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Effects of Aging on the Regulation of Mitochondrial Biogenesis and Aerobic Function in Skeletal Muscle

by

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Abstract

The inevitable age-related decline in skeletal muscle mass and muscle aerobic capacity can impact the mobility and health of older individuals resulting in lower quality of life, independence and increased risk of frailty. These declines are relatively small until late middle age (~20%), but are more severe between late middle age and senescence (~50%), suggesting that this part of the lifespan deserves particular focus on strategies to attenuate these aging effects.

Previously, we found that exercise training at late middle age improved muscle aerobic capacity, restoring it to young adult levels. Based on this finding, we hypothesized that regular exercise beyond late middle age until senescence could attenuate the declines in muscle aerobic capacity. To determine the effects of aging and exercise on skeletal muscle aerobic capacity, we combined measures of functional running capacity, *in situ* muscle aerobic capacity, *in vitro* mitochondrial enzyme activity and mitochondrial content. Secondly, since one of the major regulators of mitochondrial biogenesis (PGC-1α) declines with age, we wanted to determine if aged muscle had a similar capacity as young muscle to increase PGC-1α in response to perturbations that normally increase PGC-1α (i.e. exercise and muscle damage). Mitochondrial enzyme activity, mRNA and protein content and PGC-1α mRNA and protein content were measured in exercise trained animals and in young and senescent animals following cardiotoxin injection, which induces severe muscle damage, to assess changes in mitochondrial biogenesis.

The main findings are that 7 months of exercise training beyond late middle age resulted in a greater capacity to exercise, lower percent body fat and increased survival rates compared to sedentary animals. However, at the muscle level there were no improvements in muscle and mitochondrial aerobic capacity and PGC-1α content, suggesting a diminished plasticity of senescent muscle. In contrast, following muscle damage there was not an impairment of senescent muscle to increase PGC-1α and mitochondrial biogenesis. A significantly larger perturbation (stimulus) from the muscle damage or the recruitment of satellite cells and formation of new myofibres may explain the disparate findings between the exercise training and the muscle damage on the capacity for senescent muscle to induce mitochondrial biogenesis.

Preface

Two main observations led to the design of this thesis. Firstly, skeletal muscle aerobic capacity decreases significantly between late middle age and senescence. Secondly, mitochondrial biogenesis and mitochondrial protein synthesis decline with age and these declines likely explain the decline in muscle aerobic capacity. In a previous study that is not included in this thesis, we showed that exercise training of the F344BN rat at late middle age was able to increase muscle aerobic capacity to levels similar to young adult, thus eliminating any age-related decline observed at this time point (Betik *et al.* 2008, Exercise training in late middle aged male F344BN rats improves skeletal muscle aerobic function *Experimental Physiology*, **93**, 863-871.) We wanted to determine if extending this exercise program beyond late middle age and into senescence would attenuate the declines in skeletal muscle aerobic capacity that normally occurs over this part of the lifespan. Furthermore, since one of the primary mediators of mitochondrial biogenesis decreases with age, we wanted to determine if aged muscle had an impaired capacity to increase the presence of this mediator which would help explain the diminished muscle aerobic capacity that is observed with increasing age. As such, the primary purposes of this thesis were to determine if exercise training could attenuate the declines in muscle aerobic capacity in senescent animals and to determine if senescent muscle had a diminished capacity to increase mitochondrial biogenesis.

Chapters 2, 3, and 4 were written to be stand alone manuscripts, and as such there may be some redundancy within the introductions, discussions and methods. Chapter 2 is based on the recently accepted publication:

Betik, A.C., Thomas, M.M., Wright, K.J. Riel, C.D. and Hepple, R.T. (2009) Exercise training from late middle age until senescence does not attenuate the declines in skeletal muscle aerobic function, *American Journal of Physiology* (in press).

Chapters 3 and 4 are manuscripts that were written with the intention of submitting these for publication. For this thesis I have elaborated substantially in most sections, which would likely be too lengthy to be acceptable for publication.

Acknowledgements

As much as a doctoral dissertation is a representation of a significant amount of work that was done and a summary and interpretation of the findings, it also serves as a celebration of many years of hard work, learning, growth and maturity. In addition it is a celebration of many relationships and experiences that were essential in achieving this final product, and perhaps more importantly, were instrumental in forming the type of researcher, academic and person that the candidate has become. There is no way that what I have done, where I am today, and the person that I am, could have been achieved without the help and inspiration of many people. This is a unique opportunity to thank those people, for which I am truly grateful.

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the 12 hour experimental days and surgeries! Thank you for your quick feedback on papers and for the many, many discussions about our experiments, the results and the day-to-day functioning of the lab. More importantly, I am thankful for your great friendship, kindness and positive attitude, it has been a lot of fun. To Kathryn Wright, who has worked with me since the beginning of the training study and has continued to be a huge help with lab work, assays, data and most recently your great feedback and critique on my thesis chapter. Your (unique) sense of humour has made life in the lab fun while your positive and generous attitude has been hugely supportive. I thank you so much.

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To Russ, thank you for providing the opportunities to perform these experiments, and many not included in this thesis, and the opportunities to participate in many scholarly activities that will without a doubt benefit my career. This has been a phenomenal growing and learning experience.

Finally, to some very special people that have contributed to this PhD career in non-academic ways. Mom and Dad - you have taught me important things about life and about being a quality person with integrity, work ethic and morals. These are qualities and characteristics of which I am most proud, and are more than a PhD could ever teach me. I cannot imagine where I would be today without your endless amount of encouragement and support, throughout my life, but especially the last 12 months when I was so ready to let go; you supported me, encouraged me and convinced me to persevere. I am very, very thankful and I am very proud that you are my parents (oh, and Hallmark thanks you too). I also thank my siblings (Rebecca, Aaron, Matthew and Nick) their families, and Ezio, Lois and Zen for their support, help and encouragement. I especially appreciate your understanding when I became selfish and self-absorbed. Thanks Aaron for the golf and ski distractions!

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Dedication

This final product is dedicated to the people who helped me get to the finish line when challenges in health and life were so severe that I was believing it was not worth it to continue. Your words and/or actions, of support, encouragement and belief in me, kept me moving forward and persevering. This thesis would not be complete if it were not for you. Mom, Dad, Nick, Ann, Marina and Ame-Lia, this is for you!

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
34 mo Con	34 month old sedentary group
34 mo Train	34 month old trained group
36 mo Con	36 month old sedentary group
36 mo Train	36 month old trained group
ACC	Acetyl CoA carboxylase
AMPK	AMP-activated protein kinase
CAMK	calcium calmodulin-dependent protein kinase
Complex IV	Cytochrome oxidase
COX	Cytochrome oxidase
COXI	Cytochrome oxidase subunit I
COXIV	Cytochrome oxidase subunit IV
CS	Citrate synthase
CTX	Cardiotoxin
F344BN	Fisher344 x Brown Norway F1-hybrid rat
GAPDH	Glyceraldehyde phosphate dehydrogenase
GASr	Gastrocnemius red portion
GLUT4	Glucose transporter 4
GXT	Graded exercise test
LMA	Late middle age (~70% survival; 28-30mo in F344BN rat)
MEF2	myocyte enhancer factor 2
mtDNA	Mitochondrial DNA
NRF	Nuclear respiratory factor
OEX	Senescent exercise group (34-36 mo)
OSED	Senescent sedentary group (34-36 mo)
p160myb	p160myb binding protein
p38 MAPK	p38 mitogen-activated protein kinase
PGC-1α	peroxisome proliferator-activated receptor-γ coactivator-1α
PLAN	Plantaris
RIP140	Receptor-interacting protein 140
ROS	Reactive oxygen species
SEN	Senescence (>35% mortality, 34-36 mo for F344BN rat)
SEN+10	Senescent group 10 days post cardiotoxin injection
SEN+21d	Senescent group 21 days post cardiotoxin injection
SIRT1	Silent information regulator 2 homolog
SOL	Soleus
TA	Tibialis anterior
Tfam	Mitochondrial transcription factor A
TNF- α	Tumor necrosis factor alpha
TNF-R1	Tumor necrosis factor receptor-1
VO_2 max	Maximal oxygen uptake
YA	Young adult (<99% mortality, 7-13 mo for F344BN)
YA+21d	Young adult group 21 days post cardiotoxin injection
YSED	Young adult sedentary group

Epigraph

Voici mon secret. Il est très simple: on ne voit bien qu'avec le cœur. L'essentiel est invisible pour les yeux.

Antoine de Saint Exupery, Le Petit Prince (1943)

Chapter One: BACKGROUND AND INTRODUCTION

1.1 GENERAL INTRODUCTION

Consequences of aging include a decrease in skeletal muscle mass, muscle contractile function and skeletal muscle aerobic capacity, all of which negatively affect the quality of life of older individuals due to decreased mobility and decreased independence. Sarcopenia, the term used to describe the progressive decline in skeletal muscle mass due to aging (32; 94; 94; 111; 116), also increases the risk of disability and frailty, along with other health problems that are associated with low mobility (e.g. osteoporosis, cardiovascular disease) (112). Sarcopenia does not become relevant until around the 6^{th} decade in life, but its prevalence and severity increases drastically thereafter (8; 56). As societal trends indicate that the population in general is getting older and that people are living longer, there is an increased burden on the health care system and public services related to frailty infrastructure (i.e. chronic care, senior care facilities), not to mention the humanitarian aspect for all citizens to have a satisfactory quality of life, regardless of age. As such, there is an increasing need and importance to delay the onset and reduce the severity of sarcopenia.

1.2 POTENTIAL MECHANISMS OF SARCOPENIA

The decline in muscle mass with aging is multi-factorial, complex and still not well understood. The progressive decline in muscle mass with age is due to a progressive decline in muscle fibre number (15; 71), and perhaps smaller muscle fibre cross-sectional area, although this latter point is controversial since there appears to be some fibres that are quite a bit smaller, but also some fibres that are larger and may have hypertrophied in compensation for the smaller fibres (53; 71). There are several mechanisms that are postulated to explain the decrease in muscle mass with aging, and likely each of these contributes to the entire effect (see Figure 1.1 for an overview of mechanisms related to sarcopenia). Firstly, muscle protein synthesis rates decline with age and thus there is a lower capacity to make new proteins (110; 116; 127). Secondly, aging results in cumulative oxidative damage to skeletal muscle proteins (36; 38; 62) that results in

increased apoptosis (6; 29; 132). Damaged proteins either need to be repaired or if the damage is significant, they need to be replaced by new fibres. In this regard, there appears to be a decreased ability to repair damaged proteins with age (88; 103), and also a decreased ability to successfully complete muscle regeneration due to deficiencies in nerve re-innervation (19), satellite cell recruitment and proliferation (25; 106) and differentiation into myotubes (76). The combined effect of increased oxidative damage and increased apoptosis with age along with a decrease in protein synthesis and muscle regenerative capacity contribute to lower fibre number and decreased muscle mass with age.

The progression of sarcopenia is accompanied by decreases in contractile function (17; 18; 126) and muscle aerobic capacity (26; 46; 51) even when normalized to the existing muscle mass or cross-sectional area. A contributing reason for decreased contractile function is increased oxidative damage to proteins with age, as described in the paragraph above. The accumulation of oxidatively damaged proteins is due to both an age-related increase in reactive oxygen species (ROS) production and DNA damage (42; 67; 120; 121), as well as a lower capacity to degrade oxidatively damaged proteins (16; 41; 68). Decreases in proteosome function with age (35; 52; 55) contributes to the inability to remove damaged proteins and ultimately the accumulation of oxidative damage. As such, the increases in oxidative damage can affect the contractile function of the muscle by impairing any component of the excitation-contraction coupling system (107; 129). For example, Viner et al. (129) showed increased levels of nitrotyrosine damage to Ca^{2+}-ATPase, which could affect the contraction and relaxation times that are observed with aging (87; 96). In terms of overall muscle performance, both the declines in muscle mass and impairments within the existing muscle mass contribute to lower contractile function and decreased mobility that is associated with aging.

Declines in muscle mass are relatively moderate until late middle age (survival 70%), but become more severe after late middle age and into senescence (survival < 50%) (see Table 1.1, for relative age comparisons and survival rates). This progression of sarcopenia is similar in humans (71) and Fisher 344 x Brown-Norway F1-hybrid (F344BN)

rat (18; 46), making this animal model useful for studying the effects of aging on skeletal muscle.

1.3 AGE-RELATED DECLINES IN MUSCLE AEROBIC CAPACITY

The declines in muscle aerobic function follow a similar trend as the declines in muscle mass in that they are modest (10-20%) until late middle age; between late middle age and senescence the declines become much greater (40-60%) (46; 51). The rapid rate of decline of both muscle mass and muscle aerobic function after late middle age underscores the necessity of focusing research and treatments on this part of the lifespan (as it is beyond late middle age that mobility and health are most affected).

Similar to the story regarding contractile function, the decline in muscle aerobic capacity is not simply due to a decline in muscle mass, as has been shown in models in which muscle aerobic capacity is normalized to the muscle mass (26; 46; 51). This implies that there is an additive decline in aerobic function independent of the changes in muscle mass. While the decline in muscle aerobic capacity with age is likely multi-factorial (briefly reviewed in (12)), the use of the hindlimb perfusion model allows normalization of blood flow across animals so that the aerobic capacity of the muscle is primarily a function of muscle oxygen consumption and not oxygen delivery (7; 54). To explain the decline in muscle oxygen consumption, many of the enzymes involved with oxidative phosphorylation also decrease with age (20; 24; 46). The declines in mitochondrial enzyme activity may be associated with declines in total mitochondrial content or due to mitochondrial dysfunction (decrease in activity relative to enzyme content). Support for both of these scenarios is present in the literature, but not without controversy. A recent investigation of changes in mitochondrial content by looking at fibre mitochondrial volume density showed that there was no decrease in mitochondrial volume relative to fibre size (78). Given the declines in mitochondrial enzyme activity, integrating these findings suggests the mitochondria are present at normal levels, but the enzymes are not functioning as well as in young muscle. Again, a likely culprit for age-related declines in protein function is the accumulation of oxidative damage with aging. In particular, mitochondria

are more susceptible to oxidative damage from ROS because this is where most of the ROS are produced (complex I and III of the electron transport chain) and mitochondria have a relatively lower capacity to scavenge ROS. Furthermore, mitochondria contain a small portion of DNA (mtDNA) that is essential for the complete assembly of many of the proteins in the electron transport chain (130). The mtDNA is thus very susceptible to oxidative damage and both ROS production and levels of mtDNA damage have been shown to increase with aging and are associated with mitochondrial dysfunction and fibre atrophy (42; 46; 67; 120; 121; 130). Studies have shown increases in mtDNA oxidation (38; 50; 108; 120), mtDNA deletions (27; 38; 72; 83; 86; 130) and decreased mtDNA content (46; 77; 104; 115; 131) with increasing age. Furthermore, mitochondrial protein synthesis rates appear to decline with age (110). Thus, the combination of increasing mitochondrial damage and decreasing rate of mitochondrial protein synthesis likely explains the decreased mitochondrial enzyme activity and oxidative capacity in aging muscle. Considerations of decreased cardiovascular capacity with age aside, the above observations show that the oxidative capacity of skeletal muscle decreases with age, and that the decline can be explained (at least in part) by factors within the aged skeletal muscle.

1.4 MODULATORS OF PGC-1α AS THEY RELATE TO MITOCHONDRIAL BIOGENESIS

Given the health implications of declining muscle aerobic capacity, it is useful to devise interventions that will prevent, delay or attenuate these declines with aging. One option is to increase mitochondrial content. The primary means of increasing mitochondrial content is through a nuclear transcription cofactor, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), which has been dubbed the "master regulator of mitochondrial biogenesis" (101; 143). As noted earlier, mitochondrial content and/or function decreases with age, as does mitochondrial protein synthesis. Not surprisingly, PGC-1α mRNA (5) and protein (21) also decreases with age, suggestive of a decreased drive for mitochondrial biogenesis which will contribute to the age-related decline in aerobic capacity. Up-regulation of PGC-1α gene and protein expression have been shown

to increase mitochondrial content in a number of pharmacological (5-aminoimaidazole-4-carboxamide riboside (AICAR), an activator of AMP-activated protein kinase (AMPK), Ca^{2+}, resveratrol), exercise (electrical stimulation and voluntary exercise) and diet (calorie restriction, beta guanadinopropionic acid (ß-GPA) feeding which causes an increase in phosphagen levels and activates AMPK) interventions. Targeting PGC-1α seems to be a reasonable strategy to increase mitochondrial content in aged muscle to combat the declines in aerobic function.

Activation of PGC-1α and induction of mitochondrial biogenesis is complex, and probably still not fully understood. In terms of muscle contractile events leading to mitochondrial biogenesis, there are a number of factors that have been shown to increase with contractile activity and also shown to activate PGC-1α (see Figure 1.2 for an overview of contractile events that may activate PGC-1α and lead to mitochondrial biogenesis). Activation of PGC-1α can have the effect of increasing its promoter activity, increasing its own expression and increasing its stability (i.e. less degradation). During muscle contraction, there is an activation of several kinases (see review by (113)) that occurs by way of metabolic perturbations, namely an increase in cytosolic Ca^{2+} and alterations in the AMP/ATP ratio. Both Ca^{2+} and AMPK activation have been shown to increase mitochondrial biogenesis independent of contractile activity, providing a mechanistic link between muscle contraction and mitochondrial biogenesis (58; 91; 113; 124). Increases in AMPK activity are achieved by increases in the AMP/ATP ratio (49; 97) and possibly Ca^{2+} (61). AMPK is activated during muscle contraction (58; 134; 138), also see review by Winder et al. (136), and may be regulated in an intensity-dependent fashion (22; 57; 105). Studies using AMPK activators such as AICAR (90; 124; 135) and ß-GPA (10; 146) have shown that treatment with these AMPK agonists results in PGC-1α activation and mitochondrial biogenesis. Furthermore, two AMPK phosphorylation sites on the PGC-1α promoter region have been identified and provide evidence of a direct relationship between AMPK and PGC-1α (60). Recently, it has been shown that reactive oxygen species (ROS) can also activate AMPK (59), and since ROS production tends to increase with increasing

metabolic demand, this represents another possible mechanism by which AMPK is activated with muscle contraction.

Contractile activity also increases phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) (14; 39; 58) and its phosphorylation has also been shown to increase PGC-1α transcriptional activity (1; 64; 99; 100; 113; 139). Phosphorylation of p38 MAPK can occur *via* calcium calmodulin-dependent protein kinase (CAMK), which is also activated during muscle contraction (74), presumably by increases in Ca^{2+} (139). Furthermore, some evidence suggests AMPK can also phosphorylate p38 MAPK (63; 70), illustrating another level of redundancy by which metabolic events can lead to PGC-1α activation.

CAMK activation has also been shown to activate PGC-1α and induce mitochondrial biogenesis (91; 141; 146), although others have shown that CAMK is not necessary for the exercise-induced effects of mitochondrial biogenesis (2). It has been suggested that, at least in response to Ca^{2+} (and not exercise), CAMK effects on PGC-1α are mediated by phosphorylating p38 MAPK (139). Whether p38 MAPK activation can occur with contractile activity independent of Ca^{2+}-mediated activation of CAMK is uncertain.

Another important regulator of PGC-1α worth mentioning is the myocyte enhancer factor 2 (MEF2) which is involved in an autoregulatory loop with PGC-1α. PGC-1α binds to and coactivates MEF2 (48; 85), and MEF2 expression in turn increases PGC-1α expression (48). MEF2 may be activated during muscle contraction or exercise (81; 117; 119; 142) which is mediated through activation of calcineurin (23; 142) which is activated by Ca^{2+} (30), CAMK (82; 92; 119), AMPK (92) and p38 MAPK (80; 145). (For clarity, all of these associations were not demonstrated by arrows in Figure 1.2).
Silent information regulator 2 homolog (SIRT1) has also been shown to deacetylate and activate PGC-1α (65; 89; 109). Some studies have shown that increases in SIRT1 results in increased mitochondrial biogenesis (34; 65; 109), while knockdown of SIRT1 results in a

decrease in mitochondrial gene expression (37). It should be noted that the effects of SIRT1 on PGC-1α and mitochondrial biogenesis are not without controversy as some observations suggest that SIRT1 over-expression leads to decreases in mitochondrial biogenesis (44; 89). The differences in SIRT1 expression levels, types of tissue used, and cell lines versus *in vivo* models likely contributes to these discrepancies (44) and more work is warranted. Only one study to our knowledge has shown that exercise increases SIRT1, and this may have a positive effect on mitochondrial biogenesis (122). It has been suggested that AMPK can activate SIRT1 providing a mechanistic link between contractile activity and SIRT1 activation.

While much focus on PGC-1α has been related to the positive modulators, recently receptor-interacting protein 140 (RIP140) (98; 114) and p160myb binding protein (p160myb) (33) have been identified as repressors of genes of oxidative phosphorylation. When these protein levels are elevated, genes and proteins related to oxidative phosphorylation are decreased, working in opposite fashion as PGC-1α (33; 93; 114). Conversely, lower levels of RIP140 or p160myb levels are associated with an increase in mitochondrial gene expression (33; 93; 114; 133). An inhibitory domain on PGC-1α has been identified in which p160myb binds, evidence of a direct effect on PGC-1α (33). Interestingly, these authors also showed that p38 MAPK blocks p160myb binding to PGC-1α, suggestive of a redundant mechanism by which p38 MAPK induces mitochondrial biogenesis (as in Figure 1.2, direct phosphorylation of PGC-1α and by suppressing binding of p160myb). Meanwhile, RIP140 appears to act on nuclear receptors exerting an antagonistic effect to that of PGC-1α (47; 114). RIP140 also directly interacts with PGC-1α (47) and decreases its transcriptional activity, suggesting that the repressor actions of RIP140 exist by nuclear receptor dependent and independent means. There appears to be some fibre type specificity in RIP140 content, regulation and its effect on mitochondrial content. RIP140 content has been shown to be greater in muscles with glycolytic fibres and lower in muscles that are rich in oxidative fibres (114). Deletion of RIP140 caused increases in mitochondrial enzyme activity in the extensor digitorum longus (rich in glycolytic fibres), but not the soleus (rich in oxidative fibres) (114). Metabolic perturbation

by ß-GPA feeding induced decreases in RIP140 content in the triceps (rich in glycolytic fibres), but not in the soleus (133), which had the resultant effect of increases in mitochondrial proteins in the triceps, but not in the soleus.

1.5 TIMING OF PGC-1α AND CO-ACTIVATION OF TRANSCRIPTION FACTORS

PGC-1α induction can be very rapid and follows a time course that is important in affecting mitochondrial regulation. Upon activation, PGC-1α first appears to translocate from the cytoplasm to the nucleus where initial induction of mitochondrial genes can take place, without an increase in PGC-1α protein *per se* (140). It should be noted that in cell lines some have suggested that PGC-1α is in the nucleus, and thus some controversy of its location remains. Secondly, there is an increase in PGC-1α mRNA (within 2-6 hours following muscle activation) (1; 95; 125) which is then followed by an increase in PGC-1α protein, peaking between 6-18 hours after muscle activation (4; 124; 140). As Wright and colleagues elegantly demonstrated (140), both binding of transcription factors to nuclear genes encoding mitochondrial proteins and increases in mRNA of mitochondrial proteins occurs prior to increases in PGC-1α protein, explaining how mitochondrial adaptations can occur before PGC-1α protein is substantially elevated (i.e. 1-6 hours following exercise). This is also an example of the complexity of the actions of PGC-1α and the challenges in interpreting PGC-1α protein data as it relates to activation and execution of mitochondrial biogenesis. Adding to this complexity is a very recent study that showed that PGC-1α knock-out mice displayed normal increases in mitochondrial gene expression following exercise (69) suggesting that PGC-1α is not necessary for exercise-induced adaptations and that likely there are redundant mechanisms present for exercise to increase mitochondrial content.

In terms of mitochondrial biogenesis, the primary role of PGC-1α is as a co-factor to bind to and activate nuclear transcriptional factors (eg. nuclear respiratory factors-1 and -2, NRF-1, NRF-2; peroxisome proliferator-activated receptor-γ, PPARγ) which then induce gene expression for mitochondrial proteins (101; 102; 143). In a similar and co-ordinated

fashion (40; 58), mitochondrial transcription factor A (Tfam) is also activated in the nucleus followed by import into the mitochondria and mitochondria DNA (mtDNA) binding. This leads to mtDNA transcription and replication (66), an increase in gene expression and finally increases in mitochondrial protein expression (40). Much like the action of PGC-1α, the first events upon activation are a translocation, then a binding and activation of co-receptors initiating the activation cascade, which is then followed by an increase in Tfam gene expression and subsequent protein expression (Figure 1.2). This is an important event because some of the mitochondrial proteins are encoded by genes in the mitochondria (i.e. not the nucleus), and this allows for a coordinated activation of nuclear genes (*via* NRF-1, NRF-2) and mitochondrial genes (*via* Tfam), as is presumably necessary to build functional mitochondria (40; 66). After a single bout of exercise, provided the stimulus is sufficient, PGC-1α mRNA and protein will peak and then return to baseline levels by approximately 24 hours following exercise. However, if exercise is continued long-term (i.e. several weeks), PGC-1α will remain elevated, or continue to rise, as will mitochondrial protein content (123).

As mentioned earlier, exercise is a potent stimulus that increases PGC-1α and mitochondrial biogenesis, at least in young muscle. Improvements in aerobic capacity (VO_2 max) (11; 45; 79; 84) and mitochondrial enzyme activity (11; 20; 144) with exercise have been observed in late middle age, suggesting that at this age skeletal muscles are capable of adapting similarly to young muscle. However, few studies have investigated the adaptive response of exercise at senescence (>70yrs). While there have been some observed benefits of exercise at this age, several of these studies have suggested a lack of muscle plasticity at senescence (13; 32; 75; 94; 118). The study by Ehsani et al. (32) observed small increases in whole body VO_2 max that were explained by increases in cardiac output with no change in the muscle's ability to utilize oxygen (no change in arterio-venous O_2 difference). The study by Orlander and Aniansson (94) observed increases in mitochondrial enzyme activity in the absence of increases in mitochondrial content, suggesting the adaptation occurred within the existing mitochondrial framework. This study in particular led us to hypothesize that senescent muscle may not be able to

increase mitochondrial content and this may be due to an inability to induce PGC-1α. To date, no one has measured PGC-1α in senescent muscle following exercise training. Also, as with many exercise studies in the literature and in particular these two previously mentioned studies with aging, the reported data is usually VO_2 max or mitochondrial enzyme activity, but rarely both. This forces readers to try to integrate and interpret results from various different studies to understand the changes (or lack of) in mitochondrial biogenesis and how they relate to muscle oxidative capacity. For example, the study of Ehsani et al. (32) did not report mitochondrial enzyme activity and the study by Orlander and Aniansson (94) reported changes in mitochondrial enzyme activity with no information on how this impacted the overall capacity of the muscle (i.e. Did this result in an improvement in muscle VO_2 ?). Therefore, to describe the changes (or lack of) with exercise in senescent muscle, we wanted to include functional measures, muscle specific aerobic capacity (*in situ*), mitochondrial capacity, mitochondrial content as well as gene and protein expression of the mitochondrial biogenesis pathway to generate an comprehensive description of the effect of exercise at old age on the muscle's ability to increase aerobic capacity.

1.6 OBJECTIVES AND DESCRIPTION OF STUDIES

The overall objective of this thesis project was to better understand the effect of aging on mitochondrial biogenesis, both in terms of basal conditions and in response to stimuli that normally induce mitochondrial biogenesis in young muscle. We were interested in the pathway of inducing mitochondrial biogenesis (i.e. PGC-1α regulation), to its downstream effectors and ultimately mitochondrial content and mitochondrial function. We used two methods, long-term exercise via treadmill running and acute muscle injury invoking immediate muscle regeneration, both of which are known to activate PGC-1α and induce mitochondrial biogenesis in young muscle (31; 123).

Long-term exercise training involved running on a treadmill 4-5 times per week for 5-7 months starting at late middle age (29 mo, 65% survival for F344BN rat) and continuing until senescence (34 and 36 mo, <50% survival). The importance of this age

range is two-fold. Principally, the declines in muscle aerobic capacity and mitochondrial enzyme activity from young adulthood until late middle age are minimal (<20%), whereas more severe declines are observed between late middle age and senescence (~50%) (46). Thus the latter part of the lifespan is the most relevant to focus on attenuating the declines in aerobic function. Secondly, we have previously shown that 7 weeks of treadmill running at late middle age was effective in restoring muscle aerobic function and mitochondrial enzyme activity to typical levels observed in young adulthood (11). If we would like to consider exercise as a modality to combat the age-related declines in muscle function, it is necessary to extend these findings by continuing the exercise into senescence to determine if, and to what extent, long-term exercise can attenuate the dramatic declines that are observed after late middle age.

The second study employed a short-term model of muscle adaptation (in contrast to long term exercise training) to assess the effects of age on acute induction of mitochondrial biogenesis. Muscle regeneration following acute damage requires functional mitochondria in order to supply the energy necessary to regenerate and re-build the muscle. Duguez et al. (31) found that starting from days 5-35 following bupivacaine injection (muscle damage), there was a measureable increase in differentiation of satellite cells into muscle fibres. Responses in the early phase (day 5-10) included: a) a rise in PGC-1α mRNA and Tfam protein peaking around day 10; and b) increases in mitochondrial content and state III respiration that reached control levels by day 10. This suggests that mitochondrial biogenesis and function are necessary to allow for differentiation and muscle growth. Most of the regeneration was complete around 21 days after muscle damage, as demonstrated by similar muscle mass (9; 31) and muscle fibre-specific force (43) compared to contralateral control muscles. Thus, the fact that mitochondrial biogenesis and function are induced much earlier, and reach control levels much earlier than complete muscle restoration, is further support for the necessity of functional mitochondria early in the regrowth phase. On this premise, we used cardiotoxin, a snake venom that induces rapid and severe fibre necrosis (excess Ca^{2+} release) (28), to determine if mitochondrial biogenesis is blunted in aged muscle compared to young adult, following acute muscle damage and regeneration.

Tibilalis anterior muscle was injected and subsequently harvested 21 days following injection. A sub-group of senescent animals had their tibialis anterior muscle harvested only 10 days after muscle damage to assess the early time course of PGC-1α induction and mitochondrial biogenesis.

In general, the principle measures used were :

- Muscle specific VO_2 max as a functional measure of whole muscle aerobic capacity;
- Citrate synthase (CS) and cytochrome c oxidase (COX) activities as markers of mitochondrial oxidative capacity;
- CS and COX protein as markers of mitochondrial content;
- PGC-1α as an assessment of the signalling for mitochondrial biogenesis;
- NRF-1 and Tfam as downstream drivers of mitochondrial gene expression.

There are a few key strengths of this study that merit particular attention. Firstly, *in vitro* assessment of muscle oxidative capacity (CS and COX activity) was combined with *in situ* muscle aerobic function (VO_2 max) as a means of evaluating effective changes at the mitochondrial level compared to the whole muscle level. Secondly, the pathway from induction of PGC-1α right through to the end of the pathway (mitochondrial content) was measured. In theory, positive adaptations by means of mitochondrial biogenesis should be accompanied by improvements in whole muscle VO_2 max. Thirdly, measures of both enzyme content and enzyme activity (for the same enzymes) were obtained, to be able to address the issue of changes in activity relative to changes in total protein content (i.e. mitochondrial function). Finally, functional measures such as body composition, exercise capacity and muscle function were assessed, to relate changes with long-term exercise to functional, realistic benefits that could be translated to the human population.

1.7 SURVIVAL RATES AND RELATIVE AGES OF F344BN ANIMAL MODEL COMPARED TO HUMANS

One of the challenges with aging research is normalizing ages to some reference point from which comparisons can be made. Even different strains within a species have different survival rates and survival characteristics. For the benefit of this thesis, we will use the survival rates of humans (3) and the Male Fisher 344 x Brown Norway (F344BN) of rats (128) that we will be using in our experiments to compare these two species. In general, young adult refers to maturity with 100% survival rate, late middle age refers to a survival rate of ~70% and senescence refers to a survival rate of less than 50%, although our experiments will use an age in which the survival rate is ~35% (Table 1.1). The F344BN hybrid strain of rat is a suitable model to study aging because it has a relatively low incidence of age-related pathologies (73), has known and profound reductions in skeletal muscle mass and contractile function (18; 137) and there are published survival curves for this strain of rat (128). Further benefits of this model for aging research are described in a recent publication (11).

Table 1.1 Classification, survival rates and corresponding age ranges for male F334BN rats that will be used in the proposed experiments, and relative ages for male humans.

Age Classification	Survival rate (%)	F344BN rat (male) (age in months)	Human (male) (age in years)
Young adult	100%	8-12	18-30
Late middle age	70%	28-30	60-68
Old	55%	32	70-73
Senescent	35%	35	82-85

Ages are based on survival curves for male F334BN rats (128) and human males (3).

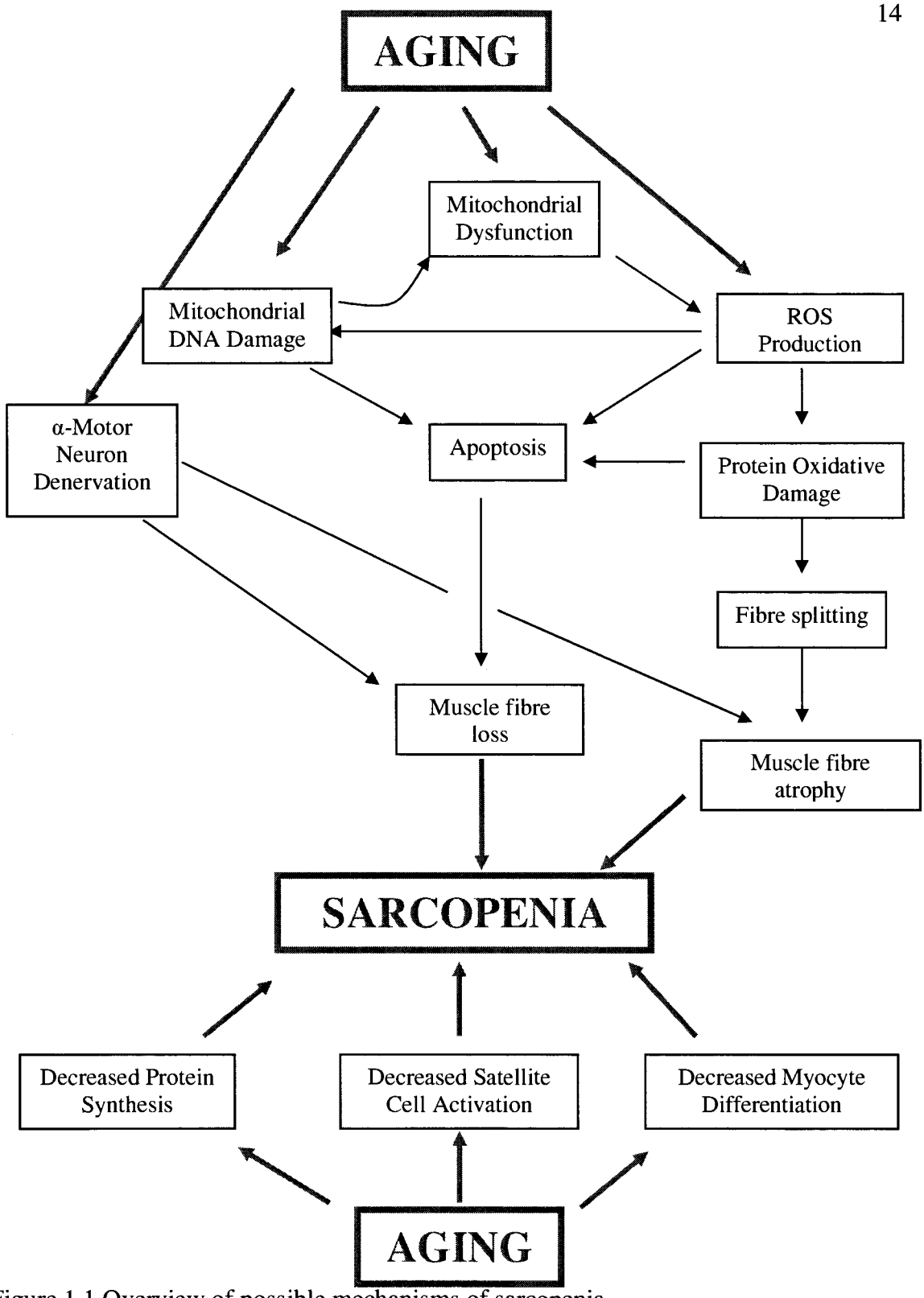

Figure 1.1 Overview of possible mechanisms of sarcopenia.

ROS = Reactive oxygen species

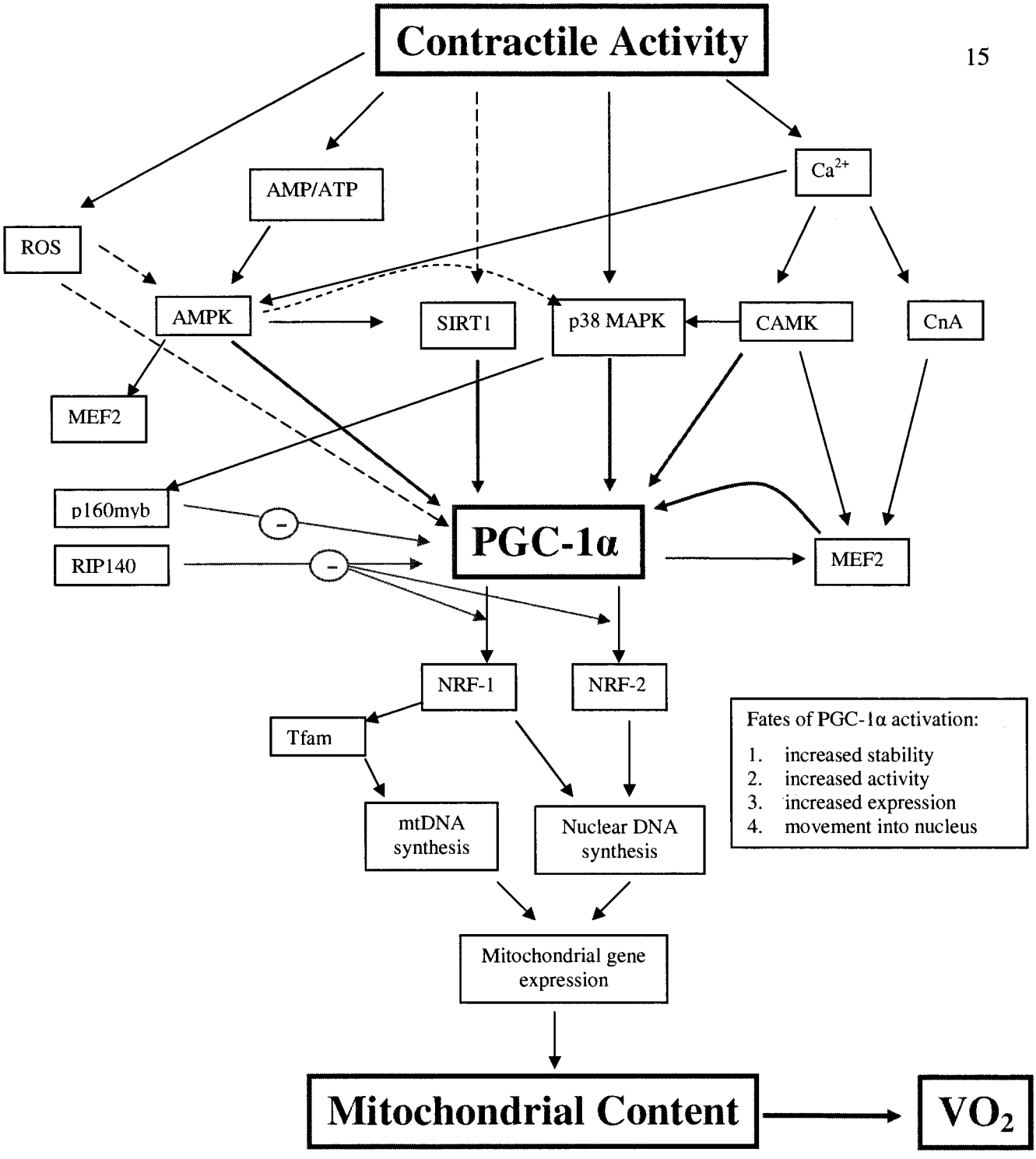

Figure 1.2 Schema of events coordinating contractile activity to PGC-1α activation and subsequent mitochondrial biogenesis.

ROS = Reactive Oxygen Species; AMPK= AMP activated protein kinase; CAMK=Ca^{2+}/calmodulin dependent protein kinase; CnA = calcineurin; SIRT1 = silent information regulator 2 homolog 1; p38 MAPK =p38 mitogen activated protein kinase; MEF2=myocyte enhancer factor 2; PGC-1α = peroxisome proliferator-activated receptor-γ coactivator-1α; NRF = nuclear respiratory factor; Tfam = mitochondrial transcription factor A; mtDNA = mitochondrial DNA

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Chapter Two: EXERCISE TRAINING FROM LATE MIDDLE AGE UNTIL SENESCENCE DOES NOT ATTENUATE THE DECLINES IN SKELETAL MUSCLE AEROBIC FUNCTION

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2.1 ABSTRACT

We previously showed that 7 weeks of treadmill exercise training in late middle aged rats can reverse the modest reductions in skeletal muscle aerobic function and enzyme activity relative to values in young adult rats (Exp Physiol. 93.7: 863-871, 2008). The purpose of the current study was to determine if extending this training program into senescence would attenuate the accelerated decline in the muscle aerobic machinery normally seen at this advanced age. For this purpose, 29 mo old Fisher 344 Brown-Norway rats underwent 5 or 7 months of treadmill exercise training. Training resulted in greater exercise capacity during an incremental treadmill exercise test and reduced percent body fat in 34 mo old and 36 mo old rats, and improved survival. Despite these benefits at the whole body level, *in situ* muscle aerobic capacity and muscle mass were not greater in the trained groups at 34 mo or 36 mo of age. Similarly, the trained groups did not have higher activities of citrate synthase or complex IV in homogenates of either the plantaris (fast twitch) or the soleus (slow twitch) muscles at either age. Finally, protein expression of citrate synthase (a marker of mitochondrial content) and PGC-1 (relating to the drive on mitochondrial biogenesis) were not higher in the trained groups. Therefore, although treadmill training from late middle age into senescence had significant benefits on running capacity, survival and body fat, it did not prevent the declines in muscle mass, muscle aerobic capacity or mitochondrial enzyme activities normally seen across this age, revealing a markedly diminished plasticity of the aerobic machinery in response to endurance exercise at advanced age.

Key Words: aging, oxygen uptake, sarcopenia, mitochondria, endurance training

2.2 INTRODUCTION

Aging is accompanied by declines in skeletal muscle mass (13; 24), contractile function (12; 13; 61) and aerobic metabolic capacity (14; 16; 29) that contribute to health

problems, impaired mobility and reduced quality of life. Furthermore, the declines in muscle mass (13; 29; 40) and muscle function (29; 33) accelerate dramatically between late middle age and senescence, both of which play an important role in the increased risk of frailty in advanced age (23). Age-related declines in physical activity do not entirely explain the decreases in aerobic capacity at the whole body level, since chronically trained older individuals and Masters athletes still show evidence of a decline with aging (27; 39; 46-48; 62). Nonetheless, many studies in middle to late middle aged individuals have shown a maintained capacity to increase whole body maximal oxygen uptake (VO_2 max) (28; 42; 43) and mitochondrial enzyme activity (14; 64) following exercise training relative to young adults. Very few studies have examined whole body aerobic responses to endurance training in very advanced age (≥80 y of age; senescence) (20; 41), and none has assessed adaptability of the skeletal muscle aerobic machinery specifically. Since improvements in muscle and whole body VO_2 max are accompanied by increases in mitochondrial enzyme activity in both young adult and late middle aged muscle (9; 14; 17), it is important to establish the efficacy of endurance training on the skeletal muscle aerobic machinery at these advanced ages because such adaptations are important to whole body aerobic capacity and mobility in the elderly.

The Fisher 344 Brown-Norway F1 hybrid rat (F344BN), a popular rodent model for studying skeletal muscle aging because it lives long enough to experience sarcopenia in a pattern similar to humans (40), experiences hindlimb muscle mass declines of 15-20% between young adult and late middle age (28 mo), but more drastic declines (a further 30-60%) between late middle age and senescence (36 mo, 35% survival) (13; 29). Similarly, declines in peak tetanic force (13) and muscle mass-specific VO_2 (29) are modest (< 20%) until late middle age, but decline 30-50% between late middle age and senescence. Given the fact that age-related declines are most severe after late middle age, it is critical that we focus our efforts in this part of the lifespan to combat these effects.

Recently, we showed that 7 weeks of treadmill training of 28 mo old male F344BN rats resulted in a 20% increase in muscle mass-specific VO_2 max and ~25% increase in

mitochondrial enzyme activity, illustrating that at this age, this rat strain remained sufficiently responsive to a training program to restore skeletal muscle aerobic function to young adult levels (9). The efficacy of a prolonged exercise program from late middle age until senescence on muscle aerobic function and mitochondrial oxidative capacity is unknown. The purpose of this study was to determine if a program of exercise training starting at late middle age and continuing into senescence would better maintain skeletal muscle aerobic function and mitochondrial oxidative capacity. Based on our previous results showing treadmill exercise increased running capacity, muscle mass-specific VO_2 max and mitochondrial enzyme activity at late middle age (9), we hypothesized that the same exercise training program executed from late middle age until senescence would result in a higher running capacity, muscle mass-specific VO_2 max and muscle mitochondrial enzyme activity compared to sedentary control animals.

2.3 METHODS

2.3.1 Animals.

57 F344BN male rats (29 mo of age) were acquired from the National Institute of Aging (Bethesda, MD). All rats were allowed food and water *ad libitum* and were housed in the same room, with 2-3 animals per cage. 24 rats were randomly assigned to the training group and the remaining 33 rats were allocated to the sedentary control group. These sample sizes were chosen on the basis of several criteria. Firstly, because the decline in muscle mass and function rapidly accelerates between 29 and 36 mo of age in the F344BN rat (29), and because we wished to obtain insight into the trajectory of changes between these ages, we determined that we would subsample a group of rats for hindlimb contractile and metabolic performance, and *in vitro* muscle analyses at 34 mo of age (9 in each group), with the remaining animals studied at 36 mo of age (11 in the control group, 9 in the trained group). Secondly, our sample size was based upon survival curves for this strain of rat (63) and the expectation that there would be greater survival rates in the trained group since exercise has been shown to prolong mean lifespan (34; 35). All experimental procedures were approved by the University of Calgary Animal Care Committee.

2.3.2 Food Intake and Body Mass.

To provide an estimate of food intake per animal, food consumption (grams) was measured weekly for each cage and divided by the number of animals in that cage. Body mass was also measured weekly throughout the entire experiment.

2.3.3 Graded Exercise Test.

All animals were habituated to the treadmill (Columbus Instruments, Columbus, OH, USA) over five consecutive days, by walking on the treadmill (10% grade) twice per day for 5 min at 5 $m \cdot min^{-1}$. After this time, all animals performed a graded exercise test (GXT) to voluntary exhaustion which consisted of starting at 5 $m \cdot min^{-1}$ for one minute and then increasing the speed by 1 $m \cdot min^{-1}$ each minute until the animal could no longer keep up with the treadmill speed and continuously sat on the shock grid. This protocol was performed on all rats after 7 weeks (31 mo of age), 5 months (34 mo of age) and 7 months (36 mo of age), except that the 34 and 36 mo old rats began at 4 $m \cdot min^{-1}$ because some of them had difficulty at 5 $m \cdot min^{-1}$.

2.3.4 Training.

Training was modeled after our recent study in late middle aged F344BN rats showing that 7 weeks of treadmill running increased mass specific VO_2 max and mitochondrial enzyme activity by 20-25% (9). Specifically, the training program consisted of running on the treadmill 5 days per week and progressively increasing the duration so that by week 3 the animals were running for 60 min per day (10% grade). Each training session was broken down into 6 bouts of 10 min with 2 min rest in between bouts. Within the 10 min segments, 8 min was performed at a base velocity and a higher velocity was used for the last 2 min. The base velocity was slowly increased from 5 $m \cdot min^{-1}$ to 7 $m \cdot min^{-1}$ at week 3, with increments of 0.5 $m \cdot min^{-1}$ each week until the animals could not tolerate an increase in velocity (coincided with week 9). The 2 min interval was performed at 2.0 $m \cdot min^{-1}$ faster than the base velocity. After 8 weeks the training sessions were reduced to 4 days per week with the goal being to maintain any adaptations that occurred during the

initial training sessions and to allow more recovery time as the animals aged into senescence.

2.3.5 Surgical Procedures.

Muscle mass specific VO_2 max was assessed by a hindlimb pump perfusion technique that permits matching skeletal muscle O_2 delivery for each animal so that differences in aerobic capacity reflect differences in the contracting muscles (7; 37). Animals were anesthetized (following at least 2 d rest after last exercise bout) with sodium-pentobarbital (50-65 $mg \cdot kg^{-1}$ i.p.) and supplemented with 10 $mg \cdot kg^{-1}$ as necessary throughout the surgical procedure. Body composition was measured in each animal by dual-energy x-ray absorptiometry (Hologic, Bedford, MA). Muscles from the right lower limb and quadriceps were removed, weighed and frozen in liquid nitrogen. The mass of the distal hindlimb muscles was used for the calculation of muscle blood flow for the perfusion experiments on the left leg. The left lower limb was prepared for hindlimb perfusion using a similar method to that described previously (31; 37), with the exception that the femoral artery and vein were surgically isolated to permit perfusion of only the distal hindlimb, as described by Baker *et al.* (8). All branching vessels from the femoral artery and vein prior to their entry into the gastrocnemius muscle were ligated with silk thread or cauterized. The portion of the Achilles tendon originating from the soleus muscle was separated from that of the gastrocnemius-plantaris muscles, and each portion of tendon was attached to individual force transducers (FT-10, Grass Instruments, West Warwick, RI) with 6.0 non-compliant silk thread such that force development could be independently measured in the soleus muscle versus the gastrocnemius-plantaris muscles. The femoral artery and vein were cannulated in the femoral area with Intramedic™ Polyethylene tubing (I.D. 0.58 mm, Becton Dickinson, Sparks, MD) that was advanced distally in each vessel to the apex of the gastrocnemius muscle to ensure the perfusate was going directly into the lower limb (8). As previously reported (29), the perfusion medium consisted of isolated bovine erythrocytes reconstituted with Krebs-Henseleit bicarbonate buffer containing 4% bovine serum albumin, 5mM glucose, 100 $mU \cdot ml^{-1}$ insulin, 1000 $mU \cdot ml^{-1}$ heparin, 0.15 mM pyruvate to achieve a hematocrit of ~45%. Once the cannulation was complete, the distal

hindlimb was perfused (0.5 $mL \cdot min^{-1}$), by a peristaltic pump (Gilson Minipuls 3, Villiers Le Bel, France) and the animal was sacrificed via cardiac removal. The sciatic nerve was cut proximal to the gastrocnemius muscle and was placed over a hook electrode to provide stimulation of the muscles in the distal hindlimb only. The hindlimb was loosely wrapped in saline-soaked gauze, cellophane and aluminum foil and was kept at 37°C with a heat lamp and thermistor probe that was inserted inside the cellophane. A pressure transducer (PT-300 Grass Instruments), in-line with the hindlimb perfusion apparatus, was positioned at the height of the hindlimb muscles to determine total perfusion pressure. Net pressure was calculated by subtracting the pressure through the arterial tubing from the total pressure recorded during the muscle contractions.

2.3.6 In situ Aerobic Capacity.

After at least 30 min of perfusion, perfusate flow was incrementally increased over a 10-12 min period to the desired flow rate, which was calculated from the masses of the entire distal hindlimb of the right leg to ensure similar mass-specific muscle blood flow for each animal. After 2 min at the peak perfusate flow, an arterial and venous resting blood sample was collected and the muscle length was adjusted to yield maximal force. The distal hindlimb muscles were stimulated (Grass S48, Grass Instruments, Warwick, RI) via the sciatic nerve with square wave electrical pulses (200 ms trains, 0.05 ms duration, 100 Hz) to induce tetanic contractions at a rate of 7.5, 15, 30 and 60 $tetani \cdot min^{-1}$ for one min each, and 90 $tetani \cdot min^{-1}$ for 2 min to elicit a maximal VO_2 response, as reported previously (32). Force from the soleus and gastrocnemius-plantaris muscles, and total pressure were recorded continuously (DATAQ DI-720, DATAQ Instruments, Akron, OH). Venous blood was sampled every 30 s throughout the contraction bout and was analyzed for PO_2, PCO_2, O_2 saturation (SO_2), hematocrit and hemoglobin concentration by a blood-gas analyzer (Rapidlab 865, Siemens, Deerfield, IL). Blood oxygen content was calculated using the formula: $[O_2] \times SO_2 \times 1.39 + 0.003 \times PO_2$. VO_2 across the distal hindlimb was calculated as the product of the rate of blood flow (held constant) and the arterio-venous O_2 content difference, and was normalized to the mass of the contracting muscles of the distal hindlimb.

2.3.7 Biochemistry.

The plantaris (primarily fast twitch) and soleus (primarily slow twitch) muscles were chosen based upon their contrasting phenotypes as representatives of the distal hindlimb musculature (3) and because these muscles adapt in response to treadmill running in rats (44). Citrate synthase and complex IV enzyme activities were assessed in crude homogenates of muscle, as we have done previously (30). The entire muscle was pulverized with mortar and pestle under liquid nitrogen and an aliquot of this powder was homogenized 1:20 (mass/volume) with a potassium phosphate buffer (pH 7.5). The homogenate was freeze-thawed three times to rupture cellular and mitochondrial membranes, centrifuged (900 G, 10 min, 4°C) and the supernatant aliquoted into eppendorf tubes. For citrate synthase activity, this homogenate was further diluted with the homogenizing buffer to achieve a final concentration of 1:400. Citrate synthase activity was measured according to the method of Srere (58). The rate of mercaptide ion formation was measured spectrophotometrically (412nm wavelength, 37°C; DU 800 Spectrophotometer, Beckman Coulter) for 3 min after the addition of 100 μL of homogenate, 650 μL Tris Buffer (pH 8.0), 50 μL 3mM Acetyl CoA, 100 μL 1 M DTNB and 100 μL of 0.5 mM oxaloacetate. Complex IV activity was determined by measuring the rate of cytochrome *c* oxidation spectrophotometrically (550nm, 37°C) for 3 min after the addition of 4 μL of homogenate, 30 μL of 1 mM reduced cytochrome *c* to 970 μL of 10 mM potassium phosphate buffer (pH 7.0, 37°C). Each sample was measured in duplicate and enzyme activities determined from the average change in absorbance over time and was normalized to the amount of tissue added to the cuvette.

2.3.8 Protein Expression.

Frozen powder aliquots of plantaris and soleus muscle samples were mechanically homogenized (1 part muscle powder to 9 parts buffer) in an extraction buffer containing 50 mM Tris-HCl, 250 mM mannitol, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100 with the following inhibitors added just prior to use (1 mM DTT, 1 mM benzamidine, 0.5 mM AEBSF, 100 uM leupeptin, 1 ug/ml soybean trypsin

inhibitor). The samples were left on ice for 30 min, followed by centrifugation for 10 min at 700 g. Supernatants were removed and placed into an eppendorff tube and protein concentration was determined using a Bradford assay (11). Samples were then diluted by half with 2x sample buffer containing Glycerol, Tris pH 6.8, SDS, DTT, Bromophenol Blue and β-mercaptoethanol. Equal quantities of protein for each sample, and a pre-stained marker (Fermentas), were loaded onto 10 % SDS-PAGE mini-gels and separated by electrophoresis at 100 V for 90 minutes. Proteins were then electro-transferred to nitrocellulose membranes in a transfer buffer containing 48 mM TRIS, 39 mM Glycine, and 20 % methanol at 100 V for 80 minutes. Blots were blocked for 1 hr at room temperature with 5 % non-fat milk PBS-0.05 % Tween solution, followed by overnight incubation (4°C) with antibodies against peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (1:1000, Calbiochem), citrate synthase (1:10,000, kind gift from J. Holloszy) and α-actin (1:2000, Santa Cruz). The following day, blots were washed three times for 15 min each with PBS-0.05 % Tween and probed with an appropriate HRP-conjugated secondary antibody (1:2000, Pierce, Rockford, IL) for 1 hr at room temperature. Blots were washed a further two times for 15 min each with PBS-0.05 % Tween, a final wash of PBS (no Tween) and then treated with chemilluminescent developing solution (Pierce). Chemiluminescence was digitally captured (Syngene Bio-Imager, Frederick, MD) and densitometry measured using the Bio-imager software (Syngene Tools, Frederick, MD). Protein data was normalized to α- actin, and expressed relative to the 34 mo sedentary group.

2.3.9 Statistics.

Comparisons within groups over time were performed with one-way repeated measures ANOVA. Comparisons of food intake between groups over time were assessed by a two-way ANOVA (Training, Time), with a Student-Neuman Keul post-hoc multiple comparison test. All other comparisons were by two-way ANOVA (Age, Training), with a Student-Neuman Keul post-hoc multiple comparison test. Values are expressed as means ± SE.

2.4 RESULTS

2.4.1 Body and Muscle Masses.

Body mass was lower in the trained groups compared to the control groups at 31, 34 and 36 mo (Figure 2.1). This was largely due to the lower body fat percentage in the trained animals versus the control animals, with no difference between the 34 and 36 mo old age groups within either trained or sedentary animals. It was not possible to obtain body composition at 29 or 31 mo. However, previous results in our lab found the percent body fat in 30 mo old sedentary animals to be 31 ± 1% (D.J. Baker, unpublished), meaning that the trained rats halved their body fat with training, whereas there was little change in the sedentary group. The body mass in the 36 mo trained animals was significantly lower than the 34 mo trained animals (406 ± 13 and 464 ± 13 g, respectively) due to lower muscle and fat masses, since the body fat percentage was the same (Table 2.1). While only the masses of the distal hindlimb muscles are reported here, significantly smaller muscles masses were observed in the 36 mo trained group compared to the 36 mo control group for the gastrocnemius, plantaris, soleus, tibialis anterior, extensor digitorum longus, adductor longus and rectus femoris muscles (A.C. Betik and M.M. Thomas, unpublished). With the exception of the 31 mo old control group, the initial body mass (29 mo) of both groups was significantly higher than all other groups and time points (583 ± 6 g).

2.4.2 Food Consumption.

Absolute food consumption in the trained group tended to be slightly lower than the control group throughout the entire experiment. However, when food consumption was normalized to body mass, food intake was actually higher at some time points in the trained animals (Figure 2.2).

2.4.3 Survival.

The survival was tracked from the beginning of the study (29 mo of age) until 34 mo of age, at which point some animals were culled from both groups for muscle

performance, biochemistry and molecular measurements. The trained group had a higher rate of survival (82%) compared to the control group (52%) between 29 and 34 mo of age.

2.4.4 Graded Exercise Tests.

Treadmill training improved performance on the GXT at 31 mo, with no change in the control group. After 7 weeks of training, the trained group increased exercise endurance time by 25% (9.2 vs 7.4 min for pre-training versus 7 week training points, respectively) and peak velocity by 17% (13.7 vs 11.8 $m \cdot min^{-1}$ for pre-training versus 7 week training points, respectively). However, performance declined in the trained group at 34 and 36 mo compared to pre-training (36 mo compared to 29 mo, velocity -18%, total time -12%) but was always better than the control group (Figure 2.3). There was no difference in the control group after 7 weeks, but their performance was lower at 34 and 36 mo, declining by ~35% in peak velocity and total time at 36 mo compared to 29 mo.

2.4.5 In situ Aerobic Capacity.

In situ aerobic capacity was assessed using a modified hindlimb perfusion model in which the muscles of the lower limb were perfused at rates proportional to their muscle masses between groups at a given age, such that convective O_2 delivery was matched at a given age between control and trained groups (Table 2.2). At both 34 and 36 mo of age, muscle VO_2 max did not differ between the trained and sedentary groups (34 mo: 212 ± 31 vs 220 ± 21; 36 mo 212 ± 39 vs 209 ± 19 $\mu mol \cdot min^{-1} \cdot 100g^{-1}$ for control and trained groups respectively, Figure 2.4). The response to the increasing frequency of the contractions was the same between all groups, resulting in a peak VO_2 between 4 and 4.5 min. Similarly, there were no differences between the control and trained groups in peak lactate concentration, peak specific force of the gastrocnemius-plantaris complex, peak specific force of the soleus muscle, or the force at the end of the stimulation protocol for either muscle, in support of a lack of training benefit for VO_2 max (Table 2.3).

2.4.6 Citrate Synthase and Complex IV Activity.

For the plantaris muscle, CS activity normalized to muscle mass was lower in the trained groups (Figure 2.5A), but this difference was eliminated after normalizing to muscle protein (Figure 2.5C). For the soleus muscle, there was no difference in CS activity between trained and control groups at 34 or 36 mo at both the whole muscle level (Figure 2.5B) and after normalizing to muscle protein. Within a given group there were no differences for CS activity for either muscle between 34 and 36 mo of age.

Complex IV activity was not different between control and trained groups at 34 and 36 mo of age in either muscle and this was the same regardless of whether the activity was expressed relative to whole muscle mass or muscle protein. As for CS activity, within a given group there was no difference between 34 and 36 mo for either muscle (Figure 2.6).

2.4.7 CS and PGC-1 Protein Content.

CS protein content was not different between trained and sedentary control groups for either muscle, regardless of age (Figure 2.7 A+B). Only in the plantaris muscle was there an effect of age, as CS protein was lower ($p<0.05$) in the 36 mo control group compared to the 34 mo control group only (not seen in trained groups). Similarly, PGC-1 protein content in plantaris muscle was not different in the trained groups compared to the control groups, regardless of age. On the other hand, for the soleus muscle, PGC-1 protein was lower in the 36 mo trained group compared to 36 mo control group ($p<0.05$). Within each group there was no difference in PGC-1 protein between 34 and 36 mo old animals for either muscle (Figure 2.7 C+D). There was no effect of age or training on actin protein levels (Figure 2.8 A+B).

2.5 DISCUSSION

The purpose of this study was to determine the efficacy of a chronic exercise program starting in late middle age and continuing into senescence on the aerobic function of skeletal muscle. Since this period of the lifespan is associated with accelerated declines in muscle mass and mass-specific oxidative capacity (29), it was hypothesized that regular

exercise would attenuate the declines in whole body exercise capacity, muscle oxidative enzyme activity, muscle VO_2 max and muscle mass. Previously, we have shown that 7 weeks of the same training program at late middle age increased mitochondrial enzyme activity and muscle VO_2 max (20-25%). Our current results show that while body fat was lower in the trained groups and whole body exercise capacity was higher at all time points after the initiation of training in the trained animals compared to the sedentary groups, whole body exercise capacity still declined (after an initial improvement over the first 2 mo of training) in the trained groups, and neither 5 mo nor 7 mo of training benefited muscle oxidative enzyme activity, muscle VO_2 max or muscle mass in this critical period of the lifespan. Indeed, muscle mass declined to a greater extent in the trained animals by 36 mo of age.

The current study design differs from our previous study (9) in that we reduced the training frequency from 5 days per week to 4 days per week after the initial 8 weeks of training. As such, it is possible that the reduction of weekly training frequency could account for some loss of training benefits after the initial 8 week period (at which point it is reasonable to assume the muscle adaptations were similar to our previous study because the training stimulus was identical). However, it seems highly unlikely that this alone would completely abrogate the training benefits for the skeletal muscle aerobic machinery, particularly relative to the sedentary control animals which were cage-bound and had no training stimulus. That the trained animals maintained a significantly lower % body fat and superior whole body running performance versus the sedentary controls to the end of the study supports this view. Therefore, we have combined measurements at the whole body level, muscle level and cellular level to demonstrate a diminished plasticity of the skeletal muscle aerobic machinery in response to endurance exercise training in very old age and to underscore the need to identify effective interventions at this advanced age.

2.5.1 Systemic Responses to Endurance Training

Despite the lack of improvement in muscle VO_2 max, there are other measures that demonstrate the exercise was beneficial. Firstly, the higher exercise intensity and longer

exercise duration in the graded exercise test for the trained animals demonstrates a higher capacity for exercise. The higher exercise capacity at 34 and 36 mo despite no difference in muscle mass specific VO_2 max is likely explained by the lower body mass of the trained animals. The trained animals had ~ 20% less body mass to support while treadmill running, meaning that at the whole body level, the same muscle VO_2 max would translate to a higher VO_2 max per body mass in the trained animals. However, despite significant improvements in running capacity after 2 mo of training, between 31 and 36 mo of age the declines in the training group (-27%) were of a similar rate as the control group (-33%), suggesting that exercise cannot prevent the aging-related decline at this critical period of the life span, and that the decline during this period is due to aging processes. This is in support of human studies that have tracked chronically trained and/or Masters athletes and observed a decline in whole body aerobic and running capacity with age despite a maintained exercise program (39; 47; 48; 62). A second benefit of the training program was that it improved life expectancy, as there were fewer deaths in the trained group over the 5 mo of training from 29 to 34 mo of age. An improvement in mean lifespan has been shown in male and female rats that voluntarily exercised (running wheel in cage) throughout their entire lifespan (34; 35), and in humans who are habitually active (2). Significantly, this study demonstrates that improvements in survival can be achieved even if the training only begins at late middle age. A third benefit of the exercise training program was a lower body fat percentage. Specifically, the trained rats demonstrated a body fat that was about half that seen in sedentary control animals, a change that likely has positive implications on health risk factors that are related to obesity (e.g., insulin resistance, cardiovascular function, atherosclerosis). For example, surgical removal of adipose tissue via liposuction improves whole body insulin sensitivity (26; 51). In our study, the trained rats did not increase their food intake sufficiently to maintain body mass, a typical response of male rats that are chronically exercising (18; 35). Although absolute food intake was slightly lower in the trained animals, when normalized to body mass (since body mass was lower in the trained groups at all points after the initiation of training), food intake was not significantly different from control animals. Therefore, the higher exercise capacity, greater survival and lower body fat show valuable benefits from the exercise program.

2.5.2 Impact of Endurance Training on Skeletal Muscle Aerobic Function

There are many human (28; 43; 45; 54), and rodent studies (14; 53; 64) that have shown beneficial improvements from endurance exercise training in late middle age, both in terms of whole body aerobic capacity and muscle mitochondrial enzyme activity. We have also previously shown that treadmill exercise training in late middle aged F344BN rats can restore muscle aerobic function and enzyme activity levels to young adult levels (9). Only a few studies have investigated endurance or resistance exercise training effects in very old humans (≥79 yrs) and although there are clear benefits to exercise training (22), overall these studies suggest that in very old age (20; 41; 57) and in frail elderly (21; 25) there is a diminished capacity for adaptation at these advanced ages. For example, Slivka and colleagues recently demonstrated a complete lack of adaptation to resistance training at the single fiber level in men ≥ 80 years of age (56). Blough and Linderman (10) also observed an inability of 36 mo old F344BN rat plantaris muscle to adapt to a functional overload (gastrocnemius muscle ablation) in which young adult rats experienced a 50% increase in muscle mass, again suggesting an impaired muscle adaptability at very old age. It is noteworthy, however, that no prior studies have examined the plasticity of the muscle aerobic response specifically to endurance exercise training in the transition from late middle age to senescence. We have previously shown that exercise training in late middle aged F344BN rats was able to increase muscle VO_2 max and muscle oxidative enzyme capacity (9), essentially restoring these values back to the levels seen in young adult rats (30). The exercise capacity from the treadmill running tests and training intensity of the rats reported here was nearly identical to our previous study (9), and thus it is reasonable to assume that the initial improvement in muscle VO_2 and muscle enzyme activity were also similar to the previous study over the first 2 mo of the training period. In contrast to the demonstrated benefits of exercise training for skeletal muscle aerobic function at late middle age, the data in the present study shows that continuing exercise into senescence does not attenuate the decline in muscle aerobic function, and at the muscle level there is no apparent benefit from exercise compared to the sedentary controls. In light of the findings

from our previous study (9), the adaptations that occurred in the first 2 mo were completely lost after 5 mo of training (34 mo of age).

The fact that muscle VO_2 max was not higher in the trained animals was surprising and unexpected given the plethora of endurance training studies in young adult to late middle aged subjects showing improvements in muscle oxidative capacity (43; 49; 54). However, no one to our knowledge has undertaken a training study that starts in late middle age and ends in senescence, and thus the effects of chronic exercise throughout this part of the lifespan were previously unknown. One particular human study on octogenarians showed only subtle improvements in arterial–venous O_2 difference in response to 3 months of endurance training, implying minimal improvements in muscle oxidative capacity, and leaving the authors to conclude that this age group has a diminished capacity for adaptation (20). However, as noted above, no prior study has considered the impact of endurance training on the muscle aerobic machinery specifically at this advanced age. Other aging studies showing positive adaptations to endurance training were only short term in duration (less than 6 mo) and did not involve very old/senescent humans.

It is possible to approximate the ages and training duration of this study relative to ages in humans based on survival data on humans (4) and the F344BN rat (63). In relation to human years, 7 mo of training in this study with rats corresponds to 18 years for a human, which would roughly equate to starting training at 65 years of age and ending around 83 years of age. Clearly conducting a human study over a similar age-range would be a major undertaking. The significance of examining this age range is that both humans (40) and rats (13; 29) exhibit a marked acceleration of sarcopenia and functional decline across this age range. Animal endurance exercise training studies, like the human studies, have also shown an adaptive capacity at older ages; however, these have also largely examined late middle aged animals (14; 53; 64). Thus the main findings from this study suggesting that beyond late middle age the capacity for endurance exercise training to attenuate the declines in aerobic function is lost, is novel in both the study design and the result, and does not refute any prior work. Another unique and advantageous aspect of this

study is the use of the hindlimb perfusion technique to match oxygen delivery to the muscles between groups at a given age, allowing for the assessment of skeletal muscle aerobic function without the confounding influence of changes in central effects such as cardiac output (7). Using this approach we observed that there is no benefit of endurance exercise training from late middle age to senescence at the muscle level specifically. This does not preclude other systemic benefits, such as the improved exercise performance, greater survival and lower body fat that we observed.

2.5.3 Effect of Endurance Training on Mitochondrial Enzyme Activities

There was no training effect on CS or complex IV activity in either the plantaris or soleus muscles, which represent mixed fast twitch and predominantly slow twitch muscles, respectively (3), showing that the responses observed are not specific to a particular fiber type. CS and complex IV are key enzymes of the Kreb's cycle and the electron transport chain, respectively, and reflect the oxidative capacity of the mitochondria. Maximal activity of these enzymes increases after training in late middle aged rodents (9; 19; 44; 53) and humans (17; 45; 54). Similar to the point raised above, we are unaware of any study examining mitochondrial enzyme adaptations in response to endurance training in senescent muscle, but it was expected that these would be higher in the trained group compared to sedentary controls, as we have shown at late middle age for this strain of rat (9). In further support of the lack of mitochondrial proliferation with training, we also measured CS protein, another marker for mitochondrial content, and it too, was not affected by training in either muscle. The fact that mitochondrial content was not increased with exercise training plays an important role in explaining why muscle specific VO_2 max was also not higher with training compared to sedentary controls.

In attempting to explain why we did not observe the expected increases in mitochondrial enzyme activity from the exercise training, we measured peroxisome proliferator-activated receptor gamma co-activator 1 (PGC-1) protein in the muscles to determine if this was up-regulated by the exercise training. PGC-1, a master regulator of mitochondrial biogenesis (36), increases significantly following both a single bout of

muscle activation (5; 60) and after chronic muscle activation (1; 38; 54; 59). However, in this study, PGC-1 protein was not higher in the 34 or 36 mo trained groups compared to the sedentary groups, which provides a credible explanation for why the trained rats did not demonstrate higher mitochondrial content versus controls. We are unaware of any other study investigating PGC-1 protein with endurance training in aged skeletal muscle, although one study in humans observed similar increases in PGC-1 mRNA across a range of ages from 21 to 87 y of age following 16 weeks of endurance exercise training (54). Note, however, that there were only two subjects aged 80 y or older in this prior study (54) and it appears that these two subjects had smaller increases in transcripts related to mitochondrial biogenesis with endurance training (e.g., PGC-1, NRF-1, Tfam). Thus, these data do not refute our point that the aerobic machinery in senescent muscle has a blunted response to endurance exercise training.

Previous studies have observed lower PGC-1 mRNA (6) and protein (15) when compared to young adult muscle. Whereas Short et al. (54) did not find PGC-1 mRNA to decline with age, in a later investigation the same group found transcript levels of some mitochondrial proteins were reduced in skeletal muscle with aging (55). A reduced rate of skeletal muscle mitochondrial protein synthesis has been observed with aging in humans (52), which is consistent with lower mitochondrial biogenesis with aging. It has been shown that AMP-activated protein kinase (AMPK) activation is diminished in late middle aged muscle (50), and since AMPK is an activator of PGC-1 (38; 65), this may partly explain why PGC-1 was not up-regulated with training in our senescent animals. Although reducing the training frequency from 5 to 4 days per week could have modestly attenuated the protective effects of the training, it is highly unlikely that this would be sufficient to explain the complete lack of benefit for skeletal muscle aerobic function and mitochondrial adaptation in the trained group relative to cage-bound sedentary rats.

2.6 PERSPECTIVES

In contrast to previous findings of a skeletal muscle metabolic adaptation to exercise training at late middle age, during the phase of the lifespan from late middle age

and into senescence there is growing evidence of a diminished plasticity in the skeletal muscles. A key element in this study was that we combined measures at the whole muscle level (aerobic function in response to electrically-evoked muscle contractions, biochemistry in muscle homogenates) and at the cellular level (molecular signals involved in mitochondrial biogenesis) to describe the muscle adaptations with endurance training in senescence. To that end, all of these measures support the notion that the aerobic machinery in senescent muscle becomes much less responsive to endurance exercise training. Skeletal muscle specific VO_2 max was not higher in the trained muscles, which is explained at the mitochondrial level by no difference in CS or Complex IV enzyme activity, and CS protein content. The significance of these findings is that they underscore the point that exercise training alone may be insufficient to prevent age-related declines in skeletal muscle mass and function in advanced age. As such, the current results suggest that further work determining whether other perturbations can induce skeletal muscle mitochondrial biogenesis at very advanced age is warranted. Despite the lack of adaptation at the muscle level, training did result in better maintained exercise capacity, greater survival and lower body fat, showing that significant benefits of endurance training can be obtained in senescence independent of a diminished plasticity in skeletal muscle.

2.7 TABLES AND FIGURES

Table 2.1. Percent body fat (% Fat), and muscle masses of the lower limb.

	Fat (%)	Gas (mg)	Plan (mg)	Sol (mg)	TA (mg)	EDL (mg)
34 mo Con	25 ± 1	1265 ± 90	247 ± 15	143 ± 8	550 ± 31	142 ± 8
34 mo Train	16 ± 2[a,b]	1216 ± 70	250 ± 11	138 ± 9	473 ± 26	134 ± 4
36 mo Con	25 ± 1	1252 ± 33	236 ± 11	124 ± 4[a]	551 ± 40	148 ± 2
36 mo Train	15 ± 1[a,b]	910 ± 64[a,b,c]	197 ± 12[a,b,c]	111 ± 6[a,b,c]	339 ± 29[a,b,c]	104 ± 6[a,b,c]

Gas=Gastrocnemius; Plan=Plantaris; Sol=Soleus; TA=Tibialis Anterior; EDL = Extensor Digitorum Longus; Significantly different ($p<0.05$) from [a]34 mo control group, [b]36 mo control group, [c]34 mo trained group.

Table 2.2 Perfusion conditions for hindlimb perfusion experiments.

	34 mo Con	**34 mo Train**	**36 mo Con**	**36 mo Train**
Contracting/Perfusion mass (mg)	3261 ± 259	3074 ± 148	3303 ± 122	2429 ± 171^{*+}
Blood flow (ml·min^{-1})	1.72 ± 0.10	1.64 ± 0.06	2.33 ± 0.14^{+}	1.70 ± 0.21^{+}
Muscle blood flow (ml·min^{-1}·g^{-1})	0.53 ± 0.02	0.53 ± 0.01	0.71 ± 0.05*	0.70 ± 0.06*
Net Perfusion Pressure (Torr)	99 ± 7	95 ± 10	161 ± 13*	131 ± 23^{+}
Arterial O_2 content (volume %)	21.6 ± 0.3	20.8 ± 0.3	21.7 ± 0.4	22.1 ± 0.3
Muscle QO_2 (μmol·min^{-1}·g^{-1})	4.5 ± 0.2	4.3 ± 0.1	6.1 ± 0.5*	6.2 ± 0.5*

Significantly different ($p<0.05$) from * 34 mo group of the same training status and +control group for the same age.

Table 2.3 Contractile and metabolic characteristics from the hindlimb perfusion VO_2 max tests.

		34 mo Con	34 mo Train	36 mo Con	36 mo Train
Peak VO_2 ($\mu mol \cdot min^{-1} \cdot 100g^{-1}$)		212 ± 31	221 ± 21	212 ± 39	209 ± 19
Peak force (N)	G-P	14.4 ± 2.0	16.5 ± 2.5	15.5 ± 3.3	10.2 ± 0.6*+
	Sol	0.94 ± 0.16	0.96 ± 0.16	0.96 ± 0.17	0.80 ± 0.11
Peak force ($N \cdot g^{-1}$)	G-P	9.2 ± 1.0	11.0 ± 1.2	9.8 ± 1.8	9.5 ± 0.6
	Sol	6.1 ± 0.9	6.6 ± 0.9	7.0 ± 1.0	7.3 ± 0.8
Final Force (N)	G-P	4.9 ± 0.7	5.4 ± 0.4	4.1 ± 0.6	4.1 ± 0.4
	Sol	0.78 ± 0.18	0.70 ± 0.11	0.80 ± 0.15	0.62 ± 0.08
Final Force (% of peak)	G-P	34 ± 2	36 ± 4	31 ± 8	40 ± 2
	Sol	78 ± 7	78 ± 8	83 ± 5	78 ± 3
Peak lactate (mmol/L)		5.2 ± 0.7	5.4 ± 0.5	4.8 ± 0.9	4.5 ± 0.3

Force values are from the gastrocnemius-plantaris muscle (G-P) complex and from the soleus (Sol). Significantly different ($p<0.05$) from * 34 mo group of the same training status and +control group for the same age.

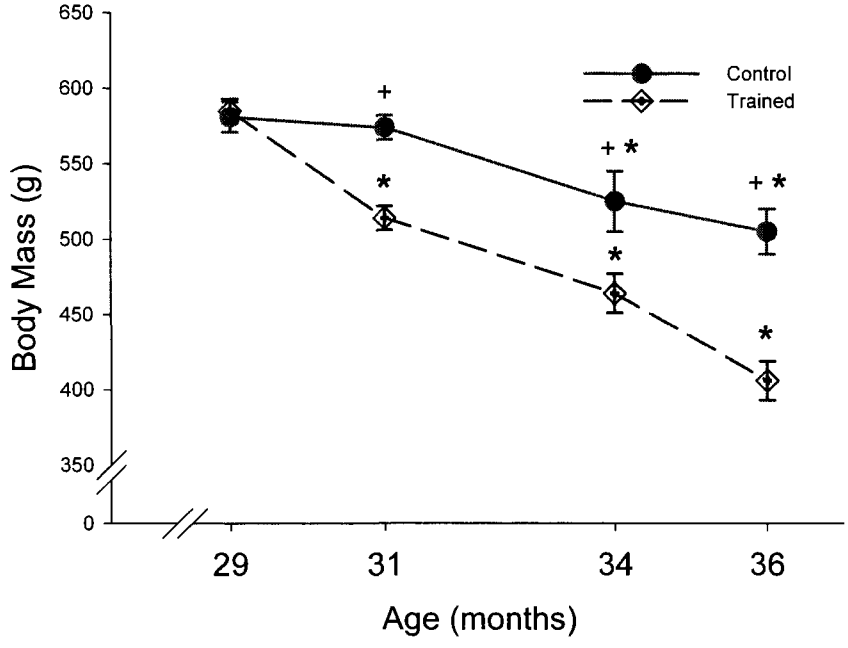

Figure 2.1 Body mass of sedentary and trained groups at four time points.

Significantly different ($p<0.05$) from * 29 mo group and +trained group for the same age.

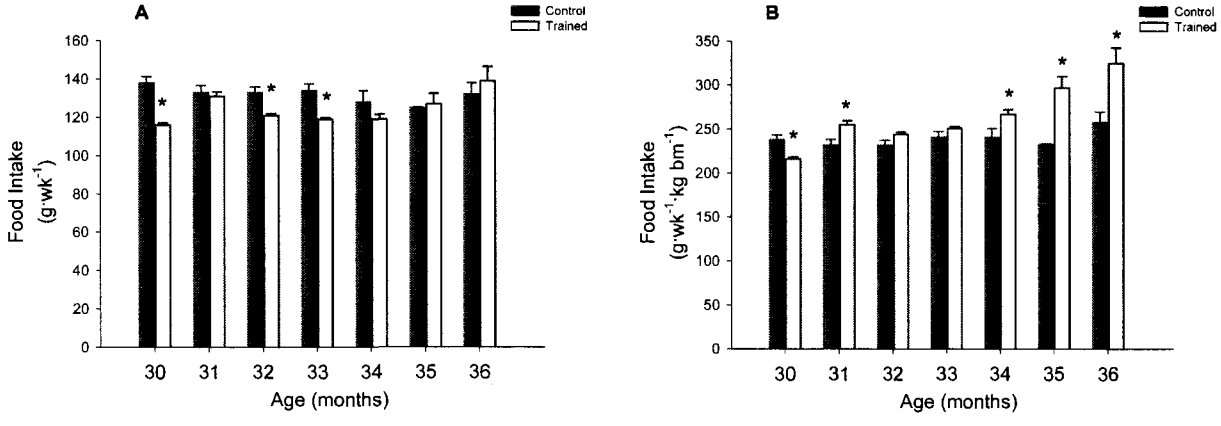

Figure 2.2 Average weekly food intake per animal expressed in absolute (A) and normalized (B) to the body mass (kg) measured at monthly intervals. Food intake was not measured in the first month.

Significantly different ($p<0.05$) from *control group for the same age.

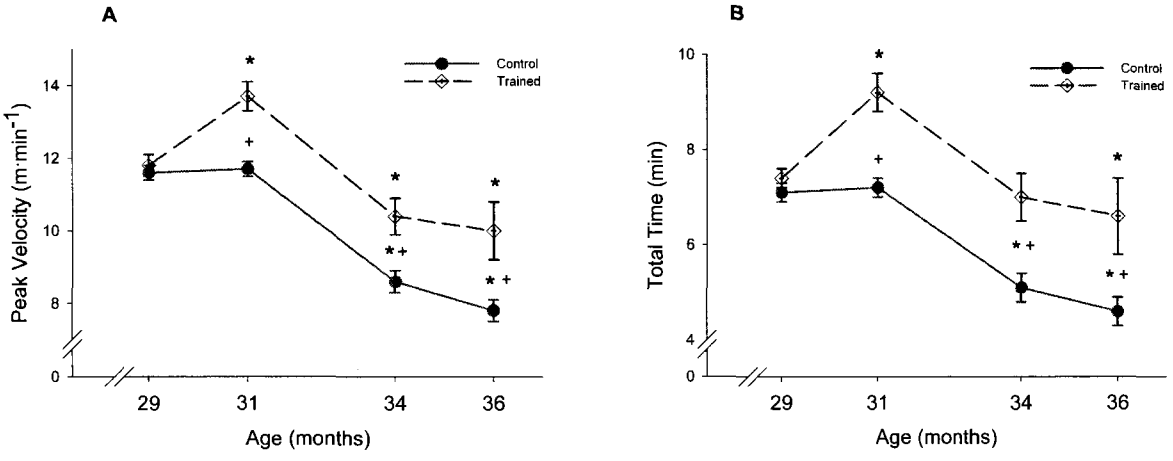

Figure 2.3 Peak velocity (A) and maximum time (B) achieved during the graded exercise test for sedentary and trained groups at four time points.

Significantly different ($p<0.05$) from * 29 mo group and +trained group for the same age.

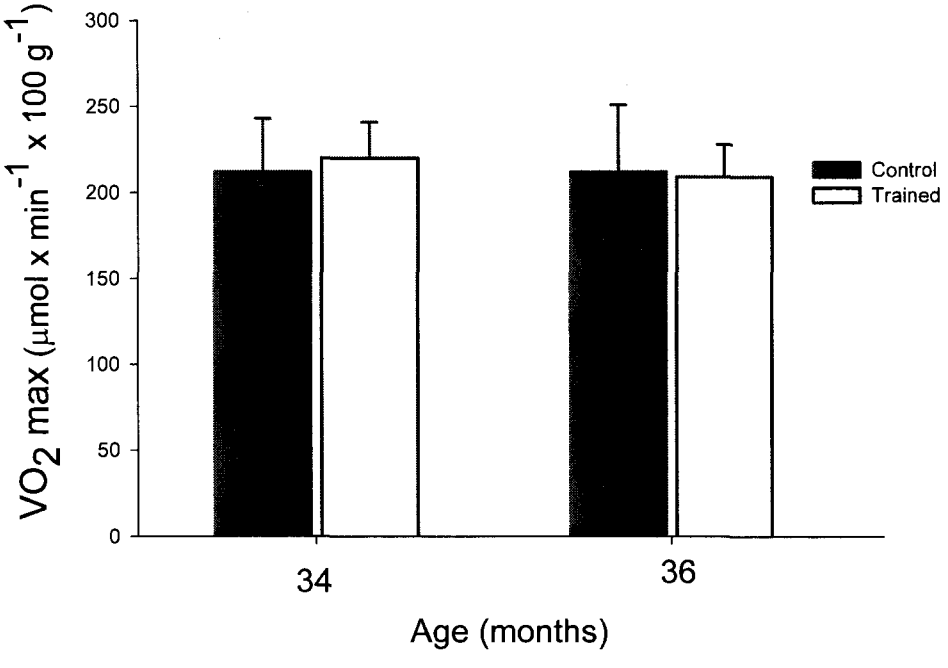

Figure 2.4 Hindlimb muscle VO_2 max achieved during increasing electrical stimulation test (pump-perfusion experiments).

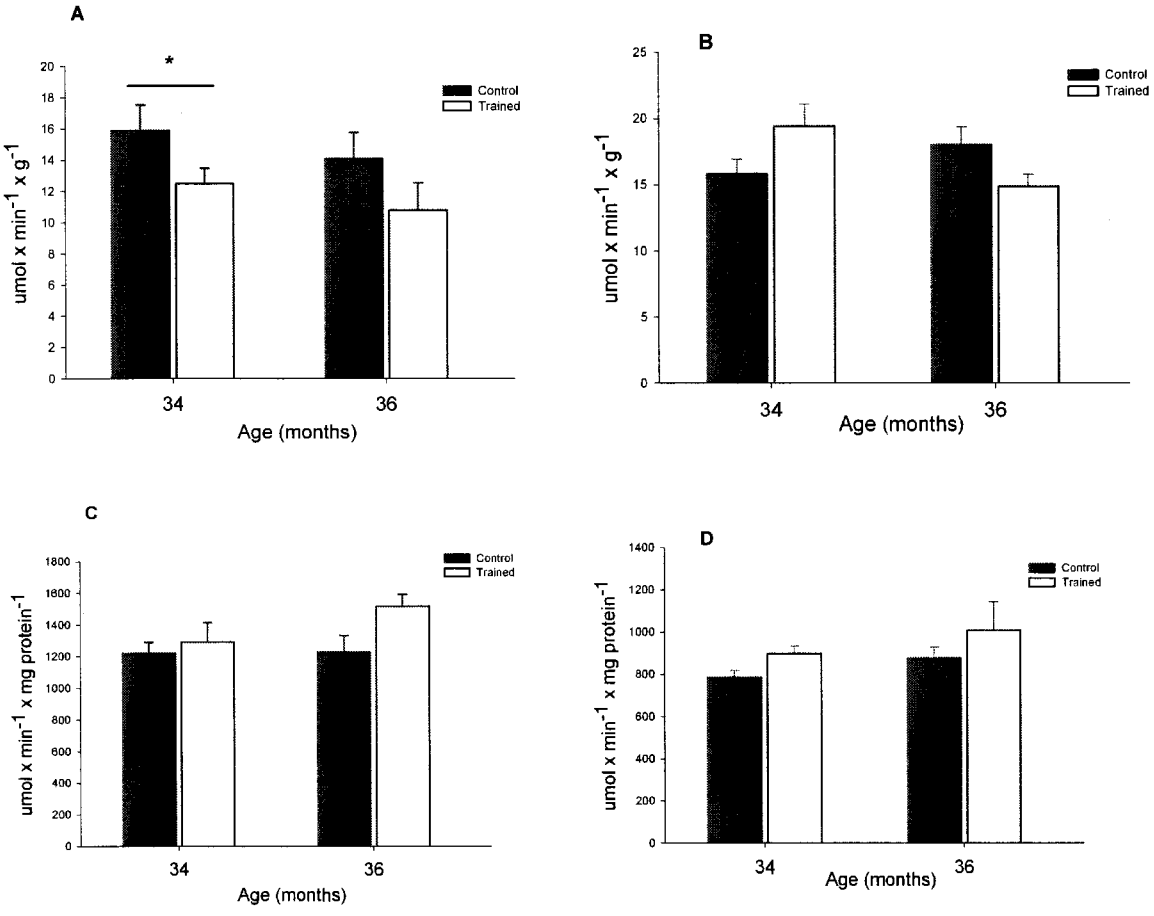

Figure 2.5 Citrate synthase enzyme activity for the plantaris and soleus.

Enzyme activity is normalized per g muscle (A, B) or per mg protein (C, D) for the plantaris (A, C) and soleus (B, D) muscles for 34 and 36 mo age in the sedentary control and trained groups. *$p<0.05$.

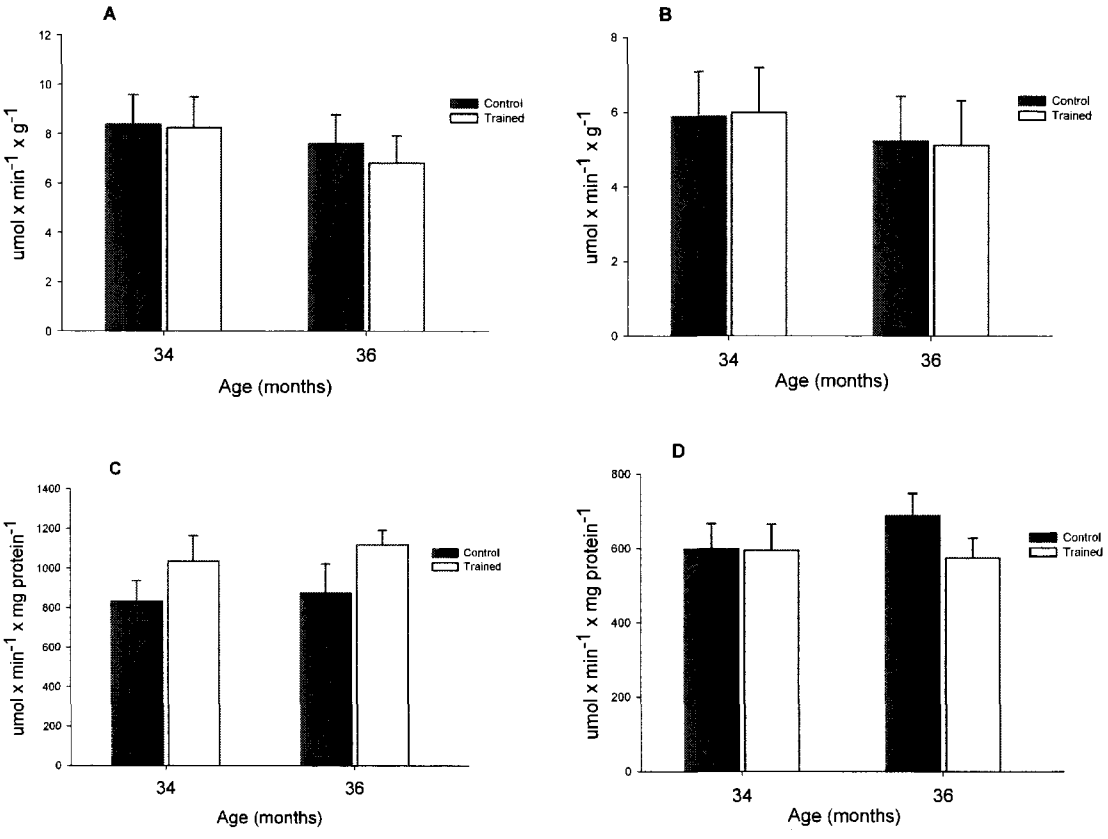

Figure 2.6 Complex IV enzyme activity for the plantaris and soleus.

Enzyme activity normalized per g muscle (A, B) or per mg protein (C, D) for the plantaris (A, C) and soleus (B, D) muscles for 34 and 36 mo age in the sedentary control and trained groups. *$p<0.05$

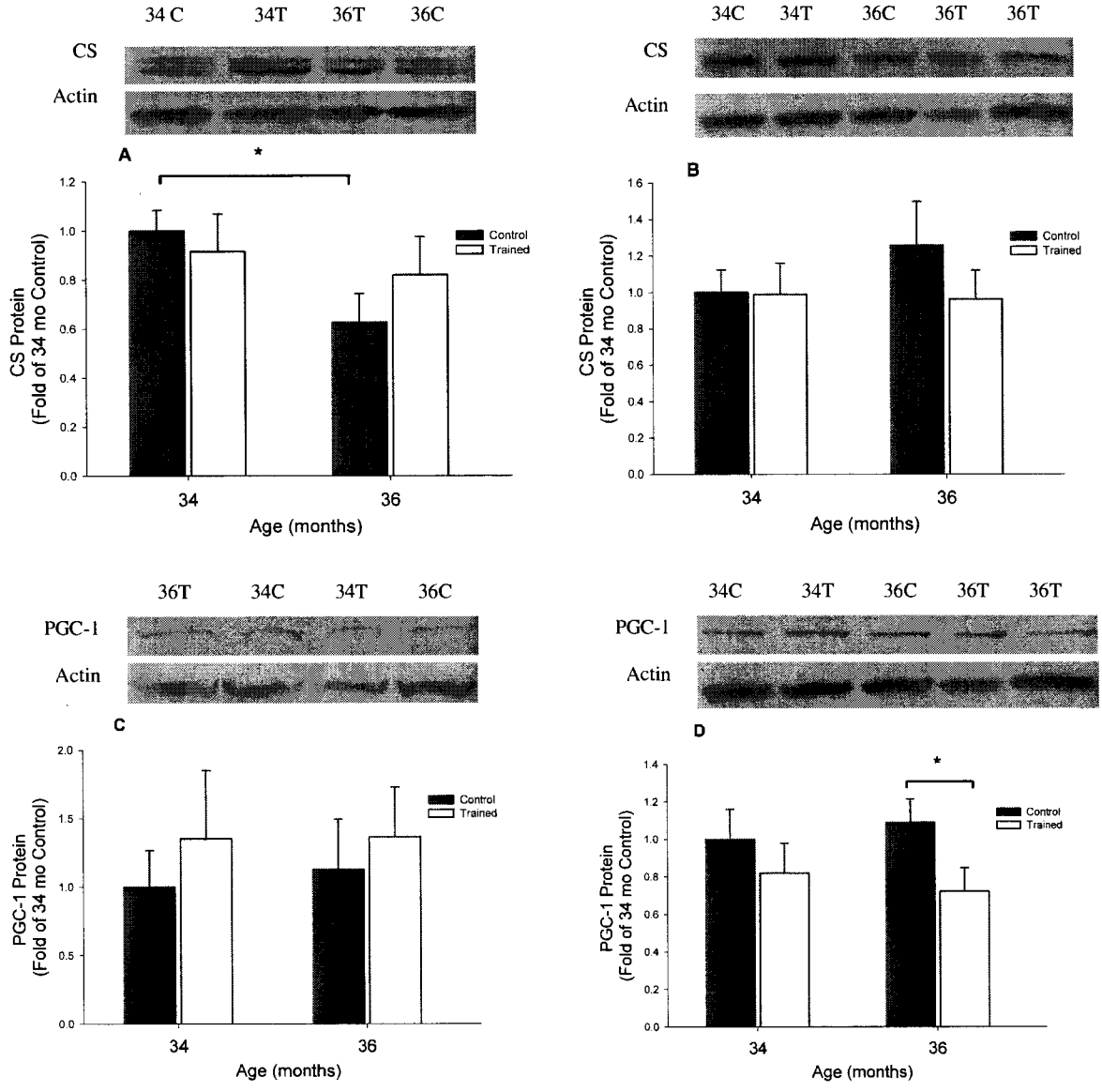

Figure 2.7 CS protein for the plantaris (A) and soleus (B) and PGC-1 protein for the plantaris (C) and soleus (D).

*$p<0.05$

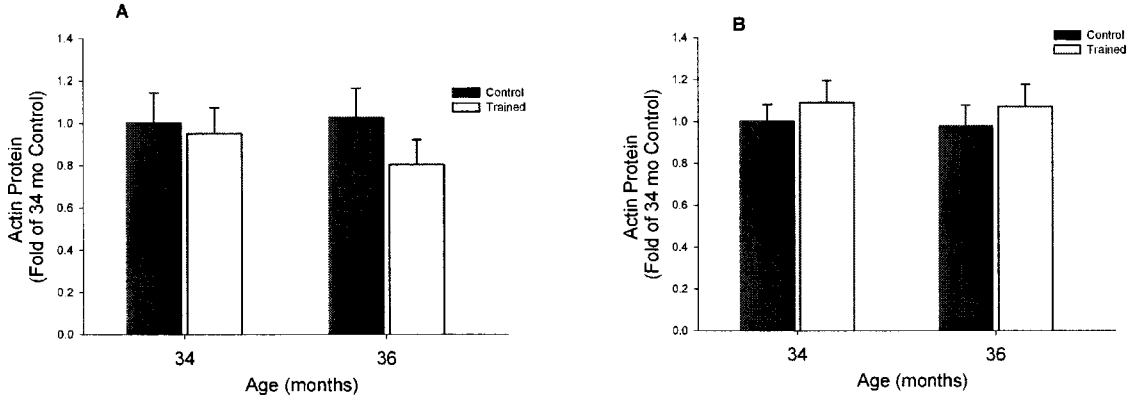

Figure 2.8 Actin protein for the plantaris (A) and soleus (B) for 34 and 36 mo age in the control and trained groups, showing no difference between any of the groups.

Representative actin blots are presented in Figure 2.7.

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Chapter Three: AGE-RELATED DECLINE IN MITOCHONDRIAL BIOGENESIS IS NOT ATTENUATED BY LONG-TERM EXERCISE TRAINING

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3.1 INTRODUCTION

Aging is related to decreases in skeletal muscle mitochondrial oxidative capacity (11; 12; 24; 31; 51) that both impair the muscle's ability to undergo oxidative phosphorylation, but also contribute to the production of reactive oxygen species (ROS) which may increase apoptosis, accumulation of oxidative damage to proteins and mitochondrial DNA damage. In turn, mitochondrial DNA damage (66; 69) and oxidative damage to proteins (8) negatively impact the synthesis and function of mitochondrial proteins, increasing the proportion of dysfunctional mitochondria. The functional implications of these events are a decrease in skeletal muscle aerobic function (12; 24), decreased muscle mass (10; 36; 69) and decreases in contractile function (10; 19; 68).

There is evidence that mitochondrial turnover decreases with age, and one hypothesis is that mitochondrial damage accumulates since the mitochondria are present in the cell for a longer time period. It has also been shown that the mitochondrial protein synthesis rate is lower in aged muscle compared to young muscle (51). Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a master regulator of mitochondrial biogenesis (46; 76) and has been shown to decrease in skeletal muscle as a function of age (4; 11), thus supporting a decreased drive for mitochondrial biogenesis in aged muscle. This lower drive contributes to the declines in muscle aerobic function which are only modest at late middle age (~20%) but decline dramatically (a further 50%) between late middle age and senescence (35% survival in the F344BN rat) (10; 24), underscoring the necessity to understand the mechanisms and design interventions against aging in this part of the lifespan.

Since exercise increases muscle mitochondrial capacity (20; 28) subsequent to increases in mitochondrial biogenesis via PGC-1α activation (3; 59; 73), it was believed that exercise training at older ages could be used to combat the age-related declines in PGC-1α and mitochondrial capacity. To this effect, we exercised late middle aged (28 mo)

F344BN rats for 7 weeks and observed 20-25% increases in muscle mass specific maximal aerobic capacity (VO_2 max), citrate synthase (CS) activity and cytochrome oxidase (COX) activity (6), such that it restored these to the levels observed in young adult. In a follow-up study, we extended the training program from late middle age until senescence to determine if the benefits of continuous exercise would be maintained until this late part of the lifespan. In contrast to the 7 week study at late middle age, continuing exercise into senescence did not have any benefit on muscle mass specific VO_2 max, CS or COX activity in the plantaris or the soleus (Chapter 2). Since the trained animals had exercise benefits such as increased survival, decreased body fat and improved running capacity, the lack of benefit at the mitochondrial level was surprising, and left us with the hypothesis that aging must be accompanied by an attenuation of the mitochondrial biogenesis pathway. We did not see an increase in PGC-1α protein with exercise in the soleus (SOL) or the plantaris (PLAN) (Chapter 2), in contrast to what is typically observed in young muscle (3; 59; 60). The mitochondrial biogenesis pathway is complex, with many upstream and downstream events necessary for PGC-1α activation to result in an increase in mitochondrial synthesis.

There are many signals that relate muscle contractile activity to activation of PGC-1α and subsequent mitochondrial biogenesis. Refer to related paragraphs in the Introduction as well as Figure 1.2 for a more detailed description of these pathways. Briefly, with muscle contraction, increases in intracellular Ca^{2+} and/or AMP:ATP ratio can activate several kinases such as AMPK, CAMK, p38 MAPK (54) which themselves can activate (directly or indirectly) PGC-1α (1; 34; 42; 45; 72; 75; 77). PGC-1α can subsequently bind to and coactivate NRF-1, which can increase nuclear DNA gene expression and also activate Tfam which subsequently can activate mitochondrial DNA gene expression to achieve a coordinated increase in mitochondrial proteins (16; 21; 73; 76).

The purpose of this study was to determine to what extent the regulatory mechanisms mediating mitochondrial biogenesis are attenuated with aging and to what extent long term exercise into senescence can mitigate the age-related changes. We expand

on our previous findings (Chapter 2) of a lack of mitochondrial benefit with exercise in senescence by taking a closer look at PGC-1α regulation with age, and exercise at old age. These goals are achieved by 1) including a young group to assess the effects of aging on all of the mitochondrial biogenesis and mitochondrial function parameters (to determine if exercise can combat the effects of age); 2) including gene expression of the mitochondrial biogenesis pathway (to determine if a mismatch between transcription and translation can explain the lack of protein induction with exercise); 3) including gene and protein expression of complex IV of the electron transport chain as a marker of mitochondrial content and to evaluate mitochondrial function; 4) investigating upstream activators of PGC-1α that may play a role in the observed changes (or lack of) in PGC-1α with aging and exercise; and 5) including red portion of the gastrocnemius, to better represent a range of metabolic phenotypes and to include the major hindlimb muscles used in our muscle VO_2 max measurements.

We hypothesized that mitochondrial oxidative capacity, mitochondrial content, PGC-1α and its downstream co-activators (NRF-1, Tfam) would be lower with aging and that exercise training would not be able to restore the mitochondrial biogenesis pathway to young adult levels. Furthermore, we hypothesized that content of PGC-1α would be lower with aging, and the activators of PGC-1α are not increased with exercise in aged muscle, to explain the lower PGC-1α with age and the lack of induction of PGC-1α with exercise.

3.2 METHODS

3.2.1 Animals.

67 Fisher344xBrown-Norway-F1 (F344BN) male rats were acquired from the National Institute of Aging (Bethesda, MD). 10 of these were 7 mo old (100% survival), and were assigned to the young adult sedentary group (YSED). The others were 28 mo of age upon arrival (65% survival) (67), and were randomly assigned to an exercise group (OEX) or sedentary group (OSED). The training program was described in detail (Chapter 2), but a brief overview of the exercise program is described below.

3.2.2 Exercise Program.

The OEX group started treadmill running at 29 mo of age beginning with 15 min per day in the first week and gradually increasing to 60 min per day by week four. Each 60 min session consisted of 6 x 10 min running bouts (2 min rest in between), in which a base velocity was used for the first 8 min and then treadmill velocity was increased for the final 2 min. Treadmill velocity was generally increased to the tolerance of the animals so as to maximize the exercise stimulus. This program has been shown to elicit improvements in running capacity, muscle VO_2 max, and mitochondrial enzyme activity in this age and rat strain (6). After 8 weeks, exercise was reduced to 4 days per week so as to maintain any beneficial adaptations that had occurred and to provide more rest for the animals as they age into senescence (50% survival, 33 mo for F344BN). In both the OEX and OSED groups, after 5 months of training (34 mo) 10 animals were randomly removed for muscle harvest and data analysis, while the rest continued in their respective groups for another 2 mo, thus 7 mo of total exercise (final age of 36 mo, 35% survival). It was our original goal to have two time points to gain a perspective of the changes that are occurring at this part of the lifespan. However, in the skeletal muscle measures relating to mitochondrial capacity and mitochondrial biogenesis, these age groups were not significantly different (Chapter 2) and thus it was decided to combine these age groups into one OSED and one OEX group.

3.2.3 Tissue harvest.

Following at least 2 d of rest, animals were anesthetized with sodium-pentobarbital (50-65 $mg \cdot kg^{-1}$ i.p.), and the following muscles of the right lower limb were collected, weighed and frozen in liquid nitrogen: soleus (SOL), plantaris (PLAN), gastrocnemius separated into red (GASr), white and mixed portions, tibialis anterior, extensor digitorum longus, vastus intermedius, vastus medialis, vastus lateralis separated into red, white and mixed portions, adductor longus and rectus femoris. Fresh portions (~50mg) of GASr, PLAN, vastus intermedius, adductor longus and mixed gastrocnemius were quickly submerged in 750μL RNA*later*™ (Ambion, Austin, Texas), stored overnight at 4°C and then at -30°C as per manufactures instructions.

Whole muscle tissue was pulverized under liquid nitrogen from which powder aliquots were homogenized in 10X volume of extraction buffer containing 50 mM Tris-HCl, 250 mM mannitol, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100 with the following inhibitors added just prior to use (1 mM DTT, 1 mM benzamidine, 0.5 mM AEBSF, 100 μM leupeptin, 1 μg/ml soybean trypsin inhibitor). Following 30 min on ice, samples were centrifuged for 10 min at 700g to remove impurities.

3.2.4 Biochemistry.

For biochemical analysis, an aliquot of these samples was further diluted 1:20 in potassium phosphate buffer (pH 7.5), freeze-thawed 3 times to rupture cellular and mitochondrial membranes and further centrifuged (900G, 10 min, 4°C). For COX activity, supernatants were collected and frozen at -80°C for subsequent analysis. For CS activity, the supernatants were further diluted to 1:400 (potassium phosphate buffer) and also frozen at -80°C.

3.2.5 Protein Expression.

Protein concentration was first measured by Bradford assay (9). Protein concentration in the homogenates was normalized for all samples by diluting in sample buffer containing Glycerol, Tris pH 6.8, SDS, DTT, Bromophenol Blue and β-mercaptoethanol. Equal quantities of protein were loaded into precast gels (Bio-Rad, Hercules, USA) and separated by electrophoresis for 90 min at 100 Volts. Proteins were then electro-transferred to nitrocellulose membranes in a buffer containing 48 mM TRIS, 39 mM Glycine, and 20 % methanol at 100 V for 80 minutes in 4°C with slight agitation. Membranes were blocked for 60 min at room temperature with 5% non-fat milk (Carnation®, Nestlé, Switzerland) in phosphate buffered saline and 0.05% Tween (PBST) with pH 7.4 . Membranes were then incubated overnight at 4°C with primary antibodies diluted in 5% milk-PBST with gentle agitation. Membranes were then washed 3 times with PBST, incubated with appropriate secondary antibody diluted in 5% milk-PBST (1:5000

Pierce, Rockford, IL) for 60 min at room temperature followed by 3 more rinses with 5% milk-PBST and a final rinse with PBS (no Tween). Finally, membranes were treated with chemilluminescent developing solution (Pierce) and chemiluminescence was digitally captured (Syngene Bio-Imager, Frederick, MD) and densitometry measured using the Bio-imager software (Syngene Tools, Frederick, MD). Each protein band (sample) was normalized to its corresponding α-actin band from the same gel to get a single value for each sample. The expression of α-actin did not change between any of the treatment conditions (Figure 3.1) and is thus a suitable internal control. Samples from each group were equally loaded onto gels to ensure equal representation of each group per gel to avoid any biases that may occur due to differences in transfer efficiency (normalizing to α-actin within each gel also helped avoid biases due to transfer efficency). For presentation purposes (graphs) the group means were normalized to the young sedentary group. The following primary antibodies were used: α-actin (1:5000, Santa Cruz), CS (1:10,000, kind gift from J. Holloszy), GLUT4 (1:1000 Abcam), p-ACC (1:500, Cell Signaling), PGC-1α (1:1000, Calbiochem), SIRT1 (1:1000 Millipore), TNF-R1 (1:1000 Abcam).

3.2.6 Real Time PCR.

RNA was extracted from GASr and PLAN samples using a Fibrous RNeasy kit (Qiagen, Mississauga, Canada) as per manufacturer's instructions. The quality and purity of the RNA was assessed by Nanodrop (using the ratios of 260/280 and 260/230) and the RNA concentration was also determined at the same time. The homogenate was diluted to yield 2 μg of total RNA from which complimentary DNA (cDNA) was synthesized by reverse-transcription using SuperScript II Reverse Transcriptase, random primers, dNTP, 5x FSB, RNase Out and DTT (all from Invitrogen, Burlington, Canada). Real time PCR was performed using a 7900HT Fast-Real Time PCR system (Applied Biosystems Inc., Foster City, USA). TaqMan® Gene Expression Assays (Applied Biosystems Inc.) were used for beta-actin, GAPDH, 18s, GLUT4, SIRT1, NRF-1, Tfam, COXI and COXIV. Primers for PGC-1α, COXI and COXIV were designed using Primer Express 3.0 software and provided by Integrated DNA Technologies (Coraville, USA) and the probes were supplied by Applied Biosystems. Samples were run in duplicate on a 96 well plate with

each well containing 1 μL gene expression assay, 1 μL of cDNA (most genes), 10 μL of TaqMan® Fast Universal PCR Master Mix and 8 uL of RNAse free water. For COXI and COXIV 2 μL of cDNA was used, while for PGC-1α 3 μL of cDNA was used and less water to achieve a total volume of 20 μL per well.

All gene expression data was normalized to an endogenous control (beta-actin or 18s) which was verified to not change with aging or training conditions accordingly ($\Delta CT_{gene} = CT_{gene\ of\ interest} - CT_{control\ gene}$; CT= cycle threshold). Beta-actin was used for the GASr since it did not change with age or training (CT values for: YSED: 21.1 ± 0.08, OSED: 21.0 ± 0.17, OEX: 20.9 ± 0.08) and 18s was used for the PLAN (CT values for: YSED: 15.9 ± 0.19, OSED: 16.1 ± 0.20, OEX: 15.9 ± 0.19). Beta-actin increased with age in PLAN while 18s increased with training in the GASr. Statistical analysis was performed on the ΔΔCT data (37), while relative differences in gene expression were determined from the log transformed data ($2^{-\Delta\Delta CT}$ method) (37) as done previously (57; 70). Data are expressed relative to 7 mo controls.

3.2.7 Plasma Adiponectin, Leptin and TNF-α.

Blood was collected upon heart dissection and was mixed with EDTA and centrifuged (2 min, 900G) after which the plasma (supernatant) was aliquoted and frozen (-30°C). Plasma leptin and adiponectin were analyzed in triplicate on a microplate reader using appropriate rat specific ELISA assay kits, according to manufacturers instructions (Millipore, Billerica, Mass, USA). We have reported previously that the coefficient of variation of these assays is < 5% (57). TNF-α was also measured in triplicate using a rat specific ELISA kit from Pierce Biotechnology Inc. (Rockford, USA).

3.2.8 Statistical Analysis.

Data are presented as means ± standard error (SEM). Comparisons between groups for each muscle were performed with a one way analysis of variance and Student-Newman-Keuls post hoc analysis. Statistical significance is accepted for a p value less than 0.05. In the figures, letter superscripts were used to denote significant differences between

groups, and this nomenclature was kept consistent for all graphs in this chapter. In cases where no difference exists, the superscript nomenclature was retained in the figure legend for consistency and as identification that the statistical test was performed.

3.3 RESULTS

3.3.1 Muscle Mass Specific VO_2 max.

Muscle mass specific VO_2 max was assessed with the hindlimb perfusion model in which blood flow and convective oxygen delivery are proportional to the contracting muscle mass. As reported previously (Chapter 2), there was no difference between 34 and 36 mo age groups in either the sedentary or trained groups and as such these were averaged together to have one old sedentary and one old exercise group. Compared to YSED, VO_2 max was 37% lower in the OSED (Figure 3.2), confirming the expected age-related decline in aerobic capacity. As noted previously (Chapter 2), there was no benefit of exercise training (OSED: 212 ± 23, OEX: 212 ± 14 $\mu mol \cdot min^{-1} \cdot 100g^{-1}$) (Figure 3.2).

3.3.2 CS and COX: Activity, Protein and mRNA.

CS and COX activity for the three muscles are summarized in Figure 3.3. CS activity was significantly lower in the OSED group compared to the YSED group for PLAN (-52%), GASr (-31%) and SOL (-24%) (Figure 3.3A). Regular exercise training was unable to attenuate these declines as there were no differences between the exercise and trained groups for any of these muscles (SOL and PLAN as previously reported (Chapter 2); GASr reported here for the first time). Similar findings of no benefit of training on CS activity were also observed in the tibialis anterior (Chapter 5, Figure 5.2) and the heart (data not shown).

COX activity decreased in the GASr (29%) between YSED and OSED, but there was not a difference between age groups for the SOL or PLAN (Figure 3.3B). COX

activity in the GASr was not different between the OEX and OSED groups, consistent with our previous finding of no training effect on COX activity in PLAN and SOL (Chapter 2).

CS protein and COXIV protein did not decrease with age (Figure 3.4). There was also no increase in either protein in any of the muscles in the OEX compared to OSED.

COXIV mRNA was not different with age or training in the GASr or the PLAN (Figure 3.5A). COXI mRNA was not different with age or training in the GASr, but was significantly higher with age in the PLAN, with no difference between sedentary and trained groups (Figure 3.5B).

3.3.3 PGC-1α Protein and mRNA.

PGC-1α protein decreased with age ~ 45% in the GASr ($p=0.014$; Figure 3.6A). PLAN PGC-1α protein was not statistically different ($p=0.059$). PGC-1α mRNA was ~50% lower in the OSED compared to YSED for both the GASr and the PLAN. There was no effect of training on the gene expression in either muscle.

3.3.4 NRF-1 and Tfam mRNA.

NRF-1 and Tfam mRNA were significantly (~50%) higher with age in the PLAN, but there was no age effect in the GASr (Figure 3.7). There was no significant difference in NRF-1 or Tfam in either muscle with training (OEX) compared to the OSED groups

3.3.5 GLUT4 Protein and mRNA.

GLUT4 protein did not change with age or training in any of the muscles. As seen in Figure 3.7A, there was a tendency for GLUT4 to increase with training; however, this never reached statistical significance. GLUT4 mRNA was significantly lower with age in both the GASr and the PLAN (Figure 3.8B). There was no effect of training on the gene expression in either muscle.

3.3.6 SIRT1 Protein and mRNA.

SIRT1 protein was unchanged by age (YSED compared to OSED) for SOL and GASr, but increased in PLAN (Figure 3.9A). In contrast, SIRT1 mRNA was reduced in PLAN OSED and unchanged in GASr OSED (Figure 3.9B). Training had no effect on SIRT1 protein or mRNA, except for a significant increase in GASr protein (Figure 3.9A).

3.3.7 Phospho-Acetyl CoA Carboxylase (p-ACC) and p-AMPK.

Phospho-ACC was measured as a marker of AMPK activation (7). Interestingly, p-ACC significantly increased with age (~2.5-fold) in both the PLAN and the GASr, although there was no difference in the SOL (Figure 3.10A). We have observed an age-related increase in p-ACC in the tibialis anterior muscle (similar magnitude) as well (see Chapter 4). There was no effect of training in the SOL, PLAN or GASr. Phospho-AMPK in GASr was significantly higher with age (60%, OSED vs YSED) and not affected by training (Figure 3.10B), similar to p-ACC.

3.3.8 TNF-R1 and RIP140.

To get an idea of other proteins that may affect PGC-1α regulation with aging, we measured tumor necrosis factor receptor 1(TNF-R1) in the PLAN and SOL (Figure 3.11A) and RIP140 in the SOL (Figure 3.11B). TNF-R1 was not significantly lower in the PLAN ($p=0.08$) and was significantly lower with aging in the SOL (-29%, $p=0.026$), with no training effect in either muscle. Similiarly, RIP140 also decreased significantly in the SOL (-55%) with age, with no effect of training.

3.3.9 Plasma TNF-α, Adiponectin and Leptin.

Results for plasma TNF-α, adiponectin, leptin, and percentage body fat are displayed in Figure 3.12. There were no differences between any of the groups for TNF-α or adiponectin, although there was a high amount of variability in the TNF-α measures. Plasma leptin was significantly higher in the OSED group compared to YSED and OEX ($p<0.05$ for all comparisons). There was no difference between the YSED and OEX

groups. These group differences are the same as observed for percent of body fat that was measured in all animals by DEXA (Young: 14.1 ± 0.5%; OSED 24.9 ± 1.1%; OEX: 15.9 ± 1.3%). Leptin was positively correlated with % body fat (r^2=0.62, $p<0.05$) (Figure 3.12D).

3.4 DISCUSSION

The goal of this study was to determine what factors in the mitochondrial biogenesis pathway were affected with aging, and to determine how exercise from late middle age into senescence affects the mitochondrial biogenesis cascade that could explain why mitochondrial enzyme activity was not higher in trained compared to sedentary rats.

In this study three different muscles displaying a range of metabolic phenotypes of the hindlimb were examined to get a broad perspective of the effects of aging and exercise in senescent hindlimb skeletal muscles. The 3 muscles chosen are also relevant because they have been shown to respond to treadmill running exercise (6; 15; 49; 52) and they are also the muscles used in the hindlimb perfusion experiment to measure muscle mass specific aerobic capacity, thus facilitating comparisons between whole muscle aerobic capacity and mitochondrial oxidative capacity (these muscles represent ~50% of the total perfused muscle mass in these experiments). Finally, levels of PGC-1α also vary amongst muscle phenotypes (5; 30) and thus PGC-1α regulation and sensitivity to perturbations may also vary amongst muscle phenotypes (30; 53). PGC-1α mRNA was also measured to determine if gene expression was upregulated with exercise; a disconnect between translation and transcription could explain the lack of altered PGC-1α protein induction in OEX animals.

3.4.1 Effect of Age on Aerobic Function and Mitochondrial Biogenesis

The effect of age (YSED versus OSED) was compared for the oxidative capacity of skeletal muscle *in situ*, the regulation of mitochondrial biogenesis and the changes in mitochondrial enzyme activity of the three muscles of the rat hindlimb that represent a

range of metabolic phenotypes. To our knowledge, this is the first study to investigate the effects of aging on Tfam, NRF-1 and PGC-1α mRNA along with PGC-1α protein in more than two muscles.

3.4.2 Muscle Aerobic Capacity with Age

A modified version of the hindlimb perfusion model was used to normalize O_2 delivery to the mass of the hindlimb muscles that would allow for comparisons of the aerobic capacity of the muscle independent of changes in O_2 delivery. This modified version in which only the distal hindlimb muscles were perfused, in contrast to the entire hindlimb (explained in detail in Chapter 2), provided similar results as we have shown previously (24; 26) in that muscle specific VO_2 max declined ~40% between young adult and senescence. We believe that this modified protocol is more accurate since it requires fewer assumptions about non-contracting muscle mass contributing to the internal consistency in age-related decreases in muscle aerobic capacity.

3.4.3 Mitochondrial Enzyme Activity with Age

CS and COX activity were measured *in vitro* in all three muscles as representative enzymes of the Kreb's cycle and electron transport chain respectively. These are also widely used markers of mitochondrial capacity and mitochondrial content. CS activity was consistently lower with age in all three muscles, in agreement with previously published data (24; 27). Surprisingly, we only observed a decrease in COX activity with age in the GASr and not the PLAN or SOL, which is not in agreement with previous work (11; 27; 51). The lack of an aging effect in the SOL and PLAN is difficult to reconcile, and further investigation is warranted. There are reports in the literature that age related declines in mitochondrial respiration and COX activity from muscle homogenates are not observed when using isolated mitochondrial preparations (11; 65). The interpretation of this discrepancy is that perhaps during the mitochondrial isolation there is a bias to collect only healthy mitochondria, and that damaged mitochondria are not harvested (65). Alternatively, one may be tempted to speculate that, in terms of the mitochondria that are present, there is not such an evident dysfunction, but the problem is just less total

mitochondrial content in aged muscle. However, when Chabi et al. (11) measured state III and state IV respiration, they did observe a significant decrease in respiration rates in the aged groups, which is probably a more indicative measure of mitochondrial function since it incorporates the coordinated action of all of the complexes of the electron transport chain, and not just maximal activities of just one or two enzymes. In light of this, we interpret our COX activity with some caution, with the conclusion that no decrease in maximal COX activity in the PLAN or SOL does not necessarily mean that the mitochondria in the aged muscles are equally functional as young muscle. Taken together, the decline in CS activity, and COX activity in the GASr, are contributors to the decrease in muscle aerobic capacity that was observed *in situ.*

Figure 3.3 shows the enzyme activity normalized to both muscle mass and to protein content. Of special note is that the age effects are eliminated when normalizing to protein content (Figures 3.3C and 3.3D). This is because the actual protein concentration decreases with age (Figure 5.3), so the lower activity in the OSED group is offset by a lower protein concentration in this group, relative to YSED. For the PLAN in particular, COX activity is higher in OSED compared to YSED, and this again can be explained by a significantly lower protein concentration in the OSED group for this muscle (Figure 5.3).

3.4.4 Mitochondrial Content with Age

CS and COXIV protein content were measured as markers of mitochondrial content based on the assumption that changes in mitochondrial volume parallels changes in mitochondrial proteins (28; 47; 62). An age-related decline in either protein was not observed, suggesting that mitochondrial content does not decline in aged muscle. This is in agreement with another study, using the same rat strain and same ages, which also concluded that fibre mitochondrial volume density did not change with age (39). Similarly, Reznick et al. (48) did not observe a decrease in cytochrome *c* content in aged EDL muscle, which is a similar phenotype as the PLAN. Often enzyme activity (CS or COX) is used as markers of mitochondrial content. This may be appropriate in certain conditions (eg. exercise training) in which it is believed that increases in activity are proportional to

increases in mitochondrial content, but in the context of aging when content and activity may change disproportionately, measuring protein content is a more appropriate marker of mitochondrial content.

3.4.5 Mitochondrial Biogenesis with Age

There are very few studies that have investigated the effects of age on the mitochondrial biogenesis pathway, with one study showing no change with age on the mRNA of PGC-1α, NRF-1 and Tfam in humans (56) and another showing significant declines in PGC-1α protein in SEN F344BN rats (11) . In the current study, PGC-1α protein declined with age only in the GASr, but not in the SOL or PLAN. This is in contrast to a recent study, which observed ~65% decline in PGC-1α protein in the SOL, and ~25% decrease in the PLAN (11). It is difficult to reconcile this marked difference in observations. Chabi et al. (11) used glyceraldehyde phosphate dehydrogenase (GAPDH) as a loading control, which has been shown to decrease with age in the plantaris (but not the soleus) (38) which would bias their results towards an increase in PGC-1α in the plantaris. For this research, the same amount of total protein was loaded for each sample and differences in α-actin were not observed (Figure 3.1), in agreement with others (63). However, this does not explain the discrepancy in the soleus data. PGC-1α mRNA was lower in the aged groups for both the GASr and the PLAN, in agreement with previous findings from our lab (4). The lower GASr mRNA in the SEN group fits well with the protein data; however, a decrease in PLAN mRNA did not result in any change in PLAN PGC-1α protein. It is possible that PGC-1α protein is present, but not functional due to the combination of increased oxidative damage (19; 33) and lower proteosome function (18; 29) to remove damaged proteins that occurs with aging. Since PGC-1α coactivation of transcription factors can be modulated by many other factors within the cell, it is conceivable that the transcriptional activity may be lower with aging, despite non-significant changes in the total protein. That being said, due to the lack of a decrease in CS or COXIV protein, there is no evidence to suggest that mitochondrial biogenesis (by way of lower PGC-1α transcription activity) is lower in these aged muscles.

Levels of both Tfam and NRF-1 which are transcriptional targets of PGC-1α and are important for activation of mitochondrial genes in the mitochondria and nucleus respectively, were investigated. The rationale for these measurements was in case there was a discrepancy between PGC-1α and mitochondrial content: one possible explanation may be due to deficiencies in these transcription factors. Interestingly, an age-related decline for Tfam or NRF-1 mRNA in the PLAN or SOL (as well as the tibialis anterior, see Chapter 4) was not observed, suggesting that there is not an age impairment in this part of the mitochondrial biogenesis pathway which is in agreement with another study looking at similar constructs in human muscle (56) . This is particularly interesting given the 50% lower PGC-1α mRNA that was observed in the same muscles, illustrating a disconnect between PGC-1α and its downstream targets. In fact, for the PLAN, both Tfam and NRF-1 had higher mRNA levels in the OSED compared to the YSED group. The notion of this disconnect is also supported by an earlier finding that aged muscles had higher Tfam protein, yet lower PGC-1α protein (11). This surprising increase with age may be a compensatory response to improve the capacity for mitochondrial biogenesis in face of lower PGC-1α and/or mitochondrial protein content. The higher NRF-1, Tfam (and even COXI) mRNA may be sufficient to minimize any age related declines in this muscle, as it was observed that COX activity and protein content were not lower in this muscle.

3.4.6 Modulators of PGC-1α with Aging

We also investigated a few regulators of PGC-1α to determine the effects of aging on these proteins and to see if that could provide more information on the mitochondrial biogenesis results with aging and exercise (see Figure 1.2 of Thesis Introduction). Principally, we embarked on some of these questions to explain why PGC-1α was not upregulated in the aged animals following exercise training. However, we found some interesting results that pertain to aging (without exercise). RIP140 is a nuclear corepressor of genes involved in oxidative metabolism and mitochondrial biogenesis (25; 55), such that when its levels are elevated, mitochondrial biogenesis is diminished and when its levels are lowered, there is an increase in mitochondrial biogenesis. It was hypothesized that its levels would increase with aging, contributing to the decline in oxidative metabolism and

mitochondrial enzyme activity. Similarly, TNF-α has been implicated in reducing PGC-1α expression (43) and thus we hypothesized that increased TNF-α in the plasma and/or content of its receptor may contribute to age related declines in mitochondrial biogenesis. Contrary to our hypothesis, in the SOL we observed decreases in TNF- α receptor protein and in RIP140 protein which in theory would increase PGC-1α protein and mitochondrial biogenesis. Although an increase in either PGC-1α or mitochondrial biogenesis was not observed, we suspect that these may be compensatory events to modulate PGC-1α and essentially minimize any declines on mitochondrial biogenesis that would normally occur with aging. Given that there was no evidence of a decrease in mitochondrial content or biogenesis in the SOL, this seems to be a reasonable explanation.

We also measured levels of phosphorylated AMPK (GASr only) and phosphorylated ACC (as markers of AMPK activation), since AMPK is a significant activator of PGC-1α (32), with the hypothesis that decreases in AMPK activation with age may contribute to decreases in PGC-1α expression. Here we present a few observations that suggest AMPK activation may be higher with aging, in contrast to this hypothesis. In particular, p-ACC levels were substantially higher in the OSED group compared to YSED in the GASr and PLAN, as well as the tibialis anterior (Chapter 4). We only measured p-AMPK in the GASr, and it too was higher with aging. Again, this may be a compensatory response to minimize decreases in PGC-1α content or transcriptional activity, and may explain why in the PLAN, age related declines in PGC-1α or mitochondrial content were not observed. We did see decreases in PGC-1α in the GASr which may not necessarily argue against this point since it could be speculated that perhaps without the AMPK activation, there would be a more severe age-related decline in this muscle. However, while AMPK is activated by changes in energy status, we are unaware of what factors in aged muscle are responsible for increased AMPK activation. A recent study showed that the ROS H_2O_2 activated AMPK (31), and since ROS production is generally higher in aged tissues, this is one possible mechanism. That being said, these authors also showed that ROS increased PGC-1α activation *via* AMPK dependent and independent mechanisms, so with the age induced increase in ROS one would expect an increase in AMPK activity and

also PGC-1α expression in aged muscle (the latter is clearly not the case). This area warrants further investigation. In particular it will be interesting to manipulate AMPK in aged muscle by inhibiting or upregulating its activity and then to determine its effects on PGC-1α. Alternatively, the increased phosphorylation of AMPK and ACC with aging may be due to decreased phosphatase activity and/or due to protein modification (such as oxidative damage) to p-AMPK so that its actual activity is not higher with aging, despite the phosphorylation status being higher (i.e. although it is phosphorylated, it may not necessarily be activated and thus phosphorylation status in this case is not a good indicator of actual AMPK activity). Measuring actual AMPK activity will also be a helpful measure in this regard.

3.4.7 Summary of the Effects of Aging

Consistent decreases in CS activity across all 3 muscles is the best predictor/ measure to explain the age related decline in muscle VO_2 max. No change in CS or COXIV content was observed, suggesting that mitochondrial content does not change with age. The observation that PGC-1α protein was not lower with age in the PLAN or SOL suggests that the mediation of mitochondrial biogenesis is not diminished with age, as has been previously postulated (4; 11).

3.4.8 Effect of Long-Term Exercise on Mitochondrial Biogenesis and Function

Late middle aged rats were exercised on a treadmill 4 times a week for 5-7 months until senescence after which the SOL, PLAN and GASr muscles were harvested for analysis of the effects of aging and exercise on mitochondrial biogenesis and function. As shown previously in Chapter 2, long-term exercise training from LMA into SEN did not attenuate the age-related decline in muscle aerobic function and mitochondrial enzyme activity, despite benefits in these same measures when the same exercise program was used over 7 weeks at LMA (6). Although PGC-1α is an important regulator of exercise induced mitochondrial biogenesis (3; 59; 60), PGC-1α protein was not upregulated in the SOL or the PLAN following exercise training in senescence (Chapter 2). This inability to

upregulate PGC-1α likely explains the lack of exercise effect on mitochondrial enzyme activity.

The previous observations in Chapter 2 were expanded by including the gastrocnemius muscle because: (i) it is a larger muscle than the SOL and PLAN and likely contributes more to the muscle VO_2 that is measured in our hindlimb perfusion muscle preparation;(ii) it may be recruited differently during treadmill running; and (iii) it is an intermediate metabolic phenotype to the SOL and PLAN (2). This was a precaution on our part, to be sure that the findings of the SOL and PLAN were representative of the entire musculature. The other important addition to this study was the measurement of mRNA of PGC-1α, NRF-1 and Tfam. The rationale for including gene expression is that the increases in PGC-1α mRNA following exercise (or other interventions) are often many fold higher (44; 61) than increases in PGC-1α protein (3; 59; 60; 72), and thus mRNA may be a more sensitive marker to indicate if PGC-1α induction is initiated. This presented two scenarios that would help explain this data and guide future research, which merited this extra analysis. Firstly, if PGC-1α mRNA was increased in the exercise group, then we could conclude that signaling systems involved in increasing PGC-1α expression is not a problem, but there may be a problem in translating the gene transcripts into functional protein. Secondly, if PGC-1α mRNA was not increased, then the speculation that there was not sufficient induction of PGC-1α from the exercise would be supported more strongly. It was hypothesized that PGC-1α mRNA would be increased in the exercise group with an impairment of aged muscle to increase PGC-1α protein.

Previous findings that mitochondrial content (CS protein content) was not increased with exercise in the SOL or PLAN (Chapter 2) was expanded by showing here that CS protein was also not increased in the GASr. In addition to CS protein, COXIV subunit of the electron transport chain was measured as another marker of mitochondrial content to convincingly show that exercise at this part of the lifespan is ineffective in increasing mitochondrial content. The other mitochondrial results in the GASr were identical to those of the SOL and PLAN (Chapter 2), in that there was no effect of training on CS or COX

activity, CS or COXIV content, or PGC-1α content. Thus, the 3 primary muscles of the hindlimb have been studied, and the data is in line with no improvement in hindlimb muscle VO_2 max, providing good confidence that the training program was ineffective at this age. Whether it is because the stimulus was not strong enough or aged muscle is deficient in responding to the normal exercise stimuli is uncertain and warrants further work. Some aspects of this will be addressed in the following sections, as well as Chapter 5.

PGC-1α mRNA was measured as a more sensitive marker of exercise-induced activation of mitochondrial biogenesis. However, there was no increase in the mRNA levels (in agreement with the protein), providing a clear indication that exercise was unable to induce mitochondrial biogenesis. This now presents two possible explanations; (i) the exercise was not sufficient enough to induce mitochondrial biogenesis (i.e. the metabolic signals resulting from exercise were not significant enough), or, (ii) assuming the exercise stimulus was sufficient enough, there is a blunted response to induce PGC-1α. This may be due to something intrinsic within the PGC-1α protein or due to impairments of the signaling events that normally would communicate muscle contractile events to PGC-1α (eg. AMPK, p38 MAPK, CAMK, see Figure 1.2 of the Thesis Introduction).

As additional support that the muscle did not respond to the exercise training, GLUT4 protein and mRNA was measured and no induction of either was observed in the trained groups. GLUT4 is normally robustly increased with exercise training in young and in late middle age (3; 13; 56). This is further evidence of an inability for the muscle to adapt to regular exercise in SEN. The other advantage of GLUT4 is that it is not a mitochondrial enzyme, but its expression is increased by PGC-1α activation (3; 32). GLUT4 expression is increased in response to either AMPK activation or Ca^{2+} mediated activation of CAMK (or both) (42; 74) , and thus can be interpreted as a sensitive marker of muscle contraction.

3.4.9 Modulators of PGC-1α with Aging and Exercise

The finding that exercise from late middle age until senescence was completely ineffective in attenuating the age-related declines in muscle aerobic capacity was surprising. PGC-1α was expected to be higher in the trained animals with the anticipation that this would result in higher mitochondrial content. To help explain a lack of effect on PGC-1α, a few upstream regulators of PGC-1α were investigated to determine if they changed with exercise (or aging). Since AMPK activation is one of the primary signals that activates PGC-1α (30; 77), and AMPK is activated with exercise or muscle contraction (30; 48; 74), we measured p-AMPK in the GASr and p-ACC in all three muscles as markers of AMPK activation (14; 23; 71). None of these markers were elevated in the exercise group, suggesting AMPK activity was not increased with exercise. This represents a very strong explanation for a blunted PGC-1α response. While this study was in progress, a study from Schulman's group was published in which they showed diminished capacity to activate AMPK in aged extensor digitorum longus muscle, a phenotype very similar to the PLAN (48). In their study, aged muscles displayed blunted activation of AMPK following AICAR administration, ß-GPA feeding and exercise compared to young muscle. Furthermore, mitochondrial biogenesis was also blunted following ß-GPA feeding in the aged rats, concluding that AMPK activation is blunted in aged muscle and this likely contributes to blunted mitochondrial biogenesis (48). It is worthwhile to note that basal activity of AMPK was not altered with aging, but the ability to upregulate its activity was blunted. It is also worthwhile to note that while this manuscript was in progress, a recent study contradicted the findings of Reznick et al. (48). Thomson et al. (64) did not observe an age-related deficiency in AMPK activation following high frequency electrical stimulation. In fact, they observed a hyperactivity of AMPK to this type of muscle activation. They do point out several differences in methodology to explain the different observations, such as rat strain, absolute and relative age and type of perturbation. If trying to apply these findings to the current study, there is a further confounder of muscle type, since both of these studies used exclusively the extensor digitorum longus. Furthermore, in the study of Thomson et al. (64), using the same F344BN rat as we have done, their old group was 30 mo old, which we consider late middle age (see Table 1.1) and we have

observed that i) the declines in muscle mass, muscle aerobic capacity and mitochondrial enzyme capacity are relatively small (~20%) at this age (24), and thus this is not a very old phenotype; ii) that late middle aged F344BN rats are able to respond positively to regular treadmill exercise such that the improvements in muscle VO_2 max and mitochondrial enzyme activity are restored to young adult levels (6). The difference between our training study at late middle age and the data presented here is that the beneficial response at late middle age appears to be lost at senescence, suggesting that there is an inherent difference between late middle age and senescence. While they correctly identify possible differences between the F344, used in the Reznick study, and F344BN rat, it would have been informative, for comparison reasons, if the Thomson et al. (64) study included a senescent group. For selfish reasons for this manuscript, it would have also been useful if this study include measures of mitochondrial biogenesis following high frequency stimulation to be able to determine if the "hyperactivity" of AMPK in old muscle resulted in increased PGC-1α and mitochondrial content.

A similar line of evidence for a blunted activation of upstream signals related to muscle contraction comes from a recent study showing that MAPK activation following stretch was altered in senescent animals (F344BN, 36 mo) (40). As mentioned earlier, one of the explanations of a lack of training effect is that the training stimulus may not have been sufficient enough to invoke a response when training was extended to senescence. This could still hold true, given that we did not see an increase in AMPK activation. However, it seems equally plausible that the exercise could have been sufficient enough, but age-related impairments in AMPK activation may have prevented any activation of PGC-1α and subsequent mitochondrial biogenesis. It is interesting to note that p-ACC (marker of AMPK activation) was elevated in two of the three muscles. Since these markers of AMPK activation were measured in resting muscle (>48 hours after last exercise bout), another explanation could be that the higher AMPK activation at rest blunts any further induction of AMPK that would be expected from each exercise bout. Other factors are also relevant for the induction of mitochondrial biogenesis from muscle contraction (refer to Figure 1.2 in Chapter 1), and future work on these is warranted.

The interest in measuring SIRT1 protein and mRNA was that SIRT1 can interact (deacetylate) with and activate PGC-1α (35; 41; 50) and SIRT1 has been highly implicated to increases in lifespan that are observed with calorie restriction. Since SIRT1 has been shown to increase with exercise (17; 58) and since improved survival in the exercise trained rats was observed (Chapter 2), it was hypothesized that SIRT1 would be higher in the exercise groups. SIRT1 protein was indeed elevated in the OEX group in the GASr, but not in the SOL or PLAN. Since PGC-1α protein or mitochondrial biogenesis were not elevated in this muscle, it is difficult to interpret any benefits of higher SIRT1 protein in the GASr. Factors affecting SIRT1 activity and its location within the cytoplasm or nucleus can modulate its effects, and as such SIRT1 protein in muscle homogenates may not necessarily imply increased SIRT1 activity or activation of PGC-1α. Adding complexity to this issue is the finding that overexpression of SIRT1 can actually cause a reduction in mitochondrial content (22; 41). This suggests that a delicate balance between magnitude of SIRT1 expression, location (cytoplasm or nucleus) and activity likely interplay to result in benefits or consequences at the mitochondrial level. Nonetheless, the hypothesis of increased SIRT1 protein in all muscles with exercise was not supported by this data. Its relevance to survival with exercise warrants further investigation. The increased survival rate of the trained animals is either due to other factors, or the positive influence of SIRT1 may be observed in other tissues such as the brain, liver or heart that may be more related to survival or mortality with age than skeletal muscle.

3.5 CONCLUSION

In conclusion, this study shows that when regular exercise training is extended from late middle age until senescence, there is not an induction of mitochondrial biogenesis that is typically seen in young and late middle aged muscle. Regular exercise at this part of the lifespan was unable to attenuate the age-related decline in mitochondrial enzyme activity and skeletal muscle aerobic capacity. The lack of mitochondrial adaptation is likely due to an inability to upregulate PGC-1α. Signals that normally would increase PGC-1α and mitochondrial biogenesis and were hypothesized to decrease with age (AMPK activation,

SIRT1, Tfam and NRF-1) were actually elevated in the aged muscle. Similarly, negative regulators of PGC-1α (TNF-R1 and RIP140) were expected to increase with age, but in fact were either not different or were lower with age (SOL). This may represent a compensatory mechanism to maintain some positive influence for mitochondrial biogenesis. These (unsuspected) findings despite no increase in PGC-1α, support the notion that regulation of mitochondrial biogenesis, both upstream and downstream of PGC-1α, is particularly complex with aging. There appear to be some discrepancies between the regular signaling of mitochondrial biogenesis and resulting downstream effect (i.e. mitochondria content and function). The age-related decline in mitochondrial function and inability to upregulate mitochondrial biogenesis in senescence is likely the result of a number of factors that are involved in a coordinated effort to initiate mitochondrial biogenesis and achieve improvements in mitochondria content and function. Chronic exercise from late middle age into senescence was ineffective in increasing mitochondrial biogenesis and unable to attenuate the declines in muscle aerobic capacity, likely due to a blunted activation of PGC-1α. Either the exercise stimulus was not sufficient enough to activate the normal contractile signals that elicit cellular perturbations and subsequent adaptive response, or aged muscle has a deficiency to respond to these signals. In light of the recent literature and data from this work, we suspect both of these scenarios are correct and contribute to the blunted response.

3.6 ACKNOWLEDGEMENTS

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as well as providing the financial means and ethics approval necessary for this experiment. Dr. Hepple also provided input on the data collection and interpretation of some of the data.

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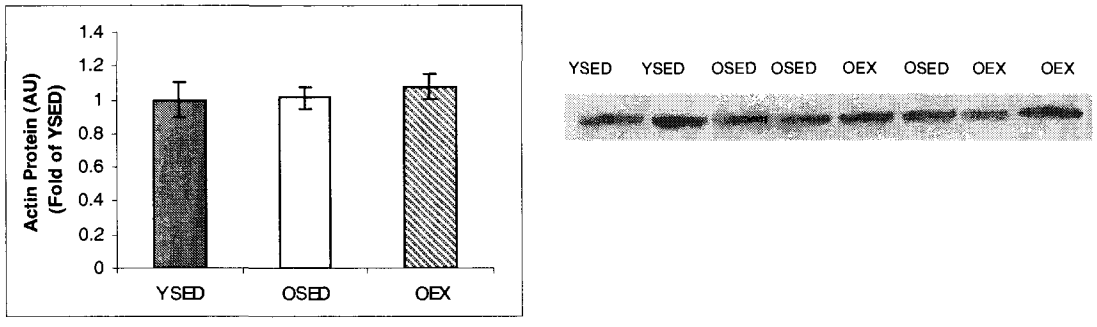
3.7 TABLES AND FIGURES

A



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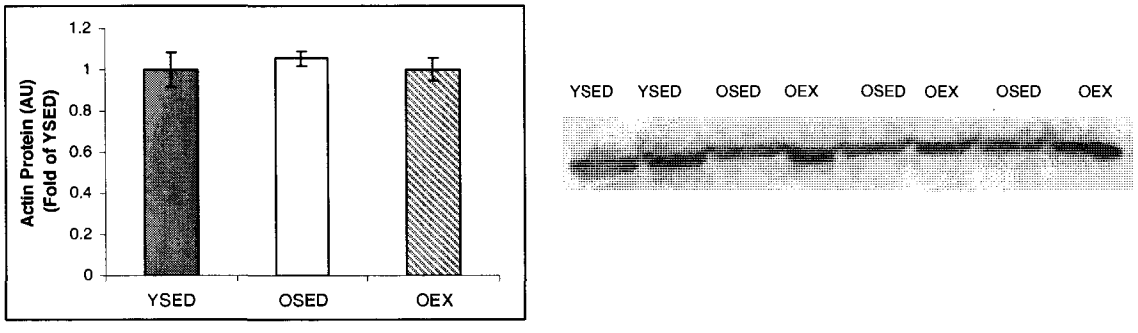

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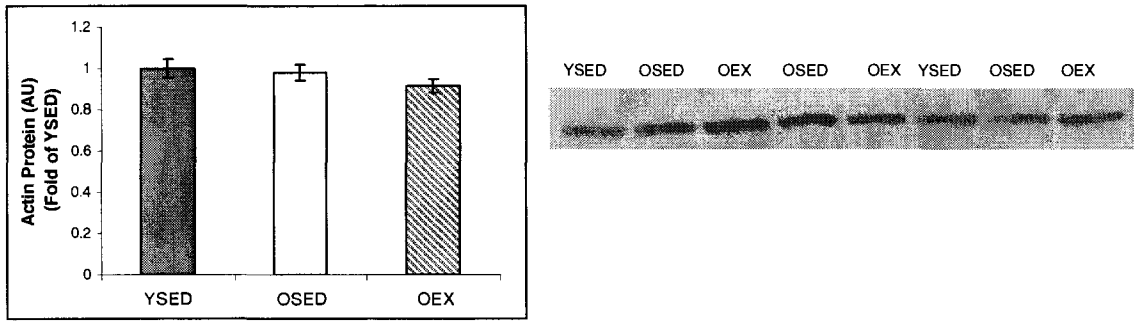


Figure 3.1 Actin protein for the soleus (A), red gastrocnemius (B) and plantaris (C) with representative Western blots showing no differences between aging or exercise groups. YSED = young sedentary group; OSED = old sedentary group; OEX = old exercise group

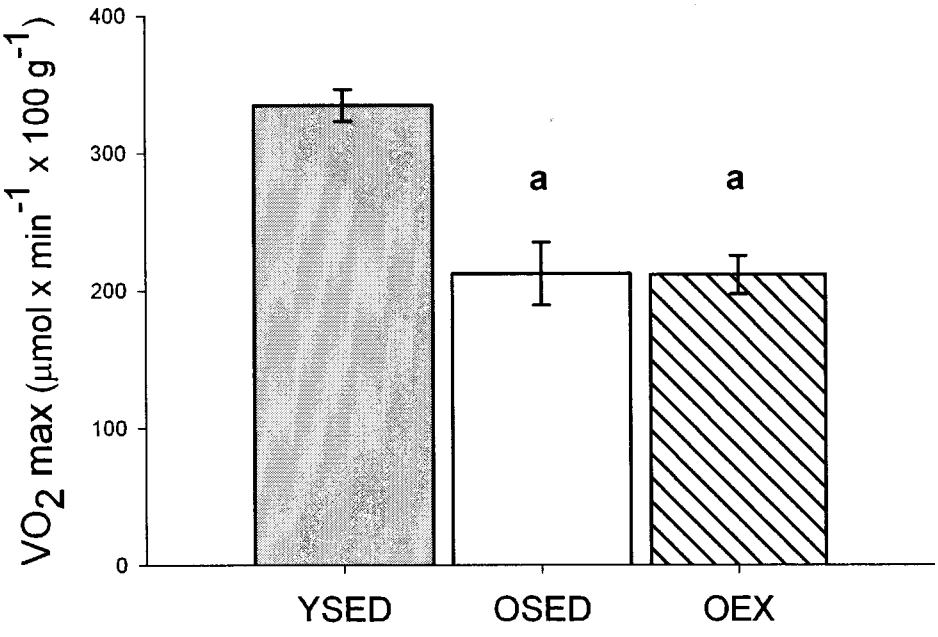


Figure 3.2 Hindlimb muscle VO_2 max achieved during increasing electrical stimulation test (pump-perfusion experiments).

Note: For comparisons to young sedentary (YSED), old sedentary (OSED) and old exercise (OEX) values were combined from 34 and 36 month values reported individually in Chapter 2.

a, $p<0.05$ vs YSED; b, $p<0.05$ versus OEX

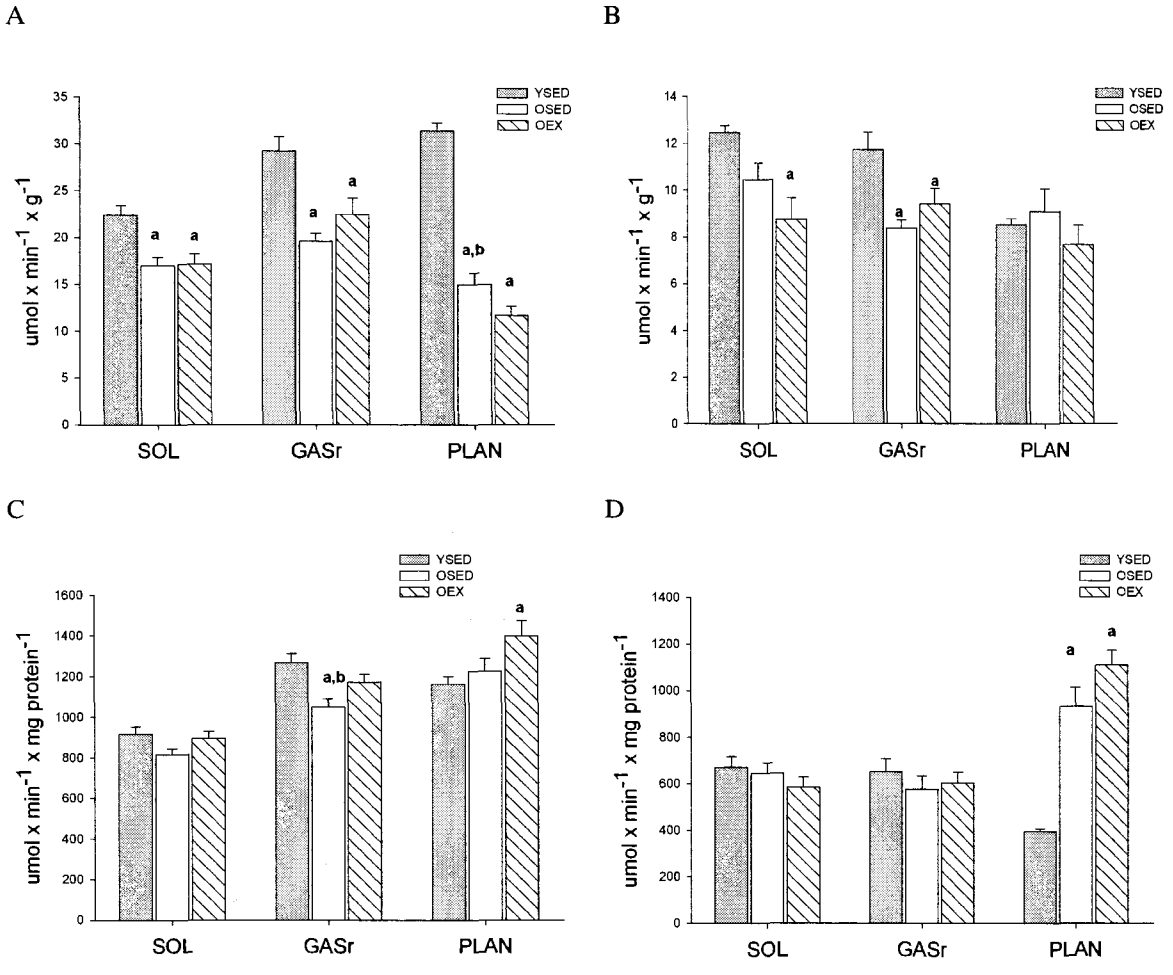


Figure 3.3 Citrate synthase enzyme activity (A,C) and cytochrome oxidase activity (B,D) for the soleus, red gastrocnemius and plantaris.

New results for young sedentary (YSED) (all muscle groups) and red gastrocnemius (GASr). Values for old sedentary (OSED) and old exercise (OEX) for soleus (SOL) and plantaris (PLAN) are from combined 34 and 36 month values reported individually in Chapter 2. Figures A and B are normalized to muscle mass while C and D are normalized to protein content. See section 5.2.3 for a discussion on normalization procedures.
a, $p<0.05$ vs YSED; b, $p<0.05$ versus OEX

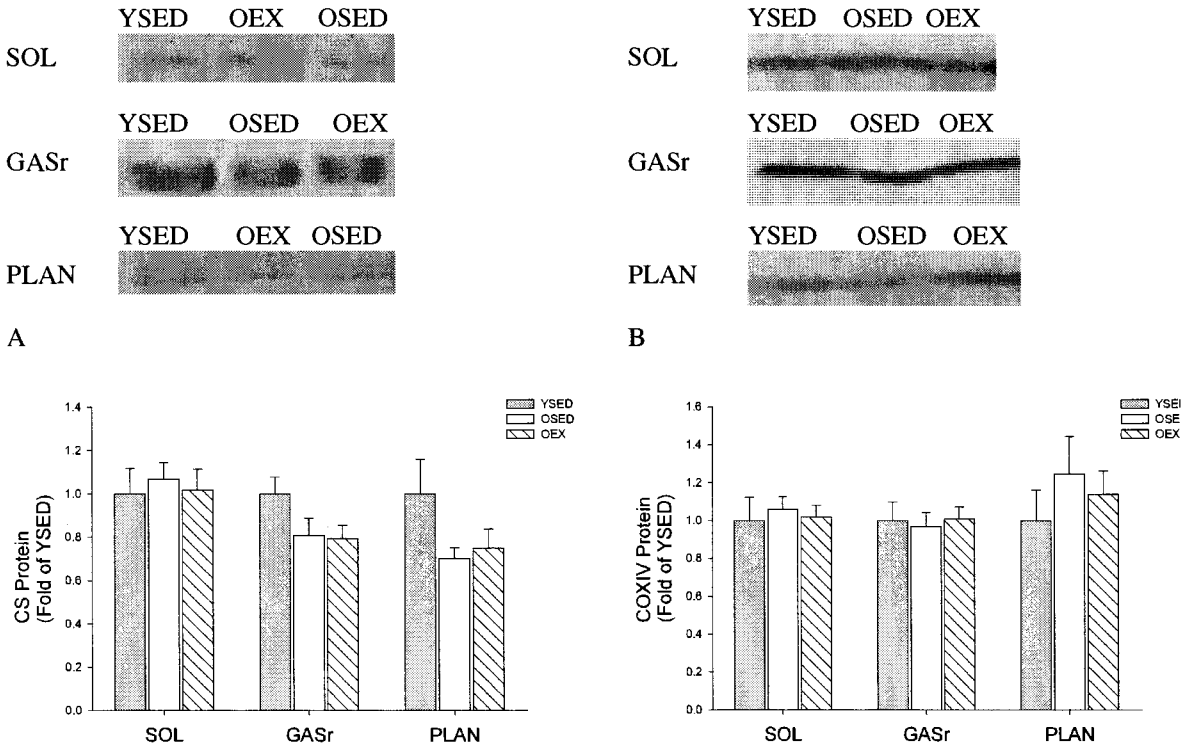


Figure 3.4 CS protein (A) and COXIV protein (B) for the soleus (SOL), red gastrocnemius (GASr) and plantaris (PLAN).

Values are normalized to the young sedentary (YSED) group for each muscle. a, $p<0.05$ vs YSED; b, $p<0.05$ versus OEX. For CS protein, YSED vs OSED $p=0.10$ in the red gastrocnemius (GASr) and $p=0.09$ in the plantaris (PLAN).

YSED = young sedentary group; OSED = old sedentary group; OEX = old exercise group

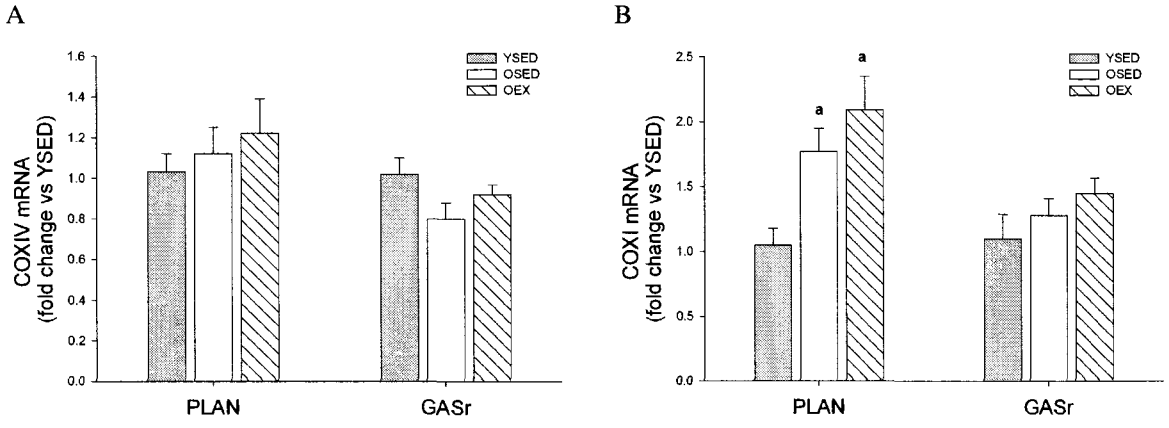


Figure 3.5 COXIV (A) and COXI mRNA (B) for the plantaris (PLAN) and red gastrocnemius (GASr).

Values are normalized to the young sedentary (YSED) group for each muscle. a, $p<0.05$ vs YSED; b, $p<0.05$ versus OEX. YSED = young sedentary group; OSED = old sedentary group; OEX = old exercise group

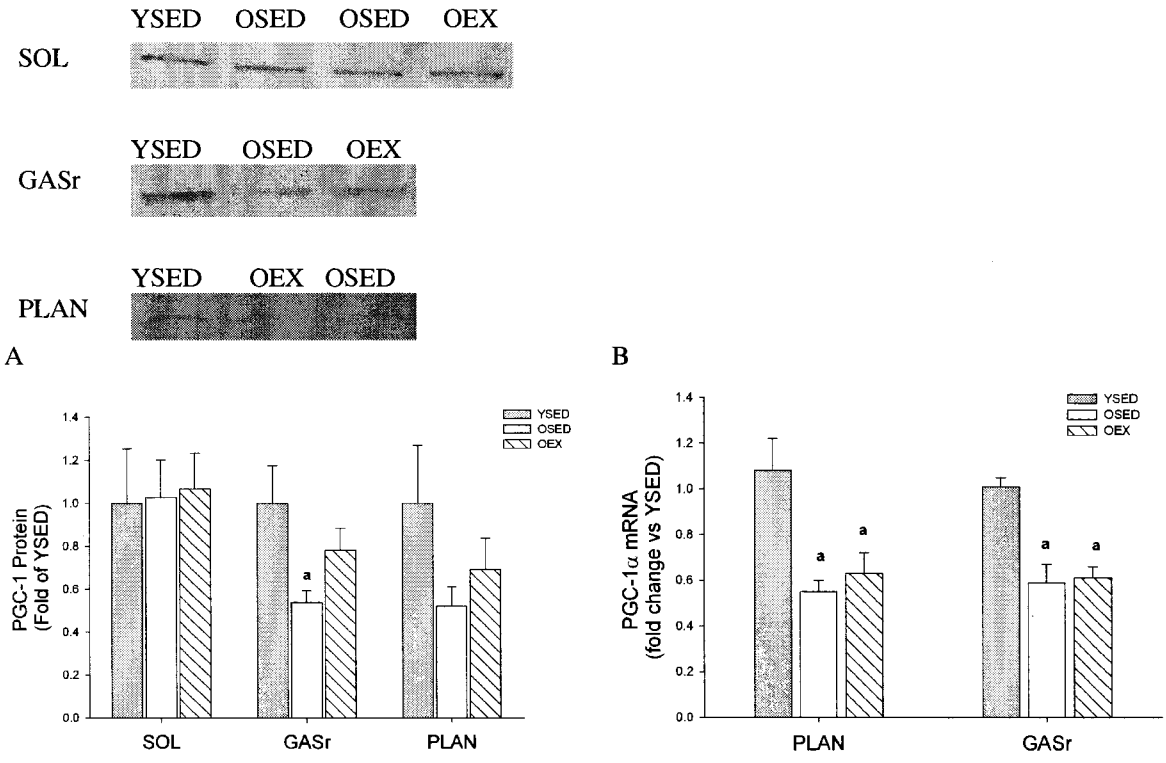


Figure 3.6 PGC-1α protein (A) for the soleus (SOL), red gastrocnemius (GASr) and plantaris (PLAN) and mRNA (B) for the plantaris (PLAN) and red gastrocnemius (GASr).

Values are normalized to the young sedentary (YSED) group for each muscle. a, $p<0.05$ vs YSED; b, $p<0.05$ versus OEX. For PGC-1α protein in the red gastrocnemius, $p=0.087$ OSED vs OEX; for plantaris, $p=0.06$ for YSED vs OSED. YSED = young sedentary group; OSED = old sedentary group; OEX = old exercise group

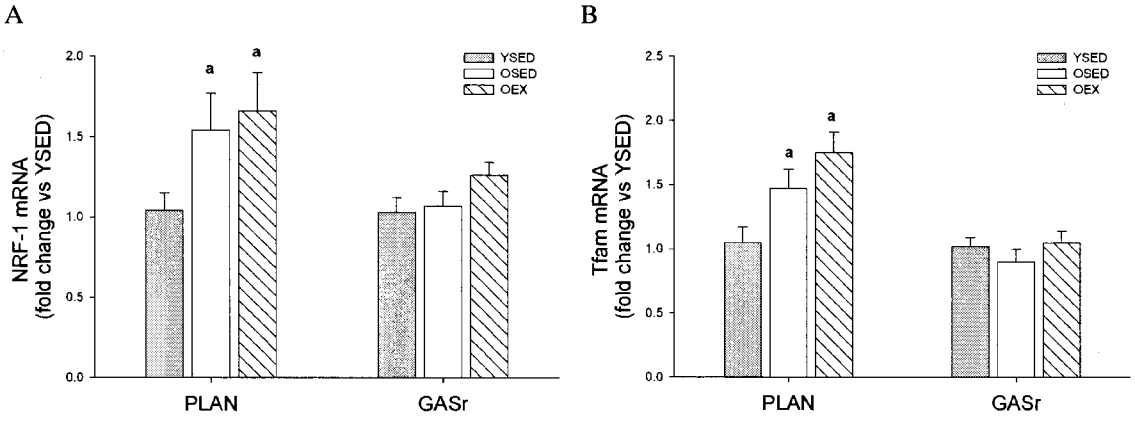


Figure 3.7 NRF-1 (A) and Tfam (B) mRNA for the plantaris (PLAN) and red gastrocnemius (GASr).

Gene expression was first normalized to an endogenous control that was shown not to change with age or exercise (18s for plantaris, ß-actin for red gastrocnemius) and values are represented as a fold change compared to the young sedentary (YSED) group. a, $p<0.05$ vs YSED.

YSED = young sedentary group; OSED = old sedentary group; OEX = old exercise group

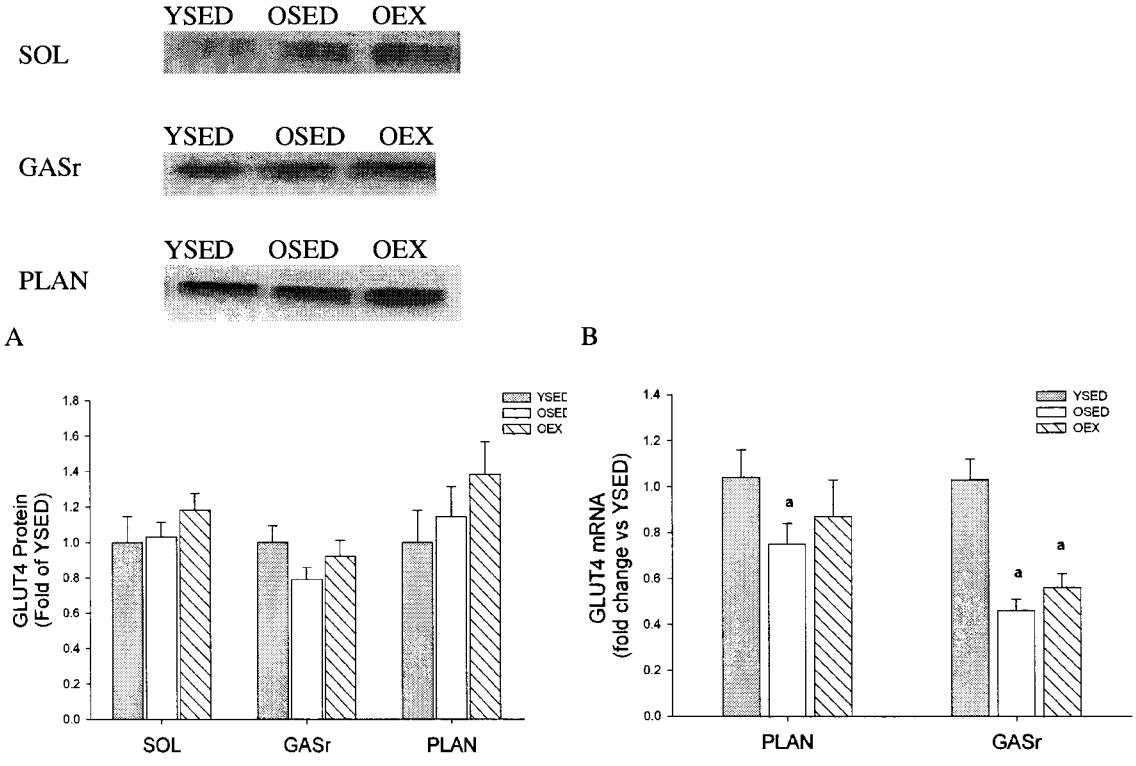
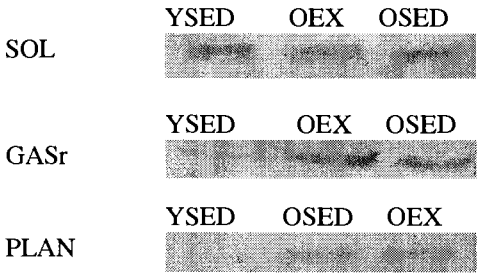


Figure 3.8 GLUT4 protein (A) and GLUT4 mRNA (B) content.

Values are normalized to the young sedentary (YSED) group for each muscle. a, $p<0.05$ vs YSED; b, $p<0.05$ versus OEX. YSED = young sedentary group; OSED = old sedentary group; OEX = old exercise group; SOL = soleus; GASr = red gastrocnemius; PLAN = plantaris



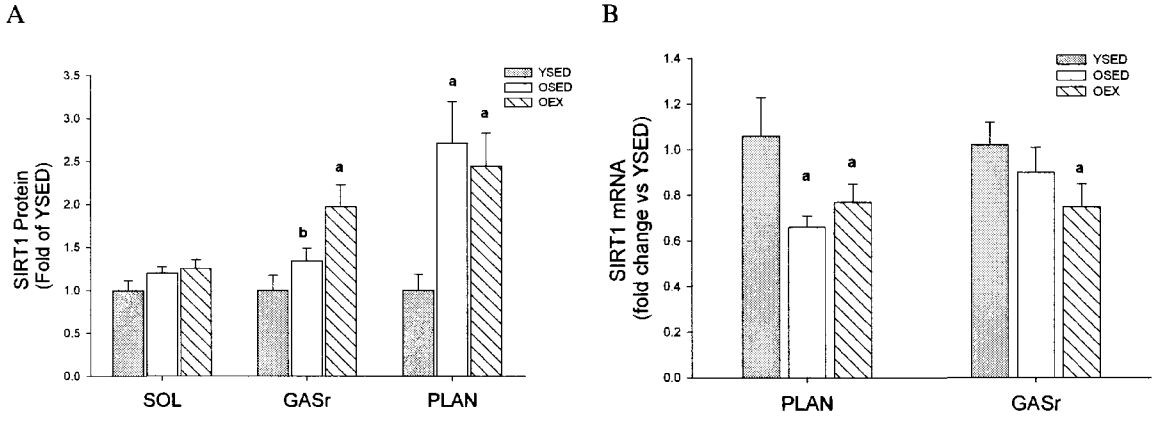


Figure 3.9 SIRT1 protein (A) and SIRT1 mRNA (B) content.

Values are normalized to the young sedentary (YSED) group for each muscle. a, $p<0.05$ vs YSED; b, $p<0.05$ versus OEX.

YSED = young sedentary group; OSED = old sedentary group; OEX = old exercise group; SOL = soleus; GASr = red gastrocnemius; PLAN = plantaris

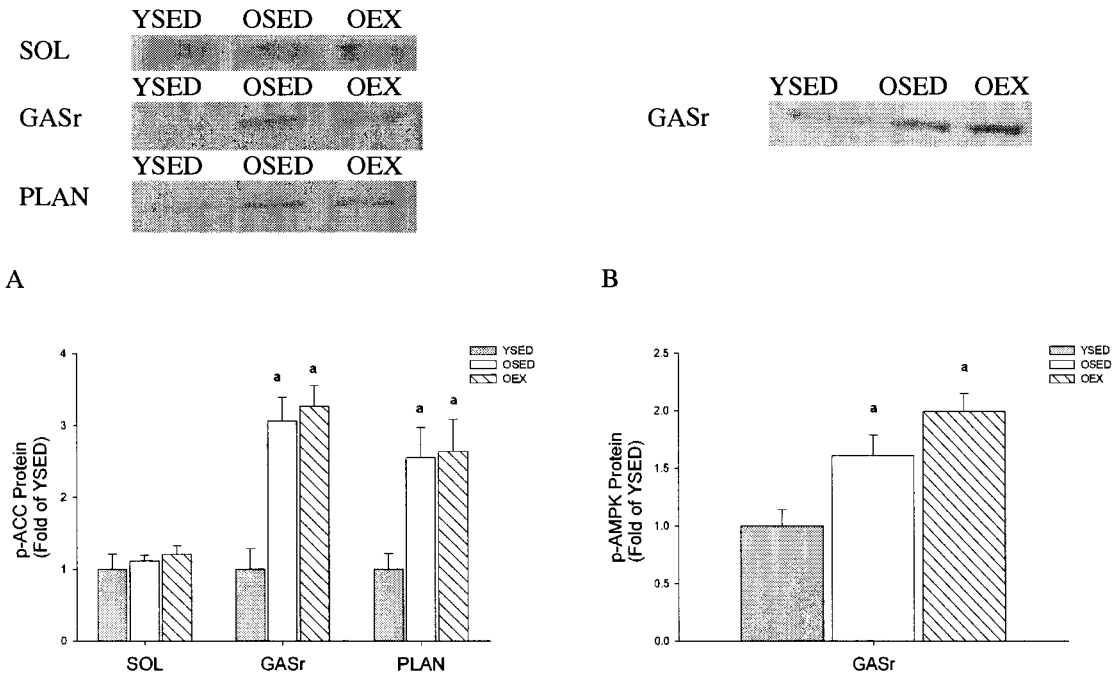


Figure 3.10 p-ACC protein (A) in the soleus (SOL), red gastrocnemius (GASr) and plantaris (PLAN) and p-AMPK protein in the red gastrocnemius (B).

Values are normalized to the young sedentary (YSED) group for each muscle. a, $p<0.05$ vs YSED; b, $p<0.05$ versus OEX.

YSED = young sedentary group; OSED = old sedentary group; OEX = old exercise group; SOL = soleus; GASr = red gastrocnemius; PLAN = plantaris

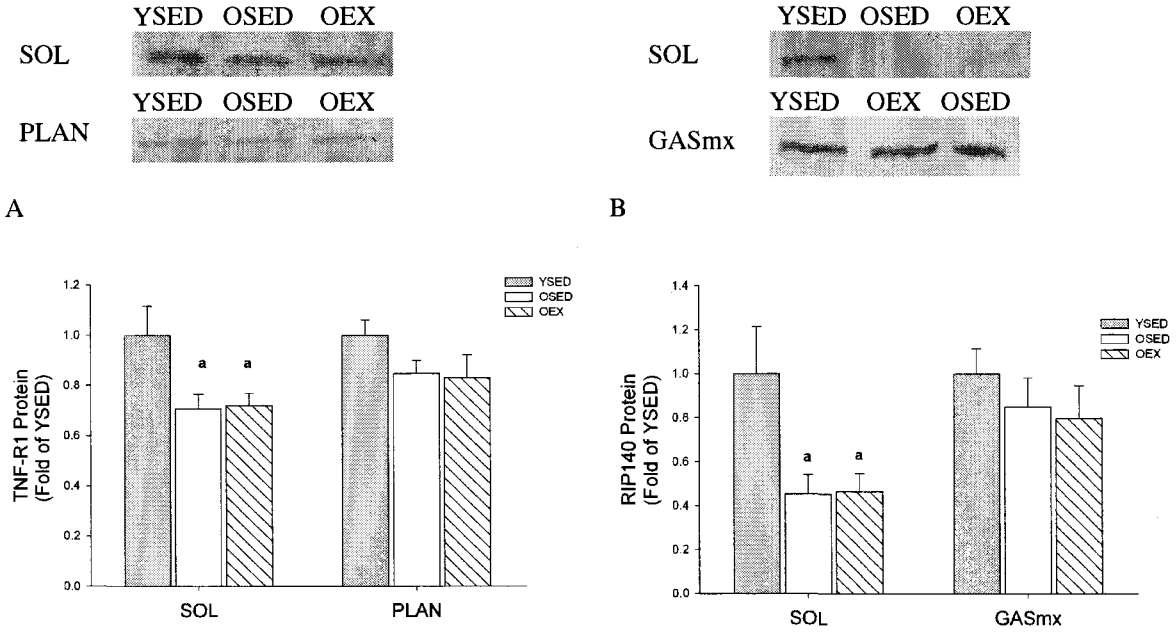


Figure 3.11 TNF-R1 protein (A) in the soleus (SOL) and plantaris (PLAN) and RIP140 protein in the soleus (SOL) and mixed gastrocnemius (GASmx) (B).

Values are normalized to the young sedentary (YSED) group for each muscle. a, $p<0.05$ vs YSED; b, $p<0.05$ versus OEX. For TNF-R1 in plantaris, $p=0.079$ for YSED vs OSED. For RIP140 for the mixed portion of the gastrocnemius (GASmx), it was not possible to normalize this to actin, but we have seen equal actin values for all groups from this homogenate).

YSED = young sedentary group; OSED = old sedentary group; OEX = old exercise group; SOL = soleus; GASr = red gastrocnemius; PLAN = plantaris

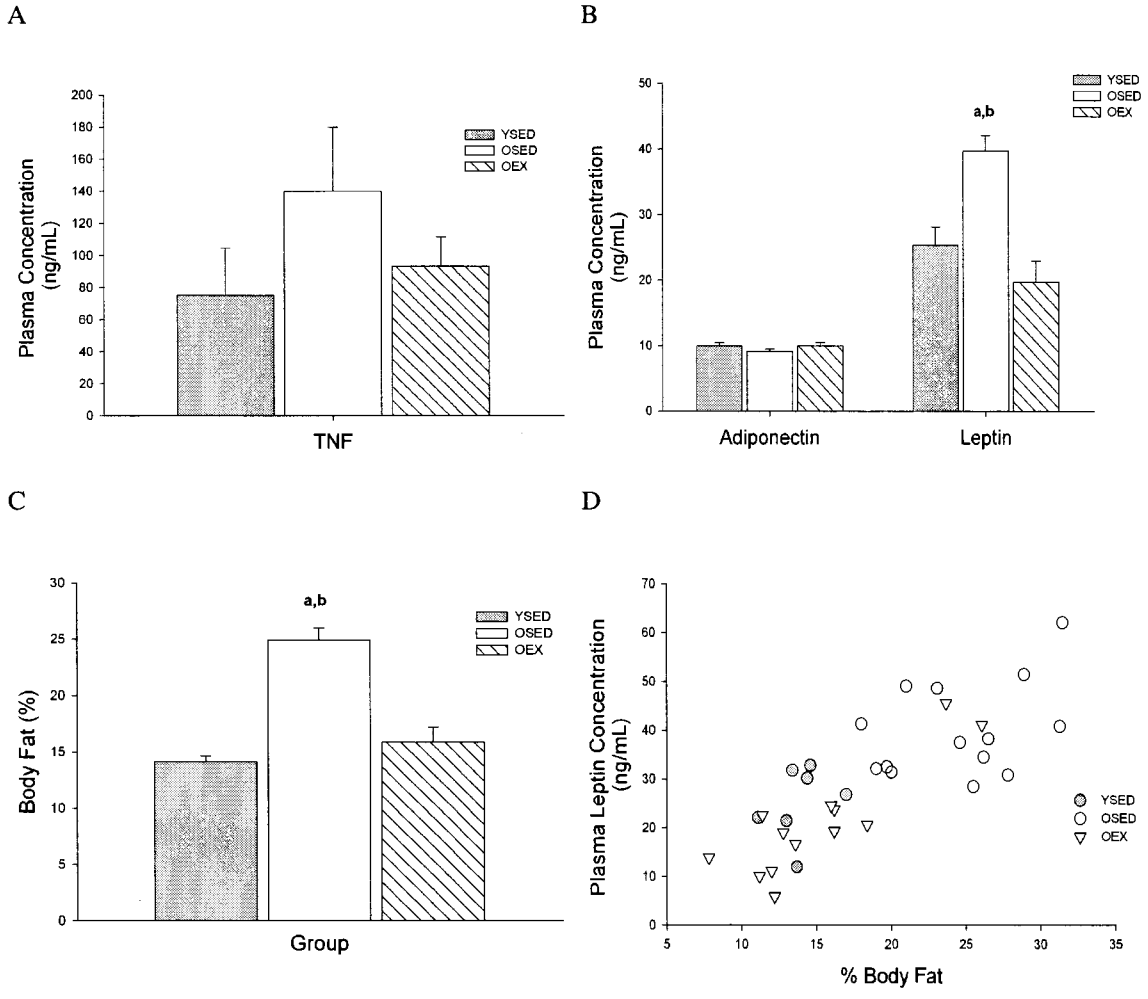


Figure 3.12 Plasma concentration for TNF-α (A) and adiponectin and leptin (B). Percent body fat measured by DEXA for each group (C) and the correlation between plasma leptin concentration and percent body fat (D) (r^2=0.62, $p<0.05$).

a, $p<0.05$ vs YSED; b, $p<0.05$ versus OEX

YSED = young sedentary group; OSED = old sedentary group; OEX = old exercise group

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Chapter Four: NO IMPAIRMENT OF SENESCENT MUSCLE TO INDUCE MITOCHONDRIAL BIOGENESIS FOLLOWING MUSCLE INJURY IN F344BN RATS

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4.1 INTRODUCTION

Aging is associated with lower peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) content suggesting a lower rate of mitochondrial biogenesis (1; 8), that along with lower mitochondrial protein synthesis rates (38) likely contributes to decreases in mitochondrial oxidative enzyme activity (9; 25) and consequently the reduced oxidative capacity of skeletal muscle (11; 20; 25). A diminishing capacity for aged muscle to perform aerobic work can, and eventually will, affect the quality of life of aged individuals by limiting mobility and independence (34) and increasing health risk factors such as insulin resistance, osteoporosis and cardiovascular disease that are associated with aging and a sedentary lifestyle.

PGC-1α has been termed a master regulator of mitochondrial biogenesis (35; 47) due to its central role in integrating a large variety of signals into activation of transcription factors that are responsible for mitochondrial biogenesis. The fact that PGC-1α decreases in aged skeletal muscle is evidence of a lower stimulus to synthesize mitochondria. To combat the age related declines in mitochondrial enzyme activity, we hypothesized that if we were able to increase PGC-1α in aged muscle we would improve the mitochondrial capacity of skeletal muscle and attenuate the declines in aerobic capacity. Unlike young muscle, we recently found that chronic exercise training in senescent animals was unable to increase PGC-1α (Chapter 2). Subsequently, there were no training benefits at the mitochondrial level, which resulted in no attenuation of the reduction in aerobic capacity of skeletal muscle with aging. This is in stark contrast to a plethora of studies that have shown that exercise in young and even late middle age is able to robustly increase mitochondrial content, enzyme activity (9; 42; 48) and muscle aerobic capacity (3; 26; 42). Specifically, work from our lab showed that treadmill training of late middle aged rats for 7 weeks was able to restore mitochondrial enzyme activity and muscle aerobic capacity to young adult levels (3). Given this blunted PGC-1α response to voluntary exercise in senescence, we followed-up with an acute intervention of muscle damage to invoke acute and robust muscle regeneration to determine if, on an acute level, induction of PGC-1α is blunted in aged muscle.

We employed a method of muscle injury that has been shown to increase PGC-1α early in the regeneration phase (17). These authors showed evidence of a clear time course relating activation and proliferation of satellite cells early in the regeneration phase (days 3-14) followed by differentiation into muscle fibres (days 5-35). Evidence of significant mitochondrial biogenesis (citrate synthase activity and state III respiration) early in the differentiation phase (days 5-10) suggests that mitochondrial function is important to the regeneration response, in particular in the differentiation of satellite cells into myofibres. The transcriptional coactivator PGC-1α and mitochondrial transcription factor Tfam were also elevated in this phase, peaking at 10 days, explaining the increases in mitochondrial biogenesis. We employed a similar model of acute muscle injury to determine if PGC-1α and mitochondrial biogenesis were equally activated in senescent and young muscle. In this study we used cardiotoxin to induce muscle damage (not bupivacaine as used by Duguez et al.(17)due to familiarity and experience (dosages, timing) with this agent and its effectiveness at inducing muscle damage without damaging satellite cells (12; 15; 21; 23).

With aging there is a decrease in muscle fibre number which largely explains the age-associated decrease in muscle mass (4; 27). One likely reason for this decline with age is a decrease in satellite cell number or function (19; 37) and a resultant decrease in the muscle's ability to replace damaged fibres. Satellite cells are quiescent cells located on the periphery of myofibres (32; 32) which remain quiescent until activated, for example, upon myotrauma or muscle injury. Upon activation, these satellite cells proliferate and eventually fuse to existing muscle fibres, to replace damaged regions (39) or fuse together to form new myofibres (reviewed by (22; 41). As such, satellite cells exist and function to repair or replace the finite number of existing terminally differentiated myofibres. Support for a reduced ability to replace damaged fibres comes from evidence that aged muscle has an impaired regenerative response following injury, likely due to impairments of reinneveration (6) but also impairments in the myogenic response within the muscle (10; 31). Deficiencies in myogenesis may be due to decreased satellite cell activation (36; 37), satellite cell proliferation (10; 40) or an impairment in the differentiation phase of the myogenic response (31). This impairment is compounded by the observation that with age

there is also an increase in apoptosis (2; 16; 45), and as such there is a greater need to replace these lost fibres. With the observations that mitochondrial biogenesis appears to be necessary early in the differentiation phase (17), and that PGC-1α appears to be lower in aged muscle (8), the lower presence of this nuclear co-activator may explain in part the impaired differentiation phase of the myogenic response. Although basal levels of PGC-1α appear to be lower with aging, it is unknown if aged muscle is able to upregulate PGC-1α to a similar extent as in young muscle.

The purpose of this study was to investigate the effect of age on mitochondrial biogenesis in regenerating muscle. Specifically, to determine if acute induction (i.e. increases in expression) of PGC-1α is blunted in aged skeletal muscle to compliment, and contrast, our long-term training study which asked if PGC-1α and mitochondrial biogenesis could be induced by chronic exercise training from late middle age until senescence. We also measured mitochondrial enzyme activity and content to assess the downstream effects of the PGC-1α response. Namely, if PGC-1α protein is not increased in senescent muscle compared to young muscle, does that result in substantially lower mitochondrial oxidative capacity? And, if PGC-1α is increased, is it as robust as in young adults? Also, does the increase in PGC-1α result in an increase in mitochondrial content and mitochondrial oxidative capacity? It was hypothesized that, 1) PGC-1α will increase in the young and aged group; 2) in the aged group there will be an attenuated increase in PGC-1α relative to the young group; and 3) mitochondrial content and enzyme activity will be lower in the aged muscle compared to young muscle consequent to the lower PGC-1α content.

4.2 METHODS

4.2.1 Animals.

All procedures were approved by the University of Calgary Animal Care Committee following CCAC guidelines. Male Fisher 344 Brown-Norway F1 hybrid rats (F344BN) were acquired from the National Institute of Aging (Bethesda, MD). The ages of the rats used for this study were young adult (7-13 mo, YA) and senescent (35 mo,

SEN). The 7 mo old animals used were from the sedentary young group of the training study (Chapter 3), while the 13 mo old animals were new animals that were used for cardiotoxin injection. These ages are associated with 95% survival and 35% survival, respectively, in ad libitum (AL) fed animals (44).

4.2.2 Cardiotoxin Injection.

Animals received an injection of snake venom cardiotoxin (CTX) (Latoxan, Valence, France) which is a protein kinase C inhibitor that results in skeletal muscle cell depolarization, excess release of calcium and rapid fibre necrosis which is followed by muscle fibre regeneration (13; 14; 18). CTX injection causes reproducible and severe muscle cell degeneration (22) but does not impact blood vessels or satellite cells (13). CTX was mixed with phosphate buffer saline solution (10 μM) and injected into the Tibialis Anterior (TA) muscle. This muscle was chosen for its ease of access and isolation from other muscles due to the fibrous sheath that encompasses the muscle. Three injections (~15 μL each) were made along the length of the muscle with a 25 gauge needle starting in the deep portion of the TA and slowly retracting the needle while injecting CTX so as to disperse CTX throughout the muscle. CTX was injected into both legs in a similar fashion; however, all analysis in this chapter was performed on the right TA only.

4.2.3 Muscle Harvest and Allocation of Animals into Groups.

TA muscles were harvested 3 weeks following CTX injection in 13 mo AL (n=7) and 35 mo AL (n=5) rats. To assess the progression of mitochondrial biogenesis throughout the regeneration phase, an additional group of SEN animals had their TA's harvested only 10 days following CTX injection (35 mo AL, n=5). To obtain an early indication of the degree of fibre necrosis, 3 YA AL animals and 2 SEN AL animals had their TA's harvested only 2 days after the injection. A representative H+E stain shows that the degree of fibre necrosis (Figure 4.1, provided by R.T. Hepple, unpublished) was substantial (Day 2), confirming the acute necrotic action of cardiotoxin.

After the designated number of days had elapsed post-injection, animals were anesthetized with isofluorane and the TA was harvested, removed from any fat and weighed. A section was cut through the mid-belly for histochemistry, another cross-section was dissected and cut into 20-30 mg pieces, placed into 750 μL RNAlater™ (Ambion, Austin, Texas), stored overnight at 4°C and then at -30°C as per manufacturer's instructions, while the remaining TA was quickly frozen in liquid nitrogen and stored in -80°C. The plantaris, gastrocnemius (separated into red, white and mixed fibre portions) and the soleus were also excised and frozen in liquid nitrogen. The animals were euthanized by cardiac removal.

The frozen TA was prepared for biochemistry and protein assays (Chapter 2). Briefly, the entire aliquot of frozen TA was powdered under liquid nitrogen from which approximately 80-100 mg was homogenized in 10X volume of extraction buffer containing 50 mM Tris-HCl, 250 mM mannitol, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100 with the following inhibitors added just prior to use: 1 mM DTT, 1 mM benzamidine, 0.5 mM AEBSF, 100 μM leupeptin, 1 μg/ml soybean trypsin inhibitor. Following 30 min on ice, samples were centrifuged for 10 min at 700g to remove impurities. From this supernatant, an aliquot was removed and diluted in 2X volume of potassium phosphate buffer (pH 7.5), freeze thawed three times (liquid nitrogen and room temperature), then centrifuged at 900g for 10 min at 4°C. The supernatant was collected and a portion was frozen at -80°C (1:20 dilution, mass:volume), while another aliquot was further diluted with potassium buffer to attain a 1:400 dilution and was subsequently stored at -80°C. The remaining supernatant was stored at -80°C for subsequent protein analysis.

4.2.4 Biochemistry.

Citrate synthase (CS) and cytochrome oxidase (COX) activity were measured spectrophotometrically at a wavelength of 412 nm and 550 nm respectively (37°C; DU® 800 Spectrophotometer, Beckman Coulter Canada Inc., Mississauga, Canada) (Chapters 2

and 3). COX activity was measured on the homogenates with a 1:20 (mass:volume) dilution and the CS activity was measured on homogenates that were diluted 1:400. CS activity was measured by following the rate of mercaptide ion formation over a 3 minute period. Tris buffer (650 μL, pH 7.5), Acetyl CoA (3 mM, 50 μL), DTNB (1 mM, 100 μL) and 100 μL of homogenate were mixed into a cuvette. Oxaloacetate (0.5 mM, 100 μL) was then added and allowed to incubate for 2 min before data measurement. COX activity was measured by following the rate of oxidation of cytochrome c over 3 min, immediately following the mixture of 10 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.0, 37°C), homogenate (4 μL) and reduced cytochrome c (30 μL). Reduced cytochrome c (1 mM) was prepared by mixing cytochrome c into 10 mM potassium phosphate buffer containing 2.7 mM L-ascorbic acid and placing it in dialysis tubing surrounded by phosphate buffer (10 mM) containing 1mM EDTA (pH 7.0) in 4°C with gentle mixing. The outside buffer was changed three times over 24 hours and sufficient reduction of cytochrome c (> 65%) was verified by the ratio of reduced to oxidized cytochrome c as determined spectrophotometrically at 550nm and 565nm respectively. For both CS and COX activity, all samples were run in duplicate and averaged, except if the difference between the 2 samples was greater than 10% in which case a third sample was measured.

4.2.5 Western Blotting.

Protein concentration was determined in the homogenates using the Bradford method (5), and Western blotting was performed as described previously (Chapter 3). Subsequently, aliquots of this homogenate were diluted to achieve equal protein concentration in all samples with sample buffer containing glycerol, Tris pH 6.8, SDS, DTT, bromophenol blue and ß-mercaptoethanol. Equal protein amounts and a PageRuler™ Prestained Protein Ladder (Fermentas International Inc., Burlington, Canada) were loaded in precast mini-gels (Bio-Rad, Hercules, USA) and separated by electrophoresis (90 min, 100 Volts). Proteins were then electro-transferred onto PVDF membranes (90 min, 100 Volts, 4°C). Membranes were blocked for 60 min at room temperature with 5% non-fat milk (Carnation®, Nestlé, Switzerland) in phosphate buffered saline (pH 7.4) and 0.05%

Tween (PBST). Membranes were then incubated overnight in 4°C with primary antibodies diluted in 5% milk-PBST with gentle agitation. Membranes were then washed 3 times with PBST, incubated with appropriate secondary antibody diluted in 5% milk-PBST (1:5000 Pierce, Rockford, IL) for 60 min at room temperature followed by 2 more rinses with in 5% milk-PBST and a final rinse with PBS (no Tween). Protein detection was achieved by measuring the chemiluminescence (Syngene Bio-Imager, Frederick, MD) and densitometry measured with Bio-imager software (Syngene Tools, Frederick, MD). The densitometry value for the protein of interest for each sample was normalized to its own α-actin band from the same gel and the normalized data was used for group comparisons. In the figures, data are displayed as normalized data to the YA+AL21d group, unless otherwise specified. Primary antibodies used were: CS (1:10,000, a kind gift from J. Holloszy), COXIV (1:5000, Molecular Probes), PGC-1α (1:1000, Calbiochem), p-ACC (1:500, Cell Signaling), and α-actin (1:1000, Santa Cruz).

4.2.6 Gene Expression.

Real time PCR was performed on TA aliquots that were stored and frozen in RNAlater™ as done previously (46). RNA was extracted using a Fibrous RNeasy kit (Qiagen, Mississauga, Canada) and was diluted to yield 2 μg of total RNA. From this, complimentary DNA (cDNA) was synthesized by reverse-transcription using SuperScript II Reverse Transcriptase, random primers, dNTP, 5x FSB, RNase Out and DTT (all from Invitrogen, Burlington, Canada). Real time PCR was performed using a 7900HT Fast-Real Time PCR system (Applied Biosystems Inc., Foster City, USA). All data was normalized to 18s (ΔCT= CTgene of interest – CT18s; CT= cycle threshold), which was verified not to differ between any of the comparison groups and thus a suitable internal control (CT for 18s: YA: 16.4 ± 0.34, YA+21d: 15.9 ± 0.29, SEN: 16.7 ± 0.23, SEN+21d: 16.2 ± 0.41, SEN+10d: 15.9 ± 0.24). TaqMan® Gene Expression Assays (Applied Biosystems Inc.) were used to determine the expression of 18s, NRF-1, Tfam and SIRT1. Primers for PGC-1α, COXI and COXIV were designed using Primer Express 3.0 software and provided by Integrated DNA Technologies (Coraville, USA) and the probes were supplied by Applied Biosystems. Samples were run in duplicate on a 96 well plate with each well containing 1

μL gene expression assay, 10 μL of TaqMan® Fast Universal PCR Master Mix and 6-8 uL of RNAse free water depending on the volume of cDNA that was used for a total of 20 μL per well (3 μL for PGC-1α, 2 μL for COXI and COXIV, and 1 μL for all other genes). Statistical analysis was performed on the ΔΔCT data while relative differences in gene expression were determined from the 2-ΔΔCT method (29), as done previously (43; 46). Data are presented as fold change compared to YA+AL 21d group unless otherwise specified.

4.2.7 Statistics.

Comparisons between groups was performed by one way analysis of variance and Student-Newman-Keuls post hoc analysis where necessary, with statistical significance set at $p<0.05$. All data are presented as means ± standard error (SEM). Graphs of protein and gene expression are expressed relative to YA group, unless otherwise noted. In the figures, letter superscripts were used to denote significant differences between groups, and this nomenclature was kept consistent for all graphs in this chapter. In cases where no difference exists, the superscript nomenclature was retained in the figure legend for consistency and as identification that the statistical test was performed.

4.3 RESULTS

4.3.1 Muscle Mass.

Muscle masses are summarized in Table 4.1. TA muscle mass was significantly lower (~40%) in the SEN animals compared to YA, demonstrating sarcopenia. Following 21 d of recovery after CTX injection, the muscle mass had returned to within 10% of control muscles, in both the YA and SEN such that the muscle masses were not different from control muscle mass ($p>0.05$).

4.3.2 CS and COX: Activity, Protein and mRNA.

CS and COX activity are summarized in Figure 4.2. There was not an age-related decline in CS activity of non-injured TA muscle. A small cohort of animals were assessed only 2 days following CTX injection to get an idea of the degree of muscle damage that had occurred at this time point (statistics could not be performed on these small samples). At two days, CS activity was markedly reduced compared to non-injured, age matched muscle (YA 14.4 ± 3.9 vs 24.9 ± 0.77, n=3; SEN 11.9 ± 6.3 vs 26.1 ± 0.83, n=2, μmol .min-1 .g-1). At the 10 day point following CTX injection, CS activity in the SEN+10d group was significantly lower than SEN and SEN+21d (17.6 ± 1.3 μmol .min-1 .g-1). Twenty-one days after CTX injection CS activity was not different between the YA and SEN (YA+21d: 24.9 ± 1.7, SEN+21d 25.7 ± 1.5 μmol .min-1 .g-1). The 21 day values also were not different from control muscles ($p>0.05$) suggesting that CS activity had returned to basal levels.

Similar to CS activity, COX activity appeared to be markedly reduced 2 days following CTX injection (YA 10.8 ± 4.1; SEN 10.3 ± 4.8 μmol .min-1 .g-1). Twenty-one days following CTX injection, the YA group had significantly higher COX activity than the SEN group (16.3 ± 0.6 and 12.0 ± 1.1 μmol .min-1 .g-1 respectively). When comparing COX activity in uninjured TA muscles, there was not a statistically significant difference between YA and SEN ($p=0.06$). At the 10 day time point, SEN+10d was significantly lower than SEN and SEN+21d. However, when comparing COX activity in the 21d groups to control groups, COX activity was significantly lower in the 21d groups for both YA and SEN (17% and 33% respectively), suggesting an incomplete restoration of COX activity by 21 days for both groups.

CS and COXIV protein expression were not different between the YA and SEN groups (Figure 4.3), suggesting there is not an age-related decline in these proteins. By 10 and 21 days post CTX injection, these proteins had returned to uninjured levels for their corresponding age groups (i.e. SEN+21 not different from SEN).

Similarly, there were no significant differences in mRNA of COXI or COXIV between young and old for both the control muscles and in the muscles that were injected with CTX (Figure 4. 4).

4.3.3 PGC-1α, p-ACC and SIRT1 Expression.

PGC-1α protein was not different between YA+21 and SEN+21 (Figure 4.5) which suggests an equal induction PGC-1α response in both ages, similar to the CS and COXIV protein response (Figure 4.3A). PGC-1α protein was not measured in uninjured muscles due to technical issues; however, we were mostly interested in determining if the SEN+21 group had lower PGC-1α protein compared to the YA+21 group to support our hypothesis that aged muscles have a blunted capacity to upregulate PGC-1α. There was not a significant difference in PGC-1α mRNA between the young and old groups for both uninjured TA and 21 days post CTX injection (Figure 4.5B). Tfam mRNA (but not NRF-1 mRNA, p=0.086) was significantly higher in the SEN+21d group compared to the YA+21d group, despite similar levels in the uninjured muscles of YA and SEN (Figure 4.6).

Phospho-ACC protein expression was significantly higher in SEN+21d compared to YA+21d (Figure 4.7). This was not measured in uninjured muscles; however, we have seen consistently that p-ACC is significantly elevated in aged muscle compared to young muscle (see Chapter 3). Finally, SIRT1 mRNA was not different between YA and SEN for uninjured muscles and also not different between YA+21d and SEN+21d for CTX injected muscles. In the young muscles, YA+21d was not different than YA controls. However, for the SEN muscles, SEN+10d was significantly lower than SEN+21d and SEN. There was not a significant difference between SEN+21d and SEN (p=0.3).
α-actin protein expression was compared across the five groups to ensure this protein does not change amongst groups. By one way analysis of variance, α-actin was not significantly different between groups (p=0.067), although there appears to be greater α-actin protein in the YA+21d group compared to the YA group (Figure 4.8). By simple t-test, the groups are still not significantly different (p=0.055); discussion of this findings and implications of a difference in α-actin is included in the limitations section in Chapter 5.

4.4 DISCUSSION

The purpose of this study was to determine if aged skeletal muscle had a blunted induction of PGC-1α and mitochondrial biogenesis following acute muscle damage. The main findings were that aged muscle had similar PGC-1α levels as young muscle 21 days after muscle damage. With the exception of COX activity, the levels of CS activity, CS and COXIV protein, COXI and COXIV mRNA were not different between the YA+21d and SEN+21d. This suggests that mitochondrial biogenesis is not impaired in aged muscle, at least not under the conditions of muscle regeneration following acute muscle damage.

4.4.1 Regenerated Muscle Mass.

There were no apparent differences in muscle regeneration as both YA and SEN groups had very similar relative regeneration of muscle mass as their age-matched control groups. This is in agreement with another study that used the extensor digitorum longus (7), as well as a similar study as this one (same muscle, rat strain, time course) that showed a similar recuperation of muscle mass in adult (18 mo) and late middle age (31 mo) animals (31). Interestingly, when they compared a very young group (3 mo), the young group had a much more robust regeneration response (rate of muscle mass recovery) than the adult (18 mo) group. However, there were no differences between the adult and old groups, as seen in the present study.

4.4.2 Mitochondrial Biogenesis

Our primary interests were in the aged muscle's ability to induce PGC-1α and upregulate mitochondrial biogenesis during muscle regeneration. Based on our prior study that PGC-1α induction following exercise was blunted in aged muscle (Chapters 2 and 3), we expected to see a blunted induction in the aged muscles following acute muscle injury. However, this was not the case, as we show here that PGC-1α protein levels are the same in the YA+21d and SEN+21d groups. Because control levels of this protein were not measured, we were unable to compare the PGC-1α content at 21d to what would be normal.

If anything, we would expect the control values for PGC-1α to be lower (8) or not different (Chapter 2 and 3) in the SEN animals compared to YA. The fact that SEN+21d PGC-1α protein and mRNA was similar to YA+21d, suggests that the capacity to increase PGC-1α following muscle damage is not impaired in SEN animals compared to YA. Although our training study showed no induction of PGC-1α in SEN muscle, it may be argued that the contractile stimulus was not sufficient enough to induce such a response. This is perhaps a fair argument seeing that at the end of the training study the animals were exercising at only 6 metres per minute, which is a slow walk (Chapter 2). Recent unpublished work on the same animal and age showed that with a more robust contractile intervention than our treadmill exercise (7 days of 3 hrs/day electrical stimulation), the SEN animals were able to increase PGC-1α and mitochondrial biogenesis, but the magnitude was blunted compared to young muscle for the same contraction intensity (30). We are currently unaware of any other study that has investigated PGC-1α responses in senescence. Based upon the graded nature of the muscle response (no induction with treadmill running, small induction yet blunted following electrical stimulation), the fact that we see no impairment in the aged muscles after muscle damage may be due to the fact that the stimulus is much greater than that of the electrical stimulation model and even more so than the treadmill exercise model. As such, the conclusions we can draw from this is that given a very extreme perturbation, aged muscle can induce PGC-1α. However, aged muscle appears to have a blunted induction of PGC-1α with a lower intensity stimulus.

The next question was to determine if the increases in PGC-1α in both age groups were equally able to induce mitochondrial biogenesis. In this regard, we observed similar levels of CS enzyme activity, CS protein content and COXIV protein content in YA and SEN, all markers of mitochondrial content, suggesting mitochondrial biogenesis is not impaired in aged skeletal muscle during muscle regeneration. This implies that the induction of PGC-1α was able to successfully execute mitochondrial biogenesis, and there was no impairment in the aged muscle. This is a very interesting finding, because in our previous work (Chapter 2) and that of Dr. Hood's group presented in abstract form (30), aged muscle had a diminished mitochondrial response to contractile activity and this could

be explained in part by a diminished PGC-1α induction. The data in this chapter suggests that, so long as PGC-1α can be induced, the aged muscle is able to increase mitochondrial biogenesis to a similar extent as young muscle.

We did observe slightly lower activity of COX in the SEN+21d group, relative to the YA+21d group, in contrast to the three measures above, and thus some caution in this interpretation is warranted. It is difficult to reconcile this difference between the groups given that the three other markers of mitochondrial content were not different. COX activity in the YA+21d had not reached control levels, and thus the lower COX activity in the SEN+21d group relative to SEN may not be an age-related impairment *per se*. Alternatively, it may be that allosteric factors influencing maximal activity of COX activity are lower in the aged muscles under these conditions. Alternatively, since the COX enzyme has both nuclear and mitochondrial coded subunits, it is possible that the coordination of the synthesis of these subunits is impaired such that complete function of the enzyme may not be possible (or is delayed), despite seeing equal amounts of COX subunit IV protein (which is nuclear encoded). In line with this, one of the postulations of aging is that there is an increase in mitochondrial DNA damage (20; 24; 28; 33; 49) that can impair the synthesis of functional electron transport chain complexes that rely on mitochondrial DNA for synthesis of its subunits. Because COX has three subunits that are coded by mitochondrial DNA, this is a possibility. Given that the regenerated myofibres originated from satellite cells, it is unknown if the satellite cells of aged muscle contain higher levels of mtDNA oxidative damage than that of normal muscle cells. Taken together, we interpret the results of our study to suggest an equal ability to induce mitochondrial biogenesis in SEN muscle following severe muscle damage and that the change in COX activity is likely due to enzyme function and is not representative of lower mitochondrial content.

4.5 CONCLUSION

There was no impairment of the aged animals to regain muscle mass, suggesting muscle regeneration is not impaired in these animals. However, further work on contractile

function will be necessary to determine if the quality of the regenerated muscle is as good as the young animals. Contrary to our hypothesis and results from the previous exercise training study (Chapters 2 and 3), we show here that aged muscle is equally able to induce PGC-1α and mitochondrial biogenesis following severe muscle damage invoked by CTX injection. It may be of interest in future studies to examine a longer time course to determine if improvements in mitochondrial enzymes are still ongoing, and if at a later time point there is an impairment in the aged muscle. It is also of interest to determine if the regenerated muscle of the old animals, which at the mitochondrial level appears to be similar to that of young animals, will display an aging phenotype a few months after the muscle damage, or if it will maintain similar mitochondrial capacity as young muscle.

4.6 ACKNOWLEDGEMENTS

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Betik and helped with most of the muscle harvest. Muscles from this study are being used for other experiments being undertaken by Dr. Hepple, and thus the injections and muscle harvest was a collaborative project. While assistance was provided, Andrew Betik participated in all levels of this experiment including cardiotoxin injections, muscle harvest and data collection. Andrew performed all the experiments measuring biochemistry, gene expression and protein expression and performed all of the data analysis. Andrew wrote the original draft of this manuscript and is responsible for the final draft, but it is duly acknowledged that he received advice and suggestions for revisions from Dr. Severson, Dr. Wright and Dr. Syme for which he is truly grateful and appreciative.

4.7 TABLES AND FIGURES

Table 4.1 Muscle masses (mg) of the right tibialis anterior following injection with cardiotoxin.

	Con	**2 d**	**10 d**	**21 d**
YA	807 ± 13	796 ± 71	No data	759 ± 44 (94% of Con)
SEN	551 ± 23[a]	466 ± 62	410 ± 28[a,b]	508 ± 76[a] (92% of Con)

a, $p<0.05$ vs YA-Con; b, $p<0.05$ vs SEN-Con; c, $p<0.05$ vs SEN+21d; there were only 2 animals in the 2 day group and thus were not included in any statistical analysis.
YA = young adult; SEN = senescent; Con = no injection; 2 d, 10 d, 21 d = number of days after cardiotoxin injection

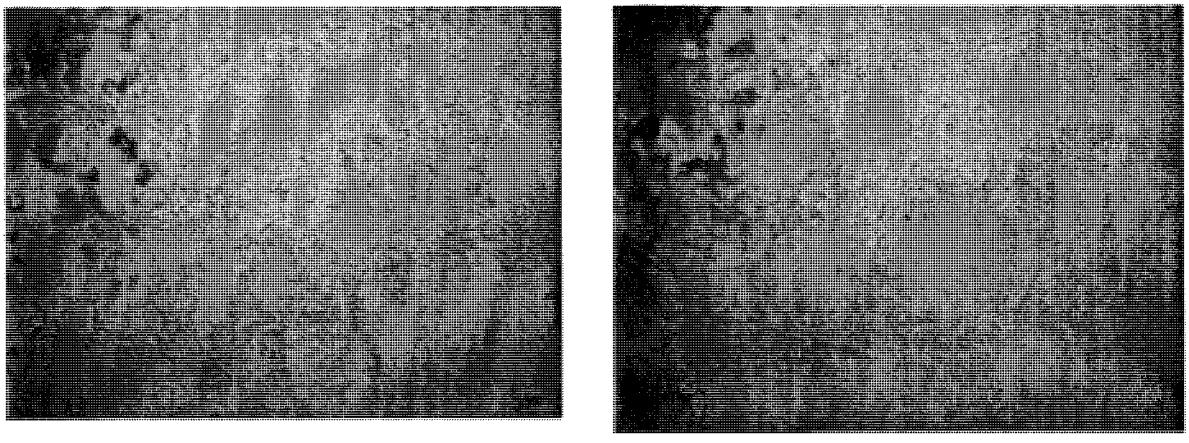
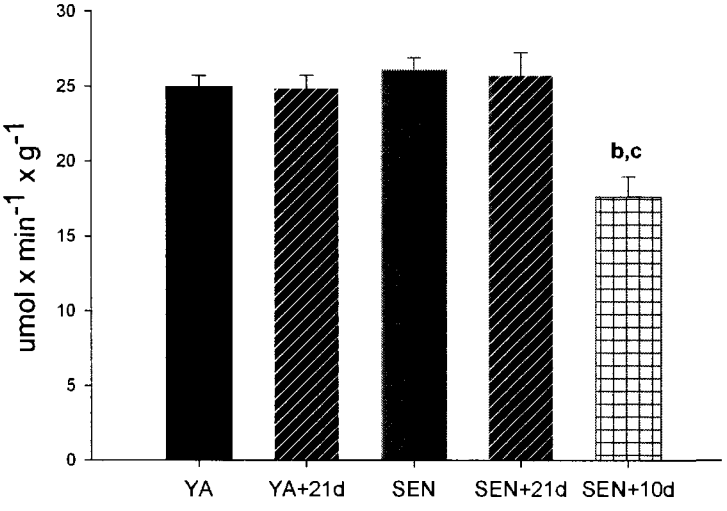


Figure 4.1 Representative H+E stains of two different tibialis anterior muscles 2 days after cardiotoxin (CTX) injection showing significant muscle fibre necrosis

Photos were provided by R.T. Hepple, unpublished.

A



B

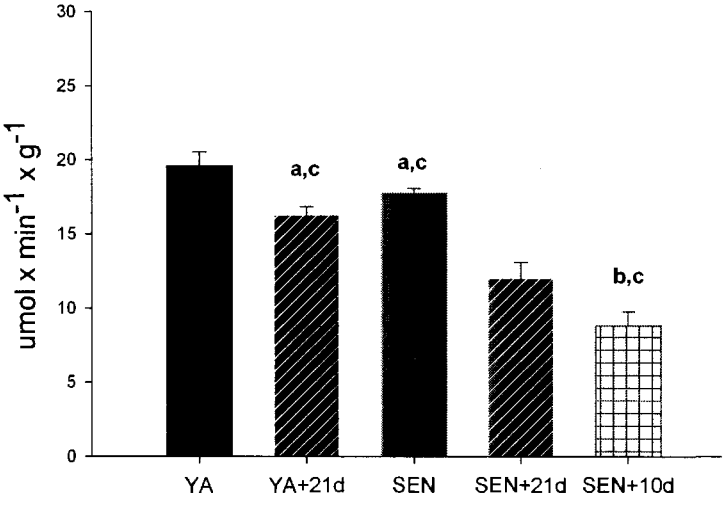


Figure 4.2 Citrate synthase enzyme activity (A) and cytochrome oxidase activity (B) for the tibialis anterior (TA).

a, $p<0.05$ vs YA; b, $p<0.05$ vs SEN; c, $p<0.05$ vs SEN+21d

YA = young adult; YA+21d = young adult 21 days after cardiotoxin injection; SEN = senescent; SEN+21d = senescent 21 days after cardiotoxin injection; SEN+10d = senescent 10 days after cardiotoxin injection

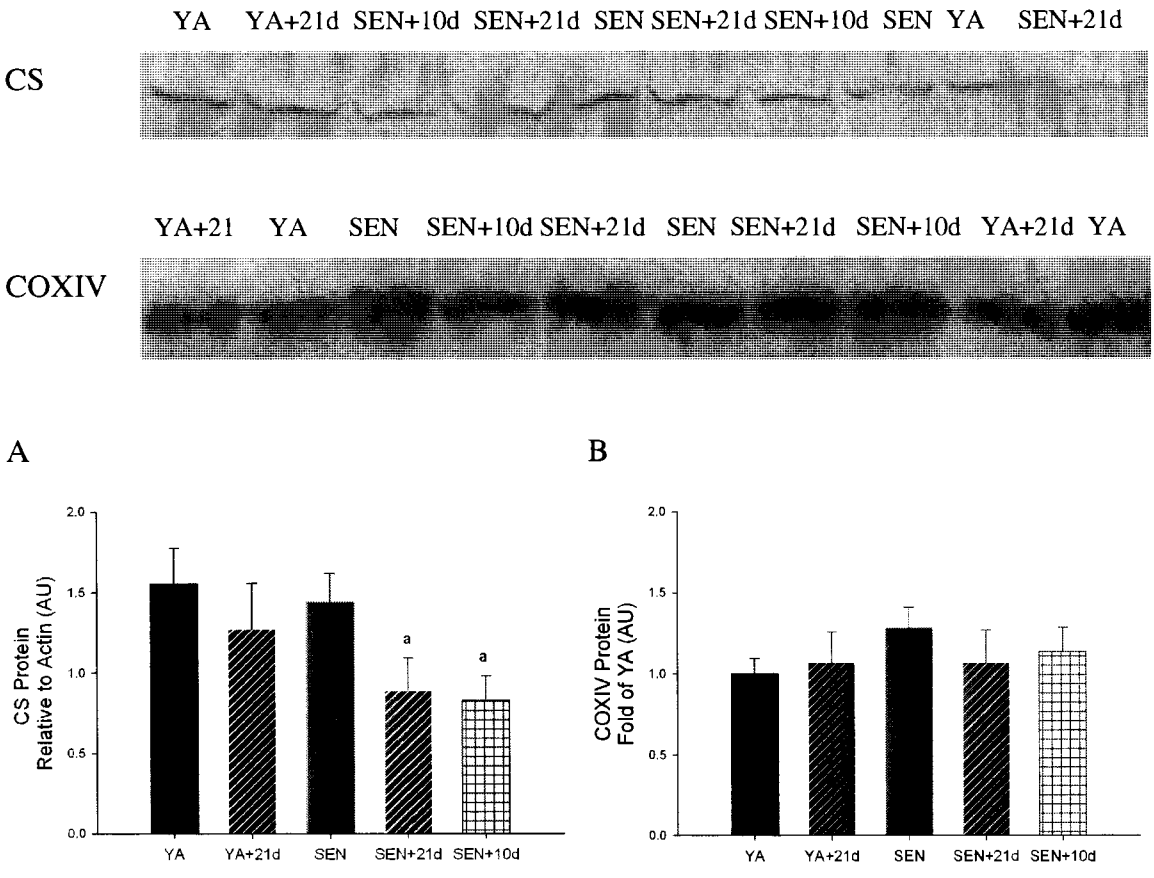


Figure 4.3 Protein expression for CS (A) and COXIV (B).

For CS, p=0.11 for SEN vs SEN+21d. a, p<0.05 vs YA; b, p<0.05 vs SEN; c, p<0.05 vs SEN+21d

YA = young adult; YA+21d = young adult 21 days after cardiotoxin injection; SEN = senescent; SEN+21d = senescent 21 days after cardiotoxin injection; SEN+10d = senescent 10 days after cardiotoxin injection

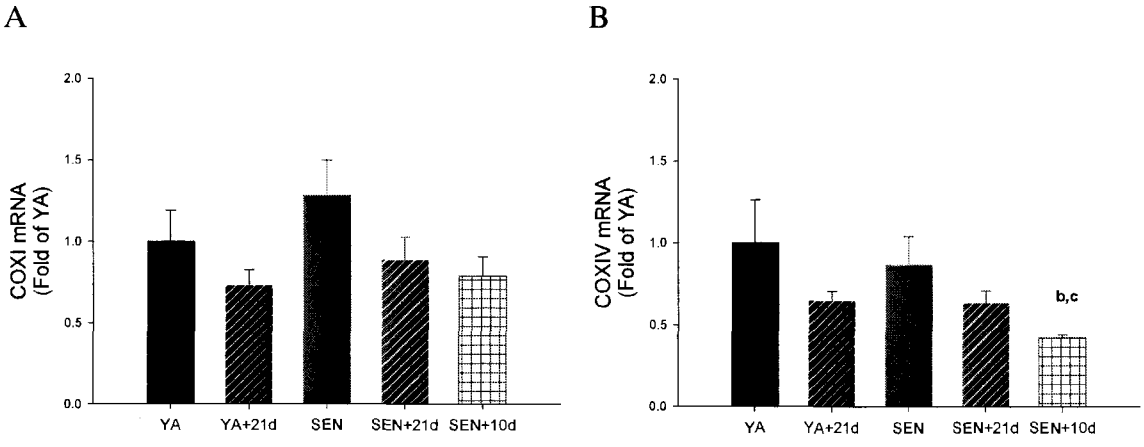


Figure 4.4 Relative gene expression for COXI (A) and COXIV (B).

mRNA is expressed as a fold change relative to the young adult (YA) group. a, $p<0.05$ vs YA; b, $p<0.05$ vs SEN; c, $p<0.05$ vs SEN+21d

YA = young adult; YA+21d = young adult 21 days after cardiotoxin injection; SEN = senescent; SEN+21d = senescent 21 days after cardiotoxin injection; SEN+10d = senescent 10 days after cardiotoxin injection

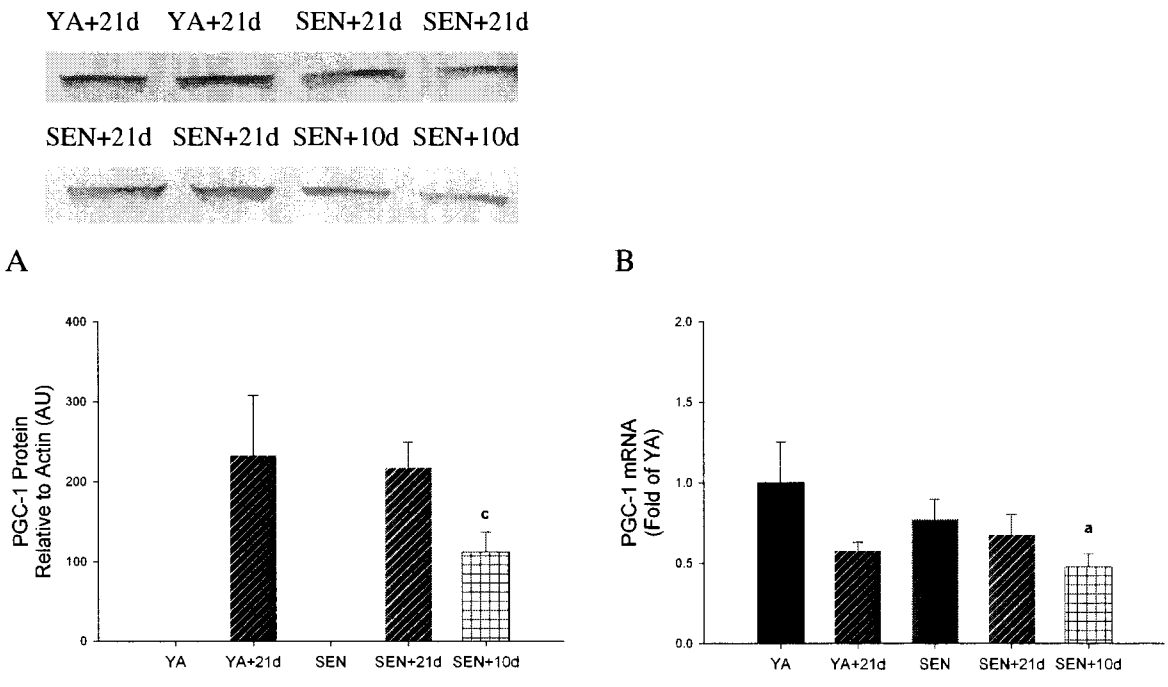


Figure 4.5 Protein (A) and mRNA (B) expression for PGC-1α.

For PGC-1α, protein content was only measured in the muscles that had received cardiotoxin (CTX).

a, $p<0.05$ vs YA; b, $p<0.05$ vs SEN; c, $p<0.05$ vs SEN+21d

YA = young adult; YA+21d = young adult 21 days after cardiotoxin injection; SEN = senescent; SEN+21d = senescent 21 days after cardiotoxin injection; SEN+10d = senescent 10 days after cardiotoxin injection

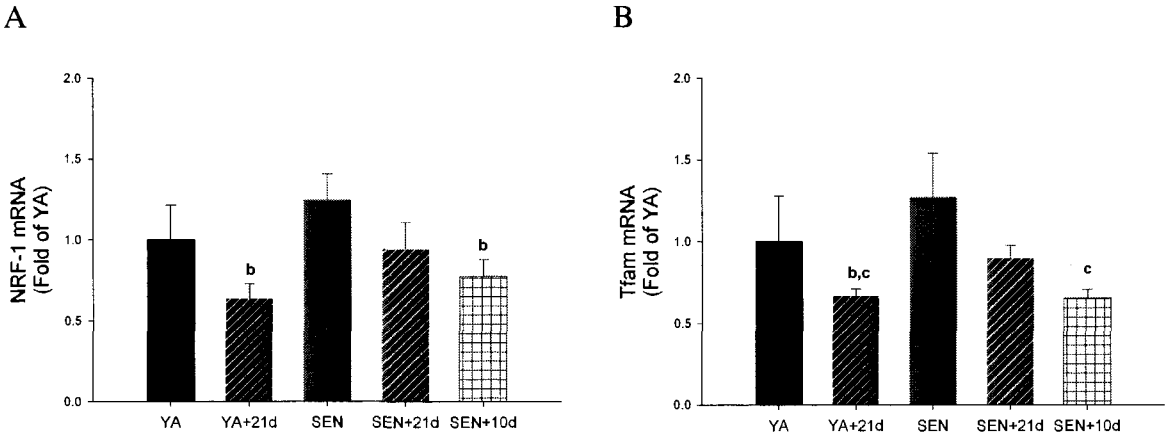


Figure 4.6 Relative gene expression for NRF-1 (A) and Tfam (B) expressed as a fold change relative to the young adult (YA) group.

a, $p<0.05$ vs YA; b, $p<0.05$ vs SEN; c, $p<0.05$ vs SEN+21d

YA = young adult; YA+21d = young adult 21 days after cardiotoxin injection; SEN = senescent; SEN+21d = senescent 21 days after cardiotoxin injection; SEN+10d = senescent 10 days after cardiotoxin injection

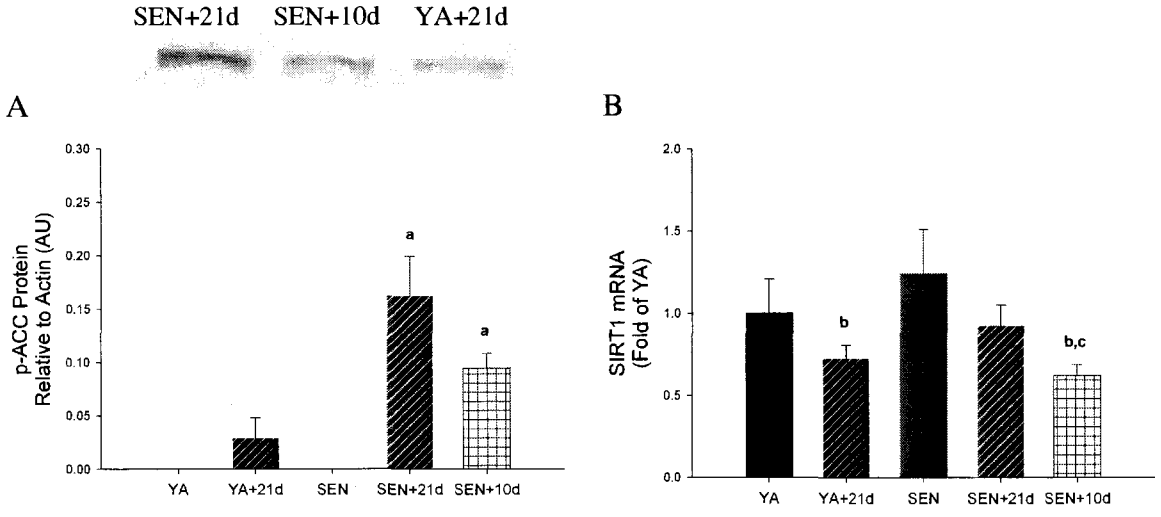


Figure 4.7 Protein expression for p-ACC (A) and relative gene expression for SIRT1 (B) expressed as a fold change relative to the young adult (YA) group.

For p-ACC, protein was only measured in the muscles that had received CTX

a, $p<0.05$ vs YA+21d; b, $p<0.05$ vs SEN; c, $p<0.05$ vs SEN+21d

YA = young adult; YA+21d = young adult 21 days after cardiotoxin injection; SEN = senescent; SEN+21d = senescent 21 days after cardiotoxin injection; SEN+10d = senescent 10 days after cardiotoxin injection

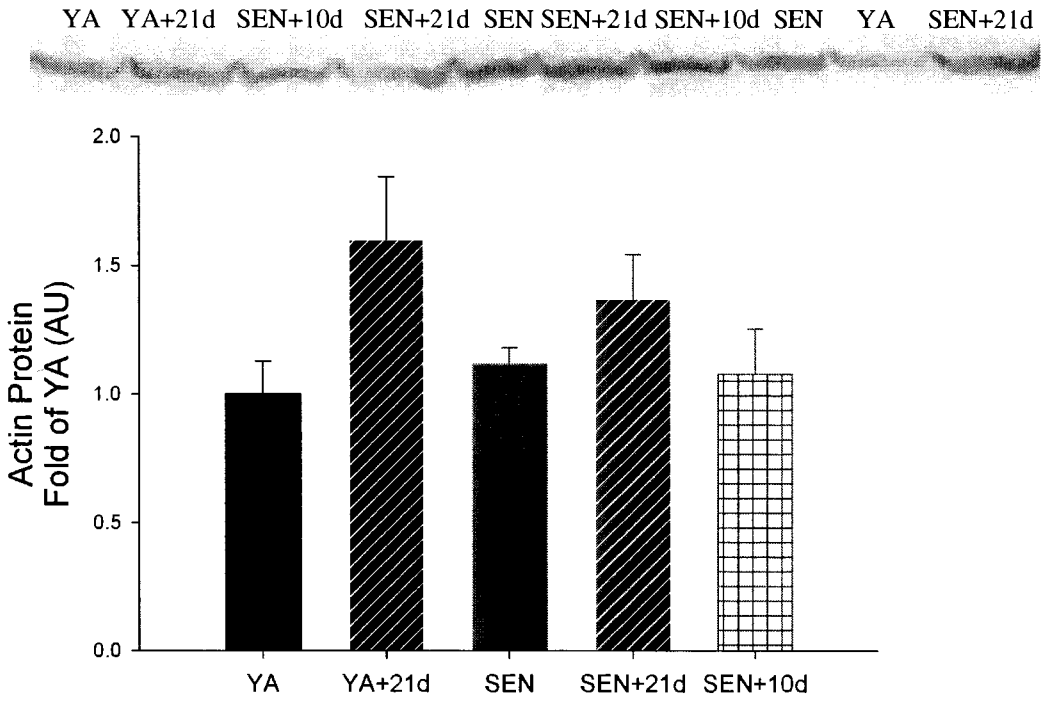


Figure 4.8 Actin protein expression for all groups.

No statistically significant differences between groups (p=0.067).

YA = young adult; YA+21d = young adult 21 days after cardiotoxin injection; SEN = senescent; SEN+21d = senescent 21 days after cardiotoxin injection; SEN+10d = senescent 10 days after cardiotoxin injection

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Chapter Five: DISCUSSION, LIMITATIONS AND CONCLUSIONS

5.1 DISCUSSION

The overarching goals of this thesis were to better understand the relationships between PGC-1α and mitochondrial function and the declines in skeletal muscle aerobic capacity with aging, and then to determine if long term exercise can be beneficial in attenuating any aging effects. First, we determined the changes of muscle aerobic function with aging *in situ* by measuring muscle mass-specific oxygen consumption using the hindlimb pump-perfusion technique in which oxygen delivery is normalized to muscle mass. This was done in three different muscles which represent a range of metabolic phenotypes. In the same muscles, we then determined the *in vitro* changes in mitochondrial enzyme activity of two important enzymes that are involved in and reflective of the oxidative capacity of the muscle. Second, we employed a long-term exercise training regimen to late middle aged rats to determine the ability of exercise to increase mitochondrial biogenesis, in particularly *via* PGC-1α, an important mediator of mitochondrial biogenesis. Subsequently, we measured muscle oxidative capacity, mitochondrial enzyme activity and mitochondrial enzyme protein content to relate the mediators of mitochondrial biogenesis to actual changes in mitochondrial content and oxidative capacity. Third, to compliment the long-term training model, we investigated the short-term, acute response of PGC-1α expression with aging using a model of muscle damage that is followed by acute and rapid muscle regeneration.

The main questions were:

1) What are the consequences of aging on some signaling pathways that promote mitochondrial biogenesis, mitochondrial content, mitochondrial oxidative capacity and muscle aerobic function ?

2a) Is aged muscle able to increase PGC-1α in response to exercise or muscle damage, and does an induction of PGC-1α result in increases in mitochondrial content, function and muscle aerobic function?

2b) If there is a deficiency, is it related to PGC-1α expression, mitochondrial gene expression, protein expression and/or downstream co-activators of PGC-1α?

3) Does the induction of PGC-1α and downstream mitochondrial biogenesis differ between a short-term (muscle damage) or long-term (exercise training) stimulus in senescent compared to young adult muscle?

To assess the regulation of mitochondrial biogenesis, we measured PGC-1α protein and mRNA, NRF-1 mRNA and Tfam mRNA. To assess mitochondrial content we measured CS protein and COX subunit IV protein, which are enzymes in the Kreb's cycle and electron transport chain which are often used as markers of mitochondrial content. To assess mitochondrial oxidative capacity, we measured *in vitro* the maximal rates of CS enzyme activity and COX enzyme activity. Finally, to assess whole muscle aerobic function, we measured *in situ* muscle mass-specific oxygen consumption by matching oxygen delivery to the muscle mass to ensure all groups were receiving the same relative amount of blood flow and oxygen delivery.

5.1.1 Lack of a Training Effect in Aged Muscles

It was very surprising that long term exercise training in senescent rats did not have a positive effect on muscle aerobic capacity, mitochondrial enzyme activity or mitochondrial content, especially given the fact that we, and others, have shown that regular exercise improves muscle aerobic capacity at least until late middle age (1; 3; 5; 14). Here we show a diminished plasticity of senescent skeletal muscle to respond to exercise. There are a few reports that support a diminished muscle plasticity at very old age. Slivka *et al.* (19) observed that in men over the age of 80 years, a diminished capacity to adapt to resistance training at the single fibre level. Ehsani *et al.* (8) used aerobic training, but improvements in whole body VO_2 were explained by improvements in cardiac output and not the muscle's ability to extract and utilize oxygen, suggesting a diminished adaptation at the muscle level. In senescent F344xBN rats, Blough & Linderman (2) showed that the plantaris muscle did not hypertrophy following mechanical

overload (induced by removing the gastrocnemius muscle), yet hypertrophy was observed in young animals.

The question arises, why did the training regimen at late middle age work (1), but when extended into senescence exhibit no benefit? Figure 5.1 integrates the findings of the 7 week training study (1) with those of Chapter 2 to show that at late middle age (28-30 mo) exercise training increased VO_2 max and restored VO_2 max to young adult levels, but in senescence the exercise training was not able to attenuate any of the age related declines. One possibility is that the training stimulus was not sufficient to induce a response. The trained animals exercised regularly and the exercise was sufficient to have some effect at the whole body level because the trained animals had nearly 50% less body fat , they were

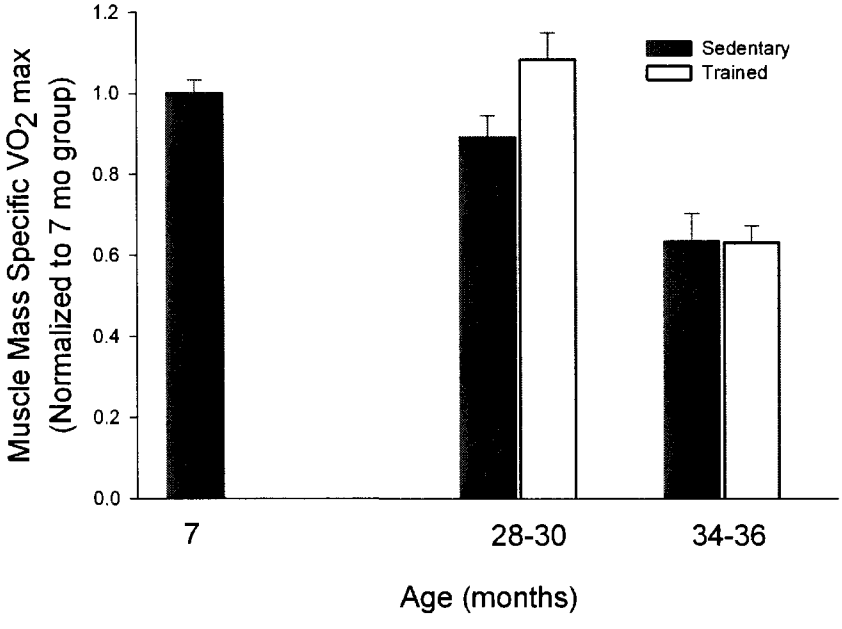

Figure 5.1 Comparison of the effect of age and exercise on muscle mass specific VO_2 max for young adult, late middle age and senescent F344BN rats.

The data in this graph is for presentation purposes only and was generated by integrating the data from Chapter 3 with the 7 week training study (1) which showed benefits of exercise training at late middle age.

able to run longer and faster during a treadmill running test and they had higher survival rates than the sedentary controls (Chapter 2). However, it is possible that the exercise intensity was still not sufficient to attenuate the age-related declines that occurred after 31 mo of age. While the training regimen was designed to maximize the effort and metabolic stimulus for each animal, after 3 months of training the treadmill velocity had to be reduced because the animals could no longer tolerate the target velocity; at the end of the study animals were exercising at ~5 metres per minute, which is a rather slow walk. As such, it is possible that this is an insufficient exercise stimulus to attenuate the normal age-related declines that were observed. As mentioned in the Introduction (Chapter 1), metabolic perturbations such as changes in intracellular Ca^{2+} and AMP:ATP ratio are the primary activators of PGC-1α (*via* a number of different kinases). As such, it is conceivable that the slow walking intensity near the end of the training program did not require sufficient muscle activation to invoke changes in Ca^{2+} or the AMP:ATP ratio, and thus PGC-1α was not activated.

In contrast to the exercise study, PGC-1α expression and mitochondrial biogenesis did not seem to be blunted in the senescent animals in response to acute muscle injury. This may be explained by the "stimulus" (i.e. metabolic perturbation) being much more substantial, and thus effective in activating PGC-1α. Cardiotoxin injury is substantial in that an estimated 80% - 90% of muscle fibres are affected (9); the acute effects are a release of calcium and a subsequent inflammatory response (6; 7), both of which can activate PGC-1α and increase mitochondrial biogenesis (12; 16; 17; 20). This explanation of differences in response to damage versus training based on differences in the intensity of the stimulus is also supported by the preliminary observation that with 3 hours per day for 7 days of electrical stimulation to the tibialis anterior (a relatively intense stimulus), senescent muscle was able to induce mitochondrial biogenesis, although the relative change that occurred was lower than which was observed in young animals (13). One could postulate that there is a relationship, although not linear, between the intensity of stimulus and degree of mitochondrial biogenesis, and that this relationship is age dependent. In the training study, the exercise stimulus was low and there was not an increase in mitochondrial biogenesis.

In the chronic stimulation model (13), the stimulus is greater than our exercise training and there was an induction of mitochondrial biogenesis in both young and aged muscle, although slightly blunted in aged compared to young muscle. Finally, with cardiotoxin injection the stimulus is even more substantial than the electrical stimulation, and the mitochondrial biogenesis response is larger and not significantly different between young and aged muscle.

Another possibility is that there is a blunted activation of the kinases that relate metabolic perturbations (Ca^{2+} and AMP:ATP ratio) to PGC-1α activation in aged muscle (Figure 2, Chapter 1). This could explain the findings by Ljubicic *et al.* (13) in which the electrical stimulus applied to the tibialis anterior was the same in old as young muscle, yet the mitochondrial response was attenuated in the old muscle. In this regard, there is evidence of decreased AMPK and MAPK activation in senescent muscle (15; 18). Reznick *et al.* (18) used ß-GPA feeding, AICAR and acute exercise to show that activation of AMPK was blunted in senescent muscle, while Mylabathula *et al.* (15) used muscle stretch to activate several MAPKs and found attenuated activation in senescent muscle. Given these findings it might be speculated that in the training study, even if the exercise was sufficient to invoke a metabolic perturbation, there may be an age-related impairment in activating the kinases which are responsible for activating PGC-1α. Thus, the combination of insufficient metabolic stimulus and impaired kinase activation may both contribute to the lack of a signalling response and subsequent metabolic adaptation in the exercise trained senescent animals.

5.1.2 Differences between Exercise Training and Muscle Damage from Cardiotoxin

Mitochondrial biogenesis was increased in the aged (SEN) muscles following cardiotoxin injection but not in the aged muscles subjected to exercise training. A number of explanations may be proposed. As above, the exercise intensity may not have been sufficient to cause metabolic perturbations that would induce a response whereas the metabolic perturbation (stimulus) following cardiotoxin injury was sufficient. Second, because cardiotoxin injury recruits satellite cells to form new myofibres, these new

myofibres may not display the same aging phenotype as pre-existing myofibres and thus have similar capacity to increase mitochondrial biogenesis in both young and aged muscle. This is assuming that there is some inherent impairment in inducing and/or increasing mitochondrial biogenesis in senescent muscle. Third, the fact that the TA did not display decreases in CS or COX enzyme activity with age (unlike the declines in CS activity in the SOL, GASr and PLAN) suggests that this muscle does not have the age-related declines as the muscles used in the training study (despite similar declines in muscle mass). Perhaps the TA simply ages differently, although it is not clear why. The SOL, GASr and PLAN represent a wide range of metabolic phenotypes; the TA is a mixed muscle that compares with the GASr and PLAN in terms of metabolic phenotype, so it is unlikely that it is a phenotype issue. Perhaps there is a difference in activation patterns between the muscles such that the TA is activated more while sitting in the cage (e.g. for balance) or walking such that it does not age metabolically in the same manner as the plantar flexors used in the training study. Not all muscles age similarly, as I have observed no decrease with age in muscle mass or CS enzyme activity in the adductor longus muscle (A.C. Betik, unpublished).

5.1.3 Actin Protein data in the CTX muscles

Upon close investigation of α-actin protein, α-actin appears higher in the 13 mo AL 21d group compared to the YA group ($p=0.067$ when included in one way ANOVA, $p=0.055$ for an unpaired t-test). It is doubtful that this is an experimental error because the groups were evenly represented in three gels and the average α-actin data per gel was very similar (within 7% of each other), suggestive of similar transfer efficiency. Since equal amounts of total protein were loaded in each lane, one interpretation of this data is that within the 3 weeks of regeneration, α-actin represents a greater proportion of the total protein pool. Although not statistically significant, the same trend holds true for the 35 mo groups in that SEN+21d is slightly higher (22%) than SEN, lending some support for this interpretation. One possibility is that in the regenerating muscle there is a greater focus, by the 3 week time point, on building the myofibrillar protein network, since α-actin is part of the structural component of the myofibre.

The challenge and limitation of this finding is that the protein data was normalized to α-actin, but if α-actin changes between groups this could affect the interpretation of the protein of interest. Firstly, and most importantly, since α-actin was not different between YA+21d and SEN+21d, then we assume that comparisons between these groups are still valid and true. Comparisons of these groups were our primary interest at the study onset, since we wanted to know if PGC-1α and mitochondrial content were the same in young and senescent muscle 21 days after muscle damage. We hypothesized that SEN+21d would have lower PGC-1α protein and lower mitochondrial content. Secondly, for comparing the non-damaged muscles to the 21d post injection muscles, assuming that α-actin is higher in the 21d post injection groups, normalizing to α-actin would underestimate the values of the protein of interest, and thus the protein values for YA+21d and SEN+21d would actually be higher than presented here. This does not impact any of the PGC-1α (or p-ACC) protein data or interpretation because we do not have YA or SEN (control) data and thus are not comparing these groups. However, for CS, the interpretation of no difference between the uninjured control and the 21d groups does not change since these were slightly lower than their respective control groups. Thus even if this is biased by higher α-actin protein, it is unlikely that the CS protein value would become statistically significantly higher than the control groups. For COXIV protein, the same scenario (as CS) would likely hold true for the SEN *versus* SEN+21d. However, for the young group, there is the possibility that the YA+21d would become significantly higher than YA. In comparing YA+21d and SEN+21d, there is still no difference in COXIV protein since α-actin does not change, thus the interpretation of similar COXIV protein content (and hence mitochondrial content) between the two groups still holds. One potential twist to the story could be that if YA+21d is higher than YA this would represent an increased mitochondrial content relative to the control group that is not observed in the SEN+21d group. This would suggest a diminished capacity to increase mitochondrial content in senescence. This scenario is consistent with the COX activity data, in which SEN+21d was lower than SEN and YA+21d. Nonetheless, some caution in this interpretation is prudent, since this is not supported by CS protein or activity, and therefore more work on this is warranted. Perhaps

other markers of mitochondrial content as well as mitochondrial respiratory data, in which assessment of the electron transport chain can be made, would be beneficial in this regard.

The other option is not to normalize to α-actin. Ideally it would have been nice to have another protein for normalization, with the assumption that equal protein was in fact loaded and differences in transfer efficiency are controlled by representing all groups on each gel. When this is done the following results come to light. 1) COXIV protein is not different between any of the groups. A slight trend of higher (~20%) COXIV protein in the 21d group compared to the control group is similar in both young and senescent groups, suggesting there is no impairment in the senescent muscle. 2) CS – no difference between YA and SEN 21d groups, and no difference between control and 21d groups for YA and SEN. Again, this suggests no impairment in the senescent muscle. No difference between YA+21d and SEN+21d was also observed on a previous set of gels (data not reported because we were unable to achieve quality α-actin bands).

5.2 LIMITATIONS

While there are a number of limitations to these studies, a few of the important ones as they relate to the discussion and interpretation of the data will be presented below.

5.2.1 Mitochondrial Protein and Enzyme Activity as Markers for Mitochondrial Content

There are differences between using mitochondrial protein or mitochondrial enzyme activity as markers of mitochondrial content, and each has its advantages and disadvantages. We used CS and COXIV protein content, measured by Western blot, as a marker of mitochondrial content. One of the challenges of the Western blot technique is the high variability and non-quantitative nature of the data. In this regard, the data was normalized to α-actin as a measure of total protein content, and thus the interpretation of the CS and COXIV protein content is only relative to the total protein content of the muscle. As such, total mitochondrial content could decrease with age, yet relative to the

total protein content in the muscle cell there could be no difference between young and old. However, our measures of CS and COX activity were normalized to muscle mass and not to muscle protein, and for the most part a decrease in activity in SEN muscle was observed. This decrease is likely due to a lower total protein content, of which the mitochondrial proteins are a subset of the total protein pool. In cases when protein content may change (e.g. with aging), cautious interpretation of the data is necessary.

5.2.2 Different Effects of Aging on Muscles from the Training Study Compared to the CTX Study

One interesting finding from the cardiotoxin study (Chapter 4) was that mitochondrial enzyme activities for the TA in the SEN animals were not substantially lower than that of the young group. Despite substantially lower muscle mass in the SEN group, which is evidence of sarcopenia, at the level of mitochondria, only COX activity was slightly lower in the SEN group, with no other evidence of an aging phenotype noted. Yet while CS activity was not lower in the SEN groups in the TA, we saw significant declines in CS activity in the SOL, GASr and PLAN (Chapter 3). Another study also showed no age-related decline in CS or COX activity of isolated mitochondria from the TA (4), yet COX activity was decreased in SOL and PLAN homogenates. Because integration of the results from Chapters 2 and 3 and Chapter 4 involve different muscles, and given an apparent lack of aging phenotype in the TA but not the other muscles, there is the possibility that effects are muscle specific.

As stated in Chapter 4, the TA was chosen for the muscle injury experiments because it is large and superficial, it is very easy to access with a needle, and it is isolated from other muscles due to a sheath that encompasses the muscle. It is possible that the TA, for unknown reasons, does not have a blunted capacity to induce PGC-1α and mitochondrial biogenesis in aged animals, whereas some other muscles may have a blunted capacity. To confirm this, it would be necessary to inject the SOL, GASr, and/or the PLAN with cardiotoxin and compare the response to the TA. Alternatively, one could use an electrical stimulation protocol that could activate the plantar flexors and extensors to the

same degree and compare the mitochondrial biogenesis response in the muscles. I recently measured CS and COX activity in the TA of the sedentary and exercise trained animals (see Figure 5.2 below) and did not see a beneficial training effect in this muscle, similar to the SOL, GASr and PLAN, suggesting that this muscle is not different than the others, at least in response to exercise. If the disparity in the findings is simply due to muscle specificity in that the TA was inherently different than the other muscles (being more responsive to perturbations) then we would have expected to see a training effect in this muscle. The limitation with this interpretation is that it is difficult to know how much the TA was recruited during treadmill running / walking, and so lack of a training effect in this muscle may be due to recruitment issues. Standardizing the level of contractile activity is necessary for such comparisons.

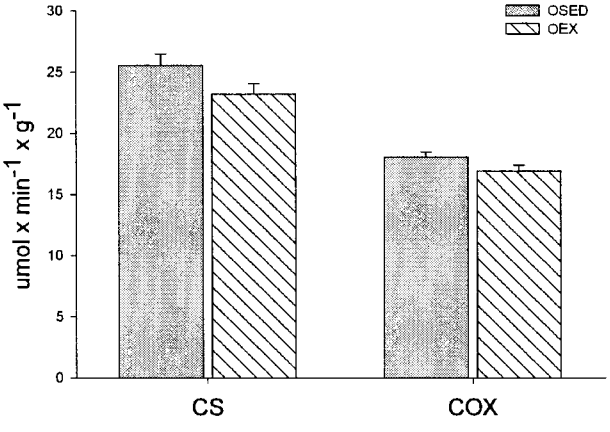

Figure 5.2 Citrate synthase (CS) and cytochrome oxidase (COX) activity for the tibialis anterior (TA) showing no training benefit.

For CS p=0.07 and for COX p=0.08.
OSED = old sedentary group; OEX = old exercise group

5.2.3 Normalizing Enzyme Activity to Muscle Mass Versus Protein Content

There is evidence throughout the literature of normalizing enzyme activity to both muscle mass and protein content (1; 4; 10; 11). The important issue is the effect on interpretation and this merits some discussion. Normalizing enzyme activity to muscle

mass allows a direct comparison with the VO_2 measures, which themselves are normalized to muscle mass. Normalizing VO_2 to muscle mass is important for controlling blood flow to ensure similar oxygen delivery, relative to muscle mass, between all groups. As such, the VO_2 represents the oxygen consumption of the entire muscle. For the same reason, enzyme activity normalized to muscle mass represents the mitochondrial capacity for the entire muscle and allows interpretations/comparisons with the VO_2 data as well as an indication of the capacity of the entire muscle. For functional interpretation this is important, particularly in the context of aging, because we want an idea of the capacity of the entire muscle and not necessarily of a specific protein pool as this can relate to the capacity of an elderly person to be active.

One limitation in this analysis is that it is not related to protein and thus does not give any idea of the functionality of proteins. Nor does it recognize that a lower mitochondrial enzyme activity may be due to lower protein concentration or lower protein function (i.e. activity relative to content), or both. A second limitation of this approach is that it is not possible to compare enzyme activity with enzyme content, measured by Western blot, because protein content is normalized to total muscle protein and not muscle mass. In fact, for measures of protein content, *via* Western blotting, samples are first normalized to muscle protein to load equal amounts of protein per sample, then normalized again to α-actin to correct for differences in loading or transfer efficiencies, under the assumption that α-actin does not change between groups, as shown in Chapter 3. It was originally planned to assess mitochondrial function with aging and exercise; however, for these methodological reasons this would be incorrect, and the analyses for mitochondrial function were not continued.

Normalizing enzyme activity to total protein is also very common, and allows changes in enzyme activity to be related to a given amount of total protein. But as such, changes in total protein content are not accounted for which has advantages and disadvantages in the interpretation. An advantage of this approach is that it allows an estimation of enzyme function (i.e. activity relative to content); although this does have its

own limitation because activity of a specific enzyme is normalized to total protein and not to the protein content of the enzyme measured. This may be addressed by measuring the specific protein by Western blot, as we have done for CS and COXIV; however, the challenge is comparing quantitative data with semi-quantitative data from Western blotting. A similar yet more informative approach, than normalizing to total protein, is to normalize to mitochondrial protein content. But again, isolating mitochondria is methodologically challenging and labour intensive.

Where the protein concentration does not change, normalizing to muscle mass or protein concentration would give the same result. As noted in Chapter 2 there was no difference regardless of the normalization procedure (see Figures 2.5 and 2.6), because there was no difference in protein concentration between sedentary and trained groups (see Figure 5.3 below). With aging, protein concentration decreases (Figure 5.3) and thus normalizing to protein concentration would obscure the loss of protein per unit of muscle and the loss of absolute activity or protein present. We would not observe a significant difference between young and old muscles (i.e. CS activity), and would interpret that there is not an age-related affect on CS enzyme capacity. It is clearly important to define how content or activity was standardized, and the impact on the strengths and weaknesses of interpretation.

If normalizing to muscle mass, as done in throughout the thesis, in accordance to standard practices of our laboratory, one cannot interpret the data in terms of specific activity (i.e. activity per unit protein), and we do not have an appreciation for the function/quality of the mitochondrial proteins within the total protein pool. Alternatively, the data provides an assessment of the capacity of the entire muscle, which as stated above can be related to VO_2 measures and interpreted in terms of functional capacity of the muscle. The interpretation in this case is that for a given unit of muscle, CS enzyme activity decreases with age. If normalized to muscle protein then I can also conclude that for a given amount of protein there was not an age-related decline in CS enzyme activity. It appears as if the decline in enzyme activity with age is due to a decline in total protein.

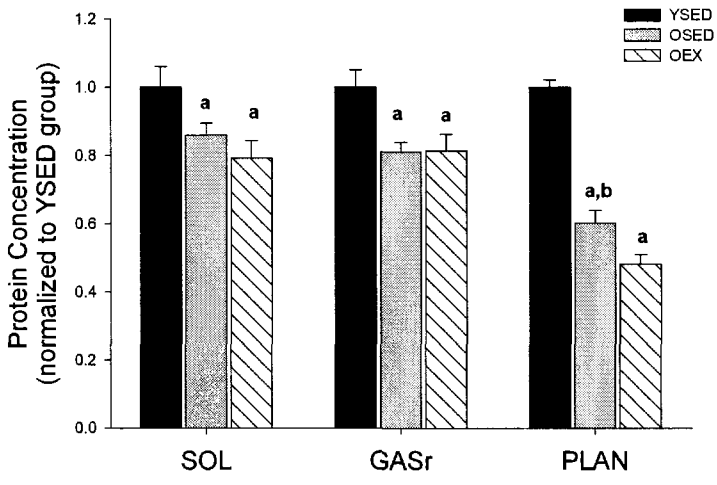

Figure 5.3 Protein concentration for the soleus (SOL), red gastrocnemius (GASr) and plantaris (PLAN) with age and training.

Values are normalized to the young sedentary (YSED) group for each muscle. a, $p<0.05$ vs YSED; b, $p<0.05$ versus OEX

YSED = young sedentary group; OSED = old sedentary group; OEX = old exercise group

5.2.4 Lack of Control Group for all Measures in CTX Study

One of the limitations with the regeneration study is a lack of a control group for some of the measures. At the onset of the experiment, the primary goal was to compare YA+21 compared to SEN+21, and it was not believed that a control group was necessary, allowing for injection of both TA's. For logistical reasons (e.g. cost of animals, statistical power, etc.) relative to this series of experiments, both TA's were injected with CTX and thus there was not an internal control group. As noted in the methods of Chapter 4, for this thesis only one TA per animal was used in the analysis. After many of the analyses were performed, it was realized that a control group might have been useful and as a result, the TA's from the YA and OSED groups from the training study were used as a control group for some of the comparisons. We were limited by tissue or methodological reasons to have a control group for all of the measures. While it was attempted, as much as possible, to include a control group, it was not possible to include a control group for all measures.

Ideally, and for the next series of experiments, it is recommended to inject only one muscle and use the contralateral limb as a control group.

5.3 CONCLUSIONS

In summary, whole-muscle aerobic capacity decreased ~40% between young adult and senescence and this was accompanied by declines in whole-muscle mitochondrial enzyme activity, although protein-specific activity was maintained with age. There was a similar decline in PGC-1α mRNA and protein, in some muscles, and a likely explanation for the decline in mitochondrial capacity is a lower presence of this key regulator for mitochondrial biogenesis. Interestingly, NRF-1 and Tfam did not decline with aging, and are not likely the culprits of lower mitochondrial biogenesis. Exercise training from late middle age until senescence did not attenuate the declines in muscle aerobic capacity or mitochondrial enzyme capacity with aging, counter to the upregulation that is typically observed with exercise in young muscle. PGC-1α was not elevated in the trained animals compared to the sedentary group, thus at this age range with this exercise intervention there was an inability to upregulate PGC-1α (as well as NRF-1 and Tfam); this offers a possible explanation for the lack of mitochondrial biogenesis in the exercise group. Since we have seen improvements in muscle and mitochondrial aerobic function at late middle age with the same exercise intervention, to the extent that the muscles were restored to young adult levels (1) (see Figure 5.1), this lack of response in aged muscle appears to be specific to the latter part of the lifespan, beyond late middle age. Regular treadmill exercise over this part of the lifespan is ineffective in attenuating the age-related declines in aerobic capacity. Finally, in contrast to the effects of long-term exercise, there is no observable detriment in aged muscle, as compared to young muscle, to restore mitochondrial function after damage. Following cardiotoxin induced muscle damage, aged muscles showed similar levels of PGC-1α (mRNA and protein) and higher levels of NRF-1 and Tfam mRNA compared to young adult muscle. Aged muscle also had similar maximal rates of CS activity, but slightly lower COX activity (22%). However, if we normalize COX activity to control (no damage) levels, the relative differences between CTX injected and control are similar for young (72%) and aged muscles (64%), suggesting that there is not necessarily a detriment

with age. For mitochondrial content, 21 days after CTX injection CS protein and COXIV protein were not different in the aged group compared to the young group. Similarly, COXI and COXIV mRNA were the same for both young and aged muscle 21 days after muscle damage. Furthermore, the protein contents of both of these enzymes were similar to their respective control groups, suggesting by 21 days mitochondrial content had been restored to control levels.

The response to exercise training and muscle damage are different in terms of the ability to induce mitochondrial biogenesis and to increase mitochondrial content/capacity. Although this was not our hypothesis at the outset, given the current findings we suspect that aged muscle does in fact have a deficiency in its capacity to induce mitochondrial biogenesis, particularly with moderate exercise as the stimulus. Why this is not supported by the muscle damage experiment may be due to the fact that muscle damage recruits satellite cells in its response and perhaps these satellite cells, forming new myotubes and myofibres, are similar in phenotype to young muscle. As such, the PGC-1α and downstream mitochondrial response is similar to young muscle, as our observations show here. One option to test this would be to induce the muscle damage in aged animals as done here, then follow that with an exercise program to see if these "new" and "young" myofibres display the same adaptive response as typical young muscles with treadmill exercise.

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