ALTERATIONS IN SKELETAL MUSCLE STRENGTH AND MITOCHONDRIAL FUNCTION INDUCED BY AGING AND EXERCISE

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By

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ABSTRACT

Introduction: Mitochondria are important organelles for skeletal muscle function. Mitochondria are susceptible to many forms of stress that alters their morphology, energy generation and reactive oxygen species (ROS) production, which collectively promote degeneration and dysfunction in skeletal muscle. These processes are implicated in many health disorders, particularly in the aging process itself. Exercise is well established to increase muscle mitochondrial content and thus may attenuate several aspects of mitochondrial deterioration. **Methods:** Both human and animal models of mitochondrial stress (aging, ROS) were utilized in order to determine their effects on mitochondrial and muscle function. Additionally, exercise training was used in order to assess its therapeutic potential in ameliorating defects in oxidative capacity, muscle atrophy and metabolic adaptation in skeletal muscle. **Results:** Aging resulted in reduced strength, aerobic capacity, larger intramyocelluar lipid droplets and fewer mitochondria in skeletal muscle. These changes were related to suppressed lipid metabolism, mitochondrial dynamics and organelle turnover. Habitual aerobic exercise partially attenuated the age-related loss of muscle strength and aerobic capacity, presumably due in part to improved mitochondrial function. Persistent mitochondrial oxidative stress prevented mitochondrial adaptations to exercise training in mice, a phenomenon that may explain why exercise cannot fully counteract the effects of aging in skeletal muscle. **Conclusions and significance:** This work furthers our knowledge of the mitochondrial consequences of aging and the therapeutic potential of aerobic exercise within skeletal muscle. These results can be applied to other differentiated tissues that are severely affected by aging (brain, heart) and

the effects described here are likely relevant to other conditions that result in muscle atrophy and energetic insufficiency.

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ABBREVIATIONS

AMP- adenosine monophosphate
ADP- adenosine diphosphate
AMPK- AMP-activated protein kinase
ATP- adenosine triphosphate
CAMK- Ca^{2+}/calmodulin-dependent protein kinases
COPD- chronic obstructive pulmonary disease
COX- cytochrome c oxidase
CS- citrate synthase
CT scan- computer tomography scan
DEXA- dual-energy X-ray absorptiometry
DNA- deoxyribonucleic acid
Drp1- dynamin-1-like protein
ETC- electron transport chain
$FADH_2$- flavin adenine dinucleotide
GTP- guanosine triphosphate
IMCL- intramyocellular lipid
MAPK- mitogen activated protein kinase
MHC- myosin heavy chain
Mfn1- mitofusin 1
Mfn2- mitofusin 2
MRI- magnetic resonance imaging
mRNA- messenger RNA
mtDNA- mitochondrial DNA
NADH- nicotinamide adenine dinucleotide
NADPH- nicotinamide adenine dinucleotide phosphate
NRF1- nuclear respiratory factor 1
NRF2- nuclear respiratory factor 2
OPA1- optic atrophy 1
PPARα- peroxisome proliferator-activated receptor alpha
PPARγ- peroxisome proliferator-activated receptor gamma
PGC-1α- peroxisome proliferator-activated receptor gamma co-activator 1-alpha
PolG- polymerase gamma
POLRMT- mitochondrial DNA-directed RNA polymerase
ROS- reactive oxygen species
RNA- ribonucleic acid
RNS- reactive nitrogen species
rRNA- ribosomal RNA
Sod2- superoxide dismutase 2
TFAM- mitochondrial transcription factor A
TFB1M- mitochondrial transcription factor B1
TFB2M- mitochondrial transcription factor B2
tRNA- transfer RNA

STATEMENT OF CONTRIBUTION

Manuscript 1: The principal investigator of this study was MAT. All data was collected and analyzed at McMaster University in the laboratory of MAT. Study design and conception were done by JDC and MAT, and the human ethics proposal was written by MAT. MCD and MH recruited the participants and samples were collected by MCD, MH and MAT. The electron microscopy was done by JDC, MCH and MH. All qPCR analysis was performed by CW. Data interpretation and analysis was done by JDC. The manuscript was written and prepared by JDC.

Manuscript 2: The principal investigator of this study was MAT. All data was collected and analyzed at McMaster University in the laboratory of MAT. Study design and conception were done by JDC and MAT and the animal ethics proposal was written by JDC and BPH. Mice were exercised by JDC and BPH. Samples were collected by JDC, AA, BPH and DIO. JDC performed most of the western blotting, and all DNA qPCR, RNA qPCR and exercise testing. AA performed the remainder of the western blotting. LGM performed the TAC assay. DIO and AA performed the 8-hydroxy mtDNA analysis. Data interpretation and analysis was performed by JDC. The manuscript was written and prepared by JDC.

Manuscript 3: The principal investigator of this study was MAT. All data was collected and analyzed at McMaster University in the laboratory of MAT. Study design and

conception were done by JDC and MAT, and the human ethics proposal was written by JDC and LGM. The participants were recruited by JDC and LGM. All testing of the subjects was performed by JDC and LGM. Data interpretation and analysis was performed by JDC. The manuscript was written and prepared by JDC.

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CHAPTER 1: GENERAL LITERATURE REVIEW

1.0.1 Introduction

Aging is the progressive deterioration of cellular function that results in disease, frailty and eventually death in all organisms. Advances in science and medicine have improved lifespan dramatically, so much so that the proportion of adults greater than 65 years is expected to increase from 12.5% currently to over 20% of the population by the year 2030 (1). Given that end of life health demands considerably more medical care than any other point in the lifespan, this population re-distribution will place a significant stress on the existing healthcare system. Current therapies aimed at extending the years of functional, healthy life in older adults are limited and often impractical, particularly in the very frail. Reduced skeletal muscle function is a major factor in the decline in mobility and independence in older adults. Various factors contribute to muscle weakness during aging including lower levels of physical activity, increased tissue stress and impaired cellular energy production. A central theme of these issues is mitochondrial dysfunction, which appears to be countered to some degree by aerobic exercise training. However, the precise factors that regulate mitochondrial function and their resultant physiologic effects during aging remain to be fully clarified. Thus, a more thorough understanding of skeletal muscle physiology and mitochondrial dysfunction is necessary in order to attenuate degeneration and promote a high quality of life in an expanding aging population.

1.1.1 Muscle mass and contractile changes with age

Skeletal muscle function generally declines with age. While it is difficult to precisely determine when this deterioration begins, most reports suggest 20-50 years of

age depending on the facet of muscle function studied, with an accelerated rate thereafter (2, 3). One aspect of this functional decline is the age-related loss of muscle mass throughout the body, known as sarcopenia. The magnitude and prevalence of sarcopenia differs considerably depending on the specific criteria used and according to the population studied (i.e. community dwelling vs. institutionalized elderly). Rates of sarcopenia in adults over the age of 65 years can be as low as 1% in otherwise healthy individuals or may approach 50% in those with health complications and high medication consumption (4-6).

Skeletal muscle is composed of 3 different types of fibers known as type I, IIa or IIx according to their predominant myosin heavy chain (MHC) isoform composition. These fiber types vary in their power production and force generating capabilities such that IIx>IIa>I (7, 8). Age-related muscle atrophy is due to both a decline in the size and in the number of skeletal muscle fibers (3) and accordingly rates of protein synthesis of the myosin heavy chains are lower in older adults (9). The age-related wasting of skeletal muscle occurs in both the type I fibers and type II fibers, however type II fibers appear to exhibit more pronounced atrophy (3, 10). Since type II fibers have 5-6 times the power production of type I fibers (8), their selective deterioration and loss is believed to be responsible for a disproportionate reduction in muscle power in older adults that exceeds the decline in muscle mass (11). Furthermore, there are reports of increased co-expression of MHC isoforms with age within the same muscle fiber (i.e. I/IIa, IIa/IIx) and homogenous fiber grouping (10, 12-14), which may mitigate the power generation of muscle compared with more homogenous MHC expression. The MHC isoform of a

muscle fiber is thought to be directly influenced by the type of motor unit that innervates it, as demonstrated by cross-innervation nerve experiments in cats (15). These changes imply that age-related muscle loss is driven by some degree of neuropathy upstream of the affected skeletal muscle fibers.

It is speculated that α-motor neuron loss is responsible for the reduction in motor units and subsequent muscle mass with age (16). Muscle from individuals over 70 years of age displays ~50% fewer functioning motor units within the upper and lower limb muscles compared to young individuals (17-20). While a longitudinal analysis has not confirmed that this loss occurs over time, the existing cross-sectional results have been supported by reports of a decline in spinal cord neural cells with age (21-24). Individual motor units innervate muscle fibers of the same fiber type and aging appears to predominantly affect a loss of fast-twitch motor units in rodents (25, 26), resulting in preferential type II fiber atrophy. Further, motor neuron sprouting from slow-twitch motor units to denervated fast-twitch motor units has been observed with age (25), likely as a compensatory mechanism to maintain muscle function. Despite a loss of motor units, maximal activation of the motor neuron pool does not appear to be a factor in the decline in force production and muscle strength in the elderly (27, 28).

Muscle strength is critical for mobility and activities of daily living in older adults. A certain amount of strength is required for dynamic motions such as rising from a chair or climbing stairs. Muscle strength is reduced by ~30% or greater in adults over the age of 70 years in both the upper (29-31) and lower limbs (11, 32-35), and thresholds for muscle strength have been reported that predict dependence and the risk of falls in the

elderly (36-38). Therefore, maintenance of a critical amount of muscle strength is necessary for independent living. While the relative amount of strength loss appears similar between men and women with age, absolute amounts of strength are generally higher in men (32, 33). Since women have a greater life expectancy and have a lower initial amount of muscle mass, they are particularly susceptible to sarcopenia and frailty as they age.

Strength appears proportional to muscle mass throughout the lifespan. Whole muscle cross-sectional area obtained via MRI or CT scans is often highly associated with absolute muscle strength of the indicated muscle groups (34, 39-41). The analyses of chemically skinned human muscle fibers, where only the contractile apparatus remains intact, showed that both type I and II fibers from older adults exhibit were atrophic and had reduced peak force, but similar normalized force (force/diameter) to fibers from young individuals (7). These findings collectively suggest that the quantity of muscle fibers and their size is responsible for losses in whole muscle strength, rather than alterations in the quality of the contractile apparatus. Other work has proposed that higher amounts of muscle collagen and other extracellular matrix proteins can impair muscle quality independent of mass during aging, typically using rodents or rabbits (42, 43). However, evidence in humans is lacking that collagen and other glycation products are increased with age (44).

Muscle specific fatigue has been routinely studied in elderly and young individuals as a corollary to the limits of functional ability. Typically, subjects are asked to complete isometric or isokinetic contractions and the deterioration of force production

over time or the maximum number of contractions to fatigue is assessed. Paradoxically, older adults exhibit resistance to muscular fatigue in both the upper (45, 46) and lower limbs (47-50) which has been attributed to a greater relative type I fiber area in elderly muscle that reduces absolute muscle force generation but results in fatigue resistant muscle. Fatigue and premature exhaustion during activities of daily living may be related to the relatively high effort necessary for task completion in older adults due to their lower absolute force production. However, there is also a clear reduction in maximal aerobic capacity with age, suggesting that cardiovascular as well as muscular factors limit mobility in the elderly.

1.1.2 Oxidative capacity and energy homeostasis during muscle aging

In addition to the previously noted reductions in muscle strength and mass, whole body aerobic capacity (VO_{2max}) declines ~10% per decade of life after age 30, severely limiting the functional abilities of older adults (51-53). As noted for muscle strength, thresholds for dependence and mortality have been defined according to aerobic capacity throughout the adult lifespan that suggest a VO_{2max} of 17.5 ml/kg/min (5 mets) or greater is necessary for functional independence (54). VO_{2max} is dependent on several central and peripheral factors, including cardiac output (the product of heart stroke volume and heart rate) and tissue oxygen extraction (arteriovenous O_2 difference). The predominant central cause of the reduction in VO_{2max} in the elderly is a reduction in heart stroke volume rather than reduced maximum heart rate (55-57). Several alterations to the cardiovascular system account for these changes. Over the lifespan, blood volume is lowered such that it

reduces atrial preload prior to ventricular contraction (58) and the myocardium loses elasticity with age due to myocyte removal and expanding fibrosis that reduces myofilament contractility (59, 60). This stiffening of the heart wall reduces the passive stretch-rebound from cardiac filling at low pressures (Frank-Starling mechanism) and thus lowers stroke volume. Apart from these cardiac alterations, there are peripheral changes to the cardiovascular system that limit the reactivity of the system to rapid changes in tissue oxygen requirements. The elasticity and distensibility of the vasculature is important for preventing athlerosclerotic plaque formation and hypertension, which strain the heart and predisposes individuals to increased risk of stroke, thrombosis or myocardial infarction. Older adults demonstrate reduced endothelium dependent dilation in response to acetylcholine or increased shear stress (61, 62). Additionally, the aorta and carotid arteries progressively stiffen with age (63, 64), even in the absence of atherosclerosis (65, 66). Nevertheless, blood oxygen delivery is only a part of the overall picture regarding VO_{2max}; muscle oxygen uptake and metabolism are also clearly important components of overall aerobic capacity.

Muscle oxidative capacity and the generation of aerobic energy plays a large role in determining VO_{2max} and these are significantly reduced with age (67). Mitochondria are intracellular organelles that are the primary generators of aerobic energy within body cells. Mitochondrial content is high in metabolically active tissues such cardiac muscle, skeletal muscle, and liver, and is proportional to the oxidative capacity of a given tissue. Each of the skeletal muscle fiber types exhibits different amounts of mitochondrial content, with an inverse relationship to their power producing qualities such that

I>IIa>IIx (68). Mitochondria utilize reducing equivalents derived from ingested macronutrients as a source of electrons to drive the formation of ATP from ADP during the process of oxidative phosphorylation (for more detail see section 1.2.1). Skeletal muscle has a relatively high abundance of mitochondria and accounts for approximately 20-30% of an individuals resting metabolic rate (69). Furthermore, muscle is responsible for 20% of basal whole body glucose uptake and this contribution increases to 38% during hyperglycemia (70). Since timely uptake of blood macronutrients is necessary to prevent toxicity (dysglycemia, ectopic lipid accumulation), mitochondrial content is particularly important for hormonal signaling from nutrients in the blood. Furthermore, the appropriate generation of aerobically derived energy during movement and exercise is a primary factor regarding the onset of fatigue and maximal work capacity. Aged skeletal muscle typically exhibits fewer mitochondria resulting in impaired exercise capacity and lower ATP generation and substrate oxidation (67, 71-74). In parallel, elderly adults have often have lower insulin sensitivity and impaired glucose tolerance in skeletal muscle due largely to an excess of caloric intake and reduced flux through mitochondrial metabolism (71, 75). Despite these broad physiologic effects, the cellular events mediating these changes to aging skeletal muscle mitochondria remain to be fully clarified. A more thorough understanding of mitochondrial structure and its regulation are necessary in order to generate effective therapies for aging.

1.2.1 Mitochondrial electron transport and dynamics

Mitochondria are double membrane structures composed of 1,013 proteins that

are encoded within nuclear or mitochondrial DNA (mtDNA). The majority of mitochondrial proteins are transcribed from nuclear DNA and imported. In contrast, mtDNA is a 16 kb circular structure that contains only 37 genes, and is located in the mitochondrial matrix (for review see (76)). Even though mitochondrial encoded genes compose only a small minority of the total, mtDNA is essential for respiratory function. Thirteen protein subunits of complexes I, III, IV and V are encoded by mtDNA and these are highly conserved in mammals. Mitochondria extract electrons from oxidative substrates (e.g. NADH + H^+, $FADH_2$) resulting from glucose, fatty acid, or protein metabolism using a chain of five enzymes (the electron transport chain, ETC). Each of complexes I through IV of the ETC undergo redox reactions during electron transfer and eventually donate the electrons to oxygen to form water. During electron flux complexes I, III and IV pump protons from the matrix into the inner membrane space, forming an electrochemical gradient. Specifically, the reducing equivalents NADH + H^+ and NADPH donate electrons to complex I of the ETC, while $FADH_2$ donates an electron to complex II. Both of these complexes converge electron flux onto complex III and subsequently complex IV. The fifth complex is the ATP synthase, which utilizes the cumulative proton gradient to drive ATP synthesis when protons are returned to the matrix.

Mitochondria are dynamic organelles that continuously fuse and divide such that if two or more cultured cells are artificially fused, their mitochondrial populations will be fully mixed within hours (77, 78). Surprisingly, the mixing of mtDNA populations appears to be more delayed and takes several days (79). Mitochondrial fusion involves

the combination of the inner, outer and matrix membranes and their associated proteins. Fusion is regulated by the GTPases mitofusin 1 and 2 (Mfn1, Mfn2) and removal of the coding sequences for these proteins is gestational lethal in mice (77). The GTPase OPA1 is also essential for mitochondrial fusion, and mutations in *Mfn2* and *OPA1* genes are associated with human neurological or metabolic diseases resulting from respiratory insufficiency (80, 81). Mitochondrial fission depends on the GTPase Drp1, which marks future sites of division and initiates the process (82). Interestingly, mediators of mitochondrial fusion/fission machinery appear to regulate apoptosis (83) and cell cycle arrest (84), suggesting they are important for the maintenance of cellular function and responses to cellular stress.

1.2.2 The role of mitochondria in aging

During the process of electron transfer, it is estimated that approximately 0.1-0.4% of electrons escape from the ETC as superoxide ($O_2^{\cdot -}$) radicals that can react with other molecules in a chain reaction (85). While *in vivo* rates of free radical generation have not been described in mammals, studies using isolated mitochondria have demonstrated that superoxide can be released from various sites of complexes I, II or III (86-89). Superoxide is highly reactive but is short-lived and is generally not believed to diffuse out of the mitochondria. Superoxide can undergo progressive reactions to form other free radicals such as hydrogen peroxide or peroxynitrite, and these molecules are collectively termed reactive oxygen or nitrogen species (ROS/RNS). These species in turn can combine with proximal lipid, protein and DNA molecules to form oxidative

damage. Additionally, cellular components containing Fe-S clusters are more chemically prone to ROS/RNS reactivity and modification, including the Krebs Cycle and ETC enzymes (90).

While the generation of radicals from mitochondria is relatively low, the accumulation of oxidative damage over time is associated with cellular degeneration and morbidity, termed the mitochondrial free radical theory of aging (91). The exposure of cells and organelles to oxidative stress induces damage to proteins and lipids, alters enzyme function and reduces lipid bilayer fluidity (92-94). These defects may reduce the fidelity of electron transfer in the ETC and generate even more ROS, resulting in a "vicious cycle". mtDNA is particularly susceptible to high rates mutagenesis from ROS/RNS reactions since it is adjacent to a radical generator (the ETC), has limited base-excision repair capacity, and lacks histone protection. In contrast, nuclear DNA is separated from radicals within the nuclear membrane, has extensive DNA proofreading and repair mechanisms, and is tightly bound in histones that prevent overt exposure to harmful agents. Indeed, mitochondrial DNA (mtDNA) is subject to far more oxidative damage over the lifespan than nuclear DNA that induces a wide array of large-scale deletions, point mutations, and tandem duplications have been characterized in multiple tissues of aged individuals (95-97). The respiratory alterations induced by mtDNA mutations are a mild phenotypic version of the dysfunction present in human mitochondrial disease, and similarly manifest to the greatest extent within differentiated skeletal muscle and brain (76). Several lines of evidence support the regulatory role of mitochondrial ROS on lifespan and the aging process. Complete ablation of the *Sod2*

gene, encoding the only matrix superoxide scavenging enzyme, results in perinatal mortality due to neuropathy and cardiomyopathy (98, 99). Conversely, overexpression of catalase in mitochondria, but not in the peroxisomes where it is normally located, increases murine lifespan and attenuates age-related mitochondrial dysfunction and insulin resistance (100, 101). However, a comprehensive analysis of transgenic mice lacking or overexpressing a variety of antioxidant enzymes has failed to yield changes in lifespan (102). Therefore the role of mitochondrially derived ROS in the aging process remains unclear, but there may be specific benefits derived from the unnatural localization of catalase to the mitochondria.

Mitochondrial dysfunction has also been linked to the loss of muscle mass with aging. Atrophic portions of myofibers in aged rats have been shown to coincide with mitochondrial oxidative damage and respiratory dysfunction (103). Additionally, muscle over expression of a transcriptional regulator of mitochondrial biogenesis, PGC-1α, appears to attenuate muscle loss in PolG mice (104) as well as aged wild-type mice (105). Thus, a high degree of mitochondrial function appears important for maintaining skeletal muscle mass with age. It seems likely that therapies that increase PGC-1α and subsequently muscle mitochondrial content could be used to reduce rates of sarcopenia in humans.

It is well established that aged muscle exhibits fewer copies of mtDNA (71, 106). Since multiple copies of mtDNA are present per mitochondria (~1 to 10), there are thousands more copies of mtDNA in skeletal muscle when expressed per copy of nuclear DNA. The transcription and maintenance of mtDNA in the face of oxidative damage and

mutations is important for appropriate mitochondrial function with age (for review see (107)). The two strands of mtDNA are denoted heavy and light strands, as the heavy strand is more guanine rich. The mitochondrial genome in mammals does not contain introns (108), however there are several non coding sites containing control regions for transcription and replication. Each strand contains a single promoter region and replication and transcription of the entire genome occurs at once similar to what occurs in bacteria, known as polycistronic replication (109). The transcription and replication of mtDNA are controlled by several enzymes not found in the nucleus. Transcription is performed by the RNA polymerase POLRMT and requires the transcription factors TFAM and either TFB1M or TFB2M (110). The resulting precursor RNA strands are then processed to their respective individual mRNA, tRNA or rRNA species (109). Replication of mtDNA requires at a minimum the action of DNA polymerase γ (PolG), the helicase TWINKLE and mitochondrial single stranded binding protein (mtSSB) (111), although TFAM can also enhance the replication of mtDNA without altering mitochondrial mass (112). The observed reduction in mtDNA copy number with age is believed to be caused by defects in mtDNA replication driven by age-related mutations in the control regions of mtDNA (113, 114). However, poor maintenance of mtDNA structure may also be responsible for age-related mitochondrial dysfunction.

mtDNA is organized into structures that group several mtDNA molecules together, called nucleoids. In addition to its role in transcription and replication, TFAM is a major component of nucleoid structure that is essential for the maintenance of mtDNA and initiates partial coiling and compaction akin to nuclear histones (115). TFAM coordinates

these nucleoids (116) and appears to coat mtDNA in a protective fashion (117). TFAM binds preferentially to areas of DNA containing oxidative lesions (118), but may prevent repair enzymes such as the glycosolase OGG1 from excising damaged bases (119). Since aging is known to increase oxidized mtDNA bases (8-OH-2dG), this represents a plausible mechanism by which mtDNA damage accumulates and prevents appropriate replication. However, the regulation of replication is often studied *in vitro* using supra-physiologic amounts of ROS at atmospheric oxygen levels. The effects of ROS on mtDNA replication and transcription *in vivo* remain to be characterized.

More recently, the direct role of mtDNA mutations in the aging process was assessed by the generation of the transgenic polymerase γ mouse (PolG) with an impaired mtDNA proofreading capacity (120, 121), that results in a considerably higher mtDNA mutation rate in all cells. Generally the effect of mtDNA mutations in mitotic cells is poorly understood because somatic tissues harbor very low rates of mutation, far below the threshold necessary for respiratory insufficiency. The elevation in mtDNA mutations in PolG mice results in systemic mitochondrial dysfunction and premature death that can be interpreted as accelerated aging (120). Specifically, PolG mutator mice have muscular atrophy, curvature of the spine, bone loss, cardiomyopathy, hair loss, and reduced lifespan (120, 121). These physiologic effects are thought to result from increased cellular apoptosis driven by the high rate of clonal mutations in mtDNA (120, 122), but not from point mutations (123). In contrast, a high prevalence of mtDNA deletions does not result in a similar degree of physiological deterioration (124). More work is necessary to clarify the role and extent of mtDNA defects in the aging process.

Only recently has the importance of proper maintenance of mitochondrial dynamics during aging been identified. Abnormally large mitochondria accumulate with age in murine skeletal muscles that exhibit triple membranes and other structural abnormalities and fail to fuse with the general pool of cellular mitochondria (125). This phenomenon has been attributed to the low membrane potential of "giant" mitochondria that prevents mixing with neighboring organelles (125, 126). In addition, impaired lysosomal removal of biological "waste" (senescent mitochondria, lipofuscin, aggregated proteins etc.) is a hallmark of aged, post-mitotic cells (127). Thus, mitochondria with compromised function may be sequestered from interacting with the general mitochondrial network, which might serve to buffer an accumulation of stochastic mutations in mtDNA or dysfunctional mitochondrial proteins. Recently, this concept has been demonstrated using a cross of the PolG mouse that had the *Mfn1* and *Mfn2* genes deleted in skeletal muscle (128). This combination induced accelerated muscle atrophy and reduced mtDNA stability, due to a lower tolerance of the mtDNA mutations and deletions present in the PolG mouse. Similarly, mitochondrial fusion and fission proteins are reduced in aged skeletal muscle in concert with mitochondrial ETC proteins (129). The impact of fewer fusion/fission events is likely greater in aged muscle that has accrued higher levels of oxidative and DNA damage, and this process may induce a viscous cycle that generates even more respiratory dysfunction. Thus, the combined effects of age-associated mtDNA mutations, ROS damage, and respiratory defects may generate or interact with alterations in mitochondrial dynamics such that they cumulatively exacerbate mitochondrial deterioration.

In summary, mitochondrial dysfunction can result from several molecular factors inherently tied to aging and metabolism and result in atrophy and reduced oxidative capacity. Skeletal muscle is particularly affected by the mitochondrial consequences of aging because it does not divide and slowly renews its mitochondria. In spite of the strong evidence to support stochastic mitochondria-centered aging in skeletal muscle, it is difficult to separate the primary causes of mitochondrial dysfunction and its secondary consequences on cellular health. Furthermore, the extent to which augmenting mitochondrial content in skeletal muscle can correct many of these issues remains to be fully evaluated.

1.3.1 The influence of aerobic exercise on muscle strength and size

Exercise has been suggested as a countermeasure to delay aging and improve health (71). Specifically, short-term and longitudinal studies in humans have demonstrated that initiation of exercise training extends life expectancy, reduces morbidity (e.g., cardiovascular disease, type 2 diabetes, metabolic syndrome, and cancer) and attenuates physical disability in later life (71, 130-132). Thus, physical fitness has emerged as the single most important predictor of overall health and well being in elderly individuals (132). However, the idea that lifelong exercise promotes an extension of lifespan is contentious. Some groups have demonstrated a minor increase in mean lifespan in lifelong exercised rodents (133, 134), while others have not (135, 136). In this context the notion that exercise predominantly improves “healthspan” has emerged that proposes that exercise extends the healthy, highly functional years of life rather than life

expectancy. Since an individual's healthspan would be more directly related to their quality of life, this may be a more important outcome than lifespan.

Since muscle strength is so paramount to physical function in the elderly, many studies have sought to determine whether habitual exercise could attenuate age-related reductions in muscle force production or muscle size. Strength athletes exhibit a slower decline in muscle strength with age (137-140), and weight training consistently improves muscle strength following a short-term period of training (141-145). However, there is some evidence that very old (>90y) individuals may have more limited gains in strength from resistance exercise training (146-148), perhaps because they start the training program with such a low amount of muscle mass. Unfortunately, weight training does not appear to significantly augment aerobic capacity or cardiovascular function in healthy adults. Therefore, some focus has been placed on aerobic exercise to examine its efficacy in remediating both muscle strength and aerobic capacity simultaneously. Aerobically active individuals (runners and orienteerers) exhibit higher aerobic capacity, but similar muscle mass and strength to inactive subjects (137, 149-152), despite having larger muscle fibers (153-155). Surprisingly, short-term cycling (156) and treadmill running/walking (157, 158) in previously sedentary elderly adults increases dynamic knee extensor muscle strength and muscle size, which is inconsistent with the cross-sectional findings regarding aerobic athletes. It remains possible that muscle strength and size adaptations due to aerobic exercise might only be evident in previously sedentary older adults, suggesting that any increase in physical activity beyond a sedentary lifestyle can partially remediate the hallmarks of sarcopenia. However, the influence of exercise mode should be considered, particularly since some sports are weight bearing (running), while others are low impact (cycling) or may activate

a greater amount of muscle mass (rowing, cross country skiing).

1.3.2 Aerobic exercise and vascular aging

A hallmark adaptation of aerobic exercise is the greater VO_{2max} observed in endurance athletes (71, 159, 160) and short-term exercise interventions consistently increase VO_{2max} in individuals of all ages (74, 156, 161). However, long-term aerobic exercise adherence does not appear to attenuate the relative decline of VO_{2max} with age (57). As described previously, a stiffening of the arteries and reduced endothelial responsiveness are commonly observed in older adults. However, elderly, lifelong aerobic athletes have less pronounced stiffening of the carotid artery and improved compliance compared to their sedentary peers (162-164). This age-related vascular stiffening appears reversible, as a short-term program of walking/jogging can improve arterial compliance to similar levels of that seen in habitually active elderly (162, 165). Endothelial dependent dilation is also greater in aerobic athletes (61, 166), and similarly improves with short-term training (61). These exercise-induced improvements have been attributed to greater bioavailability of the vasodilator nitric oxide, but may also depend on a reduced vascular oxidative stress or inflammation (167, 168). Despite the improvements in vascular function, maximum cardiac output is reduced with age, and although this effect is attenuated in aerobic athletes, it suggests a persisting effect of age on heart function irrespective of exercise history.

1.3.3 Effects of aerobic exercise on cardiac aging and cardiovascular disease risk factors

In contrast to the beneficial effects of exercise on vascular aging, age-associated changes to the heart may be less dramatically altered by exercise or take longer to manifest. Athletes are commonly noted to have greater left ventricular heart mass than sedentary individuals (169). However, the prevalence of arterial hypertension is commonly associated with aging and results in a similar degree of left ventricular hypertrophy due to increased vascular loading. It remains unknown if hypertension induced remodeling is as coordinated an expansion of ventricular space as exercise stimulated remodeling. Exercise appears to attenuate the reduction in left ventricular mass of the heart with age (169, 170). Furthermore, resting measures of diastolic dysfunction are improved in the exercise-trained elderly compared to sedentary adults (171), which is though to occur via increased left ventricular compliance (169). The changes in myocardial compliance with habitual exercise may be due to an attenuation of age-related extracellular matrix crosslinking as seen in rats (172). While cardiac responses to exercise, such as the ventricular filling rate, are improved by exercise training (173), maximal stroke volume, heart rate and arteriovenous difference are reduced with age, even in habitually active aerobic athletes (174). Therefore, the cardiovascular benefits of exercise at old age may be primarily attributed to vascular, rather than cardiac, alterations. However, exercise also positively modifies several components of cardiovascular health typically associated with heart disease. Habitual exercise reduces several risk factors associated with cardiovascular disease including

body fatness, LDL cholesterol, fasting blood glucose and arterial blood pressure (175, 176). The amelioration of these risk factors is believed to underlie a significant portion of the cardiovascular health benefits derived from aerobic exercise. Furthermore, the greater absolute reserve of aerobic capacity resulting from aerobic exercise training has significant functional ramifications for older adults.

1.3.4 Exercise-induced muscle mitochondrial and metabolic signaling

Acute aerobic exercise results in a robust increase in signaling directed towards mitochondrial biogenesis in skeletal muscle. This response is specific to the mode of exercise, where contraction intensity, fiber type recruitment and the metabolic requirements of contraction dictate the molecular signal (177). Muscle contractions during aerobic exercise induce rapid changes in intracellular calcium, rates of ATP turnover and ROS generation that signal the transcription of mitochondrial genes. Subsequent bouts of exercise are believed to amplify this signal and eventually increase muscle mitochondrial mass, resulting in energetic adaptation and resistance to metabolic stress.

While the precise events that regulate exercise-induced mitochondrial biogenesis are not completely clear, several main pathways have been identified that coordinately regulate the necessary DNA transcription factors. The energy sensitive AMP activated protein kinase (AMPK), calcium sensitive calcium/calmodulin-dependent protein kinase (CAMK), and mitogen activated protein kinase (MAPK) p38, all regulate mitochondrial biogenesis, among other signaling molecules (178-180). With repeated bouts of exercise,

activation of these kinases generates a net accumulation of mitochondrial proteins that improves muscle oxidative capacity and glucose uptake (177). Specifically, the downstream result of kinase signaling is induction of transcriptional activators and co-activators that coordinate the expression of mitochondrial gene expression. The major transcription factors that bind DNA and drive mitochondrial gene expression are the nuclear respiratory factors 1 and 2 (NRF1, NRF2), PPARγ and PPARα (181-183). These transcriptional regulators can be activated by the PPARγ co-activator-1α (PGC-1α) to dramatically increase tissue mitochondrial content (184, 185). PGC-1α mRNA is robustly increased post-exercise (186-188), as is it's nuclear localization (189). Both young and elderly subjects increase mitochondrial enzyme activities within skeletal muscle to a similar extent after participating in an aerobic exercise-training program (161), suggesting that exercise-induced mitochondrial biogenesis is preserved with age. However, basal PGC-1α protein expression and mtDNA copy number is reduced with age even in elderly adults that have maintained high levels of aerobic exercise training, in spite of preserved mitochondrial respiration (71). Conversely, age-related insulin resistance appears to be primarily dependent on physical activity as elderly athletes exhibit no deterioration in insulin sensitivity compared to young athletes (71). Therefore, age-associated accumulations in oxidative damage, muscle wasting, and/or a slow-twitch fiber type shift may prevent complete maintenance of muscle mitochondrial content with chronic exercise training. However, muscle maintains its plasticity to increase mitochondrial content following exercise training to a somewhat limited extent in aged skeletal muscle.

As has been noted, mitochondria are a major site of ROS and RNS generation. Generally the production of ROS/RNS is considered highest when the proton motive force and membrane potential across the mitochondrial inner membrane is high (190). This notion dictates that resting or basal mitochondrial respiration is responsible for the majority of oxidative damage observed during aging. During exercise, muscle mitochondria likely produce fewer free radicals as high ETC flux would reduce the mitochondrial membrane potential. Therefore exercise is not believed to be a major source of mitochondrial-derived free radicals (191). However, muscle contractions do produce ROS and RNS that impact force generation, likely resulting from cytosolic or extracellular sources, such as NADPH oxidase (192). Likewise, exercise training increases multiple antioxidant enzymes in young and old skeletal muscle (134, 193), and contraction-induced ROS generation may play an important part in adaptation (194, 195). Even though mitochondria may not play a large role in exercise generated ROS, individuals with chronic obstructive pulmonary disease (COPD) and some forms of mitochondrial disease can exhibit high rates of basal ROS production due to underlying mitochondrial dysfunction, and this condition seems to be exacerbated by exercise training (196). Further, the mitochondrial dysfunction present in aged individuals, particularly the very frail, may prevent complete restoration of age-related mitochondrial function to that of young individuals, possibly resulting from ROS/RNS interference.

Therefore, aging and the associated mitochondrial effects within skeletal muscle represent an area of research that demands attention in order to improve mobility and reduced mortality in elderly adults. Exercise holds great promise in attenuating the

decline of skeletal muscle during the lifespan, but a more thorough understanding of the molecular processes of aging and mitochondrial biogenesis are necessary in order to achieve complete preservation of mitochondrial and muscle function.

1.4.1 **Purpose of thesis**

The purpose of this research was to further our understanding of skeletal muscle function and mitochondrial homeostasis. This work utilizes models of aging and oxidative stress as conditions that stimulate mitochondrial dysfunction in order to more fully understand the cellular and physiological ramifications of these processes in skeletal muscle.

I ***hypothesize*** that aging will induce structural changes to mitochondria and decrease their association with lipid droplets in parallel to reductions in strength and muscle mass. Additionally, habitual aerobic exercisers will have greater muscle strength at an advanced age (>65 years), but not at younger ages. Finally, excess mitochondrial superoxide and increased oxidative stress will result in impaired adaptation to endurance exercise in mice by exacerbating oxidative damage to electron transport chain enzymes, similar to what occurs during human muscle aging.

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CHAPTER 2: MANUSCRIPT 1

The Effect of Aging on Human Skeletal Muscle Mitochondrial and Intramyocellular Lipid Ultrastructure

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The Effect of Aging on Human Skeletal Muscle Mitochondrial and Intramyocellular Lipid Ultrastructure

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The purpose of this study was to determine whether ultrastructural changes in intramyocellular lipid (IMCL) and mitochondria occur with aging. Muscle samples were analyzed from 24 young and 20 old, equally active, individuals for IMCL and mitochondria quantity and size as well as their association. Old men had larger IMCL droplets than all other groups in the total muscle area. Old individuals showed higher IMCL content in the subsarcolemmal area. Young participants had a greater number of mitochondria compared with old participants in both fiber regions and greater enzyme activities of cytochrome c oxidase and citrate synthase. The fraction of IMCL touching mitochondria was lowest in old women in the total area and in old men in the subsarcolemmal region. In summary, older adults have larger IMCL droplets, fewer mitochondria, and a lower proportion of IMCL in contact with mitochondria. These factors likely contribute to age-related reductions in mitochondrial function and lipid metabolism.

Key Words: Mitochondrial dysfunction—Oxidative stress—Insulin resistance—Subsarcolemmal.

THE progression of aging is well known to result in a reduction in skeletal muscle mitochondrial content and whole-body muscle mass (sarcopenia), dramatically altering lipid metabolism. In accordance, the prevalence of diabetes in developed countries is highest in individuals older than 65 years (1). Skeletal muscle intramyocellular lipid (IMCL) content has been implicated in the development of the metabolic syndrome as higher IMCL in elderly individuals is associated with reduced mitochondrial ATP production and peripheral insulin resistance (2,3). However, trained endurance athletes also have elevated IMCL content (4), whereby an increased abundance of mitochondria likely mitigates the negative aspects of augmented intramuscular lipids. This paradox points to physical activity as a major determinant of the consequences of increased net muscle lipid uptake. Because older adults are typically more sedentary than young individuals, investigations studying the physiological effects of human aging must avoid the confounding effects of physical activity when interpreting their results. Currently, the hypothetical association between IMCL accumulation and insulin resistance with aging remains to be fully characterized in healthy adults who participate in an active lifestyle.

In combination with alterations in muscle lipids, mitochondrial content is well established to decline with advancing age (5,6). An overall diminished flux through metabolic pathways and chronic activation of inflammation, factors that are exacerbated in older individuals, have been implicated in the downregulation of mitochondrial biogenesis (7). Furthermore, a reduced efficiency of Lon protease, a mitochondrial specific protease, has been demonstrated across the life span in conjunction with an overall increase in proteosome dysfunction (8,9). Although investigations have described a reduced volume density of mitochondria in elderly human skeletal muscle (5,10,11), only one has simultaneously explored changes in the size and number of mitochondria (10), with conflicting results. Additionally, specifying subpopulations of mitochondria within the cell appears to be important when evaluating age-related changes in mitochondrial function (12). The content of subsarcolemmal mitochondria is lower in insulin-resistant type 2 diabetic individuals compared with lean individuals (13), and this fraction is particularly responsive to exercise in older adults (14). Thus, the simplistic view of diminished mitochondrial content in aged skeletal muscle may overshadow more specific aspects of mitochondrial dysfunction including subcellular localization.

Studies involving mitochondrial dynamics (fusion and fission) have recognized mitochondria as mobile organelles that can respond to diverse stimuli (15–17). The location of mitochondria within a cell is constantly changing, and the balance of fission and fusion results in the size, abundance, and location of these organelles (16). Recent work has shown that exercise training causes a shift in subcellular localization of IMCL, such that lipid droplets are more closely associated with mitochondria (18). As physical activity is reduced in older individuals, structural changes may interfere with mitochondrial access to IMCL and result in increased storage within skeletal muscle. Previous observations of IMCL accumulation with aging using ^{1}H-NMR have been limited in their ability to discern changes in IMCL and mitochondrial morphology or their proximity to one another (3,19,20). Therefore, we sought to evaluate the alterations

Table 1. Participant Characteristics

	Young		Old				
	Men	Women	Men	Women	Age Effect	Gender Effect	Interaction
N	12	12	10	10			
Age (y)	23 ± 2	22 ± 1	71 ± 2	70 ± 2	$p < .05$	NS	NS
Weight (kg)	78 ± 3	63 ± 2	81 ± 3	65 ± 3	NS	$p < .05$	NS
Height (cm)	177 ± 1	166 ± 1	176 ± 2	160 ± 2	$p < .05$	$p < .05$	NS
BMI (kg/m^2)	25 ± 1	23 ± 1	26 ± 1	25 ± 1	$p < .05$	$p = .083$	NS
% Body fat	20 ± 2	29 ± 1	19 ± 1	36 ± 2	$p < .05$	$p < .05$	$p = .014$
FFM (kg)	63 ± 2	45 ± 1	65 ± 2	41 ± 1	NS	$p < .05$	$p = .049$
% FFM	80 ± 2	71 ± 1	81 ± 1	64 ± 2	$p < .05$	$p < .05$	$p = .015$
Plasma glucose (mmol/L)	4.9 ± 0.1	5.0 ± 0.2	5.0 ± 0.3	4.8 ± 0.3	NS	NS	NS
Plasma insulin (μIU/ml)	6.5 ± 1.0	5.7 ± 1.0	5.7 ± 0.9	4.5 ± 0.4	NS	NS	NS
HOMA-IR	1.4 ± 0.2	1.3 ± 0.2	1.3 ± 0.3	1.0 ± 0.1	NS	NS	NS
Physical activity							
Total (mins/wk)	170 ± 31	187 ± 24	223 ± 55	176 ± 45	NS	NS	NS
% RE	39 ± 12	25 ± 7	68 ± 9	78 ± 9	$p < .05$	NS	NS
% AE	27 ± 13	53 ± 6	29 ± 8	22 ± 9	NS	NS	$p = .089$
% Rec	17 ± 9	22 ± 7	3 ± 3	0 ± 0	$p < .05$	NS	NS

Note: *p* Values refer to significant main effects ($p < .05$) identified by two-way (age by gender) analysis of variance. See text for details. $N = 14$ (9 men/5 women) for young and $N = 10$ (6 men/4 women) for older adults for plasma insulin and glucose. Values are mean ± *SE*. AE = aerobic exercise; BMI = body mass index; FFM = fat-free mass; HOMA-IR = homeostasis model assessment of insulin resistance; NS = nonsignificant; RE = resistance exercise; Rec = recreational activity.

in mitochondria and IMCL structure that occur with natural human aging in skeletal muscle.

In this investigation, we assessed the independent and interactive effects of aging and gender on the size and abundance of IMCL and mitochondria, as well as their subcellular localization and association in human skeletal muscle. In addition, we evaluated basal messenger RNA (mRNA) content of transcripts involved in lipid metabolism, mitochondrial function, and cellular oxidative stress, as well as mitochondrial enzyme activities to assess mitochondrial content. We intentionally studied young and older adults who were equivalently active in order to discern changes in skeletal muscle that are inherently related to the aging process and not inactivity.

Methods

Participants

Muscle samples were acquired from 24 young (12 men/12 women) and 20 older (10 men/10 women) adults at rest, at least 48 hours removed from strenuous physical activity. The samples from young participants are a subset from a recent investigation in our laboratory (21). Young adults were considered recreationally active and exercised an average of 3 hours per week in a variety of modes, including resistance and aerobic exercise, as well as recreational sports such as volleyball, soccer, or basketball. Physical activity details are shown in Table 1. The older adults are a subset of participants from a 26-week resistance exercise training study (22), and samples were acquired at the conclusion of the training program to try to match the physical activity patterns of the young and old participants. This program consisted of whole-body resistance exercise training twice per week as described in detail (22). All data presented in the current study from both age groups have not been previously published. Prospective participants were informed of the procedures and risks involved in the study and gave their informed consent, which was approved by the McMaster Research Ethics Board. Participants were screened by telephone, followed by a medical history evaluation and consent from their family physician. Criteria for exclusion from the study included evidence of one or more of the following: coronary heart disease, congestive heart failure, hypertension, chronic obstructive pulmonary disease, diabetes mellitus, major orthopedic disability, renal failure, and smoking. Young women were eumenorrheic and were biopsied during the mid-follicular phase of their menstrual cycle. Older women were postmenopausal and were not taking hormone replacement therapy.

Experimental Design

Participants arrived in the neuromuscular clinic after an overnight fast. After supine rest, blood was drawn for hormone and metabolite determination. All samples were collected into heparinized tubes, centrifuged at 1,200*g* for 10 minutes, and the plasma fraction was aliquoted into 1.5-ml tubes and stored at −80°C for later analysis.

Following the blood draw, a biopsy was obtained from the vastus lateralis under local anesthetic as previously described (23). All visible fat and connective tissue were removed, and the samples were divided into sections for analysis. Approximately 5 mg was immediately transferred to ice-cold 2% glutaraldehyde (buffered with 1.0 M sodium cacodylate, pH 7.4) and stored for less than 24 hours at 4°C to retain muscle ultrastructure for electron microscopy (EM) analysis. The remaining muscle portion

was snap-frozen in liquid N_2 and stored at −80°C. Body composition was assessed using dual energy x-ray absorptiometry (QDR 1000W; Hologic, Waltham, MA) using whole-body software for adults (v5.63).

Analytical Methods

Metabolites and hormones.—Plasma glucose concentration was determined using a glucose analyzer (Model 2300 Stat Plus; Yellow Springs Instrument Co. Inc, Yellow Springs, OH). Insulin concentrations were determined using a commercially available radioimmunoassay kit (Coat-a-count, TKIN5; Diagnostic Products, Los Angeles, CA). Plasma samples were only available for 14 young (9 men/5 women) and 10 old (6 men/4 women) participants.

Enzyme activity.—Citrate synthase (CS) and cytochrome c oxidase (COX) maximal enzyme activities were determined following homogenization of 15–25 mg of wet muscle using a UV spectrophotometer (model 8453; Hewlett Packard, Wilmington, DE), as previously described by our group (24,25). Samples were run in duplicate. Due to a limited amount of sample, only 12 young (6 men/6 women) and 8 old individuals (5 men/3 women) were evaluated for enzyme activity.

Gene expression.—RNA was extracted, and samples were treated with DNase I for 25 minutes to remove any contaminating DNA. Primers and probes for each gene of interest were designed as previously described by our laboratory (26). Primer sequences are as follows: cytochrome c oxidase subunit IV (COX IV), forward-cgagcaatttccacctctgt, reverse-ggtcacgccgatccatataa; CPT1, forward-ctctcatggtgaacagcaacta, reverse-gtccagtttacggcgatacat; Dynamin-related protein 1 (Drp1), forward-tttgtgaaatggctgctgac, reverse-aaaggactcgaagtgggtaatc; Forkhead box O1A (FOXO1A), forward-agatctacgagtggatggtcaa, reverse-acacgaatgaacttgctgtgta; HSL, forward-gagcggatcacacagaacct, reverse-ccagagacgatagcacttcca; LCAD, forward-ttatcaagggacgaaagctaca, reverse-cactagctggcaaccgtatatc; Lon protease, forward-tcaatgtcacccgcaactac, reverse-ccaggttctcgttgctgtact; mitofusin-2 (Mfn2), forward-gcagcttgtcatcagctacac, reverse-caatttcctgctccaggttct; PPARα, forward-gacacgctttcaccagctt, reverse-gcagattctacattcgatgttc; PPARγ, forward-cctccttgatgaataaagatg, reverse-gggctccataaagtcaccaa; PPARδ, forward-actgagttcgccaagagcat, reverse-gtgcacgccatacttgagaa; Sirt1, forward-tatgctcgccttgctgtaga, reverse-gcaaacttgaagaatggtcttg; superoxide dismutase 1 (SOD1), forward-ctcaggagaccattgcatca, reverse-cagcgtttcctgtctttgtact; superoxide dismutase 2 (SOD2), forward-ggacaaacctcagccctaac, reverse-gccgtcagcttctccttaaac; SREBP-1, forward-cagactcgctgcttctgaca, reverse-ggactgttgccaagatggtt; TFPβ, forward-aaacaagcaatgtggctagaga, reverse-ggcttggttggcagagatac; UCP3, forward-gaaaggaactttgcccaacat, reverse-ttgtcagtgagcaggtggtagt. Duplicate reverse transcription polymerase chain reaction was performed on an iCycler real-time PCR system (Bio-Rad Laboratories, Hercules, CA) in the One-step Taqman Master Mix reagents (Roche, Branchburg, NJ) according to the manufacturer's instruction with the gene of interest and housekeeping gene (β2-microglobulin) analyzed within the same run.

EM analysis.—Muscle samples were fixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Spurr's resin. Thin sections (70 nm) were cut using an ultramicrotome (Ultracut E; Reichert, Vienna, Austria), placed on Cu/Pd grids (200 mesh size), and stained for 5 minutes in uranyl acetate followed by 2 minutes in lead acetate. Sections were visualized at a magnification of ×6500 using a transmission electron microscope (Jeol 1200 Ex; Jeol, Tokyo, Japan). Two micrographs each from four different muscles fibers (eight images per person) were taken randomly and sequentially while blinded to gender and age. Images were taken from the subsarcolemmal region (subsarcolemmal area) and another containing a portion of the subsarcolemmal region adjacent to the nucleus with most of this image containing the intermyofibrillar area (total area). Therefore, the total area is a combination of both the intermyofibrillar and subsarcolemmal areas of the muscle samples. The images were photographed using a 1-s exposure time and digitized using a white light illuminator (C-80 Epi-illumination UV Darkroom; Diamed, Mississauga, ON, Canada).

Lipid and mitochondrial analysis was determined using a computerized image analysis system (Image Pro Plus, ver. 4.0; Media Cybernetics, Silver Spring, MD). Criteria were set for lipid droplet and mitochondrial identification, and correct identification was confirmed by reviewing 12 random micrographs with a pathologist trained in EM (Dr. K. Chorneyko, MD). Lipid droplets and mitochondrial fragments were circled and converted to actual size using a calibration grid. Values are reported as mean individual IMCL or mitochondrial size (μm^2), total number of IMCL droplets or mitochondria per square micrometer of tissue (#/um^2 tissue), and percentage IMCL or mitochondrial area density as previously described (18). Because of limitations in sample quantity, EM analysis for three participants from the older adult group (one man/two women) was not included in the data set.

Statistical Analysis

All data were compared using a two-way analysis of variance with gender and age as the between group effects (Statistica, Version 5.0; Statsoft, Tulsa, OK). When significance was attained, Tukey's honestly significant difference post hoc analysis was utilized to determine specific differences. Participant characteristics, mRNA content, and enzyme activities were compared to IMCL and mitochondrial size, number, and their association with one another using Pearson's correlation. Data are expressed as mean ± *SE*. Significance was set as $\alpha = .05$.

Results

Descriptive Data

The participant characteristics are provided in Table 1. Older adults were significantly older, shorter, and had greater adiposity compared with young individuals ($p < .05$). Overall, men were taller and leaner than women ($p < .05$). There were no differences between groups for resting plasma glucose, insulin or calculated homeostasis model assessment of insulin resistance (HOMA-IR, $p > .05$). The total duration of physical activity did not differ between groups; however, older adults spent a greater proportion of time performing resistance exercise than young adults ($p < .05$) and less time engaged in recreational activities ($p < .05$). In addition, young women tended to spend a greater proportion of time performing aerobic exercise than all other groups ($p = .089$).

Enzyme Activities

Enzyme activities from young and old individuals are provided in Figure 3. The maximal activities of COX and CS were 43% and 33% lower, respectively, in older adults compared with young individuals ($p < .05$). Women tended to have a higher CS activity than men ($p = .053$). COX activity was positively correlated with the number of mitochondria (#/μm^2, $r = .65$, $p = .002$) and mitochondrial area density ($r = .70$, $p < .001$) in the subsarcolemmal area (see below).

Electron Micrograph Data

An overview of the important morphological findings in old vs. young muscle is provided in Figure 1. Representative images of young and old muscle are shown in figure 2.

Intramyocellular lipid.—Table 2 summarizes results from our analysis of electron micrographs in muscle tissue. Older men had IMCL droplets that were larger than those of the other groups when considering the total fiber area ($p = .017$). However, older adults, as a group, had larger lipid droplets than young individuals in the subsarcolemmal area ($p = .009$). IMCL number in the subsarcolemmal area, expressed as #IMCL/μm^2, was greater in women than in men ($p = .019$) but was not different between the young and old groups ($p > .05$). Lipid area density was significantly higher in older men compared with older women, young men, and young women in the total fiber area ($p = .017$). In contrast, women had a greater lipid area density in the subsarcolemmal area of the muscle fiber ($p < .05$), independent of age ($p > .05$). The fraction of IMCL droplets in direct contact with mitochondria was significantly lower in older women than in other groups ($p < .001$) when considering the total fiber area. However, in the subsarcolemmal region, older men had the lowest proportion of IMCL droplets in direct contact with mitochondria compared with all other groups; this was significantly higher in older women and greatest in young men and women ($p = .018$). Finally, the percent body fat of the participants was positively correlated with lipid area density within the subsarcolemmal region ($r = .439$, $p = .005$).

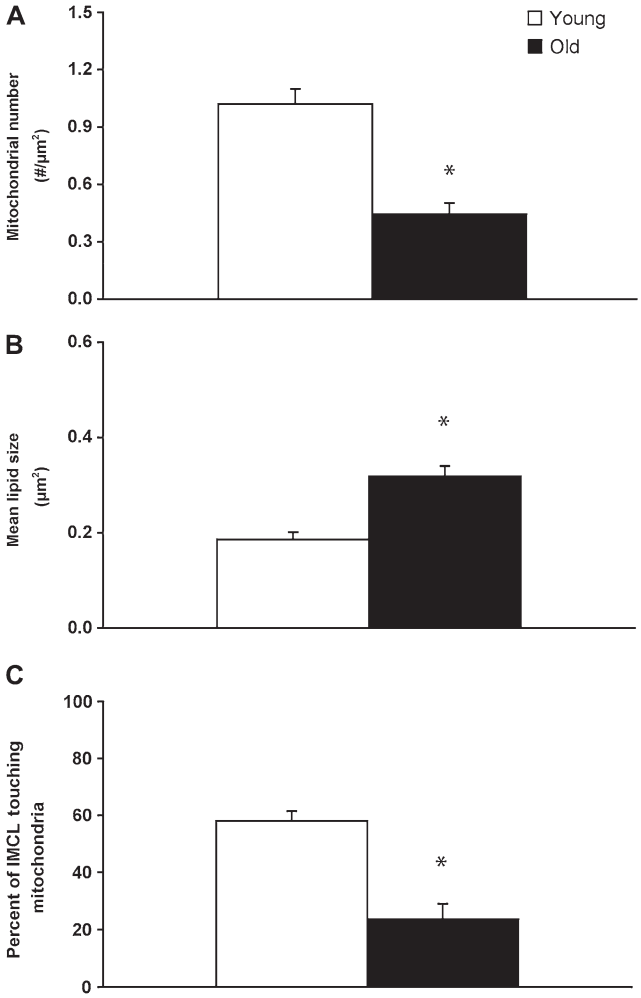

Figure 1. Electron micrograph quantification demonstrating changes in (**A**) mitochondrial number, (**B**) intramyocellular lipid (IMCL) size, and (**C**) the fraction of IMCL touching mitochondria within the subsarcolemmal area of skeletal muscle in young and old participants. Values are mean ± *SE*. *Significantly different from young individuals ($p < .05$).

Mitochondria.—There were no significant effects of age or gender on mitochondria size in the total or subsarcolemmal fiber regions ($p > .05$). The number of mitochondria per square micrometer was greater in young adults compared with old in the total and subsarcolemmal regions (SS: 132%, $p < .001$; total: 132%, $p < .001$). The mitochondrial area density in the total fiber area tended to be highest in young men, followed by young women, and lowest in old men and women ($p = .062$). Young adults had a significantly higher mitochondrial area density in the subsarcolemmal region compared with the older adults (126%, $p < .001$).

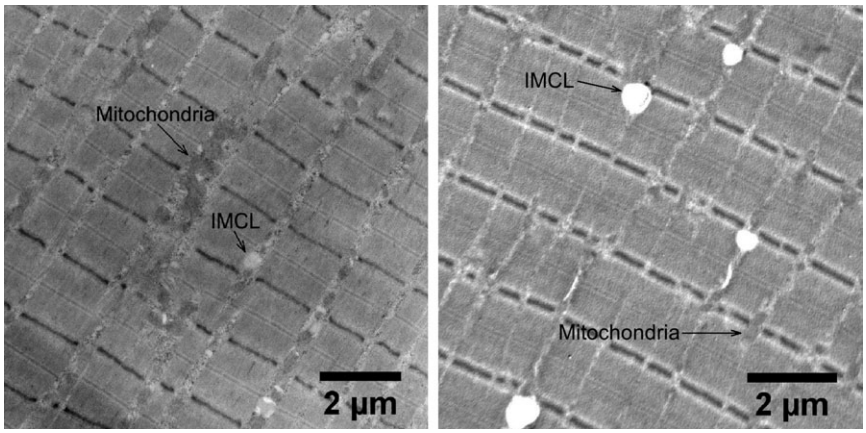

Figure 2. A representative electron micrograph of skeletal muscle from young (left) and old (right) individuals showing the size and abundance of mitochondria and the associated intramyocellular lipid (IMCL) droplets. Note the greater density and size of IMCL as well as the reduced number of mitochondria in aged skeletal muscle.

mRNA Abundance

Lipid metabolism.—All graphs are provided in Figure 4. mRNA content of HSL was significantly higher in older adults compared with young individuals ($p < .05$) and tended to be higher for SREBP-1 ($p = .057$). HSL mRNA was inversely correlated with the fraction of mitochondria in contact with IMCL in the total fiber area ($r = -.33$, $p = .041$). mRNA content of PPARα, PPARγ, and UCP3 were significantly lower in the old compared with young ($p < .05$). PPARα was negatively correlated with IMCL size in the subsarcolemmal area ($r = -.38$, $p = .015$). In addition, there was a trend for PPARγ mRNA to be greater in women ($p = .052$) and for UCP3 to be greater in men ($p = .091$). LCAD tended to be higher in women than in men ($p = .056$).

Mitochondrial function.—mRNA content of COX IV, Lon protease, and Mfn2 mRNA was significantly lower in older adults compared with young individuals ($p < .05$) and tended to be lower for Drp1 ($p = .096$). Mfn2 was positively

Table 2. Summary of Mitochondrial and IMCL Morphology in Young and Old Men and Women

	Young		Old				
	Men	Women	Men	Women	Age Effect	Gender Effect	Interaction
IMCL							
Mean lipid size (μm²)							
Total area	0.269 ± 0.034	0.208 ± 0.019	0.483 ± 0.073	0.219 ± 0.028	$p < .05$	$p < .05$	$p = .017$
Subsarcolemmal area	0.173 ± 0.019	0.197 ± 0.023	0.292 ± 0.077	0.347 ± 0.076	$p < .05$	NS	NS
No. of IMCL/μm²							
Total area	0.031 ± 0.005	0.040 ± 0.003	0.035 ± 0.004	0.038 ± 0.004	NS	NS	NS
Subsarcolemmal area	0.125 ± 0.014	0.211 ± 0.020	0.137 ± 0.029	0.168 ± 0.035	NS	$p < .05$	NS
Lipid area density (%)							
Total area	0.732 ± 0.147	0.840 ± 0.102	1.679 ± 0.336	0.830 ± 0.140	$p = .061$	$p < .05$	$p = .017$
Subsarcolemmal area	2.5 ± 0.5	4.1 ± 0.5	3.9 ± 1.3	7.3 ± 2.8	NS	$p < .05$	NS
% of lipids touching mitochondria							
Total area	85.3 ± 3.5	85.7 ± 3.5	85.0 ± 1.5	32.5 ± 2.5	$p < .05$	$p < .05$	$p < .001$
Subsarcolemmal area	61.2 ± 6.1	55.1 ± 3.6	12.2 ± 3.8	35.0 ± 8.8	$p < .05$	NS	$p = .018$
Mitochondria							
Mean mitochondrial size (μm²)							
Total area	0.178 ± 0.011	0.149 ± 0.012	0.155 ± 0.007	0.159 ± 0.010	NS	NS	NS
Subsarcolemmal area	0.196 ± 0.016	0.188 ± 0.014	0.210 ± 0.015	0.197 ± 0.009	NS	NS	NS
No. of mitochondria/μm²							
Total area	0.462 ± 0.055	0.414 ± 0.031	0.190 ± 0.019	0.187 ± 0.019	$p < .05$	NS	NS
Subsarcolemmal area	1.00 ± 0.150	1.04 ± 0.080	0.434 ± 0.080	0.454 ± 0.090	$p < .05$	NS	NS
Mitochondria area density (%)							
Total area	7.9 ± 1.0	5.2 ± 0.5	2.9 ± 0.3	2.9 ± 0.3	$p < .05$	$p = .054$	$p = .062$
Subsarcolemmal area	17.7 ± 2.0	18.8 ± 1.3	8.7 ± 1.6	9.3 ± 2.1	$p < .05$	NS	NS

Note: p Values refer to significant main effects ($p < .05$) identified by two-way (age by gender) analysis of variance. See text for details. $N = 24$ (12 men/12 women) for young and $N = 17$ (9 men/8 women) for older adults. Values are mean ± *SE*. IMCL = intramyocellular lipid; NS = nonsignificant.

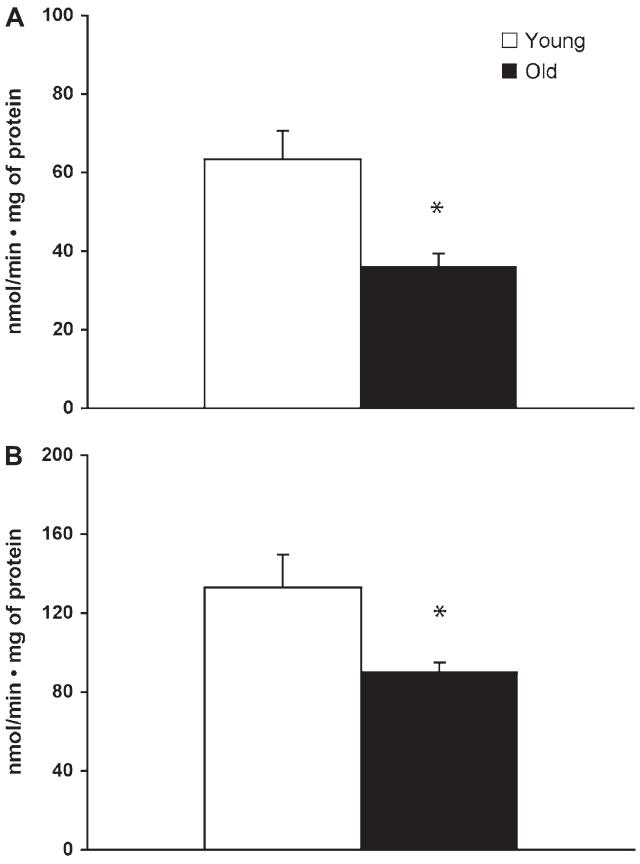

Figure 3. Enzyme activities of (**A**) cytochrome c oxidase and (**B**) citrate synthase in young and old individuals. $N = 12$ (6 men/6 women) for young and $N = 8$ (5 men/3 women) for older adults. Data are mean ± *SE*. *Significantly different from young individuals ($p < .05$).

correlated with the fraction of IMCL touching mitochondria when considering the total fiber area ($r = .33$, $p = .033$). Sirt1 mRNA content was significantly greater in the old ($p < .05$). The only gender difference observed for mitochondria-related genes was in COX IV mRNA, which was significantly higher in men than in women ($p < .05$).

Antioxidant enzymes and the oxidative stress response.—The mRNA content of FOXO1A was significantly greater in old compared with young individuals (data not shown, $p < .05$), and this was inversely correlated with the number of mitochondria (#/μm^2, $r = -.42$, $p = .009$) and the fraction of mitochondria in contact with IMCL in the total fiber area ($r = -.32$, $p = .046$). The mRNA content of SOD1 and SOD2 was significantly lower in older adults (data not shown, $p < .05$) and was positively correlated with mitochondrial area density in the total fiber area (data not shown, SOD1: $r = .71$, $p = .001$;SOD2: $r = .32$, $p = .041$). In addition, SOD1 mRNA content was significantly greater in men compared with women and was negatively correlated with IMCL area density in the total fiber area ($r = -.35$, $p = .031$).

Discussion

The present study confirms that older adults have higher IMCL content and provides novel morphological evidence that this is due to greater IMCL size (independent of quantity) combined with a reduced association with mitochondria. Furthermore, this is the first report, to our knowledge, to show that the decline in skeletal muscle mitochondrial content commonly observed with human aging is due to a reduction in the number but not the size of the existing mitochondria.

Previous studies have demonstrated elevated IMCL content in older individuals using ^{1}H-NMR spectroscopy, although these investigations do not provide any insight regarding size, subcellular localization, or proximity relationships within skeletal muscle (3,19,20). The present results indicate that the age-related increase in IMCL area density is due to a greater size, but not abundance, of lipid droplets, and that this is specific to the subsarcolemmal region in women. Recent work from our laboratory has shown that aerobic exercise training results in an increased number of lipid droplets, presumably as an adaptation to maximize surface area for lipolysis during exercise (18). Because our results indicate that mitochondrial oxidative capacity was lower and IMCL were larger in elderly adults, it is likely the changes in IMCL content observed here reflect increased storage of excess free fatty acids as triglycerides within the IMCL. This would limit the accumulation of harmful lipid intermediates (diacylglycerol, ceramide) that have direct lipotoxic effects on cellular function (27,28). This is consistent with our observed elevation of mRNA transcripts involved in triacylglycerol synthesis (SREBP-1) and breakdown (HSL) in older participants. However, older adults also had diminished gene expression of PPARα and PPARγ, indicating a downregulation of transcripts involved in lipid transport and oxidation. Although we did not observe differences in the young and old participants in the HOMA-IR, perhaps related to the high relative fitness of the elderly participants, previous work in mice has demonstrated an age-related decrease in PPARγ related to reduced insulin sensitivity (29). Interestingly, PPARα was negatively correlated with IMCL size in the subsarcolemmal area but not to the other aspects of IMCL content: abundance and area density. The lack of a relationship between HOMA-IR and IMCL content in the current study combined with the lower mitochondrial activity and content of the older adults brings into question the hypothetical relationship between IMCL and insulin resistance put forth by others (3,20) with regard to the geriatric population. Moreover, this reinforces the importance of mitochondrial function in relation to IMCL content in the pathology of insulin resistance and highlights the incomplete data available when using whole-muscle measurements.

We have also confirmed that skeletal muscle from women contained a greater amount of IMCL than men, which appears to be retained with age when the entire fiber area is

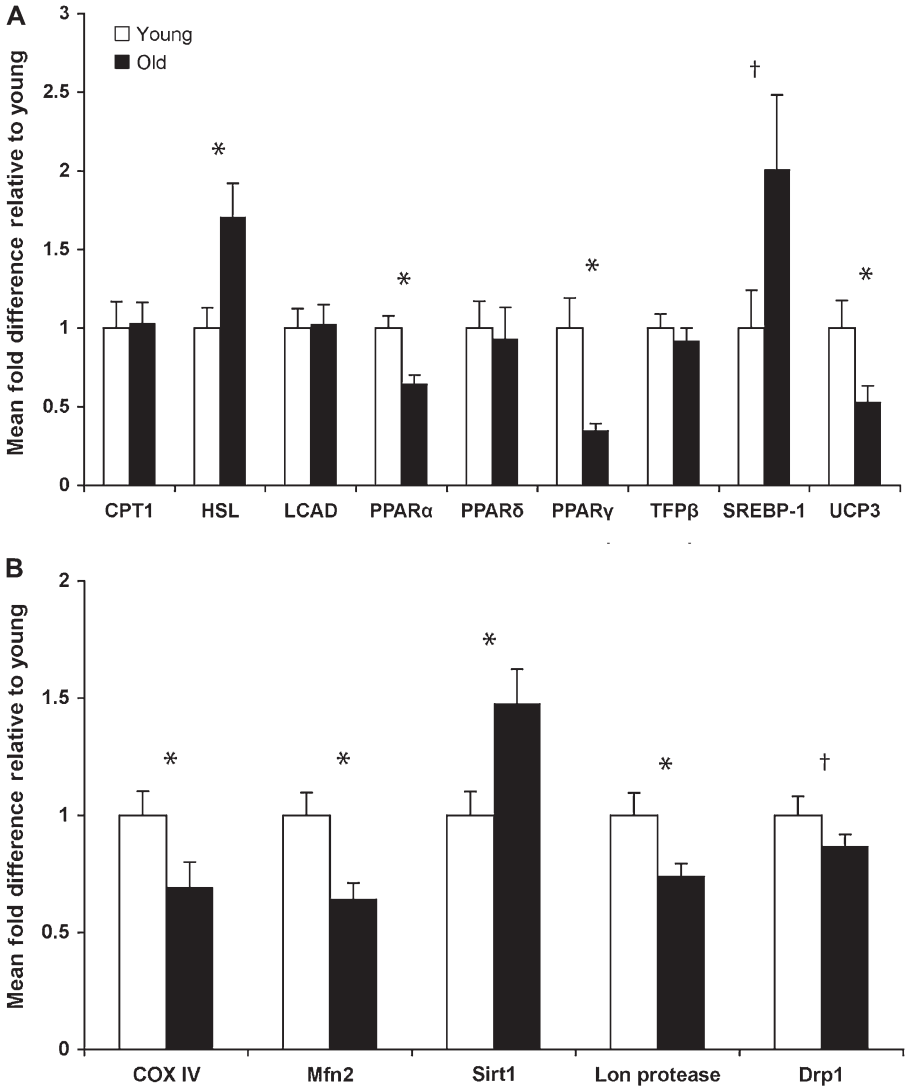

Figure 4. Gene expression of transcripts related to (**A**) lipid metabolism and (**B**) mitochondrial biogenesis and function in young and old individuals. CPT1 = carnitine palmitoyltransferase-1; HSL = hormone sensitive lipase; LCAD = long-chain acyl-CoA dehydrogenase; PPARα = peroxisome proliferator–activated receptor alpha; PPARγ = peroxisome proliferator–activated receptor gamma; PPARδ = peroxisome proliferator–activated receptor delta; TFPβ = trifunctional protein beta; SREBP-1 = strerol regulatory element–binding protein-1; UCP3 = uncoupling protein-3; COX IV = cytochrome c oxidase subunit IV; Mfn2 = mitofusin 2; Sirt1 = sirtuin 1; Drp1 = dynamin-related protein 1. N = 24 (12 men/12 women) for young and *N* = 20 (10 men/10 women) for older adults. Data are mean ± *SE*. *Significantly different from young individuals ($p < .05$). †Trend for a difference from young individuals ($p < .10$).

taken into account. Women have a greater area density of IMCL (18,21) and oxidize more fat during endurance exercise (30,31). The augmented IMCL stores in female skeletal muscle may result from the influence of sex hormones, as has been shown in rats (32). The age of the older adults precludes this possibility as the older women were beyond menopause and estrogen would play a minimal role. It is tempting to speculate that the conservation of increased IMCL content in women with aging is due to greater adiposity in the old women compared with young women. However, old men had the highest IMCL content in the total fiber area despite having the lowest body fat of all groups. Furthermore, the weak correlation between body fat and IMCL area density is only significant in the young participants when they are separated from the older adults, indicating that body fat is not necessarily the best predictor of IMCL content and does not explain our observations with aging.

We have uniquely demonstrated that the decreased mitochondrial content commonly observed with human aging is due to a reduction in the number, but not size, of the existing mitochondria, concomitant with reductions in mitochondrial enzyme activities. These findings are similar to those recently observed in aged mice (33) and are in contrast to the reduced mitochondrial size seen in obesity and type 2 diabetes (34). Some authors have postulated that impairments in insulin sensitivity are due simply to reduced mitochondrial content that results in an imbalance of fatty acid

utilization, and thus accumulation of lipid, in young individuals (13,35). We did not find any associations between HOMA-IR and mitochondrial enzyme activity or morphology, indicating that deficits in muscle oxidative capacity may not be directly attributable to parameters of insulin sensitivity in older adults. We have previously shown that increased physical activity leads to an increase in the size, but not number, of mitochondria (18), and fewer mitochondria in the current study were not correlated with increased numbers of IMCL droplets, which collectively reinforces the fact that our current observations in older adults are not simply due to a lower activity level. Thus, the defects in mitochondrial content/morphology with aging appear to be distinct from those of insulin resistance and changes in physical activity.

Some prior investigations in humans have postulated that mitochondrial size decreases with aging (10,11). However, in the work of Orlander et al. the changes in mitochondrial number were limited to the subsarcolemmal region, and only when some age groups are excluded from their analysis were the differences significant. Because their youngest and oldest age groups were significantly more active than the other age groups, some of their conclusions may be questionable, especially considering that they did not detect any age-related changes in oxidative enzyme activities or IMCL content. Many studies concerning mitochondrial dysfunction with aging have failed to take into account the location of specific populations of mitochondria within skeletal muscle, which can differ with regard to oxidative capacity and adaptability to contractile activity (36,37). In the current study we considered a distinct portion of the muscle fiber, the subsarcolemmal area, as well as the total fiber area, which appear to have markedly different IMCL and mitochondrial ultrastructure.

The cause of reduced mitochondrial content has been extensively studied in relation to accumulations in DNA and protein damage resulting from reactive oxygen species (ROS) production. Higher levels of FOXO1A expression appear to contribute to an enhanced ROS response (38), and this may be due to an elevated level of oxidative damage in the current study that has been previously established to occur with aging (39). Although indirect, reduced gene expression of SOD1 and SOD2 could underlie increased oxidative stress due to an impaired antioxidant defense system. In support of this, SOD1 and SOD2 expression was positively correlated with mitochondrial number. Moreover, the age-related decline in the expression of Lon protease, a mitochondrial matrix enzyme, results in greater levels of oxidatively damaged proteins (8), compounding the influence of reduced cellular antioxidants. Age-related increases in DNA and protein damage also have negative effects on mitochondrial biogenesis (40), further contributing to a decreased number of mitochondria. Several investigations have reported "giant" mitochondria in aged skeletal muscle with malformed cristae and an extremely large size (41). We did not observe these mitochondria in older adults, potentially because they were healthy and active for their age.

Physical associations between IMCL and mitochondria with aging have not been previously investigated. We have ascertained that aging results in a reduced number of IMCL touching mitochondria and that this occurs in the subsarcolemmal region for men and throughout the entire fiber area in women. Aerobic exercise training produces the opposite effect, whereby mitochondria and IMCL become closely associated and are therefore more optimally situated for oxidation (18). Mitochondrial dynamics (fission and fusion) may explain changes in subcellular localization and associations with IMCL. It has been shown that proteins regulating mitochondrial fusion, mitofusins, are upregulated in response to acute exercise (15), while the inverse is demonstrated in obese individuals (42), indicating a potential regulation of mitochondrial dynamics by physical activity. The observed decrease in the gene expression of Mfn2 and Drp1 with aging, proteins involved in mitochondrial fusion and fission, respectively, could partially explain the diminished association of mitochondria with IMCL droplets. Indeed, we find a significant positive correlation between Mfn2 and the fraction of IMCL in contact with mitochondria. Thus, the aging process may downregulate fusion and fission events, retarding motility and reducing the metabolic need for mitochondria to be in close proximity with IMCL stores.

A potential shortfall of our findings is the inability to determine fiber type–specific differences in relation to mitochondria and IMCL ultrastructure. Although we cannot discount the influence of fiber type on our observations, increases in the proportion of type I fibers with aging are not likely to be the sole cause of the changes in IMCL and mitochondrial morphology. Previous studies have used the soleus muscle to demonstrate an age-related increase in IMCL content (3,19,20). Being primarily of the type I myosin heavy chain isoform in young adults and heavily recruited for posture and daily ambulation, this muscle would not be expected to present a dramatically different fiber composition in older adults. In addition, because type I fibers are more insulin sensitive (43) and contain a greater content of mitochondria (44), a larger fraction of these fibers in the elderly should serve to counter a decline in muscle oxidative capacity and insulin sensitivity, strengthening the current findings in relation to subcellular structure. Furthermore, inclusion of the changes that occur in the total area of the muscle fibers, independent of fiber type, are likely to be more representative of whole-muscle function and metabolism, which is of primary importance for the elderly population. While alterations in mRNA are not always representative of changes at the protein level, we do observe significant agreement between morphological assessments and several mRNA transcripts as well as mitochondrial enzyme activities. The aforementioned differences and relationships with age were observed in spite of both groups

being evenly matched for physical activity, and these changes would likely be exacerbated in sedentary older individuals.

In summary, we have demonstrated that reductions in the association of larger IMCL droplets with a fewer number of mitochondria occur with aging. These alterations appear to be independent of changes in insulin sensitivity and physical activity and are likely an inherent aspect of the aging process. Furthermore, structural changes to IMCL and mitochondria were correlated with transcriptional alterations related to aberrant lipid metabolism, mitochondrial dysfunction, and oxidative damage, implying that morphology is a critical component of cellular homeostasis with senescence. These findings have important implications in the rapidly expanding field of mitochondrial dynamics, as well as for exercise or pharmaceutical treatments that seek to attenuate the effects of aging on skeletal muscle.

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CHAPTER 3: MANUSCRIPT 2

Elevated Mitochondrial Oxidative Stress Impairs Metabolic Adaptations to Exercise in Skeletal Muscle

In Review

TITLE: Elevated mitochondrial oxidative stress impairs metabolic adaptations to exercise in skeletal muscle

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SUMMARY

Mitochondrial oxidative stress is a complex phenomenon that is inherently tied to energy provision but is implicated in many metabolic disorders. Exercise training increases mitochondrial oxidative capacity in skeletal muscle yet it remains unclear if oxidative stress plays a role in regulating these adaptations. We demonstrate that the elevation in mitochondrial oxidative stress present in *Sod2*$^{+/-}$ transgenic mice impairs the functional and biochemical mitochondrial adaptations to exercise. Following exercise training *Sod2*$^{+/-}$ mice fail to improve exercise performance, maximal aerobic capacity, muscle mitochondrial respiration, and mtDNA copy number, despite a normal augmentation of mitochondrial proteins. Additionally, exercised *Sod2*$^{+/-}$ mice cannot compensate for their higher amount of basal mitochondrial oxidative damage and exhibit poor electron transport chain complex assembly that accounts for their compromised adaptation. Overall these results demonstrate that skeletal muscle mitochondrial oxidative stress does not impact exercise induced mitochondrial biogenesis, but impairs the resulting mitochondrial protein function and can limit metabolic plasticity.

HIGHLIGHTS

- Elevated mitochondrial oxidative stress prevents improvements in exercise capacity.
- Mitochondrial function is impaired by oxidative damage despite an increase in mitochondria from exercise training.
- mtDNA transcription is blunted under conditions of oxidative stress and results in enhanced binding of the chaperone GRP75 to TFAM.
- Mitochondrial complex assembly is compromised under conditions of oxidative damage.

INTRODUCTION

Mitochondria are a major source of reactive oxygen species (ROS) and reactive nitrogen species (RNS), directly releasing a small portion of the product superoxide from the electron transport chain during the process of oxidative phosphorylation. It is believed that protein subunits of complex I, II and III can each release superoxide during electron transfer (Barja, 1999; Muller, Liu and Van Remmen, 2004; Quinlan et al., 2012). The accumulation of oxidatively modified proteins, lipids and DNA resulting from spontaneous reaction with superoxide and other ROS/RNS is proposed to contribute to the mitochondrial dysfunction associated with a host of health problems including insulin resistance (Houstis, Rosen and Lander, 2006), neurodegeneration (Lin and Beal, 2006) and sarcopenia (Wanagat et al., 2001). Post-mitotic tissues such as skeletal muscle and brain are particularly susceptible to mitochondrial oxidative damage because they are terminally differentiated, have a relatively slow cellular turnover and a high metabolic rate. However, many health interventions that intend to augment mitochondrial content and metabolism in differentiated tissues in order to counter disease progression must contend with an environment of mitochondrial oxidative stress (i.e. aging, obesity). Therefore, insight regarding the consequences of mitochondrial oxidative damage is necessary in order to understand its broader role in altering mitochondrial function and adaptation.

Several antioxidant enzymes exist within cells for the conversion of ROS into less harmful species. In order to delineate the cellular role of these enzymes, several research groups have produced transgenic mice that exhibit a continual state of oxidative stress by

partial or complete removal of a specific antioxidant enzyme. Superoxide dismutase (Sod) converts the highly labile and reactive superoxide radical into hydrogen peroxide in the cytosol (Sod1), mitochondria (Sod2) or extracellular space (Sod3), although Sod1 may also be present in the mitochondrial inner membrane space (Okado-Matsumoto and Fridovich, 2001; Sturtz et al., 2001). Sod2 has been established as a particularly important scavenger of ROS as this enzyme is essential for life beyond perinatal development in mice (Lebovitz et al., 1996; Li et al., 1995) in contrast to the antioxidant enzymes Sod1, Gpx1 or Gpx4 (Ho et al., 1998; Ran et al., 2007; Zhang et al., 2009). Sod2 heterozygous (*Sod2*$^{+/-}$) mice exhibit elevated mitochondrial oxidative stress and minor mitochondrial dysfunction (Kokoszka et al., 2001) without any impairment in physical function or lifespan (Van Remmen et al., 2003). Moreover, the *ex vivo* respiratory issues previously observed in liver mitochondria from these mice may have been overestimated due to the mitochondrial isolation procedure (Picard et al., 2010). Overall, intramitochondrial superoxide production represents a relatively indefensible problem as there is no other enzymatic superoxide scavenger besides Sod2 within the mitochondrial matrix. Furthermore, mitochondrial DNA (mtDNA) is uncoiled, unsheltered and proximal to the electron transport chain sites of ROS generation.

Habitual exercise is well known to improve health and enhance the mitochondrial content and oxidative capacity of skeletal muscle. In healthy, young individuals exercise training facilitates an increase in antioxidant enzymes (Gomez-Cabrera, Domenech and Viña, 2008), presumably from increased ROS generation. Mitochondrial ROS, however, are not believed to contribute significantly to acute, exercise-induced ROS production

(Powers and Jackson, 2008). It is hypothesized that a greater proportion of mitochondrial superoxide is generated under basal (state 4) conditions than with high flux (state 3) mitochondrial respiration (St-Pierre et al., 2002), and thus exercise training may paradoxically increase basal ROS generation by virtue of elevating total mitochondrial mass. In several conditions where persistent oxidative stress accompanies disease progression, such as chronic obstructive pulmonary disease (COPD), exercise interventions may actually increase levels of oxidative damage in skeletal muscle (Barreiro et al., 2009) by increasing mitochondrial ROS (Puente-Maestu et al., 2012). Dietary antioxidant compounds have been utilized to elucidate the role of ROS in mediating skeletal muscle adaptations, with evidence that antioxidants can blunt the positive metabolic benefits of exercise within muscle (Gomez-Cabrera et al., 2008; Ristow et al., 2009). There is, however, some contention as to these effects (Higashida et al., 2011). Nevertheless, uptake of these compounds in various tissues, their cellular compartmentalization, as well as complex dosage dependent pro-oxidant/anti-oxidant properties (e.g. as with vitamin C, (Gutteridge and Halliwell, 2010)) hamper definitive conclusions regarding their mechanism of action. It remains necessary to clarify how exercise, mitochondrial function and oxidative stress interact within skeletal muscle to regulate metabolic adaptation.

In this study we demonstrate that the *Sod2*$^{+/-}$ mouse has impaired aerobic adaptations to exercise *in vivo,* which is due to compromised electron transport chain function and mtDNA maintenance in skeletal muscle. These results are the first to specifically implicate mitochondrial radicals in altering exercise adaptation and serve to

expand our understanding of the detrimental effects of ROS and RNS within post-mitotic tissues.

RESULTS

Sedentary *Sod2*$^{+/-}$ mice have equivalent running performance to wild-type mice, but fail to functionally adapt to exercise training

In order to study the specific effects of mitochondrial oxidative stress on exercise adaptations, we monitored *Sod2*$^{+/+}$ and *Sod2*$^{+/-}$ mice at rest before initiating any exercise training. Prior to the training intervention, there were no differences between genotypes with regard to resting metabolic and behavioral parameters (VO_2, VCO_2, food intake and accumulated x-axis ambulatory activity, Figure S1A-D). However, after mice had been separated into sedentary (SED) or forced-exercise training (EX) groups for four months, *Sod2*$^{+/+}$ EX mice improved several aspects of running performance (work capacity, total distance run, and VO_{2max}) compared to their sedentary counterparts, while *Sod2*$^{+/-}$ mice did not exhibit differences in these characteristics (Figure 1A-C). In addition, there was a significant association between VO_{2max} and total distance run in the *Sod2*$^{+/+}$ mice regardless of training group, but not in *Sod2*$^{+/-}$ mice, indicating an exercise-induced dissociation between oxygen consumption and muscle performance (Figure 1D). This contrasts with studies showing predominantly normal adaptations in bone, cartilage and heart with exercise training in *Sod2*$^{+/-}$ mice (Baur et al., 2011; Richters et al., 2011). None of the basal metabolic or behavioral measurements performed prior to beginning the study were different between training groups or genotypes following the training

intervention (Figure S1E-H), demonstrating that resting metabolism was not affected by exercise training-induced alterations in mitochondrial oxidative stress.

Mitochondrial function is disrupted in *Sod2*$^{+/-}$ mice and fails to improve in spite of an exercise-induced increase in mitochondrial protein content

Oxidative stress has been shown to induce mitochondrial biogenesis *in vitro* to compensate for impaired mitochondrial function (Lee and Wei, 2005), however, we did not find basal differences in skeletal muscle mitochondrial protein content or enzyme activity between *Sod2*$^{+/+}$ and *Sod2*$^{+/-}$ SED mice (Figure 2A-C). It remains possible that mitochondria in the *Sod2*$^{+/-}$ mice are maintained below an undefined threshold whereby oxidative stress would induce compensatory mitochondrial biogenesis. Exercise training resulted in a similar increase in the protein content of complex I, II, and IV in both genotypes (Figure 2A) while complex III and V remained unchanged. Nevertheless, the maximal activity of the mitochondrial proteins citrate synthase, complex I+III, and complex IV was augmented only in *Sod2*$^{+/+}$ mice with exercise (Figure 2C). This suggests that while increased mitochondrial oxidative stress in *Sod2*$^{+/-}$ mice did not alter the exercise-induced augmentation of mitochondrial protein expression, it did significantly disrupt their assembly into functional complexes. Further, mitochondrial respiration in permeabilized muscle fibers from *Sod2*$^{+/-}$ mice was elevated in the presence of glutamate and malate without ADP addition, which is indicative of proton leak or redox "slip" (only between SED groups, Figure 2D). Maximal coupled respiration (complex I+II substrates with ADP addition) was reduced in *Sod2*$^{+/-}$ SED compared to

Sod2$^{+/+}$ SED mice and increased with training only in *Sod2*$^{+/+}$ EX mice (Figure 2E). Additionally, there was a general reduction in respiratory control in *Sod2*$^{+/-}$ mice (Figure 2F) accounting for the impairments in muscle oxidative capacity (VO_{2max}) in the *Sod2*$^{+/-}$ EX mice. In contrast, complete ablation of muscle Sod2 impairs acute exercise capacity and only resulted in reduced activity of complex II (Kuwahara et al., 2010), however mitochondrial function or oxidative damage were not comprehensively evaluated in this study.

A clear physiologic discrepancy in the *Sod2*$^{+/-}$ mice is the lack of a positive relationship between oxygen uptake and exercise performance (Figure 1D). Several proteins, such as uncoupling protein 3 (UCP3) and adenine nucleotide translocase 1 (ANT1), can act as channels across the mitochondrial inner membrane in skeletal muscle and facilitate proton leak, which uncouples oxygen utilization and ATP generation (Boss et al., 1997; Brand et al., 2005) and UCP3 activity is sensitive to superoxide levels (Echtay et al., 2002). However, UCP3 protein content per amount of mitochondria was unaltered in the current study (Figure 2G), and given that there were no differences in resting metabolic rate between genotypes after the training intervention (Figure S1A-H) gives little support for a role of UCP3 in impairing the oxidative capacity of the *Sod2*$^{+/-}$ EX mice. There were differences in the non-ADP stimulated "leak state" of mitochondrial respiration between *Sod2*$^{+/-}$ and *Sod2*$^{+/+}$ SED mice (Figure 3D), so it is possible that UCP3 may play a minor role in untrained muscle in a preliminary attempt to deal with excess oxidative stress. Regardless, UCP3 may be more relevant to alterations in metabolic flux induced by acute exercise in skeletal muscle (Jiang et al., 2009). ANT1

content, on the other hand, was elevated in response to exercise training in *Sod2*$^{+/-}$ mice (Figure 2H). ANT1 proton conductance is related to its content, but not its ADP/ATP translocase activity (Brand et al., 2005). ANT1 did not differ in its basal expression between SED *Sod2*$^{+/+}$ and *Sod2*$^{+/-}$ mice and since reduced coupling was observed in both *Sod2*$^{+/-}$ SED and EX mice (compared to *Sod2*$^{+/+}$) it seems unlikely that the training-induced elevation ANT1 accounts for the lack of aerobic adaptation in the *Sod2*$^{+/-}$ mice. It is probable that elevated ANT1 is at least partly compensating for impaired ATP generation in the *Sod2*$^{+/-}$ EX group due to upstream mitochondrial complex dysfunction. Overall, these results indicate that increasing mitochondrial protein expression in concert with elevated oxidative stress seems to exacerbate minor mitochondrial dysfunction rather than correct it.

Exercise in *Sod2*$^{+/-}$ mice does not abrogate mitochondrial oxidative damage

Exercise is known to stimulate compensatory mechanisms to alleviate oxidative stress including increasing the expression of several antioxidant enzymes, importing non-enzymatic antioxidant compounds (i.e. vitamin C, E) and upregulating repair mechanisms in response to macromolecular and DNA oxidative damage (Ji, 2008; Radak, Chung and Goto, 2008). As expected, Sod2 protein in skeletal muscle was lower when comparing SED mice (Figure 3A-B) but *Sod2*$^{+/-}$ EX mice were not different different from *Sod2*$^{+/+}$ mice, indicating increased expression with exercise. However, we determined that exercise reduces non-enzymatic antioxidant activity in isolated mitochondria (Figure 3C), a phenomenon that would impair free radical scavenging and be particularly detrimental

within *Sod2*$^{+/-}$ muscle. Since *Sod2*$^{+/-}$ mice have a higher abundance of the mitochondrial oxidative modification nitro-tyrosine regardless of exercise status (Figure 3D-E), it is unlikely there was a sufficient compensation in any of the mitochondrial antioxidant enzymes due to exercise. Importantly, while nitro-tyrosine levels per amount of mitochondria were similar between the *Sod2*$^{+/-}$ SED and EX groups, the EX group had ~40% more mitochondria on the whole cell level implying a higher absolute burden of oxidatively modified proteins. Previous work has shown protein carbonyl levels to be elevated in *Sod2*$^{+/-}$ mice in liver mitochondria (Williams et al., 1998) and in aged skeletal muscle (Bota, Van Remmen and Davies, 2002), but neither the lipid peroxidation product 4-hydroxy-nonenal or protein carbonyls were elevated in *Sod2*$^{+/-}$ skeletal muscle mitochondria (Figure S2A-B). Isolated mitochondria from skeletal muscle exhibits higher complex I, II, III and IV activity than liver (Kwong and Sohal, 2000) and may be susceptible to different cellular radicals than liver because it is differentiated. Given that muscle has a high bioavailability of nitric oxide (NO), it is likely that the nitro-tyrosine adducts result from reacting NO and superoxide to form peroxynitrite, which can reduce respiration of complex I and II in submitochondrial particles (Lizasoain et al., 1996). In parallel to the elevated nitro-tyrosine levels in mitochondria, *Sod2*$^{+/-}$ mice had greater levels of 8-hydroxy-2-deoxy guanosine (8-OH-dG) damage to mtDNA (Figure 3F), amounting to approximately 5-fold higher levels when comparing *Sod2*$^{+/+}$ SED vs. *Sod2*$^{+/-}$ EX mice. This suggests that DNA is a more susceptible macromolecule to superoxide induced oxidative damage than protein or lipid in skeletal muscle. Correspondingly, the expression of the base excision repair enzyme OGG1 was higher in

Sod2$^{+/-}$ SED vs. *Sod2*$^{+/+}$ mice but was not elevated further by exercise in *Sod2*$^{+/-}$ EX mice (Figure 3G-H), indicating dysregulation of DNA repair in the presence of higher amounts of oxidative damage.

Mitochondrial oxidative stress disrupts mtDNA transcription by altering the stoichiometry of mtDNA copies:TFAM protein

mtDNA is vulnerable to oxidative damage from byproducts of mitochondrial electron transport and this is exacerbated by the lack of protective histones akin to nuclear DNA. Mitochondrial transcription factor A (TFAM) is essential for the transcription and maintenance of mtDNA and is believed to coat a significant portion of its length at all times in order to protect mtDNA from ROS and other damage (Kang and Hamasaki, 2005). Exercise training predictably induced an increase in mtDNA copy number in *Sod2*$^{+/+}$ but this did not occur in *Sod2*$^{+/-}$ mice (Figure 4A). Conversely, *TFAM* mRNA was basally elevated in *Sod2*$^{+/-}$ mice and TFAM protein expression in whole muscle was only increased in *Sod2*$^{+/-}$ mice following exercise training (Figure 4B-D). This demonstrates that with typical levels of mitochondrial oxidative stress there is sufficient TFAM protein to maintain and replicate mtDNA in response to exercise, and that elevations disrupt TFAM function. Consequently, we observed that the ratio of mtDNA per amount of TFAM is reduced in *Sod2*$^{+/-}$ EX mice (Figure 4E). Recombinant TFAM binds less efficiently to synthesized 8-OH-dG DNA adducts (Yoshida et al., 2002), and since 8-OH-dG damage to mtDNA is increased with exercise training in *Sod2*$^{+/-}$ mice, greater expression of TFAM protein might be an attempt to preserve

mtDNA maintenance. Unfortunately, TFAM-bound mtDNA can prevent base excision repair enzymes such as OGG1 from accessing mtDNA (Canugovi et al., 2010) and explains lower, rather than higher, OGG1 expression in *Sod2*$^{+/-}$ EX vs. *Sod2*$^{+/-}$ SED mice (see Figure 3G-H) as unbound OGG1 would be degraded. Moreover, the activity of the mitochondrial Lon protease, which catabolizes TFAM, is lower in *Sod2*$^{+/-}$ mice (Bota, Van Remmen and Davies, 2002) and thus damaged or malfunctioning TFAM may be incompletely removed in these mice. When TFAM was immunoprecipitated from isolated mitochondria we found more GRP75, but not HSP60, associated with TFAM in the *Sod2*$^{+/-}$ EX vs. SED mice (Figure 4F-H), possibly reflecting a response to sequester dysfunctional TFAM molecules that have evaded proteolysis. This appears to be a targeted chaperone response rather than a general unfolded protein response as GRP75 was not altered in isolated mitochondria (data not shown). mtDNA transcription is therefore sensitive to local oxidative stress, which may explain reduced mtDNA copy number in pathologies associated with mitochondrial ROS damage such as COPD or Alzheimer's disease (Coskun, Beal and Wallace, 2004; Puente-Maestu et al., 2011).

Electron transport chain complexes from oxidatively damaged mitochondria exhibit increased protein misfolding and mis-assembly

Since mitochondrial protein function is clearly disrupted in the *Sod2*$^{+/-}$ SED mice and exacerbated in the *Sod2*$^{+/-}$ EX mice, we sought to examine whether ETC complex assembly contributed to the impairments in exercise adaptation. Exercise training generally appears to improve assembly of the ETC complexes I, III, IV and V in *Sod2*$^{+/+}$

mice as immunoblotting indicates more punctate migration in the native direction in EX vs. SED mice (Figure 5). Conversely, exercise training appears to worsen complex formation in the *Sod2*$^{+/-}$ mice particularly in the case of complex I and IV. It is likely that the increased levels of damaged proteins in *Sod2*$^{+/-}$ mice (Figure 3) interfere with the assembly process within the mitochondrial membrane space. The mis-assembly of mitochondrial complexes is similarly observed in pathologies linked with mitochondrial dysfunction and oxidative stress, including Parkinson's disease (Distelmaier et al., 2009) and Alzheimer's disease (Fukui and Moraes, 2008). While the causative events of these diseases remain unclear, we propose that excess superoxide can independently disrupt mitochondrial complex assembly under conditions of mitochondrial expansion or remodeling.

DISCUSSION

Mitochondrial oxidative stress has been proposed to contribute to a host of diseases by nature of inducing ETC dysfunction and impairing cellular bioenergetics. Prior reports have demonstrated basal mitochondrial respiratory dysfunction in *Sod2*$^{+/-}$ mice coincided with elevated oxidative damage, but have failed to establish the relevance of these changes to physiologic function or their effect in concert with increases in mitochondrial mass. Moreover, while the consequences of mitochondrial oxidative stress and damage are often observed in disease, rarely can one conclude ROS/RNS is the primary cause of cellular dysfunction since release of these radicals often coincides with mitochondrial protein and mtDNA defects that can in turn augment ROS generation.

Within this context, we provide evidence of two mechanisms by which excess mitochondrial superoxide impairs mitochondrial function *in vivo* using exercise training as a model to stimulate mitochondrial biogenesis in skeletal muscle: (1) by impeding proper ETC complex assembly and (2) via disruption of mtDNA maintenance.

While we reaffirm several aspects of basal mitochondrial insufficiency in *Sod*$^{+/-}$ mice, it should be noted that there were no phenotypic differences present between genotypes prior to the exercise intervention. This is an important distinction and possibly reflects a "threshold effect" in which physiologic function is only limited beyond a certain level of oxidative stress or damage. This threshold remains unclearly defined, but is highly relevant in differentiated tissues that must cope with a continual accrual of oxidative modifications. Since mice overexpressing *Sod2* do not exhibit signs of a metabolic advantage within differentiated skeletal muscle or in lifespan (Lee, Van Remmen and Csete, 2009; Pérez et al., 2009), it seems likely that mitochondrial antioxidants are only relevant to mitochondrial adaptation in deficient states. Reports suggesting that antioxidants disrupt mitochondrial exercise adaptation have failed to properly demonstrate a role of mitochondrial oxidative stress (and its subsequent attenuation), or to implicate potential cellular signaling pathways that prevent adaptation. It is entirely possible that the "antioxidants" used in these studies actually acted as pro-oxidants *in vivo* and compromised metabolic function in skeletal muscle via the mechanisms observed in the current study or via additional oxidative modifications. Furthermore, these findings suggest caution in the implementation of exercise interventions in populations that might have persistent mitochondrial oxidative stress or

that might be sensitive to mitochondrial stressors. More research is necessary regarding interplay of mitochondria-derived free radicals in order to maximize the adaptive potential of skeletal muscle for health promotion.

EXPERIMENTAL PROCEDURES

Animal Handling and Care

Sod2$^{+/-}$ male and female mice were acquired from Jackson Laboratories and bred to produce Sod2$^{+/-}$ and Sod2$^{+/+}$ littermates. Mice were housed in micro-isolator cages and maintained under controlled environmental conditions (12/12 h light/dark cycle, 23^{o}C). Mice received chow food (Diet 8640, Harlan Teklab, Madison, WI) and water *ad libitum.* Food intake and body weight were recorded biweekly during the study. At 6 months of age, mice were randomly allocated into sedentary or forced-endurance exercise training interventions for 16 weeks. At ~10 months of age all mice were sacrificed, after an overnight fast, by cervical dislocation. Sacrifice was performed one week following any *in vivo* testing procedures and the final exercise bout. All animal procedures were conducted under the approval of McMaster University's Animal Research Ethics Board and the Canadian Council on Animal Care.

Exercise Training Protocol

Mice in the exercise groups ran on level treadmills (Eco 3/6 treadmill; Columbus Instruments, Columbus, OH, USA) three times per week for 16 weeks. Initially the speed was set at 12 meters/minute (m/min) for 30 minutes, but gradually increased to 20 m/min for 60 minutes by the conclusion of the study. Mice were encouraged to run by the use of

electrical shock bars at the ends of the treadmill lanes. All mice had similar adherence to the training regimen and completed ~98% of the training volume.

Basal Metabolic Measurements

Resting oxygen consumption (VO_2), carbon dioxide output (VCO_2), respiratory exchange ratio (RER), food intake, water intake and spontaneous activity (beam breaks, XAMB) were monitored under a consistent temperature (25^{o}C) using an indirect calorimetry system (Columbus Instruments Comprehensive Lab Animal Monitoring System (CLAMS), OH, USA). Mice were acclimatized to the metabolic chamber for 12h prior to commencing data collection. VO_2, VCO_2 and RER were measured in individual mice at 20 minute intervals during a 48h period, 12h/12 h light-dark cycle with lights on at 07:00.

Exercise Testing

At the conclusion of the study, mice were subjected to a maximal exercise test to exhaustion using a metabolic treadmill (Exer4-Oxymax; Columbus Instruments, Columbus, OH, USA). The test consisted of running on a 10-degree uphill gradient at 11 m/min for 5 min prior to an increase in speed by 2 m/min every 2 min. The experimenter was blinded to the mouse genotype during testing. Volitional exhaustion was defined as spending 10 seconds or more on the shock bar without attempting to re-engage the treadmill. Total work performed during the exercise test was calculated as the product of cumulative distance run (meters) and body weight (kg).

Whole muscle homogenization

Approximately 20 mg of *tibialis anterior* muscle was homogenized in potassium

phosphate buffer (50 mM K_2HPO_4/KH_2PO_4, 1 mM EDTA, 0.1 mM DTT, pH 7.4) supplemented with protease inhibitors (Roche) using an electric homogenizer (Pro Scientific). Insoluble proteins were removed by differential centrifugation at 700×*g*. A portion of the supernatant was supplemented with phosphatase inhibitors (Roche) for use in immunoblotting and both aliquots were stored at -86^{o}C until use. Protein concentration was determined in homogenates with the bichinconic acid method as per the manufacturer's recommendations (Pierce) with a spectrophotometer (Benchmark Plus, Biorad).

Mitochondrial Isolations

Mitochondrial fractions were prepared using differential centrifugation. Briefly, ~150 mg of freshly dissected skeletal muscle (*quadriceps femoris*) was homogenized on ice in 1:10 (wt/vol) ice-cold isolation buffer A (10 mM sucrose, 10 mM Tris/HCl, 50 mM KCl, and 1 mM EDTA, and 0.2% fatty acid free BSA, pH 7.4, supplemented with protease inhibitor cocktail (Roche)) using an electric homogenizer (Pro Scientific). The homogenates were then centrifuged for 15 min at 700×*g* and the resulting supernatants were centrifuged for 20 min at 12,000×*g* to pellet mitochondria. All centrifugation steps were carried out at 4°C. Mitochondrial pellets were resuspended in potassium phosphate buffer (50 mM K_2HPO_4/KH_2PO_4, 1 mM EDTA, 0.1 mM DTT, pH 7.4) plus protease inhibitors (Roche) and, following determination of protein concentration, were used for mtDNA isolation, SDS-PAGE or co-immunoprecipitations.

SDS-PAGE

Equivalent amounts of protein from each sample were run on acrylamide gels with molecular weight standards, transferred to nitrocellulose and developed using appropriate primary and secondary antibodies. Proteins were separated at 120 volts for approximately 2 hours and then transferred at 110 volts for 1 hour to nitrocellulose membranes (GE Healthcare). Membranes were blocked with milk or bovine serum albumin diluted in tris-buffered saline with tween (TBS-T) for 1 hour and then incubated in primary antibody overnight at 4°C. Antibodies and their dilution used with whole muscle or isolated mitochondria immunoblots are as follows: Anti-Tfam was from Santa Cruz (sc-23588, 1:800). The MitoProfile total OXPHOS antibody (ab110413, 1:1000), anti-nitro tyrosine (ab7048, 1:1000), 4HNE (ab48506, 1:1000), anti-Sod2 (ab13534, 1:3000), anti-Ogg1 (ab204, 1:1000) and anti-UCP3 (ab3477, 1:3000) were from Abcam. Anti-VDAC #4661, 1:3000 was from Cell Signaling. Anti-ANT1 (MSA02, 1:1000) antibody was acquired from MitoSciences. For determination of protein carbonyls, samples were derivatized and detected using a kit from Millipore (Oxyblot). After primary incubation, all blots were washed 3 times in TBS-T and incubated in respective anti-mouse, anti-rabbit or anti-goat secondary (1:10000; GE Healthcare) antibodies at room temperature for 1 hour. Subsequently, membranes were developed with ECL plus (GE Healthcare) and exposed to x-ray film (GE Healthcare). All films were digitized and band density was determined using ImageJ (NIH).

Enzyme activities

All enzyme activities were assessed on homogenates that contained protease

inhibitors only. Cytochrome c oxidase activity was assayed as described (Parise, Brose and Tarnopolsky, 2005), while citrate synthase and complex I + III activity was determined as described (McKenzie et al., 2000) with minor modifications to CI+III: spectrophotometer absorbance was monitored using a continual read for 2 minutes with auto correction for rotenone insensitive reactions using a reference cell.

Total Antioxidant Capacity (TAC)

Isolated mitochondrial fractions were analyzed for TAC using an assay based on the decolorizing of a solution of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cations ($ABTS^{\cdot+}$) by antioxidant solutions as described (Walker and Everette, 2009). All results are expressed as mM Trolox equivalents per mg protein (mM TE/mg protein).

RNA isolation and mRNA analysis

RNA was isolated, reverse transcribed to cDNA and gene expression analyzed by qPCR as described (Ogborn et al., 2012). Primers for mouse *β2-microglobulin* and *TFAM* are as follows: *β2-microglobulin*, forward: cccgcctcacattgaaat; *β2-microglobulin*, reverse: gaaagaccagtccttgctgaa; *TFAM,* forward: aacaggacatggaaagcagat; *TFAM*, reverse: gaagggaatgggaaaggtaga.

Mitochondrial 8-OH-2dG

mtDNA was isolated from resuspended mitochondria using the QIAamp DNA mini kit (Qiagen). DNA concentration was determined using PicoGreen dye and a lambda DNA standard curve (Invitrogen), followed by digestion to single bases using a one-step Benzonase protocol as described (Quinlivan and Gregory, 2008). Digested bases

were analyzed for 8-hydroxy-2-deoxyguanosine (8-OH-dG) in duplicate using a commercially available kit (Cayman Chemical) and the amount of 8-OH-dG present in each sample was normalized to initial mtDNA content.

mtDNA Copy Number Analysis

Total DNA was isolated from 20 mg of *tibialis anterior* muscle using the QIAamp DNA mini kit (Qiagen). The abundance of a mitochondrial DNA gene (COXII) relative to a nuclear gene (β–globin) was determined using quantitative real-time PCR according to the $2^{-\Delta Ct}$ method. Primer sequences are as follows: COXII, forward: gccgactaaatcaagcaaca, reverse: caatgggcataaagctatgg; β–globin, forward: gaagcgattctagggagcag, reverse: ggagcagcgattctgagtaga.

Muscle Fiber Permeabilization and Mitochondrial Respiration

A portion of freshly isolated *quadriceps femoris* muscle was placed into ice-cold permeabilization solution (10 mM Ca^{2+}/EGTA buffer, 0.1 μM free calcium, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$, 20 mM Taurine, 15 mM Na_2Phosphocreatine, 20 mM Imidazole, 0.5 mM DTT, and 50 mM MES; BIOPS) to isolate fiber bundles as described (Boushel et al., 2007). Muscle was carefully dissected free of any fat and connective tissue and separated into bundles under a dissection microscope with fine forceps for 20 minutes. Fibers were then collected, immersed in fresh BIOPS solution supplemented with saponin (50 μg/ml) and incubated at 4°C on a rotator for 30 mins. Bundles were then washed twice in respiration buffer (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1 g/L BSA (fatty acid free), pH 7.1) for 5 mins on a rotator at 4°C to remove any residual

permeabilization solution. Permeabilized fiber bundles were blotted dry, weighed (1-2 mg) and transferred to a high-resolution respirometer (Oroboros Instruments) containing air saturated respiration buffer at 37°C and the chamber was closed. Standardized calibrations to correct for background oxygen flux were completed prior to performing the experimental procedures. All experiments were performed in duplicate, simultaneously.

Resting, routine respiration without adenylates was obtained by the addition of 10 mM glutamate and 2 mM malate as substrates of complex I. This was followed by injection of 2.5 mM ADP to assess oxidative phosphorylation through complex I. The subsequent addition of succinate (10 mM) provided the measurement of convergent electron flux through complexes I and II. Mitochondrial outer membrane intactness was tested by the addition of 10 μM cytochrome c; no change in respiration was observed in our preparations in the presence of cytochrome c. Experiments were performed at oxygen concentrations greater than 80 μM to prevent diffusion limitations present in permeabilized fibers. Measurements were acquired using steady state regions of oxygen flux following substrate addition.

Co-immunoprecipitation

Isolated mitochondria lysates (200 μg) were solubilized using 1% n-dodecyl maltoside prior to performing immunoprecipitations and stored at -80°C. Subsequently, 20 μl of protein A/G plus agarose beads was added to spin columns (Pierce), washed twice with PBS, washed twice with 200 μl of wash buffer (PBS/0.05% n-dodecyl maltoside with protease inhibitors (Roche)), spun, and incubated with 2 μg of anti-TFAM

antibody (Santa Cruz, sc-23588) in PBS for 2 hours at 4°C to conjugate the antibody to the beads. After 2 additional washes in wash buffer, the solubilized mitochondrial lysate was added to the columns and allowed to rotate overnight at 4°C. The following morning, the columns were spun to collect the proteins not bound to the beads (IP supernatant). This fraction did not contain any detectable TFAM on western blots, indicating that all of the TFAM protein had been depleted from the original sample (Figure S3A). After collecting the supernatant, the beads were washed twice with 200 µl of wash buffer, plugged, and incubated with 50 µl of 1% SDS and spun to elute any immunoprecipitated proteins. A second elution produced no detectable TFAM protein from the beads, indicating complete extraction. All spins were done at 3,000 rpm for 1 minute at 4°C. The immunoprecipitates were run on gels using SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed using anti-TFAM (Cell Signaling #7945), anti-GRP75 (Abcam, ab2799) and anti-HSP60 (Abcam, ab59457) to detect the amount of HSP60 and GRP75 associated with TFAM according to the SDS-PAGE methods. No signal was present when using an IgG control antibody for immunoprecipitations (Figure S3B). Additionally, only TFAM was detected in sample that had been heat denatured at 95°C prior to performing the TFAM immunoprecipitation, indicating that the HSP60 and GRP75 protein detected in the immunoprecipitates was due to a specific protein-protein interaction with TFAM (Figure S3C).

2-dimensional Blue Native PAGE

Mitochondrial lysates (50 µg) were sedimented by centrifugation at 19,200 ×*g* for 10 minutes at 4°C. After removing the supernatant, mitochondria were solubilized in 40µl of 0.75 M aminocaproic acid/0.05 M Bis-tris and xul of 10% n-dodecyl lauryl maltoside as described (Schägger and von Jagow, 1991). Prior to gel loading, 2.5 µl of 0.05% coomasie blue/1M aminocaproic acid was added to each sample. Samples and a native protein standard ladder (Invitrogen) were loaded onto a single 10% first dimension native acrylamide gel with an empty lane separating samples and run overnight at 100 volts at 4°C. After the gel run, sample lanes were excised from the gel, soaked in denaturing buffer (10% glycerol, 2% SDS, 50 mM Tris (pH 6.8), 50 mM DTT, and 0.002% Bromophenol blue) turned 90 degrees and placed in an open well at the top of a 12.5% second dimension denaturing gel. The native ladder was soaked in coomasie blue and destained until bands were visualized in order to identify the extent of native mitochondrial complex migration. A pre-stained molecular weight ladder was also run in the second dimension SDS-PAGE gel. Blots were transferred to nitrocellulose membranes at 110 volts for 1 hour and then blocked in 5% milk for 3 hours. Subsequently membranes were incubated in a mitochondrial cocktail primary antibody that recognizes a subunit of each of the 5 complexes of the electron transports chain (MitoProfile total OXPHOS antibody; ab110413, 1:1000 in 1% milk/TBS-T) overnight at 4°C. Complex 2 was not well visualized using this relatively low amount of protein and thus was not included in the results. Blots were then incubated in an anti-mouse HRP conjugated secondary antibody and visualized using ECL.

Statistical Analyses

Groups were compared using ANOVA. Significance was accepted as $p < 0.05$).

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FIGURE LEGENDS

Figure 1. Elevated mitochondrial oxidative stress impairs exercise performance resulting from exercise training. Mice were tested for exercise capacity on a treadmill at a 10-degree angle after 4 months of exercise training or remaining sedentary as stated in the Experimental Procedures. (A) Total distance run, (B) VO_{2max}, and (C) total work performed during an exercise test to exhaustion. (D) Correlations between VO_{2max} and total distance for each genotype. Data are mean±SE. N = 7-8. *Indicates a significant difference ($p < 0.05$) from the indicated group as determined by ANOVA. †Indicates a significant correlation using Pearson's regression. NS, non-significant.

Figure 2. Mitochondrial function is compromised with exercise training in Sod2+/- mice without influencing organelle biogenesis. Total protein lysates of *tibialis anterior* muscle were probed by immunoblotting for individual complex subunits (I: NDUFB8, II: SDHB, III: UQCRC2, IV: COXI, V: ATP5A) or assayed for enzyme activity. (A) The protein expression of mitochondrial proteins determined by densitometry relative to LDHA, (B)

representative immunoblots of mitochondrial proteins and (C) enzyme activities of citrate synthase (CS), complex I+III, and complex IV (CIV) per unit of protein (N = 8-10). Data are expressed relative to the Sod2+/+ SED group. Mitochondrial respiration rates in saponin permeabilized muscle fibers from *quadriceps femoris* muscle using (D) glutamate and malate without adenylates to indicate a respiratory leak state, (E) glutamate, malate, succinate and ADP to measure maximal coupled oxidative phosphorylation capacity and (F) respiratory coupling ratio of ADP stimulated and non-stimulated respiration using glutamate and malate as substrates (N = 4-5). (G) UCP3 and (H) ANT1 protein expression in isolated mitochondrial lysates from *quadriceps femoris* muscle relative to VDAC and (I) representative immunoblots for each group (N= 8-10). All data are mean±SE. *Indicates a significant difference ($p < 0.05$) from the indicated group as determined by ANOVA. NS, non-significant.

Figure 3. Mitochondrial oxidative stress persists with exercise training in Sod2+/- mice. Total protein lysates were prepared from *tibialis anterior* muscle and isolated mitochondrial lysates from *quadriceps femoris* muscle (A) Sod2 protein in total lysates relative to LDHA and (B) representative immunoblots. (B) Non-enzymatic anti-oxidant capacity of mitochondrial lysates per amount of protein measured expressed as Trolox Equivalents. (D) Quantification of mitochondrial nitro-tyrosine and (E) representative immunoblots. (F) Levels of the oxidative lesion 8-hydroxy-2-deoxyguanosine in isolated mitochondrial DNA digested to single bases. (G) the DNA repair enzyme Ogg1 in isolated mitochondrial lysates relative to VDAC and (E) representative immunoblots. N = 8-10 per group for all measurements. All data are mean±SE. *Indicates a significant

difference ($p < 0.05$) from the indicated group(s) as determined by ANOVA.

Figure 4. mtDNA transcription is disrupted by oxidative stress due to impaired TFAM function. (A) Mitochondrial DNA copy number in *tibialis anterior* muscle relative to copies of genomic DNA (N = 8-10). (B) *TFAM* mRNA expression using *β2-microglobulin* as the housekeeping gene in *quadriceps femoris* muscle, (C) TFAM protein content relative to LDHA in *tibialis anterior* muscle and (D) representative immunoblots (N = 8-10). (E) Copies of mtDNA expressed relative to TFAM protein (N = 8-10). (F) GRP75 and (G) HSP60 co-immunoprecipitated with TFAM in isolated mitochondria and (H) representative immunoblots for each group (N = 3). All data are mean±SE. *Indicates a significant difference ($p < 0.05$) from the indicated group(s) as determined by ANOVA. NS, non-significant.

Figure 5. Mitochondrial complexes are poorly assembled in the presence of oxidative damage. Lauryl-maltoside solubilized mitochondria were subjected to two-dimensional Blue-Native PAGE in order to visualize native species migration of each of four electron transport chain complexes. Samples were run simultaneously on the same native gel. Image frames are identically sized for each complex and were acquired from the same exposure. Note the divergent response to exercise between the two genotypes.

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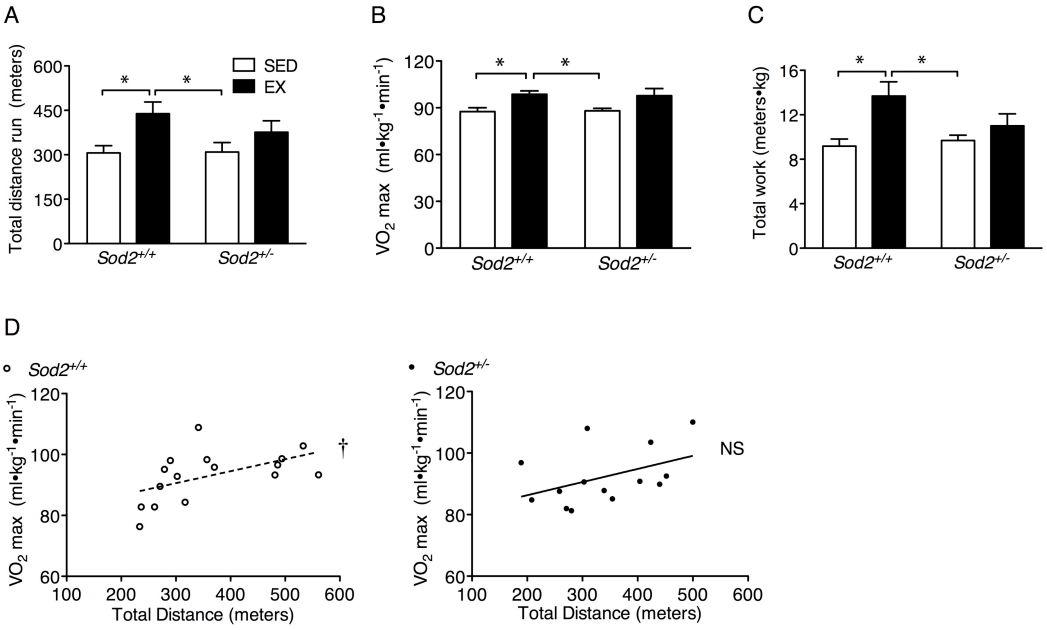
Figure 1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D
SED
EX
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Total work (meters•kg)
Sod2+/+
Sod2+/-
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†
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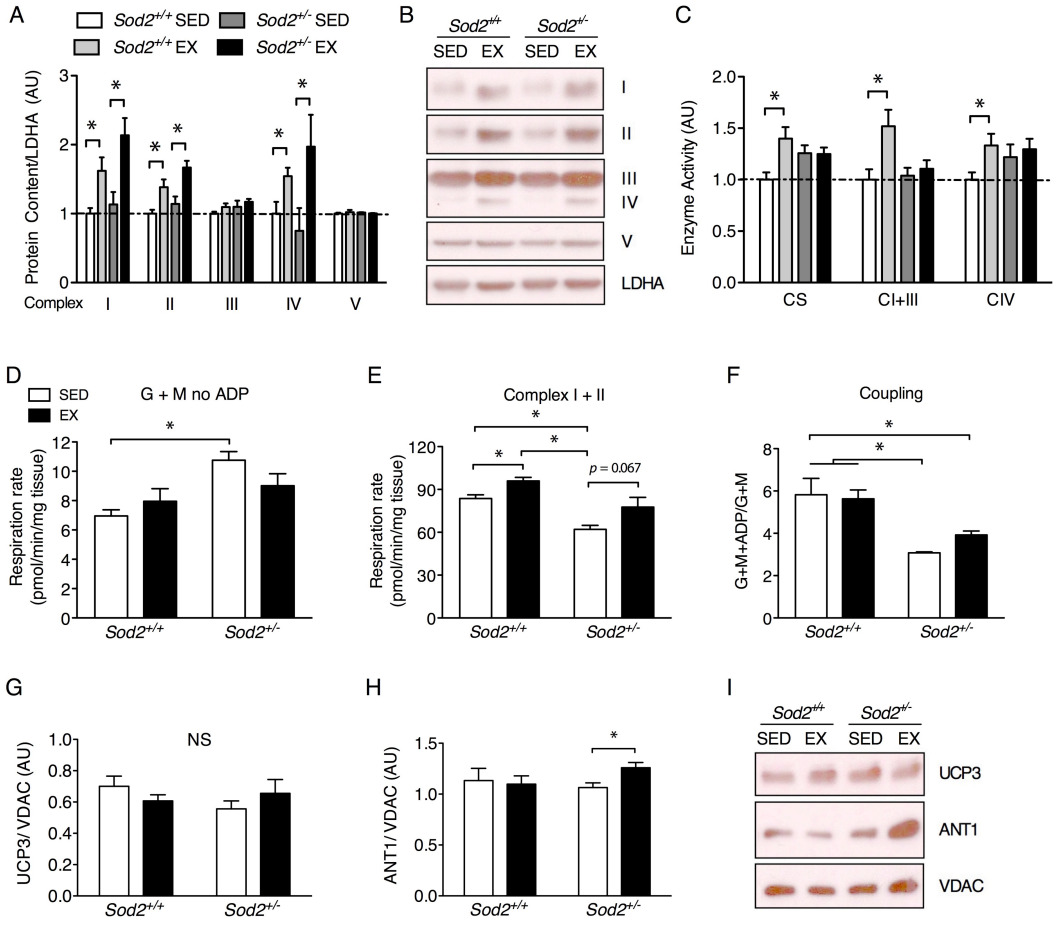
Total Distance (meters)

Figure 2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II
III
IV
V
LDHA
C
Enzyme Activity (AU)
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CI+III
CIV
D
SED
EX
G + M no ADP
Respiration rate (pmol/min/mg tissue)
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Sod2+/-
E
Complex I + II
p = 0.067
Respiration rate (pmol/min/mg tissue)
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Coupling
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UCP3/ VDAC (AU)
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ANT1/ VDAC (AU)
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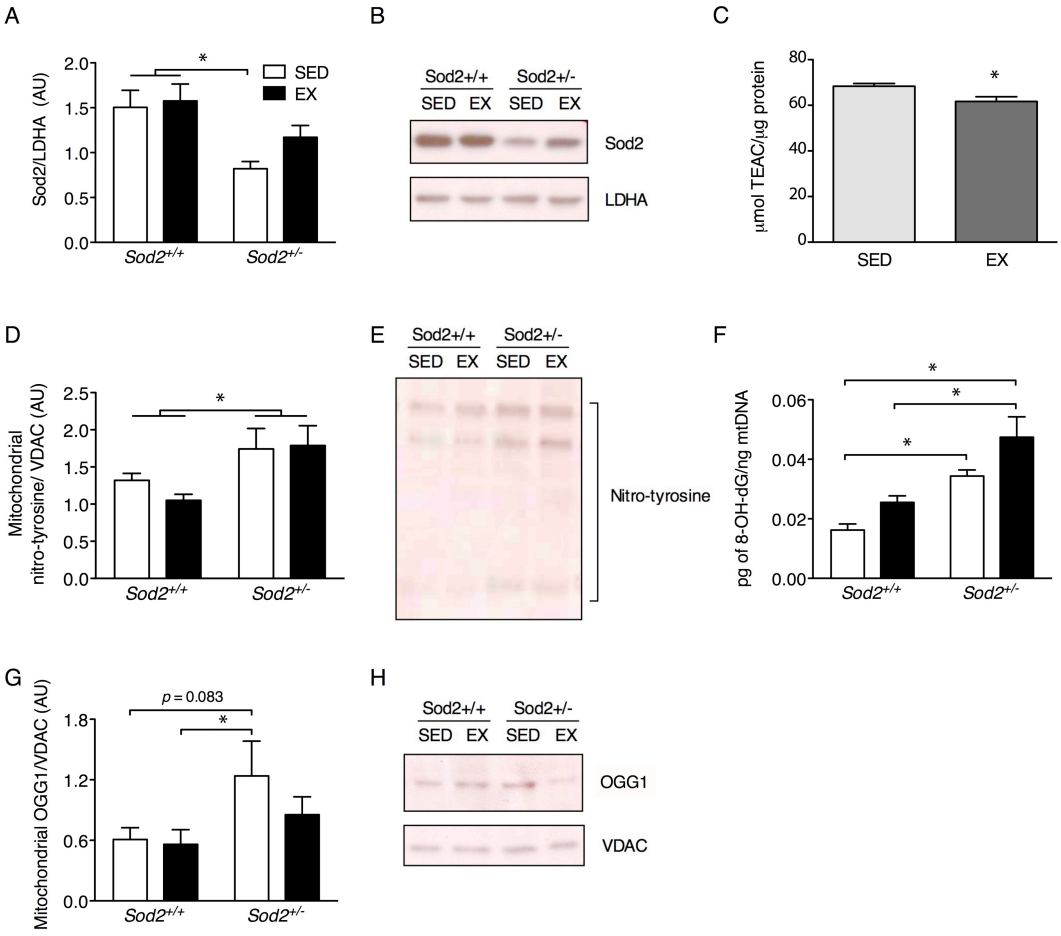
UCP3
ANT1
VDAC

Figure 3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LDHA
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D
E
F
Mitochondrial nitro-tyrosine/ VDAC (AU)
Nitro-tyrosine
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H
p = 0.083
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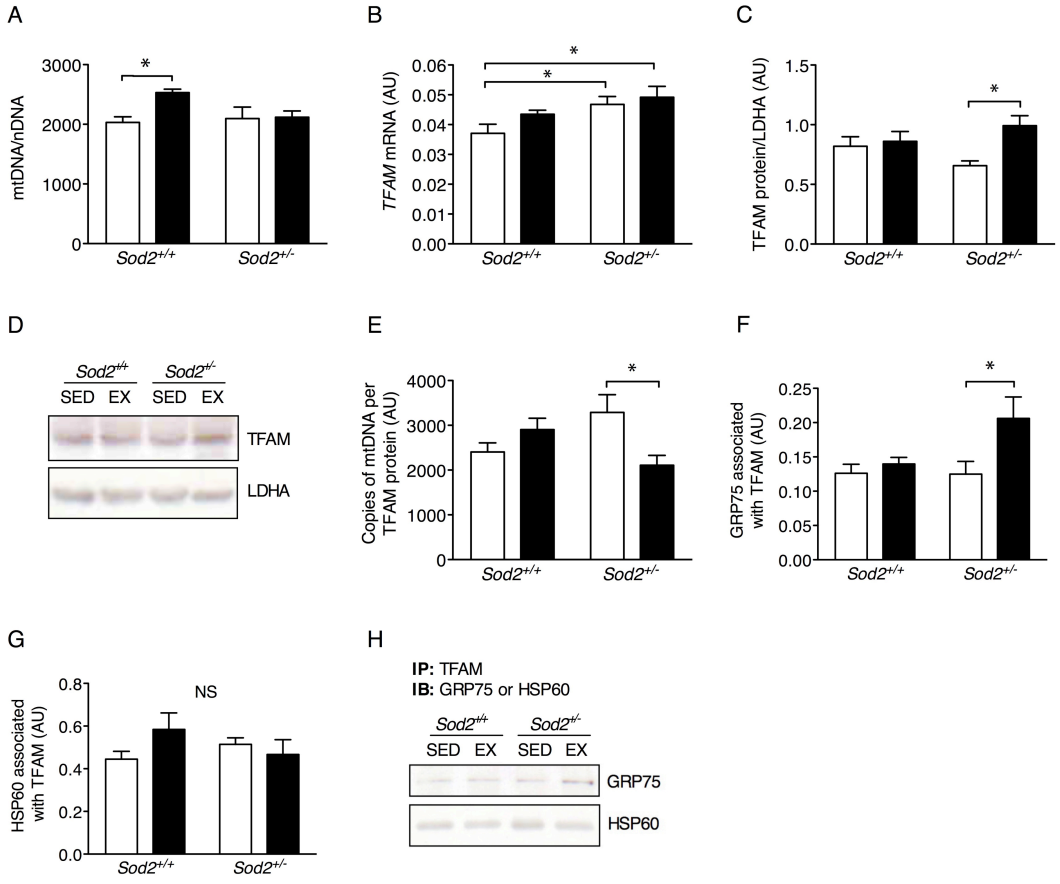
OGG1
VDAC

Figure 4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0.02
0.01
0.00
*
*
Sod2+/+
Sod2+/-
C
TFAM protein/LDHA (AU)
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D
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TFAM
LDHA
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Copies of mtDNA per TFAM protein (AU)
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3000
2000
1000
0
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Sod2+/-
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GRP75
HSP60

Figure 5

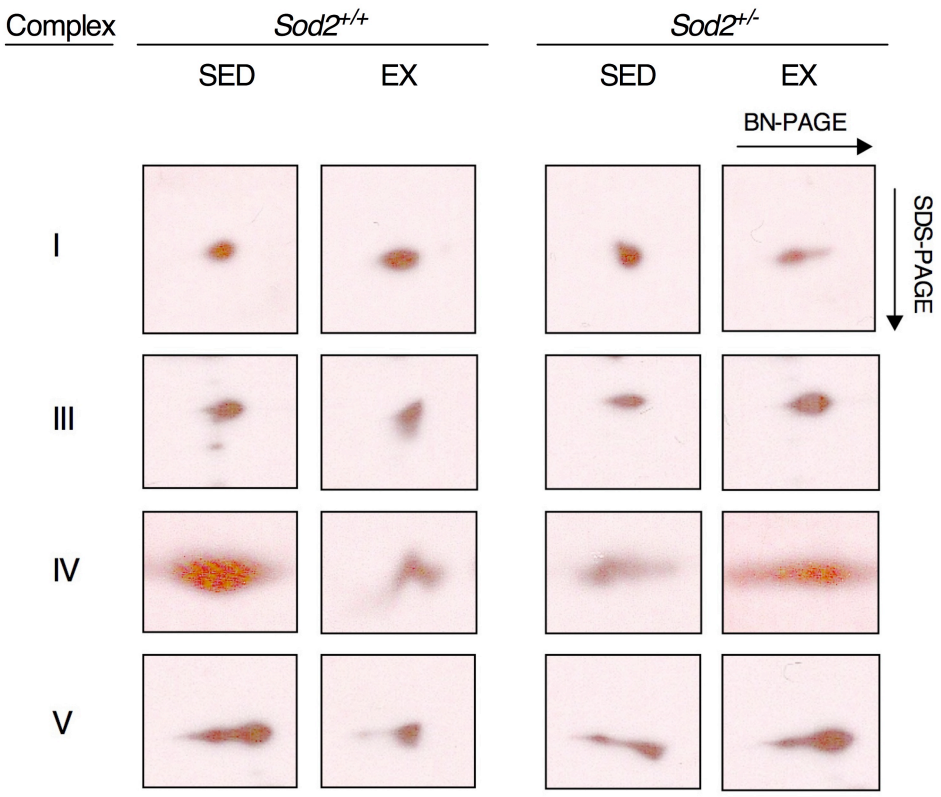

Fig. S1

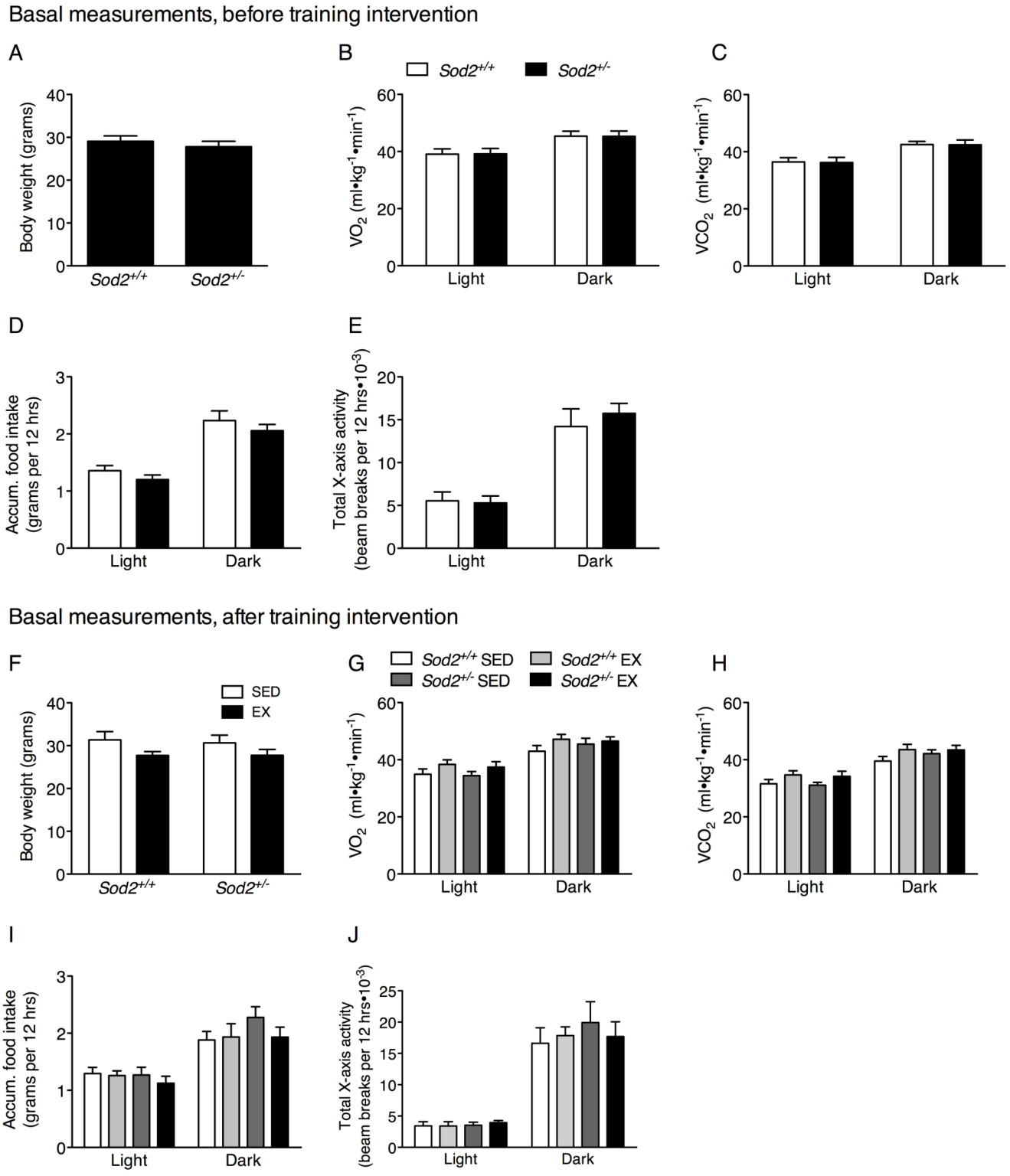

Figure S1. Metabolic and behavioural measurements before and after separation into study groups. Initial measurements of (A) body weight, (B) VO_2, (C) VCO_2, (D) total food intake and (E) X-axis ambulatory activity during light and dark cycles in *Sod2*$^{+/+}$ and *Sod2*$^{+/-}$ mice. Measurements in the same mice after separation into groups of (F) body weight (G) VO_2, (H) VCO_2, (I) total food intake and (J) X-axis ambulatory activity. Data are mean±SE and contain no significant differences between groups.

Fig. S2

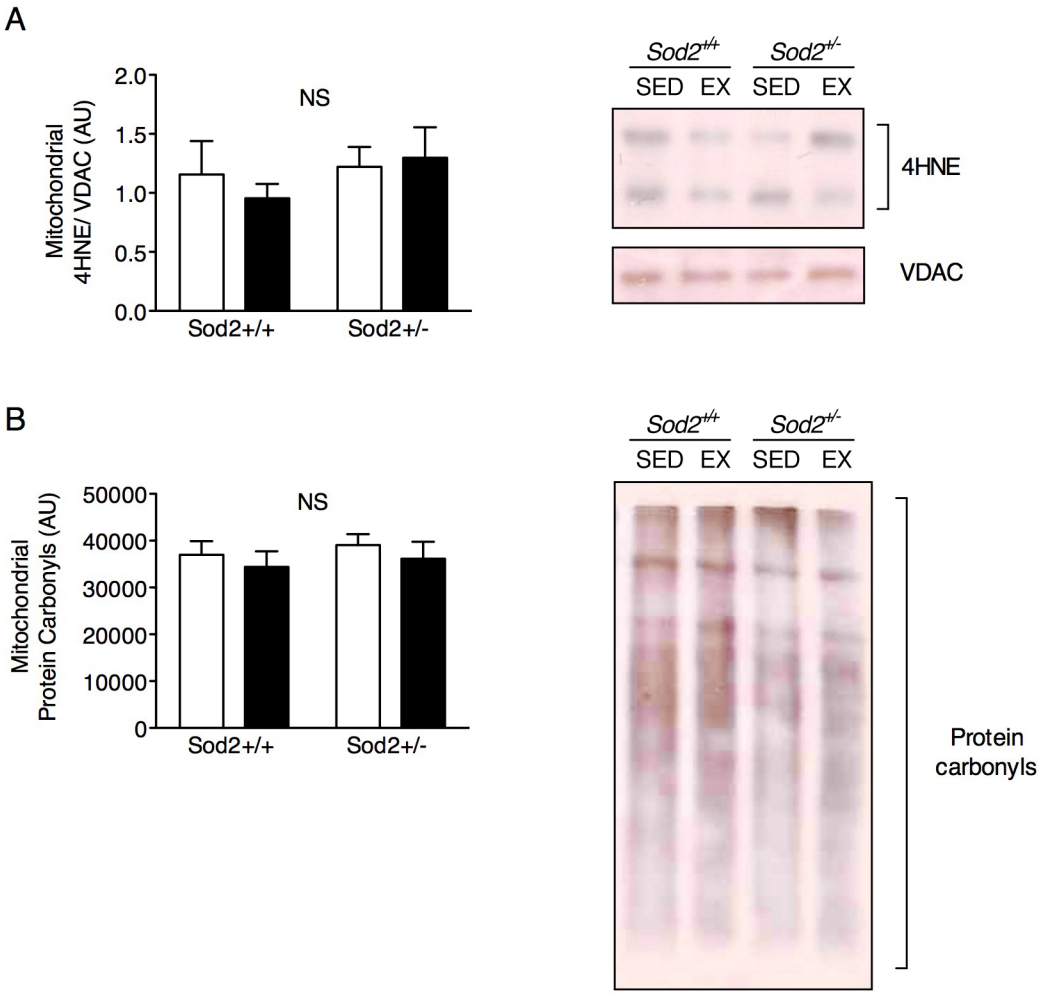

Figure S2. Non-significant mitochondrial oxidative damage analysis. Densitometry and immunoblots of (A) 4-Hydroxynonenal (4HNE) normalized to VDAC and (B) protein carbonyls in isolated mitochondria from *quadriceps femoris* muscle in *Sod2*$^{+/+}$ and *Sod2*$^{+/-}$ SED and EX mice. Data are mean±SE. NS, non-significant.

Fig. S3

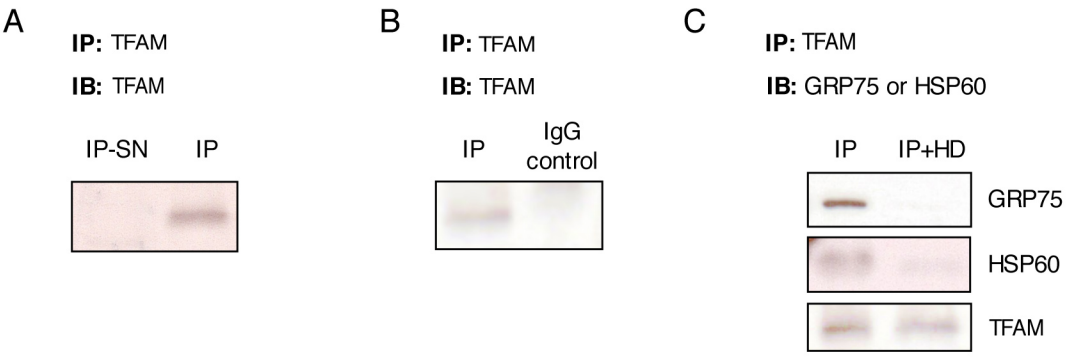

Figure S3. Immunoprecipitation (IP) control experiments. (A) The extent of TFAM depletion from mitochondrial lysate by comparing the sample after IP (IP-SN) with the pulled-down sample in terms of TFAM content. (B) Immunoprecipitation with anti-TFAM antibody or IgG control antibody to confirm that no non-specific interactions occurred. (C) Normal Immunoprecipication of TFAM using normal conditions (IP) and with heat-denaturing of the sample at 95°C for 5 minutes prior to immunoprecipitation (IP+HD) to confirm that the signal on immunoblots was due to a specific protein-protein interaction with TFAM.

CHAPTER 4: MANUSCRIPT 3

Long-term Aerobic Exercise is Associated with Greater Muscle Strength Throughout the Lifespan

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TITLE: Long-term aerobic exercise is associated with greater muscle strength throughout the lifespan

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RUNNING TITLE: Aerobic exercise and muscle strength

ABSTRACT

Aging is associated with a progressive decline in muscle strength, muscle mass and aerobic capacity which reduces mobility and impairs quality of life in elderly adults. Exercise is commonly employed to improve muscle function in individuals of all ages, however chronic aerobic exercise is believed to largely impact cardiovascular function and oxidative metabolism, with minimal effects on muscle mass and strength. To study the effects of long-term aerobic exercise on muscle strength, we recruited 74 sedentary (SED) or highly aerobically active (ACT) men and women from within three distinct age groups (young: 20-39 y, middle: 40-64 y, and older: 65-86 y) and tested their aerobic capacity, isometric grip and knee extensor strength, and dynamic 1 repetition maximum (1RM) knee extension. As expected, ACT subjects had greater maximal oxygen uptake and peak aerobic power output compared to SED ($p < 0.05$). Grip strength relative to body weight declined with age ($p < 0.05$) and was greater in ACT compared to SED subjects in both hands ($p < 0.05$). Similarly, relative maximal isometric knee extension torque declined with age ($p < 0.05$) and was higher in ACT vs. SED individuals in both legs ($p < 0.05$). Absolute and relative 1RM knee extension declined with age ($p < 0.05$) and was greater in ACT vs. SED groups ($p < 0.05$). Knee extensor strength was associated with a greater amount of leg lean mass in the ACT subjects ($p < 0.05$). In summary, long-term aerobic exercise appears to attenuate age-related reductions in muscle strength in addition to its cardiorespiratory and metabolic benefits.

INTRODUCTION

A loss of muscle strength and function is a defining feature of aging that contributes to frailty and disability in elderly adults (1). Muscle mass begins to decline at approximately age 25 (2), and is accompanied by similar reductions in maximal dynamic and isometric force of the upper and lower limbs (3-5). These strength changes compromise several important daily tasks, such as opening jars, holding rails, rising from a chair, or climbing stairs. At the same time, aerobic capacity (VO_{2max}) is reduced by about 9% per decade of life in inactive adults (6) such that a loss of strength is compounded with increasing fatigability. Thus, suitable interventions that can simultaneously target all of these detriments are necessary in order to promote health and well being in an aging population.

Exercise is frequently recommended as a means to improve muscle function. A common paradigm in exercise prescription is that short-term resistance exercise increases muscle size and strength, whereas aerobic exercise primarily improves oxidative capacity and cardiovascular function with minimal overlap between the modes (7). Typically, strength trained individuals have relatively high muscle mass and force production, but a similar VO_{2max} compared to their sedentary peers (8). Conversely, aerobic athletes (runners and orienteerers) have relatively high aerobic power production and maximal oxygen uptake, but similar muscle mass and strength to inactive subjects (8-12), despite having larger muscle fibers (13-15). Short-term cycling (16) and treadmill running/walking (7,17) in previously sedentary elderly adults increases dynamic knee extensor muscle strength, although it is unclear why older aerobic athletes do not exhibit an attenuation of strength and muscle mass due to long-term aerobic exercise participation. It is possible that these adaptations may be unique to formerly inactive older adults and there is a lack

of evidence demonstrating that these same benefits would be present in individuals of all ages that chronically perform aerobic exercise.

In addition to the loss of muscle strength, an accelerated onset of fatigue is typically observed in older adults. Aged individuals complete fewer cycles of repetitive tasks and exert a greater relative effort when performing activities of daily living (18). Somewhat paradoxically, older adults exhibit higher muscular fatigue resistance relative to young individuals in both upper (19,20) and lower limbs (21-24) presumably due to a fiber type shift in their muscle favoring more oxidative, type 1 fibers. Notably, this shift does not appear to counter an age-related decline in aerobic power production and suggests that the fatigability in the elderly may be largely due to their lower cardiovascular fitness and not muscle specific mechanisms. Therefore strength and aerobic capacity, but not muscle fatigue, appear to be the primary determinants of disability and immobility in older adults. Aerobic exercise may simultaneously address these issues when performed on a regular basis, but thus far this phenomenon has not been described.

Studies involving highly competitive athletes may not be an ideal characterization in order to identify optimal training interventions for the general public as these individuals may have genetic physiologic advantages that muddle conclusions regarding the natural aging process. It is more relevant to contrast individuals that are recruited according to their physical activity level rather than their performance, competitiveness or standing in a given sport. Therefore, we compared strength and aerobic capacity in two groups of individuals across the lifespan with very distinct physical activity patterns: one that frequently engaged in intense aerobic exercise and a largely sedentary cohort that served as age- and gender-matched controls. We chose grip and knee extensor strength as our primary outcomes since these measurements are

considered highly predictive of physical function and potential disability in the elderly (1,3,25,26).

METHODS

Seventy-four (42 men, 32 women) individuals were recruited from the southern Ontario region according to two distinct habitual physical activity levels and were gender and age-matched across activity groups for comparison. Participants in the active group (ACT) regularly performed 4 hours or more of moderate to vigorous endurance exercise (cycling, running, cross-country skiing, orienteering, in-line skating, rowing or swimming) per week and had done so for at least the past 10 years. An exception was made for those under the age of 25, such that they had to have at least 5 years of aerobic training history. Those that engaged in running exercise did so in combination with another sport at least half of their training time. In contrast, participants in the sedentary group (SED) engaged in no more than 60 minutes of moderate to vigorous physical activity per week and had not exceeded this level of activity for at least the last 10 years. No limitations were placed on the amount of low intensity exercise (walking, yoga, stretching) within each group. Each group was further divided into three distinct age groups: young- 20-39 y, middle- 40-64 y, older- 65-86 y. Additionally, the active group was subdivided into lower body (running, cycling, in-line skating) and whole body (swimming, triathlon, x-c skiing, rowing) exercise groups and compared to one another in order to an assess the effect of recruited vs. non-recruited muscles on the functional measures.

Criteria for exclusion from the study included any evidence of one or more of the following: coronary heart disease, congestive heart failure, chronic obstructive pulmonary disease, diabetes mellitus, major orthopedic disability, renal failure, smoking or any condition that impaired ones ability to perform a single session of aerobic exercise. Additionally, individuals could regularly consume one blood pressure medication and/or one cholesterol medication to be considered for the study. This study was approved by the McMaster University Research Ethics Board (REB# 11-114).

Study Procedures

Prior to their arrival for the study trial, subjects completed a self-reported 3-day diet record typical of their habitual diet (1 weekend day and 2 weekdays), a physical activity history to characterize their past exercise patterns, the Baecke physical activity questionnaire (27), and signed a consent form that described the study procedures. Subjects were asked to adhere to the following requests prior to each visit: abstain from moderate to intense exercise for 48h, abstain from caffeine for 12h and to eat according to their normal diet for 48h (except for the morning of). Participants were allowed to continue to consume a multivitamin and vitamin D if they normally did so leading up to the trial, but any other antioxidant supplements were stopped at least 2 weeks prior to the study date. Fat mass, lean mass, and bone density were determined using a whole-body dual energy x-ray absorptiometry (DEXA) scan.

Exercise Testing

On the day of the study trial, subjects arrived in the morning following an overnight fast (starting at 2200h the prior evening). Quadriceps muscle strength was

assessed by measuring maximal isometric voluntary knee extension torque at 90 degrees, taking the highest value of three attempts for each leg (Biodex). 1-repetition maximum knee extension was ascertained using a traditional weight stack machine (Cybex Eagle) by incrementally increasing the weight until full knee extension could not be completed. Additionally, maximal grip strength was determined using a grip dynamometer (Jamar) for each hand using the highest value of three attempts. Peak aerobic capacity (VO_{2peak}) was ascertained by monitoring oxygen uptake during an incremental cycling test on an electronically braked cycle ergometer (Lode) until 2 of 3 criteria were met for peak oxygen uptake: (1) RER $\geq$ 1.15, (2) a plateau in oxygen consumption in two consecutive thirty second measurement intervals and (3) a maximal heat rate >90% of age-predicted (220-age). Initially ergometer resistance was set at 30 watts and increased by 15 watts per minute in the sedentary group; alternatively, resistance was initially set at 100 (women) or 150 (men) watts and increased by 30 watts per minute when testing the active group.

Statistics

All data were initially compared using a two-way ANOVA using age (young, middle, or older) and physical activity level (SED or ACT) as variables (Statistica 4). Significance was set at $p < 0.05$. When significance was attained, the pertinent effect was assessed using Tukey's Honest Significant Difference post-hoc analysis. Associations between variables were assessed using Pearson's Correlation. Functional data from the active group that was subdivided into lower body and whole body groups was compared within each age group using an unpaired *t-test* with significance set at $p < 0.05$. All values are reported as mean±*SE*.

RESULTS

Subject Characteristics and Diet Composition

Taken together, sedentary subjects were significantly heavier than active individuals and had greater total and relative fat mass and lower relative lean mass (Table 1, $p < 0.05$). The ACT group had higher leg lean mass ($p < 0.05$), but not arm lean mass ($p > 0.05$). There were no age-related differences in weight, height, body composition or bone density ($p > 0.05$). However, SED young and ACT middle-aged subjects had significantly lower total bone density than SED older individuals. ACT subjects ate significantly more daily calories than the SED group, due to higher fat and protein intake ($p < 0.05$), although carbohydrate intake was similar ($p > 0.05$).

Physical Activity Habits and Baecke Activity Index

At the time of testing, ACT subjects habitually performed a higher amount of moderate to vigorous activity compared to SED subjects (Table 2, $p < 0.05$), but there were no differences in light activity ($p > 0.05$). Similarly, during prior decades of life the ACT group consistently performed a greater amount of moderate-to-vigorous activity than the SED group ($p < 0.05$), while light activity was not different between groups for any prior decade ($p > 0.05$). The standardized activity index indicated that work activity significantly increased with age ($p < 0.05$), but there were no group differences ($p > 0.05$). The sport and leisure activity indices were greater in the ACT vs. SED subjects ($p < 0.05$), but did not differ with age ($p > 0.05$).

Aerobic Capacity and Muscle Strength

Peak oxygen uptake (VO_{2peak}) was significantly higher in ACT vs. SED individuals, regardless of age (Fig. 1A, $p < 0.05$). Similarly, absolute and relative (normalized to total body weight) peak aerobic power output was greater in ACT subjects compared to SED throughout the lifespan (Fig. 2B-C, $p < 0.05$). VO_{2peak}, as well as absolute and relative aerobic peak power declined with age (older lower than young and middle, $p < 0.05$). When comparing lower body (LB) and whole body (WB) modes of exercise, there were no differences with regard to VO_{2peak} or peak aerobic power (Fig. 2D-E, $p > 0.05$). However, the middle-aged LB had higher relative peak aerobic power than the WB group (Fig. 2F, $p < 0.05$).

Peak isometric grip strength of the dominant hand tended to be higher in the ACT group (Fig. 2A, $p = 0.063$), but no differences were seen in the non-dominant hand ($p > 0.05$). Absolute grip strength of both the dominant and non-dominant hands positively correlated with total arm lean mass ($r = 0.699$ and $r = 0.669$, respectively, $p < 0.05$). Relative grip strength of both the dominant and non-dominant hands was significantly greater in the ACT compared to the SED group (Fig. 2B, $p < 0.05$) and declined with age (middle vs. young, $p < 0.05$). Absolute peak isometric torque of the knee extensors tended to be greater in the ACT group in the dominant (Fig. 2D, $p = 0.099$) and non-dominant hand ($p = 0.078$). Relative voluntary peak isometric torque of the knee extensors in both the dominant and non-dominant leg was significantly higher in the ACT vs. SED group (Fig. 2E, $p < 0.05$) and declined with age (young vs. older, $p < 0.05$). Absolute and relative 1-repetition maximum (1RM) knee extension were higher in the ACT vs. SED group (Fig. 2G-H, $p < 0.05$) and were lower with advancing age (absolute:

young vs. older, $p < 0.05$; relative: young and middle vs. older, $p < 0.05$). There were no differences between LB and WB active adults with regard to absolute or relative grip strength, isometric knee extension or 1RM strength (relative data shown in Fig 2C, F and I respectively, $p > 0.05$). Absolute 1RM knee extension was positively correlated with leg lean mass ($r = 0.693$, $p < 0.05$).

DISCUSSION

Interventions that counter the age-related decline in physical function are an important consideration for promoting a high quality of life in older adults. Quality of life is largely reliant on mobility and daily task completion, both of which depend on adequate muscle strength. Several studies have shown no differences in strength between individuals that engage in aerobic exercise and similarly aged, sedentary subjects (8-12). In comparison to individuals with little to no life history of regular exercise, we demonstrate that long-term aerobically active individuals have higher absolute 1RM knee extension strength in addition to exhibiting greater aerobic capacity and aerobic power output commonly associated with habitual aerobic training.

The mobility of older adults is limited primarily by weight-bearing tasks such as rising from a chair or ascending stairs. Therefore, we chose to normalize strength and aerobic capacity to body weight as a better relative metric of functional strength. This analysis revealed that aerobically trained subjects had greater relative isometric grip and isometric and dynamic knee extensor strength than inactive individuals ranging from 20-86 years of age. Importantly, the strength characteristics that displayed significant differences between the ACT and SED groups were the only strength determinations that also significantly declined with age, confirming that

aerobic exercise is attenuating age-related detriments in muscle force production. Strength of the knee extensors is a strong predictor of fall incidence in the elderly (1,28), and grip and knee extensor strength normalized to body weight is an excellent predictor of physical function (25,29). Thus, the observed differences in muscle strength between the ACT and SED groups imply a considerable advantage for the prevention of falls and disability in older adults. While the higher muscle strength in young or middle-aged individuals might not have as profound a functional benefit as it would within older adults, mitigating muscle loss and strength at a younger age might extend the years of functional life considerably provided physical activity is kept constant. A limitation of the current study is that we do not know how long these adaptations take to manifest within muscle and it is possible that this plasticity may be altered with age.

The higher lean body mass in the active subjects from the current study seems to be driven primarily by the young and middle aged groups, although as a proportion lean mass was consistently higher in the active group regardless of age. While a decline in absolute muscle mass with age might be expected in the older age group, the prevalence of sarcopenia can vary widely between 1-34% depending on the specific definition of sarcopenia, the cohort studied, and the young reference population (30-33). Further, several studies report no change in lean mass with age in healthy, community dwelling individuals (34-36). Our careful subject recruitment sought to minimize potential confounding factors regarding age-related physical function in order to examine the impact of habitual exercise. Thus our sedentary cohort had an unusually low prevalence of medical complications and consumed fewer medications relative to the general elderly population (37). In this context, it is not surprising that this group did not

exhibit significantly lower lean mass with age compared to previous investigations. Additionally, since the sedentary older group weighed, on average, 9 to 14 kilograms more than either young group, their higher adiposity may be partially attenuating a loss of lean mass by increasing loading on the lower limbs. In agreement, a higher BMI (>30) has been associated with a lower prevalence of sarcopenia (38) and it has been shown that reduced strength, but not muscle mass, is associated with increased mortality in older adults (39). If the greater muscle strength in the older active group cannot be entirely attributed to changes in lean mass, it brings to question what might be causing the functional changes in muscle strength? Both habitual and short-term aerobic exercise training appear to improve single fiber power production predominantly by increasing the shortening velocity of skeletal muscle fibers (15,16), but aging does not influence this parameter (40). In contrast, resistance exercise seems to augment power production by increasing fiber force production via hypertrophy (41), although shortening velocity may also be altered (42). Thus aerobic exercise seems to promote unique adaptations beneficial for improving muscle strength beyond simply altering muscle size.

Previous studies comparing aerobically trained athletes have largely shown no benefits with regard to whole muscle strength (8-12), likely because these studies have predominantly focused on distance runners. Only one cross-sectional study utilized elderly competitive cross-country skiers in addition to runners and reported higher absolute and relative isometric strength of upper and lower body muscle groups compared to non-exercising individuals of the same age (43). Unfortunately, this investigation failed to demonstrate that absolute and relative isometric strength is lower with advanced age because it lacked a young cohort, making it difficult to conclude if chronic exercise is altering characteristics susceptible to aging. It is known that run

training reduces fiber diameter in young individuals following 13 weeks of training (44) and is associated with reduced single fiber peak power in Master's runners (45) possibly because running directs fiber adaptations towards efficiency and weight savings rather than hypertrophy and increased strength. The active individuals in the current study performed multiple modes of exercise and spent, at most, half of their exercise time running (in many instances active subjects did not run at all, e.g. cycling, rowing) and had appreciably greater knee extensor and grip strength. It seems sensible that running, given its high rate of injury and potential to mitigate strength adaptations, should not be exclusively recommended in order to derive the optimal benefits of aerobic exercise training.

In addition to the benefits to knee extensor strength, we observe higher amounts of grip strength within active individuals where upper limb utilization may appear to be minor to non-existent during exercise (runners, cyclists). However, cycling does regularly utilize the forearms via braking and engagement of the handlebars, which requires activation of the upper limb muscles. Even running requires relatively constant elbow flexion and shoulder activation during the course of a complete stride. While this degree of upper limb muscle activation might be lower than swimming or rowing in active adults, there is at least minor upper limb muscle activation for a considerable portion of their exercise training. Thus, the higher relative grip strength of the active subjects is likely derived from greater upper limb habitual activity. There may also be an influence of exercise-induced circulating factors that support muscle growth such as growth hormone (46). Moreover, reduced levels of inflammatory cytokines are typically observed in active older individuals (47), which may be advantageous for maintaining muscle contractile function in peripheral muscle groups with age.

The current investigation has implications for exercise prescription in individuals across the adult lifespan and counters the long-standing notion that chronic aerobic exercise does not augment muscle strength, particularly in young individuals. Since long-term aerobic exercise is also associated with improved glucose tolerance, insulin sensitivity and cardiovascular function (6,35,48) in addition to our findings regarding muscle strength, it represents an ideal mode for health promotion and rehabilitation.

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FIGURE LEGENDS

Figure 1. Aerobic capacity and power are higher in long-term aerobically active individuals. (A) VO_{2peak}, (B) peak power output and (C) relative peak power output attained during a graded cycling exercise test to volitional exhaustion in all groups. Comparisons of (D) VO_{2peak}, (E) peak power output and (F) relative peak power output in lower body (LB) and whole body (WB) exercisers within each age group. SED: sedentary control group; ACT: aerobically active group. Values are mean±*SE*. *Indicates a significant difference (p <0.05) from the indicated group. †Indicates a significant difference from young and middle age groups (p <0.05).

Figure 2. Aerobically active individuals are stronger than their sedentary peers regardless of age. (A) Absolute and (B) relative peak grip strength between SED and ACT groups across the lifespan. (C) Relative grip strength of lower body (LB) and whole body (WB) exercisers. (D) Absolute and (E) relative maximal isometric knee extension torque within activity groups. (F) Relative isometric knee extension of LB and WB exercisers. (G) Absolute and (H) relative 1RM knee extension in SED and ACT age groups. (I) Relative 1RM knee extension in LB and WB exercisers. SED: sedentary control group; ACT: aerobically active subject. Y: young, M: middle, O: older. Values are mean±*SE*. †Indicates a significant age effect between the indicated groups (p <0.05). ‡Indicates a significant activity group effect between the SED and ACT subjects (p <0.05).

Fig. 1

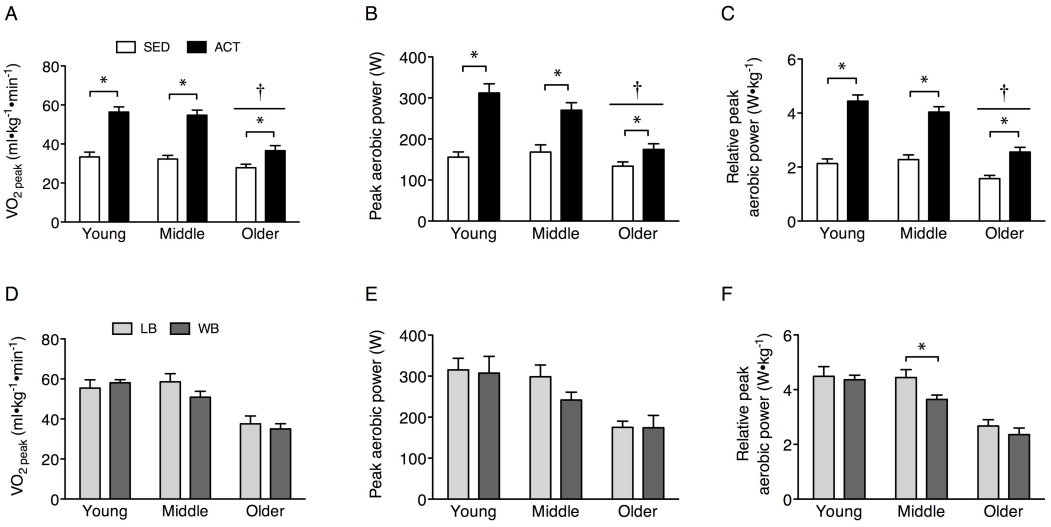
A
SED
ACT
$VO_{2\,peak}$ (ml•kg^{-1}•min^{-1})
Young
Middle
Older
B
Peak aerobic power (W)
C
Relative peak aerobic power (W•kg^{-1})
D
LB
WB
E
F

Fig. 2

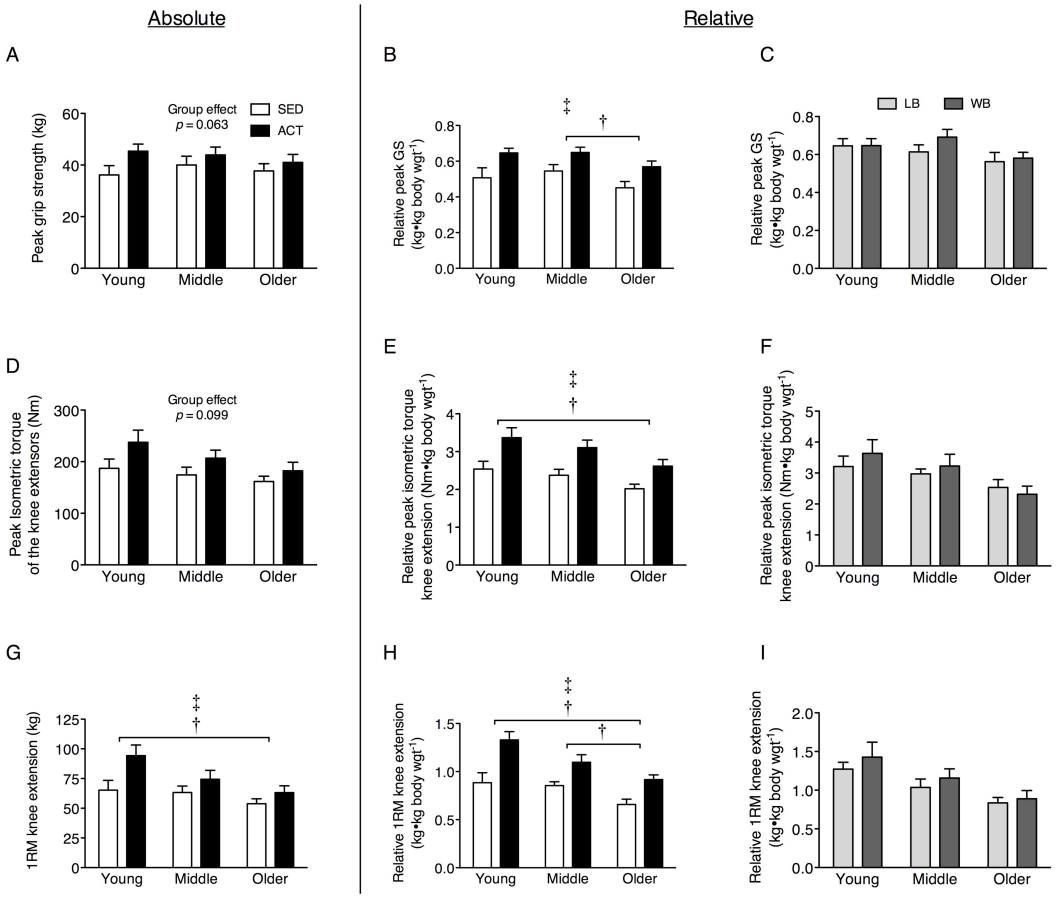
Absolute
Relative
A
B
C
D
E
F
G
H
I
Peak grip strength (kg)
Group effect
p = 0.063
SED
ACT
Relative peak GS (kg•kg body wgt-1)
LB
WB
Peak Isometric torque of the knee extensors (Nm)
Group effect
p = 0.099
Relative peak isometric torque knee extension (Nm•kg body wgt-1)
1RM knee extension (kg)
Relative 1RM knee extension (kg•kg body wgt-1)
Young
Middle
Older
‡
†

Table 1. Subject Physical Characteristics and Diet Composition

	Young		Middle		Old				
	SED	**ACT**	**SED**	**ACT**	**SED**	**ACT**	**Age Effect**	**Group Effect**	**Interaction**
N	11 (5M/6F)	11 (5M/6F)	12 (6M/6F)	12 (6M/6F)	14 (10M/4F)	14 (10M/4F)			
Age (y)	30±2	31±2	53±2	53±2	72±2	72±2	$p < 0.05$	NS	NS
Height (cm)	170±3	175±2	167±2	173±1	172±2	172±2	NS	NS	NS
Weight (kg)	75±5	70±3	74±3	67±3	84±3	68±3	NS	$p < 0.05$	NS
Total Body fat (kg)	25±3	12±2	23±3	11±1	28±2	15±1	NS	$p < 0.05$	NS
Body Fat (%)	33±3	18±3	33±3	18±2	35±2	23±2	NS	$p < 0.05$	NS
Total Lean Mass (kg)	47±3	55±3	47±2	52±2	52±2	51±3	NS	NS	NS
Lean Mass (%)	64±3	78±2	63±2	79±2	63±2	75±2	NS	$p < 0.05$	NS
Arm Lean Mass (kg)	5.2±0.4	5.9±0.5	5.3±0.5	5.7±0.4	5.8±0.4	5.7±0.4	NS	NS	NS
Leg Lean Mass (kg)	16.5±1.1	20±1.2	16.1±1.0	18.9±0.8	17.7±0.9	17.8±1.1	NS	$p < 0.05$	NS
Total Bone Mineral Density (g/cm^2)	1.18±0.02	1.23±0.02	1.23±0.02	1.18±0.02	1.25±0.02	1.17±0.03	NS	NS	$p < 0.05$
DEXA Z-score	0.08±0.33†	0.78±0.34	0.93±0.24	0.27±0.21†	1.49±0.26	0.50±0.32	NS	NS	$p < 0.05$
Dietary Habits									
Carbohydrate (g/day)	295±39	333±18	229±12	322±18	245±28	293±22	NS	NS	NS
Fat (g/day)	81±16	77±6	73±6	89±8	59±8	104±10	NS	$p < 0.05$	NS
Protein (g/day)	90±13	111±8	82±6	97±9	73±12	96±9	NS	$p < 0.05$	NS
Total calories (kcal/day)	2266±316	2471±109	1953±98	2474±136	1823±178	2523±149	NS	$p < 0.05$	NS

Note: *p* Values refer to significant main effects ($p < 0.05$) identified by two-way (age by activity group) analysis of variance. Data are presented as mean±*SE*. NS = non-significant ($p > 0.05$). †Significantly different from SED old group ($p < 0.05$). DEXA = dual-energy x-ray absorptiometry.

Table 2. Current and Past Physical Activity Patterns.

	Young		Middle		Old				
	SED	**ACT**	**SED**	**ACT**	**SED**	**ACT**	**Age Effect**	**Group Effect**	**Interaction**
Current (hours/week)									
Mod.-Vig. PA	0.02±0.02	9.10±1.18	0.33±0.12	7.34±1.11	0.03±0.03	5.99±0.92	NS	$p < 0.05$	NS
Light PA	0.95±0.52	0.45±0.45	1.87±0.61	1.81±1.35	1.02±0.33	1.47±0.45	NS	NS	NS
Past Habitual PA (hours/week)									
20-29 y									
Mod.-Vig. PA	0.62±0.45	9.10±1.73	1.03±0.38	6.41±1.51	0.56±0.38	4.84±1.99	NS	$p < 0.05$	NS
Light PA	1.25±0.66	0.00±0.00	1.06±0.72	0.13±0.13	2.18±0.96	0.28±0.14	NS	NS	NS
30-39 y									
Mod.-Vig. PA	0.22±0.13	9.90±1.52	0.35±0.18	10.23±3.84	2.38±1.98	4.87±1.04	NS	$p < 0.05$	NS
Light PA	1.13±0.43	1.00±1.00	1.17±0.71	0.09±0.09	2.90±1.24	0.44±0.21	NS	NS	NS
40-49 y									
Mod.-Vig. PA	N/A	N/A	0.60±0.24	7.64±0.96	0.23±0.16	6.40±1.18	NS	$p < 0.05$	NS
Light PA	N/A	N/A	2.03±0.88	0.38±0.17	1.76±0.91	0.81±0.41	NS	NS	NS
50-59 y									
Mod.-Vig. PA	N/A	N/A	0.31±0.62	8.63±1.15	0.50±0.50	7.32±1.10	NS	$p < 0.05$	NS
Light PA	N/A	N/A	1.56±0.79	3.71±2.18	2.08±0.97	1.73±0.58	NS	NS	NS
60-69 y									
Mod.-Vig. PA	N/A	N/A	0.18‡	4.5‡	0.50±0.41	8.28±1.31	N/A	$p < 0.05$	N/A
Light PA	N/A	N/A	4.32‡	2‡	2.29±0.85	1.49±0.63	N/A	NS	N/A
70-79 y									
Mod.-Vig. PA	N/A	N/A	N/A	N/A	0.45±0.37	4.61±0.90	N/A	$p < 0.05$	N/A
Light PA	N/A	N/A	N/A	N/A	2.66±1.27	1.16±0.65	N/A	NS	N/A
Baecke Index									
Work	2.5±0.2†	2.2±0.2†	2.4±0.2	2.5±0.2	3.1±0.2	2.5±0.2	$P < 0.05$	NS	NS
Sport	1.7±0.2	4.7±0.1*	2.4±0.2	3.9±0.2*	1.8±0.2	4.2±0.2*	NS	$p < 0.05$	$p < 0.05$
Leisure	2.4±0.2	3.2±0.2	2.7±0.1	3.3±0.2	2.8±0.2	3.0±0.2	NS	$p < 0.05$	NS

Note: *p* Values refer to significant main effects ($p < 0.05$) identified by two-way (age by activity group) analysis of variance. †Indicates age effect compared to the old age group ($p < 0.05$). *Significantly different from age-matched, sedentary group ($p < 0.05$). NS = non-significant ($p > 0.05$); PA = physical activity; N/A = data not available or applicable. ‡N=1; statistics cannot be run on these data points.

CHAPTER 5: GENERAL DISCUSSION

5.0 Introduction

Skeletal muscle mitochondrial dysfunction is commonly observed in elderly individuals, but can vary significantly depending on physical activity levels, body composition and genetic background. The studies outlined in this thesis were designed to investigate several novel aspects of aging and mitochondrial dysfunction in order to generate an understanding of their impact on muscle function. We utilized several techniques to test mitochondrial function that ranged from mitochondrial respiration in permeabilized fibers to peak whole body aerobic capacity. By maintaining this connection with physical function, these investigations have aimed to be as physiologically relevant as possible.

5.1 Skeletal muscle mitochondria and IMCL droplets undergo morphological changes with age in association with altered mitochondrial dynamics, lipid metabolism and degradation.

It is well established that muscle mitochondrial content is reduced with age. Mitochondrial content is typically assayed using tissue homogenates, which gives little specific information regarding the location and proximity of the affected organelles. Since mitochondria are so dynamic, it becomes necessary to determine what structural changes occur to mitochondria across the lifespan in order to understand the underlying causes of muscle aging. We found that mitochondrial number is reduced with age by about 60%, yet size is maintained (1). Similarly, since aerobic capacity declines ~10% per decade of life, there would be an estimated 50% reduction in VO_{2max} in these subjects

due to their age difference. Combined with the observed 50% lower maximal activity of citrate synthase (CS) and cytochrome c oxidase (COX) activities in older adults, it would appear that oxidative capacity is considerably dependent on the amount of mitochondria present in skeletal muscle (i.e. quantity) and not quality issues. This has been substantiated by other studies demonstrating similar relative rates of mitochondrial respiration between young and elderly subjects that maintain a highly active lifestyle, despite an overall reduction in mtDNA copy number and mitochondrial proteins (2). The larger IMCL droplets seen in aged skeletal muscle are likely secondary to the reduction in mitochondrial content and muscle oxidative capacity that impairs the absolute rate of lipid metabolism. Obviously either the synthesis or degradation of mitochondria is being affected by aging such that there is net removal of mitochondria over time. Unfortunately, it is difficult to assess the independent rates of these processes simultaneously.

One potential cause of the age-related reduction in the number of mitochondria is that mediators of mitochondrial biogenesis are suppressed with age (SirT1 mRNA in this study, PGC-1a protein in another (2)), suggesting a failure to continually form an active pool of mitochondria. It appears that overexpression of PGC-1α may be enough to mitigate the decline in muscle mitochondria with age (3). However, we also discovered that the association of mitochondria with IMCL droplets is reduced in aged subjects, implying a restructuring process that might be related to mitochondrial fusion and fission dynamics. Correspondingly, elderly subjects displayed reduced mRNA levels of the fusion regulator Mfn2 and the fission regulator Drp1, while another study has subsequently observed reduced levels of the fusion regulator Opa1 (4). While the

contribution of mitochondrial dynamics to muscle oxidative capacity is not well understood, subtle reductions in the mixing of mitochondrial populations may exacerbate defects in mitochondrial biogenesis by harboring mtDNA defects, rather than maintaining a natural dilution. Furthermore, there may be some influence of reduced degradation of mitochondria with age, but its relative contribution to aging compared to mitochondrial biogenesis has yet to be fully explored.

5.2 Mitochondrial oxidative stress impairs typical mitochondrial adaptations to aerobic exercise training in mice by impairing enzyme function and mtDNA maintenance.

Many studies have observed reductions in mitochondrial function in parallel to higher levels of oxidative damage within numerous disease states such as Alzheimer's, COPD and sarcopenia. However, the true consequences of oxidative stress are difficult to assess since in disease it is unknown whether mitochondrial dysfunction drives oxidative stress or vice versa. Furthermore, there is evidence that oxidative stress can alter muscle fatigue and that antioxidants can mitigate adaptations to exercise (5-7), suggesting that redox reactions are a critical component of cellular hormesis in response to muscle contractions. We demonstrate that an excess of mitochondrial superoxide due to reduced Sod2 protein is sufficient to limit the adaptive potential of skeletal muscle to aerobic exercise training. Not only was mitochondrial enzyme activity and respiration adversely affected, but mtDNA copy number failed to increase, suggesting adaptive defects in both protein and DNA due to mitochondrial oxidative stress.

The limitations to mitochondrial adaptation during volume expansion are relevant in several ways: 1) A persistent elevation in mitochondrial oxidative stress may be responsible for the limited adaptation in octogenarian and older individuals to both aerobic and resistance exercise at an advanced age due to a vicious cycle of metabolic defects. Muscle restructuring and appropriate contractile function are energy intensive and failure to enhance ATP generation would likely mitigate contractile adaptations. 2) In a general sense, since exercise capacity was unaffected in sedentary mice that exhibit oxidative stress, relatively low levels of macromolecular damage seems to precede disruptions in oxidative capacity. Moreover, oxidative damage was not exacerbated per unit of mitochondria, suggesting that ROS generation was not overtly altered by the accrued mitochondrial defects, even though complex assembly was affected. It is possible that electron slip only increases after a certain threshold of ETC deterioration has occurred from oxidative modifications. More precise measurements of ROS generation *in vivo* and the resulting protein modifications are necessary to determine this stress threshold and its relevance in disease.

5.3 Habitual aerobic exercise attenuates the loss of muscle strength with age.

Traditional exercise paradigms have dictated the widespread use of resistance exercise training to combat age-related reductions in muscle mass and strength rather than aerobic exercise. Since greater mitochondrial function has recently been linked to an attenuation of sarcopenia (3), we assessed the impact of long-term aerobic exercise in mitigating the effects of aging on muscle mass and strength. We found that individuals

that habitually perform aerobic exercise have greater absolute and relative knee extension strength as well as higher relative grip strength. As expected, the aerobically active subjects displayed these strength improvements in conjunction with considerably higher whole body VO_{2peak} and peak aerobic power production, suggesting more comprehensive anti-aging adaptations to aerobic exercise than others have described. The functional implications for these adaptations are very clear in terms of extending the adult "healthspan" in terms of the years of high mobility and independent living.

While we can ascribe some of the strength benefits to greater fat free mass in the active subjects, particularly at young and middle ages, the older adults had similar lean mass regardless of activity level. This brings two factors into consideration: 1) that aerobic exercise qualitatively changes the contractile apparatus and/or 2) that DEXA measurements may not provide enough resolution to accurately determine muscle mass or be able to discern intramuscular fat depots within or around muscle tissue. It seems likely that the discrepancy could be related to both factors. It is well established that fat accumulates around in and within fascicles with age (8). Also, contractile velocity can be increased by aerobic exercise training and in combination with subtle shifts in fiber type proportions, can significant influence force and power generation even with a similar amount of muscle mass. However, these changes in muscle quality are extremely difficult to assess objectively and different types of analysis each have individual drawbacks. For example, single fiber experiments that assess the contribution of the contractile elements within a specific fiber type fails to test the effect of age on calcium handling or neurologic activation. To make matters worse, it is unknown if MHC hybrid fibers (I/IIa,

IIa/IIx or a triple hybrid) or even "pure" fibers differ in their MHC content throughout the length of the entire fiber. It seems highly likely that this heterogeneity would increase during aging due to reduced activation of muscle mass and α-motor neuron loss. Thus, the factors influencing absolute whole muscle force generation, independent of muscle mass are considerably complex and require further investigation.

Our findings regarding muscular adaptation to long-term adherence to aerobic exercise suggest that this mode be more widely prescribed in elderly adults. Of note, aerobic exercise can be performed in a group setting, may be less intimidating and "foreign" than resistance exercise to initiate as an exercise program, and simultaneously remediates cardiovascular and muscular aspects of aging. While aerobic exercise may still not be considered the optimal countermeasure for aging, clearly some amount of aerobic training should be included to more comprehensively complement power or strength based exercise interventions. A growing segment of scientific research is investigating signaling pathways and activating compounds that can mimic the effects of exercise within skeletal muscle, with obvious implications for aging and many forms of metabolic disease (9). However, targeting the signaling cascades necessary for muscle mitochondrial and growth signaling will very likely require some stimulation of ATP turnover or calcium flux. These processes are difficult to manipulate in a muscle specific fashion without adversely influencing other tissues types that rely on similar processes for cellular function such as calcium oscillations in smooth and cardiac muscle. It is not difficult to imagine the health complications resulting from heart or vascular muscle hyperactivity. Perhaps more importantly, initiating a growth stimulus (whether

specifically mitochondrial or generally hormonal) in sedentary elderly individuals that have accrued a proportion of premalignant or transformed cells may initiate or accelerate cancer development. Some degree of muscular activation by contractions will likely be a necessary component of sarcopenia interventions and disease prevention within the general population in the near future. Therefore, extending our knowledge of the specific adaptations to exercise and the limits of metabolic plasticity will be important in order to continually improve exercise training prescription in the elderly.

5.4 Conclusions and future directions

In a broad sense, the deterioration of mitochondrial bioenergetics is evident in many age-associated diseases that parallel sarcopenia such as neurodegeneration, hepatic steaosis, and heart failure. Therefore, the cellular events ascribed to aging in this thesis and its related work can be applied to a spectrum of health concerns. Moreover, there is increasing evidence that exercise is not only considerably beneficial to skeletal muscle, but also positively affects peripheral tissues (kidney, liver, brain, etc.) that "age" in parallel, due in part to augmented tissue mitochondrial content (10, 11). If these adaptations are dependent to some degree on skeletal muscle contractions, it is likely that maintenance of a certain amount of muscle oxidative capacity might be necessary to provide these benefits to the whole body. Furthermore, while simply increasing muscle mitochondria pharmacologically shows promise in improving muscle health, the regulation of oxidative stress, biological waste products, and cellular damage must also be dealt with in order to optimally prevent tissue degeneration. Again, exercise may

prove a fruitful model in which to simultaneously address the complexities of aging, since it also lowers oxidative stress and increases cellular turnover in muscle. However, each mode of exercise (resistance or aerobic) cannot independently remediate all aspects of the aging process, and so exercise prescription should be appropriately tailored to individuals based on their lifestyle and goals.

In summary, skeletal muscle aging is an important health concern that will continue to worsen to if not adequately addressed. While exercise is a promising countermeasure to muscle aging, not all individuals are able to exercise and it is not reasonable to expect a major increase in physical activity in the elderly in the short-term. This work helps to define some of the defects inherent in aged muscle and presents some of the persisting functional limitations in spite of life-long exercise. It remains quite clear that developing therapies that prohibit muscle atrophy are necessary that could be used in conjunction with exercise to augment existing muscle strength. Adequately addressing dysfunction is aged muscle will require novel techniques aimed at studying the human condition rather than simply using aged rodents. Further, because physiology is complex and multi-faceted, it is likely that several approaches will be required to optimally maintain whole body function and quality of life in the elderly.

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