DISSERTATION

TARGETING SKELETAL MUSCLE MITOCHONDRIAL FUNCTION WITH A NRF2 ACTIVATOR IN A NOVEL MODEL OF MUSCULOSKELETAL DECLINE

Submitted by

Robert Vincent Musci

Department of Health and Exercise Science

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Doctoral Committee:

Advisor: Karyn L. Hamilton

Kelly S. Santangelo
Daniel S. Lark
Matthew S. Hickey

ABSTRACT

TARGETING SKELETAL MUSCLE MITOCHONDRIAL FUNCTION WITH A NRF2 ACTIVATOR IN A NOVEL MODEL OF MUSCULOSKELETAL DECLINE

This dissertation describes a series of three experiments with an overall objective to understand how targeting mitochondrial function with a phytochemical Nrf2 activator can prevent the onset of or mitigate the progression of mitochondrial dysfunction and sarcopenia in a novel model of musculoskeletal aging. The specific aims of the three experiments were to 1) characterize the age-related changes in skeletal muscle in Dunkin-Hartley guinea pigs; 2) assess the effect of Nrf2 activator treatment on skeletal muscle energetics by measuring mitochondrial function; and 3) determine how Nrf2 activator treatment influences components of skeletal muscle proteostasis. Dunkin-Hartley guinea pigs exhibit several characteristics reflective of human musculoskeletal aging including a decline in the proportion of type II muscle fibers, a shift towards a smaller myofiber size distribution, and a decline in muscle density in the gastrocnemius, as well as a decline in protein synthesis in both the soleus and gastrocnemius. In the second experiment, Nrf2 activator treatment improved mitochondrial respiration in both 5- and 15-month-old male and female guinea pigs. Moreover, Nrf2 activator treatment attenuated the age-related decline in mitochondrial respiration. In the third experiment, Nrf2 activator treatment attenuated the age-related decline in protein synthesis in Dunkin-Hartley guinea pigs. Altogether, these data demonstrate 1) Dunkin-Hartley guinea pigs experience age-related changes in skeletal muscle consistent with the aged musculoskeletal phenotype in humans 2) this phytochemical Nrf2 activator can improve mitochondrial function and 3) targeting mitochondrial dysfunction is an efficacious intervention to mitigate age-related declines in components of proteostasis in skeletal muscle and improve overall musculoskeletal function.

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CHAPTER 1 – INTRODUCTION AND EXPERIMENTAL AIMS

INTRODUCTION

Musculoskeletal aging broadly describes the progressive, age-related decline in skeletal muscle, bone, tendon, and articular cartilage that contributes to disability, chronic disease, and impaired quality of life in older adults[1,2]. One facet of musculoskeletal aging is sarcopenia, which was classically defined as the age-related loss of muscle mass[3]. Few studies have measured the economic burden of sarcopenia, but one study in 2000 estimated that it cost $18.5 billion in the United States[4], with similar burdens likely in European communities[5]. Sarcopenia affects 28% of men and 19% of women over 60 years of age[6], and over half of individuals over the age of 80[7]. Over the past decade, the definition of sarcopenia has evolved to incorporate the age-related loss of muscle function as well[8,9]. However, muscle mass loss cannot, by itself, account for the age-related decline in muscle function[10,11], other terms may more appropriately encompass the multifactorial contributors to skeletal muscle decline with aging. As one example, Manini introduced the concept of dynapenia[12,13] to account for factors outside of muscle mass that contribute to the failure to maintain force-generating capacity with age. Taking into consideration the variety of factors that contribute to the age-related functional deficit in skeletal muscle is important to help frame and develop new potential strategies to treat age-related skeletal muscle dysfunction.

In recognition of the importance of an integrative approach, the definition of sarcopenia, has evolved. In 2016, the World Health Organization has established an ICD-10 code for sarcopenia broadly defining it as “age-related skeletal muscle dysfunction,” which has led to commentary on the need for clearer terminology to develop interventions and address the various factors that contribute to age-related skeletal muscle dysfunction[14,15]. Past review articles have predominantly focused on the use of resistance exercise and amino acid

supplementation, with some consideration to timing of supplementation, to minimize or reverse the loss of muscle mass with age[16–19]. However, maintaining muscle mass is not the only, or perhaps most effective, way to slow age-related declines in skeletal muscle function. Here, I build the case for targeting one factor of sarcopenia, mitochondrial dysfunction, and highlight the consequent changes in cellular energetics as a potential treatment for sarcopenia (henceforth referring to age-related skeletal muscle dysfunction unless otherwise specified). To do so, I detail how cellular energetics determine protein synthetic responses, how protein synthetic responses in turn affect skeletal muscle function, and how we should therefore target mitochondrial function and protein homeostasis to minimize sarcopenia.

THE ROLE OF PROTEOSTASIS IN MUSCULOSKELETAL FUNCTION

Proteostasis refers to the maintenance of protein homeostasis through mechanisms that involve the location, concentration, conformation, and turnover of individual proteins[20]. Of particular importance to the current review is the role of protein turnover in maintaining proteostasis. In skeletal muscle, impaired protein turnover with age leads to the loss of contractile protein quality and quantity because of the accumulation of protein damage (Figure 1.1)[21,22]. For example, with age there is an increase in non-enzymatic modifications of proteins such as advanced glycation end products (AGEs)[23,24]. Through cross-bridge formation, AGEs and the modified proteins they are attached to, are resistant to breakdown leading to further accumulation[25,26]. In addition to AGEs, oxidatively modified proteins also accumulate in muscle with age leading to enzymatic dysfunction[22,27–30]. Since there is a limited capacity to enzymatically repair proteins[31,32], protein turnover is the primary mechanism to prevent or reverse age-related accumulation of modified or damaged proteins. Age-related increases in skeletal muscle oxidatively modified proteins and AGEs contribute to declines in strength, independent of actin and myosin concentration, emphasizing the importance of protein quality and its effect on skeletal muscle function independent of protein quantity[33]. Therefore,

degradation of damaged proteins and synthesis of new functional proteins (protein turnover) represents an important mechanism to maintain skeletal muscle protein quality with age.

PROTEIN TURNOVER AND CELLULAR ENERGETICS

Protein turnover is an energetically costly process. The energy required to synthesize new proteins represents approximately 20% of basal metabolism[34,35], while protein breakdown accounts for another 5-15% of basal metabolism[36]. Protein synthesis is an energy intensive series of processes that involves translating mRNA into amino acids and assembling the amino acids into peptide chains. Amino acid synthesis is energetically costly, requiring 12-72 ATP per amino acid[37–39]. The synthesis of peptide bonds between amino acids requires 4 high energy phosphates from ATP or GTP hydrolysis per peptide bond[37–39]. The energetic cost of protein breakdown is more difficult to ascertain. Breakdown occurs through protein ubiquitination or direct lysosomal degradation. Ubiquitination for subsequent proteasome-mediated breakdown requires 2 ATP per ubiquitin tag[39]. Subsequent proteasome activity requires the hydrolysis of between 100-200 ATP per protein depending on the length and other characteristics of the protein[39]. Lysosomal degradation costs approximately 1 ATP for every 3-4 amino acids in a given protein[39]. The cost of protein turnover explains a substantial (30-50%) portion of the basal metabolism not accounted for by mitochondrial proton leak[37,40]. Because the energy demands of protein turnover are substantial, protein synthesis and breakdown are tightly regulated processes.

The Dynamic Energy Budget theory posits that, in a given a moment, organisms have a finite amount (or budget) of energy to sustain cellular processes[41,42]. Even though the supply of energy in the form of lipid and carbohydrate stores is enough to last a human for weeks, in a given moment, there are energetic constraints related to the rate of oxidizing stored energy to usable ATP on demand, which is limited by mitochondrial function[43,44]. Therefore, limited energetic resources (i.e. usable ATP) must be allocated based on cellular priorities[45]. While

energetic accounting is challenging, there appears to be a hierarchy of cellular energetic processes broadly categorized as metabolism, growth, or somatic maintenance[46]. The category of metabolism encompasses processes that sustain life such as energy production, locomotion, feeding, and ion channel and pump maintenance. The growth processes involve synthesis of new biomass, and are usually accompanied by DNA replication to form new cells or to maintain a DNA to cytoplasm ratio[47]. Finally, somatic maintenance processes preserve existing biomass and include protein turnover[48,49]. The latter two categories, growth and somatic maintenance, often compete for energetic resources[50].

To understand supply and demand in cellular energetics, using terms from the field of economics is helpful. Elasticity refers to the degree to which demand for a good or service is sensitive to changes in its supply. Demand for inelastic commodities are constant or inflexible regardless of supply because inelastic commodities are essential or indispensable. For example, commodities such as water and petrol are inelastic whereas brand-name clothes and specialty cheeses are elastic. In cells, metabolic processes such as maintaining proton pumps are indispensable, or inelastic, whereas repair and growth are elastic. When cellular demand for energy exceeds the rate of energy production, cells will allocate energy toward inelastic cellular processes. Under energetic constraints, somatic maintenance and growth are elastic processes that can be sacrificed for the inelastic metabolic processes[37,38,45]. In addition, there can be tradeoff between the elastic processes so that as growth increases, for example, less energy can be allocated to somatic maintenance and vice versa. Therefore, energy provision for metabolic processes are maintained, while growth and somatic maintenance compete for the remaining energetic budget[50].

MITOCHONDRIAL DYSFUNCTION CONTRIBUTES TO A LOSS OF PROTEOSTASIS

While it is controversial whether mitochondrial dysfunction is a cause or a consequence of aging and age-related chronic diseases, it is clear that it is a characteristic[51–54]. While there

are many components of mitochondrial function, such as managing inflammation, regulating calcium signaling, and retrograde signaling, this project focuses predominantly on mitochondrial respiration. When there is dysfunction within a mitochondrial unit, increasing the size of the mitochondrial reticulum can compensate for the lack of energy producing capacity. By this mechanism, total electron transport capacity is increased rather than the relative capacity of individual components[55–58]. However, the ability to expand the reticulum is limited implying that this compensatory mechanism is constrained[59,60]. The inability of mitochondrial reticulum expansion to fully compensate may further compound mitochondrial dysfunction and its consequences.

Age-related declines in mitochondrial bioenergetics contribute to a decline in skeletal muscle proteostatic processes[44]. When mitochondrial function is decreased, provision of reducing equivalents such as NADH in excess of electron transport system capacity can lead to an imbalance that contributes to reactive oxygen species (ROS) formation[61]. This dysfunction is associated with the aging process and exacerbates ROS production and oxidative damage[62,63]. In addition to ROS-induced damage, mitochondrial dysfunction leads to the accumulation of metabolic byproducts that also cause cellular damage. For example, decreases in free fatty acid flux from impaired mitochondrial function, leads to the accumulation of lipotoxic intermediates such as diacylglycerides (DAGs) and ceramides[64]. DAG and ceramide accumulation leads to inflammation and oxidative stress that damage myofibrillar and mitochondrial proteins[65–67], membrane lipids, and DNA[68].

An additional problem associated with mitochondrial dysfunction is the shortage of readily available energy (i.e. ATP). As mentioned, protein quality control is an energetically costly process. Therefore, dysfunctional mitochondria may not be able to provide enough ATP to meet the energetic demands for both metabolism and cellular repair[69–72]. When faced with this problem, cells compromise elastic energetically costly proteostatic processes, such as protein

turnover, in favor of inelastic metabolic processes[37,38]. Accordingly, impaired mitochondrial function contributes to the loss of skeletal muscle proteostasis and sarcopenia in two ways: by increasing the accumulation of damaged proteins and by decreasing the ability of the mitochondria to generate energy for somatic maintenance.

Whereas mitochondrial dysfunction contributes to protein damage, maintaining mitochondrial function facilitates proteostatic mechanisms. First, the maintenance of mitochondrial proteins facilitates the coupling of electron transport system to ATP production[73,74], thus decreasing ROS production. Further, functional mitochondria readily adapt to fluctuating energy demands with efficient energy production[75]. Efficient aerobic energy production facilitates the maintenance of proteostatic processes by not having to compromise elastic processes such as somatic maintenance. Key to this somatic maintenance is the ability to maintain protein turnover, which minimizes accumulation of protein damage and further improves mitochondrial function[76–78].

It is worth making the point that mitochondrial function can improve without a change in mitochondrial content. However, increasing mitochondrial protein turnover, even in the absence of an increase in content, can also improve mitochondrial function[79–81]. It is even possible that with aging, improving the turnover of existing mitochondrial proteins is a better strategy to improve muscle function because increasing mitochondrial content without improving function may just lead to greater ROS production[82]. Therefore, when examining strategies to mitigate sarcopenia, assessments of mitochondrial function are equally, if not more important than mitochondrial content.

To summarize, damaged proteins accumulate in skeletal muscle with age and there is an impairment in cellular energetics. The increased protein damage and decreased energy availability constrains somatic maintenance and mechanisms of proteostasis[43,44], which exacerbates the decline in protein quality[68]. To counter this downward spiral, improving the rate

of energy production in skeletal muscle by improving mitochondrial energetics could relieve energetic constraints[77,80], improving proteostatic mechanisms and thus somatic maintenance[82]. Therefore, interventions that improve mitochondrial function could be useful for mitigating sarcopenia.

TARGETING MITOCHONDRIA TO MITIGATE SARCOPENIA

Improving mitochondrial function is a promising target to prevent sarcopenia by increasing the efficiency of energy production to match energy demand, minimizing oxidative damage and improving capacity for proteostatic processes[53,77,83,84]. Restoring mitochondrial energetics, even at older ages, improves ATP production and improves skeletal muscle function[80]. The ability to efficiently produce ATP in a given moment directly facilitates somatic maintenance by increasing the energy budget. Allocation of energetic resources to somatic maintenance may, in turn, facilitate an environment that is conducive to growth. Although it is true that treatments that improve mitochondrial function do not always also increase muscle size, they likely have the potential to do so. Below, we discuss how aerobic exercise and mechanistic target of rapamycin (mTOR) inhibition allow for improved proteostatic mechanisms and thus somatic maintenance (Figure 1.2).

Aerobic exercise has well known benefits on mitochondria. These benefits occur primarily via an increase in mitochondrial content[85] and mitochondrial function[74,79,86]. Increases in mitochondrial content and function are mediated through increases in mitochondrial protein synthesis[87,88], and mitochondrial-specific autophagy (mitophagy)[89]. These changes increase the capacity for mitochondrial ATP production and VO_2max. The increased capacity to produce energy on demand improves the cellular energetic budget allowing cells to allocate energetic resources to elastic processes[90,91]. There is no direct evidence of improvements in mitochondrial energetics both precede and facilitate improvements in skeletal muscle function and size. However, there is support of this concept from data showing that, in older adults, aerobic

exercise training improves single muscle fiber size and function[92], whole muscle size and strength[93,94], and whole muscle power[95]. In healthy young adults, aerobic exercise training improved myofibrillar protein synthesis at rest[96]. In addition, lifelong, predominantly aerobic, physical activity can delay the loss of skeletal muscle[97].

In addition to improving energy production, mitochondrial adaptations from aerobic exercise training facilitate important metabolic improvements. First, the increased energetic demands of aerobic exercise increase the flux of fatty acid substrates through beta oxidation. As demonstrated in previous studies, increased flux of fatty acid substrates diminishes the accumulation of lipotoxic intermediates[67,98–101]. Improvements in mitochondrial electron flux through the electron transport chain also decrease formation of AGEs and oxidatively modified proteins[33,102–104]. Finally, aerobic exercise stimulates endogenous antioxidant production, protecting myofibers from oxidative damage[105]. Therefore, in addition to increasing the capacity for maintaining proteostasis, increased mitochondrial function and metabolic flux decreases the demand for somatic maintenance.

mTOR inhibition

Inhibition of mTOR (e.g. through rapamycin treatment and caloric restriction) also mitigates age-related skeletal muscle dysfunction, but by different mechanisms than aerobic exercise training. The prolonged activation of 5' adenosine monophosphate-activated protein kinase (AMPK) and inhibition of mTOR, provide a cellular signal that energy is restricted. As a result, the cell dedicates more resources towards somatic maintenance instead of growth. For example, whether calorie restriction increases mitochondrial biogenesis was controversial[106–108]. However, by accounting for rates of growth our group confirmed that mitochondrial biogenesis increases in a variety of energy-restricted states[78,109–111]. Prolonged mTOR inhibition causes cells to allocate energy toward somatic maintenance at the expense of growth. Therefore, under

these conditions, growth is restricted, but ATP dedicated to processes related to somatic maintenance increases which improves the integrity of the cell and overall organismal function.

The positive effects of activating energetic signaling on muscle function seem somewhat underappreciated. For example, rapamycin treatment, which inhibits mTOR, attenuates the age-related losses of strength, lean body mass, and endurance capacity in mice[112,113]. In addition, three months of rapamycin treatment directly mitigates sarcopenia as measured by improved grip strength and rotarod performance in already aged mice[114]. Further, 5 months of calorie restriction in 21-month-old rats improves ATP production and grip strength compared to *ad libitum* fed rats[73]. Calorie restriction also mitigates oxidative stress, preserving the neuromuscular junction and contributing to the maintenance of skeletal muscle function[73,115]. Therefore, despite inhibition of growth, both calorie restriction and mTOR inhibition via rapamycin treatment attenuate age-related musculoskeletal dysfunction.

While it may seem untenable to translate interventions such as caloric restriction or rapamycin treatment in older adults, these interventions provide insight into potential mechanisms that may improve or maintain muscle function. There is a current clinical trial using metformin, an AMPK activator and mTOR inhibitor, to augment strength training[116]. The trial highlights a strategy of improving metabolic health and bioenergetics to improve muscle function and potential to gain muscle mass. It may seem counterintuitive to restrict growth as a means of improving skeletal muscle function; however, it is important to remember that muscle size is not the sole determinant of function. Muscle quality (i.e. force divided by cross sectional area) is determined by such factors as neuronal integrity, oxidatively modified proteins, and AGE accumulation[12,33]. Further, restricting growth by calorie restriction or rapamycin treatment does not constrain mitochondrial ATP production, but rather puts in motion a series of stress-related mechanisms that preserve energy production to maintain cellular integrity[43,117,118].

TARGETING MITOCHONDRIAL PROTEOSTASIS TO MITIGATE SARCOPENIA

Skeletal muscle mitochondrial function declines with age, constraining the energetic budget available for cellular processes. As a result, cellular energy allocation to proteostatic mechanisms, and consequently somatic maintenance, declines resulting in skeletal muscle dysfunction. Therefore, the maintenance of mitochondrial proteostasis has a central role in preventing sarcopenia in two ways; preventing damage to cellular components, and improving the efficient production of ATP for elastic cellular processes. Future studies should focus on the importance of protein turnover, independent of hypertrophy, for mitigating sarcopenia. Further, there should be an effort to translate interventions that target skeletal muscle mitochondria to specifically target sarcopenia in humans. Finally, aerobic exercise training should be viewed as an important adjunct or even primary form of exercise to help maintain skeletal muscle function with aging.

IMPROVING MITOCHONDRIAL PROTEOSTASIS WITH A NRF2 ACTIVATOR

While exercise remains the most effective intervention to maintain and improve health, adherence to exercise guidelines remains remarkably low both in the United States (less than 10%) and Europe (less than 50%)[119,120]. Thus, alternative and/or complementary therapies with better adherence rates could be utilized to maintain or increase healthspan. Given the detrimental role of age-related increases in chronic ROS production on health, there has been emphasis on antioxidant supplementation to mitigate those age-related increases in oxidative stress to prevent, delay the onset of, and mitigate the severity of chronic diseases[121].

Aerobic exercise, as discussed, is a potent stimulator of endogenous antioxidant upregulation, resulting in the transcription of endogenous antioxidants, such as SOD1 and SOD2, which is mediated by activation of transcription factors such as Nrf2[122–124]. As opposed to exogenous antioxidant supplements, there are compounds, which are often comprised of phytochemical components, that enhance cellular antioxidant capacity by upregulating the

expression of antioxidant enzymes such as SOD1 and catalase. While exogenous antioxidant supplements seem to abrogate important redox signaling leading to beneficial adaptations, because they directly scavenge oxidants, enhancing endogenous antioxidant capacity permits redox signaling while simultaneously preventing ROS from reaching a tipping point in which a stress becomes maladaptive[125].

Recent research has demonstrated that upregulation of endogenous antioxidants has beneficial effects on skeletal muscle function and overall organismal health. Phytochemical Nrf2 activators are one approach used to upregulate endogenous antioxidants. Nrf2 activators stimulate the translocation of Nrf2 into the nucleus leading to the transcription of the endogenous antioxidant genome[126]. As opposed to exogenous antioxidant supplements, Protandim, a Nrf2 activator, has been shown to extend median lifespan in male heterogenous mice[127]. Our lab has demonstrated that treatment with Protandim protects coronary endothelial cells and cardiomyocytes from oxidative stress challenges[128,129]. Again, in contrast to exogenous antioxidant supplements, treatment with Protandim also enhanced proteostatic mechanisms and permitted the mitohormetic adaptations to physical activity[130]. Finally, our lab has demonstrated that treatment with a similar phytochemical Nrf2 activator enhanced the proteostatic maintenance of skeletal muscle contractile proteins in sedentary, healthy older adults[131]. Together, these findings suggest that Nrf2 activators may improve skeletal muscle quality and help maintain muscle function with age. Other Nrf2 activators, such as sulforaphane, demonstrate similar results including improved mitochondrial and skeletal muscle function[132–134]. Altogether, the use of compounds that activate Nrf2 represent a promising intervention to target mitochondrial energetics to maintain skeletal muscle homeostasis throughout age.

THE USE OF PRECLINICAL MODELS TO ASSESS THE EFFICACY OF INTERVENTIONS

The average duration required to translate an intervention into clinical practice is 17 years from basic science[135]. While there are a multitude of factors that contribute to this factor,

one factor is the use of pre-clinical models that fail to adequately resemble human physiology or the disease process being tested. In humans, sarcopenia is slow, developing over decades, and heterogenous in nature[10,11,136]. Testing interventions in rodent models have offered insight into the process of sarcopenia and interventions to slow or reverse that process in much less time [137]. However, muscle loss in most aging rodent models fails to resemble the nature of sarcopenia in humans that will be discussed in greater detail in Chapter 2. The first goal of this overall project is to examine the age-related changes in the Dunkin-Hartley guinea pig to determine whether or not they resemble changes in skeletal muscle that is more reflective of musculoskeletal aging in humans. Dunkin-Hartley guinea pigs spontaneously develop primary knee osteoarthritis[138], which is associated with sarcopenia[139–141], similarly to humans. Thus, if this strain of guinea pigs exhibit age-related declines in muscle function similarly to humans, then it may be a valid pre-clinical model to test interventions for sarcopenia and accelerate the translation of interventions for the disease to clinical practice.

SUMMARY AND AIMS

Targeting mitochondria appears to be a valid approach to mitigate age-related musculoskeletal dysfunction. In particular, Nrf2 activators seem to be a promising candidate to improve mitochondrial proteostasis and subsequently function, which, in turn, should improve skeletal muscle function in older adults. Thus, the goal of these subsequent studies is to determine whether or not treatment with a Nrf2 activator can prevent the onset or mitigate the progression of mitochondrial dysfunction and sarcopenia in a pre-clinical model. These studies are part of larger project which goal is to accelerate the pace of development of novel therapeutics for preventing health issues affecting the elderly.

The aim of the larger project is to use a Nrf2 activator to slow the progression of primary osteoarthritis and age-related loss in muscle function in a guinea pig model that is similar to the musculoskeletal aging of humans. To accomplish this, we treated guinea pigs before (2 months

of age) and after (5 months of age) the initiation of knee osteoarthritis to determine whether or not a Nrf2 activator could prevent and mitigate, respectively, the age-related decline in musculoskeletal function in the Dunkin-Hartley guinea pig. The primary outcome of this study was mobility, which is an integrative measure of musculoskeletal health, because of the goal to assess multiple systems including skeletal muscle, bone, and joints. The studies below 1) characterize the age-related declines in skeletal muscle in Dunkin-Hartley guinea pigs; 2) assess the effect of Nrf2 activator treatment on skeletal muscle energetics by measuring mitochondrial function; and 3) determine how Nrf2 activator treatment influences components of skeletal muscle proteostasis.

Specific Aim for Experiment 1: Evaluate the age-related changes in the skeletal muscle of the Dunkin-Hartley guinea pig to determine whether or not the exhibit similar changes in skeletal muscle as humans.

Specific Aim for Experiment 2: Examine the effect of long-term Nrf2 activator treatment on skeletal muscle mitochondrial function in young and old, male and female Dunkin-Hartley guinea pigs.

Specific Aim for Experiment 3: Assess the efficacy of Nrf2 activator treatment in improving components of skeletal muscle proteostasis in young and old Dunkin-Hartley guinea pigs.

OVERALL HYPOTHESES

We hypothesize that Dunkin-Hartley guinea pigs will develop a skeletal muscle phenotype as they age consistent with musculoskeletal aging in humans. Treatment with a Nrf2 activator in young guinea pigs will improve mitochondrial function while long-term treatment will mitigate the decline mitochondrial function in older guinea pigs. Furthermore, Nrf2 activator treatment-mediated improvements in mitochondrial function will attenuate the declines in protein synthesis of skeletal muscle. Additionally, Nrf2 activator treatment will increase the allocation of

protein synthesis dedicated to the maintenance of the proteome. In context of the larger parent project, we hypothesize that these Nrf2 activator-mediated improvements in skeletal muscle, along with changes in other components of the musculoskeletal system, will ultimately be reflected in improvement of the primary, integrative outcome of the parent project: mobility.

FIGURES

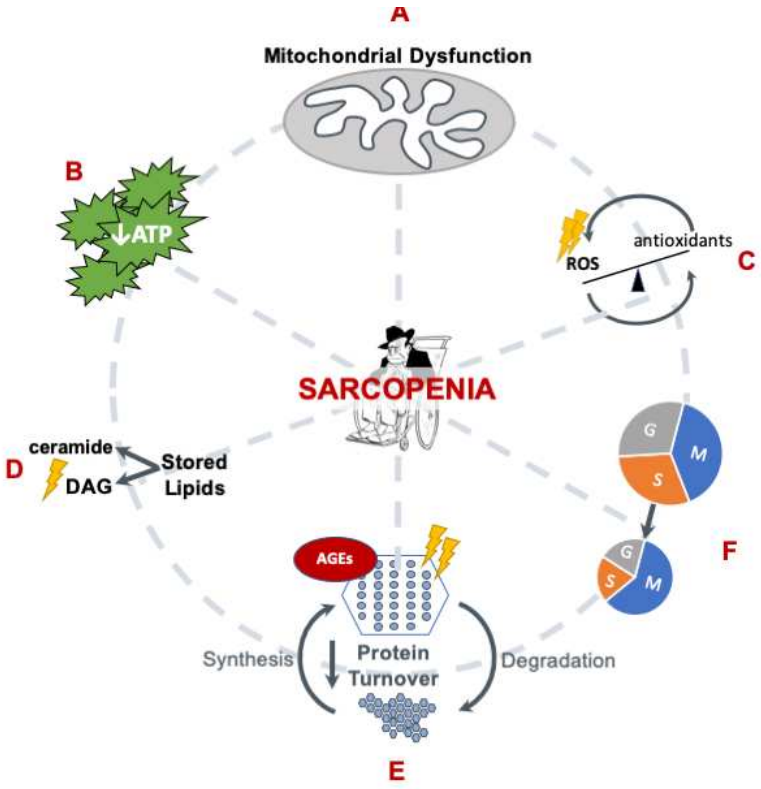

Figure 1.1 Conceptual role of mitochondrial dysfunction in the development of sarcopenia. (A) Aging skeletal muscle is characterized by a mitochondrial dysfunction that includes both decreased ATP availability (B) and a chronic mismatch between reactive oxygen species (ROS) produced and scavenged by endogenous antioxidants (C). A decline in metabolic flux also contributes to accumulation of lipotoxic intermediates which cause greater oxidative stress (D). Damaged proteins, including those modified by advanced glycation end products (AGEs) and ROS, accumulate; this accumulation is exacerbated by a decline in protein turnover (E), a primary component of skeletal muscle dyshomeostasis. The age-related decline in the rate of ATP production constrains energy available for cellular function. (F) This energetic constraint forces cells to increase the proportion of energy allocated toward metabolism “M”, while sacrificing processes related to growth “G” and somatic maintenance “S”. Collectively, these inter-related aspects of mitochondrial dysfunction contribute to sarcopenia.

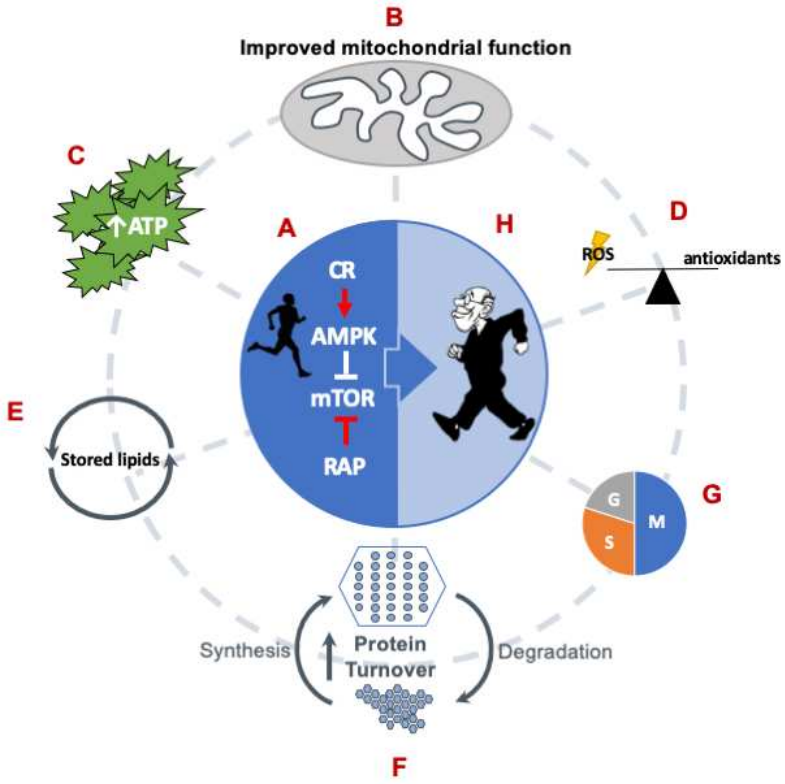

Figure 1.2 Improving mitochondrial dysfunction mitigates sarcopenia. (A) Interventions such as aerobic exercise and mTOR inhibition induce adaptations that improve mitochondrial function, which results in improved rates of ATP production (B) and a better balance between ROS production and antioxidant scavenging (C). Aerobic exercise stimulates metabolic flux of free fatty acids, decreasing the accumulation of lipotoxic products (D). Because of decreases in chronic oxidative stress and improved antioxidant scavenging, oxidative modifications to proteins decrease (E), leading to increased proteostasis. The improvement in ATP production in addition to induction of signaling cascades that cause cells to allocate energy more toward somatic maintenance ”S” than growth “G” (F) The relief of energetic constraint in addition to the maintenance of the rate of energy production facilitates the maintenance of the skeletal muscle proteome. Altogether, interventions that improve mitochondrial function maintain skeletal muscle and mitigate sarcopenia.

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CHAPTER 2 – DUNKIN HARTLEY GUINEA PIGS ARE CHARACTERIZED BY EARLY ONSET MYOFIBER REMODELING THAT RESEMBLES HUMAN MUSCULOSKELETAL AGING

INTRODUCTION

Musculoskeletal aging broadly describes the progressive, age-related decline in skeletal muscle, bone, tendon, and articular cartilage that contributes to disability, chronic disease, and impaired quality of life in older adults[1,2]. One facet of musculoskeletal aging is sarcopenia, which was classically defined as the age-related loss of muscle mass[3]. However, loss of muscle mass does not always explain the age-related decline in muscle function[4] and, correspondingly, sarcopenia is now broadly used to describe age-related skeletal muscle dysfunction[5–8]. Diagnostic criteria vary widely but can include a loss in lean muscle mass alongside a decrease in function such as slower gait speed or impairment in the ability to complete activities of daily living[6]. Skeletal muscle dysfunction increases fall risk by nearly 50% in older adults[9] and accompanies many other age-related chronic diseases such as diabetes, Alzheimer's disease, cancer, and cardiovascular disease[10,11]. In addition, skeletal muscle strength is a predictor of increased all-cause mortality[12]. The estimated prevalence of sarcopenia varies widely due to the lack of consensus criteria for sarcopenia. However. estimates are that 75% of men and 35% of women over the age of 60 years have age-related skeletal muscle dysfunction; these percentages increase to 88% and 53% of men and women over 80 years old[11]. Undoubtedly, the projected increase in the aged population will lead to a growing number of individuals living with marked skeletal muscle, and overall musculoskeletal decline[13], while the economic burden of managing it will grow[14]. Given both the health and economic burden, understanding the etiology and discovering therapies to prevent or mitigate age-related skeletal muscle decline, and promote overall musculoskeletal health with aging, is critical.

Currently, effective established therapies for treating age-related musculoskeletal decline are lacking[15] due to the heterogeneous and multifactorial nature. Common disease

drivers of aging[16,17] including inflammation[18], impaired mitochondrial function[19], loss of proteostasis[20], and macromolecular damage[21] also contribute to the musculoskeletal decline. Decreases in physical activity[22], and loss of motor neurons driving skeletal muscle dysfunction[23] also collectively contribute to the development and progression of the aging muscle phenotype in humans. In addition to the diverse etiology of the disease, sarcopenia develops over decades and with great inter-individual variability. The average human over the age of 65 loses 1% muscle mass per year, which does not always equate to functional losses in every individual[4]. The insidious and heterogeneous nature of age-related musculoskeletal dysfunction poses a challenge to progress in identifying the most promising therapeutic targets in humans and mandates reliance on laboratory animal models.

Rodent models provide insight into the underlying mechanisms and potential interventions to treat age-related skeletal muscle dysfunction. However, as covered in a comprehensive review[24], there are several shortcomings to current rodent models of skeletal muscle loss. One important limitation is that rodents have a sudden and precipitous decline in muscle mass compared to the progressive decline (~1% of muscle mass/year) in humans. It seems rodents experience an abrupt increase in muscle protein breakdown rates at much later stages in their lifespan (i.e. 20 months of age)[25], whereas protein degradation in human muscle declines slowly or remains fairly constant with age likely contributing to protein dyshomeostasis[26,27]. Models that trigger loss of muscle through genetic manipulation, hindlimb suspension, or limb immobilization offer insight into mechanisms that play a role in muscle loss, but may not recapitulate the progressive decline in muscle function in aging humans[28,29] and may involve signaling pathways generally not implicated in human musculoskeletal aging. For example, ubiquitin ligases play a role in atrophy related to hindlimb suspension[30], but not necessarily in aging human skeletal muscle[31]. Similarly, transgenic models, such as the PolgA mutant mouse which increases mtDNA mutations, lead to a sarcopenic or frail phenotype but

may also have unwanted systemic effects and comorbidities not present in human musculoskeletal aging[32]. While these models provide valuable insights, their limitations underscore the need for other preclinical models that more closely and comprehensively model the human musculoskeletal aging phenotype.

Identifying a comprehensive model of age-related musculoskeletal decline is important given the interconnectedness of the primary components of the musculoskeletal system. That is, muscle, bone, tendon, and cartilage must work in conjunction to promote musculoskeletal function. Additionally, evidence suggests that with advancing age, skeletal muscle dysfunction does not occur isolation. For example, age-related loss of skeletal muscle strength/function increases the risk for knee osteoarthritis (OA)[33] (Lee et al. 2016), and may contribute to knee OA progression[34]. Knee OA also imposes a greater risk of developing skeletal muscle dysfunction[35], establishing the two as key musculoskeletal system components that contribute to age-related decline. Therefore, a preclinical model that mimics the systemic age-related musculoskeletal decline in humans is needed.

The Dunkin Hartley guinea pig is a model of spontaneous (also considered primary or idiopathic) OA that closely resembles the onset and disease progression in humans[36]. When fed *ad libitum*, this strain of guinea pig begins developing knee OA at approximately 3-4 months of age. By 9 months, there are clear decrements in mobility and, by 18 months, mobility is severely constrained[37]. While the exact cause(s) of OA in this model is still under investigation, inflammation is one factor associated with disease progression[38]. Dunkin Hartley guinea pigs develop OA accompanied by increased systemic inflammation a relatively early age compared to the lifespan of other outbred strains of guinea pigs (15 months compared to a maximal lifespan of 12 years)[39]. Importantly, an age-related increase in inflammation and oxidative stress (i.e. inflammaging) is also associated with human musculoskeletal aging[40]. However, age-related skeletal muscle changes have yet to be characterized in these guinea pigs. Thus, we

sought to determine if Dunkin Hartley guinea pigs develop a muscle phenotype similar to skeletal muscle from aging humans. If the skeletal muscle phenotype in this strain of guinea pigs with OA indeed closely models muscle aging in humans, it would serve as a valuable preclinical model for investigating the etiology of musculoskeletal decline and for evaluating intervention efficacy, with greater potential for translation of findings to improve musculoskeletal health in humans. The purpose of this study was to characterize the gastrocnemius and soleus muscles of Dunkin Hartley guinea pigs at 5, 9, and 15 months of age, a time course encompassing the onset and progression of OA. We hypothesized that, with age, there would be shifts in the skeletal muscle phenotype that resemble those that occur during musculoskeletal aging in humans.

METHODS

Husbandry, euthanasia, and tissue acquisition

All procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (Protocols 16-6755AA and 19-9129A) and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. 18 male Dunkin Hartley guinea pigs were obtained from Charles River Laboratories (Wilmington, MA, USA) at 3.5, 7.5, and 13.5 months of age (mo) (n=6 at each age). Animals were maintained at Colorado State University's Laboratory Animal Resources housing facilities and were monitored daily by a veterinarian. All guinea pigs were singly-housed in solid bottom cages, maintained on a 12-12 hr light-dark cycle, and provided ad libitum access to food and water. At the time of harvest, the guinea pigs were 5, 9, or 15 mo (n=6/group). In accordance with the standards of the American Veterinary Medical Association, animals were anesthetized with a mixture of isoflurane and oxygen; thoracic cavities were opened, and blood was collected via direct cardiac puncture; immediately afterward, the anesthetized animals were transferred a chamber filled with carbon dioxide for euthanasia.

Skeletal muscle mass, volume, and density and tibia length

Magnetic resonance imaging (MRI) was used to obtain volume in both heads of the gastrocnemius and soleus, which had been formalin fixed with the knee and ankle joints at 90^{o} prior to being individually dissected at their attachments to the bone. MRI scans were performed at Rocky Mountain Magnetic Resonance of Colorado State University. MRI measurements were performed with a 2.3 T Bruker BioSpec, equipped with a 20.5 cm, 100 mT/m gradient system, using a custom built 3.4 cm internal diameter, single channel RF Litz coil (Doty Scientific Inc, Columbia, SC, USA) tuned to detect ^{1}H at 100.3 MHz. The excised muscle tissue was first weighed and then imaged in groups of 8. In the T1- weighted gradient echo images, a fast low-angle shot sequence was used to acquire volumetric images resolved with 0.5 mm isotropic resolution in three-dimensions: echo time = 4.73 ms; repetition time = 15 ms; field of view = 96.0 x 33.5 x 29.5 mm^{3}; matrix size = 192 x 67 x 59.

The volumetric images were exported as DICOM and Analyze 11® was used for segmentation and ROI analysis of total volume, muscle volume, tendon volume, and cross-sectional areas of the muscle volume. Results included the percent of muscle and tendon within each image. Muscle mass and volume were utilized to calculate density – mass divided by volume (mg/mm^{3}).

Tibial length was determined using calibrated digital calipers. Measurements were collected on the posterior/caudal aspect of the bone from the intercondylar eminence to the articular surface of the medial malleolus. Measurements were taken in triplicate with the mean recorded.

Skeletal muscle fiber type and size distribution

We employed immunohistochemistry to measure fiber type and size distribution in the gastrocnemius and soleus muscles. During tissue harvest, portions of both the soleus and gastrocnemius were embedded in OCT and frozen in isopentane cooled by liquid nitrogen. We then mounted 5 μm skeletal muscle cryosections on microscope slides, allowed them to air dry

for 10 min, fixed them in -20^{o} C acetone for 10min, and rehydrated them in 1X phosphate buffered saline (PBS). We then blocked the samples in 10% normal goat serum (NGS) for 1 hr and rinsed them in 1X PBS for 30 seconds. Samples were incubated in the following primary antibodies diluted in 10% NGS for 2 hr at room temperature protected from the light: Laminin: Abcam 11576, 1:500; MyHC I: DHSB BA-F8, 1:50; MyHC IIB: DHSB 10F5, 1:50; MyHC IIA: DHSB 2F7 1:50. Following 3, 5 min rinses in 1X PBS, we incubated the cross sections with secondary antibodies also in 10% NGS for 1 hr (ThermoFisher AlexaFluor 350 A21093; 647 A21242; 555 A21426; 488 A21121; concentration: 1:500), applied an anti-fade reagent (Prolong Gold Antifade, ThermoFisher), and adhered cover slips to the microscope slides.

The slides were imaged by the Center of Muscle Biology at the University of Kentucky as described[41]. Briefly, images were acquired using an upright microscope at 20x magnification (AxioImager M1, Zen2.3 Imaging Software; Zeiss, Göttingen, Germany), which automatically acquires consecutive fields in multiple channels. These fields were stitched together in a mosaic image. The different fiber types were visually identified based on color differences in the merged image. The merged images were then analyzed using MyoVision, software developed by the Center for Muscle Biology at the University of Kentucky. The software used the anti-laminin immunofluorescence to establish line and edge structures, generating fiber outlines to provide the cross-sectional area (CSA) of each fiber. Within each fiber, the software then qualified the fiber type based on the fluorescence. Type I fibers were fluorescent at 647 nm, Type IIA fibers at 488 nm, Type IIB fibers at 555 nm. Fibers that were negative under all channels were classified as Type IIX. An average of over 1000 myofibers were analyzed per animal for each muscle. To analyze fiber size distribution, fibers were categorized into 250 μm^2 bins. The number of fibers in each bin was then divided by the total number of fibers analyzed to determine the percent distribution of each bin.

Pennation angle

During tissue harvest, one hindlimb was fixed with knee and ankle joints fixed at 90°, placed in formalin, and then moved to PBS 24 hr later. Both the medial and lateral gastrocnemius heads were stained in India ink, imaged, and analyzed for pennation angle (θ), defined as the angle of the fiber from the muscle's line of action[42]. Measurements were made in four different regions of the muscle using ImageJ and an average angle was recorded. This technique allowed for unbiasing regions of muscle due to the heterogeneity of the gastrocnemius, as previously performed in other rodent muscle[42]. Measurements were quantified in a blinded and randomized fashion by a single observer. Repeatability testing was completed by performing the measurements twice ($r^2 = 0.5953$).

Skeletal muscle collagen content

Portions of the gastrocnemius and soleus were fixed in formalin and then switched to PBS 24 hr later. Tissue was paraffin embedded, cross sectioned, and stained with Masson's Trichrome following an established protocol at the Colorado State University Diagnostic Medicine Center. Masson's Trichrome staining results in collagen fibers stained blue, nuclei tissue stained black, and background tissue stained red. We imaged the stained cross sections using an upright microscope at 10x magnification and used ImageJ to determine the percentage of area stained blue. To do this, two reviewers set a threshold to quantify the amount of blue present in the cross section and results were subsequently averaged.

Additionally, we used a spectrophotometric method to quantify relative collagen content in tissue using Sirius Red and Fast Green (Chondrex, Inc., Redmond, Washington, USA). Briefly, portions of the gastrocnemius and soleus were frozen at the time of tissue harvest and 5 μm skeletal muscle cryosections were mounted on microscope slides. Sections were rinsed with 1X PBS, immersed in the Dye Solution for 30 min at room temperature, and covered to prevent evaporation of the solution. After incubation, we aspirated the solution and then rinsed the tissue section with distilled water. We then applied the Dye Extraction Buffer and then transferred the Buffer to a 96 well plate for spectrophotometric analysis. We measured the OD

values at 540 nm and 605 nm and calculated collagenous and non-collagenous using manufacturer instructions.

Skeletal muscle protein synthesis

We used the stable isotope deuterium oxide (^2H_2O) to measure long-term skeletal muscle protein synthesis rates. 30 days prior to euthanasia, all guinea pigs were given a subcutaneous injection of 0.9% saline enriched with 99% ^2H_2O equivalent to 3% of their body weight. Drinking water was then enriched with 8% ^2H_2O to maintain deuterium enrichment of the body water pool during the 30-day labelling period.

During tissue harvest, approximately 50 mg of gastrocnemius and soleus were collected and frozen immediately in liquid nitrogen. Tissue was later homogenized and fractionated following an established differential centrifugation protocol[43] with one extra step to acquire a collagen-enriched fraction validated by both western blot and proteomic analysis[44].

Body water enrichment was determined from plasma as previously described[43,45,46]. To determine percentage of deuterium enriched alanine in muscle fibers we followed previously published procedures[43,45–47]. Approximately 25-50 mg of skeletal muscle was bead homogenized (Next Advance, Inc., Averill Park, NY, USA) in an isolation buffer containing 100 mM KCl, 40 mM Tris HCl, 10 mM Tris base, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM ATP (pH 7.5), with phosphatase and protease inhibitors (HALT; ThermoScientific, Rockford, IL, USA). After homogenization, the samples were centrifuged at 800 g for 10 min at 4°C. The resulting pellet was enriched with collagen and myofibrillar proteins. The supernatant contained mitochondrial and cytosolic proteins. To separate the collagen and myofibrillar proteins, we isolated and washed the pellet with 500 µL of 100% ethanol and rinsed with 500 µL of Milli-Q water twice. We then added 0.3 M NaOH, incubated for 30 min 37° C, centrifuged at 16,300 g for 10 min at 4° C, and transferred the supernatant, which contains myofibrillar proteins, to another tube. We repeated the 0.3 M NaOH incubation to extract any remaining myofibrillar proteins. The remaining collagen pellet was rinsed with Milli-Q water and ethanol. The pellet was then

resuspended in 250 µL of 1M NaOH and placed on a heat block for 15 min at 50° C shaking at 900 rpm. The collagen protein enriched fraction was then incubated in 6 M HCl for 24 hr at 120° C for protein hydrolysis.

To precipitate the myofibrillar proteins from the previous supernatant, we added 500 µl of 1 M perchloric acid and centrifuged at 3000 g, 4° C for 20 min. After removing the supernatant, we rinsed the pellet with ethanol and Milli-Q water, resuspended in 250 µL of 1M NaOH, and placed on a heat block for 15 min at 50° C shaking at 900 rpm. The myofibrillar protein enriched fraction was then incubated in 6 M HCl for 24 hr at 120° C for protein hydrolysis.

To isolate the mitochondrial and cytosolic proteins, we centrifuged the supernatant from the initial centrifugation at 9000 g in 4° C for 30 min. The resulting supernatant, containing cytosolic proteins, was removed and placed in a new tube. The resulting pellet, enriched with mitochondrial proteins, was washed in a buffer containing 100 mM KCl, 10 mM Tris HCl, 10 mM Tris Base, 1 mM $MgCl_2$, 0.1 mM EDTA, and 1.5% BSA. We then rinsed the pellet in ethanol and Milli-Q water, resuspended in 250 µL of 1 M NaOH, and placed on a heat block for 15 min at 50° C shaking at 900 rpm. The mitochondrial protein enriched fraction was then incubated in 6 M HCl for 24 hr at 120° C for protein hydrolysis.

The supernatant containing cytosolic proteins was divided into two 400 µl aliquots. We added 400 µl of 14% sulfosalicylic acid to one aliquot and incubated it on ice for 1 hr. After incubation, we centrifuged at 16,000 g at 4° C for 10 min, rinsed the pellet with ethanol and Milli-Q water, resuspended the pellet in 250 µL of 1 M NaOH, and placed on a heat block for 15 min at 50°C shaking at 900 rpm. The cytosolic protein enriched fraction was then incubated in 6 M HCl for 24 hr at 120° C for protein hydrolysis.

All hydrolysates were ion exchanged, dried in a vacuum, and resuspended in 1 mL of molecular biology grade water. Half of the suspended sample was derivatized by a 1 hr incubation of 500 µL acetonitrile, 50 µL K_2HPO4, pH 11, and 20 µL of pentafluorobenzyl bromide. Ethyl acetate was added and the organic layer was removed, dried under nitrogen

gas, and reconstituted in 200 – 600 µL ethyl acetate for analysis on an Agilent 7890A GC coupled to an Agilent 5975C MS as previously described[43,45,46]. The newly synthesized fraction of myofibrillar proteins was calculated from the enrichment of alanine bound in muscle proteins over the entire labelling period, divided by the true precursor enrichment, using the average plasma deuterium enrichment over the period of measurement with MIDA adjustment [48].

Statistics

A one-way ANOVA was performed when outcome measures were assessed (body mass, muscle mass and density, pennation angle, and protein synthesis) at all three ages of 5, 9, and 15 mo to analyze the main effect of age. If there was significant main effect of age, we conducted a Tukey's post-hoc test to specifically compare ages. For outcomes comparing only in 5 and 15 mo guinea pigs (histological outcomes i.e. CSA, fiber type, and collagen), a Student's independent t-test was used. GraphPad Prism 8.0 (La Jolla, CA, USA) was used for statistical analysis and to create figures. While statistical significance was set *a priori* at $p<0.05$, because of the relatively small sample size, we also chose to report values of $p<0.10$ as noteworthy observations. Data are normally distributed and are presented as mean ± standard error.

RESULTS

Age related changes in body mass and tibia length

As anticipated, there was a significant effect of age ($p<0.001$) on body mass. Body mass increased with age, with 9 mo guinea pigs weighing significantly more than 5 mo, and 15 mo guinea pigs tending ($p=0.07$) to weigh more than 5 mo (Fig 2.1A). However, there were no significant differences among ages in tibia length (Fig 2.1B).

Age related, muscle-specific, and strain-specific differences in muscle mass and density

There was a significant effect of age ($p<0.05$) on soleus mass, with 15 mo guinea pigs having greater mass than their 5 mo counterparts (5 mo: 212.35 ± 12.23 mg, 15 mo: 274.85 ± 16.43 mg, $p<0.05$) (Fig 2.2B). In the gastrocnemius, there was a trend ($p=0.06$) for a significant effect of age on mass (Fig 2.2A).

Because there was a significant effect of age on body mass, we also expressed muscle mass relative to body mass. There were no age-related differences in either relative gastrocnemius or soleus masses relative to body mass (Figs 2.2C, 2.2D).

There was a significant effect of age on gastrocnemius volume, with volume increasing with age (5 mo: 1529.39 ± 84.53 mm^3, 9 mo: 1800.76 ± 65.44 mm^3, 15 mo: 1956.06 ± 60.39 mm^3; $p<0.05$) (Fig 3A). Similarly, there was a significant increase in soleus volume with age (5 mo: 225.22 ± 12.05 mm^3, 15 mo: 303.56 ± 10.19 mm^3; $p<0.05$) (Fig 3B). There was a trend for a significant effect of age on gastrocnemius density, with lower density in 15 mo guinea pigs compared to 5 mo (5 mo: 1.18 ± 0.05 mg/mm^3, 15 mo: 1.09 ± 0.03 mg/mm^3; $p=0.07$) (Fig 3C). There was a significant main effect of age in the soleus (5 mo: 0.70 ± 0.03 mg/mm^3, 9 mo: 0.91 ± 0.03 mg/mm^3, 15 mo: 0.90 ± 0.04 mg/mm^3; $p<0.001$).

Age-related changes in myofiber cross sectional area, size, distribution, and fiber type composition

Average cross-sectional area of all gastrocnemius myofibers did not change as a consequence of age (Fig 2.4). This was observed in all fiber types of the gastrocnemius. We also saw no difference in myofiber CSA in the soleus (Fig S2.1).

Despite the lack of difference in the average myofiber CSA, there was a change in fiber size distribution in the gastrocnemius. 15 mo guinea pigs had a greater proportion of 1750 μm^2 myofibers and less 2750 μm^2 myofibers compared to 5 mo guinea pigs ($p<0.05$) (Fig 2.5A). In the soleus, there was no change in myofiber size distribution (Fig 2.5B). 15mo guinea pigs had significantly less type II myofibers (5 mo: 72.9 ± 2.86%, 15 mo: 59.73 ± 7.71%; $p<0.05$) and more type I myofibers (5 mo: 13.58 ± 0.99%, 15 mo: 30.07 ± 3.89%; $p<0.05$) in the

gastrocnemius compared to 5 mo (Fig 6A). The lower proportion of type IIB myofibers seems to have accounted for the difference in fiber type composition (5 mo: 17.11 ± 10.5%, 15 mo: 2.34 ± 1.63%; p=0.07) (Figure 2.6B).

Skeletal muscle architecture and collagen

There were no significant age-related differences in the gastrocnemius pennation angle (5 mo: 13.6^{o} ± 0.56; 9 mo: 14.8 ± 0.97^{o}; 15 mo: 14.9 ± 0.99^{o}) (Fig 2.7). Using Masson's Trichrome staining, we observed no significant age-related differences in collagen content in either the gastrocnemius (5 mo: 8.7 ± 0.71%; 9mo: 6.8 ± 1.28%; 15 mo: 6.7 ± 0.83%) (Fig 8A) or soleus (5 mo: 7.3 ± 0.90%; 9 mo: 6.7 ± 1.10%; 15 mo: 5.8 ± 1.01%) (Fig 2.8B). Results of the spectrophotometric assay, however, indicated that 15 mo guinea pigs had lower relative collagen content than 5 mo guinea pigs in both the gastrocnemius (5 mo: 3.8 ± 0.04%, 15 mo: 3.3 ± 0.11%; p<0.05) (Fig 2.8D) and soleus (5 mo: 4.0 ± 0.07%, 15 mo: 3.4 ± 0.12%; p<0.05) (Fig 2.8E).

Skeletal muscle protein synthesis

In the gastrocnemius, myofibrillar, cytosolic, mitochondrial, and collagen fractional synthesis rates (FSR) were all lower in 15 mo guinea pigs compared to 5 mo (p<0.05) (Fig 2.9). Myofibrillar protein synthesis was lower in 15 mo compared to 5 mo guinea pigs (5 mo: 2.45 ± 0.13%/day, 15 mo: 1.80 ± 0.16%/day; p<0.05) (Fig 2.9A). Cytosolic FSR was significantly lower at each time point (5 mo: 2.55 ± 0.08%/day, 9 mo: 2.23 ± 0.05%/day, 15 mo: 1.85 ± 0.16%/day; p<0.05) (Fig 2.9B). The same was also observed in the mitochondrial fraction (5 mo: 2.25 ± 0.10%/day, 15 mo: 1.61 ± 0.14%/day; p<0.05) (Fig 2.9C). Collagen FSR was significantly lower in 9 mo and 15 mo guinea pigs compared to 5 mo (5mo: 0.59 ± 0.08%/day, 9 mo: 0.30 ± 0.03%/day, 15 mo: 0.13 ± 0.004%/day; p<0.05) (Fig 2.9D).

Similar to the gastrocnemius, soleus synthesis rates were slower in the older guinea pigs in each fraction except for collagen (Fig 2.10). Myofibrillar FSR was lower in the 9 mo and 15 mo compared to the 5 mo guinea pigs (5 mo: 3.44 ± 0.11%/day, 9 mo: 2.52 ± 0.18%/day, 15

mo: 2.81 ± 0.12%/day; $p<0.05$) (Fig 2.10A). There was a trend for cytosolic FSR to be lower in 9 mo and 15 mo compared to 5 mo guinea pigs (5 mo: 3.87 ± 0.18%/day, 9 mo: 3.13 ± 0.19%/day, 15 mo: 3.04 ± 0.30%/day; $p=0.06$ for 5 mo vs. 9 mo; $p=0.0558$ for 5 mo vs. 15 mo) (Fig 2.10B). Mitochondrial FSR was also lower in 15 mo guinea pigs compared to their 5 mo counterparts (5 mo: 2.63 ± 0.11%/day, 15 mo: 1.96 ± 0.212%/day; $p<0.05$) and tended to be lower between 9 mo and 15 mo guinea pigs (9 mo: 2.54 ± 0.18%/day; $p=0.08$) (Fig 2.10C). There were no significant age-related differences in collagen protein synthesis in the soleus ($p>0.10$) (Fig 2.10D).

DISCUSSION

Musculoskeletal decline is a key contributor to disability and chronic disease during aging. Obtaining a full understanding of mechanisms contributing to the decline is hampered by complex contributions from systemic components including skeletal muscle, bone, articular cartilage, and tendon, and by the lack of preclinical models that closely and comprehensively model the human musculoskeletal aging phenotype. The overall goal of this study was to characterize changes in the skeletal muscle phenotype during a time course that encompasses the onset and progression of joint degeneration in Dunkin Hartley guinea pigs, a model of idiopathic OA. Our findings document, for the first time, that from 5 to 15 months of age, there is evidence of skeletal muscle remodeling that is consistent with those observed during musculoskeletal aging in humans. During this period, we observed a decline in type II myofibers, a shift in fiber size distribution favoring smaller muscle fibers, progressive declines protein synthesis rates, and a decline in gastrocnemius density, which are all observed in human skeletal muscle aging. Moreover, these skeletal muscle changes are accompanied by impairments in other components of the musculoskeletal system, specifically degeneration of articular cartilage[36]. The current study, combined with previous work detailing progression of joint degeneration and inflammation[38], suggest that Dunkin Hartley guinea pigs are a viable

model to study age-related musculoskeletal changes and interventions to improve musculoskeletal function with aging.

Similarities between skeletal muscle remodeling in Dunkin Hartley guinea pigs and aging humans

Over the time course of the current study, we observed that Dunkin Hartley guinea pigs have skeletal muscle remodeling that is consistent with the aged skeletal muscle phenotype in humans. While we, and others[49], failed to see age-related declines in muscle mass from 5 to 15 months, other characteristics indicative of muscle aging including muscle fiber size distribution and fiber type composition, muscle density, and rates of protein synthesis suggest age-related decrements in skeletal muscle quality in these guinea pigs. Most interestingly, these observations occur by an age (15 mo) that is young relative to the maximal predicted lifespan of other strains/breeds of guinea pigs (~10% of the predicted 12 years[39]). In comparison, murine models only begin demonstrating signs of sarcopenia well after 20 months of age, much closer to their maximal predicted lifespan (50% of 40 months[50]). The skeletal muscle remodeling observed in the current study occurred within 10 months, which offers a convenient model to test long-term interventions that might modulate the progression of skeletal muscle decline.

In humans, muscle with mixed muscle fiber types (such as the gastrocnemius or vastus lateralis) are more susceptible to loss of mass compared to muscles comprised primarily type I muscle fibers, such as the soleus, which are generally unaffected by age[51,52]. This is likely related to the fact that sarcopenia is associated with the loss of larger, type II muscle fibers and an increase in type I muscle fibers[53]. In humans, type II myofibers atrophy and type I myofibers remain largely unchanged resulting in a shift in myofiber size distribution toward smaller myofibers[53,54]. We observed the same phenomenon in the gastrocnemius of Dunkin Hartley guinea pigs. From 5 months to 15 months of age, there was a shift toward a smaller myofibers. Moreover, there was a lower percentage of type II myofibers and a greater percentage of type I myofibers, which is consistent with studies investigating MHC expression in whole muscle

homogenates from these guinea pigs[49]. More research is necessary to determine if these changes in muscle composition and myofiber size result in loss of strength and other functional decrements as observed in aging humans.

As humans age, there is generally an increase in fat deposition within (intermuscular triglycerides, IMTG) or around (intra-muscular adipose tissue, IMAT) skeletal muscle. In Dunkin Hartley guinea pigs, we observed lower gastrocnemius density in 15 month guinea pigs compared to 5 month, which may be indicative of increased fat deposition given that fat has lower density than muscle. However, there were no age-related differences in soleus density between young and older guinea pigs. The contrasting outcomes in gastrocnemius and soleus densities in young and older guinea pigs could be explained by the fact that type II fibers are at greater risk of lipid spillover due to an inability to switch to from carbohydrate to lipid oxidation (i.e. metabolic inflexibility)[55]. Both IMTG[56,57] and IMAT[58] are negatively associated with skeletal muscle function and seem to contribute to diabetes. It remains unclear, however, if lower density in gastrocnemius is associated with greater concentrations of specific lipid species that impart deleterious effects on skeletal muscle function.

There is a relative paucity of data on age-related changes in collagen content in human skeletal muscle. However, most data suggest that there is no change in collagen content with age in human[59–61]. Despite this lack of change, there seems to be an impairment in collagen turnover[59], which in turn may lead to the accumulation of oxidatively damaged collagen in skeletal muscle[60]. In our guinea pigs, the two methods we employed to measure collagen content demonstrated either no difference or lower collagen levels between the 5 month and 15 month guinea pigs. Despite the lack of, or small, change in collagen content, there was a precipitous decline in muscle collagen synthesis rate with age. As such, for there to be similar collagen content while collagen protein synthesis decreased, breakdown of collagen proteins must have sharply declined as well. Accordingly, a decline in both synthesis and degradation

would be indicative of decreased collagen protein turnover. As a consequence of such a decline in turnover, we hypothesize that the accumulation of oxidatively damaged/crosslinked collagen in the extracellular matrix of skeletal muscle increases with age, which is associated with impairments in skeletal muscle strength in humans[60]. However, further work is needed to measure the age-related differences in oxidatively damaged collagen proteins and muscle strength in these guinea pigs.

In humans, pennation angle has been shown to decrease in the gastrocnemius and soleus with age. Such architectural changes are likely due to decreases of sarcomeres both in series and in parallel[62]. We did not see an age-related decline in pennation angle. These were the first such measures to be assessed cross-sectionally in Dunkin Hartley guinea pigs and the angles quantified within the gastrocnemius were similar to those previously measured in guinea pigs of a different strain[63]. Given differences in quantification methods[62,64] and the paucity of longitudinal data, potential relationships between pennation angle and force production, and how they change with age in this model of musculoskeletal decline, should be addressed in future studies.

Humans experience a decline in skeletal muscle mass at a relatively slow rate (~1% decline per year)[65]. In order for there to be a loss of muscle mass, protein breakdown rates must exceed protein synthesis rates. Consensus suggests that protein breakdown in skeletal muscle remains unchanged throughout age[26], though some evidence suggests impairments in autophagy[66]. Thus, the current paradigm for the underlying mechanism of age-related loss in muscle mass is that there is impairment in protein synthesis. Resting protein synthesis rates between young and old adults appear not to be different[26], suggesting that there is impairment in protein synthesis in response to anabolic stimuli such as protein ingestion and exercise[57,67–69]. As a consequence, protein synthesis rates in human skeletal muscle likely decline very slowly with age[65]. This is in contrast to other rodent models, such as mice and rats, that experience

abrupt and sharp changes in protein turnover[24]. Using the stable-isotopic tracer deuterium, we were able to measure the cumulative protein synthesis over 30 days, capturing both resting and anabolic episodes of protein synthesis in these guinea pigs. We found that with increasing age, there were lower rates of protein synthesis in all the subfractions of both gastrocnemius and soleus muscles. By 15 months of age, FSR of both myofibrillar proteins (i.e. contractile proteins) and mitochondrial proteins were significantly lower than observed in 5 month old guinea pigs. While we did not observe lower muscle mass in the older guinea pigs, the age-related declines in protein synthesis are consistent with the smaller myofiber size distribution observed through immunohistochemistry as well as lower gastrocnemius muscle density. While statistically significant, the difference in FSR between 5 and 15 month old guinea pigs is small (<1%), which may better reflect the progressive decline in muscle protein synthesis with age in humans.

Potential mechanisms underlying the skeletal muscle remodeling in Dunkin Hartley guinea pigs

At the moment, there is no clear, established mechanism for the early onset of a skeletal muscle aging phenotype we observed in the Dunkin Hartley guinea pigs. This strain of guinea pig develops primary OA that histologically resembles human OA[36]. In humans, knee OA increases the risk for sarcopenia[35], which may be due to impaired levels of physical activity[70]. However, we did not monitor spontaneous physical activity in these guinea pigs. Physical activity is known to influence skeletal muscle mass, quality (e.g. fiber type, density), and function[71]. Given that knee OA generally decreases physical activity[72], we predict that decreases in physical activity may account for some of the skeletal muscle remodeling we observe at a relatively young age in these guinea pigs.

Another mechanism that could explain the early onset muscle aging phenotype is increased systemic inflammation. Compared to a strain of guinea pig that is not prone to primary OA at a young age, Dunkin Hartley guinea pigs have higher levels of the pro-inflammatory mediator interleukin-1β (IL-1β)[38]. Interventions such as caloric restriction[73], elicit lower levels of

systemic inflammatory markers compared to *ad libitum* fed controls and delay the onset of OA in this model. In humans, both knee OA and elevated levels of inflammatory mediators are associated with sarcopenia[34,74]. Additionally, age-related increases in IL-1β are associated with decrements in extension peak knee extension torque[75]. Inflammation is a known disease driver of aging[17,40] and implicated in most age-related chronic diseases[76]. Therefore, future inquiry will include inflammatory profiling systemically as well as locally in skeletal muscle and other tissues beyond the musculoskeletal system in these guinea pigs to determine if they are a more encompassing model of inflammaging.

Future directions and conclusions

While we assessed a number of well-recognized aspects of skeletal muscle remodeling associated with human aging, there are other important components of the aging muscle phenotype we have yet to measure in this unique guinea pig model of musculoskeletal decline, including muscle function. We predict that the changes in muscle quality we observed would be paralleled by declines in muscle function. The observed loss of type IIB fibers is known to be a key determinant of muscle strength[53]. The decline in type II myofibers, which are primarily responsible for power generation, may lead to functional decrements such as impaired gait and mobility. Additionally, age-related denervation of skeletal muscle is a primary factor in the onset of sarcopenia in humans and changes in muscle fiber type[23]. Thus, we presume that changes at the neuromuscular junction may partially explain the fiber type shift observed in the current study. Furthermore, myofiber size distribution favoring a larger percentage of smaller fibers is also a characteristic of older adults and has potential to impart decreased muscle force production[54]. We have not yet measured changes in spontaneous physical activity with age but anticipate that this strain of guinea pig likely models the age-related decline in physical activity typically observed in humans. Finally, the current observations were only in male guinea pigs. Given that the onset and prevalence of decline in muscle function differs between aging males

and females[77], investigating the age-related changes in skeletal muscle in female guinea pigs is another important focus of our follow-on studies.

Collectively, our results suggest that Dunkin Hartley guinea pigs could be a valuable translational model of musculoskeletal aging. The definition of sarcopenia has evolved with expanded understanding of skeletal muscle aging and it is difficult to distinguish strict age-related muscle dysfunction from other age-related phenomena that contribute to muscle dysfunction (e.g. inactivity, cancer, metabolic disorders, osteoarthritis, etc.)[78–80]. The strengths of the Dunkin Hartley guinea pig as a preclinical model are that it is a non-transgenic, outbred model that recapitulates a number of aspects of the human musculoskeletal aging phenotype, characterized by age-related inflammation (i.e. inflammaging), joint degeneration, and skeletal muscle remodeling. This is in contrast to other models of sarcopenia, such as hindlimb unloading/immobilization or very old mice/rats where processes unrelated to human musculoskeletal aging seem to contribute the loss of muscle function. Given that changes in skeletal muscle and articular cartilage occur within just 15 months of life, an early point in the maximal predicted guinea pig lifespan, the Dunkin Hartley guinea pig model may also be ideal for evaluating interventions within a relatively short timeframe. While more work is necessary to further characterize skeletal muscle remodeling and function, as well as to assess other components of the musculoskeletal system including bone and tendon, our data suggest that these guinea pigs are a promising model to study age-related myofiber remodeling related to overall musculoskeletal decline that mimics human aging.

FIGURES

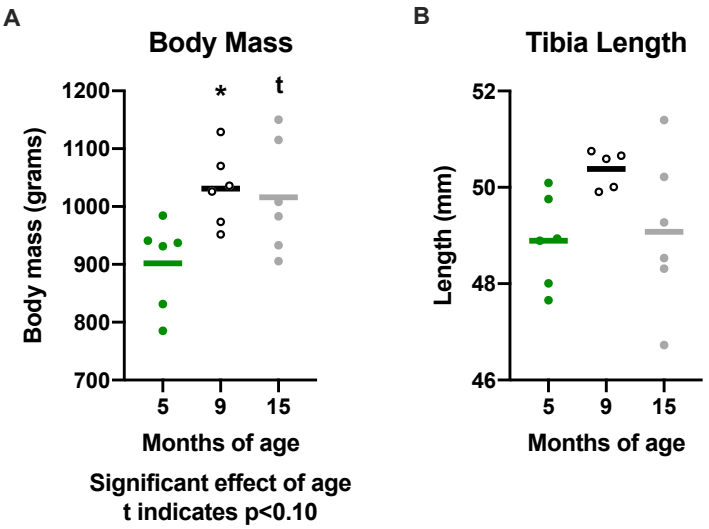

Figure 2.1: Differences in body mass and tibia length. There was a significant effect of age ($p<0.05$) on body mass in Dunkin-Hartley guinea pigs (Fig 2.1A). 15 mo guinea pigs tended to be larger than 5 mo guinea pigs ($p=0.07$). However, there was no difference in tibia length between any of the ages (Fig 2.1B). * denotes $p<0.05$ compared to 5 mo; t denotes $p<0.10$ compared to 5mo.

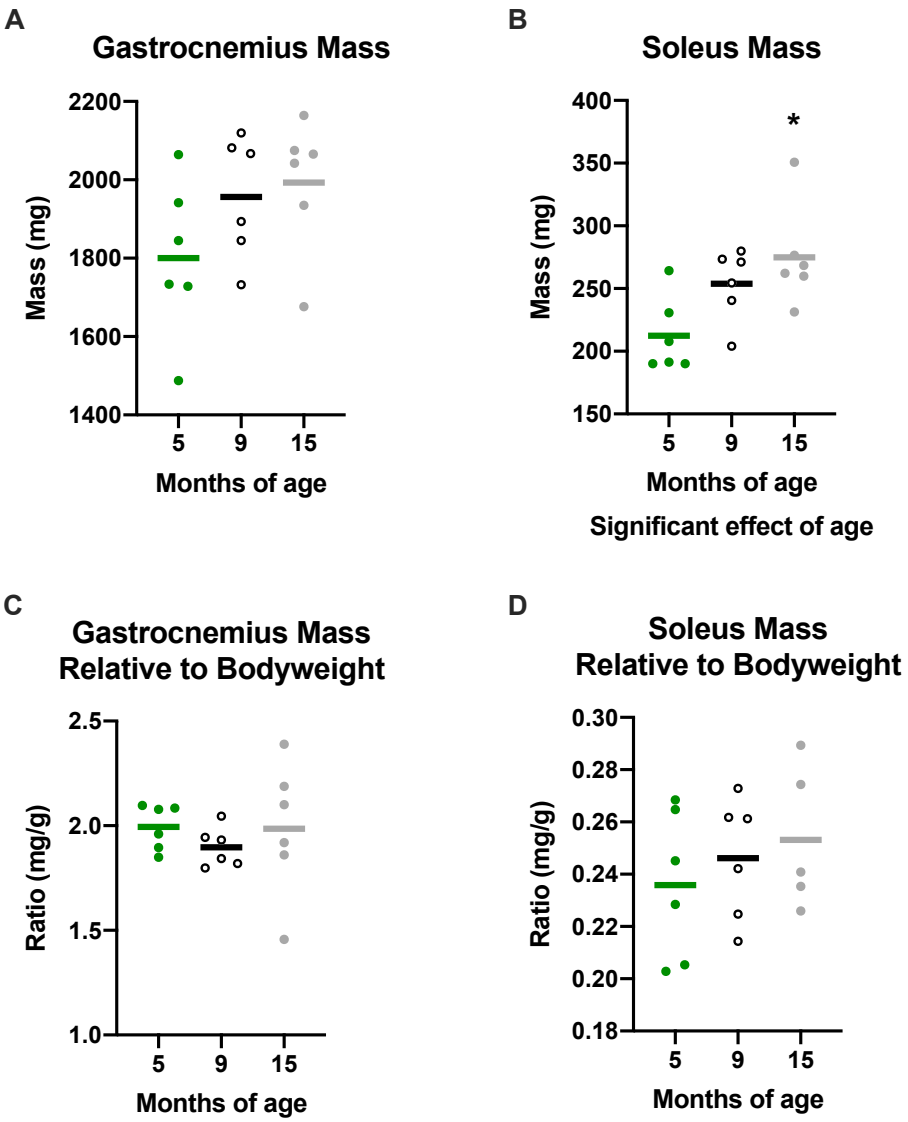

Figure 2.2: Differences in gastrocnemius and soleus mass and mass relative to bodyweight. There was no effect of age on gastrocnemius mass (Fig 2.2A), though age did have a significant effect on soleus mass (Fig 2.2B). When expressed relative to body mass, there were no age-related differences in either the gastrocnemius (Fig 2.2C) or soleus (Fig 2.2D). * denotes $p<0.05$ compared to 5 mo.

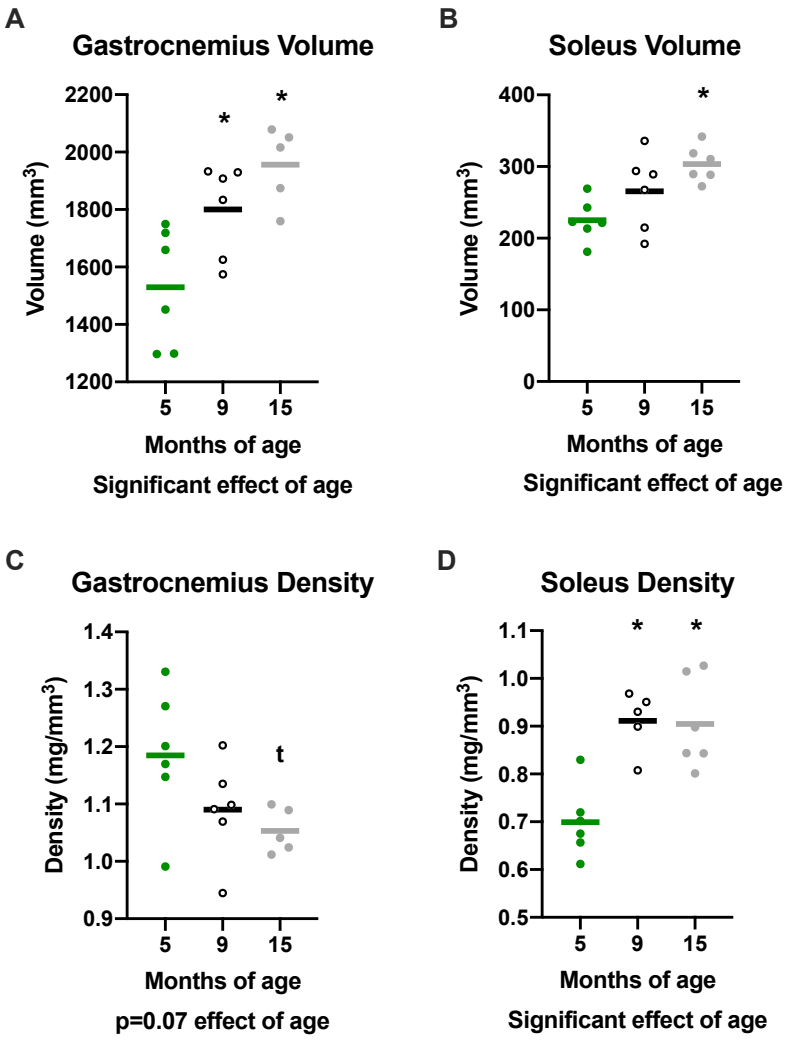

Figure 2.3: Differences in muscle volume and density. 9 mo and 15 mo guinea pigs had greater gastrocnemius volume compared to 5 mo guinea pigs ($p<0.05$) (Fig 2.3A). However, age trended to have a negative effect on gastrocnemius density ($p=0.07$) (Fig 2.3C). 15 mo guinea pigs had greater soleus volume compared to 5 mo guinea pigs ($p<0.05$) (Fig 2.3B). In constrast to the gastrocnemius, soleus density was greater in 9 mo and 15 mo guinea pigs compared to 5 mo counterparts ($p<0.05$) (Fig 2.3D). * denotes $p<0.05$ compared to 5 mo; t denotes $p<0.10$ compared to 5mo.

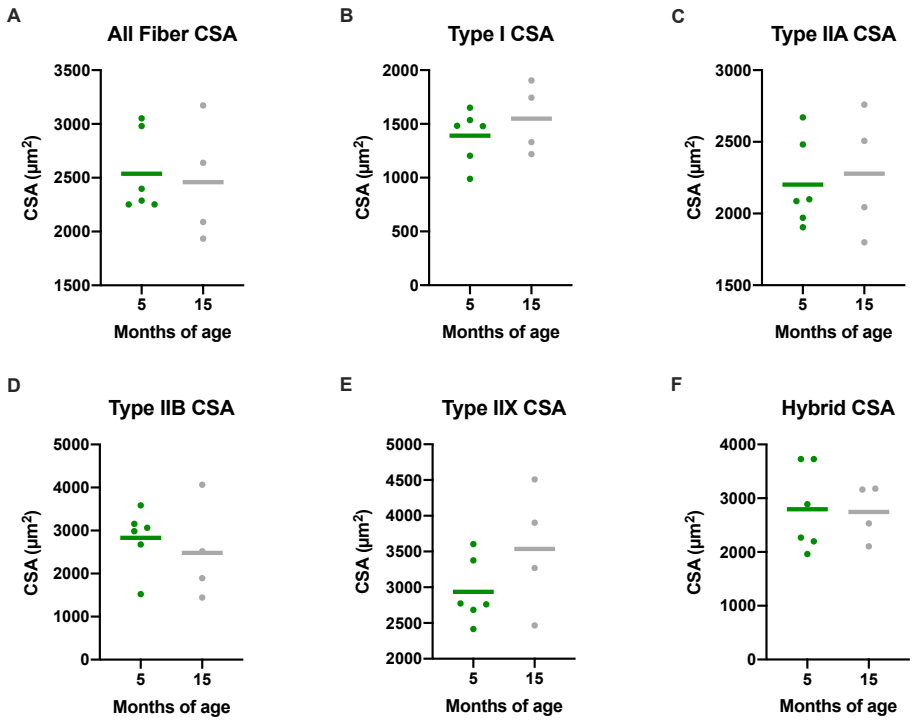

Figure 2.4 Muscle fiber type-specific CSA averages. There were no differences between the CSA of skeletal muscle fibers of any type in the gastrocnemii of 5 mo and 15 mo Dunkin Hartley guinea pigs.

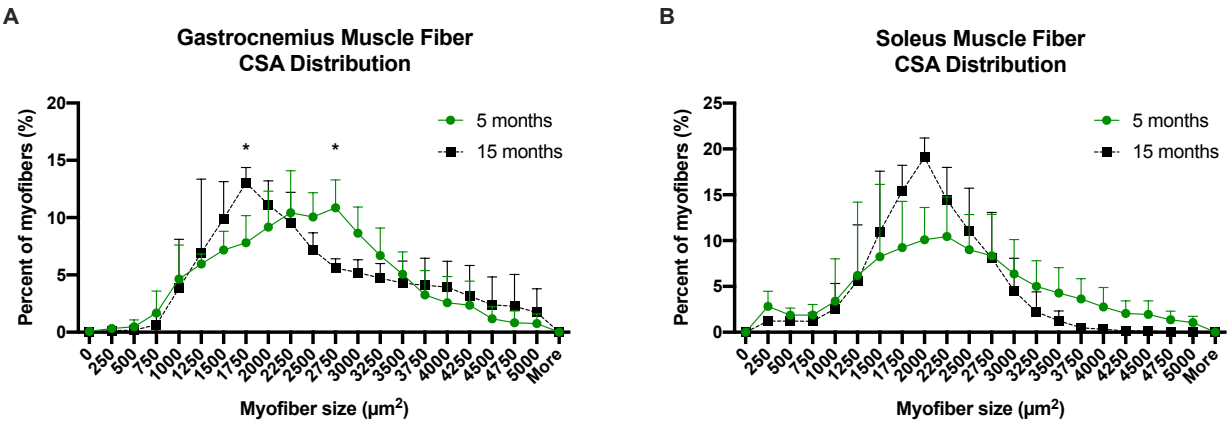

Figure 2.5: CSA distribution of all myofibers in both the gastrocnemius and soleus. 15 mo guinea pigs had a greater proportion of 1750 μm2 myofibers and less 2750 μm2 myofibers compared to 5 mo guinea pigs ($p<0.05$) (Fig 2.5A). However, there was no difference in myofiber size distribution in the soleus (Fig 2.5B). * denotes $p<0.05$ compared to 5 mo.

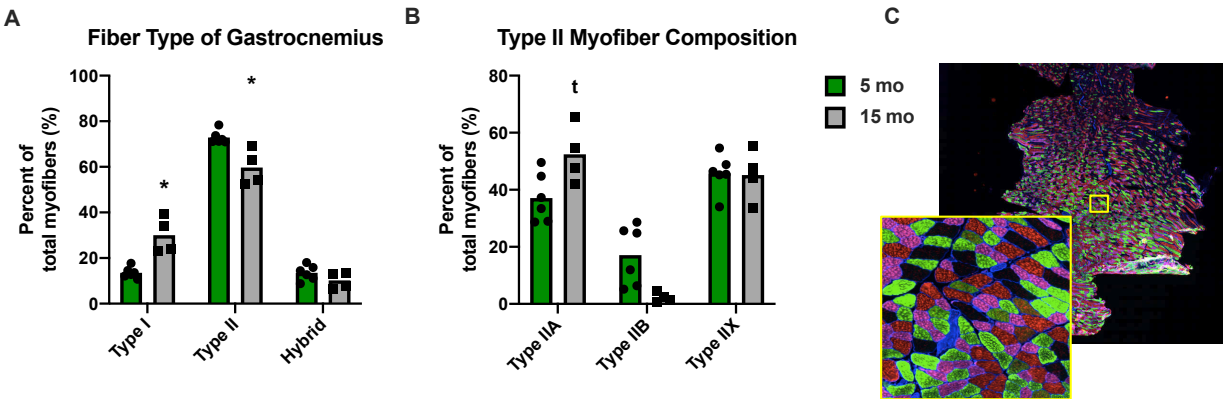

Figure 2.6: Fiber type composition of the gastrocnemius. There was a significant greater amount of type I myofibers and lesser amount of type II myofibers in 15 mo guinea pigs compared to 5 mo guinea pigs (Fig 2.6A). There trended ($p=0.07$) to be less type IIB myofibers in 15 mo guinea pigs as well (Fig 2.6B). Fig 6C is an example of fiber typing with a zoomed in portion to show detail. * denotes $p<0.05$ compared to 5 mo; t denotes $p<0.10$ compared to 5mo.

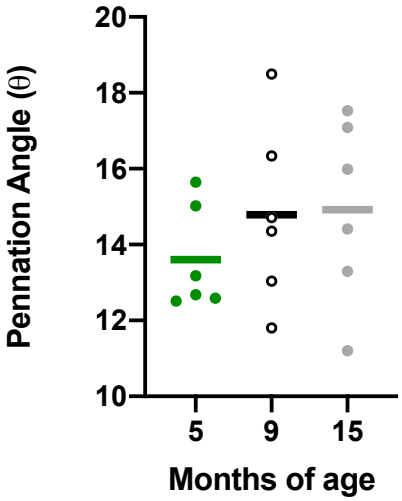

Figure 2.7: Pennation angle of the gastrocnemius. There were no differences in pennation angle between any of the ages.

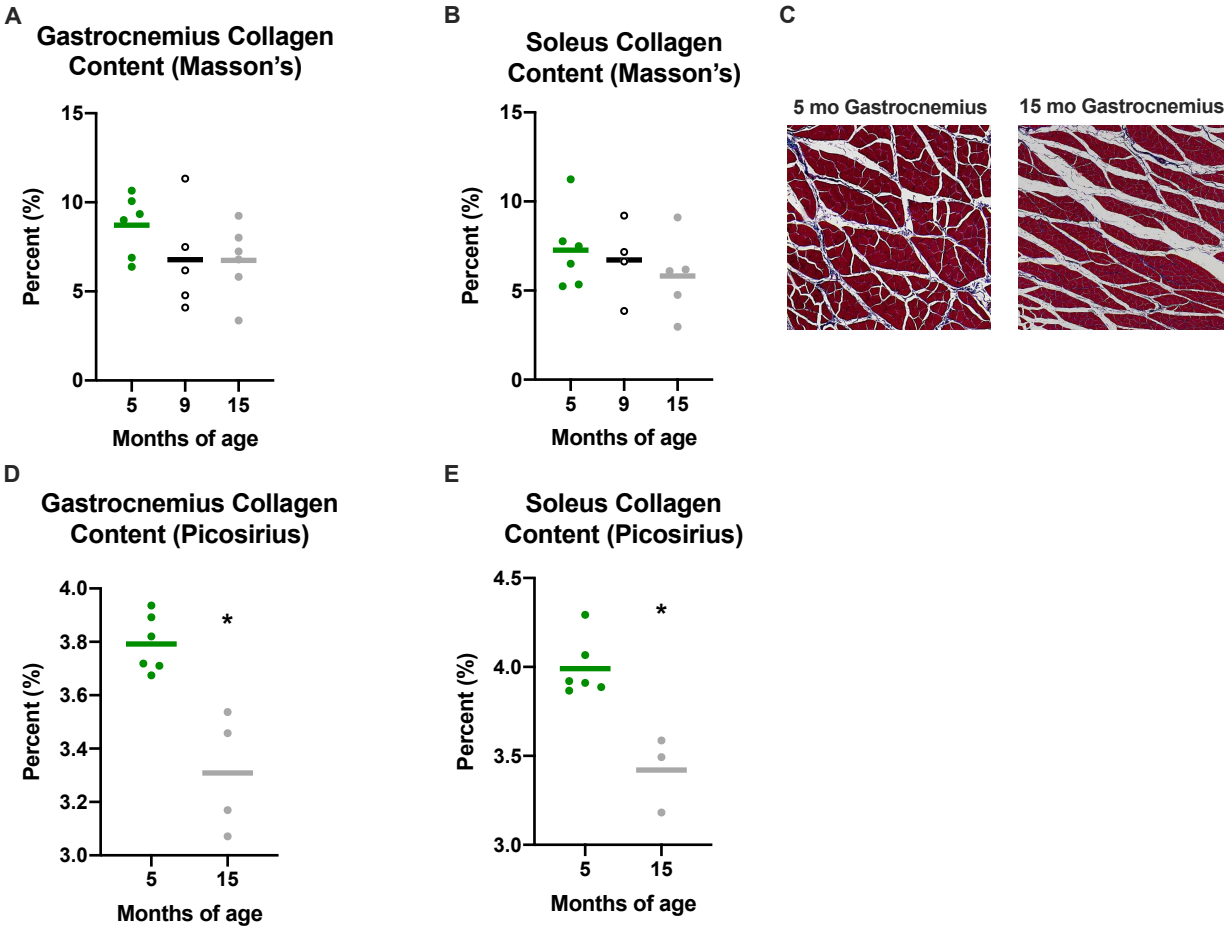

Figure 2.8: Collagen content in the guinea pig skeletal muscle. As assessed with Masson's Trichrome staining, there was no difference in the percentage of collagen in either the gastrocnemius (Fig 2.8A) or soleus (Fig 2.8B). Figure 2.8C are representative images of stained 5 and 15 mo gastrocnemius cross sections. Assessed with spectrophotometry, collagen content was significantly lower in both the gastrocnemius (Fig 2.8D) and soleus (Fig 2.8E) of 15 mo guinea pigs compared to 5 mo guinea pigs ($p<0.05$). * denotes $p<0.05$ compared to 5 mo.

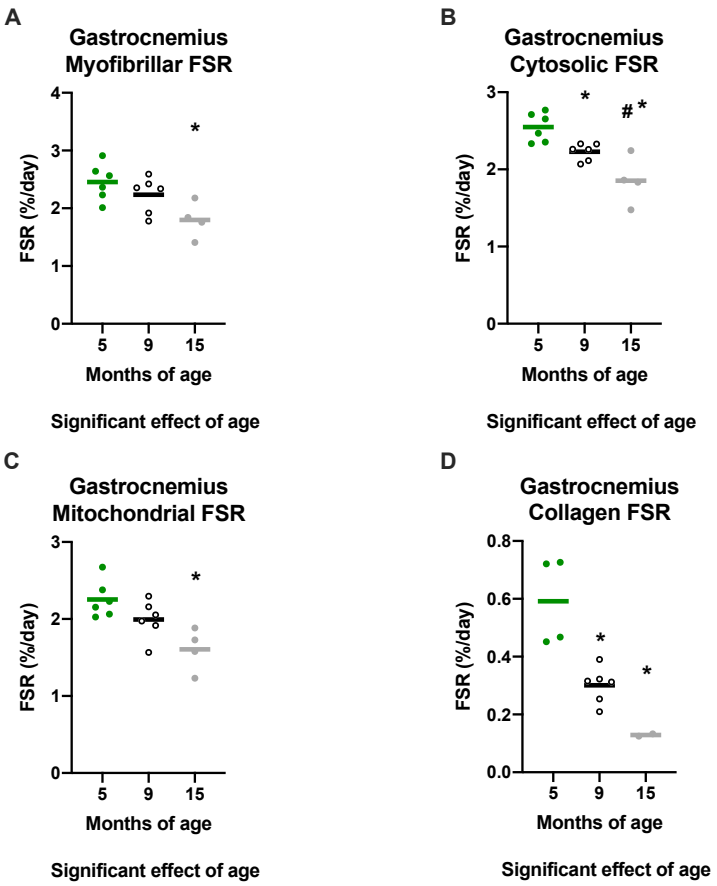

Figure 2.9: Skeletal muscle protein synthesis rates in the gastrocnemius. In all subfractions of the gastrocnemius there was a significant, negative effect ($p<0.05$) of age on fractional sythesis rates. In all subfractions, 15 mo guinea pigs had lower FSR compared to 5mo guinea pigs. There was significantly slower ($p<0.05$) rates of protein synthesis in the cytosolic (Fig 2.9B) and collagen (Fig 2.9D) fractions of the gastrocnemius between 5 mo and 9 mo guinea pigs. * denotes $p<0.05$ compared to 5 mo. # denotes $p<0.05$ compared to 9 mo.

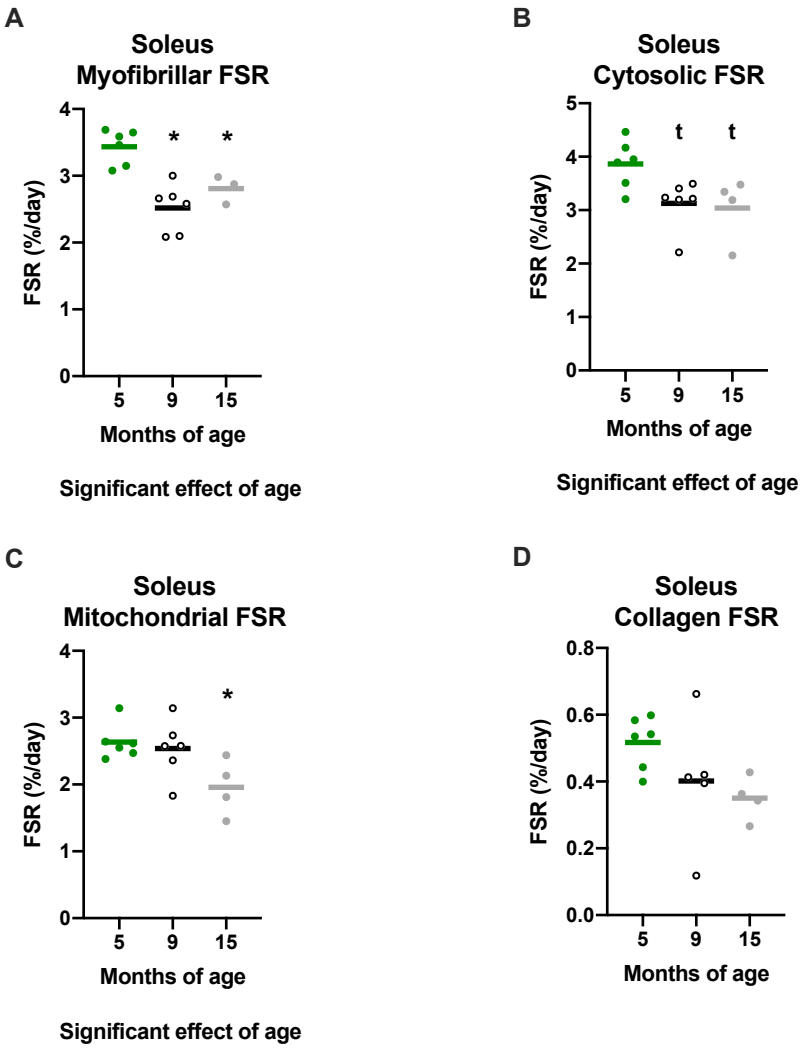

Figure 10: Skeletal muscle protein synthesis rates in the soleus. There was a significant, negative effect ($p<0.05$) of age on fractional sythesis rates in the myofibrillar (Fig 2.10A) mitochondrial (Fig 2.10C) subfractions of the soleus, with 15 mo guinea pigs having significantly slower rates of protein synthesis than 5 mo guinea pigs. Compared to 5 mo guinea pigs, 9 ($p=0.06$) and 15 mo ($p=0.06$) guinea pigs tended to have lower rates of cytosolic protein synthesis (Fig 2.10B). However, there was no effect ($p>0.10$) of age on collagen FSR (Fig 2.10D). * denotes $p<0.05$ compared to 5 mo; t denotes $p<0.10$ compared to 5 mo.

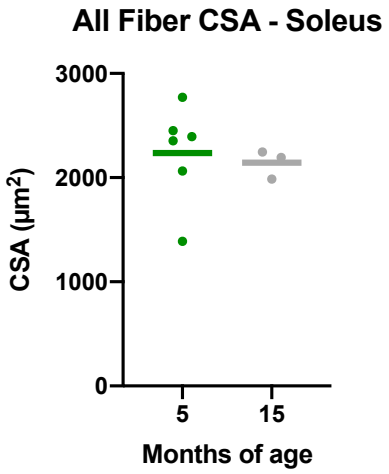

Figure S 2.1: Cross sectional area of soleus myofibers. There was no difference in average myofiber CSA between 5mo and 15mo guinea pigs.

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CHAPTER 3 – NRF2 ACTIVATOR ATTENUATES AGE-RELATED MITOCHONDRIAL DYSFUNCTION IN MALE AND FEMALE DUNKIN-HARTLEY GUINEA PIGS

INTRODUCTION

Aging is the progressive loss in function caused by a decline in physiological integrity[1]. Part of this decline is explained by the impairment in the oxidative capacity of mitochondria, which is responsible for the generation of ATP. Maintaining a high oxidative capacity supports critical cellular processes related to metabolism, growth, and somatic maintenance[2–5]. With age, there is an accumulation of damage to critical cellular components, such as protein and DNA, that leads to tissue-, organ-, and organismal-level dysfunction. This accumulation of damage leads to a decline in health and promotes disease[6], such as diabetes[7], Alzheimer's disease[8], and cancer[9]. Thus, targeting the common characteristics, or hallmarks, of the aging phenotype, should delay and mitigate the progression of disease and promote the healthspan[1].

Mitochondrial dysfunction is a hallmark of aging[1]. Mitochondrial dysfunction impairs cellular function through increased levels of reactive oxygen species (ROS) emission and impaired ability to generate ATP to support cellular function[10,11]. The loss of mitochondrial function has been implicated in a number of diseases. In skeletal muscle, age-related mitochondrial dysfunction is associated with age-related impairments in skeletal muscle function, or sarcopenia[12].

Age is not entirely determinant of mitochondrial function[13,14]. While age is certainly related to maximal oxidative capacity[15–18], factors such as fitness, physical activity, and adiposity have a stronger impact on oxidative capacity [19–21]. Indeed, aging accounts for less than 10% of the observed decline[14]. There appears to be an increase in ROS emission and decrease in ADP sensitivity as well[22–25], however exercise does seem to mitigate those

changes[22] and improve muscle function and overall health[26,27]. Because age is not determinant of mitochondrial function and that mitochondrial function is modifiable, developing interventions to target mitochondrial dysfunction is an important pursuit in extending the healthspan[10,28–30].

Aerobic exercise training is one of the most reliable interventions to improve mitochondrial function of skeletal muscle, regardless of age[26,31,32]. Aerobic exercise stimulates mitochondrial biogenesis and enhances mitochondrial function[33–37] as well as increasing antioxidant capacity[38–41], enhanced aerobic fitness[42–46], improved organismal functional[47,48], and decreased risk of age-related and chronic diseases[49,50]. However, exercise is poorly adhered to with less than half of both American[51] and European[52] populations adhering to physical activity guidelines. Thus, alternatives to exercise that enhance mitochondrial function are necessary in an effort to impart similar health benefits to exercise[53–55].

Nrf2 (nuclear factor erythroid 2-related factor 2) is a transcription factor responsible for the upregulation of cytoprotective genes[56]. Aerobic exercise is a known activator of Nrf2[57]. Nrf2 activation mediates many exercise-related adaptations including mitochondrial biogenesis and antioxidant and anti-inflammatory gene transcription[39,40,58–60]. Moreover, age-related impairments of Nrf2 contribute to impaired adaptation to stress and redox dyshomeostasis[61,62]. Thus, pharmacologically targeting Nrf2 activation is a promising avenue to impart improvements in mitochondrial and organismal function[10].

There are a multitude of phytochemical compounds that have demonstrated efficacy in activating Nrf2 including sulforaphane[63] and Protandim[64–67]. Our lab investigated Protandim, a phytochemical compound, and has found that, *in vitro,* the Nrf2 activator has cytoprotective effects in coronary endothelial cells[67] and cardiomyocytes[66] in the face of oxidative challenges. *In vivo*, Protandim improved proteostatic mechanisms and permitted the mitohormetic, the

notion that an acute mitochondrial stress subsequently improves mitochondrial function[68], adaptations to physical activity in rats[64] and also extended median lifespan in mice[69]. In humans, this Nrf2 activator improved proteostatic maintenance of contractile proteins in older, sedentary adults[70]. So far, though, our lab has only observed Protandim-mediated benefits in male organisms[64,69,70]. More work is necessary to uncover the mechanisms that mediate the improvement of function and longevity from Nrf2 activators, such as improvement in mitochondrial function. Additionally, further investigation and is necessary to discover other Nrf2 activators that improve function in both sexes.

PB125 is a second-generation phytochemical compound that stimulates Nrf2 activation more robustly than other compounds such as Protandim. Our lab sought to determine whether PB125 could enhance mitochondrial respiration in a preclinical model for musculoskeletal aging, the Dunkin-Hartley guinea pig, and prevent any age-related decrements in mitochondrial respiration. To test this, we treated both male and female guinea pigs of both young and older ages for 3 and 10 months, respectively, with a daily dose of PB125. We then measured mitochondrial respiration using high resolution respirometry. We hypothesized that there would be age-related changes in mitochondrial function in guinea pigs, and that treatment with PB125 would improve mitochondrial function and prevent age-related changes in mitochondrial function in both sexes.

METHODS

Husbandry, euthanasia, and tissue acquisition

All procedures were approved by the Colorado State University Institutional Animal Care and Use Committee and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. 28 female and 28 male Dunkin-Hartley guinea pigs were obtained from Charles River Laboratories (Wilmington, MA, USA) at 1- and 4- months of age each for a total of 112 guinea pigs. Animals were maintained at Colorado State University's Laboratory

Animal Resources housing facilities and were monitored daily by veterinary staff. All guinea pigs were singly-housed in solid bottom cages, maintained on a 12-12 hour light-dark cycle, and provided ad libitum access to food and water.

PB125 (Pathways Bioscience, Aurora, CO) is a phytochemical compound comprised of rosemary, ashwagandha, and luteolin powders mixed in a 15:5:2 ratio by mass, respectively[71]. Prior to initiation of treatment, we conducted a pharmacokinetic analysis to by measuring the three active components of PB125 in the plasma at 15, 30, 45, 60, 90, and 120 minutes after dosing of 250, 750, and 1250 PPM. Based on the analysis conducted at the NSC Analytical Pharmacology Core at the University of Texas Health Medical School (San Antonio, Texas, USA) (Figure S3.1), we selected a dosage of 250 PPM, which is approximately 0.8mg/kg of bodyweight. After one month acclimation to housing conditions, male and female guinea pigs in each age group (2 or 5 months) were randomized to receive a daily oral dose of 0.8mg/kg bodyweight of PB125 (Nrf2a) suspended in OraSweet (Perrigo, Dublin, Ireland) or an equivalent volume of OraSweet only (CON). Because the guinea pigs were growing throughout the study, we calculated dosage weekly based on bodyweight. At the time of harvest, the guinea pigs were 5 or 15 months of age (mo) (n=14/age/group/sex). We chose these ages to determine whether Nrf2a could prevent the onset (5 mo) or mitigate the progression (15 mo) of osteoarthritis[72,73] and skeletal muscle dysfunction (Chapter 2). In accordance with the standards of the American Veterinary Medical Association, animals were anesthetized with a mixture of isoflurane and oxygen; thoracic cavities were opened and blood was collected via direct cardiac puncture. Immediately after exsanguination, the anesthetized animals were transferred a chamber filled with carbon dioxide for euthanasia.

Upon euthanasia, the right leg of the guinea pig was promptly removed for the excision of the soleus muscle. A portion of the soleus muscle (~40mg) was harvested and placed in

BIOPS preservation buffer containing 12.5 μM blebbistatin to inhibit muscle contraction. The rest of the soleus was frozen in liquid nitrogen and used for other analyses.

Mitochondrial respirometry

After the soleus was placed in BIOPS, the muscle fibers were prepared for high resolution respirometry as follows. Mechanical permeabilization occurred on ice using forceps to separate the fibers from each other. After mechanical permeabilization, fibers underwent chemical permeabilization for 30 minutes in BIOPS with 12.5 μM blebbistatin and 50 μg/mL saponin. After chemical permeabilization, the fibers were rinsed in BIOPS for 15 minutes. After the rinse, approximately 2.0 mg (wet weight) of muscle fibers were placed in the Oxygraph-2k (O2K) (Oroboros, Innsbruck, Austria) for high resolution respirometry.

High resolution respirometry was performed in duplicate using two different protocols. Both protocols used mitochondrial respiration medium (MiR05, 0.5 mM EGTA, 3 mM $MgCl_2 6H_2O$, 20 mM Taurine, 15 mM Na_2Phosphocreatine, 20 mM Imidazole, 0.5 mM Dithiothreitol, and 50 mM K^+ -MES at pH 7.1).

The first protocol (SUIT 1) was an ADP titration protocol to determine ADP sensitivity (Km) and maximal oxidative capacity (Vmax) under Complex I supported respiration. We measured Complex I supported leak respiration (State $2_{[PGM]}$) with the addition of 10 mM glutamate, 0.5 mM malate, and 5 mM pyruvate. Upon acquisition of State $2_{[PGM]}$, we titrated progressively increased concentrations of ADP from 0.1mM, 0.175mM, 0.25mM, 1mM, 2mM, 4mM, 8mM, 12mM, 20mM, to 24mM (State $3_{[PGM]}$), awaiting steady-state oxygen flux prior to adding the subsequent titration to determine Complex I linked ADP Vmax and apparent Km (i.e. ADP sensitivity). After the ADP titration was completed, we added 5 mM cytochrome C to test mitochondrial membrane integrity. We excluded values that had a cytochrome C control factor of greater than 0.30 based on the threshold in which a relationship between the cytochrome C control factor and respiration existed (Figure S3.2). Based on this criterion, we excluded no trials in SUIT 1. After cytochrome C addition, we added 10 mM succinate to acquire maximal

Complex I and II supported coupled respiration (State $3_{[PGM + S]}$). We then added 0.5 µM FCCP sequentially until there was no increase in respiration to determine the capacity of the electron transport system to consume oxygen, or maximal uncoupled respiration ($ETS_{[CI-CIV]}$). Finally, we added 5 µM rotenone to measure maximal uncoupled respiration with the inhibition of Complex I ($ETS_{[CII-CIV]}$). We then added 2.5 µM Antimycin A to measure residual oxygen consumption (ROX).

The second protocol (SUIT 2) measured oxygen consumption while simultaneously measuring ROS production by using the fluorometer attachment of the O2K[74]. We added 10 µM Amplex Red, 1 U/ml horseradish peroxidase, and 5 U/ml superoxide dismutase. We then measured fatty acid supported leak respiration by adding 10 mM glutamate, 0.5 mM malate, 5 mM pyruvate, and 0.2 mM octanoylcarnitine (State $2_{[PGM + Oct]}$) and 10 mM succinate (State $2_{[PGM + Oct + S]}$). After stimulating maximal leak respiration, we added submaximal boluses of ADP (0.5mM: (State $3_{[Sub + 0.5D]}$) and 1mM: State $3_{[Sub + 1.0D]}$), followed by a saturating bolus of ADP (6.0mM: State $3_{[Sub + 6.0D]}$). We added 5 mM cytochrome C to test mitochondrial membrane integrity and excluded 9 trials that had a Cytochrome C control factor of greater than 0.30 based on (Figures S3.2B and S3.2C). We then added 5 µM rotenone to determine maximal coupled respiration in the absence of Complex I (State $3_{[Sub + D - CI]}$) followed by sequential titrations of 0.5 µM FCCP until respiration no longer increased to determine maximal fatty acid supported uncoupled respiration ($ETS_{[Sub + D – CI]}$). and added 2.5 µM antimycin A to measure ROX. The respiratory control ratio (RCR: State 3/State 2), which is an index of mitochondrial efficiency, phosphorylation control ratio (PCR: State 3/$ETS_{[CI-CIV]}$), substrate control ratio (SCR: State $3_{[PGM]}$/State $3_{[PGM + S]}$), and leak control ratio (LCR: State $2_{[PGM]}$/State $3_{[PGM + S]}$) were also evaluated.

Statistics

In line with best practices, technical replicates were averaged. The variability between technical replicates was 18%, which is standard according to the literature[75]. Apparent Km and Vmax values were determined using Michaelis-Menten kinetics in Prism 8.0 (La Jolla, California, USA). Three-way ANOVAs were used to measure the interaction of age and sex with treatment. Post-hoc analyses were performed using Tukey's HSD post-hoc test. To determine the effect of Nrf2a on age-related changes in mitochondrial respiration, when a significant effect of age was detected, a one-way ANOVA with a Dunnett's post-hoc test comparing 15mo treated and untreated guinea pigs to 5mo untreated guinea pigs. Because this study was a secondary outcome to a larger study with a different primary outcome, we did not design this study to be powered to detect differences in mitochondrial respiration at a p-value < 0.05. We nevertheless set statistical significance *a priori* at $p<0.05$. However, we also report differences with $p<0.10$ as non-significant differences to highlight potential directions for future studies. Data are presented as mean +/- SEM. All statistics were performed in Prism 8.0 (La Jolla, California, USA).

RESULTS

The effects of age and Nrf2 activator treatment on ADP kinetics in 5mo and 15mo guinea pigs

There was an age-related increase in ADP Vmax in ($p=0.05$) (Figure 3.1A). There was a significant effect of sex, with mitochondria of female guinea pigs having lower ADP Vmax values compared to males ($p=0.001$). Treatment with the Nrf2 activator (Nrf2a) had a main effect of increasing Complex I supported ADP Vmax. Post-hoc comparisons suggest the effect of Nrf2a mediated improvement of ADP Vmax occurred predominantly in young female guinea pigs $p=0.05$).

Despite an age-related increase in ADP Vmax, there was no effect of age on the apparent Km of ADP (Figure 3.1B). There were also no differences in Km between sexes. However, Nrf2a did significantly increase the apparent Km ($p<0.01$) indicating a decrease in

ADP sensitivity. There was non-significant interaction between sex and Nrf2a treatment (p=0.09), indicating the lower ADP sensitivity to be only in male guinea pigs.

Sex and age differences on mitochondrial respiration

There were several sex differences in mitochondrial respiration observed in the Dunkin-Hartley guinea pigs. Maximal coupled (State $3_{[PGM+S]}$) (Figure 3.2A) and uncoupled ($ETS_{[CI-CIV]}$) (Figure 3.2B) respiration was significantly greater in males (both $p<0.01$). The addition of rotenone also demonstrated sex differences in the absence of Complex I during coupled (State $3_{[Sub + D - CI]}$) (Figure 3.4A) uncoupled respiration ($ETS_{[Sub, CII-CIV]}$) (Figure 3.2C) (both $p<0.01$). Further, males had greater fatty acid supported Complex I-IV supported respiration at sub-saturating (State $3_{[Sub + 1.0D]}$) and saturating (State $3_{[Sub + 6.0D]}$) concentrations of ADP ($p=0.029$ and $p=0.030$, respectively) (Figure 3.3).

Age had a negative effect on several aspects of mitochondrial function in both male and female guinea pigs. 15mo male and female guinea pigs had lower coupled (State $3_{[PGM+S]}$) (Figure 3.2A) and uncoupled ($ETS_{[CI-CIV]}$) respiration (Figure 3.2B; both $p<0.01$). When Complex I was inhibited, there were also age-related declines in coupled (State $3_{[Sub + D - CI]}$) (Figure 3.4A) and uncoupled ($ETS_{[Sub, CII-CIV]}$) (Figures 3.2C and 3.4B) respiration (both $p<0.01$). Age had no effect on fatty acid oxidation supported respiration at sub-saturating levels (State $3_{[Sub + 0.5D]}$and State $3_{[Sub + 1.0D]}$) of ADP (Figures 3.3A and 3.3B), though 15mo guinea pigs had lower fatty acid oxidation supported respiration at a saturating level (State $3_{[Sub + 6.0D]}$) of ADP (Figure 3.3C; $p=0.058$).

There were other components of mitochondrial respiration that changed with age. The leak control ratio (LCR), phosphorylation control ratio (PCR), and substrate control ratio (SCR) increased with age ($p<0.01$ for each) (Figures 3.5A-C). Respiratory control ratio (RCR), however, decreased as a result of age ($p=0.01$) (Figure 3.5D).

The effect of Nrf2a treatment on mitochondrial respiration

Nrf2a treatment improved several components of mitochondrial respiration in both young and old guinea pigs, both male and female. Nrf2a non-significantly increased coupled respiration (State $3_{[PGM+S]}$) in both male and female guinea pigs (p=0.10) (Figure 3.2A), and significantly enhanced electron transport system (ETS) capacity ($ETS_{[CI-CIV]}$) (Figure 3.2B; p=0.04). However, Nrf2a did not improve ETS capacity in the absence of Complex I ($ETS_{[CII-CIV]}$) (Figure 3.2C).

Nrf2a non-significantly improved fatty acid supported Complexes I through IV coupled respiration both at sub-saturating (State $3_{[Sub + 1.0D]}$) and saturating (State $3_{[Sub + 6.0D]}$) amounts of ADP (Figures 3.3B and C; p=0.09, p=0.10, respectively). Nrf2a also improved fatty acid supported Complex II through IV coupled respiration (State $3_{[Sub + D - CI]}$) (Figure 3.4A; p=0.04), but did not affect uncoupled respiration ($ETS_{[Sub + D - CI]}$) (Figure 3.4B). Despite these general improvements in mitochondrial respiration, there was no main effect of Nrf2a on LCR, PCR, SCR, or RCR (Figure 3.5).

The effect of Nrf2a treatment on the age-related changes in mitochondrial respiration and ADP kinetics

For any age-related declines in mitochondrial respiration, we tested whether Nrf2a prevented that decline. In the case of a non-significant difference between 5mo control guinea pigs and 15mo treated guinea pigs while there is a significant difference between 5mo control guinea and 15mo control guinea pigs would indicate that Nrf2a prevented an age-related change in mitochondrial function. While there was a reported main effect of age on ADP Vmax in the Three-Way ANOVA, there was a non-significant increase in ADP Vmax between 5mo and 15mo guinea pigs (p=0.11) in the subsequent one-way ANOVA analysis (Figure 3.6A). Treated 15mo guinea pigs, however, had a significantly higher ADP Vmax compared to 5mo guinea pigs (p<0.01) (Figure 3.6A). Interestingly, this effect, though, was only observed in male guinea pigs (p=0.02) (Figure 3.6B). While ADP Vmax tended to increase with age, there was a significant

(p=0.02) decrease in maximal coupled respiration (State $3_{[CI\text{-}CIV]}$) between 5mo and 15mo guinea pigs (Figure 3.6C). Nrf2a, however, prevented that age-related decline (Figure 3.6C; p=0.53). Maximal uncoupled respiration ($ETS_{[CI\text{-}CIV]}$) also declined with age (Figure 3.6E), though further interrogation revealed that there was only age-related decline in female guinea pigs (p=0.05) in $ETS_{[CI\text{-}CIV]}$ while Nrf2a prevented that decline (p=0.29) (Figure 3.6F). Interestingly, in the absence of Complex I, Nrf2a had no effect on uncoupled respiration $ETS_{[CI\text{-}CIV]}$ and failed to recover CII-CIV_E capacity in either male (p=0.03) nor female (p<0.01) guinea pigs (Figures 3.6G and 3.6H). Additionally, Nrf2a did not affect fatty acid supported respiration (State $3_{[Sub + 6.0D]}$) (Figures 3.7A and 3.7B). Moreover, in the absence of Complex I, Nrf2a did not appear to prevent the age related decline in either fatty acid supported coupled (State $3_{[Sub + D - CI]}$) or uncoupled ($ETS_{[Sub + D - CI]}$) respiration (Figures 3.7C-F).

Nrf2a tended to prevent the age-related increase (p=0.09) in LCR, though there were no sex-specific effects observed (Figures 3.8A and B). Nrf2a had no effect on the age-related increase in PCR observed in females (p<0.01) (Figure 3.8D). Nrf2a did prevent the age-related increase in SCR in female guinea pigs (p=0.18) (Figure 3.8F). Nrf2a also tended to prevent the observed age-related decline in RCR. However, this appears to have only occurred in males where there was a significant difference (p=0.04) between 5mo and 15mo guinea pigs (Figure 3.8G). Treated 15mo male guinea pigs were not significantly different (p=0.15) compared to 5mo (Figure 3.8H). There was no effect of either age or Nrf2a on RCR in females (Figure 3.8H).

Despite the numerous effects of Nrf2a on mitochondrial respiration, there were no effects of Nrf2a on ROS emission (Figure 3.9).

DISCUSSION

Mitochondrial dysfunction contributes to many chronic diseases, including the skeletal muscle dysfunction, or sarcopenia, that is part of age-related musculoskeletal decline. Here we describe sex differences in skeletal muscle mitochondrial respiration in 5mo and 15mo Dunkin

Hartley guinea pigs. Several components of mitochondrial respiration were lower in 15mo guinea pigs compared to 5mo. Nrf2 activator treatment (Nrf2a) improved mitochondrial respiration in both male and female guinea pigs and attenuated the decline in mitochondrial function in these guinea pigs. Altogether, our results support our hypothesis that Dunkin Hartley guinea pigs experience age-related declines in mitochondrial respiration just as humans and occur concomitantly with other changes in skeletal muscle (Chapter 2). Moreover, the data support our hypothesis that Nrf2a would also attenuate age-related declines in mitochondrial respiration. In addition, some of these improvements occurred in a sex-specific manner. Collectively, these results provide insight into a potential mechanism of how Nrf2 activator treatment may improve musculoskeletal function and extend healthspan.

Differences between skeletal muscle mitochondrial respiration in male and female Hartley guinea pigs

Female guinea pigs had consistently lower rates of mitochondrial respiration compared to males at both 5 and 15 months of age. Female guinea pigs had lower rates of Complex I supported Vmax (Figure 3.1A). Additionally, females had lower maximal coupled (State $3_{[PGM+S]}$) and uncoupled ($ETS_{[CI-CIV]}$) respiration (Figures 3.2A and 3.2B). Mitochondrial respiration from female skeletal muscle was still lower compared to males in the absence of Complex I ($ETS_{[CII-CIV]}$) (Figure 3.2C). Interestingly, there were no sex differences in fatty acid oxidation supported mitochondrial respiration at lower concentrations of ADP (Figure 3.3A). However, at greater concentrations of ADP (Figures 3.3B and 3.3C), mitochondrial respiration was, again, lower in females. Altogether, these data that sex differences are indicative the female guinea pigs have overall lower oxidative capacity and that impairments in respiration are not limited to one complex. However, this does not implicate lower mitochondrial function *per se*, and could instead be reflective of lower mitochondrial density in skeletal muscle that could be the source of lower respiration values. In humans, however, it is equivocal whether or not mitochondrial

respiration differs between men and women[76], though mitochondria of women may have higher rates of respiration than mitochondria of men[77]. More work is necessary to determine the underlying causes of lower respiration in female guinea pigs compared to males.

The age-related decline in mitochondrial respiration in Dunkin-Hartley guinea pigs

The decline in mitochondrial oxidative capacity alongside age is well documented[16,17]. While chronological age may not have a direct role in decrease oxidative capacity, factors that generally correlate with age, such as inactivity and adiposity, have a significant effect on mitochondrial function[14]. Thus, determining whether Dunkin-Hartley guinea pigs have age-related declines in mitochondrial function was also of interest. There were several differences in mitochondrial respiration between 5mo and 15mo guinea pigs. Consistent with humans[14,16], we observed an age-related decline in maximal coupled (State $3_{[PGM+S]}$) (Figure 3.2A) and uncoupled ($ETS_{[CI-CIV]}$) (Figure 3.2B) mitochondrial respiration in guinea pigs. Uncoupled respiration with Complex I inhibited ($ETS_{[CII-CIV]}$) (Figure 3.2C) also declined with age, suggesting that the age does not just affect Complex I capacity in these guinea pigs, but the entire electron transport system (i.e. Complex I – Complex IV). In contrast to humans, Complex I supported ADP Vmax was higher in both aged male and female guinea pigs compared to their younger counterparts (Figure 3.1A), despite no change in ADP sensitivity (3.1B). In humans there is no age-related documented change in ADP Vmax, but a decrease in ADP sensitivity[22]. However, it should be noted that most measurements in mitochondrial respiration in humans are made in muscles with mixed fiber types instead of a muscle (i.e. soleus) comprised predominantly of type I myofibers.

Despite the observed age differences in respiration with Complex I supported substrates, there were no age-related differences in fatty acid oxidation supported coupled respiration (Figures 3.3A – 3.3C). However, the inhibition of Complex I revealed an age-related deficiency in Complex II coupled respiration (Figure 3.4A) in older guinea pigs. The addition of

protonophore FCCP suggested that this deficiency is not likely a consequence of ATP synthase (i.e. Complex V). We also observed an age-related increase in the substrate control ratio (SCR) (Figure 3.5C), which reflects the contribution of Complex II to mitochondrial respiration. The increase in SCR observed with age signifies a decrease in relative contribution of Complex II. While there is no evidence that SCR declines with age in humans[17], there is a reported decline in Complex II uncoupled respiration with age in humans[14,16].

The leak control ratio (LCR) increased with age in both male and female guinea pigs, which suggests that there are age-related intrinsic defects in the electron transport system perhaps related to membrane or protein quality[78]. However, there is no evidence that LCR changes with age in healthy humans[14]. The phosphorylation control ratio (PCR) is indicative of a reserve capacity of the electron transport system relative to the capacity of ATP synthase[79]. The PCR increased with age in guinea pigs, suggesting a decrease in reserve capacity. In humans, PCR does not appear to change with age[14]. Interestingly, both acute and chronic exercise generally increase PCR[79–81]. However, the effect of exercise on PCR may be more indicative mitochondrial remodeling (as opposed to mitochondrial network expansion) instead of an index of reserve capacity[79].

The respiratory control ratio (RCR) is reflective of mitochondria efficiency in terms of the proportion of energy that is wasted. This efficiency is measured by the magnitude of increase in respiration above leak respiration[82]. Guinea pigs experienced an age-related decline in RCR (Figure 3.5D), which suggests an age-related decline in mitochondrial efficiency. However, this is only one measure of mitochondrial efficiency. Another metric of mitochondrial efficiency is the amount of ATP generated by molecule of oxygen consumed (i.e. the P/O ratio), which we did not measure. Importantly, these two indices, in some cases, may not corroborate each other (i.e. contradict each other)[83]. Thus, it is necessary to further interrogate age-related changes in

other measures of mitochondrial efficiency, particularly as it pertains to ATP produced per oxygen consumed.

The effect of Nrf2a on mitochondrial function

Treatment with a Nrf2 activator improved mitochondrial respiration in both young and old, male and female guinea pigs. Nrf2a increased ADP Vmax in both males and females (Figure 3.1A). The significant three-way interaction among the independent variables (age, sex, and treatments) suggests that this positive effect on ADP Vmax was likely observed in old males and young females only. Nrf2a increased apparent Km suggesting there was a decrease in ADP sensitivity (Figure 3.2A). However, this interpretation should be made with caution. Theoretically, ADP sensitivity could decrease in the presence of an increase in ADP Vmax even if respiration at lower concentrations of ADP remained the same. Indeed, this is what appears to have occurred (Figure S3.4). Regardless, if Nrf2a did reduce ADP sensitivity this potentially represents one mechanism in which Nrf2a hormetically improves mitochondrial function. Reduced ADP sensitivity as a mitohormetic mechanism to improve mitochondrial function is not without precedent. Acute exercise, a potent stimulator of mitobiogenesis[35], acutely reduces ADP sensitivity[84], which is associated with subsequent improvements in overall mitochondrial function.

In addition to increased Vmax, Nrf2a also increased Complex I and II coupled and uncoupled respiration (Figures 3.2A and 3.2B) in males and female. However, the addition of rotenone, which inhibits Complex I abrogated the positive effect of Nrf2a on mitochondrial respiration (Figure 3.3C), which suggests that Nrf2a improvements are related to improved Complex I capacity. This is further supported by the fact that ADP Vmax was measured under Complex I, not II, supported respiration (Figure 3.1A). Nrf2a also increased fatty acid oxidation supported coupled respiration at sub-saturating (1.0mM) and saturating (16.0mM) amounts of ATP (Figures 3.3B and 3.3C), however, consistent with observations during the Complex I

supported ADP titrations, the positive effect of Nrf2a on respiration was not observed at lower concentrations of ADP (0.5mM) (Figure 3.3A). Nrf2a also increased fatty acid oxidation supported Complex II coupled respiration (Figure 3.4A), which seems to contradict the lack of effect of Nrf2a on Complex II supported uncoupled respiration (Figure 3.2C). However, it is important to note that these conditions are not the same and may reflect improvements in fatty acid oxidation[85]. Additionally, subsequent addition of the protonophore FCCP led to fatty acid oxidation supported Complex II uncoupled respiration which, consistent to the previous observation, was not improved by Nrf2a (Figure 3.4B).

While there were several improvements in mitochondrial respiration, there were no significant effects of Nrf2a on the control ratios. However, it is worth noting potential sex-specific effects of Nrf2a on the phosphorylation (PCR) and respiratory (RCR) control ratios. Nrf2a consistently, but non-significantly, decreased PCR in both young and old male guinea pigs, but had mixed effects in female guinea pigs. This suggests that, in male guinea pigs, Nrf2a increased mitochondrial reserve capacity. This increased reserve capacity may reflect enhancements in Complex I – IV capacity in the absence of improvements in ATP synthase. Conversely, Nrf2a non-significantly increased RCR in young and old female guinea pigs, which may reflect enhanced mitochondrial efficiency in terms of decreasing energy wasted during electron transfer. However, it is important to exercise caution when interpreting changes in mitochondrial efficiency as other metrics of mitochondrial efficiency may demonstrate otherwise[85]. One contributing factor to mitochondrial efficiency is supercomplex formation. Supercomplexes, or respirasomes, are supramolecular assemblies of mitochondrial complexes that are stabilized by phospholipids such as cardiolipin[86]. Supercomplexes offer a kinetic advantage and improves electron transfer thereby increasing respiration[87], and decreasing ROS emission[88]. The decreased leak relative to maximal ETS capacity (RCR) we observed may reflect enhanced supercomplex formation, which tends to improve mitochondrial efficiency[89,90].

While it's unclear whether Nrf2 activation has any direct effect on supercomplex integrity, Nrf2a may improve antioxidant capacity which may prevent oxidation of components to supercomplexes, such as cardiolipin.

The effect of Nrf2a on the attenuation of age-related changes in mitochondrial respiration

While the effects of Nrf2a on mitochondrial respiration have already been described here, it is important to determine whether Nrf2a prevented or attenuated age-related changes in mitochondrial respiration. Nrf2a had several moderating effects on the age-related changes in mitochondrial respiration. While we observed a main effect of age on ADP Vmax, Nrf2a increased ADP Vmax in 15mo males compared 5mo counterparts. Maximal coupled respiration (State $3_{[PGM+S]}$) significantly decreased with age (Figure 3.6C), predominantly in females (Figure 3.6D). However, Nrf2a, attenuated this age-related decline in females (Figure 3.6D). Additionally, Nrf2a blunted the age-related decline in uncoupled ($ETS_{[CI-CIV]}$) respiration (Figure 3.6E). Interestingly, Nrf2a failed to prevent the decline in uncoupled respiration with Complex I inhibited ($ETS_{[CII-CIV]}$) (Figure 3.6G). Moreover, Nrf2a also failed to attenuate the age-related decline in coupled respiration with Complex I inhibited (State $3_{[Sub + D - CI]}$) (Figures 3.7C and 3.7D). Together, these results suggest that Nrf2a improves mitochondrial function by improving Complex I related metabolism. Nrf2a also mitigated the age-related increase in the Leak Control Ratio (LCR; Figure 3.8A) as well as the increase in substrate control ratio (SCR), which was predominantly observed in female guinea pigs (Figures 3.8E and 3.8F), suggesting that Nrf2a improved the relative contribution of Complex II to coupled respiration. Given that Nrf2a attenuated the decline in Complex I respiration, it's likely that the attenuated increase in SCR is a result of improved Complex II function. However, this is somewhat contradictory to our other results that suggest Nrf2a did not prevent the age-related decline Complex II supported respiration (Figures 3.7C and 3.7D).

Nrf2a also attenuated the age-related decline in mitochondrial efficiency (i.e. RCR) (Figure 3.7G), though this attenuation was only observed in males (Figure 3.7H). This could be

a consequence of a decrease in LEAK respiration or an improvement in maximal ETS capacity. However, there were no age-related changes in LEAK respiration (Figure S3.3), suggesting that the maintenance of mitochondrial efficiency are related to the observed improvement in mitochondrial capacity (Figure 3.6E). Improved efficiency could be related to attenuating the loss of Complex I function, to general improvements in mitochondrial structure such as improved mitochondrial membrane integrity[91] or supercomplexes through antioxidant upregulation and protection from oxidative damage[60,86,88,92,93]. However, further work should be done to further understand how mitochondria in guinea pig skeletal muscle change with age and how Nrf2a may modulate those changes, particularly with regard to Complex I function.

The attenuation of age-related changes in mitochondrial function could have broader implications in overall organismal health. Protandim is another phytochemical Nrf2 activator that functions similarly to the Nrf2 activator used in this study. The Interventions Testing Program assessed the effects of Protandim on longevity in heterogenous mice and revealed that the Nrf2 activator enhanced median lifespan in males, but not females. Given that mitochondrial dysfunction is a hallmark of aging, our results suggest that Nrf2 activators may contribute to median lifespan extension through improvements in muscle mitochondrial respiration. Importantly, our data also support sex-specific effects of Nrf2a on mitochondrial respiration as well as the attenuation of age-related declines. Further research should investigate the mechanisms underlying our sex-specific observations including how sex hormones may mediate the effect of Nrf2 activator treatment[94–96].

Proposed mechanism of action

Collectively, the results of this study suggest that Nrf2a improved mitochondrial function and attenuated age-related declines in mitochondrial function through several mechanisms. Nrf2a enhances endogenous antioxidant capacity[63,97], which could explain improvements in Complex I supported respiration. Given that pyruvate dehydrogenase is a redox sensitive

enzyme that supplies NADH to Complex I[98], it is worth considering in future directions whether or not Nrf2a treatment restored age-related declines in redox balance in skeletal muscle[61,99,100] as other interventions using Nrf2 activators have done[63,101]. Nrf2a could also have protected mitochondrial structures, such as inner mitochondrial membrane and supercomplexes, from age-related oxidation[102], which might explain improved mitochondrial function. Also, we have previously reported that Nrf2 activators enhance mitochondrial biogenesis[64] and others have implicated the importance of Nrf2 in regulating mitochondrial biogenesis[58]. Because we did not control for mitochondrial content, it is possible that the improvements in skeletal muscle respiration are, in part, a consequence of greater mitochondrial density or greater complex-specific protein content (e.g. Complex I) elicited by Nrf2a stimulated mitochondrial biogenesis. Thus, further research is necessary to account for mitochondrial content as well the maintenance and turnover of mitochondrial proteins as potential mechanisms that mediated the improvements in mitochondrial respiration.

It is also unclear whether improvements in mitochondrial respiration are a consequence of direct Nrf2 activation caused by Nrf2a (and thus increased transcription of antioxidant and anti-inflammatory genes as well as upregulation to genes related to mitochondrial biogenesis) thereby protecting mitochondria from oxidative damage; or if there are other mechanisms, such as mitohormesis[68,103], in which Nrf2a directly alters mitochondrial function and thus elicits mitochondrial adaptation. For example, we reported that Nrf2a decreased ADP sensitivity (Figure 3.1B). Similarly, acute exercise, which is a potent stimulus for beneficial mitochondrial adaptations[10], causes ADP sensitivity to temporarily decrease, which leads to an increase in ROS emission[84]. This transient increase in ROS may be one of the underlying mitohormetic mechanisms in which exercise enhances mitochondrial function and stimulates mitochondrial biogenesis[10,38,104]. However, because our study focused on the long-term effects of Nrf2a, it is

unclear whether Nrf2a acutely changes ADP sensitivity and could similarly exert a mitohormetic effect as aerobic exercise. Future studies should look toward the acute effects of Nrf2a including its acute effects on mitochondrial function, particularly ADP sensitivity.

Limitations

There are several strengths in this study. The use of high resolution respirometry allowed in-depth interrogation of mitochondrial respiration. The use of two age groups also allowed us to discern if Nrf2a could mitigate age-related changes in mitochondrial function and whether or not age influenced the effect of Nrf2a. We also detected a significant sex differences in the response to Nrf2a. However, it is clear that we were not adequately powered to detect statistically significant differences. Given the calculated effect size for treatment on several components of mitochondrial respiration, we would need an additional two guinea pigs per group to be adequately powered to detect a statistically significant effect (G Power, Heinrich-Heine-Universität Düsseldorf). Further, it must be noted that the concentrations of substrates (e.g. ADP, pyruvate, glutamate, etc.) are well above concentrations observed *in vivo*. Thus, it is difficult to ascertain if differences at high concentrations of substrates are physiologically relevant when *in vivo* concentrations are much lower. For example, *in vivo* concentrations of ADP at rest are below 50 μM and at exercise may range from 0.2 mM – 1.1 mM[105]. However, it is worth noting that the capacity of mitochondrial respiration at non-physiologic concentrations are still related to physical function in humans. For example, mitochondrial respiration with ADP concentrations of up to 2mM reveal a relationship between mitochondrial function and physical function such as gait speed and grip strength[15]. Thus, despite interrogating mitochondrial function at supersaturating concentrations of substrate, the differences observed in this study are still relevant to overall organismal function.

Summary

Altogether, these data describe sex and age-related differences in skeletal muscle mitochondrial respiration of the Dunkin-Hartley guinea pig which may underly the age-related changes in skeletal muscle function (Chapter 2). Moreover, we describe the beneficial effects of Nrf2a on mitochondrial respiration and its attenuating effect on age-related declines in mitochondrial respiration. Importantly, we also describe sex-specific and age-specific effects of Nrf2a on mitochondrial function which should be further investigated to understand the mechanisms in which impart improvements in function. Given the important role mitochondrial function in overall cellular function, further research should assess whether improvements in mitochondrial function translate to improvements in cellular and organismal function.

FIGURES

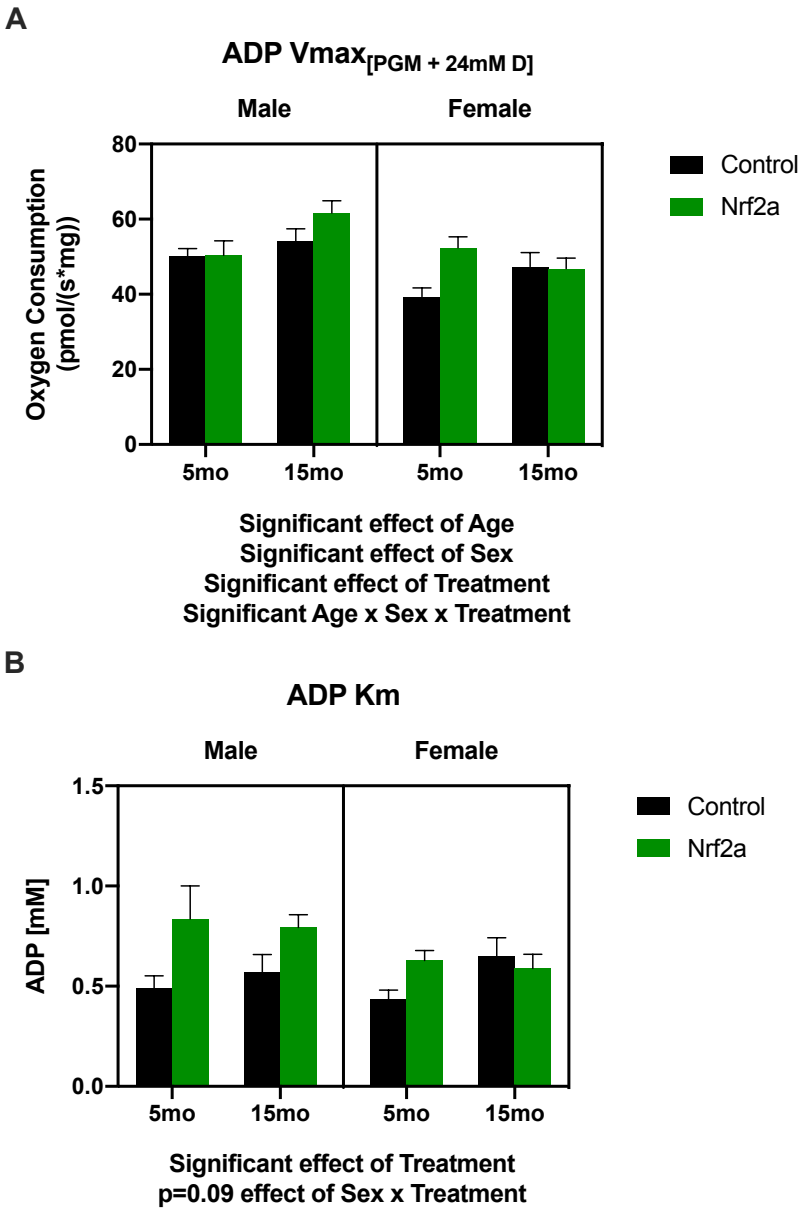

Figure 3.1: ADP kinetics of skeletal muscle mitochondria. There was a significant effect of Age, Sex, and Treatment on ADP Vmax as well as a significant, three-way interaction (all $p<0.05$) (Figure 3.1A), which indicates ADP Vmax in males was greater in females, 15mo guinea pigs had a higher Vmax than 5mo guinea pigs, and that Nrf2a increased ADP Vmax. However, this Nrf2a-mediated improvement in ADP Vmax occurred in 15mo male and 5mo female guinea pigs. Treatment increased apparent ADP Km (i.e. decreased sensitivity) ($p<0.05$). (Figure 3.1B). However, this effect was observed mostly in male guinea pigs as indicated by the non-significant interaction between Sex and Treatment ($p=0.09$).

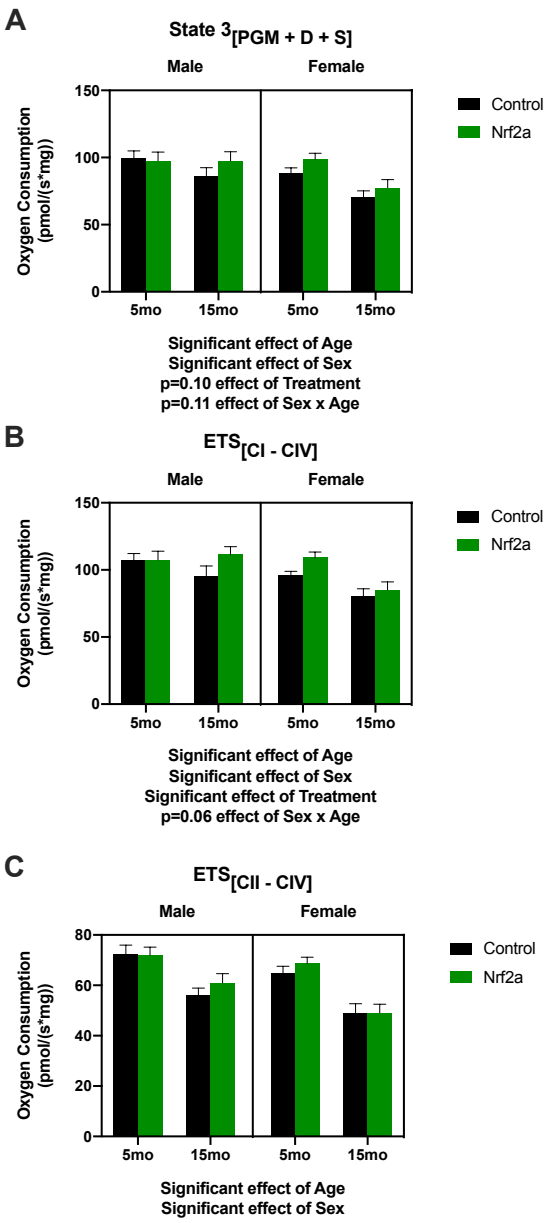

Figure 3.2: Age- sex- and treatment-related differences in mitochondrial respiration. Male guinea pigs had greater State $3_{[PGM + S]}$ respiration than females. There was also an age-related decline, though a non-significant interaction between Sex and Age suggests the decline may have occurred in females (p=0.11). Nrf2a non-significantly increased State $3_{[PGM + S]}$ in both males and females (p=0.10) (Fig 3.2A). Male guinea pigs had greater $ETS_{[CI-CIV]}$ respiration than females. There was also an age-related decline, though this may have occurred only in females (Sex x Age effect p=0.06). Nrf2a increased $ETS_{[CI-CIV]}$ in both males and females ($p<0.05$) (Fig 3.2B). Female guinea pigs had lower $ETS_{[CII-CIV]}$ than male guinea pigs ($p<0.05$). There was a significant age-related decline in $ETS_{[CII-CIV]}$ ($p<0.05$), but Nrf2a had no effect on $ETS_{[CII-CIV]}$ (Fig 3.2C).

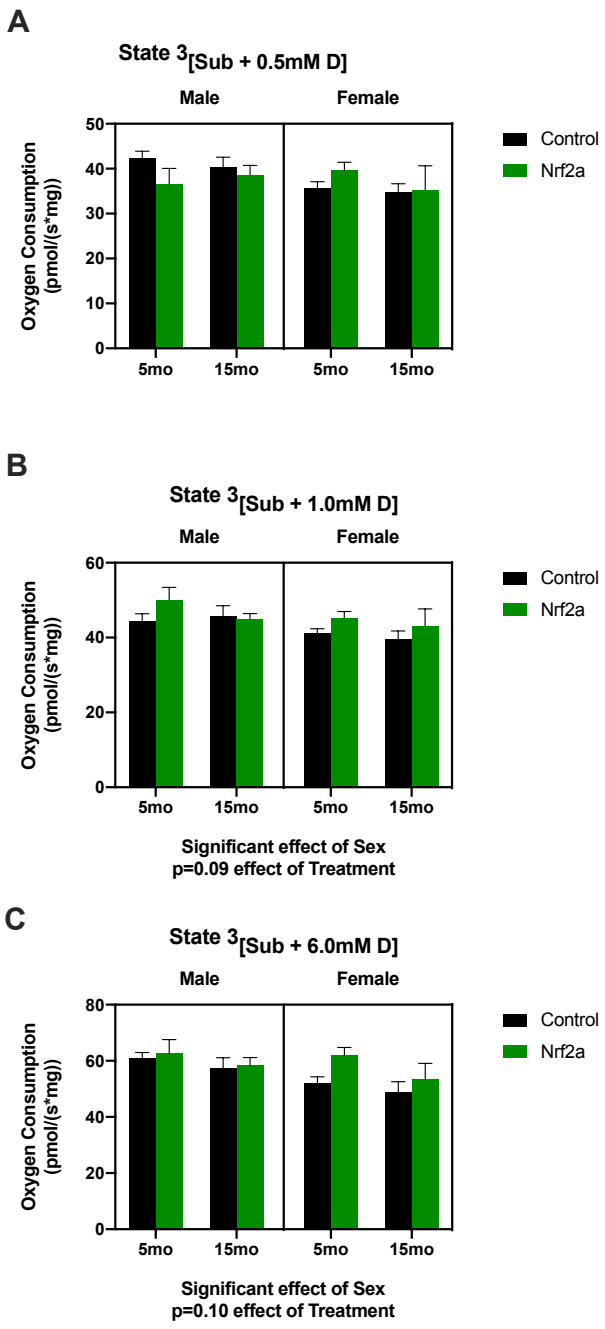

Figure 3.3: Age- sex- and treatment-related differences in fatty-acid supported respiration at sub-saturating and saturating doses of ADP. There were no differences in State $3_{[Sub + 0.5D]}$ among any groups (Fig 3.3A). However, male guinea pigs had greater State $3_{[Sub + 1.0D]}$ than females and Nrf2a had a non-significant but positive effect on State $3_{[Sub + 1.0D]}$ (p=0.09) (Fig 3.3B). These observations continued at a saturating dose of ADP (State $3_{[Sub + 6.0D]}$) with male guinea pigs have greater respiration and Nrf2a have a non-significant, positive effect (p=0.10) (Fig 3.3C)

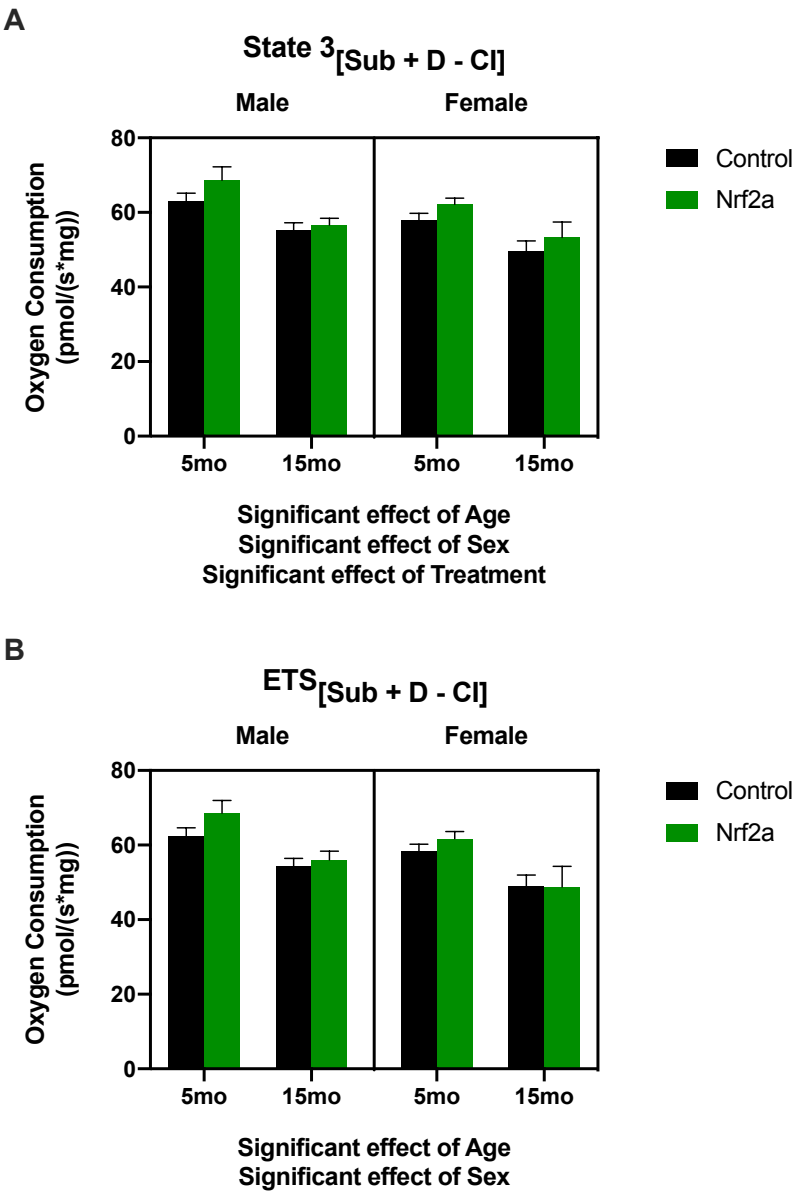

Figure 3.4: Age- sex- and treatment-related differences in fatty-acid supported coupled and uncoupled respiration with inhibition of Complex I. Male guinea pigs had greater State $3_{[Sub + D - Cl]}$ compared to female guinea pigs. Further, there was an age-related decline in State $3_{[Sub + D - Cl]}$. Additionally, Nrf2a increased State $3_{[Sub + D - Cl]}$ (Fig 3.4A). However, uncoupling respiration abrogated the positive effect of Nrf2a while the significant effects of age and sex remained (Fig 3.4B).

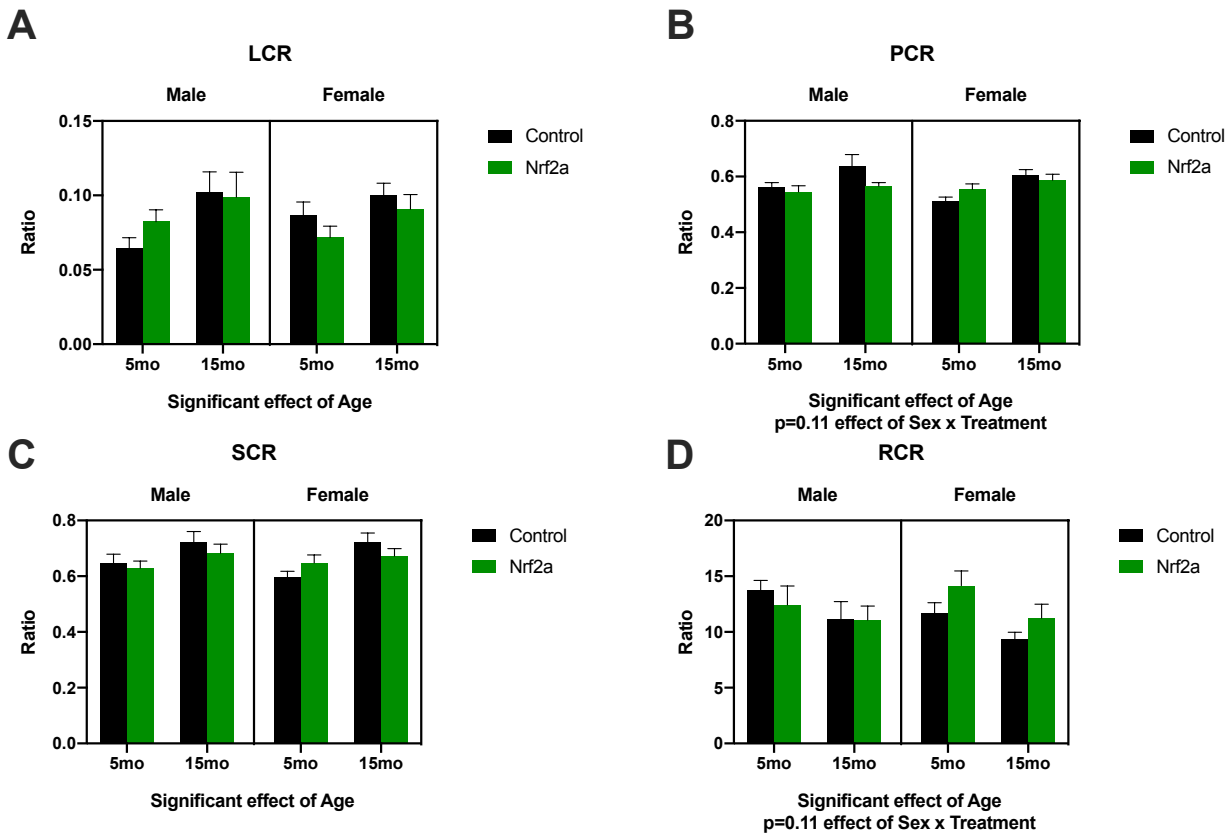

Figure 3.5: Mitochondrial control ratios in Dunkin Hartley guinea pigs. There was a significant age-related increase in the Leak Control Ratio (LCR) (Fig 3.5A), Phosphorylation Control Ratio (PCR) (Fig 3.5B), and Substrate Control Ratio (SCR) (Fig 3.5C). There was an age-related decline in the Respiratory Control Ratio (RCR) (Fig 3.5D). There was a non-significant interaction between Sex and Treatment in both PCR and RCR (Figs 3.5B & 3.6D) (p=0.11).

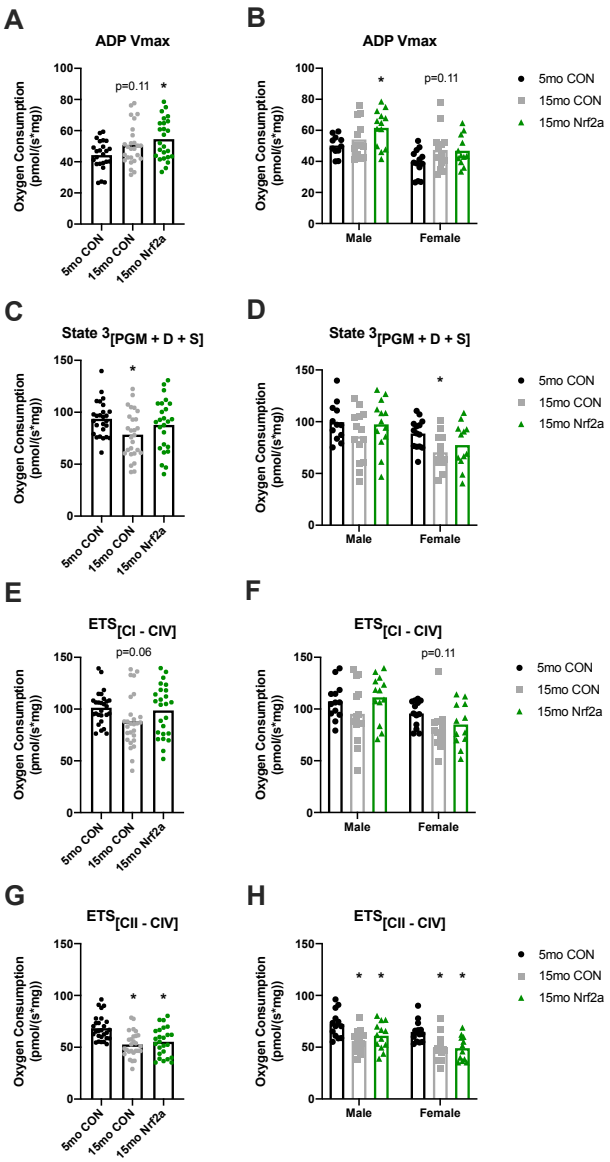

Figure 3.6: Nrf2 moderates age-related changes in mitochondrial respiration in male and female guinea pigs. There was an insignificant (p=0.11) increase in ADP Vmax between 5mo and 15mo CON guinea pigs, whereas 15mo Nrf2a guinea pigs had a higher ADP Vmax than 5mo CON guinea pigs (Fig3.6A). Broken out by sex, this Nrf2 mediated improvement, occurred in male guinea pigs only (Fig 3.6B). There was an age-related decrease in State $3_{[PGM + S]}$ between CON guinea pigs, though this difference was attenuated in 15mo Nrf2a guinea pigs (Fig 3.6C). The age-related decline though, was only observed in female guinea pigs, which was attenuated by Nrf2a (Fig 3.6D). Uncoupled respiration $ETS_{[CI - CIV]}$ non-significantly (p=0.06) decreased with age, though Nrf2a attenuated this difference (Fig 3.6E), though there were no significant differences when sex was considered (Fig 3.6F). There was a significant decrease in $ETS_{[CII - CIV]}$ between 5mo and 15mo CON guinea pigs that Nrf2a could not attenuate (Fig 3.6G) in either sex (Fig 3.6H).

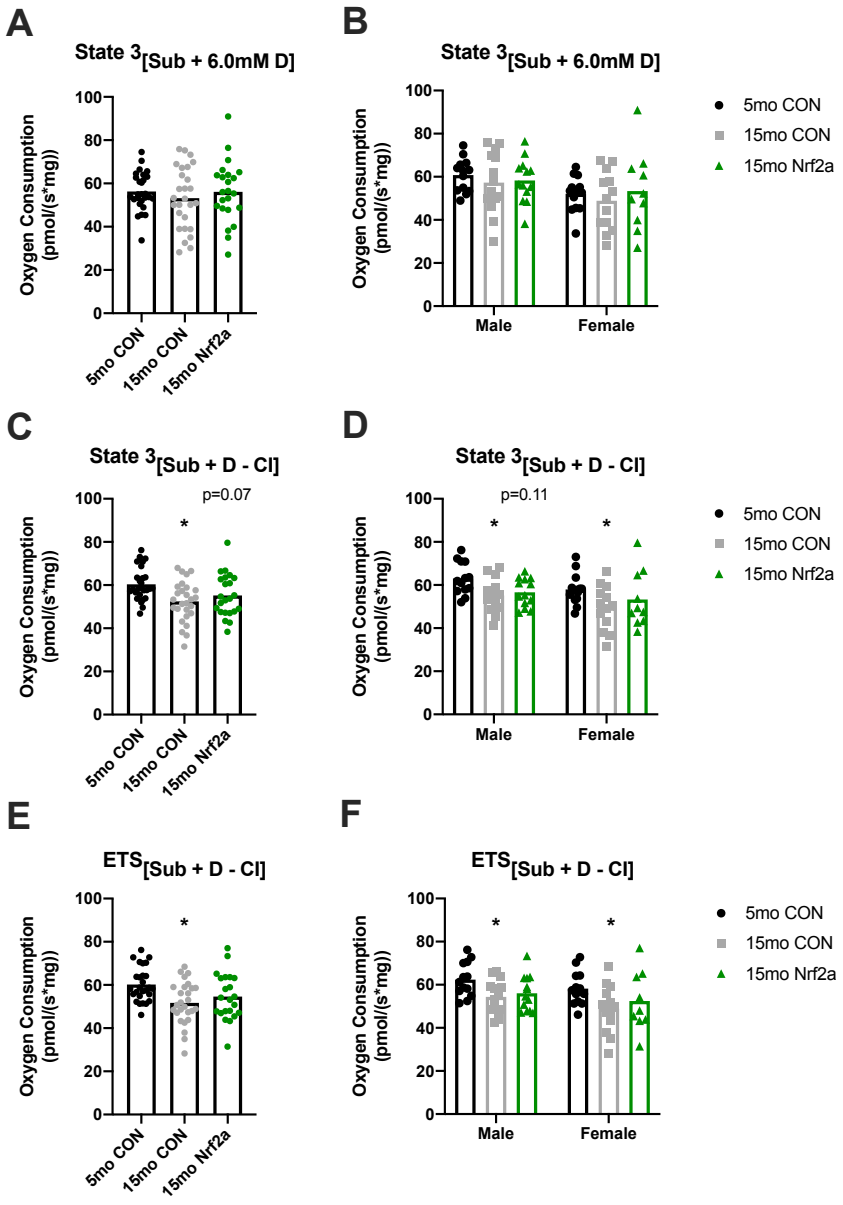

Figure 3.7: The attenuation of age-related changes in fatty acid supported mitochondrial respiration by Nrf2a. There were no age-related changes in State $3_{[Sub + 6.0D]}$, and no effect of Nrf2a (Figs 3.7A & 3.8B). There was an age-related decline in State $3_{[Sub + D - Cl]}$ that Nrf22a failed to abrogate (Fig 3.7C) in either sex (Fig 3.7D). However, 15mo Nrf2a treated guinea pigs were did not statistically lower $ETS_{[Sub + D - Cl]}$ from 5mo CON guinea pigs whereas 15mo CON guinea pigs did (Fig 3.7E) in both sexes (Fig 3.7F).

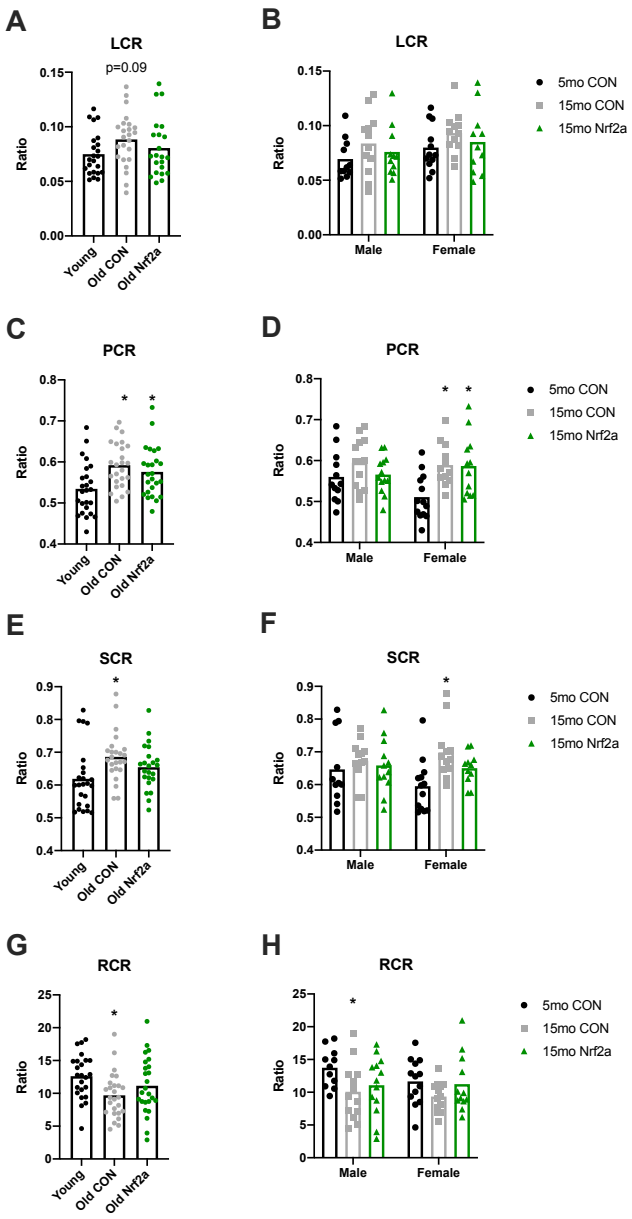

Figure 3.8: The influence of Nrf2a on age-related changes in mitochondrial control ratios. There was a non-significant (p=0.09) increase in LCR between 5mo and 15mo CON guinea pigs (Fig 3.8A), however there was no difference between 5mo CON and 15mo Nrf2a guinea pigs. There were no differences in LCR in either sex separately (Fig 3.9B). 15mo CON and 15mo Nrf2a guinea pigs both had higher PCR than 5mo CON guinea pigs (Fig 3.9C). However, this difference was observed only in females (3.9D). 15mo CON guinea pigs had higher SCR than 5mo guinea pigs though 15mo Nrf2a guinea pigs did not (Fig 3.9E). When comparing sexes separately, these changes were only observed in females and not males (Fig 3.9F). There was an age-related decline in RCR between 5mo and 15mo CON guinea pigs (Fig 3.9G). Nrf2a, however, attenuated that decline (3.9G). However, the age-related decline in RCR was observed only in males, which Nrf2a also attenuated (3.9H).

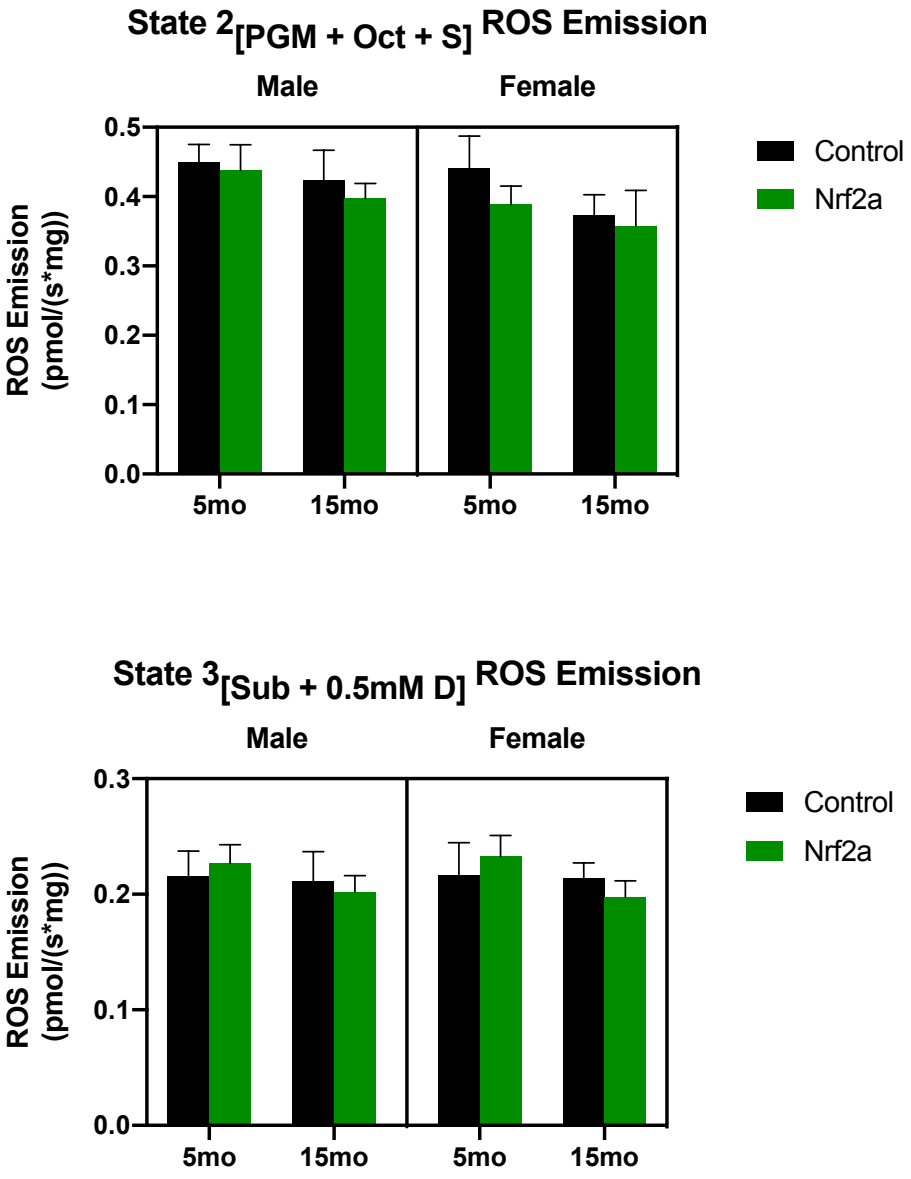

Figure 3.9: Age- sex- and treatment-related effects on mitochondrial ROS emissions. There was no age- sex- or treatment-related effects on ROS emission under either State $2_{[PGM + Oct + S]}$ or State $3_{[Sub + 0.5D]}$ respiration (Figs 3.9A & 3.9B).

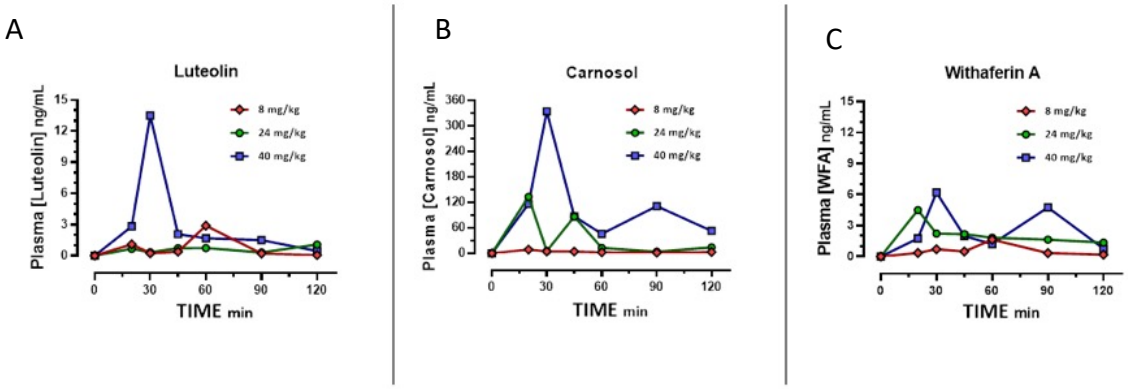

Figure S3.1: Pharmacokinetic analysis of PB125. The concentration of luteolin (Fig S3.1A), carnosol (Fig S3.1B), and withaferin A (Fig S3.1C) in plasma after dosing guinea pigs with 8, 24, or 40 mg/kg of PB125 from 0 to 120 min.

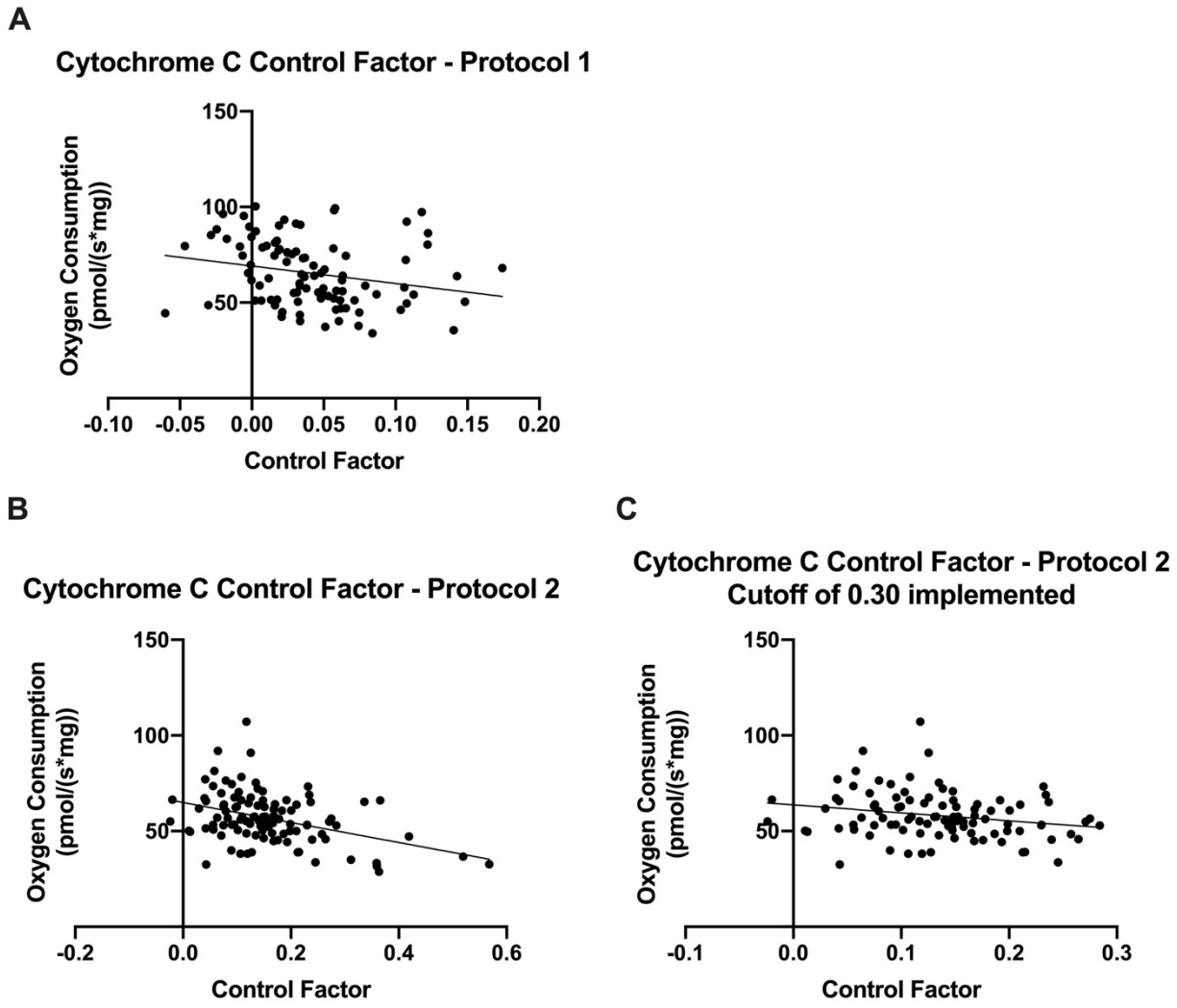

Figure S3.2: Cytochrome C Control Factor Scatterplots. Scatterplots and regression line relating Cytochrome C Control Factor to coupled respiration in Suit 1 (Fig S3.2A) and Suit 2 before (Fig S3.2B) and after (Fig S3.2C) a limit of 0.30 was implemented to establish O2K trials to exclude due to over permeabilization.

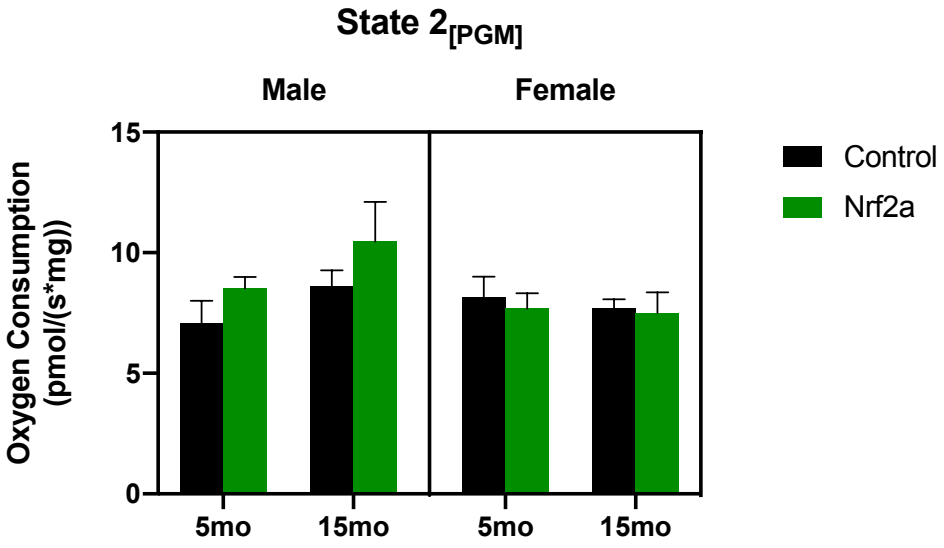

Figure S3.3: Age- sex- treatment- related differences in State 2 (LEAK) respiration. There were no differences in any treatment, sex, or age group.

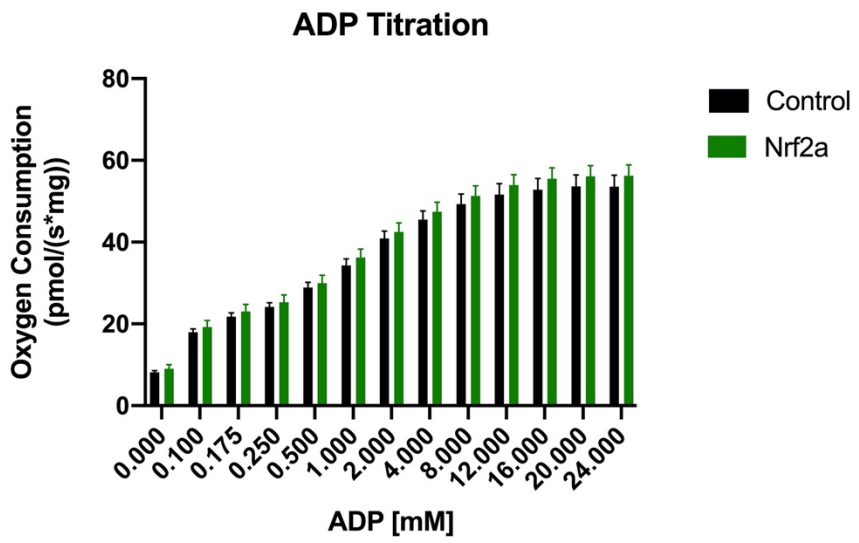

Figure S3.4: ADP Titration in young male and female guinea pigs CON and Nrf2a treated guinea pigs. There were no differences between young CON and Nrf2a treated guinea pigs.

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CHAPTER 4 – NRF2 ACTIVATOR TREATMENT MITIGATES AGE-RELATED DECLINES IN SKELETAL MUSCLE PROTEIN SYNTHESIS IN DUNKIN-HARTLEY GUINEA PIGS

INTRODUCTION

Age-related decline in skeletal muscle function, sarcopenia, contributes to disability[1–3]. Skeletal muscle dysfunction also contributes to metabolic disorders such as type II diabetes[4–8] and other chronic diseases such as cancer[9,10], cardiovascular disease[11,12], and Alzheimer's disease[13,14]. The criteria for diagnosing sarcopenia vary[1,15–21], and thus, the estimates of prevalence also vary. However, recent estimates suggest that approximately 75% of men and 35% of women over the age of 60 years are sarcopenic, and these percentages increase to 88.1% and 52.5% of men and women over 80 years old[22]. Undoubtedly, the projected increase in the aged population[23] will lead to a growing number of individuals with sarcopenia. Thus, there is a heightened need to understand the underlying factors of sarcopenia as well as interventions to prevent the onset and progression of the disease.

The underlying mechanisms of sarcopenia remain to be completely elucidated. Impaired proteostasis (protein homeostasis) is implicated in the progression of sarcopenia[24,25]. Proteostasis refers to the maintenance of the concentration, conformation, and location of proteins[26,27]. As organisms age, impaired proteostasis leads to increased concentration of damaged proteins which impair tissue function[28–32]. In muscle, impaired proteostasis leads to the accumulation of damaged proteins[24,33–36], such as those responsible for contraction[35], which in turn leads to skeletal muscle dysfunction. One critical contributor to maintenance of proteostasis is protein turnover, the synthesis and degradation of proteins, which is essential for maintaining protein concentration as well as preventing accumulation of damaged proteins[28,33].

It remains unclear how protein turnover changes with age. Generally speaking, aging is associated with a decline in skeletal muscle mass[37–40]. Therefore, at some point, muscle protein

breakdown must exceed protein synthesis[41]. Conventional dogma suggests that protein degradation is relatively similar between young and old adults[42], however more recent research in rodent models suggests protein breakdown may be impaired with age[43–45]. Under the existing paradigm, however, research on age-related changes in protein turnover focuses predominantly on the decline in protein synthesis[46,47]. Most research suggests basal protein synthesis rates are no different between middle-aged and old adults[46–48]. However, a blunting of protein synthetic response to anabolic stimuli may contribute to the age-related decline in overall protein turnover and loss of muscle mass and function[47,49–51]. The causes of impaired protein synthesis with aging remain incompletely understood.

Protein turnover is an energetically costly process, which highlights the role mitochondrial function may have in the decline in protein turnover with age. Protein turnover accounts for 35% of basal metabolism and thus requires a significant amount of energy[52,53]. Mitochondria supply the majority of energy in the cell in the form of ATP produced from mitochondrial respiration. Because mitochondrial function declines with age[54–57], it is possible that mitochondrial dysfunction contributes to the age-related decline in protein turnover and overall impaired proteostasis[58,59].

The relationship between mitochondrial dysfunction and sarcopenia is observed across species. Studies in *C. elegans* implicate mitochondrial dysfunction as a contributor to the age-related loss of proteostasis and overall muscle dysfunction[33,36]. Similar relationships between skeletal muscle mitochondrial function, proteostasis, and muscle function have been observed in higher order organisms. In mice, impaired muscle mitochondrial function precedes the development of sarcopenia[60]. Additionally, mitochondrial dysfunction blunts the recovery of skeletal muscle following hindlimb unloading[61]. Improving mitochondrial function appears to improve proteome integrity in skeletal muscle[62]. In humans, exercise-mediated enhancements in mitochondrial function lead to greater translational efficiency[58]. Altogether, it is clear that

mitochondrial function influences skeletal muscle proteostasis and function. Thus, targeting mitochondrial dysfunction holds promise for preventing age-related declines in skeletal muscle function[25,63].

Protein synthesis is required for both proteome maintenance and for cell proliferation [64]. During cell replication, protein mass essentially doubles requiring increases in the rates of protein synthesis (Grebien et al., 2005). Additionally, increased protein synthetic resources are required to maintain a functional proteome (somatic maintenance) via synthesis of proteins to replace those degraded to prevent accumulation of damage (Poppek and Grune, 2005 or others). Measuring cellular proliferation simultaneously with protein synthesis allows for the assessment of tradeoffs between new proteins allocated to proteome maintenance (i.e. proteostasis) or proliferation (growth).[64]. Using the stable isotope deuterium oxide (^2H_2O), our lab simultaneously measures DNA and protein synthesis over days[65], weeks[66–68], and months[69–71]. Using this approach, we have identified that increased protein synthesis dedicated to proteostasis is a commonly shared trait across long-lived species and/or species treated with a lifespan extending intervention[64], particularly in mitochondria[72].

As demonstrated in Chapter 3, PB125, a Nrf2 activator (Nrf2a), improved skeletal muscle mitochondrial function in Dunkin-Hartley guinea pigs in a sex and age specific manner. Since mitochondrial function has a significant role in the maintenance of the proteome, we were interested whether the improvements in mitochondrial function affected a component of proteostasis, protein turnover. The purpose of this study was to determine whether or not the improvements in mitochondrial function from PB125 treatment would translate to improvements in proteostasis, particularly in mitigating the decline in skeletal muscle protein synthesis we observed in Dunkin-Hartley guinea pigs in Chapter 2. We hypothesized that treatment with PB125 would mitigate the age-related declines in skeletal muscle protein synthesis and increase the allocation of newly synthesized proteins to maintain proteostasis.

METHODS

Husbandry, euthanasia, and tissue acquisition

All procedures were approved by the Colorado State University Institutional Animal Care and Use Committee and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. 28 female and 28 male Dunkin-Hartley guinea pigs were obtained from Charles River Laboratories (Wilmington, MA, USA) at 1- and 4- months of age each for a total of 112 guinea pigs. Animals were maintained at Colorado State University's Laboratory Animal Resources housing facilities and were monitored daily by veterinary staff. All guinea pigs were singly-housed in solid bottom cages, maintained on a 12-12 hour light-dark cycle, and provided ad libitum access to food and water.

PB125 (Pathways Bioscience, Aurora, CO) is a phytochemical compound comprised of rosemary, ashwagandha, and luteolin powders mixed in a 15:5:2 ratio by mass, respectively[73]. Guinea pigs were orally administered daily with 0.8mg/kg bodyweight of PB125 suspended in OraSweet (Perrigo, Dublin, Ireland) or OraSweet only. Because the guinea pigs were growing throughout the study, we calculated dosage weekly based on bodyweight. 30 days prior to euthanasia, all guinea pigs were given a subcutaneous injection of 0.9% saline enriched with 99% $^{2}H_2O$ equivalent to 3% of their body weight. Drinking water was then enriched with 8% $^{2}H_2O$ to maintain deuterium enrichment of the body water pool during the 30-day labelling period.

At the time of harvest, the guinea pigs were 5 or 15 months of age (n=14/age/group/sex). In accordance with the standards of the American Veterinary Medical Association, animals were anesthetized with a mixture of isoflurane and oxygen; thoracic cavities were opened and blood was collected via direct cardiac puncture. Immediately after exsanguination, the anesthetized animals were transferred a chamber filled with carbon dioxide for euthanasia. During tissue harvest, at least 70 mg of the gastrocnemius and soleus were

collected and frozen immediately in liquid nitrogen. Bone marrow was also harvested in saline from the humeri.

Protein isolation and fractionation

Tissues were fractionated similarly as described in Chapter 2. Tissues (20 – 50 mg) were homogenized in 1 : 10 isolation buffer (100mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM MgCl2, 1 mM EDTA, 1 mM ATP, pH – 7.5) with phosphatase and protease inhibitors (HALT< Thermo Scientific, Rockford, IL, USA) using a bead homogenizer (Next Advance Inc., Averill Park, NY, USA). After homogenization, subcellular fractions were isolated via differential centrifugation as previously described in Chapter 2. Once fractionated pellets were isolated and purified, 250 µl 1 M NaOH was added and pellets were incubated for 15 min at 50 ^{o}C and 900 RPM.

DNA extraction

Approximately 100 ng/µL of total DNA was extracted from 20 mg tissue (QiAMP DNA mini kit Qiagen, Valencia, CA, USA). DNA from bone marrow was extracted from the bone marrow suspension and centrifuged for 10 min at 2000 g and also yielded approximately 100 ng/µL.

Sample preparation and analysis via GC/MS: Proteins

Protein subfractions were hydrolyzed in 6 M HCl for 24 hours at 120 ^{o}C after which the hydrolysates were ion-exchanged, dried *in vacuo*, and then resuspended in 1 mL of molecular biology grade H_2O. Half of the suspension was derivatized with 500 µL acetonitrile, 50 µL 1 M K_2HPO_4 (pH = 11), and 20 µl of pentafluorobenzyl bromide and incubated at 100 ^{o}C for 60 min. Derivatives were extracted into ethyl acetate and the organic layer was transferred into vials which were then dried under nitrogen. Samples were reconstituted in ethyl acetate (200 µL – 700 µL).

The derivative of alanine was analyzed on an Agilent 7890A GC coupled to an Agilent 5975C MS as previously described[68–70,74–77]. The newly synthesized fraction (f) of proteins was

calculated from the true precursor enrichment (p) based upon plasma analyzed for $^{2}H_2O$ enrichment and adjusted using mass isotopomer distribution analysis[78]. Protein synthesis was calculated as the fraction of deuterium-labeled over unlabeled alanine[78] in proteins over the entire labeling period (30 days).

Sample preparation and analysis via GC/MS: Body water

80 µL of plasma was placed into the inner well of an o-ring screw cap and inverted on a heating block overnight at 100 ^{o}C. After incubation, 2 µL of 10 M NaOH and 20 µL of acetone were added to the samples and $^{2}H_2O$ standards (0 – 20%) and capped immediately, vortexed, and incubated at room temperature overnight. Samples were extracted with 200 µL hexane and the organic layer was transferred through pipette tips with anhydrous Na_2SO_4 into GC vials and analyzed via EI mode using a DB-17MS column.

Sample preparation and analysis via GC/MS: DNA

Incorporation of ^{2}H into purine deoxyribose (dR) of DNA was measured follow procedures already described[75,76,79,80]. DNA that was isolated from tissue and bone marrow were hydrolyzed with nuclease S1 and potato acid phosphatase at 37 ^{o}C shaking at 150 RPM overnight. These hydrolysates were derivatized with pentafluorobenzyl hydroxylamine and acetic acid and incubated at 100 ^{o}C for 30 min. After incubation, samples were acetylated with acetic anhydride and 1-methylimidazole. Dichloromethane was added and then extracted, dried *in vacuo*, and resuspended in ethyl acetate, and analyzed by GC/MS as previously described[75,79–81]. The fraction new was calculated by dividing deuterated dR of the muscle tissue by the bone marrow of the same animal, which represents a fully turned-over cell population, and thus indicative of precursor enrichment[64,75,81]. Protein synthesis rates were expressed relative to DNA synthesis rates to provide insight into allocation of synthesized proteins for newly proliferating cells versus for proteome maintenance[64,72,80,81].

Assessing protein synthesis related to mechanisms of proteostasis

To calculate the amount of protein synthesis related to protein maintenance, we calculated the ratio of protein synthesis to DNA synthesis. This ratio (PRO:DNA) represents how much of the newly synthesized protein is not related to cellular proliferation (new DNA) during the labeling period[64,72,80,81]. Increases in PRO:DNA is indicative of a greater proportion of protein synthesis related to protein turnover to maintain the proteome, with less dedicated to proliferation.

Protein content

Western blotting was used to measure relative content of Nrf2 and OXPHOS proteins. 50-70 mg portions of gastrocnemius (n=6 per treatment group) were powdered under liquid nitrogen and homogenized in a Bullet Blender with zirconium beads and 1.0 mL of radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 0.1 mM EDTA, 50 mM Tris, 0.1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, pH = 7.50) with HALT and protease inhibitors. Samples were reduced (50 µL of B-Mercaptoethanol) and heated at 50 ^{o}C for 10 min. Approximately 10 µg of protein was loaded into a 4% - 20% Criterion pre-cast gel (Bio-Rad, Hercules, CA, USA) and resolved at 120 V for 120 min. The proteins were then transferred to a PVDF membrane at 100 V for 75 min in transfer buffer (20% w/v methanol, 0.02% w/v SDS, 25 mM Tris Base, 192 mM glycine, pH 8.3). Protein transfer to membrane was confirmed with ponceau stain. Membranes were then blocked and incubated with primary antibodies Nrf2 (Santa Cruz 13032) and Total OXPHOS (Abcam 110413) diluted to 1:500 on a shaker overnight in 4 ^{o}C. Membranes were rinsed and then incubated with appropriate secondary antibodies (Santa Cruz 2004 and 2005, respectively) diluted to 1:10,000 for 45 min at room temperature. After the membranes were rinsed, SuperSignal West Dura Extended Duration Substrate (Thermo Fisher 34075) was applied and the membranes were subsequently imaged using a FluorChem E Chemiluminescence Imager (Protein Simple, San Diego, CA, USA). Analysis of

densitometry was completed using AlphaView SA Software. Units are expressed as density of primary antibody relative to density of ponceau staining.

Statistics

To compare the effect of age, sex, and treatment on protein synthesis, a three-way ANOVA was used. Post-hoc analyses were performed using Tukey's HSD post-hoc test. To determine the effect of Nrf2a on age-related changes in protein synthesis, when a significant effect of age was detected, a one-way ANOVA with a Dunnett's post-hoc test was used. To measure the difference between PRO:DNA, we used an unpaired t-test between treated and untreated guinea pigs of the same sex and age. We set statistical significance *a priori* at $p=0.05$. However, we also report differences with $p<0.10$ as non-significant differences to highlight potential directions for future studies. Data are presented as mean +/- SEM. All statistics were performed in Prism 8.0 (La Jolla, California, USA).

RESULTS

Protein synthesis in the gastrocnemius

There was an age-related decline in fractional synthesis rate (FSR) of all subfractions in the gastrocnemius in both male and female guinea pigs (Fig 4.1). However, Nrf2a did not have an effect on the fractional synthesis rate (FSR) in either the myofibrillar, mitochondrial, or cytosolic subfractions in the gastrocnemius of either young or old, male or female guinea pigs (Figs 4.1A – 4.1C). There was a non-significant interaction between age and Nrf2a in the collagen-enriched subfraction of both male and female guinea pigs, suggesting that Nrf2a increased collagen FSR in 5mo pigs, but decreased collagen FSR in 15mo guinea pigs (Fig 4.1D).

Protein synthesis in the soleus

Similar to the gastrocnemius, there was an age-related decline in FSR of all subfractions of the soleus in both male and female guinea pigs (Fig 4.2). Similar to the gastrocnemius, there

was no main effect of Nrf2a on FSR in any subfractions (Fig 4.2). However, myofibrillar FSR was non-significantly (p=0.072) greater in 15mo Nrf2a guinea pigs compared to 15mo controls (Fig 4.2A).

The effect of Nrf2a on the age-related decline in protein synthesis

As there was an age-related decline in protein synthesis in all subfractions of both the gastrocnemius and soleus, we sought to determine if Nrf2a attenuated any of those declines. In the gastrocnemius, Nrf2a had no attenuating effect on the age-related decline in protein synthesis in any subfraction (Fig 4.3). In contrast, Nrf2a attenuated age-related declines in FSR in several subfractions. Nrf2a mitigated the age-related decline in myofibrillar FSR in both males and females (Figs 4.4A and 4.4B). Additionally, Nrf2a attenuated the decline in mitochondrial FSR in the soleus (Fig 4.4C). Nrf2a also mitigated the decline in cytosolic FSR in males only (Fig 4.4F). Nrf2a, though, failed to prevent the decline in collagen FSR for both sexes (Figs 4.4G and 4.4H).

Age-related differences in skeletal muscle DNA synthesis

There was a significant age-related decline in both gastrocnemius and soleus DNA synthesis (Figs 4.5A and 4.5B) in both males and females. Moreover, Nrf2a had no effect on skeletal muscle proliferation in either young or old guinea pigs, which is perhaps reflected in the similar rates of growth (Fig S4.1) and skeletal muscle mass (Fig S4.2).

The effect of Nrf2a on protein synthesis related to proteostasis

To measure the effect of Nrf2a on the measurement of proteostasis, we used an unpaired t-test comparing its effect within an age in both male and female guinea pigs for the following reasons: 1. At 5 months of age, guinea pigs are still developing and skeletal muscle growth, and thus DNA proliferation, is much greater than at 15 months of age when guinea pigs have completed growth. Consequently, comparing the allocation of protein synthesis between two different contexts would be inappropriate. 2. We were not powered to determining the effect of sex on Nrf2a, because this was a secondary aim, the original study was not designed to

detect sex differences, and there was sample loss that precluded us from analyzing this measurement in all guinea pigs.

In 5mo guinea pigs, there was no effect of Nrf2a on the PRO:DNA in the gastrocnemius (Fig 4.6) or the soleus (Fig 4.7). While there was no effect of Nrf2a on protein synthesis rates relative to cell proliferation in young guinea pigs, Nrf2a did change the ratio of PRO:DNA in 15mo animals. In the gastrocnemius, Nrf2a non-significantly increased PRO:DNA in the myofibrillar, mitochondrial, and cytosolic subfractions (Figs 4.8A – 4.8C), though these changes were observed predominantly in the females ($p=0.097$, 0.088, and 0.054 respectively). In contrast, there was a significant decrease ($p=0.025$) in the proportion of newly synthesized proteins related to proteostasis (PRO:DNA) in the mitochondrial subfraction in the soleus in 15mo female guinea pigs (Fig 4.9B). However, there were no other effects of Nrf2a on PRO:DNA in the soleus muscle (Fig 4.9).

DISCUSSION

Our results describe the age-related changes in skeletal muscle protein synthesis in both males and females and the effect of Nrf2a on protein synthesis (Figs 4.1 & 4.2). These results corroborate the age-related decline in protein synthesis in skeletal muscle of male guinea pigs in Chapter 2. Additionally, we show that female guinea pigs experience a similar decline in protein synthesis (Figs 4.1 & 4.2). Nrf2a did not affect fractional synthesis rates (FSR) in either the gastrocnemius or the soleus in young or older guinea pigs (Figs 4.1 & 4.2). However, Nrf2a did attenuate the age-related decline in FSR in the soleus (Fig 4.4), but not the gastrocnemius (Fig 4.3). Importantly Nrf2a did not alter the rate of body mass growth (Fig S4.1) or cell proliferation in muscle (Fig 4.5) during development. We found that 15mo guinea pigs had lower rates of skeletal muscle DNA synthesis compared to 5mo guinea pigs (Fig 4.5), which likely reflects the decrease in the rate of growth (Fig S4.1). This study also highlights sex differences in protein allocation towards growth versus protein maintenance in 5mo guinea pigs still

undergoing development (Figs 4.6 & 4.7) Finally, we show that Nrf2a increased the amount of protein synthesis related to proteostasis (increased PRO:DNA) in the gastrocnemius of 15mo guinea pigs (Fig 4.8). Collectively, these results provide further insight into the musculoskeletal aging process of the Dunkin-Hartley guinea pig with greater understanding about how muscle protein synthesis related to growth and maintenance change with age. Moreover, we provide evidence that Nrf2a may attenuate deleterious age-related decreases in protein synthesis and increase the allocation of protein synthesis related to the maintenance of the proteome.

Age- and sex- differences in skeletal muscle protein synthesis

Similar to our results in Chapter 2, we observed an age-related decline in FSR in all subfractions of both the gastrocnemius and soleus in male Dunkin-Hartley guinea pigs (Figs 4.1 & 4.2). 15mo female guinea pigs also had lower FSR in all subfractions compared to 5mo, indicating for the first time that female Dunkin-Hartley guinea pigs experience age-related declines in protein synthesis (Figs 4.1 & 4.2) and may be similarly susceptible to sarcopenia as male guinea pigs (Ch. 2). Interestingly, there were no sex differences in FSR in any subfractions of either skeletal muscle (Figs 4.1 & 4.2).

There was an age-related decline in FSR in each subfraction of both gastrocnemius and soleus. It is unclear whether these decreases in FSR corresponded to lower concentrations of proteins, however, the lack of decline in muscle mass would suggest that the concentration of certain proteins (e.g. myofibrillar and cytosolic proteins) would remain unchanged. If that were the case, it would suggest protein breakdown, and therefore overall protein turnover, decreased. A decrease in turnover would suggest that there would be an increase in the accumulation damaged proteins. We also observed a decline in mitochondrial FSR in both skeletal muscles. Interested in determining whether this decline had an effect on mitochondrial protein concentration, we measured protein content of the mitochondrial complexes in the gastrocnemius and found that there was no difference in the relative abundance of Complexes I – IV between 5mo and 15mo guinea pigs (Figs S4.4A – S4.4D). However, despite the decline in

mitochondrial protein synthesis (Figs 4.1 & 4.2), there was an age-related increase in Complex V (i.e. ATP synthase) (Fig S4.4E). This suggests that concomitant with a decrease in mitochondrial FSR there was a decrease in mitochondrial autophagy (mitophagy), a process that likely occurs in humans[82,83] and contributes to mitochondrial dysfunction and disease[84–86]. Altogether, these results argue for the importance of measuring both the concentration of proteins as well as the concentration of damaged proteins to determine whether changes in protein turnover are linked to impaired proteostasis.

While there were no differences in protein FSR between males and females, there were significant differences between sexes in the allocation of protein synthesis at 5mo. In the mitochondrial, myofibrillar, and cytosolic subfractions of gastrocnemius, protein synthesis in male guinea pigs was allocated more towards to the maintenance of protein as opposed to cell proliferation in skeletal muscle (Fig 4.6). Similar, but non-significant ($p=0.0877$ and $p=0.0833$, respectively) differences were observed in the mitochondrial and myofibrillar subfractions of the soleus (Fig 4.7). These results suggest that despite no differences in protein synthesis between male and female guinea pigs, at 5mo, protein synthesis in females is related to proliferation whereas a greater proportion of protein synthesis in males is allocated towards proteostasis. Interestingly, this is not reflected by rate (k) of change in body mass (Fig S4.1), as changes in body mass can influence overall rates of protein synthesis (unpublished data). By the time Dunkin-Hartley guinea pig growth plateaus, though, there are no differences in protein allocation between male and female guinea pigs (Figs 4.8 & 4.9).

The implications of sex differences in allocation of protein synthesis are not known. The data suggest that 5mo male guinea pigs invest more energy in the maintenance of the skeletal muscle proteome compared to females. Generally speaking, investing in mechanisms promote proteostasis are associated with improved function and longevity. However, it is unclear whether or not these greater male PRO:DNA translate to improved muscle function or the maintenance

of muscle function throughout age compared to females, particularly given that there are no sex differences in PRO:DNA at 15mo.

Nrf2a attenuates age-related declines in protein synthesis

While there was no main effect of Nrf2a treatment on protein turnover or proteostasis across young and old, male and female guinea pigs, Nrf2a did attenuate the decline in myofibrillar and mitochondrial fractional synthesis rates (FSR) observed in 15mo male and female guinea pigs in the soleus (Figs 4.4A – 4.4C). Interestingly, Nrf2a had no effect on the age-related decrease in FSR in the gastrocnemius (Fig 4.3). It's unclear what would explain the discrepancy in the effect of Nrf2a between these two muscles. However, in the guinea pig the soleus is comprised predominantly of type I muscle fibers whereas the gastrocnemius is a mixed fiber type muscle (Ch. 2). Type I fibers generally have greater mitochondrial density[87–89], which may explain the disparity of effect. Importantly, in Chapter 2, we observed more detrimental, age-related changes in the gastrocnemius muscle compared to the soleus. Thus, further investigation into the mechanisms that differentially mediate the effect of Nrf2a in mixed fiber type muscles should be examined. Follow up research is required to determine if the attenuation of protein synthesis decline between 5 and 15 mo suggests that Nrf2a could aid in preventing the age-related declines in skeletal muscle proteome integrity and function.

While we observed an attenuation in the age-related decline in FSR with Nrf2a, we were also interested to determine whether Nrf2a affected the proportion of protein synthesis dedicated to protein maintenance. There was no effect of Nrf2a on the allocation of protein synthesis to either growth or maintenance in 5mo guinea pigs (Figs 4.6 & 4.7). Nrf2a did increase the amount of protein synthesis dedicated toward maintaining proteostasis in the myofibrillar (Fig 4.8A), mitochondrial (4.8B), and cytosolic (4.8C) subfractions gastrocnemius of female guinea pigs. Thus, while Nrf2a did not attenuate declines of protein synthesis in the gastrocnemius (Fig 4.3), Nrf2a did increase the relative amount of protein synthesis related to

protein maintenance in 15mo female guinea pigs. There is an age-related increase in protein damage of myofibrillar proteins, which is related to impaired function[35]. Thus, with the increase in protein turnover related to proteostasis[90], Nrf2a likely improved the integrity of the skeletal muscle mitochondrial and myofibrillar proteome. Our lab has previously observed similar improvements in myofibrillar PRO:DNA in humans treated with Protandim, which is also a phytochemical Nrf2 activator; however, the effect was only observed in males[91]. Studies using other Nrf2 activators have also observed improvements in skeletal muscle function[92], which may reflect improved proteome integrity. However, we did not measure oxidatively damaged proteins in the muscle, particularly in contractile proteins, which tend to increase with age and potentially contribute to impaired strength[35]. We also observe a decline in muscle density and a fiber size distribution in the gastrocnemius (Ch. 2), which is reflective of a decline in overall muscle quality[95–97]. Accordingly, additional assessments, including protein damage, fiber size, and muscle density, are necessary to further understand the effect of Nrf2a on skeletal muscle quality. Moreover, future research should also address the sex-specific effects we observed with Nrf2a particularly considering the sex-specific effects we observed in Chapter 3 as well as in studies using other Nrf2 activators to improve proteostasis and longevity.

The influence of Nrf2a on growth in guinea pigs

An important consideration for the use of a long-term treatment to delay the onset of a chronic disease, particularly age-related diseases, is how a treatment influences normal growth and development. In contrast to treatment with the Nrf2 activator Protandim which decreased cell proliferation rates in skeletal muscle from rats[93], the Nrf2 activator PB125 did not have any significant effect on rates of DNA synthesis in either young or old guinea pigs (Fig 4.5) or on the rate of growth (Fig S4.1), body size (Fig S4.2E), or muscle mass (Figs S4.2A-D) in Dunkin-Hartley guinea pigs. While there were no significant differences in cell proliferation proliferation in skeletal muscle, the changes in protein allocation suggest that Nrf2a may have subtle effects

on cellular growth and proteostasis. *In vitro,* other Nrf2 activators (unpublished), including as Protandim[93] decrease myoblast proliferation. Thus, it is possible that Nrf2a decreased skeletal muscle growth in these guinea pigs, but the magnitude of effect was too small to detect. At the moment, though, the underlying mechanisms how transient Nrf2 activation affects cell cycle and may reduce proliferation are unclear. However, reducing overall, global protein synthesis, particularly related to growth and proliferation, while simultaneously maintaining the synthesis of proteins to maintain proteostasis in mitochondria is a commonly shared trait of long-lived and slowed-aging interventions that activate energetic stress signaling[72].

Potential mechanisms underlying the Nrf2a-mediated improvements in proteostasis

While Nrf2a attenuated the age-related decline in FSR and re-allocated protein synthesis towards proteostatic mechanisms in 15mo guinea pigs, the mechanisms by which Nrf2a imparts these effects are unclear. The improvement in proteostatic processes from Nrf2a could be mediated by variety of mechanisms. Nrf2a could have stimulated signaling cascades that augment protein synthesis, the improvements in mitochondrial function may have facilitated greater protein translation, and/or enhanced endogenous anti-inflammatory and antioxidant enzymes may have improved skeletal muscle quality and the response to anabolic stimuli. Below we review each potential mechanism and further studies necessary to elucidate these pathways.

The mechanistic target of rapamycin (mTOR) is a key nutrient sensitive regulator of protein synthesis[94] and thus is a potential pathway for Nrf2a to have affected protein synthesis. However, it is unlikely that Nrf2a directly affects protein synthesis by stimulating mTOR or other anabolic signaling pathways as there was no main effect of Nrf2a increasing protein synthesis. Instead, given our observations that Nrf2a attenuates mitochondrial dysfunction in 15mo guinea pigs (Ch. 3), it is likely that the attenuation of mitochondrial dysfunction is related to the mitigation in the age-related declines in protein synthesis. Age-related mitochondrial oxidative

stress can initiate the integrated stress response that can lead to suppression of protein translation through phosphorylation of the translation initiation factor 2α (eIF2α)[59,95,96]. Declines in mitochondrial respiration could also induce energetic stress which activates AMPK and lead to the repression of protein synthesis[97]. Further, impairment in ATP production from mitochondrial respiration can impair chaperone[98] (e.g. Hsp70) activity which can lead to the activation of the unfolded protein response (UPR)[59,99,100], which also represses global protein synthesis and instead activates pathways related to proteostasis[101]. Importantly, Nrf2 appears to mediate many of these adaptive responses[102–104]. Interestingly, we observed an age-related decline in Nrf2 content in male guinea pigs in the gastrocnemius, but an age-related increase in female guinea pigs (Fig S4.4B). Nrf2a attenuated the decline of Nrf2 content in males, however Nrf2a failed to attenuate declines in protein synthesis in males or increase PRO:DNA in the gastrocnemius. Further investigation should assess the effect of Nrf2a on Nrf2 content in the soleus as that is where predominant changes in protein turnover occurred.

In Chapter 3, we documented Nrf2a mediated improvements in mitochondrial respiration, which we hypothesize would improve ATP production and alleviate any energetic constraints on cellular processes as a consequence of age-related declines in mitochondrial function. Combined with the fact that Nrf2a did not change content of mitochondrial complexes I, II, IV, and V (Fig S4.4), it is likely that Nrf2a increased mitochondrial efficiency and quality in the gastrocnemius. Nrf2a did decrease Complex III content (Fig S4.4C), which, combined with a lack of change in mitochondrial biogenesis, would suggest that Nrf2a stimulated mitophagy, a process that is associated with improved mitochondrial function[105]. However, because western blotting was only conducted in a subset of guinea pigs, caution should be taken when interpreting these data. Thus, further investigation is necessary to determine how Nrf2a may change mitochondrial content and whether improvements in mitochondrial respiration is related to Nrf2a-mediated increases in mitophagy.

Mitochondrial dysfunction precedes the loss of proteostasis in skeletal muscle[36]. Interventions that attenuate the decline in mitochondrial function or improve mitochondrial function improves proteostatic mechanisms and preserves overall muscle function[25,36,62,106,107]. These studies emphasize the importance of mitochondrial production of ATP to facilitate proteostatic mechanisms. In humans, aerobic exercise improves mitochondrial function through mitochondrial remodeling and improves skeletal muscle function[108]. Moreover, pharmacologically enhancing mitochondrial respiration and subsequently mitigates skeletal muscle damage and improves function in mice[62]. Our data, in combination with existing literature, support the posit that Nrf2a-mediated maintenance of mitochondrial function with age can facilitate mechanisms related to proteostasis.

Another mechanism by which Nrf2a may attenuate age-related declines in protein turnover and enhance mechanisms of proteostasis, is via improvements in anabolic responses to stimuli such as feeding or exercise. Anabolic resistance seems to contribute to declines in muscle mass and function in humans[47,51,109,110]. Importantly, interventions designed to mitigate age-related increases oxidative stress or inflammation seem to improve anabolic responses[49,111–113]. Nrf2a stimulates transcription of endogenous antioxidant and anti-inflammatory genes[73,114]. Thus, the anti-inflammatory effect of Nrf2a could have also improved the anabolic responses in 15mo guinea pigs. Additionally, in relation to potential improvements in translational efficiency and ribosome concentration, there is growing speculation that ribosomal capacity, which is impaired with age[47], is necessary to respond to anabolic stimuli such as exercise[115]. Thus, the maintenance of mitochondrial function may similarly improve the anabolic response to exercise or feeding by maintaining ribosomal capacity to adequately respond to an anabolic stimulus. However, because we measured cumulative protein synthesis

over 30 days rather than acutely in response to an anabolic stimulus such as feeding, we cannot determine if there were any changes specifically in the anabolic response to feeding.

As mentioned, Nrf2a stimulates the upregulation of antioxidants enzymes which should protect cells, particularly the proteome, from oxidative damage. While mechanisms such as protein turnover are essential for preventing the accumulation of damaged proteins and maintaining proteome integrity, upregulation of endogenous antioxidants may protect proteins and prevent oxidative damage from occurring. As such, the lack of improvement in PRO:DNA observed in the soleus (Figs 4.7 & 4.9) does not exclude the possibility that skeletal muscle proteome quality was not improved, but reflects a lack of difference in protein synthesis related to protein maintenance. Other interventions that increase endogenous antioxidants seem to improve proteome integrity and skeletal muscle function[92,116,117]. We have previously reported that treatment with phytochemical-based Nrf2 activators upregulates engenous antioxidants and protects cultured cells from oxidative insults[118,119]. Thus, it is necessary to further study whether or not Nrf2a decreased the concentration of damaged proteins.

Summary and future directions

Nrf2a attenuated age-related declines in soleus myofibrillar, mitochondrial, and cytosolic FSR in both male and female guinea pigs. Additionally, Nrf2a increased PRO:DNA in the gastrocnemius of female guinea pigs. While it is unclear how Nrf2a directly mediates these improvements in skeletal muscle, these data support our overall hypothesis that attenuating the age-related decline in mitochondrial respiration (Ch. 3) would translate to the mitigation of age-related changes in skeletal muscle. Of course, it is also possible that Nrf2a facilitated the maintenance of the mitochondrial proteome, which then in turn attenuated the age-related decline in mitochondrial respiration. It is still necessary to determine whether or not the effects of Nrf2a we observed on mitochondrial function and skeletal muscle translate to improved proteome quality and skeletal muscle function. However, these data support the notion that

Nrf2a can mitigate age-related declines in protein synthesis and that these occur alongside the attenuation of mitochondrial dysfunction in a preclinical model of musculoskeletal aging. The mechanisms and signaling pathways that link mitochondrial function and proteostasis remain to be fully elucidated, particularly connecting age-related declines in mitochondrial function and ATP production with impairments in proteostatic mechanisms and skeletal muscle function.

FIGURES

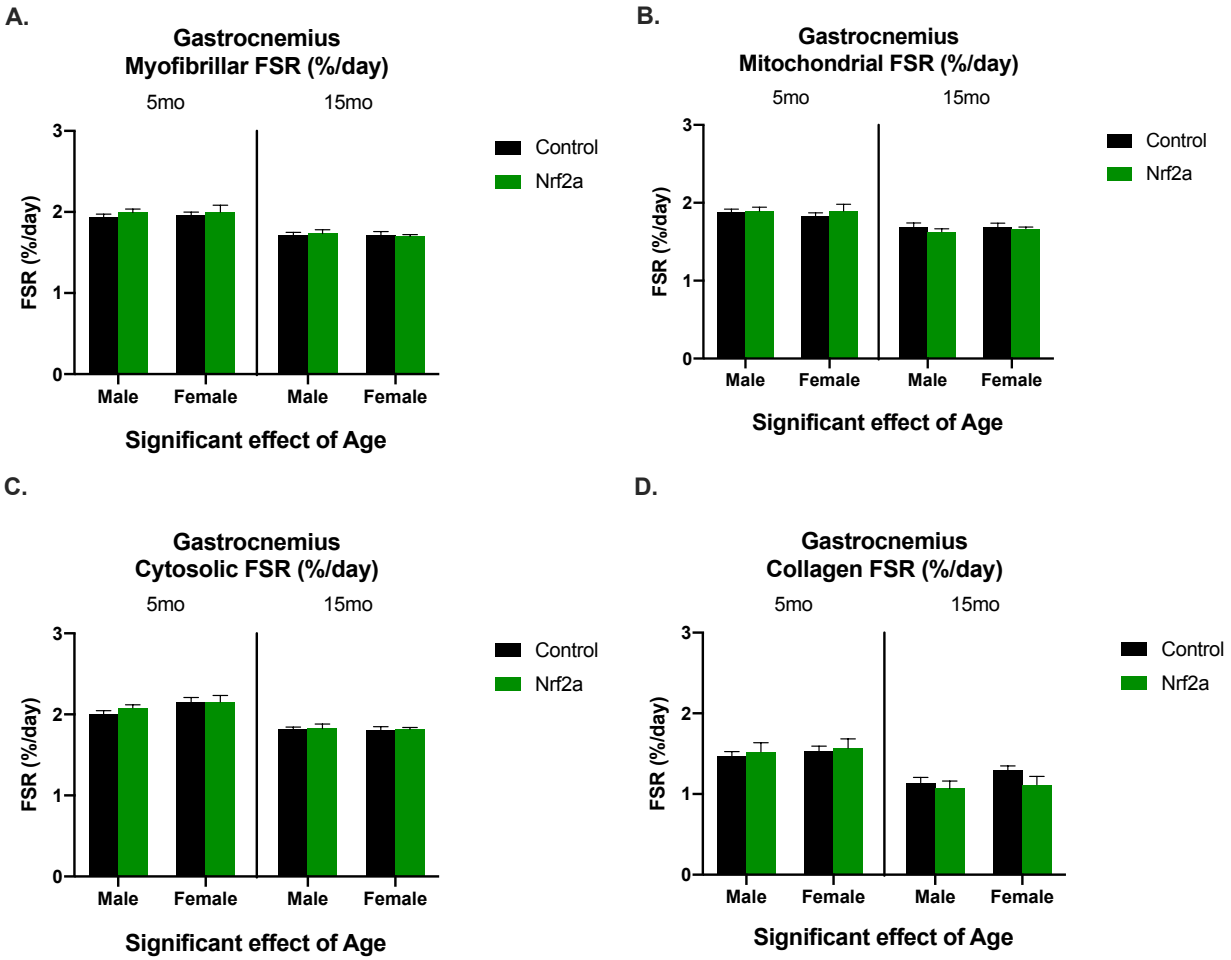


Figure 4.10: Fractional synthesis rates of the gastrocnemius. There was a significant, negative effect of age ($p<0.05$) in all subfractions of the gastrocnemius in males and females. Additionally, there was no effect of sex on FSR in any subfraction.

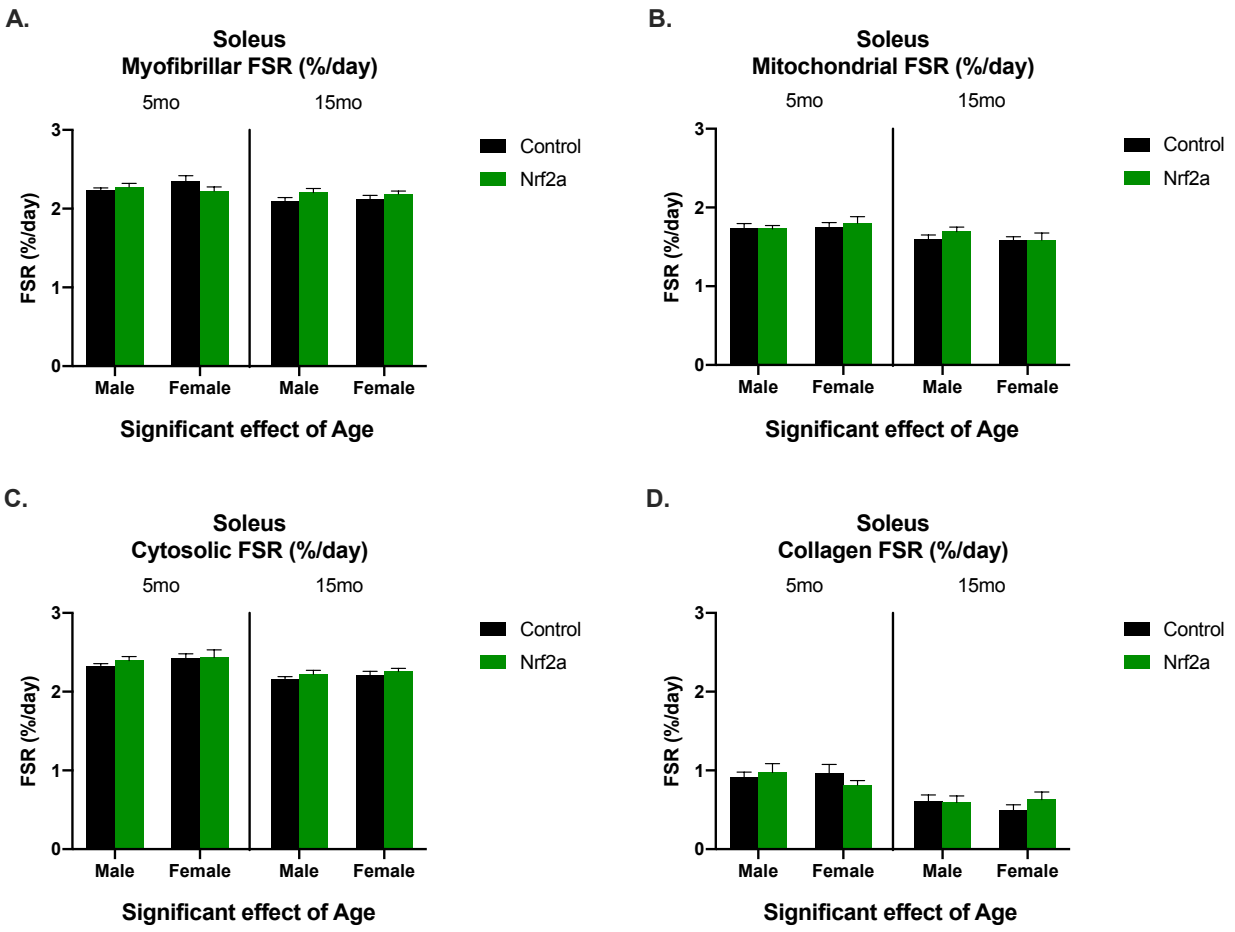


Figure 4.11: Fractional synthesis rates of the soleus. There was a significant, negative effect of age ($p<0.05$) in all subfractions of the gastrocnemius in males and females. Additionally, there was no effect of sex on FSR in any subfraction.

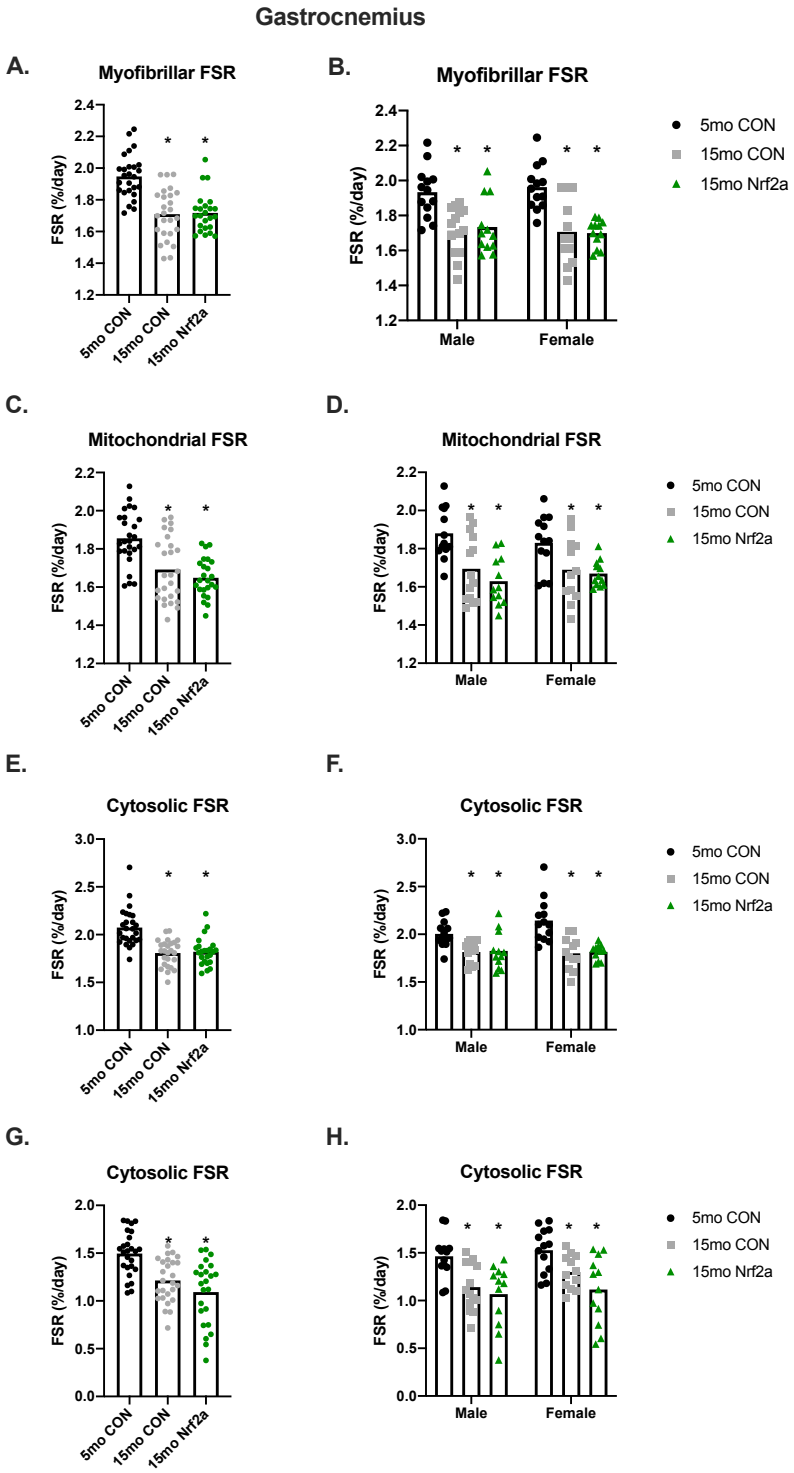


Figure 4.12: The effect of Nrf2a on the attenuation of declines in FSR in the gastrocnemius. FSR was significantly lower in 15mo CON guinea pigs compared to 5mo CON guinea pigs in all subfractions ($p<0.05$). That age-related difference also persisted with Nrf2a as treatment failed to attenuate any of those differences ($p<0.05$).

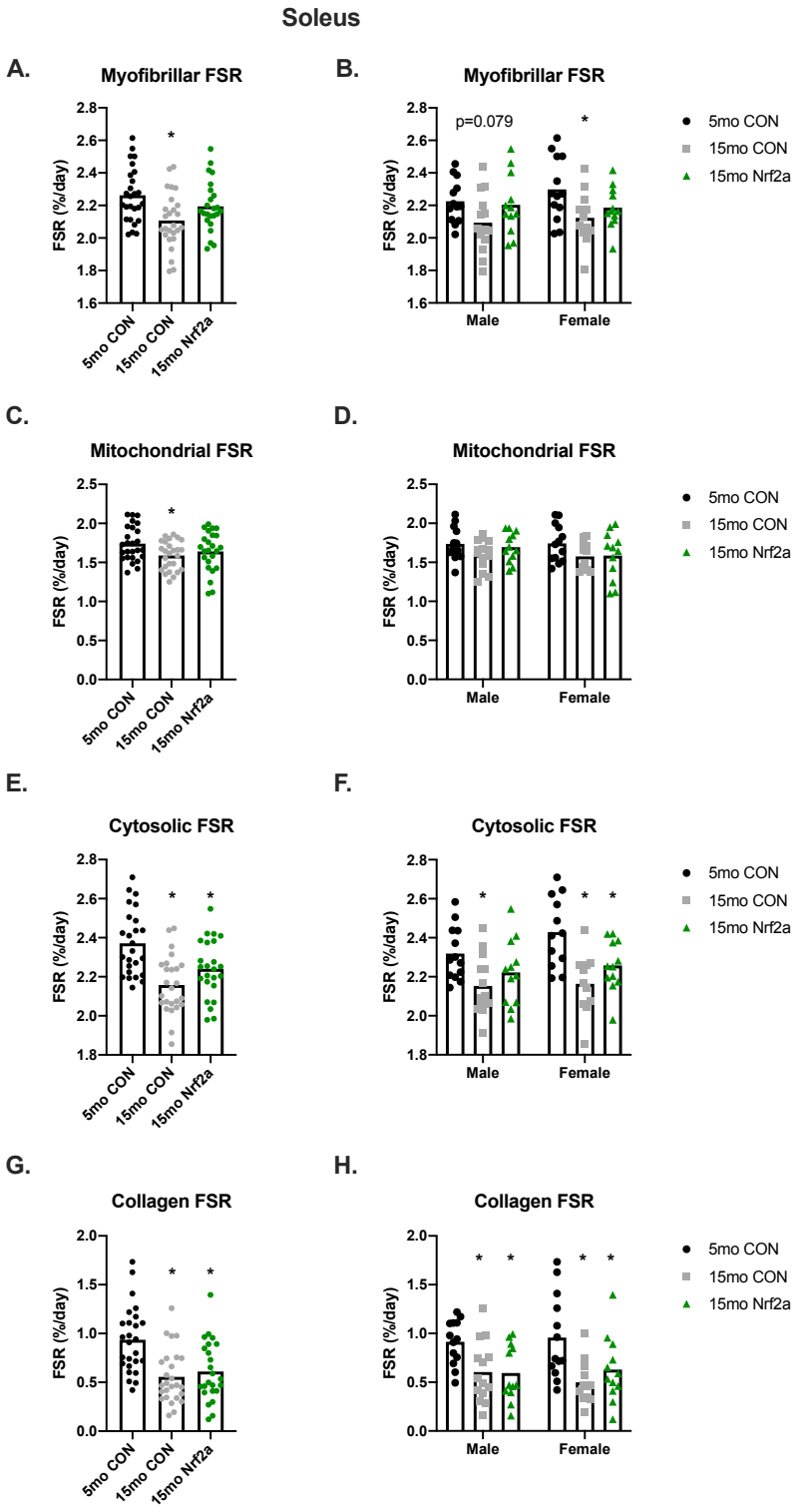


Figure 4.13: The effect of Nrf2a on the attenuation of declines in FSR in the soleus. FSR was significantly lower in 15mo CON guinea pigs compared to 5mo CON guinea pigs in all subfractions ($p<0.05$). Nrf2a attenuated the decline in FSR in all subfractions ($p>0.05$) in both males and females.

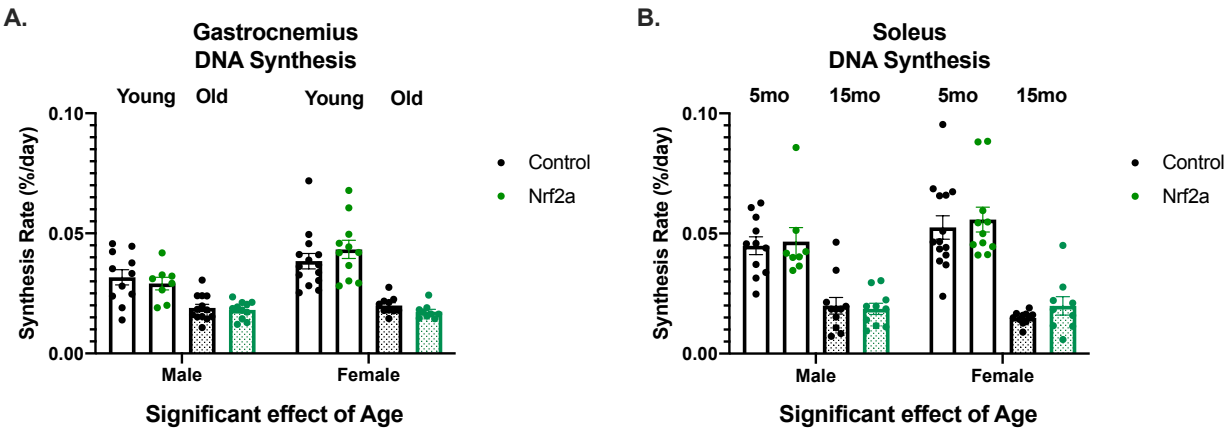


Figure 4.14: Differences in skeletal muscle proliferation. There was a significant, negative effect ($p<0.05$) of age on DNA synthesis. However, there was no effect of sex or treatment on DNA synthesis.

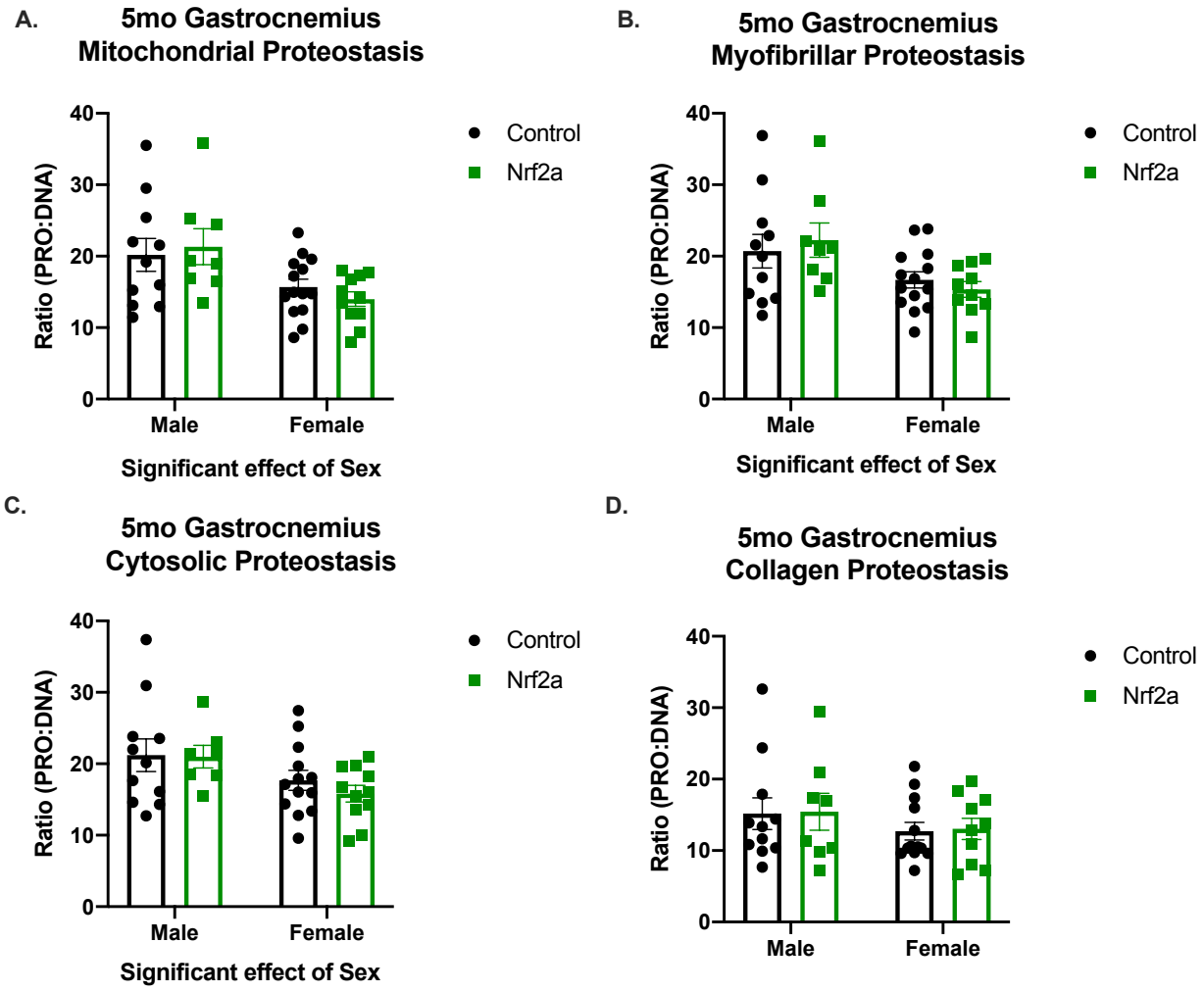


Figure 4.15: Allocation of protein synthesis towards proteostatic mechanisms in the gastrocnemius of young guinea pigs. There was a significant effect of sex, where female guinea pigs had lower($p<0.05$) PRO:DNA compared to males, in the mitochondrial, myofibrillar, and cytosolic subfractions of the gastrocnemius. However, there was no effect of treatment in either male and female young guinea pigs.

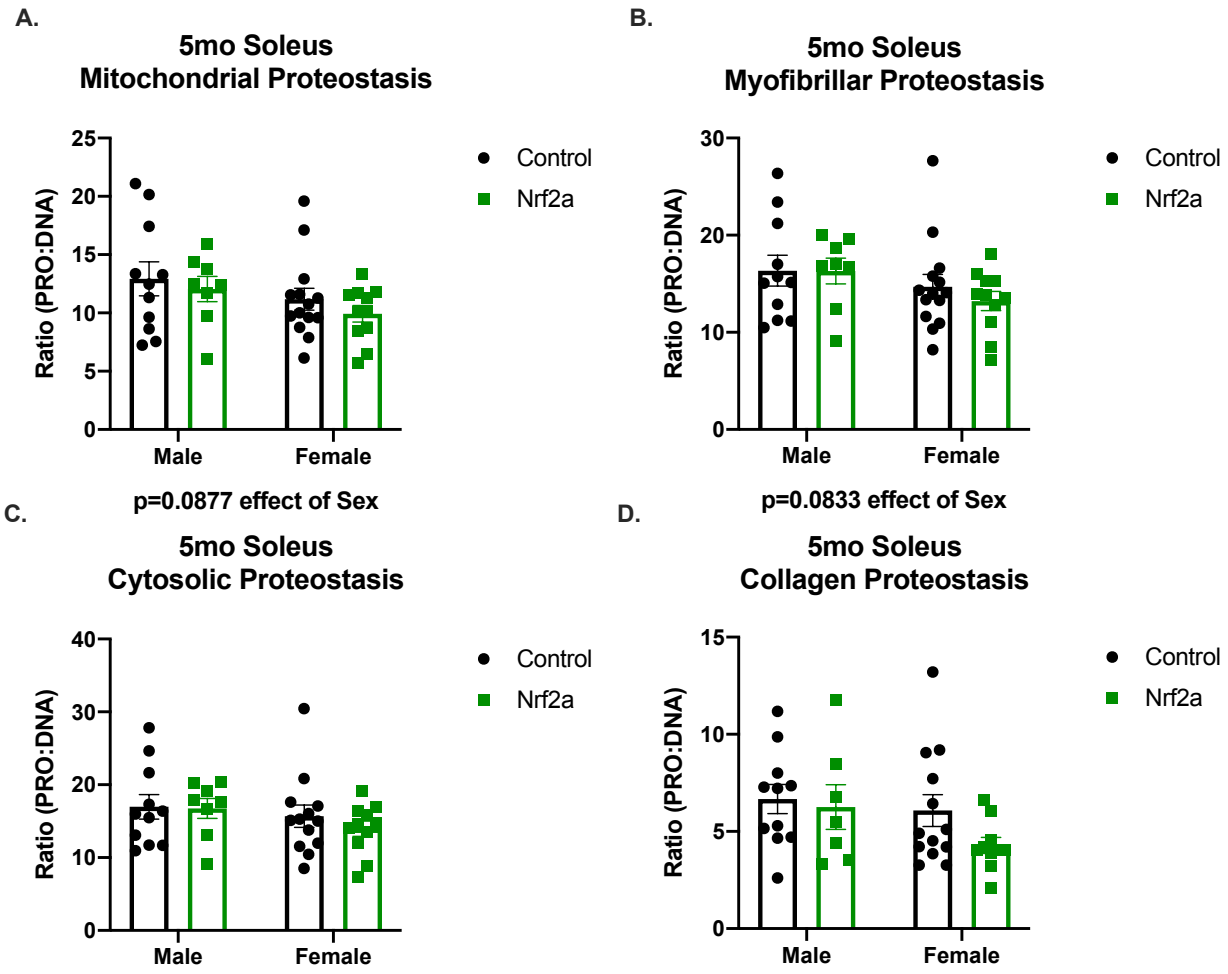


Figure 4.16: Allocation of protein synthesis towards proteostatic mechanisms in the soleus of young guinea pigs. There was a non-significant effect of sex, where female guinea pigs had lower(p=0.08) PRO:DNA compared to males, in the mitochondrial and myofibrillar subfractions of the soleus. However, there was no effect of treatment in either male and female young guinea pigs.

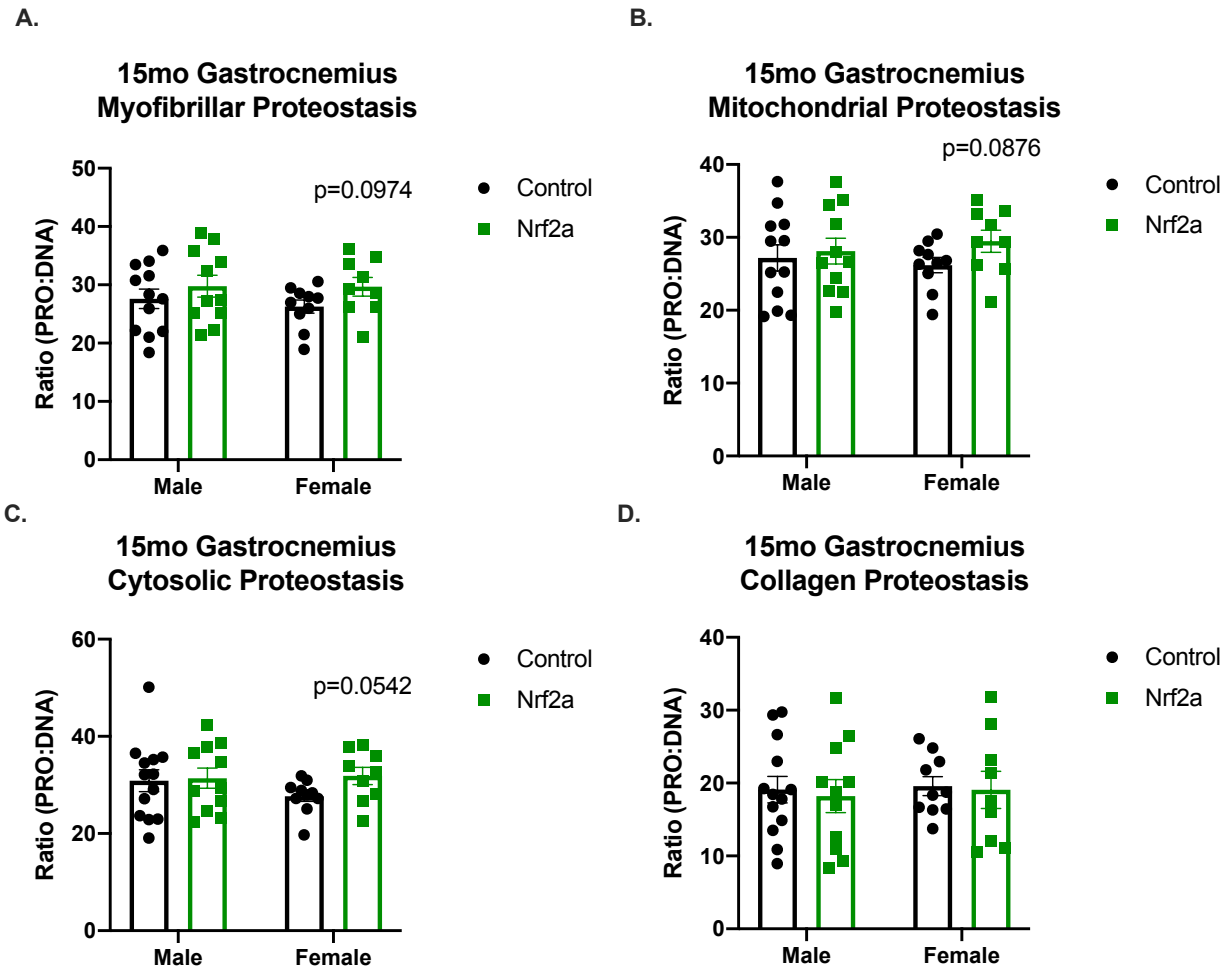


Figure 4.17: Allocation of protein synthesis towards proteostatic mechanisms in the gastrocnemius of old guinea pigs. There were no sex differences in the PRO:DNA of guinea pigs in the gastrocnemius. There were non-significant increases (p=0.097, 0.088, and p=0.054) in PRO:DNA of the myofibrillar, mitochondrial, and cytosolic subfractions in female guinea pigs, but there were no Nrf2a mediated changes in PRO:DNA observed in male guinea pigs.

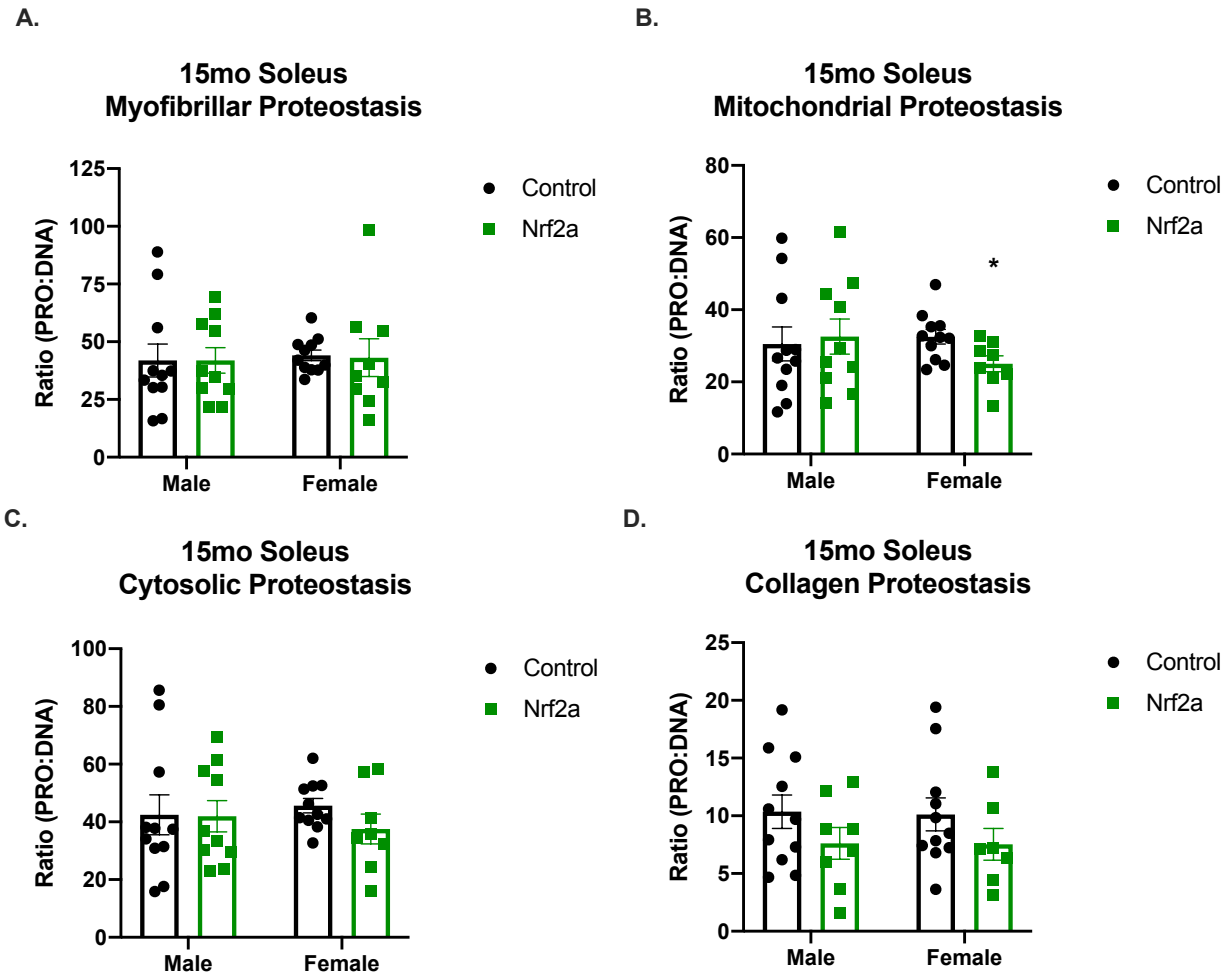


Figure 4.18: Allocation of protein synthesis towards proteostatic mechanisms in the soleus of old guinea pigs. There was no effect of sex observed on PRO:DNA in the soleus. There was no effect of Nrf2a on PRO:DNA in the soleus except for a significant ($p<0.05$) decrease in PRO:DNA in the mitochondrial subfraction of the soleus.

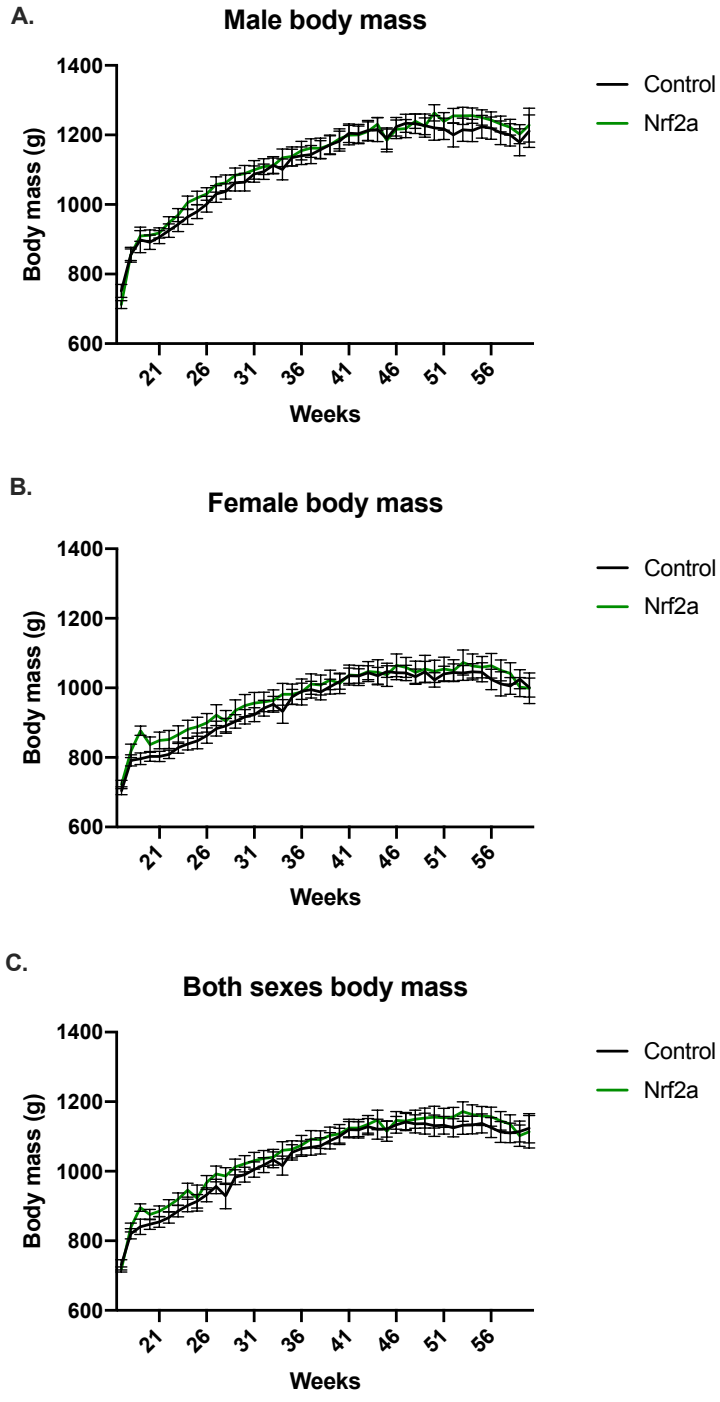


Figure S4.5: Changes in body mass in male and female guinea pigs. There was no effect of Nrf2a on the rate in growth or maximal body size compared to CON guinea pigs.

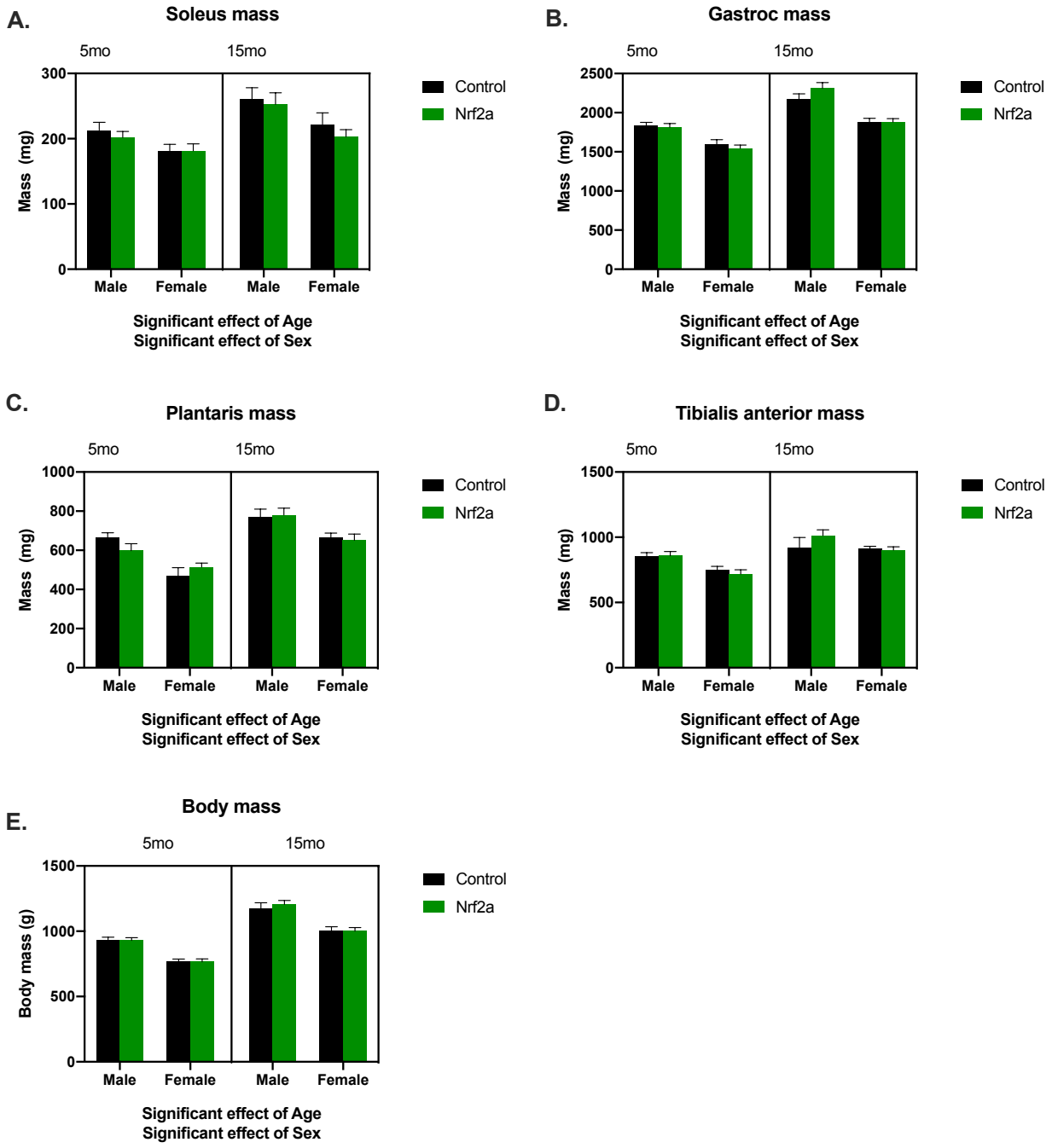
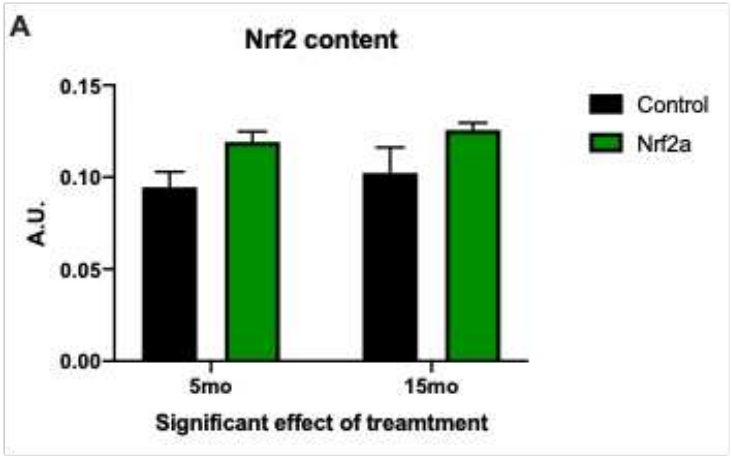
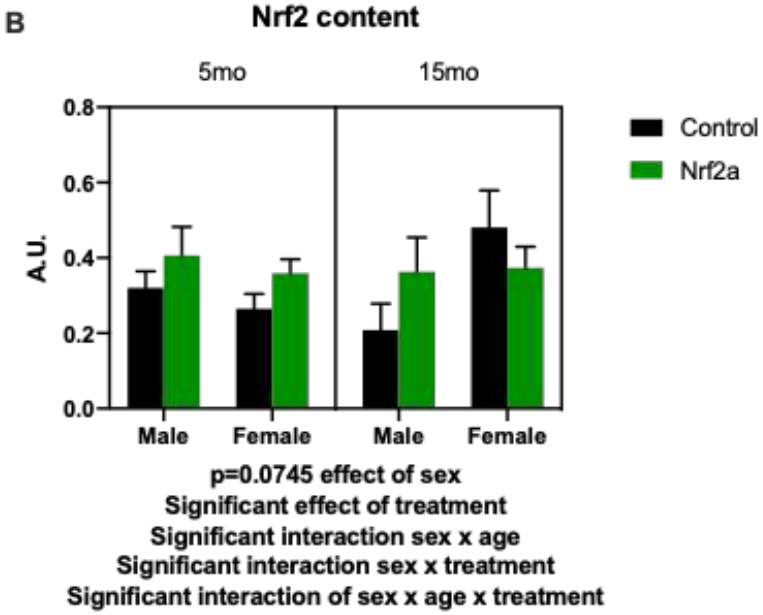


Figure S4.6: Differences in body and muscle mass between sexes and ages. There was a significant ($p<0.05$) increase in both body and muscle mass with age. Additionally, female guinea pigs had significantly ($p<0.05$) lower body mass compared to male counterparts. There was no effect of Nrf2a on body or muscle masses.





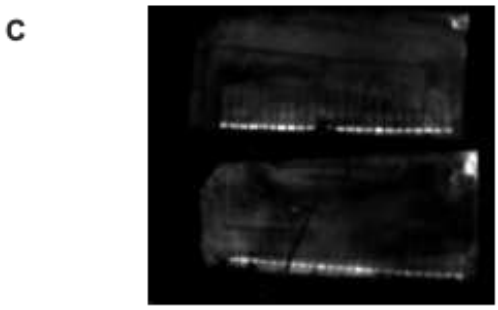


Figure S4.7: Age, sex, and treatment – related differences in Nrf2 content. There was a significant ($p<0.05$) effect of treatment (Figs S4.3A & S4.3B) to increase Nrf2 content in the gastrocnemius. Treatment mitigated the age-related decline in Nrf2 content in male guinea pigs, but not female guinea pigs (Fig S4.3B).

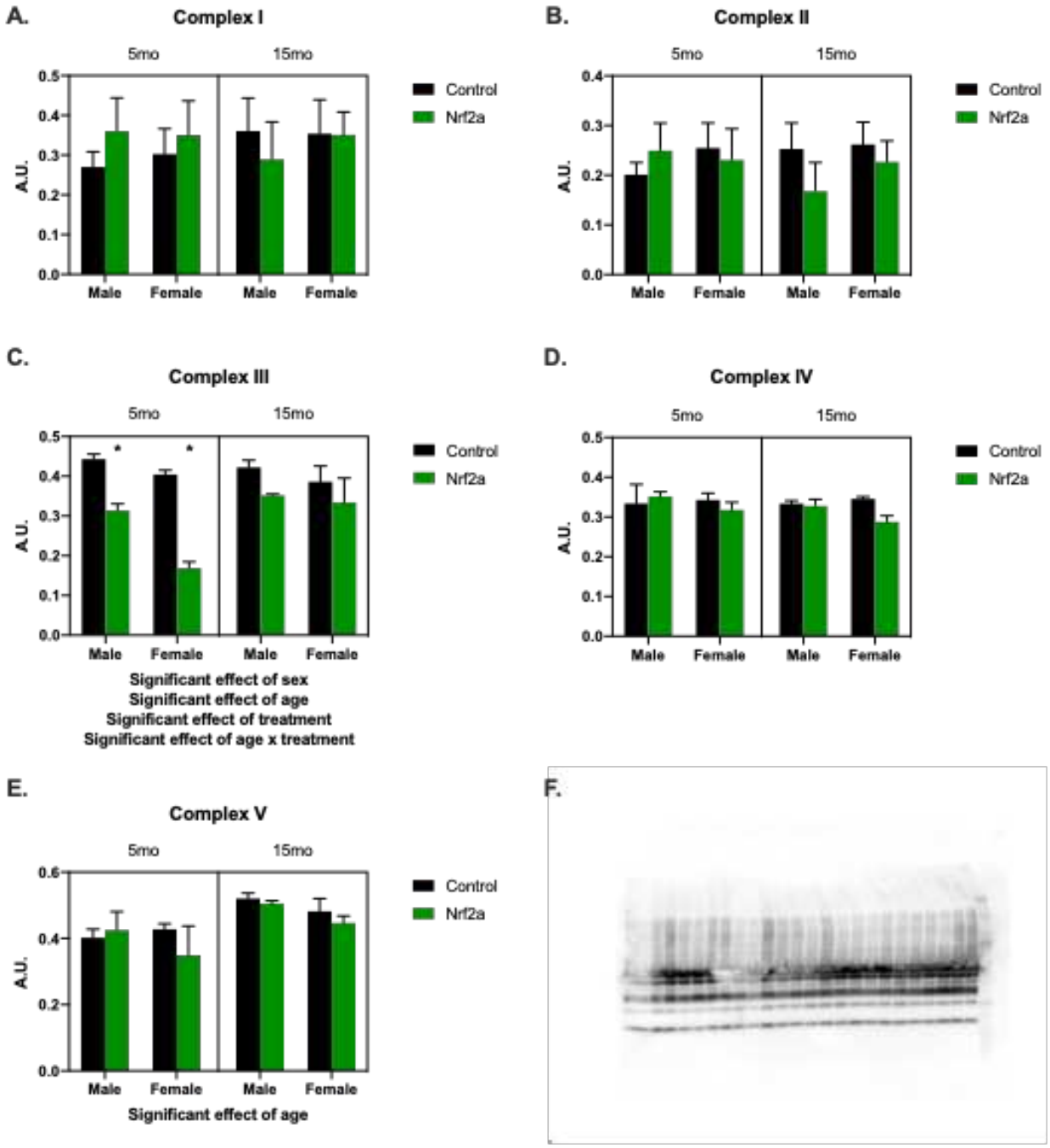


Figure S4.8: Western blot of OXPHOS proteins in the gastrocnemius. Nrf2a had no effect on content of Complexes I, II, IV, and V (Figs S4.4A, S4.4B, S4.4D, S4.4E). Nrf2a decreased Complex III content (Fig S4.4C). There was a significant age-related increase in Complex V content (Fig S4.4E). F: representative blot of treatment group in triplicate, young male and female, control and Nrf2a and old male and female, control and Nrf2a.

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CHAPTER 5 – OVERALL CONCLUSIONS

SUMMARY

In this series of studies, we examined the efficacy of targeting skeletal muscle energetics to mitigate age-related skeletal muscle changes in a preclinical model that resembles the sarcopenic phenotype in human skeletal muscle. In the first study, we established the Dunkin-Hartley guinea pig as a model for human musculoskeletal aging particularly focusing on impairments in skeletal muscle density and fiber size with underlying decrements in protein synthesis (Ch. 2). In the second study, we found that the observed age-related changes in skeletal muscle were associated with decrements in mitochondrial function. We then treated young and older Dunkin-Hartley guinea pigs with a Nrf2 activator and found that it improved mitochondrial function in age- and sex- specific manners (Ch. 3). Finally, in the last study, we determined that these improvements in mitochondrial function were associated with attenuating the age-related decline in protein synthesis and increased protein synthesis dedicated to proteostasis (Ch. 4). Altogether, this project helps establish a preclinical model for human musculoskeletal aging and supports the rationale that targeting skeletal muscle energetics could attenuate age-related decrements in skeletal muscle.

Most interventions to prevent or mitigate sarcopenia focus predominantly on the maintenance of protein anabolism and skeletal muscle size[1–5]. However, muscle size does not necessarily dictate muscle function[6] and muscle mass loss does not entirely account for loss of strength[7–9]. Mitochondrial function has a significant role in maintaining skeletal muscle function in humans. The Baltimore Longitudinal Study on Aging demonstrated that mitochondrial function explains 36% of the variability in cardiorespiratory fitness, 17% of gait speed, 11% of grip strength, as well as 17% of leg strength in humans aged 24 – 91 years[10]. Other studies investigating the role of mitochondrial dysfunction in the age-related loss in proteostasis and

skeletal muscle function highlight the central role mitochondrial dysfunction has in initiating and promoting the decline in skeletal muscle[11]. Aerobic exercise training, one of the most well-known interventions to stimulate mitochondrial biogenesis and improve mitochondrial function, delays skeletal muscle dysfunction with age[12–14]. While aerobic exercise training has a multitude of beneficial effects on health independent of improvements in mitochondrial function, there is growing evidence and consensus that targeting mitochondrial function can mitigate sarcopenia[15–18].

This series of studies demonstrates that targeting and improving mitochondrial function can mitigate the progression of sarcopenia. Nrf2a treatment attenuated the age-related decline in mitochondrial respiration in the soleus of both male and female Dunkin-Hartley guinea pigs (Figs 3.6 – 3.8). We hypothesized that because of the energetic cost of protein turnover, attenuation of mitochondrial dysfunction would mitigate declines in protein synthesis. Nrf2a indeed attenuated declines in fractional synthesis rates (FSR) in several enriched subfractions (mitochondrial, myofibrillar, and cytosolic enriched subfractions) of the soleus muscle (Fig 4.4). The attenuation of declines in the myofibrillar subfraction may have beneficial effects on overall muscle strength. In humans, a decline in proteome integrity in the myofibrillar fraction is associated with impaired muscle strength[19]. However, we did not directly measure skeletal muscle strength or myofiber force in these studies, thus future studies should assess the effect of Nrf2a on aging skeletal muscle function.

Dunkin-Hartley guinea pigs experience declines in musculoskeletal health beyond the skeletal muscle phenotype described in the current studies. They develop idiopathic, primary knee osteoarthritis as early as four months of age[20]. Both inflammation and oxidative stress contribute to osteoarthritis and sarcopenia in humans[21–23]. The Dunkin-Hartley guinea pigs also seem to experience an age-related increase in inflammation[20], which may underlie their propensity to develop an aged musculoskeletal phenotype in an accelerated manner. Thus,

because Nrf2a leads to transcription of cytoprotective genes that may decrease oxidative stress and inflammation, the overall purpose of the parent project was to determine if Nrf2a treatment could mitigate decrements in gait, an integrative and translatable outcome of human musculoskeletal health[24].

There are many components of gait that change with knee osteoarthritis and age. With age in general, one primary component of gait that changes is speed; in fact the decline in gait speed can predict mortality[10,25,26]. Compared to those without knee osteoarthritis, individuals with knee osteoarthritis have shorter stride length[27], slower walking speed and decreased range of motion[28]. In fact, stride length is one component of gait that clinicians use to assess knee and hip osteoarthritis progression[29]. We found that Nrf2a treatment improved stride length, in both male and female 15mo guinea pigs (unpublished data), signifying that Nrf2a improved musculoskeletal function in guinea pigs. Because gait is an integrative outcome, it is difficult to determine whether or not this improvement in gait is associated with improved skeletal muscle function. However, based on our study, it is probable that Nrf2a-mediated improvements in mitochondrial function and proteostasis in skeletal muscle, contributed to improvements in overall musculoskeletal function.

Few studies have linked mitochondrial dysfunction or improvements in mitochondrial function to improved proteostasis and skeletal muscle function. To date, most studies that implicate mitochondrial dysfunction in the age-related impairment of skeletal muscle proteostasis and function have been limited to *C. elegans*[11] or rodent models[30]. In *C. elegans,* mitochondrial dysfunction precedes the loss of proteostasis; and in mice, improving mitochondrial function leads to a decreased oxidatively damaged proteome. The present studies further support this notion by demonstrating an improvement in mitochondrial respiration was reflected in the maintenance of protein synthesis, a component of proteostasis, in skeletal muscle.

A strength of our study is having greater genetic variance in these outbred guinea pigs, which is reflective of the genetic heterogeneity of humans, compared to the commonly used inbred mouse strains. A trade off, though, is that this increased genetic variance perhaps translated to greater phenotypic or functional variability (e.g. mitochondrial function) which precluded us from being able to detect statistically significant differences between control and treated guinea pigs, particularly compared to inbred preclinical models. However, that we still observed significant effects of Nrf2a in these outbred animals strengthens the external validity as we translate this to more genetically diverse preclinical (e.g. dogs) and clinical models. Guinea pigs are also genetically more homologous to humans than other rodents[31] and, similar to humans, require vitamin C in their diet[32], which has important ramifications for diseases, such as sarcopenia[33,34], that are related to oxidative stress. Moreover, because we tested the Nrf2a treatment on both young and older, male and female guinea pigs, we were able to measure sex, age, and treatment effects on mitochondrial respiration and protein synthesis. Importantly, we also observed significant interactions between sex, age, and treatment that, with further research, may offer insight into the consistent sex-differences in the effects of Nrf2a in rodent models[35] as well as humans[36]. These studies provide evidence that targeting mitochondrial function with a Nrf2 activator can mitigate musculoskeletal dysfunction measured by a translatable clinical outcome (i.e. gait) in a pre-clinical model that bears greater resemblance to the aged musculoskeletal phenotype[37] (Ch. 2) and is genetically more similar to humans than other preclinical models.

MECHANISM OF ACTION

Nrf2 is a transcription factor that stimulates the expression of over 100 of cytoprotective genes[38]. This dissertation project predominantly focused on the effect of the Nrf2 activator PB125 on mitochondrial function and skeletal muscle proteostasis based on previous data from

our lab[36,39,40] its antioxidative effects[41], as well as the success of another phytochemical Nrf2 activator in extending median longevity in mice in the Interventions Testing Program (ITP) of the National Institutes of Aging[35]. PB125 is currently being testing in another arm of the ITP on its efficacy to extend longevity in heterogenous mice[42]. Our data from this project provides necessary insight into potential mechanisms in which long-term administration of PB125 could extend healthspan and/or improve longevity. Our studies show that Nrf2a improved two processes related to the aging, mitochondrial dysfunction and impaired proteostasis, which led to improved mobility (unpublished data) in these guinea pigs. However, at the moment is unclear how Nrf2a improved mitochondrial function and proteostasis.

Based on our results from Chapter 3, it is clear that Nrf2a improved mitochondrial respiration. Our working hypothesis is that Nrf2a enhanced mitochondrial respiration primarily through improvements in Complex I (Ch. 3), but it is unclear how Nrf2a elicited those improvements. Nrf2a neither attenuated the decline in mitochondrial biogenesis nor changed mitochondrial content in the gastrocnemius (Fig 4.3). While Nrf2a attenuated age-related declines in mitochondrial biogenesis the soleus (Fig 4.4), it is unclear how Nrf2a affected mitochondrial content. It is possible that Nrf2a increased mitochondrial content in the soleus, which could explain the improvements in skeletal muscle mitochondrial respiration (Ch. 3). However, if mitochondrial content remained the same in the soleus, the maintenance of mitochondrial protein synthesis in the soleus could be reflective of overall greater rates of mitochondrial protein turnover. Increased mitochondrial turnover would also indicate that Nrf2a may have attenuated age-related deficiencies in mitochondrial autophagy (mitophagy)[43,44]. Other groups have demonstrated that Nrf2 activators stimulate mitophagy[45–47], thus there is potential for Nrf2a to have sustained mitochondrial protein turnover in the present study. Mitophagy is thought to be a necessary process to maintain mitochondrial integrity and function and has been implicated in improving musculoskeletal health[43,48–51]. However further work is

necessary to measure the effect of Nrf2a on mitochondrial content and mitophagy in the soleus to better ascertain how Nrf2a maintained mitochondrial function.

Another potential mechanism in which Nrf2a could have improved and protected mitochondrial function from the aging process is through the upregulation of endogenous antioxidants. Nrf2a increased the content of antioxidant proteins[38]. Our lab has demonstrated that Nrf2 activator treatment protects endothelial cells and cardiomyocytes from oxidant-induced apoptosis[52,53] as well as increase antioxidant content in human skeletal muscle[36]. Thus, it is possible that Nrf2a in this project enhanced endogenous antioxidant capacity in the Dunkin-Hartley guinea pigs and protected from age-related oxidative damage. While we did not observe any changes in ROS emission with Nrf2a (Fig 3.9), it is possible that Nrf2a attenuated oxidative damage. Other mitochondrial-targeted antioxidant treatments enhance both mitochondrial function and cellular function[54,55]. Mitochondrial-targeted peptide SS-31 protects mitochondria from oxidative damage and enhances mitochondrial coupling and respiration[56], which we similarly observed with Nrf2a treatment (Ch. 3). Moreover, SS-31 protects mitochondria from oxidation and improves mitochondrial structural integrity and respiration and decreases ROS emission[30,57–60]. Thus, further research should investigate whether Nrf2a treatment improves mitochondrial function by protecting mitochondria from oxidative damage and improve mitochondrial integrity.

The attenuation of declines in mitochondrial respiration with Nrf2a were associated with mitigating the decline in skeletal muscle protein synthesis, though it is not possible to conclude whether or not one caused the other. However, because protein turnover is an energetically costly process[61], we hypothesize that the improvement mitochondrial respiration represents a greater capacity to generate ATP to support cellular functions related to proteostasis[61–63]. As a result of greater protein turnover and allocation of energy towards proteostasis, the integrity of the skeletal muscle proteome may have improved. Though Nrf2a may have had direct effects

on the cytosolic and myofibrillar proteome that also protected the proteome from damage by increasing antioxidant and anti-inflammatory enzymes, independent of changes in mitochondrial function. Evidence from these studies support the notion that improvements in mitochondrial function may have supported the attenuation of age-related declines in protein synthesis, which together supported maintenance of musculoskeletal function as demonstrated through improved gait.

GAPS AND FUTURE DIRECTIONS

While these studies provide promising support for targeting mitochondrial function to mitigate age-related impairments in skeletal muscle proteostasis and function, further research is necessary to discover the mechanisms in which Nrf2a improves both mitochondrial function and proteostasis in skeletal muscle. Such future endeavors include understanding how improvements in mitochondrial function and proteostasis affected the concentration of damaged proteins. As stated, improving mitochondrial function can decrease the accumulation of damaged proteins[11,30], which may prevent the accumulation of damaged proteins and improve contractile function[19,64,65]. Nrf2a could have also prevented oxidative damage from initially occurring through the upregulation of antioxidants[41]. However, it is unclear at the moment whether levels of protein oxidation changed with Nrf2a. Thus, future studies should compare measure the effect of Nrf2a on antioxidant capacity in these guinea pigs as well as relate the change Nrf2a-mediated changes in protein turnover and the concentration of oxidized proteins in skeletal muscle.

In both Chapters 3 and 4, we observed not only sex differences in mitochondrial function between guinea pigs, but also that sex influenced the effect of both age and the response to Nrf2a on mitochondrial function and protein turnover. Female guinea pigs had lower rates of mitochondrial respiration compared to male guinea pigs. Interestingly, Nrf2a increased rates of mitochondrial respiration, such as ADP Vmax, to similar levels as male guinea pigs. While, there

was an age-related decline in ADP Vmax, that decline occurred primarily in male guinea pigs and Nrf2a attenuated that decline in males only. We also observed sex differences in the relative amount of protein synthesis dedicated to proteostasis compared to proliferation (PRO:DNA). In the gastrocnemius, young female guinea pigs had lower proportion of protein synthesis allocated to proteostasis (PRO:DNA) in mitochondrial, cytosolic, and myofibrillar subfractions than young males (Fig 4.6). Interestingly, Nrf2a increased PRO:DNA in older females but had no influence in older males (Figs 4.8 & 4.9). These results suggest there may be a difference in how Nrf2a may have influenced the accumulation of damage in the proteome between these sexes. Future research should compare the difference in age-related changes in the concentration of damaged proteins between male and female guinea pigs as well as the effect of Nrf2a on these changes.

It is unclear what underlies the sex differences we observed, though estrogen is a likely candidate to mediate the effect of Nr2a treatment and the changes we observed[66], as some of the antioxidative effects of estrogen receptor beta stimulation may be partially mediated by Nrf2 activation[67]. Loss of serum estrogen post menopause in women is associated with greater levels of oxidation (GSSG/GSH)[68]. Moreover, hormone replacement therapy, which increased serum estrogen levels, led to an increase in antioxidant enzyme expression and decrease in oxidation levels (GSSG/GSH)[68]. It does not appear, though, that estrogen mediates Nrf2 activation, though the decline in Nrf2 content with age[69,70], may attenuate the antioxidative effect of estradiol and activation of estrogen receptor beta[67,71]. However, we did not find that 15mo female guinea pigs had lower Nrf2 content in the gastrocnemius compared to younger female guinea pigs (Fig S4.3). Future steps in the overall project should be to relate outcomes such as Nrf2a mediated improvements with circulating sex hormones like estrogen. It is unlikely that by 15 months of age, these guinea pigs were affected by menopause[72]. However, it is possible that the ~15 day long estrous cycle may have played a role in variance we observed in our

outcomes[72] as we did not control for the estrous cycle and when assessing mitochondrial function throughout the estrous cycle. The present project adds to the growing evidence that Nrf2 activation has sex-specific effects and thus warrants further investigation.

This collective project offers insight into a promising intervention that can mitigate age-related musculoskeletal dysfunction. Other Nrf2 activators have shown promise for extending median murine lifespan[35] and our lab demonstrated the safety and efficacy of using a Nrf2 activator to improve mechanisms of skeletal muscle myofibrillar proteostasis in humans[36]. Based in part on the findings of this project and past work, we are now conducting a pilot clinical trial to determine the effect of Nrf2 activator treatment on mitigating pain and improving mobility in both men and women with knee osteoarthritis. While this clinical trial will test the translatability of Nrf2 activator treatment into a clinical population, concurrent research should continue to address the gaps and future directions already highlighted.

LIMITATIONS

The purpose of the parent R21 project was to study the effects of long-term administration of a Nrf2 activator on musculoskeletal health. The purpose in this series of studies was to interrogate the effect of Nrf2a particularly on skeletal muscle mitochondrial function and proteostasis and whether that effect may be associated with improved musculoskeletal function. The integrative approach of targeting musculoskeletal decline is both a strength and a limitation of these studies. While we observed improvements in an important functional outcome (i.e. gait), it is difficult to discern whether or not the improvements can be attributed strictly to improvements we observed in skeletal muscle or to the other components of the musculoskeletal system. For example, our collaborators have observed that Nrf2a improved the knee joint (i.e. decreasing osteoarthritis severity) (unpublished data) as well as bone integrity (unpublished). Given the integrative nature of gait, it is likely that all three components of the musculoskeletal system mediated the improvement in stride length. However, in order to

link improvements in mitochondrial energetics and skeletal muscle proteostasis to improved muscle function, more specific measures of skeletal muscle protein damage and function, such as contractile strength, are necessary.

CONCLUSION

Nrf2 activators are a class of compounds that hold promise for healthspan extension[40]. Various Nrf2 activators have been used to protect cells from oxidative challenges[52,53], preserve skeletal muscle strength[45,73], and extend median lifespan in preclinical models[35]. This project adds to the growing literature that supports the use of Nrf2 activators to improve organismal health. In these series of experiments, we characterized the decline in skeletal muscle function in the Dunkin-Hartley guinea pig, a model for musculoskeletal dysfunction. We studied the long-term adaptations of Nrf2 activator treatment on skeletal muscle mitochondrial function and proteostasis in these guinea pigs. We found that Nrf2a concomitantly improved mitochondrial function and skeletal muscle proteostasis. More importantly, we, and our collaborators, also found that Nrf2a also improved an integrative measure of musculoskeletal health. More research is necessary to understand the effects of Nrf2a on skeletal muscle protein integrity and function. However, these studies altogether provide necessary evidence to establish the efficacy of Nrf2a treatment to improve musculoskeletal function and organismal health.

There is growing interest in targeting mitochondrial function to mitigate age-related chronic diseases, such as sarcopenia[15,17,18,74,75]. Adequate mitochondrial function is essential for overall cellular function and integrity. Mitochondrial dysfunction is a hallmark of aging as it is implicated in chronic diseases, such as sarcopenia, and leads to the impairment of other critical cellular functions, such as proteostasis[76]. Improving mitochondrial function is a characteristic of slowed aging interventions and long-lived species[77] and supports the energetic requirements to maintain other essential cellular processes, such as proteostasis[18]. The data from this

dissertation further add to the growing literature that targeting mitochondrial dysfunction is an efficacious intervention to mitigate age-related declines in components of proteostasis in skeletal muscle and improve overall musculoskeletal function.

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