Content

YC   MAC   AC   AR

Optical Density

0.75
0.60
0.45
0.30
0.15
0.00

YC MAC AC AR

PGC1   ~90kD

GAPDH   36kD

B. Citrate Synthase Activity

YC   MAC   AC   AR

CS Activity (µmol/min/mg protein)

350
280
210
140
70
0

#   #

Figure 3.3:

A.

Cytosolic Cytochrome c Protein Content

B.

Figure 3.4:

A. MnZnSOD Activity

C. CuZnSOD Activity

B. MnSOD Protein Content

D. CuZnSOD Protein Content

Figure 3.5:

A. H$_2$O$_2$ Concentration
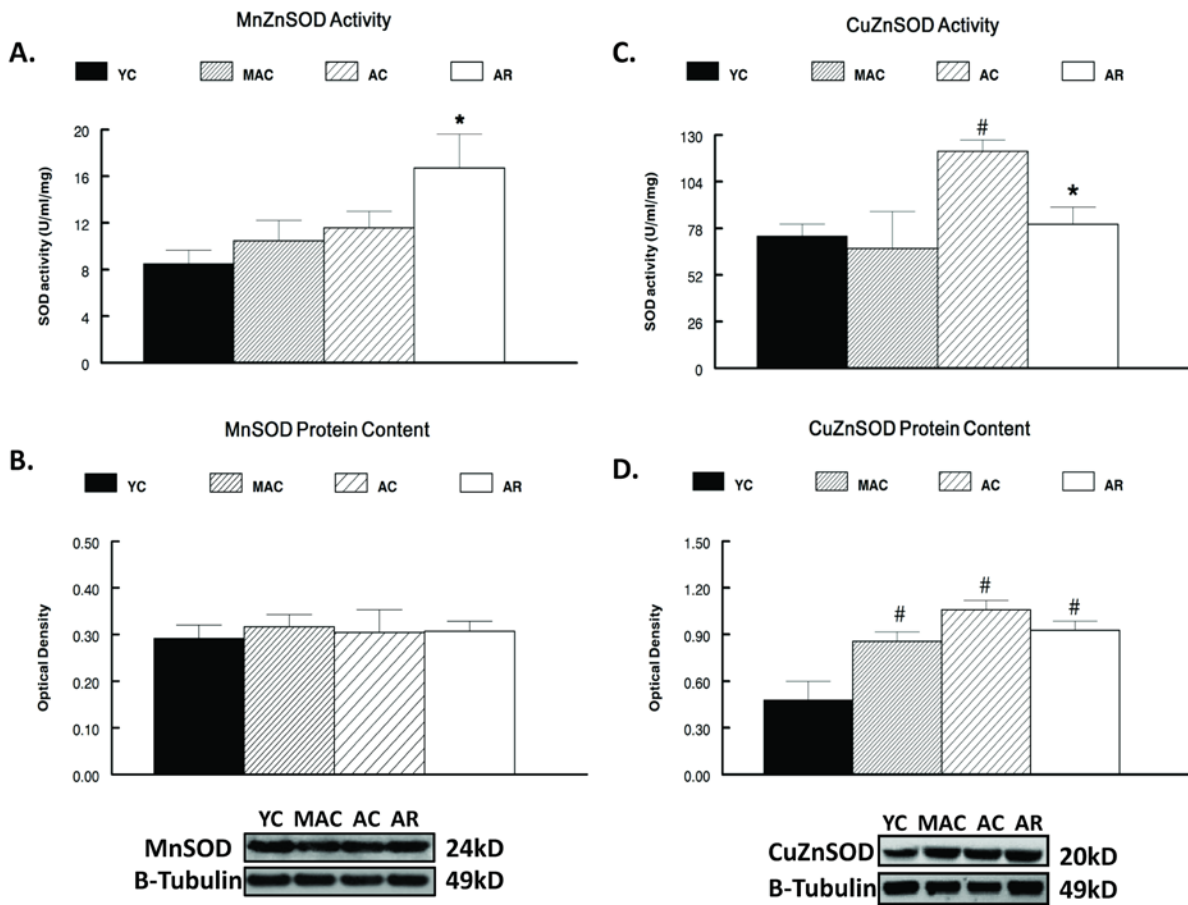
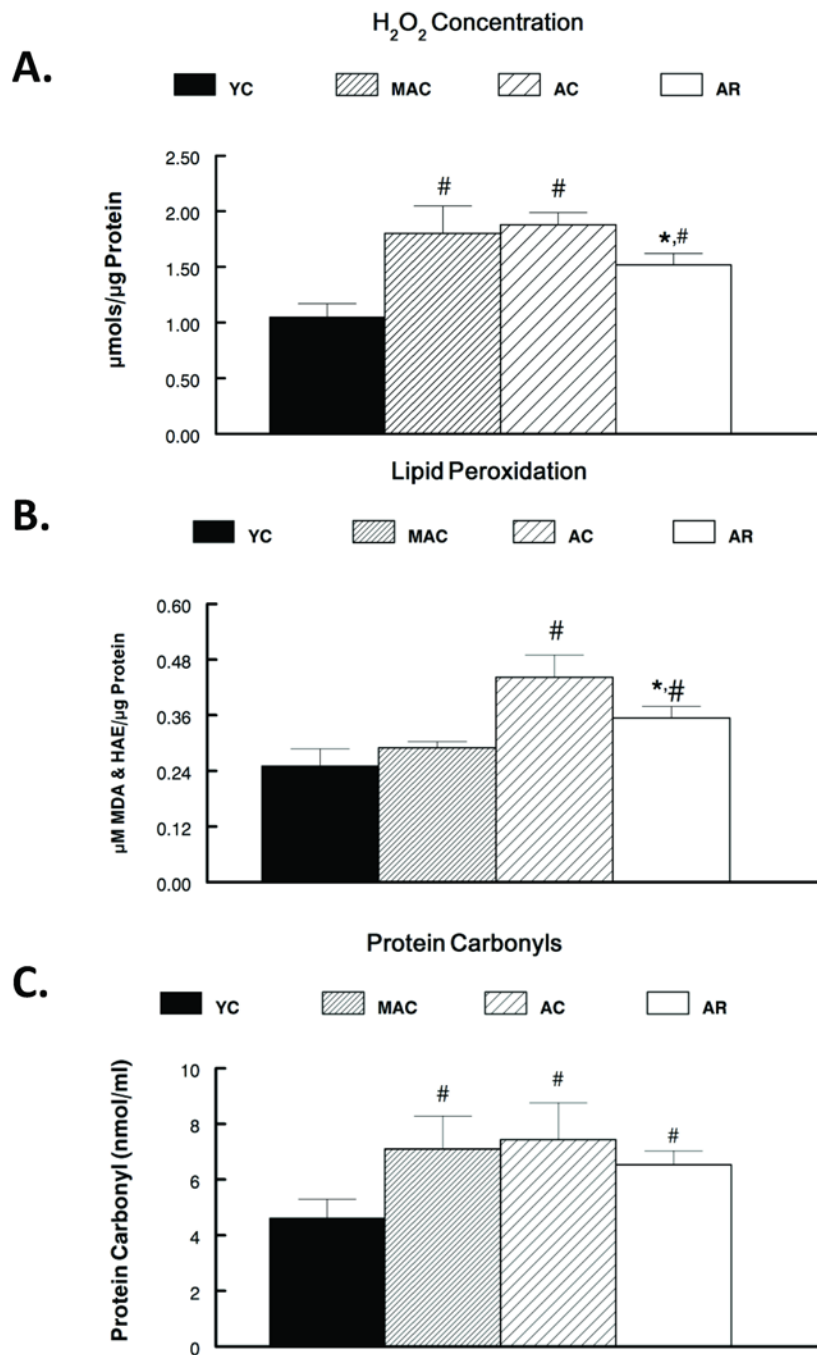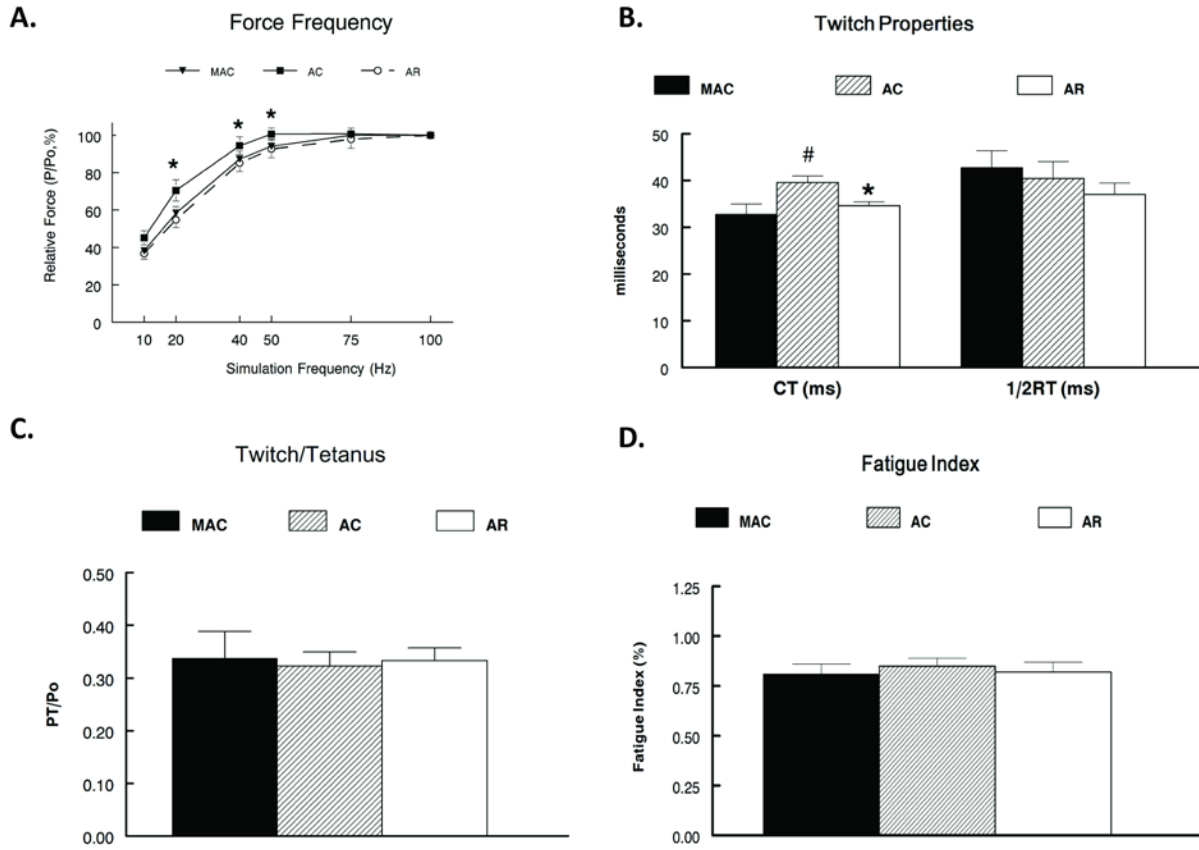B. Lipid Peroxidation

C. Protein Carbonyls
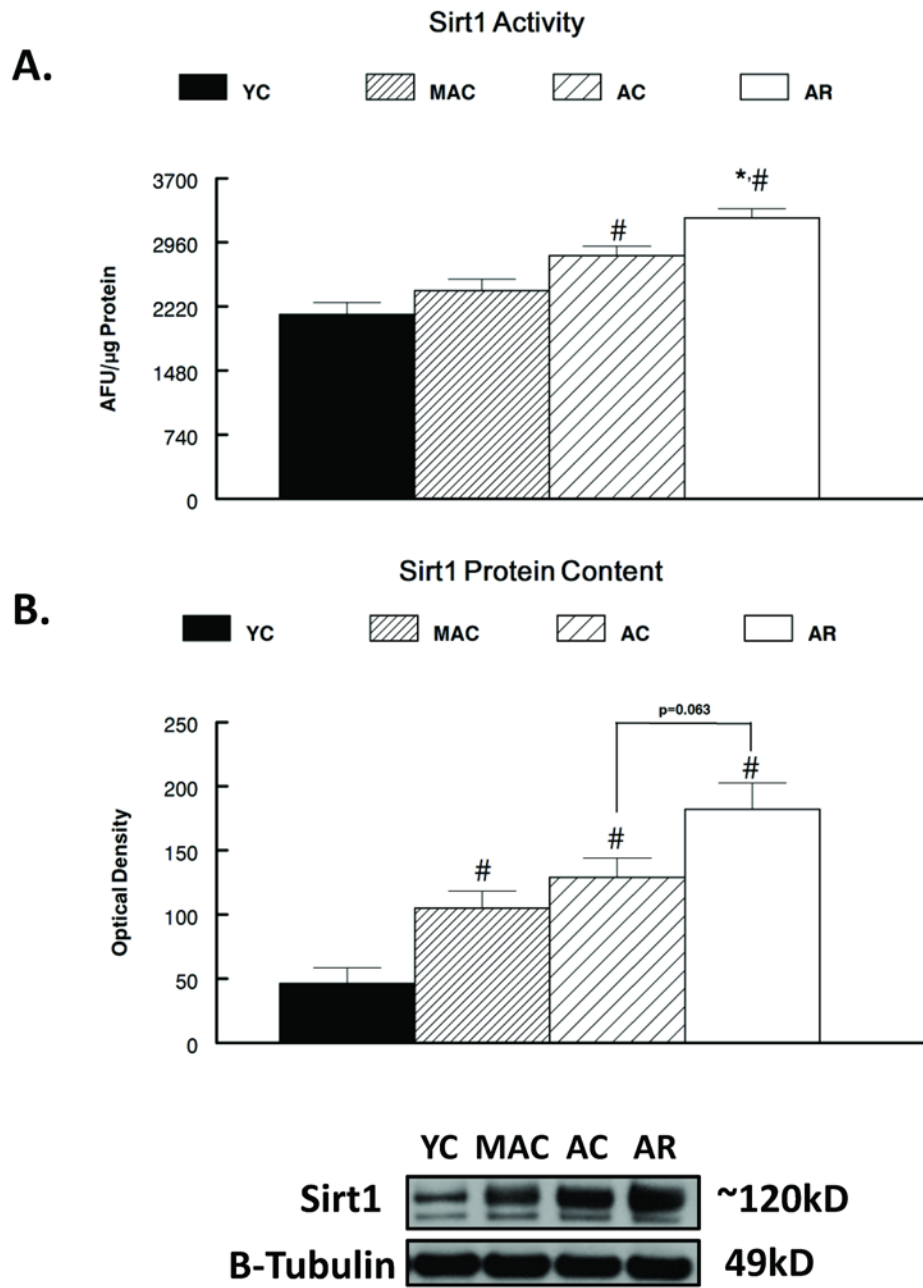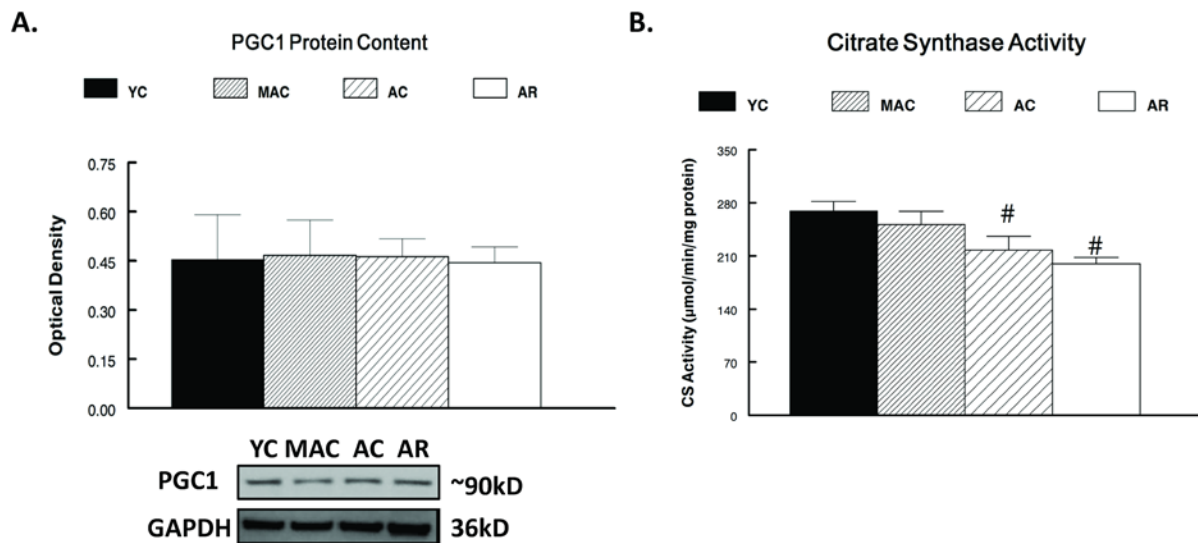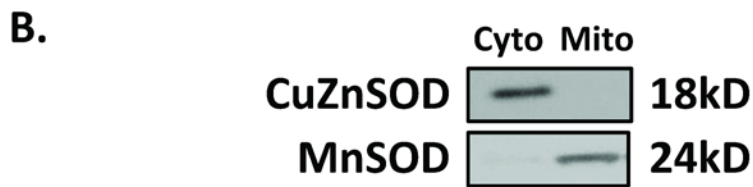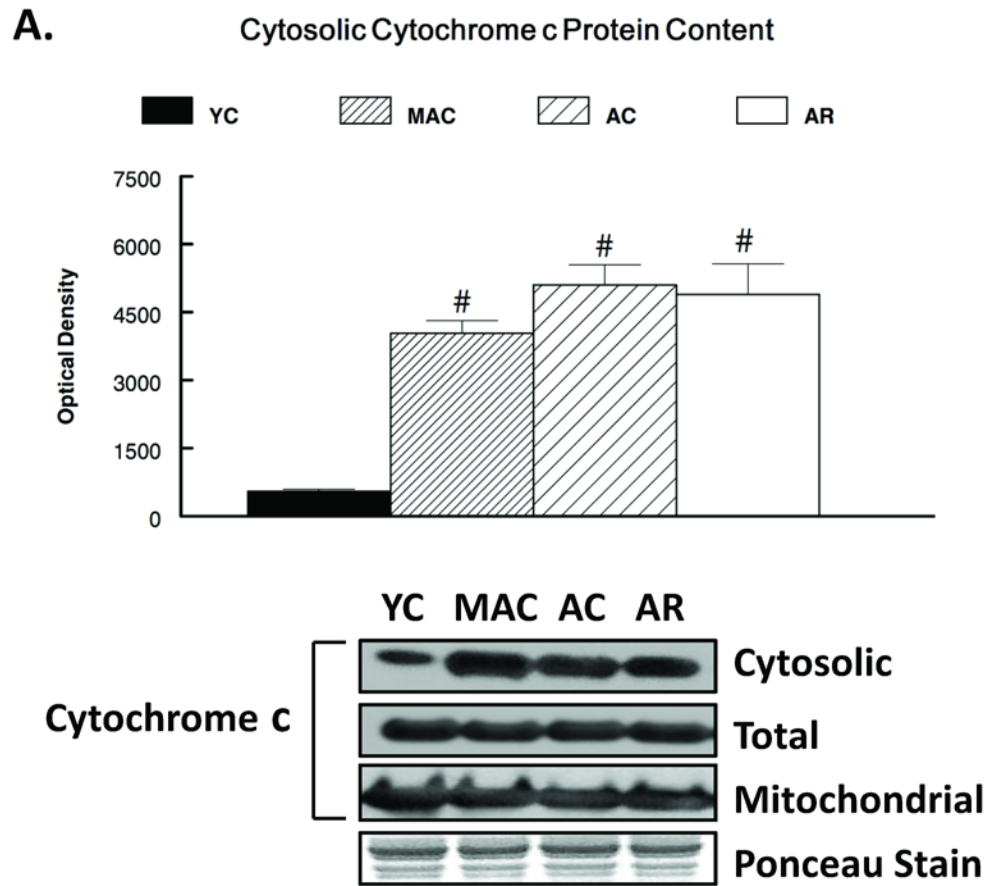
Figure 3.6:

Chapter 4: General Discussion and Conclusions:

<u>Overview:</u>

The overall goal of this dissertation was to determine the efficacy of resveratrol supplementation to ameliorate the detrimental relationship between oxidative stress, apoptosis and skeletal muscle atrophy as they pertain to both muscle disuse and aging. Furthermore, the included studies sought to elucidate resveratrol's capacity to upregulate silent mating type information regulation homolog1, (Sirt1), and to determine if upregulation of Sirt1 by resveratrol would enhance mitochondrial integrity and content. Our <u>long-term goal</u> was to determine if supplementation with resveratrol could prove to be an effective therapeutic agent to attenuate muscle atrophy associated with chronic disuse (hypodynemia and hypokinesia) and also to ascertain if long-term supplementation with dietary resveratrol could prove to be an effective countermeasure to slow the progression of sarcopenia, the age-associated loss of muscle mass and function.

The *central hypothesis* of the dissertation was that resveratrol would protect skeletal muscle from oxidative stress via upregulation of the endogenous antioxidant system and augmentation of mitochondrial membrane integrity. This would result in a reduction of both oxidant release and pro-apoptotic protein release from the mitochondria, the leading to reduction in oxidative damage and downstream apoptosis, with the expected outcome to be an attenuation of muscle atrophy. The *rational* for the project was to shed light on molecular mechanisms of muscle atrophy induced by both aging and disuse and to attempt to a possible intervention, via resveratrol

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supplementation, to help limit increased oxidant exposure and thus an atrophic muscle environment.

Taken as a whole the cumulative results of the dissertation are in agreement with the majority of the current literature confirming that increases in oxidative stress occur concomitantly with both disuse-mediated skeletal muscle atrophy (chapter 2) and sarcopenia (chapter 3). Furthermore, apoptotic signaling in the skeletal muscle environment was increased in response to both hindlimb suspension and advanced age (chapter 2). The novelty of this dissertation was to evaluate the efficacy of resveratrol supplementation to attenuate oxidative stress and thus mitigate downstream mediators of muscle atrophy.

The efficacy of resveratrol to mitigate the effects of skeletal muscle disuse:

Hindlimb suspension (HLS), which is a model that elicits both hypodynemia and hypokinesia in conjunction with unloading of the hindlimb muscles, caused significant atrophy of gastrocnemius muscles from both young and aged animals (Figure 2.5). Oxidative stress and apoptotic signaling were also elevated in response to HLS in muscles from both young and aged animals, although to a greater extent in muscles from aged animals. This study yielded mixed results with regard to the efficacy of resveratrol administration to ameliorate disuse mediated atrophy. With the exception of the upregulation of catalase activity (Figure 2.4) in muscles from young HLS animals, resveratrol had no protective, or detrimental, effects on muscles from young animals. In aged gastrocnemius muscles, twenty-one days of resveratrol administration was effective at reducing both hydrogen peroxide (H_2O_2)

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concentrations and oxidative damage in the form of lipid peroxidation (Figure 2.3) in muscles exposed to HLS. These reductions in oxidative stress indices coincided with the fact that resveratrol enhanced the endogenous anti-oxidant system in the form of increases in both MnSOD and catalase protein contents and enzymatic activities (Figure 2.2). Although not addressed in chapter 2, increases in MnSOD and catalase activity were likely mediated by resveratrols ability to upregulate Sirt1 in gastrocnemius muscles from aged animals (Figure 4.1), given that resveratrol has been repeatedly shown to upregulate endogenous antioxidant enzymes in a Sirt1-FOXO dependent manner (as discussed in Chapter 1).
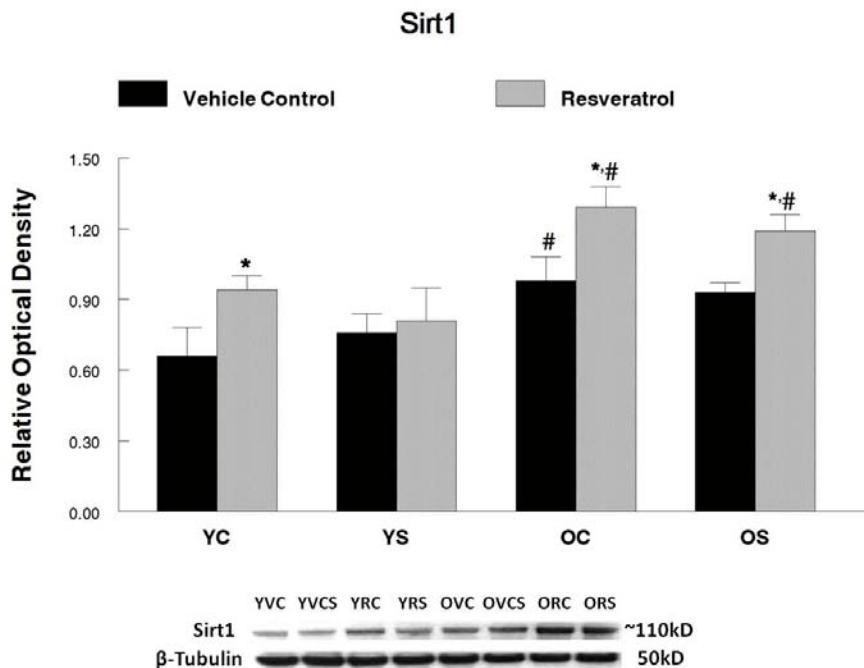

Figure 4.1- Sirtuin 1 protein content in gastrocnemius muscles. Gastrocnemius muscles from young and aged animals exposed to 21 days of resveratrol administration (12.5mg/kg/day) and 14 days of hindlimb suspension. **YC= Young Control, YS = Young Suspended, OC = Old Control, OS = Old Suspended, YVC = Young Vehicle Control, YVCS = Young Vehicle Control Suspended, YRC = Young Resveratrol Control, YRS = Young Resveratrol Suspended, OVC = Old Vehicle Control, OVCS = Old Vehicle Control Suspended, ORC = Old Resveratrol Control, ORS = Old Resveratrol Suspended.** **p $\leq$ 0.05, non-suspended control vs. suspended animals (Suspension effect). # p $\leq$ 0.05, old vs. young treatment matched (Aging effect). *p $\leq$ 0.05, vehicle control vs. age-matched and treatment matched groups (Effect of resveratrol).

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Despite reductions in pro-apoptotic signaling and the upregulation of the anti-apoptotic protein Bcl-2 (Figure 2.5), resveratrol administration was not an effective countermeasure to disuse-mediated increases in apoptotic signaling in aged skeletal muscle. However, in terms of the functional outcomes the results were quite promising. Resveratrol administration was effective in preserving the relative ratio of muscle mass to body mass and significantly attenuated the loss of maximal isometric force in aged plantarflexor muscles following HLS (Figure 2.5). Similarly to both, indices of oxidative stress and apoptosis this protective effect of resveratrol was age dependent and only present in aged animals. To the author's knowledge this is the first study presenting data that shows a clear differential effect of resveratrol in skeletal muscle that is dependent upon the age of the animal. The overall results of the study indicate that resveratrol has the potential to be a therapeutic agent, likely through a pharmacological pre-conditioning effect that acts to upregulate the endogenous anti-oxidant enzyme system potentiating the system to be able to handle the increased oxidant load that is present in acute conditions of muscle wasting.

Sarcopenia progression in long-term resveratrol supplemented animals.

Given the fact that acute resveratrol supplementation was effective at reducing oxidative stress and preserving relative muscle mass and function in aged skeletal muscle following disuse-mediated atrophy, the second study in this dissertation sought to ascertain if long-term dietary supplementation with resveratrol would reduce the chronic basal levels of oxidative stress that are often found in aged skeletal muscle. My hopes were that by reducing oxidative stress over a long period of time, resveratrol could potentially slow the progression of sarcopenia. Additionally, in light of the fact that

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resveratrol was able to upregulate Sirt1 following acute administration (figure 4.1), the second study aimed to clarify if upregulation of Sirt1 by resveratrol over prolonged periods would affect mitochondrial signaling, as measured by PGC1 signaling which is a known target of Sirt1 (as discussed in chapter 1)

Muscles from aged (28mo) mice weighed significantly less than muscles from either young (6mo), or middle-aged mice (18mo), even when normalized to body weight confirming the notion that with advanced age there is a progressive loss of muscle mass (Figure 3.1). Counter to what was hypothesized, long-term (10mo) dietary supplementation with resveratrol was unable to attenuate sarcopenia in either gastrocnemius or plantaris muscles (Table 3.1). Despite the lack of preservation of muscle mass seen in supplemented animals, dietary resveratrol supplementation was effective in reducing indices of oxidative stress including reduction of H_2O_2 levels and lipid peroxidation levels (Figure 3.4). In congruence with the effects of resveratrol on aged muscle from HLS animals, long-term resveratrol supplementation effectively upregulated MnSOD activity and decreased CuZnSOD activity. Presumably these results are due to the interplay of increased MnSOD activity effectively reducing H_2O_2 concentrations and in turn the reduction in H_2O_2 levels are likely responsible for the reductions in CuZnSOD activity which is known to become upregulated in response to oxidant load.

Although not addressed in the main body of chapter two, markers of apoptotic signaling and DNA fragmentation were assessed in vastus lateralis muscles to determine if reductions in oxidative stress would results in a reduction in the translocation of the pro-apoptotic Bax to the mitochondrial membrane. As discussed at

length in chapter 1, this translocation is initiated by an oxidant-sensitive conformational change in the Bax protein that allows Bax to disassociate from the anti-apoptotic protein Bcl-2 in the cytoplasm. This leaves Bax free to translocate to the outer-mitochondrial membrane and initiate pore formation leading to an eventual loss of mitochondrial membrane integrity, the end result being a release of cytochrome c and eventual apoptosis. Long-term resveratrol supplementation was not effective in reducing the translocation of Bax to the mitochondria, nor was it effective in reducing the total cytosolic protein content of Bax (Figure 4.2). Moreover, unlike the results from the acute administration of resveratrol, long-term supplementation with resveratrol was unable to increase the anti-apoptotic Bcl-2 protein (Figure 4.2) or lower caspase 3 activity (Figure 4.3).
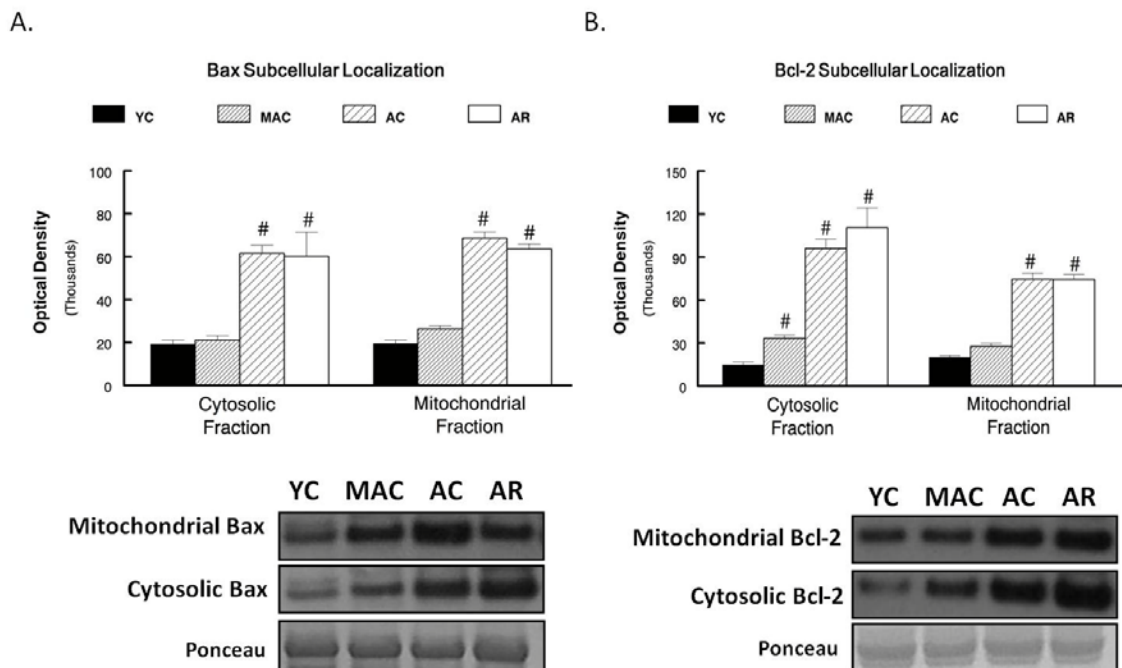

Figure 4.2- The subcellular localization of Bax and Bcl-2. Vastus lateralis muscles were fractionated into a mitochondrial pellet and a mitochondria-free cytosolic fraction for assessment of the subcellular localization of the pro-apoptotic Bax protein and the anti-apoptotic Bcl-2 protein. **A.** Immunblot analysis of Bax protein content. Data are expressed as a measure of optical density and a representative ponceau stain is displayed as qualitative assurance of equal protein loading. **B.** Immunblot analysis of Bcl-2 protein content. Data are expressed as a measure of optical density and a representative ponceau stain is displayed as qualitative assurance of equal protein loading. **YC =**
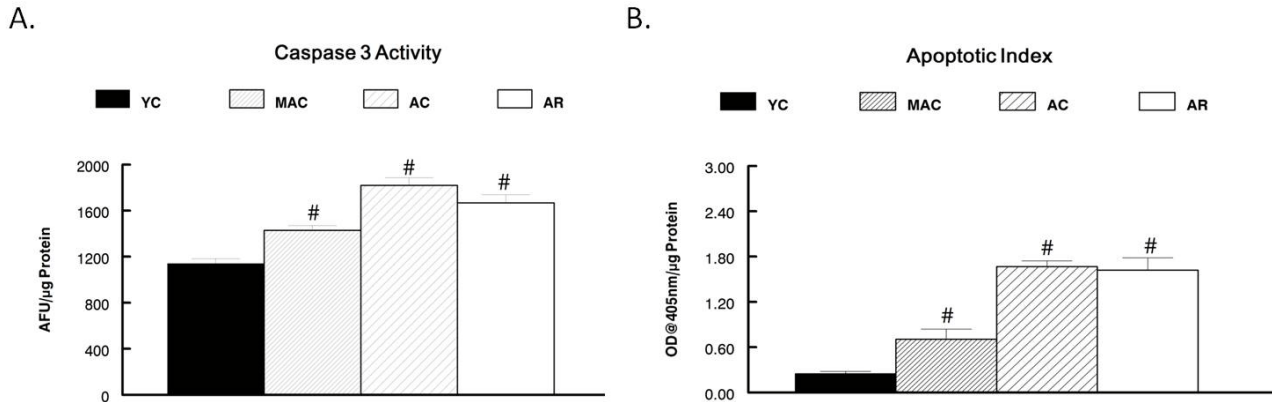
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Figure 4.3- Apoptotic Signaling: Apoptotic indices were measured in vastus lateralis muscles. **A.** A fluorescent enzyme activity assay was used to assess the activity of the executioner caspase, caspase 3, within vastus lateralis muscle homogenate. The data are presented as normalized fluorescent intensity. **B.** An ELISA quantifying DNA fragmentation was implemented to estimate the levels of apoptosis, within vastus lateralis muscles. The results are represented as normalized optical density readings and are linearly related to the amount of fragmented DNA present in the muscle homogenate. **YC = young control, MAC = middle-aged control AC = aged control AR = aged resveratrol supplemented.** Significance was set at (p ≤ 0.05) and all data are presented as mean ± standard error. # p ≤ 0.05, young control (Aging Effect).

Not surprisingly then, there was no attenuation of DNA fragmentation (Figure 4.3), the hallmark of apoptosis, by long-term resveratrol supplementation. These data highlight the differences between acute and long-term exposure to resveratrol and also suggest that a perturbation may be necessary (i.e. muscle disuse) in order to observe any protective effective that is attributable to resveratrol supplementation. Furthermore, the results of the dissertation as whole bring into question the assumed linear relationship that is often thought to exist between oxidative stress and apoptosis. It seems more likely that the increases in oxidative stress and apoptotic signaling that occur with both advanced age and muscle wasting conditions are concomitant conditions that although may be correlated are not casual to each other.

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Sirt1 activity was modestly upregulated following ten months of dietary resveratrol supplementation (Figure 3.1). Unfortunately, this augmented Sirt1 activity did not translate to increased citrate synthase activity, PGC1 protein content or improved mitochondrial membrane integrity. It was hypothesized that through the upregulation of Sirt1 and subsequently PGC1, skeletal muscles from supplemented animals would display enhanced resistance to muscle fatigue. This hypothesis was rejected since plantaris muscles from aged supplemented animals did not show any resistance to muscle fatigue (figure 3.6), it should be noted that muscle fatigue was measured *ex vivo* and therefore these data were unable to take into account neuronal, and/or systemic factors that can contribute to muscle fatigue. Furthermore, plantaris muscles from supplemented animals displayed a rightward shift of their force-frequency curves indicative of a faster muscle profile, a direct contrast to what one would expect from a muscle that had undergone a metabolic shift to a more oxidative state.

Although others have shown that resveratrol's ability to activate Sirt1 leads to a PGC1-mediated enhancement of oxidative metabolism, muscle fatigue resistance and mitochondrial biogenesis, our results do not corroborate a relationship between Sirt1 upregulation and PGC1 signaling. This may be due in part to the wide range of dosages, treatment durations and modalities of resveratrol administration present in the current body of literature. The results of this dissertation suggest that the capacity of resveratrol to protect skeletal muscle from increases in oxidative stress and consequently oxidative damage likely resides in resveratrols ability to activate Sirt1 and via this upregulation enhance components of the endogenous anti-oxidant system. It also would seem that increases in oxidative stress can influence muscle mass and

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function, but they are not causative factors mediating apoptosis in skeletal muscle. It is also possible, given that resveratrol has been shown to be an effective anti-inflammatory agent, that reductions in inflammation within aged skeletal muscle lessen the effects of exogenous stressors and therefore improve the outcomes of muscle following atrophy-mediating events. Further work is needed to determine if resveratrol has the potential to be an effective therapeutic agent to treat muscle functional decrements associated with chronic elevated oxidative stress in aged individuals. However, its effectiveness as a pharmacological pre-conditioner in response to acute stress seems to be better established and appears to be the result of improving the redox status associated with atrophic conditions.

Future directions and recommendations:

Upcoming studies that seek to further understand of the molecular effects of resveratrol on skeletal muscle may be better served to focus on pathways outside of the oxidative stress-apoptosis continuum and perhaps investigate in more detail the Sirt1-dependent effects that resveratrol can have on stress resistance (i.e. FOXO mediated upregulation of MnSOD and Catalase) and mitochondrial signaling. Furthermore, given the relative importance of insulin like growth factor (IGF-1) and mammalian target of rapamycin (mTOR) signaling with regard to muscle protein synthesis it would also be interesting and prudent to evaluate the effects, if any, that resveratrol and Sirt1 signaling may have with regard to this metabolic signaling network, given that Sirt1 is a powerful modulator of many metabolic pathways including insulin signaling. Although the current experimental protocol did not elicit enhanced PGC1 signaling, this is likely due to the

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dosage and modality of supplementation. Other investigators have shown successful augmentation of mitochondrial biogenesis and improved oxidative capacity in skeletal muscle from rodents receiving dietary resveratrol supplementation that was prepared in a high fat medium. Given that resveratrol, is fat soluble, the lack of increased fat content in our dietary model may have prevented maximal absorption of the compound and could explain the failure of resveratrol to bring about an increase in PGC1 signaling in the current study. Therefore if the chosen route of resveratrol exposure is going to be dietary, the chow should be made with a higher fat content.

To better determine if resveratrol protects against increased oxidative stress by both augmenting the antioxidant defense system and enhancing mitochondrial biogenesis, more exact measures to quantify mitochondrial function and content should be used. Fluorescent activated cell sorting analysis could be employed to more directly measure mitochondrial density, size and internal complexity, electron transport chain respiratory capacity to quantify mitochondrial capacity and mitochondrial membrane potential ($\Delta\Psi_m$) could be measured by flow cytometry using the ratiometric dye 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazol carbocyanine iodide (JC-1; Molecular Probes, Carlsbad, CA). Additionally, pinpointing the source of ROS from the mitochondria could be obtained concomitantly to mitochondrial respiratory measurements by using the oxidation-dependent compound, dihydroethidium, to measure intracellular levels of $O_2^{\bullet-}$ and ROS in isolated mitochondria by flow cytometry. With regard to skeletal muscle form and function it would be both novel and beneficial to measure the effects of resveratrol on muscle fiber composition and cross-sectional area. To date the literature has only established the effects of resveratrol

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supplementation on skeletal muscle enzyme composition and muscle wet weight. There are also several Sirt1 transgenic models to choose from that may prove to be more exact in delineating the exact molecular signaling pathways that Sirt1 targets and thus further the understanding of the potential therapeutic role(s) that resveratrol may fulfill.

Janna R. Jackson

125 Cobblestone Circle, Morgantown, WV 26505

Cell Phone (304)-288-4067 ~ Work Phone: (304)-293-1984 ~ Email: janna_jackson@hotmail.com

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EDUCATION

Ph.D. Cellular and Integrative Physiology
2005-Present West Virginia University, Morgantown WV

> **Doctoral Dissertation:** The role of resveratrol supplementation in mediating oxidative stress and apoptosis in aged skeletal muscle (Expected Graduation Date; December 2010)

2002-2004 **Masters of Science Exercise Physiology**
Midwestern State University, Wichita Falls, TX

1999-2000 **Secondary Teaching Credential Grades 6-12, Biology and Chemistry**

California Polytechnic State University, San Luis Obispo, CA

1994-1999 **Bachelor of Science Microbiology and Immunology**
California Polytechnic State University, San Luis Obispo, CA

PEER REVIEWED PUBLICATIONS

Janna R. Jackson, Michael J. Ryan, and Stephen E. Alway. Mediation of Endogenous Antioxidant Enzymes and Apoptotic Signaling by Resveratrol Following Muscle Disuse in the Gastrocnemius Muscles of Young and Old Rats. *J Gerontol A Biol Sci Med Sci (2010) 65A(8): 815-831*

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Michael J. Ryan**, Janna R. Jackson** and Stephen E. Alway. Inhibition of xanthine oxidase activity reduced oxidative stress, apoptosis and improved muscle function in the plantar flexors from young and aged mice (Submitted to *Free radical Biology and Medicine*)

Yanlei Hao, **Janna R. Jackson**, Yan Wang, Neile Edens, Suzzette L. Pereira, and Stephen E. Alway. Hydroxy-β-methylbutyrate reduces muscle mass loss and myonuclear apoptosis improving recovery following hindlimb suspension in aged rats. (In Progress)

Stephanie Wright, **Janna R. Jackson**, Joesph Gigliotti1, Stephan E. Alway and Janet C. Tou. Resveratrol Supplementation Influences Bone Properties in Ambulatory and Hindlimb Suspended Old Fisher 344 x Brown Norway Male Rats (Submitted) *Osteoporosis International*

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AWARDS

- Julie Betschart Research Convocation, Physiology Student Researcher of the Year
  - First Place Overall 2008
- NIH 2008- Current Issues and Recent Developments in Dietary Supplement Research Practicum
  - Student Attendee
  - NIH 2008- Student Travel Award ($1200)
- West Virginia University Van Liere Research Day 2008 Second place poster presentation
- West Virginia School of Medicine Graduate Student Travel Award 2008 ($500)
- West Virginia University Van Liere Research Day 2007 Second place poster presentation

LABORATORY PROFICIENCIES

Human:

VO2 max Testing
Body Composition Testing
Exercise Prescription
EKG
HR & Blood Pressure

Animal:

Small animal surgery (Birds & Rodents)
Surgical implantation
Hindlimb Suspension
Oral Gavage
In vivo Muscle Function Analyses
Ex Vivo Muscle Function Analyses
Maintenance of Transgenic Mouse Colonies

Cell Culture:

C2C12 Myoblast
Fluorescence-activated cell sorting (FACS)
Adeno-associated viral infection

Molecular Biology:

Immunoblot analysis
Total and sub-cellular protein isolation
Mitochondrial Isolation
Immunoprecipitation
Immunohistochemistry
DNA & RNA isolation
RT- PCR
ELISA
Enzyme Activity/Kinetics
Genotyping

TEACHING EXPERIENCE

2005-Present: Graduate Assistant West Virginia University School of Medicine

Mediator Human Function Problem Based Learning; First Year Medical Students

Physiology and Pharmacology Tutoring; Medical Students

Lab Mentoring to Master level research students

Student research mentor to undergraduate students in the West Virginia Idea Network of Biomedical Research Excellence (WV-INBRE) program 2006-2009

Physiology Small Group leader for first-year medical students 2007-2008 Human Function Course

Medical Student Tutoring 2007-2009

2002-2004: Teaching Assistant Midwestern State University

Laboratory Instructor for undergraduate Exercise Physiology and Exercise Testing and Prescription courses

Designed and implemented syllabus and course-work for physical health

and activity courses including Strength and Conditioning, Racquetball,

Softball, Cycling, and Introduction to Physical Fitness and Nutrition

2000-2001: Arroyo Grande High School

Grade 11-12: Advanced Placement Biology

Grades 9-10: Physical Science

REFERENCES

Stephen E Alway, Ph. D.
Professor, Chair of Exercise Physiology
820 HSS
West Virginia University
PO Box 9227
Morgantown, WV 26506
Phone: 304-293-0772
Fax: 304-293-7105
Email: salway@hsc.wvu.edu

Randall W Bryner, Ed. D.
Associate Professor
Division of Exercise Physiology
8317 HSS
PO Box 9227
West Virginia University
Morgantown, WV 26506
Phone: 304-293-0441
Fax: 304-293-7105
Email: rbryner@hsc.wvu.edu

John M. Hollander, Ph.D.
Assistant Professor
Division of Exercise Physiology
110- HSN
PO Box 9227
West Virginia University
Morgantown WV, 26506
Phone: 304-293-3683
Fax: 304-293-7105
Email: jhollander@hsc.wvu.edu