AGE-RELATED CHANGES IN CARDIAC MITOCHONDRIAL FUNCTION AND OXIDATIVE STRESS: INFLUENCE OF VOLUNTARY WHEEL RUNNING

By

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This dissertation is dedicated to my parents, Tom and Janis Phaneuf, for teaching me to believe that I could accomplish whatever I set my mind to, and for supporting and encouraging me through all my years of school (I promise this is it!). It is also dedicated to my husband Andy, for his love and support, and for never letting me lose sight of the things that are truly important in life.

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Mounting evidence indicates that mitochondrial dysfunction and oxidant production, along with an accumulation of oxidative damage, contribute to the aging process. Regular physical activity can delay the onset of morbidity, increase mean lifespan, and reduce the risk of developing several diseases. It has been hypothesized that the health benefits of chronic exercise may be at least partially due to a reduction in oxidant production and oxidative damage. Although two functionally different populations of cardiac mitochondria exist – subsarcolemmal (SSM) and interfibrillar (IFM) – few studies have examined age-related changes in oxidant production and oxidative stress in both populations. Therefore, we investigated the effects of age and long-term voluntary wheel running on mitochondrial function, oxidant production, antioxidant defenses, and oxidative stress in SSM and IFM.

We found an age-related increase in hydrogen peroxide (H_2O_2) production from SSM but not IFM. Furthermore, manganese superoxide dismutase (MnSOD) and

glutathione peroxidase (GPX) activities were increased in both populations with age, an adaptation that likely occurred as a result of chronic exposure to oxidants. Of note, IFM from old animals had higher MnSOD, GPX, and catalase (CAT) activities compared to SSM from old animals; and this may explain why we did not detect an increase in H_2O_2 production from IFM.

Long-term voluntary wheel running significantly reduced H_2O_2 production from both SSM and IFM, compared to sedentary animals. Additionally, MnSOD activity was lower in SSM and IFM from wheel runners, and this may reflect reduced mitochondrial superoxide production. Voluntary wheel running has been reported to increase mean lifespan in rodents, and our results suggest that a reduction in mitochondrial oxidant production may be a contributing factor.

Furthermore, IFM exhibited higher antioxidant enzyme activities, lower levels of glutathione, and increased oxidative damage compared to SSM; indicating that IFM are subjected to greater oxidative stress than SSM. Due to their close proximity to the myofibrils, IFM dysfunction may detrimentally impact myocardial function. Additionally, the dramatic differences observed between SSM and IFM demonstrate the importance of studying both populations when attempting to elucidate the contribution of mitochondrial dysfunction to myocardial aging.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Introduction

Aging is inevitable, and is characterized by a progressive deterioration in physiological functions and metabolic processes, ultimately leading to morbidity and mortality. The increased morbidity associated with aging is detrimental to the individual, who may experience diminished quality of life, and detrimental to society, which must spend tremendous amounts of money to treat the rapidly growing elderly population. Because of improvements in sanitation, diet, and health care over the past 100 years, the average human life expectancy has increased from 57 years (in 1900) to 80 years (in the 1980s). In fact, it is estimated that there will be more than 19 million U.S. citizens over the age of 85 by the year 2050 [1].

The incidence of cardiovascular disease is extremely high in elderly individuals and the primary cause of hospitalization, disability, and death in Americans over age 65 is heart failure [2-4]. Because the cost of treating heart failure and other cardiovascular diseases is so enormous, a vast amount of research has focused on elucidating the mechanisms that contribute to myocardial dysfunction with age. Although it is difficult to distinguish "normal" aging changes from those that are influenced by alterations in lifestyle (e.g., physical activity, nutrition, and environment) and the increased incidence of disease that often accompanies aging, it does appear that there are at least two major changes in the heart (myocardial “stiffening” and tissue remodeling) that can be attributed to normal aging [3, 5, 6]. In the myocardium of both humans and animals, it is

consistently observed that there is a decrease in the number of myocytes with age, and subsequent hypertrophy of the remaining myocytes [7-9]. One belief is that oxidative damage to myocytes ultimately triggers their death via necrotic or apoptotic processes [10]. Since adult cardiomyocytes have a limited ability to regenerate, they are generally replaced by fibroblasts, which produce collagen [3]. This results in an increased collagen-to-elastin ratio and more rigid ventricles in the aged heart. These age-related changes cause decreased cardiac distensibility and diastolic dysfunction, both of which can contribute to the development of chronic heart failure [2, 3, 6].

Despite the fact that all mammals age, there is no clear agreement as to how or why aging occurs. At least 24 different theories of aging have been proposed [11], most of which fall into one of two major categories [12]: 1) aging is the result of random damage to critical molecules or; 2) aging is a genetically controlled event. In all likelihood, the aging process probably involves some combination of the two: a genetically controlled program activated at conception that can be accelerated by random damage to macromolecules.

One of the most popular theories of aging is the free radical or oxidative stress theory of aging. This theory, originally postulated by Dr. Denham Harman in 1956 [13], proposes that free radicals (or reactive oxygen species), produced as by-products of normal metabolism, cause oxidative damage to macromolecules; and it is the accumulation of these damaged molecules that causes cellular dysfunction with age, eventually leading to death.

Another theory, one that is not mutually exclusive of the free radical theory, is the mitochondrial theory of aging [14, 15]. According to this theory, reactive oxygen

species, produced via mitochondrial respiration, attack mitochondrial DNA (mtDNA). The accumulation of oxidant-induced somatic mutations in mtDNA is therefore believed to be the underlying cause of the decline in physiological function with age. Mitochondrial respiratory complex function may be altered as a result of mtDNA mutations, leading to increased reactive oxygen species production and further damage to mtDNA, as well as other macromolecules [16]. The age-related increase in oxidative damage to DNA, lipids, and proteins [17] has been well documented, along with evidence supporting an increase in mtDNA deletions and mitochondrial dysfunction with age [15, 18]. Since cardiac myocytes are postmitotic cells that exhibit a high rate of oxygen consumption throughout an individual's lifetime, the heart may be especially prone to oxidative damage.

If the oxidative stress/mitochondrial theory of aging holds true, then experimental interventions that reduce oxidative damage should be able to extend lifespan. To date, the only intervention that has consistently been shown to slow the rate of aging and to increase mean and maximum lifespan in a variety of species is calorie restriction [17, 19, 20]. However, overexpression of antioxidant enzymes [21], antioxidant supplemented diets [22], and lifelong voluntary wheel running [23-27] have all been shown to have some degree of success in increasing mean, but not maximal, lifespan.

Most lifelong, voluntary wheel running studies have been performed for the sole purpose of determining whether it can increase lifespan. Only a handful of studies using long-term wheel running have measured any parameters of oxidative stress [28-30]; and to our knowledge, none have determined the effects on mitochondrial function. Our study determined whether long-term voluntary wheel running could improve

mitochondrial function, enhance antioxidant defenses, and reduce oxidative damage in the hearts of aged, male Fischer-344 rats.

Questions and Hypotheses

Question 1. Are there age-related changes in mitochondrial function and oxidant production?

Hypothesis 1. Mitochondrial function will decline and oxidant production will increase with age.

Question 2. Are there differences in function and oxidant production between subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) with age?

Hypothesis 2. IFM will show greater declines in function and greater oxidant production compared to SSM.

Question 3. Can long-term voluntary wheel running attenuate the age-related changes in mitochondrial function and oxidant production?

Hypothesis 3. Mitochondrial function will be better preserved and oxidant production lower in wheel-running rats compared to sedentary rats.

Question 4. Is oxidative damage increased in the aging heart, and can voluntary wheel running reduce the accumulation of oxidative damage with age?

Hypothesis 4. Oxidative damage in the heart will increase with age, and wheel runners will show attenuation of this damage.

Question 5. Are antioxidant defenses and repair systems altered with age? Will long-term voluntary wheel running enhance these defenses?

Hypothesis 5. Antioxidant enzyme activities will increase with age, and the GSH:GSSG ratio and 20S proteasome activity will decline. Wheel running will lead to

further increases in antioxidant enzyme activity, and will attenuate the decline in GSH:GSSG and 20S proteasome activity.

Literature Review

Overview of Free Radical (Oxidative Stress) and Mitochondrial Theories of Aging

As mentioned earlier, the free radical theory of aging proposes that endogenously produced oxidants damage important cellular components, such as DNA, lipids, and proteins; and proposes that this damage accumulates with time, resulting in the age-related loss of physiological function, and ultimately death. For simplicity, the term oxidants will be used (instead of free radicals) and will refer to all reactive oxygen species, including superoxide anions, hydroxyl radicals, and hydrogen peroxide. The idea that cells produced these damaging oxidants was controversial for several years. However, the discovery of superoxide dismutase in 1969 by McCord and Fridovich [31] provided the first *in vivo* evidence that cells did indeed produce oxidants and furthermore, that they had evolved an elaborate enzymatic defense system to protect against these harmful chemical species.

While there are numerous endogenous sources of oxidants, including peroxisomes, NADPH oxidase, and cytochrome P450, mitochondria appear to be responsible for producing the majority of oxidants. Classic *in vitro* studies in the 1970s showed that during mitochondrial respiration, 1-2% of the oxygen consumed was converted to hydrogen peroxide [32]. Although this may overestimate *in vivo* rates of oxidant production, mitochondria continue to be considered significant sources of oxidants since electron transport accounts for about 85% of the oxygen consumed by the cell. In addition, mitochondria are present in almost all cells, and in higher numbers than other oxidant-generating systems [33].

In addition to being sources of oxidants, mitochondria are also targets for oxidant-induced damage and those observations led to the formation of the mitochondrial theory of aging [14]. Mitochondrial DNA (mtDNA) is thought to be especially vulnerable to oxidative damage, since it is located near the inner mitochondrial membrane where oxidants are generated; it lacks protective histones; and it has relatively little DNA repair activity [14, 33]. Indeed, oxidative damage to mtDNA has been shown to increase with age in a variety of tissues, including cardiac muscle [18, 34, 35]; and if not repaired, may be converted to mtDNA mutations [5]. In fact, there is a positive correlation between the increase in oxidative damage to mtDNA and the age-associated increase in mtDNA deletions and point mutations [12]. Since mtDNA encodes for 13 of the polypeptides in the respiratory chain complex [33], mutations can result in altered coupling of electron transport and ATP production, leading to increased oxidant production, and launching a vicious cycle of ever-increasing mitochondrial dysfunction and oxidative damage with age [5, 16].

Structural Changes to Mitochondria in the Aging Heart

Although the number of cardiac mitochondria declines with age in both rats and humans, the total volume that they occupy within the cell does not change, suggesting that they increase in size [36, 37]. Additionally, the inner mitochondrial membrane becomes smoother and there is a loss of cristae [38], potentially resulting in reduced electron transport activity [5]. Paradies et al. [39] reported that the content of cardiolipin (a phospholipid located on the inner mitochondrial membrane that is crucial for mitochondrial function) decreases with age. Based on the structural changes that occur in cardiac mitochondria with age, it is not surprising that there is also a decline in mitochondrial function in the aging heart.

Age-Related Alterations in Cardiac Mitochondrial Function

Two functionally different populations of cardiac mitochondria exist [40, 41]: subsarcolemmal mitochondria (SSM), which are located beneath the plasma membrane, and interfibrillar mitochondria (IFM), which are found in parallel rows between the myofibrils [42]. Interpreting age-related changes in mitochondrial function is made difficult by the fact that most isolation procedures yield either SSM alone or a mixed population of SSM and IFM. Detecting bioenergetic changes due to aging is also problematic because of the fact that cells with extremely dysfunctional mitochondria will likely die via apoptosis or necrosis (so that only relatively healthy mitochondria are obtained upon isolation) [16].

In 1977, Palmer and colleagues [40] reported that the heart contained two structurally similar but biochemically different mitochondrial populations (SSM and IFM). They found that citrate synthase and succinate dehydrogenase activities were higher in IFM compared to SSM, and that IFM oxidized substrates quicker than SSM. In 1985, the same group reported that state 3 respiration, content of respiratory cytochromes, and activities of electron transport chain complexes were higher in IFM compared to SSM [41]. In more recent years, it has been demonstrated that IFM function is adversely affected during aging, while SSM remain relatively unaffected [42-44].

Until recently, there was little agreement in the literature on whether mitochondrial oxidative phosphorylation was adversely affected with age, since some laboratories found a decline in oxidative metabolism [45, 46] while others reported no changes [47, 48]. However, recent studies by Fannin et al. [42] found that when SSM and IFM were isolated from adult and old rat hearts and analyzed individually, only the IFM exhibited age-related declines in protein yield and oxidative phosphorylation rates. Since the

amount of IFM in a mixed population of mitochondria would be expected to vary, these results may help to explain the lack of consistency regarding age-related changes in oxidative phosphorylation. Additionally, this may account for the lack of an age-related decline in oxidative phosphorylation observed in an earlier study that used an isolation procedure that yielded only SSM [49].

There is also little agreement regarding changes in various electron transport complex activities with age. Of the five respiratory chain complexes, Complex I (NADH-ubiquinone reductase or NADH dehydrogenase) appears to be the most susceptible to age-related declines in activity [49-53], although some studies have reported no changes in Complex I activity with age [54, 55]. Given that 7 of the 13 mtDNA encoded polypeptides in the respiratory chain complex are found in Complex I, it is not surprising that this complex would be most greatly affected with aging if the mitochondrial theory of aging is true [51]. Additionally, many studies report age-associated declines in the activities of Complex III (ubiquinol-cytochrome c reductase) [43, 50] and Complex IV (cytochrome c oxidase) [42, 50, 52, 53, 55], which also contain mtDNA encoded proteins, while the activity of Complex II (succinate dehydrogenase) appears not to change [49, 52, 54] or increase [50, 55]. This finding is significant, since mtDNA does not encode for any of the polypeptides in Complex II [56]. In further support of the idea that Complexes I, III, and IV are negatively affected with age, it has recently been found that mRNA levels of NADH dehydrogenase subunit 1, cytochrome c oxidase subunit 3, and cytochrome b are reduced in old compared to young mouse hearts [57]. Additional attempts to identify some of the mechanisms responsible for these declines in activity indicate that the reduction in Complex III activity may be

due to alterations in the cytochrome c binding site [43], while Complex IV activity may be decreased due to changes in cardiolipin content [39, 42]. Further research is needed to determine whether Complex V activity (ATP-synthase) changes with age, as there is evidence showing that it declines [58, 59] and data indicating that it does not change [60]. Moreover, recent data from our laboratory indicate that heart mitochondrial ATP content and production is not changed with age [61]. Overall, the evidence supports an age-related decline in cardiac mitochondrial function, but future studies are required to determine the mechanisms contributing to these changes, and to further characterize functional differences in the two mitochondrial subpopulations with age.

Mitochondrial Oxidant Production in the Aging Heart

Soon after it was reported that mitochondria were sources of oxidants [32], Nohl and Hegner [62] found that heart mitochondria isolated from old rats generated more hydrogen peroxide (H_2O_2) than did mitochondria from young rats. Since then, numerous studies have been published supporting an increase in cardiac mitochondrial oxidant production with age [63-65]. However, some studies have found no changes in H_2O_2 production with age [61, 66, 67]. It is likely that the type and concentration of substrate used to determine the rate of H_2O_2 production may account for some of these discrepancies [67]. Additionally, since SSM and IFM are differently affected by aging [42, 43], it is possible that they may also generate different amounts of H_2O_2 with age, but this has not yet been addressed. Even if oxidant production proves not to increase with age, this does not negate the oxidative stress/mitochondrial theories of aging. Gredilla et al. [66] propose that consequences, and not causes, of aging should increase with age, since aging is a progressive process. If oxidant production is a cause of aging, then the rate of oxidant production should remain relatively constant over the lifetime of

an organism, while the consequences of this oxidant production (i.e., oxidative damage) should increase with age.

Oxidative Damage in the Aging Heart

Oxidants produced by mitochondria can damage DNA, lipids, and proteins if not effectively scavenged. Oxidized DNA may become mutated [5], lipid peroxidation can cause alterations in membrane fluidity [68], and oxidized proteins frequently lose catalytic activity [69]. All of these events can negatively impact cell function and contribute to the decline in physiological function with age. As a major source of oxidants, mitochondria themselves are believed to be especially susceptible to oxidative damage. Indeed, there is a great deal of evidence showing an increase in oxidative damage to mitochondrial DNA with age [18, 34, 35, 70]. Additionally, DNA damage in the heart (and other postmitotic tissues, such as the brain) is more extensive than that observed in mitotic tissues such as the liver [71]. This is likely a result of the limited ability of heart cells to undergo mitosis (in addition to the fact that the heart exhibits high oxygen consumption and low antioxidant capacity compared to the liver) [72].

While oxidative damage to both nuclear and mitochondrial DNA increases as a function of age [73], Barja and Herrero [34] found that levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), an indicator of oxidative DNA damage, were four-fold higher in mitochondrial DNA compared to nuclear DNA in heart tissue obtained from 8 mammalian species with varying lifespans (3.5 to 46 years). Furthermore, there was a significant inverse correlation between 8-oxodG levels in mtDNA and maximum lifespan (MLSP) among the different species. This agrees with previous work indicating that long-lived animals produce less mitochondrial superoxide and hydrogen peroxide compared to short-lived animals [74]. Since the rate of repair of 8-oxodG is similar in

nuclear and mitochondrial DNA [75], it is postulated that the increased level of 8-oxodG observed in mtDNA is related to the fact that mtDNA is located closer to the source of oxidant production than nuclear DNA. Furthermore, mitochondrial DNA repair activity has actually been shown to increase in the aging heart, further suggesting that the rate of damage to mtDNA is increased with age [76]. These observations are consistent with the free radical/mitochondrial theory of aging.

Although oxidative damage to mitochondrial DNA has been well documented, there is much less information regarding protein oxidation and lipid peroxidation in cardiac mitochondria. This is probably because most studies measure oxidative damage in whole-heart homogenate. Only one study reported an age-related increase in mitochondrial lipid peroxidation [77]. Another study did not find changes in lipid peroxidation with age, but did find that lipofuscin levels (also known as age pigment) were significantly increased in the hearts of old animals, with the mitochondrial compartment showing the greatest increase [78]. As far as mitochondrial protein oxidation is concerned, a recent study by Davies et al. [59] measured *o*-, and *m*-tyrosine, along with protein carbonyls, in the mitochondria and cytosol from hearts of young (2-3 mo) and old (24 mo) rats. They found that protein oxidative damage was not greater in the mitochondria compared to the cytosol; furthermore, they found no evidence of an age-related increase in mitochondrial or cytosolic protein oxidation. Conversely, an earlier study found that protein carbonyls were significantly increased in heart mitochondria from old (24 mo) rats compared to adult (12 mo) rats although mitochondrial lipid peroxidation was not different between the two groups [53]. However, the paucity of data regarding age-related changes in protein and lipid oxidative

damage in heart mitochondria makes it nearly impossible to draw firm conclusions on whether mitochondria exhibit age-related increases in these parameters.

An age-associated increase in oxidative damage is supported by several studies using whole-heart homogenate. Although the measurement of protein carbonyls as a marker of protein oxidative damage has been extensively criticized, it remains the most commonly used method for assessing protein oxidation. Various laboratories have reported an increase in the protein carbonyl content of the aging heart [63, 64, 79, 80]. In an attempt to identify which oxidants are involved in protein oxidation during aging, Leeuwenburgh et al. [81] used the highly sensitive technique of gas chromatography-mass spectrometry (GCMS). They found a significant increase in *o,o*'-dityrosine cross-links in cardiac muscle proteins from old mice, suggesting that tyrosyl radicals contribute to protein oxidation with age. However, in another study also using GCMS, no increases in *o*-tyrosine and 3-nitrotyrosine were observed, possibly indicating that proteins oxidized by hydroxyl radical and reactive nitrogen species do not accumulate in the aging heart [82].

The polyunsaturated fatty acids found in membrane lipids are vulnerable to peroxidation by oxidants, and lipid peroxidation has been shown to increase in the aging heart when measured in cytosol or homogenate [54, 77, 83, 84]. Chen and Yu [68] reported that lipid peroxidation is a major contributor to the age-related loss of membrane fluidity and that two aldehydic lipid peroxidation products, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), are primarily responsible for the decrease in membrane fluidity. In cell culture studies, both MDA and HNE have cytotoxic effects, possibly due to the fact that they can readily react with proteins [85]. Indeed, although HNE is an

end-product of lipid peroxidation, it is highly reactive with other biological molecules, especially proteins [86]. HNE exerts numerous effects, including inhibition of protein and DNA synthesis and enzyme inactivation, and is believed to play a major role in oxidative stress-induced cellular dysfunction [86]. Furthermore, HNE has been shown to covalently bind to and inhibit proteasome activity [86]; and HNE-modified proteins can become resistant to proteolytic degradation [87], and also act as noncompetitive inhibitors of the proteasome [87, 88].

The non-enzymatic peroxidation of arachidonic acid by oxidants also gives rise to another major class of lipid peroxidation products – the isoprostanes [89]. In humans, the measurement of isoprostanes in urine or plasma has been shown to be a reliable and noninvasive way to assess lipid peroxidation *in vivo*; and isoprostanes can also be detected in almost all human and animal tissues. Isoprostane levels have been shown to increase during oxidative stress. In rats injected with carbon tetrachloride to induce oxidative stress, liver isoprostane levels increased 55-fold within 4 h and remained elevated (21-fold increase) at 24 h [90]. Furthermore, rats with diminished copper-zinc superoxide dismutase activity (due to dietary copper deficiency) had plasma isoprostane levels that were 2.5-fold higher than control animals [91]. Finally, isoprostanes are not only markers of oxidative stress, but also exert biological effects, such as vasoconstriction and endothelin release.

Defense Mechanisms Against Oxidative Stress

The extent to which oxidized macromolecules accumulate with age is dependent on several factors including the amount and type of oxidant produced; the ability of the antioxidant defense system to prevent oxidant-induced damage; and the cell's capacity to

repair or remove oxidized DNA, lipids, and proteins [69]. To that end, cells have developed an elaborate antioxidant defense system consisting of enzymes and low-molecular-weight antioxidants that can directly scavenge oxidants [92]. Cells also contain numerous enzymes and small molecules that can effectively repair or remove oxidized DNA, lipids, and proteins [69, 92]. Although this system is highly efficient, it is not fully capable of preventing and repairing all oxidant-induced damage (as evidenced by the fact that oxidative damage can be detected in the aged heart). Nonetheless, the importance of this system in protecting tissues from excessive oxidative damage cannot be overlooked.

The major antioxidant enzymes present in cells are superoxide dismutase (SOD), of which there is a copper-zinc (CuZnSOD) and manganese (MnSOD) isoform present in the cytosol and mitochondria respectively; glutathione peroxidase (GPX); and catalase (CAT). Superoxide dismutase catalyzes the dismutation of superoxide radicals to oxygen and hydrogen peroxide, while catalase and glutathione peroxidase convert hydrogen peroxide to water.

Most cells also contain several low-molecular-weight antioxidant compounds such as glutathione, uric acid, and ascorbic acid. These compounds can directly scavenge radicals by donating an electron to them. Alternatively, they can help prevent oxidation to macromolecules by chelating transition metals, and thereby inhibiting Haber-Weiss reactions (which result in the formation of the highly reactive hydroxyl radical) from occurring [92]. Glutathione, a thiol-containing tripeptide (glutamate-cysteine-glycine), is one of the most important low-molecular-weight antioxidants and is found in millimolar concentrations in most cells [93]. It can directly scavenge radicals, acts as a chelating

agent for copper ions, is a cofactor for glutathione peroxidase, and can prevent oxidation of sulfhydryl groups in proteins [92]. Furthermore, glutathione plays an important role in redox-sensitive cell signaling [93].

There are also various systems within cells that can repair or remove oxidatively damaged molecules. DNA repair enzymes exist in both the nucleus and mitochondria; and the base excision repair (BER) pathway is responsible for repairing the majority of oxidative DNA lesions [94]. DNA glycosylases that recognize oxidized lesions remove the damaged bases and the DNA backbone is then cleaved by an apurinic/apyrimidinic (AP)-endonuclease or an AP-lyase. Finally, the DNA is fully repaired by either the short patch (1 nucleotide) or long patch (2-6 nucleotides) pathways, each of which uses different enzymes to complete DNA synthesis and ligation. Lipid hydroperoxides can be repaired via the concerted action of phospholipase A_2 and glutathione peroxidase [95]. Phospholipase A_2 releases hydroperoxides from peroxidized phospholipids and GPX then reduces them.

Presently, only two enzymes have been identified that can repair oxidized proteins: thioredoxin/thioredoxin reductase reduces oxidized disulfide bonds while methionine sulfoxide reductase converts methionine sulfoxide back to methionine [96]. Therefore, it is essential to have an effective mechanism by which to remove oxidized proteins in order to prevent the accumulation of toxic protein aggregates. The proteasome, a large multicatalytic protease found in the cytosol and nuclei of eukaryotic cells, is responsible for degrading the majority of soluble, intracellular proteins [97]. The proteasome exists in two major forms: a ~700 kDa 20S, and the much larger ~2000 kDa 26S. The 20S proteasome contains the catalytic activity, while the 26S proteasome is formed when the

20S complexes with two 19S regulatory subunits that confer the ability to hydrolyze ATP and recognize polyubiquitinated proteins [98]. The 26S is primarily responsible for degrading ubiquitinated proteins in an ATP-dependent manner; while there is compelling evidence that the 20S selectively and rapidly degrades oxidized proteins in an ATP- and ubiquitin-independent fashion [98-100].

It has been suggested that the age-related accumulation of oxidized DNA, lipids, and proteins may be a result of deficient antioxidant enzyme activity and/or declining activity of the various repair and removal systems. However, it is extremely difficult to conclude whether the accumulation of oxidative damage with age is linked to changes in antioxidant enzyme activity, since consistent changes have not been reported [101]. A large reason for this may be that changes in antioxidants vary depending on the tissue and animal studied, and which cellular compartment the activity is measured in (i.e., homogenate, cytosol, mitochondria). Data obtained in heart tissue illustrate this point, as several studies report decreases in SOD activity with age [102-104] while others report no changes [105, 106]. Additionally, myocardial GPX activity has been reported not to change [80, 102-104] or to increase [105] while CAT activity has been shown to decrease [106], increase [103], or not change with age [105]. However, two studies looked at antioxidant enzyme activity in heart cytosol and mitochondria; and found in both cases that there was an age-related decrease in cytosolic CuZnSOD, GPX, and CAT activities while mitochondrial GPX and MnSOD activities significantly increased with age [77, 107]. The situation is further complicated by the fact that antioxidant enzyme activity can be induced in response to oxidative stress. Therefore, even when consistent changes in activity are observed, the same conclusions are not always reached. Increased activity

may be interpreted as being beneficial because it provides better protection against oxidant-induced damage, or it may be viewed as negative because it could indicate a need for enhanced antioxidant defenses due to increased oxidant production [108].

By measuring the amounts and ratios of various redox couples (i.e., reduced:oxidized glutathione, NADP(H):$NADP^+$, reduced:oxidized thioredoxin), the redox state of cells can be determined. Glutathione is often used as an indicator of the redox state of the cell, since it is the most abundant redox couple. Unfortunately, the results regarding changes in reduced:oxidized glutathione (GSH:GSSG) content with age are no more consistent than the antioxidant enzyme data. When comparing very old rats (29 mo) with very young ones (4 mo), Bagchi et al. [106] found a significant decrease in heart GSH content. Vega et al. [109] reported a decline in GSH in the right and left ventricles of 12-mo-old rats compared to 3-mo-old rats, but did not find further declines between the ages of 12- and 24-mo. However, another study reported no changes in heart mitochondrial GSH or GSSG content with age (8 vs. 25-mo-old rats) [80]. In contrast, a recent report showed that there was a decline in heart mitochondrial GSH and an increase in GSSG with age, resulting in a reduced GSH:GSSG ratio [110]. Furthermore, of several tissues studied (heart, liver, brain, kidney, testes), the largest drop in mitochondrial redox potential occurred in the heart. In the same study, GSH did not change with age when measured in heart homogenate, although the GSH:GSSG ratio declined because of an increase in GSSG.

As far as repair and removal systems are concerned, there is little data regarding how they change in the aging heart. The possibility that DNA repair processes are decreased with age was refuted by de Souza-Pinto et al. [76] who reported that 8-oxodG

DNA glycosylase (OGG1) activity was significantly increased in heart mitochondria obtained from 12- and 23-mo-old rats compared to 6-mo-old rats. Uracil DNA glycosylase and endonuclease G activities were also higher in the mitochondria from old rats compared to young. However, it was recently reported that large amounts of OGG1 and uracil DNA glycosylase are trapped in the outer mitochondrial membrane in precursor form in old animals [111]. These data suggest that import of mitochondrial proteins required for DNA repair may be impaired with age; and may also explain why mitochondrial 8-oxodG levels are higher in aged animals, despite the observed increase in total activities of certain DNA repair enzymes.

Some investigators believe that the accumulation of oxidized proteins with age may be due to a decline in 20S proteasome activity. However, in the heart, very few studies have investigated whether 20S proteasome activity diminishes with age. Bulteau et al. [112] found that all three of the major proteolytic activities of the 20S proteasome (chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase) declined significantly in the aging rat heart. They also found that protein carbonyls and HNE-modified proteins were significantly elevated in the hearts from old animals, providing support for the idea that a reduction in 20S proteasome activity may contribute to the accumulation of oxidized proteins with age. However, because of the lack of consistent changes reported in antioxidant enzyme activity with age, and the paucity of data regarding oxidative damage repair and removal systems, it is clear that further information regarding age-related changes in myocardial antioxidant defenses and repair/removal systems is required.

Long-Term Voluntary Exercise as a Countermeasure to Age-Related Mitochondrial Dysfunction and Oxidative Stress

The sedentary lifestyle that many individuals lead is detrimental to their health, as indicated by the numerous benefits of chronic, moderate exercise. Regular physical activity can delay the onset of morbidity, and increase mean lifespan [24, 25, 113]. The risk of developing coronary heart disease, cerebrovascular disease, hypertension, type II diabetes, colon cancer, and osteoporosis is significantly less in individuals who are physically active compared to those who are sedentary [113]. Although it is widely agreed that exercise is beneficial for health and longevity, the molecular mechanisms through which exercise exerts these effects are not well understood. However, it has been hypothesized that the health benefits of chronic exercise may be at least partially due to a reduction in oxidant production and oxidative damage.

Paradoxically, it was shown more than two decades ago that strenuous exercise can actually increase oxidant production and tissue damage [114]. Since that time, additional evidence has been obtained indicating that acute bouts of exercise in untrained subjects can indeed elevate oxidant production and tissue oxidative damage [80, 115-117]. Despite direct evidence, it is often assumed that mitochondrial oxidant production rises in direct proportion to tissue oxygen consumption. Therefore, it has been proposed that the increase in oxidative stress with acute exercise is a result of the dramatic increases in tissue oxygen consumption (up to 23-fold in skeletal muscle and 3.6-fold in the heart) that occur during heavy exercise [118].

How is it then that moderate chronic exercise can be so beneficial? First, it was found that mitochondrial oxidant production does not dramatically increase during

ADP-supplemented (state 3) respiration [118]. Conversely, mitochondrial oxidant production measured *in vitro* actually decreases quite significantly in state 3 compared to state 4, although there is still a small amount of oxidant production. Although some oxidative damage may occur due to the low level of state 3 oxidant production, this observation helps to explain the lack of massive oxidative damage after exercise despite the tremendous increases in oxygen consumption.

Several investigators have also reported increases in antioxidant enzyme activity in skeletal and cardiac muscle after endurance exercise training [119-123], an adaptation that should help to reduce oxidative damage. Additionally, Venditti et al. [124] recently found that 10 weeks of swim training significantly reduced the basal rate of mitochondrial H_2O_2 production in rat gastrocnemius muscle compared to untrained animals. Both of these adaptations to exercise training would therefore be expected to reduce tissue oxidative damage. Lipid peroxidation has been shown to be lower in heart and liver of exercise-trained animals compared to sedentary animals [123]. A reduction in the oxidative DNA damage marker, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), has been reported in skeletal muscle after exercise training [125, 126]; and protein carbonyl content in the heart is significantly lower in trained rats compared to sedentary rats [127]. Radak et al. [125-127] also determined the effect of exercise training on DNA repair activity and 20S proteasome activity . Eight weeks of treadmill training in middle-aged (20 mo) and old (30 mo) rats increased 8-oxodG repair activity and chymotrypsin-like proteasome activity in rat skeletal muscle [125]. Nine weeks of swim training increased the chymotrypsin-like proteasome activity in rat heart [127] and both chymotrypsin- and trypsin-like activities in gastrocnemius muscle [126]. Overall, the

available data suggest that chronic exercise may serve to reduce mitochondrial oxidant production, enhance antioxidant enzyme activities, and augment certain repair/removal pathways, thereby reducing the amount of oxidative tissue damage.

There remains a great deal of debate surrounding the optimum intensity, frequency, and duration of exercise required to elicit the maximum benefits. However, there is some evidence that moderate and prolonged exercise may provide the most protection against oxidative stress [128]. In rodent models, a commonly used method of exercise training involves forced treadmill running. The obvious advantage of this type of training is that the investigator can control the duration, intensity, and frequency of the running. Furthermore, there are generally large improvements in the oxidative capacity of skeletal and cardiac muscle with this type of training [129]. On the negative side, forced treadmill running can also cause physiological adaptations that are indicative of chronic stress. Moraska et al. [129] found that 8 weeks of treadmill training in male rats resulted in adrenal gland hypertrophy and thymic involution, both of which were attributed to chronic elevations in stress hormones, which can negatively impact health. Additionally, serum levels of the corticosterone carrier protein, corticosteroid binding globulin (CBG), were reduced and this could lead to prolonged elevations in free corticosterone. Finally, lymphocyte proliferation and antigen-specific IgM production were suppressed in the treadmill-trained animals, an adaptation that could increase susceptibility to disease. The authors cautioned that investigators should be aware of the negative stress-induced changes brought on by forced treadmill running, as they may have an impact on other physiological parameters being measured and could make interpretation of results difficult.

An alternative to forced treadmill running is to place rodents in cages with running wheels and allow them to exercise voluntarily for an extended period of time. This mode of training has been found to increase mean lifespan [23-27, 130] and body weights of rats allowed to run voluntarily are lower than those of rats forced to run on a treadmill [130]. Narath et al. [130] compared survival of rats that were either allowed to run voluntarily in wheels or forced to run on a treadmill (20 minutes, twice a day, five days a week) between the ages of 5 and 23 mo. There was a significant decrease in survival of the rats in the treadmill running group (10 deaths, $n = 32$) as compared to rats in the wheel running group (2 deaths, both of which occurred in conjunction with anesthesia, $n = 32$). Furthermore, voluntary wheel running does not cause the negative adaptations associated with chronic stress that forced treadmill running does [131]. However, the duration, frequency, and intensity of exercise cannot be controlled with this mode of training, and there are large variations between rodents in the total meters run per day.

Despite the fact that several studies have examined the effects of long-term voluntary wheel running on survival, little is known about the effects it has on mitochondrial function and oxidative stress. Kim et al. [29] measured lipid peroxidation and antioxidant enzyme activity in hearts from 20-mo-old male Fischer 344 rats after 18.5 months of voluntary wheel running. Compared to the sedentary rats, long-term voluntary wheel running resulted in decreased lipid peroxidation and increased catalase activity but no changes in superoxide dismutase or glutathione peroxidase activities. These changes were observed despite the fact that the rats in this study ran, on average, only 60 meters per day. Using the same experimental protocol, this group also observed a significant increase in catalase and glutathione peroxidase activities and increased

glutathione content in liver [30]. Liver microsomal oxidant production was reduced by exercise, although no differences were seen in mitochondrial oxidant production between sedentary and exercised animals. Additionally, membrane fluidity was significantly increased in both hepatic microsomes and mitochondria obtained from the wheel running animals. In another study, levels of *o,o'*-dityrosine (a marker of protein oxidation) in urine were significantly reduced in 24-mo-old rats that had undergone 19 months of voluntary wheel running compared to sedentary control animals [28]. Glutathione peroxidase and mitochondrial superoxide dismutase activities were also elevated in skeletal muscle from the exercised animals in this study.

The recent development of gene expression microarrays allows investigators to monitor changes in mRNA transcript levels in particular tissues under various experimental conditions. Results from these studies indicate that there are numerous age-related changes in gene expression in a variety of tissues [57, 132-134], and provide a powerful tool by which to study aging. Gene expression analyses have been performed in the hearts of physically active and sedentary middle-aged (20 mo) and old (33 mo) mice [134]. Mice in the physically active group were placed in cages with running wheels at one month of age and were allowed to exercise voluntarily until the time of death. In the sedentary group, 137 genes were significantly different (up- or down-regulated by at least 50%) in the old compared to the middle-aged mice and most of these genes were associated with either the inflammatory or stress response. Exercise attenuated age-related changes in 70 of the137 genes that changed in the old, sedentary mice. It was concluded that the aging heart is subjected to oxidative stress (which leads to a pro-inflammatory state) and that long-term voluntary exercise can prevent many of

these changes. Of interest, it was found that Lon protease expression was significantly increased with age in the exercised but not the sedentary animals. Lon protease is found in the mitochondria and Bota et al. [135] reported that protein levels of this protease decline with age. This is important because Lon protease preferentially degrades oxidized proteins and therefore, may play a crucial role in preventing the accumulation of oxidized proteins in the mitochondria with age.

Summary

Most of the available data indicates that there is a decline in mitochondrial function, and an increase in oxidative damage in the aged heart. However, few studies have investigated whether the two mitochondrial subpopulations, subsarcolemmal (SSM) and interfibrillar (IFM), are differently affected with age. We measured oxygen consumption and hydrogen peroxide production in SSM and IFM isolated from young rats (6 mo) and old rats (24 mo) to determine how they are affected with age. Furthermore, inconsistent age-related changes in cardiac antioxidant enzyme defenses have been reported in the literature, and relatively little is known regarding how age influences the systems which repair and remove oxidative damage. Additionally, mitochondria and cytosol may exhibit different changes in these parameters during aging. To help clarify existing data, we measured the major antioxidant enzyme activities, reduced and oxidized glutathione levels, and markers of protein and lipid oxidative damage in SSM, IFM, and cytosol. We also measured oxidative damage to nuclear DNA and 20S proteasome activity in cytosol. Finally, despite the fact that long-term, voluntary exercise has been shown to increase mean lifespan, very few studies have investigated the possibility that it may also be an intervention that can attenuate age-related changes in cardiac mitochondrial function, antioxidant defenses, and oxidative damage. Therefore,

we measured the above-mentioned parameters in the hearts of old (24 mo) rats that performed voluntarily wheel-running exercise for 21 months.

CHAPTER 2
EXPERIMENTAL PROCEDURES

Animals

Male Fischer-344 rats were purchased from Harlan (Indianapolis, IN) at 10-11 weeks of age and were housed in our facilities until they were sacrificed at 24 months of age. One week after arriving at our facilities, rats were randomly assigned to one of three groups: sedentary, *ad libitum* fed (Old; $n = 19$); sedentary, 8% calorie restriction (SED; $n = 20$); or wheel running, 8% calorie restriction (WR; $n = 20$). All animals were singly housed in a temperature- ($20 \pm 2.5°C$) and light-controlled (12:12-h light-dark cycle) environment with unrestricted access to water. All sedentary rats were housed in standard rodent boxes supplied by the University of Florida's Animal Care Services. Rats in the wheel running group were housed in cages equipped with stainless steel running wheels (1.081 meters circumference) to which the animals had free access. Each wheel was equipped with a magnetic switch and an LCD counter that recorded the number of wheel revolutions. The number of revolutions was recorded for each animal daily and the counters then reset. Approximately two months before the scheduled sacrifice of the old animals, a group of 4-mo-old male Fischer-344 rats (Young; $n = 12$) were obtained from Harlan. These animals were singly housed in the same room as the old animals and given food and water *ad libitum*. This group of animals was compared to the old, sedentary *ad libitum* fed rats to determine changes in mitochondrial function and oxidative stress with age. All experimental procedures were approved by the University of Florida's Institute on Animal Care and Use Committee.

Feeding and Body Weights

All rats were fed a standard rodent diet (Harlan Teklad Rodent Diet, #8604) daily. Food intake was recorded daily by weighing the amount of food given to each animal and weighing the amount left the following day. Rats fed *ad libitum* tend to abruptly decrease their running activity and slight food restriction (8-10%) has been shown to help maintain running activity [136]. The *ad libitum* food intake of all animals was monitored for ~2 months at the start of the experiment and after this time, rats in the wheel running group (WR) had their food intake restricted by 8% below the *ad libitum* food intake. At the same time, a group of sedentary rats (SED) also had their food intake restricted by 8% in order to control for any changes that may occur due to the slight food restriction. Throughout the duration of the study, food intake of these two groups was adjusted accordingly (based on *ad libitum* food intake). Body weights of all rats were recorded weekly.

Isolation of Mitochondria and Cytosol

Animals were euthanized with isoflurane (administered via inhalation using a precision vaporizer at 5%) and the hearts were perfused with phosphate buffered saline (PBS), removed, rinsed in saline, blotted dry, and weighed. Subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria were isolated according to the method of Palmer et al. [40] with slight modification [42]. After trimming excess fat and removing the atria, the ventricles were minced and homogenized 1:10 (w/v) in ice-cold Chappell-Perry buffer (100 mM KCl, 50 mM MOPS, 1 mM EDTA, 5 mM $MgSO_4 \cdot 7H_2O$, 1 mM ATP, pH 7.4). The homogenates were then centrifuged at 500 *g* for 10 minutes at 4°C (all remaining centrifugation steps were also performed at 4°C). The supernatant was transferred to a new tube and the pellet re-suspended and centrifuged at 500 *g* for 10 minutes. The two

supernatants were subsequently pooled and centrifuged at 3000 *g* for 10 minutes to obtain SSM while the pellet obtained in the second low-speed centrifugation step was saved for IFM isolation. After the first 3000 *g* spin, the supernatant (crude cytosolic fraction) was aliquotted and stored at -80°C while the pellet was re-suspended and centrifuged at 3000 *g* for 10 minutes. This wash step was repeated once more and the final SSM pellet suspended in 1 ml of Chappell-Perry buffer and kept on ice until use. Meanwhile, nagarse (5 mg/g wet weight tissue) was added to the tissue pellet containing IFM, incubated on ice for 5 minutes, and homogenized. The homogenate was then diluted two-fold with Chappell-Perry buffer and centrifuged at 5000 *g* for 5 minutes. The supernatant was discarded and the pellet re-suspended in the original volume of buffer. The re-suspended pellet was centrifuged at 500 *g* for 10 minutes to yield the nuclear pellet. The supernatant was saved, and to improve recovery of IFM from the nuclear pellet, the pellet was re-suspended and centrifuged at 500 *g* for 10 minutes. This step was repeated once more with the supernatant being saved after each spin. The supernatants from the three low-speed spins were combined and centrifuged at 3000 *g* for 10 minutes to obtain the IFM pellet. The IFM pellet was washed twice and the final pellet suspended in 0.75 ml of Chappell-Perry buffer. Protein concentrations of SSM and IFM were determined using the Bradford method [137] and mitochondrial oxygen consumption and hydrogen peroxide production were measured in both fractions immediately thereafter.

Oxygen Consumption

Mitochondrial respiratory function was measured using a biological oxygen monitor system (model YSI 5300, Yellow Spring Instruments, Columbus, OH). Mitochondrial respiration was monitored at 37°C in incubation buffer (145 mM KCL, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1 % fatty-acid free albumin,

pH 7.4), 2.5 mM pyruvate, 2.5 mM malate, and 0.5 to 1 mg mitochondrial protein for a total volume of 500 ml. State 4 respiration (no ADP) was read for 2 minutes and monitored using a chart recorder. State 3 respiration (with ADP) was then measured in the presence of 500 mM ADP for 10 minutes, or until the oxygen pressure was equal to zero. Oxygen consumption was reported as nmoles O_2 consumed/min/mg protein. In addition, the respiratory control ratio (state 3/state 4) was calculated as an indicator of mitochondrial viability.

Hydrogen Peroxide Production

Hydrogen peroxide production was measured in intact mitochondria following the method of Barja [138]. Briefly, incubation buffer (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1% fatty acid-free BSA, pH 7.4) was added to test tubes followed by the addition of mitochondria (0.25 mg/ml protein), horseradish peroxidase (5.7 Units/ml), homovanilic acid (0.1 mM), and substrate (2.5 mM pyruvate, 2.5 mM malate) so that the total volume was equal to 1.5 ml. The tubes were incubated in a shaking water bath at 37ºC for 15 minutes and the reaction was stopped by placing the tubes on ice and adding 0.5 ml stop solution (0.1 M glycine, 25 mM EDTA-NaOH, pH 12.0) to each tube. Fluorescence was measured at an excitation wavelength of 312 nm and an emission wavelength of 420 nm using a SPECTRAmax Gemini XS dual-scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA). Arbitrary fluorescence units were converted to known amounts of H_2O_2 using a glucose-glucose oxidase standard curve. All measurements were performed in duplicate and results expressed as nmoles H_2O_2 produced/min/mg protein.

Antioxidant Enzyme Activity

Antioxidant enzyme activity was measured in SSM, IFM, and cytosol. Superoxide dismutase (both MnSOD and CuZnSOD) activity was assayed according to Oyanagui [139] with slight modification. One unit (U) of SOD activity was defined as the concentration of enzyme that inhibits nitrite formation from hydroxylamine in the presence of xanthine oxidase by 50%. Selenium-dependent glutathione peroxidase (GPX) activity was determined at 37ºC according to Flohe´ and Gunzler [140], with H_2O_2 as the substrate. Glutathione reductase (GR) activity was measured at 30ºC according to Carlberg and Mannervik [141]. Catalase (CAT) activity was measured at 25ºC according to Aebi [142].

Reduced/Oxidized Glutathione

Reduced and oxidized glutathione (GSH and GSSG, respectively) were measured according to the methods of Jones et al. [143] and Fariss and Reed [144]. Sample proteins were precipitated with 10% (w/v) perchloric acid (PCA) containing 10 mM of the metal chelator, diethylenetriamine pentaaceticacid (DTPA). Derivatization of thiols in the acid-soluble fraction with 40 mM iodoacetic acid (IAA) to form S-carboxymethyl derivatives was followed by fluorophore conjugation of primary amines with 75 mM dansyl chloride. GSH and GSSG were separated by normal phase high performance liquid chromatography (HPLC) using an aminopropyl column (Cel Associates, Houston, TX). Derivatives were detected using a Hitachi fluorescence detector with an excitation wavelength of 330 nm and an emission wavelength of 515 nm. Quantification was achieved relative to authentic GSH and GSSG standards and by using γ-glutamyl-glutamate as an internal control to assess completeness of derivitization.

Proteasome Activity

Chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase activities of the 20S proteasome were measured using the fluorogenic peptides, N-Succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-AMC), Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (LSTR-AMC), and Z-Leu-Leu-Glu-7-amido-4-methylcoumarin (LLE-AMC), respectively. All fluorogenic peptides were obtained from Sigma-Aldrich Co. (St. Louis, MO). Briefly, 10 μl of cytosolic protein was incubated at 37°C for 30 minutes with 25 mM Tris (pH 7.5) and either 25 μM LLVY-AMC, 40 μM LSTR-AMC, or 150 μM LLE-AMC in a final volume of 150 μl. Following 30 minutes incubation, the reaction was stopped by the addition of 150 μl of ice-cold 96% (v/v) ethanol. The proteasome inhibitor MG-132 (20 μM; Sigma-Aldrich Co., St. Louis, MO) was used to ensure that proteasome peptidase activities were being measured. Fluorescence was determined using a SPECTRAmax Gemini XS dual-scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 380 nm and an emission wavelength of 440 nm. A standard curve was constructed using known concentrations of free AMC. Measurements were performed in triplicate and specific activity expressed as μmoles/min/mg protein.

Protein Oxidation

Protein carbonyls were measured in SSM and IFM using an enzyme immunoassay (Zentech PC Test, Zenith Technology Corp., Dunedin, NZ) with slight modification. Briefly, all samples, standards, and quality controls were normalized to 1.8 mg/ml (protein concentration of the lowest sample). Next, 11 μl of each sample, standard, and control was incubated in 19 μl of dinitrophenylhydrazine (DNPH) for 45 minutes at room temperature. Following derivitization with DNPH, 7.5 μl of each sample (also standards

and controls) was diluted into 1 ml of EIA buffer. Manufacturer's instructions were then followed starting with the section entitled ELISA procedure (#3). All samples, standards, and controls were run in triplicate.

Lipid Peroxidation

For detection of 4-hydroxy-2-nonenal (HNE)-modified proteins, proteins (15 μg) were separated using 4-20% sodium dodecyl sulfate (SDS)-polyacrylamide gels under denaturing conditions and then transferred to nitrocellulose. The membranes were blocked in PBS/0.05%Tween-20/5% milk and incubated with rabbit anti-HNE antiserum (Alpha Diagnostic International, San Antonio, TX) at a 1:500 dilution overnight. The next morning, the membranes were washed in PBS/0.05% Tween-20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) at a 1:1000 dilution for 1 h. ECL detection reagents from Amersham (Amersham Pharmacia Biotech Inc, Piscataway, NJ) were used to generate a chemiluminescent signal and bands were visualized by exposing the membranes to Hyperfilm-ECL (Amersham Pharmacia Biotech Inc, Piscataway, NJ). Blots were analyzed using Kodak 1D Image Analysis software (Eastman Kodak Company, Rochester, NY). Optical density was determined by calculating the net optical density (sum of the background-subtracted pixel values) of all the bands within a given lane.

As an additional marker of lipid peroxidation, we also measured thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa [145], with some modifications. Samples and standards were incubated with 12.5% tricholoroacetic acid /0.8 N hydrochloric acid and 1% 2-thiobarbituric acid at 95 ºC for 1 h. After cooling, samples and standards were spun in a microcentrifuge tube at 10,000 rpm for 10 minutes. The supernatant was pipetted into a microplate and absorbance at 532 nm was

determined. All samples and standards were run in duplicate and results expressed as μmoles/mg protein.

Oxidative DNA Damage

8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) levels in nuclear DNA were measured according to the method of Helbock et al.[146]. DNA was extracted using a chaotropic sodium iodide precipitation method. DNA was hydrolyzed using alkaline phosphatase and nucleotidase to completely digest the DNA to its corresponding nucleosides. The DNA hydrolysates were separated using reverse-phase HPLC with a LC-DB C18 column (Supelco, Belfonte, PA). The amount of 8-oxodG in the samples was analyzed by coulometric electrochemical detection at 0.1 V and 0.45 V and quantified relative to authentic 8-oxodG standards. Oxidative damage to DNA was expressed as the ratio of nmoles of 8-oxodG to 10^5 nmoles of deoxyguanosine in the same sample.

Statistical Analysis

An unpaired t-test was used to determine whether there was a significant age effect (Young vs. Old) and a significant exercise effect (SED vs. WR). An unpaired t-test was also used to detect differences between SSM and IFM isolated from the same group of animals. Significance was set at $p < 0.05$.

CHAPTER 3
RESULTS

Morphological Characteristics

There was no difference in body weight between the young and old animals (379.5 ± 9.2 g vs. 401.0 ± 9.5 g) possibly due to the fact that male Fischer rats tend to exhibit a decrease in body mass after approximately 18 months of age (based on growth curves provided by Harlan). However, heart weight significantly increased with age (1.01 ± 0.03 g in the young animals vs. 1.12 ± 0.03 g in the old animals, $p < 0.01$).

As would be expected, wheel running (WR) animals weighed significantly less than their sedentary (SED) counterparts throughout the study (Figure 1) but there was no difference in heart weight between the two groups (1.04 ± 0.01 g in SED vs. 1.03 ± 0.02 g in WR) at the termination of the study.

Running Wheel Activity

The average daily running activity is shown in Figure 2. Although peak running activity occurred at 6 months of age (~2500 meters/day), running activity was maintained at an average of 1145 ± 248 meters/day for the remainder of the study. This is in contrast to previous studies that show a continual decline in the average distance run per day with age [25, 136].

Mitochondrial Protein Yield

The protein yield of SSM was not different between young and old animals whereas there was a decrease in IFM protein yield from old rats compared to young rats

(Table 1). This finding is in agreement with that of Fannin et. al.[42] who reported that IFM protein yield was lower in 24- and 28-mo-old rats compared to 6-mo-old rats.

No differences in SSM and IFM protein yield were observed between sedentary and wheel running rats (Table 2). Within each of the four groups, the protein yield of IFM was significantly lower when compared to SSM yield for the same group of animals.

Mitochondrial Oxygen Consumption

We measured mitochondrial respiration in the presence (state 3) and absence (state 4) of ADP. There were no age-related changes in state 4 oxygen consumption in either SSM or IFM (Table 1). In contrast, state 3 oxygen consumption was significantly increased with age in SSM, while it was significantly decreased with age in IFM.

Furthermore, in young animals, IFM exhibited higher rates of state 3 respiration compared to SSM. In both young and old animals, the respiratory control ratio (RCR) was also significantly higher in IFM compared to SSM, indicating that mitochondrial respiration was more tightly coupled in IFM than in SSM.

As seen in Table 2, wheel running had no effect on the rates of oxygen consumption (state 4 or state 3) or RCR. A similar finding was reported by Servais et al. [147] who measured oxygen consumption in SSM and IFM in young animals after 5 months of voluntary wheel running. Furthermore, differences in RCR between SSM and IFM observed in young and old animals were not detected in either SED or WR rats.

Hydrogen Peroxide Production

Hydrogen peroxide (H_2O_2) production in SSM was significantly greater in old rats compared to young rats (Figure 3A) but no age-related difference in IFM H_2O_2 production was observed. Additionally, no significant differences in H_2O_2 production

were detected between SSM and IFM isolated from young rats. However, IFM from old rats produced less H_2O_2 than SSM from old rats.

Voluntary wheel running resulted in a significant decrease in both SSM and IFM H_2O_2 production when compared to sedentary rats (Figure 3B). Furthermore, when compared to SSM, H_2O_2 production in IFM was significantly decreased in both SED and WR rats.

Antioxidant Enzyme Activity

Superoxide Dismutase Activity

Manganese superoxide dismutase (MnSOD) activity was increased with age in SSM and IFM (Figure 4A). There were no differences in MnSOD activity between SSM and IFM isolated from the same group of animals. Additionally, we measured cytosolic SOD activity and found no difference between young and old animals (503.4 ± 16.7 vs. 472.9 ± .26.4 units/mg protein).

Wheel running caused a significant reduction in MnSOD activity in SSM and IFM (Figure 4B). In both the SED and WR groups, MnSOD activity was higher in IFM when compared to SSM. In contrast to what was observed in the mitochondria, cytosolic SOD activity was not different between SED (367.9 ± 13.0 units/mg protein) and WR (350.9 ± 9.4 units/mg protein) rats.

Glutathione Peroxidase Activity

Glutathione peroxidase (GPX) activity was significantly higher in SSM and IFM from old rats compared to young rats (Figure 5A). This finding is not surprising given that we also observed an increase in MnSOD activity with age, since the reaction catalyzed by SOD produces H_2O_2 that is then converted to H_2O via GPX. IFM from

young and old rats exhibited higher GPX activity compared to SSM. In agreement with the increase in H_2O_2 observed in the old animals, there was also a significant increase in cytosolic GPX activity in old vs. young rats (114.2 ± 2.1 vs. 101.8 ± 1.7 nmoles/min/mg protein, $p = 0.0003$).

When compared to sedentary rats, there was no effect of wheel running on GPX activity in SSM or IFM (Figure 5B). However, GPX activity was higher in IFM compared to SSM in both groups (SED and WR) of rats. We also measured cytosolic GPX activity and found no difference between SED and WR rats (88.4 ± 2.5 vs. 89.2 ± 2.4 nmoles/min/mg protein).

Catalase Activity

There was a significant decline in catalase (CAT) activity in SSM from old animals compared to young animals while IFM exhibited an age-related increase in CAT activity (Figure 6A). In young rats, IFM had significantly lower CAT activity compared to SSM while in old animals the opposite effect was observed (IFM had higher CAT activity than SSM). Cytosolic CAT activity did not change with age (1.01 ± 0.08 vs. 1.11 ± 0.14 units/mg protein, young vs.old).

No differences in CAT activity were detected in SSM or IFM from SED and WR rats (Figure 6B). As observed with GPX, IFM from both groups had significantly higher CAT activity than SSM. Cytosolic CAT was also not different between the two groups (0.80 ± 0.07 vs. 0.83 ± 0.05 units/mg protein, SED vs WR).

Glutathione Reductase Activity

Although not an antioxidant enzyme per se, glutathione reductase (GR) is responsible for regenerating reduced glutathione (GSH) from oxidized glutathione

(GSSG) using NADPH as an H^+ donor. GSH can then be used by GPX to metabolize H_2O_2 to H_2O.

There were no changes in GR activity with age in SSM or IFM (Figure 7A). Additionally, no differences between SSM and IFM from the same group of animals were detected. There was also no age-related change in cytosolic GR (10.08 ± 0.46 vs. 9.17 ± 0.46 nmoles/min/mg protein, young vs. old)

GR activity in SSM and IFM was not different between SED and WR rats (Figure 7B). When comparing IFM to SSM, GR activity was significantly higher in IFM for both groups. Cytosolic GR activity was not affected by wheel running (7.85 ± 0.31 vs. 7.6 ± 0.22 nmoles/min/mg protein, SED vs. WR).

Glutathione Levels

Since glutathione is a cofactor for GPX and can also directly scavenge oxidants, we determined the levels of reduced (GSH) and oxidized (GSSG) glutathione in the cytosol as well as GSH levels in SSM and IFM. Cytosolic GSH levels were higher in the old animals compared to the young but there were no differences between the two groups in cytosolic GSSG levels or the GSH:GSSG ratio (Table 3). Furthermore, no age-related differences in GSH were detected in SSM or IFM (Figure 8A). There was, however, significantly less GSH in IFM compared to SSM for both young and old rats.

We observed no changes in GSH, GSSG, or the GSH:GSSG ratio in the cytosol of SED and WR rats (Table 3). Mitochondrial levels (SSM and IFM) of GSH were also not affected by voluntary wheel running (Figure 8B). Once again, IFM from SED and WR rats contained less GSH than SSM.

Oxidative Damage

Protein Carbonyls

Protein carbonyls, a commonly used marker of oxidative protein damage, were significantly elevated with age in both SSM and IFM (Figure 9A). Furthermore, we found that IFM exhibited higher levels of protein carbonyls compared to SSM, indicating that IFM are subjected to more extensive protein oxidation than SSM.

Compared to SED rats, there was a significant increase in protein carbonyls in SSM from WR rats while no differences between the two groups were detected in IFM (Figure 9B). Identical to what was observed in the young and old animals, protein carbonyls were higher in IFM compared to SSM in both groups of rats.

4-Hydroxy-2-Nonenal-Modified Proteins (HNE)

Although not significant, there was a tendency for HNE-modified protein content to be lower in cytosol from old animals compared to young ($p = 0.086$; Figure 10A). No differences in the amount of HNE-modified proteins in SSM were detected between young and old animals (Figure 10B). In striking contrast, IFM from old animals exhibited significantly more HNE-modified proteins compared to young animals (Figure 10C). No significant differences in the amount of HNE-modified proteins were detected in cytosol (Figure 11A), SSM (Figure 11B), or IFM (Figure 11C) from SED and WR rats.

Thiobarbituric Acid Reactive Substances (TBARS)

Malondialdehyde, an aldehydic lipid peroxidation product, forms a 1:2 adduct with thiobarbituric acid under acid conditions and high heat, and the product of the reaction can be measured spectrophotometrically. We found no difference in the amount of TBARS in SSM from young and old animals and a significant increase in TBARS in IFM from old rats compared to young rats (Figure 12A), findings identical to what was

observed with HNE. Furthermore, similar to what was observed with protein carbonyls, IFM from both young and old animals had significantly greater amounts of TBARS than the corresponding SSM.

In agreement with the HNE data, no significant differences in TBARS in SSM and IFM from SED and WR rats were observed (Figure 12B). However, TBARS were significantly increased in IFM from both groups compared to SSM.

Oxidative DNA Damage

The levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in nuclear DNA significantly increased with age (Figure 13A). There was also a significant increase in 8-oxodG in nuclear DNA from wheel running rats compared to sedentary rats (Figure 13B).

Proteasome Activity

Since some studies have reported an accumulation of oxidized proteins with age [63, 64, 79, 80], we measured the three major proteolytic activities (chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase) of the 20S proteasome since this protease is responsible for recognizing and degrading oxidized proteins [98-100]. Futhermore, 20S proteasome activity has been shown to decline with age [112], whereas there is some evidence that exercise training can increase proteasome activity [125-127].

We found that there was no change in the chymotrypsin-like and peptidylglutamyl-peptide hydrolase (PGPH) activities with age, while trypsin-like activity was significantly increased with age (Figure 14). Furthermore, there was no difference in any of the three proteolytic activities between SED and WR rats (Figure 15).

Table 1. Effect of age on mitochondrial protein yield and respiratory parameters

	Young		Old	
	SSM	IFM	SSM	IFM
Protein yield (mg/g wet wt.)	16.87 ± 0.58	13.34 ± 0.50 #	18.31 ± 0.96	9.76 ± 0.42 * #
State 4	11.27 ± 1.34	11.59 ± 2.12	13.54 ± 1.26	9.71 ± 0.83 #
State 3	31.12 ± 2.43	60.55 ± 5.53 #	43.98 ± 3.88 *	44.43 ± 3.62 *
RCR	2.67 ± 0.12	5.74 ± 1.32 #	3.31 ± 0.19 *	4.71 ± 0.38 #

Results are expressed as means ± SEM. State 4 and state 3 respiration values are in nmoles O_2 consumed/min/mg protein. RCR is calculated as the ratio between state 3 and state 4 respiration. * denotes significant ($p < 0.05$) difference between identical mitochondrial preparations from young and old rats; # denotes significant ($p < 0.05$) difference between SSM and IFM within the same group of animals. For young animals, $n = 11$ for SSM and $n = 10$ for IFM. For old animals, $n = 9$ for SSM and $n = 8$ for IFM.

Table 2. Effect of voluntary wheel running on mitochondrial protein yield and respiratory parameters

	SED		WR	
	SSM	IFM	SSM	IFM
Protein yield (mg/g wet wt.)	16.32 ± 0.38	9.69 ± 0.27 #	16.93 ± 0.59	9.65 ± 0.34 #
State 4	10.94 ± 0.71	10.99 ± 0.99	11.39 ± 1.02	11.73 ± 1.20
State 3	33.33 ± 2.09	41.60 ± 3.53	30.24 ± 2.89	35.06 ± 2.88
RCR	3.02 ± 0.22	3.36 ± 0.40	2.85 ± 0.25	3.25 ± 0.35

Results are expressed as means ± SEM. State 4 and state 3 respiration values are in nmoles O_2 consumed/min/mg protein. RCR is calculated as the ratio between state 3 and state 4 respiration. # denotes significant ($p < 0.05$) difference between SSM and IFM within the same group of animals. For both SED and WR rats, $n = 12$ for SSM and IFM.

Table 3. Effect of age and voluntary wheel running on reduced and oxidized glutathione in cytosol

	Young	Old	SED	WR
GSH (nmoles/mg prot)	19.14 ± 0.50	22.23 ± 0.67 *	17.89 ± 0.49	18.36 ± 0.49
GSSG (nmoles/mg prot)	0.303 ± 0.06	0.307 ± 0.03	0.298 ± 0.04	0.303 ± 0.01
GSH:GSSG	80.91 ± 10.47	77.70 ± 6.21	68.61 ± 6.82	61.43 ± 2.11

Results are expressed as means ± SEM. Statistical comparisons between young and old animals and between SED and WR animals were performed. * $p < 0.05$, young vs. old. For young animals, $n = 11$; for old animals, $n = 9$. For SED, $n = 12$; for WR, $n = 11$. GSH – reduced glutathione; GSSG – oxidized glutathione; GSH:GSSG is calculated as the ratio of reduced to oxidized glutathione.

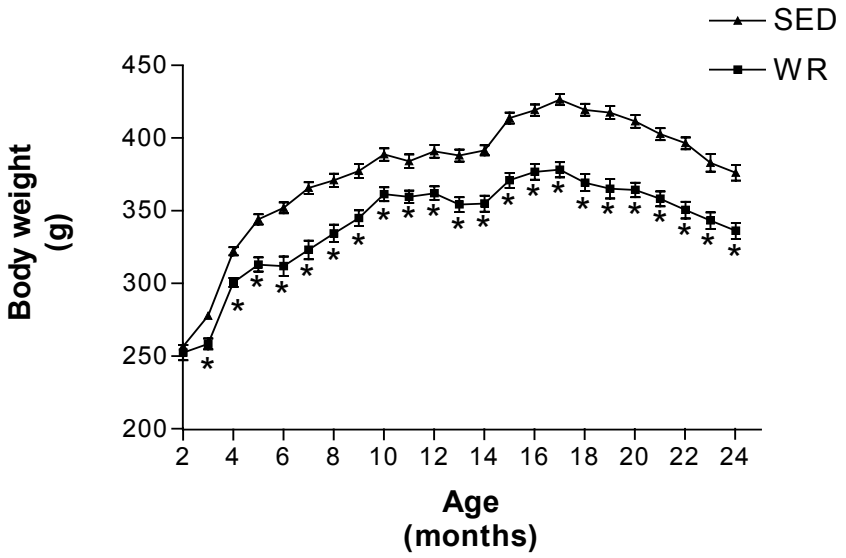

Figure 1. Average body weights of rats in the sedentary (SED) and wheel running (WR) groups. Results are expressed as means ± SEM. * $p < 0.05$.

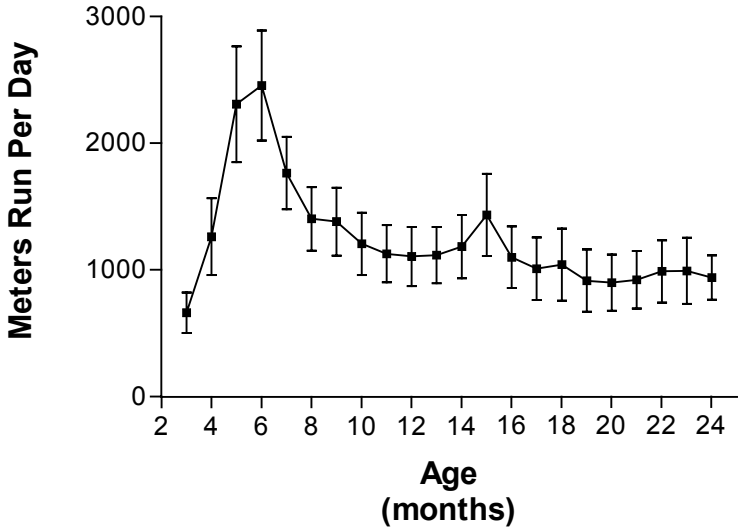

Figure 2. Average distance (meters) run per day for rats in the wheel running group. Values are means ± SEM.

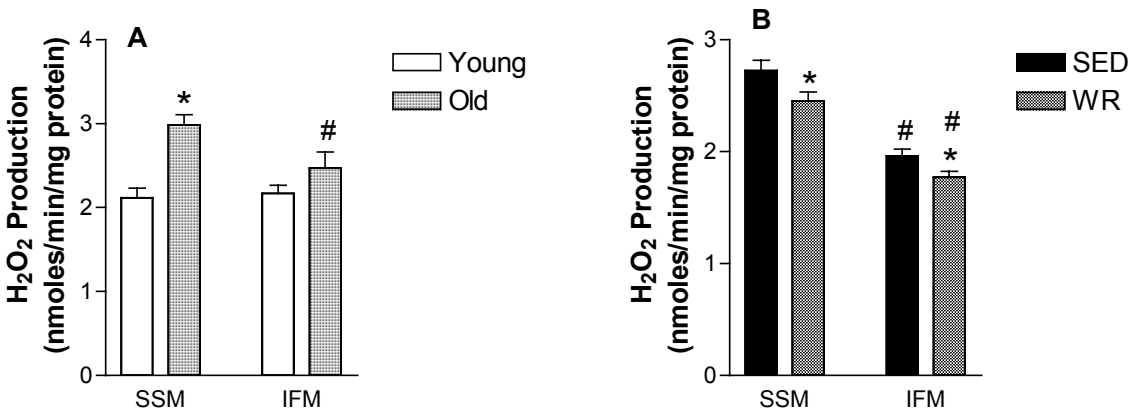

Figure 3. Hydrogen peroxide (H_2O_2) production in SSM and IFM from young and old rats (A) and SED and WR rats (B). Values are means ± SEM. * denotes significant ($p < 0.05$) difference between identical mitochondrial preparations from young and old rats and from SED and WR rats; # denotes significant ($p < 0.05$) difference between SSM and IFM within the same group of animals. For young animals, $n = 11$ for SSM and $n = 10$ for IFM. For old animals, $n = 9$ for SSM and $n = 8$ for IFM. For SED and WR animals, $n = 12$ for both SSM and IFM.

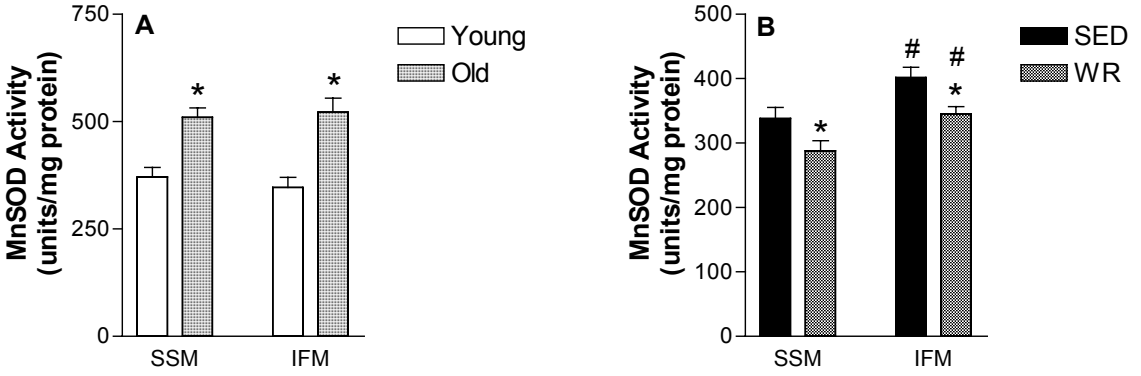

Figure 4. Manganese superoxide dismutase (MnSOD) activity in SSM and IFM from young and old rats (A) and SED and WR rats (B). Values are means ± SEM. * denotes significant ($p < 0.05$) difference between identical mitochondrial preparations from young and old rats and from SED and WR rats; # denotes significant ($p < 0.05$) difference between SSM and IFM within the same group of animals. For young animals, $n = 11$ for SSM and $n = 10$ for IFM. For old animals, $n = 9$ for SSM and $n = 8$ for IFM. For SED animals, $n = 12$ for both SSM and IFM. For WR animals, $n = 11$ for both SSM and IFM.

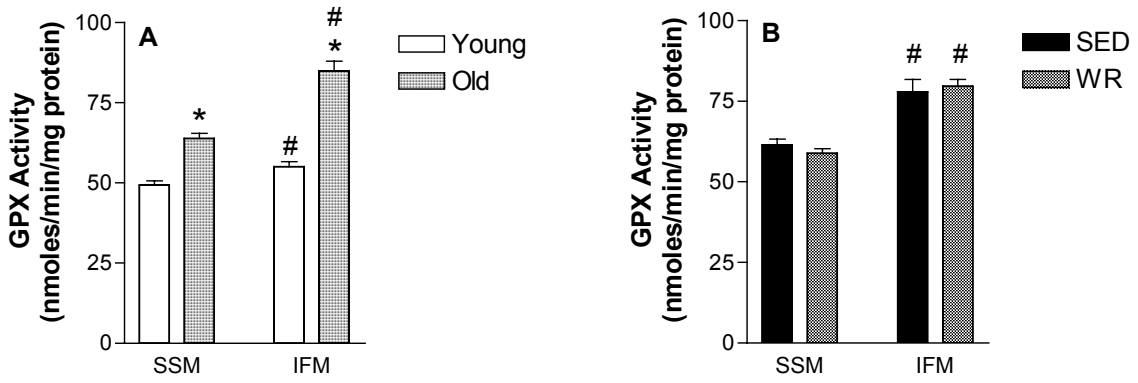

Figure 5. Glutathione peroxidase (GPX) activity in SSM and IFM from young and old animals (A) and SED and WR animals (B). Values are means ± SEM. * denotes significant ($p < 0.05$) difference between identical mitochondrial preparations from young and old rats; # denotes significant ($p < 0.05$) difference between SSM and IFM within the same group of animals. For young animals, $n = 11$ for SSM and $n = 10$ for IFM. For old rats, $n = 9$ for SSM and $n = 8$ for IFM. For SED and WR rats, $n = 12$ for both SSM and IFM.

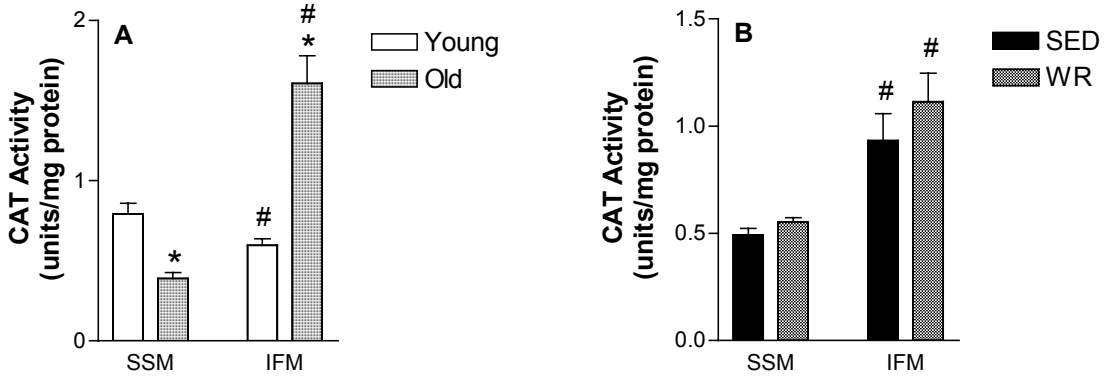

Figure 6. Catalase (CAT) activity in SSM and IFM from young and old animals (A) and SED and WR animals (B). Values are means ± SEM. * denotes significant ($p < 0.05$) difference between identical mitochondrial preparations from young and old rats; # denotes significant ($p < 0.05$) difference between SSM and IFM within the same group of animals. For young animals, $n = 11$ for SSM and $n = 9$ for IFM. For old animals, $n = 8$ for SSM and $n = 7$ for IFM. For SED animals, $n = 11$ for SSM and $n = 10$ for IFM. For WR animals, $n = 11$ for SSM and $n = 9$ for IFM.

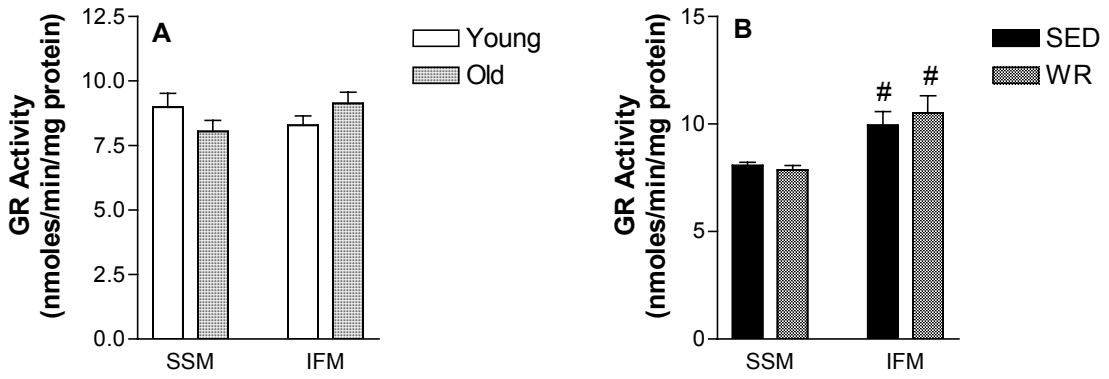

Figure 7. Glutathione reductase (GR) activity in SSM and IFM from young and old animals (A) and SED and WR animals (B). Values are means ± SEM. # denotes significant ($p < 0.05$) difference between SSM and IFM within the same group of animals. For young animals, $n = 11$ for SSM and $n = 9$ for IFM. For old animals, $n = 9$ for SSM and $n = 7$ for IFM. For SED and WR animals, $n = 11$ for both SSM and IFM.

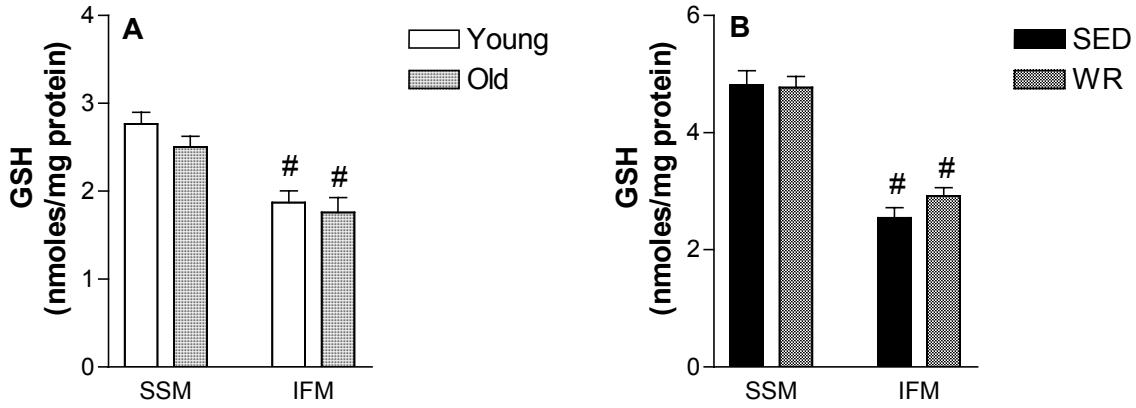

Figure 8. Reduced glutathione (GSH) content in SSM and IFM from young and old animals (A) and SED and WR animals (B). Values are means ± SEM. # denotes significant ($p < 0.05$) difference between SSM and IFM within the same group of animals. For young animals, $n = 11$ for SSM and $n = 10$ for IFM. For old animals, $n = 9$ for SSM and $n = 7$ for IFM. For SED animals, $n = 11$ for SSM and $n = 10$ for IFM. For WR animals, $n = 12$ for both SSM and IFM.

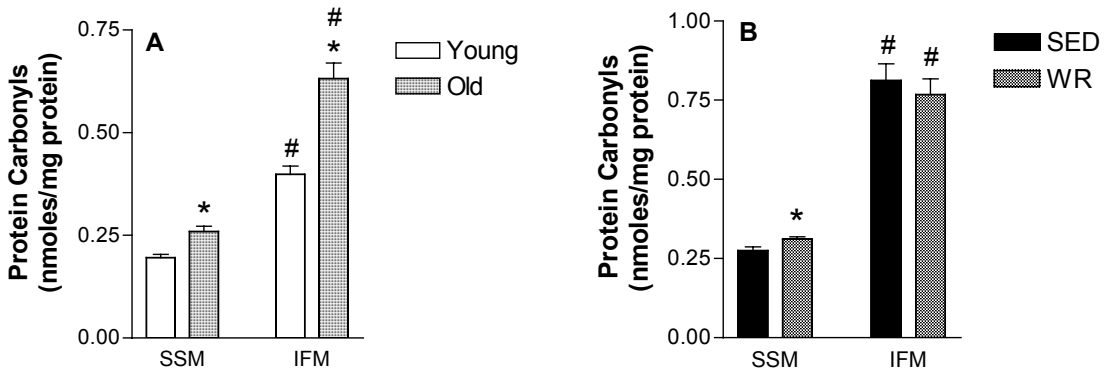

Figure 9. Protein carbonyl content in SSM and IFM from young and old animals (A) and SED and WR animals (B). Values are means ± SEM. * denotes significant ($p < 0.05$) difference between identical mitochondrial preparations from young and old rats and from SED and WR rats; # denotes significant ($p < 0.05$) difference between SSM and IFM within the same group of animals. For all groups, $n = 6$.

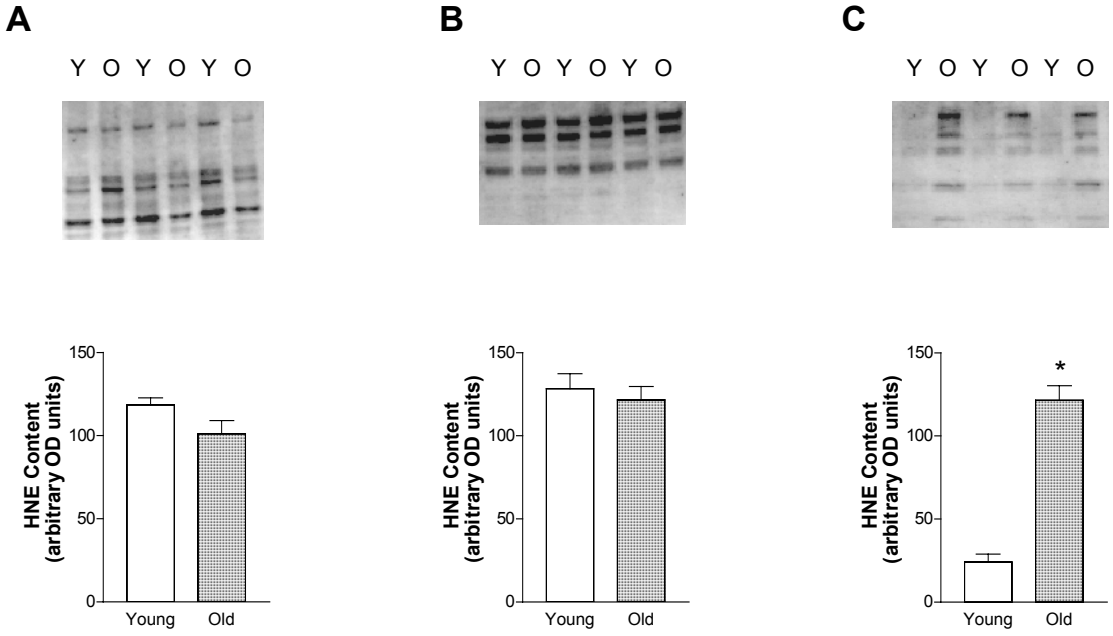

Figure 10. Western blot analysis of 4-hydroxy-2-nonenal-modified proteins (HNE) in cytosol (A), SSM (B), and IFM (C) from young and old rats. The top panel depicts a representative blot of HNE-modified proteins from young (Y) and old (O) animals. Optical densities (OD) of all the HNE-modified bands within a given lane were calculated using Kodak 1D Image Analysis software and represented graphically in the bottom panel. * $p < 0.0001$. Values are means ± SEM. For each group, $n = 5$.

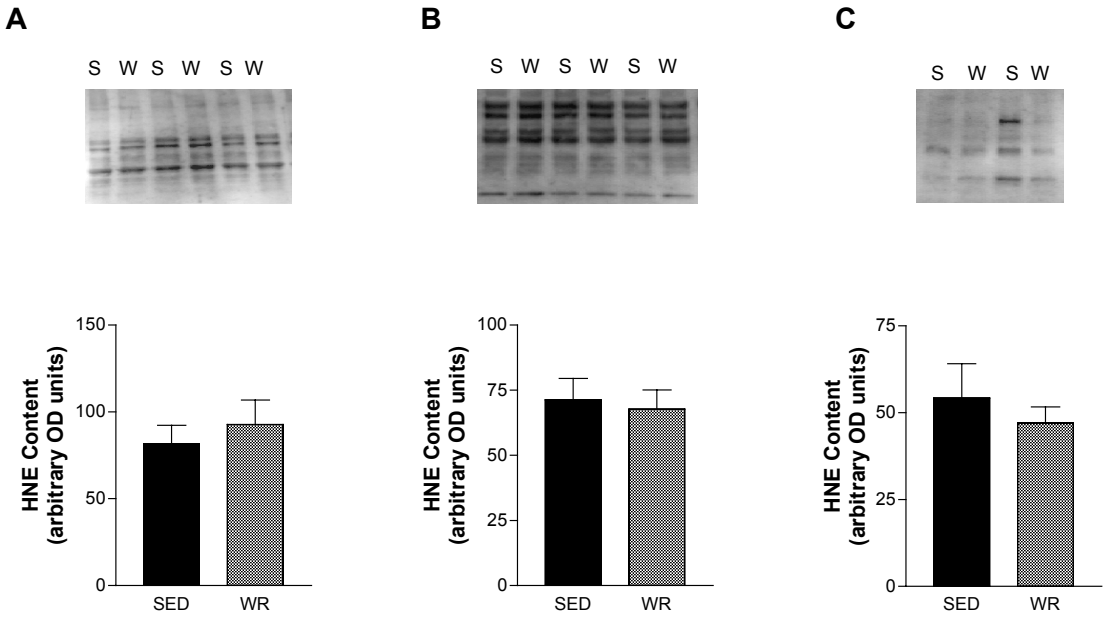

Figure 11. Western blot analysis of 4-hydroxy-2-nonenal-modified proteins (HNE) in cytosol (A), SSM (B), and IFM (C) from SED and WR rats. The top panel depicts a representative blot of HNE-modified proteins from SED (S) and WR (W) animals. Optical densities (OD) of all the HNE-modified bands within a given lane were calculated using Kodak 1D Image Analysis software and represented graphically in the bottom panel. Values are means ± SEM. For each group, $n = 5$.

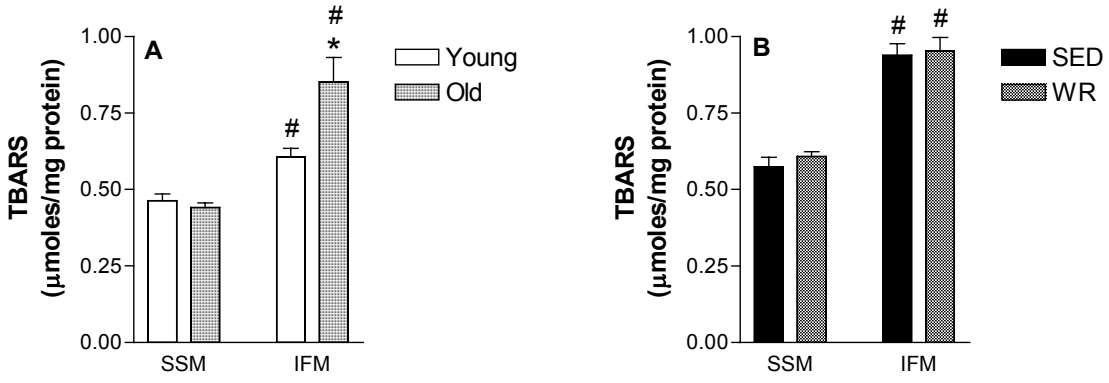

Figure 12. Thiobarbituric acid reactive substances (TBARS) in SSM and IFM from young and old rats (A) and SED and WR rats (B). Values are means ± SEM. * denotes significant ($p < 0.05$) difference between identical mitochondrial preparations from young and old rats; # denotes significant ($p < 0.05$) difference between SSM and IFM within the same group of animals. For all groups, $n = 6$.

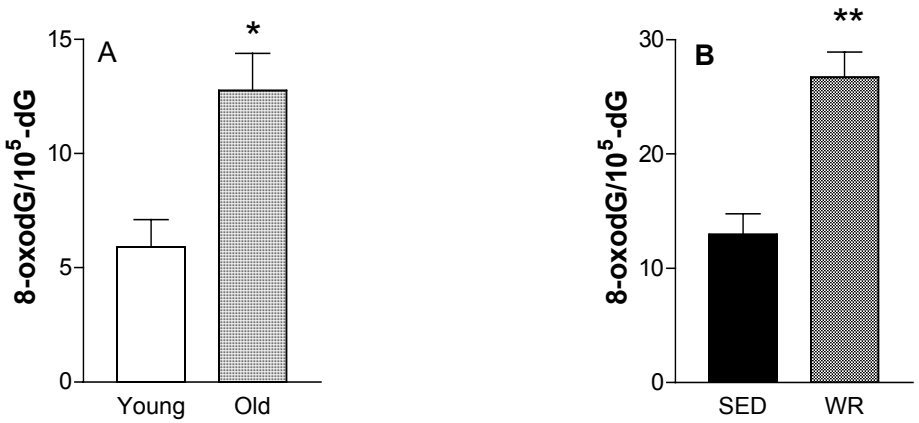

Figure 13. Nuclear oxidative DNA damage (8-oxodG) in young and old rats (A) and SED and WR rats (B). Values are means ± SEM and are expressed as the ratio of nmoles of 8-oxodG to 10^5 nmoles of deoxyguanosine. * $p < 0.05$, Young vs. Old; ** $p < 0.001$, SED vs. WR. Heart tissue from two rats was pooled for each measurement. For young and old rats, $n = 4$ and for SED and WR rats, $n = 6$.

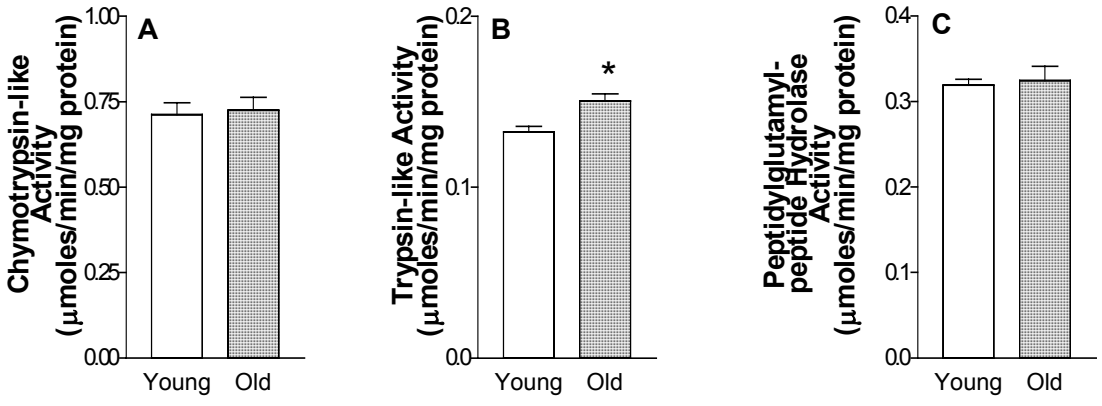

Figure 14. Chymotrypsin-like (A), trypsin-like (B) and peptidylglutamyl-peptide hydrolase (C) proteasome activities in heart cytosol from young and old rats. Values presented are means ± SEM. * $p < 0.05$. For all groups, $n = 9$.

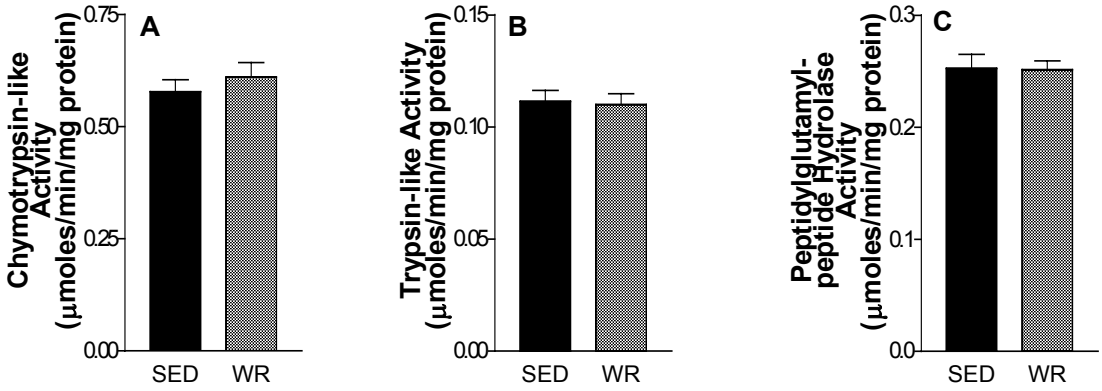

Figure 15. Chymotrypsin-like (A), trypsin-like (B) and peptidylglutamyl-peptide hydrolase (C) proteasome activities in heart cytosol from SED and WR rats. Values presented are means ± SEM. For all groups, $n = 9$.

CHAPTER 4
DISCUSSION

Aging is associated with an increase in mitochondrial DNA deletions [18, 34, 35] and a decline in mitochondrial function [42, 45, 46], leading to increased mitochondrial oxidant production [62-65, 78]. Furthermore, oxidative damage to DNA, lipids, and proteins has been shown to increase as a function of age [17, 108]. Regular physical activity can delay the onset of morbidity and increase mean lifespan [24, 25, 113], but the precise mechanisms by which exercise exerts its effects have not been determined. We explored the possibility that the benefits of long-term voluntary exercise, an intervention shown to increase mean lifespan [23-25], may be due to a reduction in mitochondrial oxidant production and oxidative damage. Additionally, since there are two functionally different populations of cardiac mitochondria – subsarcolemmal (SSM) and interfibrillar (IFM) – we investigated whether these populations were differently affected by age and exercise.

Mitochondrial Respiratory Function and Aging

It has previously been reported that there is a selective age-related decline in protein content and oxidative metabolism of IFM but not SSM [42]. Indeed, we found that with age, mitochondrial protein yield and state 3 respiration were significantly reduced in IFM but not SSM. The decline in state 3 respiration is likely to be a true effect of age and not an artifact of increased mitochondrial membrane damage during isolation, since there were no differences in state 4 respiration in IFM from young and old rats. Although it is possible that selective loss of dysfunctional mitochondria could occur

during isolation (thereby yielding an isolated population of relatively healthy mitochondria), it has also been shown that oxygen consumption in isolated cardiac myocytes declines with age [148].

A reduction in state 3 (ADP-stimulated) respiration should correspond to diminished ATP production. It is of particular concern that state 3 respiration is reduced in IFM but not SSM, since IFM are located between the myofibrils and are likely the major source of ATP for myosin ATPases [42, 44]. Since high levels of ATP are required for both systolic contraction and diastolic relaxation [149], reduced availability of ATP, as a result of IFM dysfunction, could contribute to the alterations in cardiac contractility observed with age [150, 151]. It is also of interest to note that with age, state 3 respiration was significantly increased in SSM. To our knowledge, this phenomenon has not been previously reported. The increase in state 3 respiration in SSM may reflect an adaptive response to the reduced state 3 respiration in IFM.

Mitochondrial Respiratory Function was Not Altered by Wheel Running

As noted, wheel running had no effect on mitochondrial protein yield, rates of oxygen consumption (states 4 and 3), or RCR. This is not entirely surprising given that several investigators have shown that, unlike skeletal muscle, oxidative capacity of cardiac muscle is not increased in response to treadmill training [120, 122, 152], an exercise protocol that is typically much more intense than voluntary wheel running. Indeed, Bizeau and colleagues [153] demonstrated that 6 weeks of treadmill training significantly increased state 3 respiration in skeletal muscle SSM and IFM whereas Servais et al. [147] found that 5 months of voluntary wheel running did not alter state 3 respiration in SSM or IFM isolated from quadriceps muscle. Since the heart is already highly oxidative, consuming more oxygen per gram of muscle at rest than skeletal muscle

does during heavy exercise [154], it is not expected to be as responsive to exercise-induced increases in oxidative capacity as skeletal muscle.

Hydrogen Peroxide Production Increased with Age in SSM but not IFM

Even under normal conditions, mitochondria produce hydrogen peroxide as a result of inefficient reduction of O_2 to H_2O [32, 155]. It is generally accepted that autoxidation of ubisemiquinone gives rise to superoxide ($O_2^{\bullet-}$) which is released into the mitochondrial matrix [156, 157]. Superoxide is then rapidly dismutated to H_2O_2 by manganese superoxide dismutase (MnSOD), which is abundant in the mitochondrial matrix [158]. Since $O_2^{\bullet-}$ production cannot be measured in intact mitochondria, H_2O_2 production is often used as a measure of mitchondrial oxidant production, and is believed to be an accurate reflection of mitochondrial $O_2^{\bullet-}$ production [159].

Mitochondrial dysfunction is associated with increased mitochondrial oxidant production [16]. Although mitochondrial function appears to decline with age, there are conflicting reports in the literature regarding the effects of age on mitochondrial H_2O_2 production. This may be due to the fact that most of these studies measured H_2O_2 production only in SSM, or in a mixed population of SSM and IFM. We are one of the first groups to determine age-related changes in mitochondrial H_2O_2 production in cardiac SSM and IFM.

We detected a significant increase in H_2O_2 production from SSM, but not IFM, with age. These results are in contrast to the findings recently reported by Suh et al. [44], that oxidant production increased with age in IFM, but not SSM, isolated from heart. Differences in the methods used to detect mitochondrial oxidant production may account for this discrepancy. Suh and colleagues [44] did not directly measure H_2O_2 production in isolated mitochondria, but used the rate of oxidation of 2'7'-

dihydrodichlorofluorescein (DCFH) in isolated mitochondria as an indicator of total mitochondrial oxidant production. Since no other reports have been published regarding differences in H_2O_2 production from SSM and IFM with age, it is clear that further investigation is warranted.

In addition to looking at age effects, we also determined whether SSM and IFM produced different amounts of H_2O_2. In agreement with Suh et al. [44], no changes in H_2O_2 production were observed between SSM and IFM from young rats. However, we found that H_2O_2 production was significantly lower in IFM from old rats compared to SSM from old rats. State 4 oxygen consumption was also significantly reduced in IFM from old rats, compared to SSM from the same animals. This finding is significant, since mitochondrial oxidant production is higher in state 4 than in state 3 due to greater reduction of the respiratory chain in state 4 [160]. Since we measure H_2O_2 production in state 4 conditions, the reduction in IFM H_2O_2 production may be directly related to the reduction in state 4 oxygen consumption.

Wheel Running Reduced Hydrogen Peroxide Production in SSM and IFM

A reduction in mitochondrial H_2O_2 production after exercise training has been reported in skeletal muscle [124] and 5 weeks of voluntary running reduced levels of 2,3-dihydroxybenzoic acid (an indicator of hydroxyl radical) in heart [161]. However, to our knowledge, we are the first to report a significant reduction in H_2O_2 production in SSM and IFM after 21 months of voluntary wheel running. Additionally, in both SED and WR rats, IFM produced significantly less H_2O_2 than SSM. Although H_2O_2 is not a radical per se (i.e., it has no unpaired electrons), it can be converted to the highly damaging hydroxyl radical in the presence of transition metal ions via the Fenton reaction [162]. Therefore, a

reduction in mitochondrial H_2O_2 could partially explain the reduction in oxidative damage that has been reported after exercise training [125-127].

Furthermore, since mitochondrial H_2O_2 arises as a result of the dismutation of two $O_2^{\bullet-}$ radicals, it can be speculated that voluntary exercise reduced the amount of $O_2^{\bullet-}$ being produced during electron transport. Since state 4 respiration was not altered between SED and WR animals, the exact mechanism by which this might occur is unknown. An attractive possibility is that long-term moderate exercise may reduce mitochondrial protonmotive force (Δp). A recent study by Lambert and Merry [163] found that caloric restriction (a known life-prolonging intervention) caused a significant reduction in liver mitochondrial Δp and H_2O_2 production, despite the fact that no changes in state 4 respiration were observed. It was determined that the reduction in Δp in response to CR arose as a result of decreased substrate oxidation and increased proton leak. Given that decreased substrate oxidation results in reduced oxygen consumption but increased proton leak causes increased oxygen consumption, the net effect is that state 4 respiration is not altered. In light of these findings, future studies should be performed to determine whether long-term exercise elicits changes in mitochondrial substrate oxidation and/or proton leak.

Antioxidant Enzyme Activity

Differences in the amount of oxidative damage that accrues with age or exercise may arise not only from changes in oxidant production, but may also occur as a result of alterations in the ability to scavenge oxidants and repair oxidative damage. Therefore, we also measured antioxidant enzyme activity in SSM, IFM, and cytosol to gain a better understanding as to whether alterations in antioxidant enzyme activity contribute to the

accumulation of oxidative damage with age [17, 63, 64, 80, 81] and to the reported exercise-induced attenuation of oxidative damage [125-127].

Age-Related Changes in Antioxidant Enzyme Activity

Although we did not directly measure mitochondrial $O_2^{\bullet-}$ production, we measured the activity of manganese superoxide dismutase (MnSOD), the enzyme responsible for dismutating $O_2^{\bullet-}$ to H_2O_2 in the mitochondrial matrix. We detected a significant increase in MnSOD activity in SSM and IFM with age, which likely reflects increased mitochondrial $O_2^{\bullet-}$ production. Indeed, it has been shown that mitochondrial $O_2^{\bullet-}$ production is increased in the aging heart [63].

Since MnSOD converts two $O_2^{\bullet-}$ radicals to H_2O_2 and O_2, an increase in MnSOD activity with age should result in elevated H_2O_2 production in the mitochondria. As previously discussed, we detected a significant increase in H_2O_2 production from SSM but not IFM with age. Therefore, we also examined the activities of the two enzymes in the mitochondria involved in scavenging H_2O_2 – glutathione peroxidase (GPX) and catalase (CAT).

Since GPX is believed to play a greater role in scavenging H_2O_2 in the heart than CAT [164], it was not surprising that we observed a significant increase in GPX activity with age in both SSM and IFM. Changes in CAT activity were less consistent, with SSM exhibiting an age-related decline in CAT activity while IFM showed an age-related increase in CAT activity. Although some studies report that antioxidant enzyme activities decline with age in the heart, in light of the fact that exposure to oxidants acts as a signal to increase the activity and expression of antioxidant enzymes [165], it seems more likely to expect an increase in antioxidant enzyme activity with age, as this adaptation may help to protect tissues from oxidative stress. However, it is possible that

under conditions of extreme oxidative stress, antioxidant enzymes themselves may be targets of oxidant-induced damage, and this may lead to a decline in enzyme activity. Indeed, Mayo and colleagues [166] found that free radicals could oxidatively modify and inactivate CAT activity *in vitro* while GPX activity has been shown to be reduced in pathological conditions associated with severe oxidative stress, such as doxorubicin-induced oxidative stress [167] and sepsis [168]. Furthermore, in brains of patients with Alzheimer's disease (in which oxidative stress is believed to play a role in the pathogenesis of the disease), the activities of all three major antioxidant enzymes (SOD, GPX, and CAT) were decreased compared to normal, age-matched controls with the greatest difference observed in CAT activity [169]. Given that H_2O_2 production was greater in SSM compared to IFM with age, it could be hypothesized that oxidants selectively modified CAT in SSM, and this might help to explain the age-related decrease in CAT activity in SSM.

Our findings that MnSOD and GPX activities are increased with age are in agreement with previous studies [77, 83, 107]. However, the differences we observed between SSM and IFM from old animals are unique, and may help to explain why we detected an increase in H_2O_2 from SSM but not IFM with age. In the old animals, there was no difference in MnSOD activity between SSM and IFM, but both GPX and CAT activities were higher in IFM than in SSM. Therefore, IFM appear to have a greater ability to scavenge H_2O_2 than SSM. Seeing as our assay measures the amount of H_2O_2 released from the intact mitochondria into our medium, the increased GPX and CAT activities in IFM is a likely explanation for why we did not detect an increase in H_2O_2 production from IFM with age.

Although GPX activity was increased with age, we did not find any age-related changes in glutathione reductase (GR) activity in SSM or IFM. Since GSH is required for GPX to function, it might be expected that we would see a concomitant increase in GR, the enzyme responsible for regenerating GSH. However, previous studies have also found increases in GPX activity and no change in GR activity with age [83, 105], possibly because the heart contains sufficient levels of GR to regenerate GSH from GSSG. Support for this lies in the fact that in skeletal muscle, which contains lower levels of GR than heart, GR activity only increased by 29% with age despite the fact that GPX activity increased by 45% [105].

GSH has been reported to decrease in IFM with age [44], but we did not see any age-related differences in GSH content in SSM or IFM. However, Suh et al. [44] also found an age-related decrease in GR activity in IFM and subsequently, an increase in GSSG in IFM. It is possible that we did not see a decline in GSH because of the fact that we saw no alterations in GR activity. It is also known that GSH is synthesized in the cytosol (it cannot be synthesized in the mitochondria) and must be transported into the mitochondria [170]. If GSH transport into the mitochondria were increased with age, this would also help to maintain adequate levels of mitochondrial GSH. Indeed, we saw an increase in cytosolic GSH with age and this may facilitate transport into the mitochondria.

Even if GSH levels are not changed, an alteration in the ratio of GSH:GSSG towards the oxidized state can result in glutathiolation (also known as S-thiolation) of proteins [110]. Furthermore, mitochondrial GSSG content has been correlated with oxidative damage to mitochondrial DNA [35]. Unfortunately, the GSSG peaks in SSM

and IFM were very low and could not be resolved by HPLC. Since GSSG formed in the mitochondria cannot be transported into the cytosol to become reduced [171], it would have been interesting to see if SSM and IFM from old animals contained higher levels of GSSG, thereby altering the GSH:GSSG ratio.

Effects of Voluntary Wheel Running on Antioxidant Enzyme Activity

In the wheel running animals, there was a significant decrease in MnSOD activity in SSM and IFM, suggesting that mitochondria isolated from wheel runners produced less $O_2^{\bullet-}$. We have previously shown that MnSOD activity and H_2O_2 production are reduced in SSM from hearts of young animals after 8 weeks of calorie restriction [172], a known life-prolonging intervention. Lifelong calorie restriction attenuates age-related increases in mitochondrial $O_2^{\bullet-}$ and H_2O_2 production [63, 101], and our results support the idea that voluntary wheel running, an intervention which increases mean lifespan in rodents, has similar effects.

Wheel running did not alter the activities of any other mitochondrial or cytosolic antioxidant enzymes we measured (GPX, CAT, and GR) and mitochondrial GSH levels were also not different between SED and WR rats. It is difficult to directly compare our results to other studies that have used exercise training (treadmill or swimming) since the duration and intensities of those types of exercise are typically much greater than voluntary wheel running, and are generally performed for much shorter time periods. Kim et al. [29] measured various cytosolic antioxidant enzyme activities in hearts from 20-mo-old Fischer 344 rats that ran voluntarily for 18.5 months. Similar to our results, they found no effect of wheel running on cytosolic SOD or GPX, but they did find a significant increase in cytosolic CAT activity. Another study examining the effects of short-term voluntary wheel running (1 and 7 days) found results remarkably similar to

ours in that total SOD activity was decreased in the hearts of 1- and 7-day runners, while GPX and CAT activity remained unchanged [173]. Although the reduction in MnSOD activity in WR animals may be in response to reduced mitochondrial $O_2^{\bullet-}$, additional studies are required to determine whether this is true.

Protein Carbonyls Increased in SSM and IFM with Age

Carbonyls can be formed via several mechanisms including site-specific metal-catalyzed oxidation of lysine, arginine, proline, and threonine residues; glycation reactions; and interaction of amino acid side chains with lipid peroxidation products, such as 4-hydroxy-2-nonenal and malondialdehyde [69]. Several studies have reported an increase in protein carbonyls in the aging heart [63, 64, 79, 80] and we found that protein carbonyls were significantly increased with age in both SSM and IFM. To our knowledge, we are the first to report that carbonyl content is elevated in both mitochondrial subpopulations (SSM and IFM) with age. An accumulation of oxidized proteins is believed to play a key role in the loss of physiological function with age, since oxidized proteins can lose catalytic activity and are also prone to forming large, potentially cytotoxic, protein aggregates [69, 174]. Therefore, the increase in mitochondrial carbonyls likely contributes to mitochondrial dysfunction with age.

The extent to which oxidized proteins accumulate is dependent on several factors including the amount and type of oxidant produced; the ability of the antioxidant defense system to prevent oxidant-induced protein damage; and the cell's capacity to repair or remove oxidized proteins [69]. Since mitochondrial antioxidant enzymes and GSH did not decline with age (with the exception of CAT in SSM), the observed increase in carbonyls is likely a result of increased oxidant production and/or diminished removal of oxidized proteins. Indeed, H_2O_2 production was significantly elevated in SSM with age,

although there was no age-related difference in H_2O_2 production in IFM. Additionally, it has been reported that the activity of Lon protease (the protease responsible for recognizing and degrading oxidized mitochondrial proteins) declines with age, and correlates with the accumulation of oxidized aconitase [135, 175]. Therefore, it is possible that the age-related increase in protein carbonyls we observed in SSM and IFM is a direct result of diminished Lon protease activity, and we plan to address this issue in future studies.

Wheel Running Increased Protein Carbonyls in SSM

We observed a small (12%) but significant increase in protein carbonyls in SSM from WR rats compared to SED rats. It is possible that this increase is related to the significant reduction in MnSOD activity in SSM from WR rats. We previously reported that, despite a reduction in H_2O_2 production and MnSOD activity, protein carbonyls in SSM from calorie-restricted rats were increased compared to *ad libitum* fed rats [172]. Bota et al. [135] found that young (3-6 mo) *Sod2*$^{+/-}$ mice, which exhibit a 50% decrease in MnSOD activity, display an 80% increase in skeletal muscle protein carbonyls compared to young *Sod2*$^{+/+}$ mice. Old (27 mo) *Sod2*$^{+/-}$mice had an even greater increase in carbonyls (250%) when compared to young *Sod2*$^{+/+}$ mice. Although MnSOD activity was also reduced in IFM from WR rats, no significant differences in carbonyls in IFM were observed between SED and WR rats. This may be partially explained by the fact that IFM had higher antioxidant enzyme activity compared to SSM. It will be of interest to determine whether the increase in carbonyls observed in SSM from WR rats corresponds to reduced activity of key mitochondrial proteins, such as aconitase (which has been shown to be susceptible to oxidative modification) [135].

Lipid Peroxidation was Significantly Elevated in IFM from Old Rats

4-hydroxy-2-nonenal (HNE) is a cytotoxic end-product of lipid peroxidation that is highly reactive with other biological molecules, especially proteins [85]. Using Western blotting, we found that IFM from old animals contained significantly more HNE-modified proteins compared to IFM from young animals. We also measured TBARS, an indicator of malondialdehyde levels, and found that levels were significantly increased in IFM from old animals compared to IFM from young animals. Despite the fact that there was an age-related increase in protein carbonyls in SSM, we found no differences in HNE or TBARS in SSM with age. However, this is in agreement with a previous study that reported an increase in protein carbonyls, but not lipid peroxidation, in SSM with age [53].

The finding that IFM exhibit age-related increases in lipid peroxidation is intriguing in light of recent reports that bovine heart cytochrome c oxidase incubated *in vitro* with HNE exhibits a 50% decrease in electron transport activity [176], and that there is a significant age-related increase in the amount of HNE adducted to Complex IV (cytochrome c oxidase) in IFM but not SSM [44]. Suh et al. [44] also detected a concomitant age-related decrease in Complex IV activity in IFM but not SSM.

Interestingly, Fannin and colleagues [42] also found an age-associated decline in cytochrome c oxidase activity when it was measured in freshly isolated IFM. However, when IFM were incubated *in vitro* with asolectin (a source of exogenous phospholipids) or were freeze-thawed prior to measuring cytochrome oxidase activity, age-related differences were abolished. The decline in cytochrome oxidase activity has been associated with a reduction in mitochondrial cardiolipin (an essential inner membrane phoshpholipid) [177], and administration of exogenous cardiolipin restores cytochrome

oxidase activity in old rats to levels similar to that of young rats [39]. Based on these findings, Fannin and coworkers [42] proposed that the age-related decline in IFM cytochrome oxidase activity arises as a result of alterations to the phospholipid composition of the inner mitochondrial membrane and not because of changes in, or loss of, the catalytic subunits of cytochrome oxidase. Since loss of cardiolipin is associated with oxidant-induced lipid peroxidation [178], the elevated levels of HNE and TBARS that we found in old IFM may be a major contributing factor to the decline in state 3 respiration observed in IFM with age.

Furthermore, Echtay and coworkers [179] recently proposed a role for HNE in regulating mitochondrial uncoupling and reducing oxidant production. They found that kidney mitochondria incubated with physiological concentrations of HNE exhibited increased mitochondrial proton conductance that was mediated by uncoupling proteins 1, 2, and 3 (UCPs) and adenine nucleotide translocase (ANT). Additionally, mitochondrial H_2O_2 production was significantly reduced in response to HNE. They propose that membrane phospholipids can be oxidized by $O_2^{\bullet -}$ (produced during electron transport), leading to the formation of HNE. The resultant oxidative stress may alter ANT activity and induce UCP expression, thereby increasing mitochondrial proton leak and reducing $O_2^{\bullet -}$ production. The increase in HNE we observed in IFM from old rats may therefore help to explain why mitochondrial H_2O_2 production was found to increase in SSM, but not IFM, with age.

Lipid Peroxidation was Not Different Between Wheel Running and Sedentary Rats

Voluntary wheel running did not reduce the amount of HNE-modified proteins in cytosol, SSM, or IFM, nor did it reduce the amount of TBARS in SSM or IFM. Although this was not what we had originally hypothesized, Radak et al. [126] reported

that TBARS, HNE-modified proteins, and protein carbonyls in gastrocnemius muscle from 14-mo-old rats that had been swim-trained for 9 weeks were not different compared to age-matched, sedentary rats. Navarro et al. [180] examined oxidative damage in hearts of mice that performed moderate treadmill exercise from 28 to 78 weeks of age. At 52 weeks, protein carbonyls and TBARS were significantly reduced in hearts of exercised mice compared to age-matched sedentary controls. However, at 78 weeks no differences in carbonyls or TBARS were observed between the exercised and sedentary mice. From this data, it appears that moderate exercise reduces oxidative damage in adult rodents, but with advancing age, these benefits are no longer observed. Since our rats were old (104 weeks) at the time of sacrifice, this may be one reason as to why we found no differences in lipid peroxidation between SED and WR rats.

Nuclear Oxidative Damage Increased With Age and Voluntary Wheel Running

In agreement with previous studies in heart, there was an age-related increase in the levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in nuclear DNA [34, 73]. Although we were unable to measure mitochondrial 8-oxodG (due to the large amount of tissue required to obtain sufficient amounts of mitochondrial DNA), it is tempting to speculate that mitochondrial DNA damage also increased with age, as it has been reported that mitochondrial DNA accumulates more 8-oxodG with age than nuclear DNA [34]. Hamilton and colleagues [73] reported that the increase in 8-oxodG with age is not a result of reduced DNA repair activity, as nuclear 8-oxodG lesions induced by γ-irradiation are removed at the same rate in young and old mice. However, it was noted that the levels of 8-oxodG induced by γ-irradiation were 85% higher in hearts of old mice compared to young mice, leading the authors to propose that old animals are more susceptible to oxidative stress. Indeed, Edwards et al. [181] found that in response to

paraquat-induced oxidative stress, transcription of three isoforms of GADD45 (a DNA damage responsive gene) were significantly induced in hearts from 5-mo-old mice, but not in hearts from 25-mo-old mice. It appears that the aged heart has an impaired ability to respond to oxidative stress and this may contribute to the accumulation of oxidative damage in old animals.

Unexpectedly, wheel running further increased levels of nuclear 8-oxodG, a finding that is in contrast to previous reports that treadmill running [125] and swim training [126] reduced 8-oxodG in rat gastrocnemius muscle. Additionally, Selman et al. [173] found no differences in oxidative DNA damage in lymphocytes and hepatocytes from voles that ran voluntarily for 1 or 7 days compared to sedentary voles. Although it is unclear as to why 8-oxodG levels in the heart would be significantly increased by wheel running, it is possible that it could be related to the significant decrease in MnSOD activity. Van Remmen and coworkers [182] reported that mice heterozygous for the *Sod2* gene (*Sod2*$^{+/-}$), which corresponds to ~50% reduction in MnSOD activity in all tissues, exhibit significantly greater amounts of nuclear 8-oxodG compared to wild-type mice. The old *Sod2*$^{+/-}$ mice (26 mo) also had an increased incidence of tumors compared to wild-type mice, but mean and maximum lifespan between the two groups were not different. Furthermore, in old mice, no differences were observed between the two groups in levels of carboxymethyl lysine and pentosidine in skin collagen, immune function, or cataract formation - all biomarkers of aging. However, in this study, it was proposed that a reduction in MnSOD activity could damage nuclear DNA as a result of increased mitochondrial $O_2^{\bullet -}$ and H_2O_2 production. We found that SSM and IFM from wheel

runners produced less H_2O_2, so further investigation as to why DNA damage is increased in wheel runners is clearly required.

Effects of Age and Wheel Running on 20S Proteasome Activity

Despite reports of decreased 20S proteasome activity with age [112, 183], we found no changes in chymotrypsin-like or peptidylglutamyl-peptide hydrolase (PGPH) activity and surprisingly, a significant increase in the trypsin-like activity with age. It has been reported that HNE selectively inactivates the trypsin-like activity of the 20S proteasome [183] and therefore, the tendency for HNE-modified protein to be lower in cytosol of old animals may be one possible explanation for the increased trypsin-like activity.

The fact that we did not observe an increase in any of the activities of the 20S proteasome is not entirely surprising given that we did not see a decline in proteasome activity with age. Furthermore, activity of the 20S proteasome is increased under conditions of mild oxidative stress and decreased in response to severe oxidative stress [99, 100]. Therefore, since there were no differences in cytosolic oxidative stress between SED and WR rats, it is not unexpected that 20S proteasome activity was not different between the two groups.

IFM Are Under Greater Oxidative Stress Compared to SSM

Interestingly, we found that in both the SED and WR animals, all four of the antioxidant enzyme activities we measured (MnSOD, GPX, CAT, and GR) were significantly higher in IFM compared to SSM, while mitochondrial GSH was significantly lower in IFM than in SSM. Similar changes were observed in IFM from young and old animals.

Since it has been reported that IFM exhibit more detrimental changes with age [42-44], it is likely that the elevated antioxidant enzyme activity is an adaptive response to increased oxidant production in IFM compared to SSM. Although H_2O_2 production from IFM was lower than SSM in all groups of old animals, this may simply be a result of the increased GPX and CAT activity in IFM. This would allow for more H_2O_2 to be scavenged inside the mitochondria, whereas our assay measures H_2O_2 released from intact mitochondria.

In further support of the idea that oxidative stress is greater in IFM, IFM exhibited significantly elevated levels of protein carbonyls and TBARS compared to SSM. Therefore, it appears that despite the fact antioxidant enzyme activities were increased, they were not sufficiently elevated to scavenge the oxidants being produced and prevent oxidative damage.

To our knowledge, we are the first to report that antioxidant enzyme activities and protein carbonyls and TBARS (two markers of oxidative damage) are greater in IFM compared to SSM. These findings have profound implications for future research investigating the effects of age on mitochondrial function and oxidative stress, as it is clear that numerous differences between the two subpopulations exist.

Possible Explanations for Lack of Expected Differences in Wheel Runners

Long-term voluntary wheel running did not cause as many beneficial effects as we had originally hypothesized and therefore, we have attempted to provide some explanation for the lack of significant findings. The first possibility is that the amount of exercise performed by the rats was not sufficient to cause major adaptive changes. Compared to other strains of rats, the Fischer 344 exhibits relatively low voluntary running activity. The maximum amount of running performed by the rats in our study

was 2462 ± 435 meters/day (at 6 mo) after which point, the mean running activity for the remainder of the study decreased to 1145 ± 248 meters/day. In contrast to this, it has been reported that Long-Evans rats run between 4000-6000 meters/day at 6 mo, and although running activity does decline with increasing age, they continue to run more than 2000 meters/day at 24 mo [24, 136]. However, we chose the Fischer 344 as our model because it is an inbred strain that has been extremely well characterized with respect to age-associated changes, and most of our previous work has been performed using the F344.

Another strong possibility as to why we did not see as many changes as expected, is that the very mild food restriction (8% below *ad libitum* intake) imposed on both our wheel running and sedentary rats, may have independently exerted positive effects in the sedentary group that masked any beneficial effects of the wheel running. In fact, statistical analyses performed between the old, sedentary, *ad libitum* fed group (Old) and the old, sedentary restricted group (SED) reveal significant differences in several of the parameters we measured (for example, GSH was significantly elevated in both SSM and IFM from SED compared to Old animals). Furthermore, Holloszy reported that the beneficial effects of calorie restriction (70% of *ad libitum* intake) and wheel running were not additive or synergistic in terms of lifespan – calorie restricted, wheel running rats had the same maximal lifespan of calorie restricted, sedentary rats [24]. Therefore, it is highly likely that if the food restriction used in our study did have beneficial effects, it may have hindered our ability to detect significant changes that occurred as a result of wheel running.

Summary and Significance

Mitochondrial dysfunction and the accumulation of oxidative damage to macromolecules are believed to play key roles in the aging process[13, 14]. Characterization of age-related changes to cardiac mitochondria has been complicated by the fact that two distinct populations of mitochondria (SSM and IFM) exist in cardiac muscle [40]. In agreement with previous studies [42, 43], we have shown that state 3 respiration declines in IFM, but not SSM, with age. Furthermore, we have shown that oxidative stress is greater in IFM than SSM, as evidenced by the fact that antioxidant enzyme activities and oxidative damage are increased in IFM compared to SSM. These results demonstrate the importance of studying both populations when attempting to elucidate the contribution of mitochondrial dysfunction to myocardial aging.

Despite the fact that we observed significant reductions in H_2O_2 and MnSOD activity in both SSM and IFM from wheel runners (a phenomenon we have also observed in calorie-restricted rodents), no other beneficial effects of WR were detected and possible reasons for this have been provided. Since wheel running has been reported to increase mean lifespan in rodents [23, 24, 27, 136], it is clear that further studies are needed to determine the mechanisms underlying this observation. However, based on our findings, we cannot rule out the possibility that reduced mitochondrial oxidant production is one contributing factor.

The lack of changes observed in the cytosol with age or voluntary wheel running lends further support to the idea that mitochondria are centrally involved in the aging process. Additionally, the discrepancies in the literature regarding age-related changes in antioxidant enzymes and oxidative damage may be partially explained by the fact that many studies have used tissue homogenate for their analyses. The cytosolic fraction

would comprise a much greater proportion of the total homogenate than the mitochondrial fraction, and therefore may mask the changes occurring in the mitochondrial compartment.

Finally, the notion that mitochondria are involved in numerous cardiovascular diseases is gaining more popularity. In recent years, mitochondrial dysfunction and oxidant production has been implicated in contributing to atherosclerosis and hypertension [184], ischemia-reperfusion injury [185], and heart failure [186]. The development of strategies to prevent mitochondrial dysfunction and reduce mitochondrial oxidant production may prove useful in treating various cardiovascular, and other age-associated diseases. Although this notion may seem far-fetched, mitochondria-targeted antioxidants, spin traps, and thiol compounds have been developed and used successfully *in vitro*; work regarding their efficacy *in vivo* is ongoing [187], but may someday lead to new therapies for human disease.

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BIOGRAPHICAL SKETCH

Sharon Phaneuf Judge graduated magna cum laude from Wheaton College (MA) with a Bachelor of Arts degree in biology in 1995. She was then employed as a research assistant at Ergo Science, Inc., in Charlestown, MA. While at Ergo Science, Sharon was involved in pre-clinical research designed to test and develop a new drug for Type II diabetes. In August 1998, Sharon began her graduate studies in exercise physiology at the University of Florida. She received her M.S.E.S.S. degree in May 2000 and then went on to pursue a Ph.D. in exercise physiology.