Effects of Aging and Exercise on Mitochondrial Physiology

by

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Abstract

Mitochondria are organelles which main function is to generate energy in the form of ATP, although they are involved in a myriad of cellular processes. Mitochondria form a dynamic network that responds to the energetic demands of the cell, and that undergoes constant remodeling through biogenesis, fusion, fission, and mitophagy. Proper mitochondrial function is essential for the maintenance of health, and their (dys)function is involved in aging and in the development of several pathologies. Physical exercise is one of the best non-pharmacological interventions to improve mitochondrial function. Chapter 1 reviews the skeletal muscle ribosome and mitochondrial biogenesis in response to different exercise training modalities. Previous work has suggested a possible competition between both processes. However, the available literature suggests that untrained individuals present a generic response to exercise, with either resistance training (RT) or endurance (ET) training stimulating both ribosome and mitochondrial biogenesis. As the individual becomes trained in a particular modality, mitochondrial biogenesis is prioritized with endurance training, while ribosome biogenesis is prioritized with resistance training. Chapter 2 investigated the effects of aging and long-term physical activity on mitochondrial physiology and redox state of the cortex and cerebellum of female rats. There were minimal changes in several markers of mitochondrial content, function, and dynamics in the cortex and cerebellum in response to both aging and long-term physical activity. Furthermore, the redox status of the tissues investigated remained overall unaltered. The results suggest that the brain mitochondrial physiology and redox homeostasis of females may be more resilient to the aging process than initially thought. Chapter 3 investigated the acute and chronic effects of RT on skeletal muscle markers of mitochondrial remodeling in older adults. Ten weeks

of RT increased mitochondrial protein content and markers of mitochondrial dynamics, although no changes in these markers were detected following the first training bout. Importantly, the results show that besides the known improvements in muscle mass and strength, RT could be a viable approach to improve mitochondrial health in older adults. Chapter 4 investigated the effects of RT on the redox status of skeletal muscle in older adults. Six weeks of RT significantly decreased oxidative damage to lipids and increased the activity of different antioxidant enzymes. Therefore, RT may be a viable approach to counteract a possible age-related disruption of skeletal muscle redox homeostasis in older adults. Lastly, chapter 5 investigated the effects of prior RT on the molecular and performance adaptations to subsequent ET in humans. The results showed that prior RT had no additional benefits on adaptations to ET. Even though both groups had similar endurance performance improvements, most mitochondrial adaptations to ET were blunted in the RT+ET group, but such impairments seem to be related to the cessation of RT.

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General Introduction

Mitochondria are organelles in our cells which main function is to generate energy in the form of adenosine 5'-triphosphate (ATP) through a process known as oxidative phosphorylation. Recent scientific advances have demonstrated that mitochondria are also involved in a variety of cellular functions, including regulation of the oxidative status of the cell, calcium homeostasis, and cell death (van der Bliek et al., 2017).

Because of their important role in energy production, mitochondria are highly abundant in tissues with a high energy demand, such as skeletal muscle and brain. In skeletal muscle, mitochondria form a highly complex and dynamic network that responds to the energetic demands of the cell (Glancy et al, 2015). Mitochondria undergo constant remodeling through the generation of new mitochondria (biogenesis), joining (fusion), splitting (fission), and degradation of dysfunctional portions (mitophagy) (Youle & Bliek, 2012). Adequate remodeling processes are essential for maintaining functional mitochondria (Tilokani et al., 2018; Youle & Bliek, 2012).

Reflective of their involvement in several cellular processes, proper mitochondrial function is essential for the maintenance of health. The loss of mitochondrial function and/or the impairment of mitochondrial dynamics have been implicated in the development of several pathologies, such as sarcopenia and neurodegeneration (Carter et al., 2015; De Mario et al., 2021). In addition, mitochondrial (dys)function is considered a major player in the aging process. Several studies have shown lower mitochondrial content and function in older individuals (Carter et al., 2015; Russel et al., 2014). Tightly linked to the loss of mitochondrial function, aging is

also characterized by an impaired redox state of cells, with lower capacity of the antioxidative defense systems and increased oxidative damage to molecules (Dai et al., 2014).

Physical exercise is known to positively affect mitochondrial physiology and possibly counteract age- and pathology-related decreases in mitochondrial function. Historically, there is a common distinction between resistance training (RT) and endurance training (ET), in which RT promotes increases in muscle mass and strength, and ET promotes cardiovascular and mitochondrial adaptations. Indeed, various forms of ET, including high-intensity interval training (HIIT), have been shown to increase mitochondrial content and function (Granata et al., 2018; Jacobs et al., 2013). However, emerging evidence highlights that RT may also promote positive mitochondrial adaptations (Lee et al., 2018; Lim et al., 2019).

The purpose of the present dissertation was to investigate the effects of aging and exercise on different aspects of mitochondrial physiology. Through a series of studies, each corresponding to a different chapter herein, I have investigated the effects of aging and different modalities of exercise on skeletal muscle and brain mitochondrial physiology, and the differences and interplay between RT and ET molecular and performance adaptations.

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Chapter 1 - Skeletal Muscle Ribosome and Mitochondrial Biogenesis in Response to Different Exercise Training Modalities

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INTRODUCTION

Research interest in the fields of ribosome and mitochondrial biogenesis has been growing considerably over the last decades. While the number of overall publications listed on MEDLINE has been increasing steadily during the last 20years (~200% increase when comparing 2020–2000), during the same period, there was an even greater increase (over 2,500%) in the number of publications with the search terms "ribosome biogenesis" or "mitochondrial biogenesis." Much of the research in "ribosome biogenesis" and/or "mitochondrial biogenesis" has dealt with cancer biology (Derenzini et al., 2017; Vanderveen et al., 2017; Pelletier et al., 2018), aging (Tiku and Antebi, 2018; Correll et al., 2019; Roque et al., 2020), and other disciplines unrelated to exercise physiology. However, in recent years, several exercise physiology laboratories have been utilizing more mechanistic molecular tools to study the adaptations that occur with exercise to discern the well documented health and/or performance benefits following exercise.

Ribosome and mitochondrial biogenesis are both complex processes. A detailed description of the molecular underpinnings of each process is beyond the scope of this review and readers are referred to other excellent reviews on the topics [ribosome biogenesis (Henras et

al., 2015; Kressler et al., 2017), mitochondrial biogenesis (Jornayvaz and Shulman, 2010; Bouchez and Devin, 2019)]. For the purpose of this review, ribosome biogenesis refers to the de novo synthesis of ribosomes, a process that involves the transcription and processing of rRNA and the assembly of several ribosomal proteins. The rate-limiting step of ribosome biogenesis is thought to be generation of the 45S pre-rRNA by RNA Polymerase I (Kopp et al., 2007). This precursor is then processed, yielding the 18S, 5.8S, and 28S mature rRNA transcripts. These transcripts are exported to the nucleus and associate with 5S rRNA and different ribosomal proteins resulting in the assembly of the mature ribosome (Kressler et al., 2017). Mitochondrial biogenesis is accomplished through the recruitment of newly synthesized mitochondrial proteins to existing organelles, which can grow and divide (Ryan and Hoogenraad, 2007; Miller and Hamilton, 2012). Mitochondrial biogenesis involves the transcription of proteins encoded by both nuclear and mitochondrial genomes. Considered a major regulator of mitochondrial biogenesis, peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) activates nuclear respiratory factors, increasing nuclear transcription of mitochondrial genes (Ryan and Hoogenraad, 2007). These nuclear respiratory factors activate mitochondrial transcription factor A (TFAM), which promotes the transcription and replication of mitochondrial DNA (Wu et al., 1999).

Importantly, researchers frequently use activation markers of cell signaling pathways, mRNA expression, protein levels, and/or enzymatic activity as a measure of mitochondrial biogenesis. However, as nicely discussed by Miller and Hamilton (2012), such variables present important limitations (i.e., measure of organelle content instead of biogenesis process per se, disregarding degradation processes), and although informative, are not direct measures of biogenesis. The only direct measure of mitochondrial biogenesis currently available is the

measure of mitochondrial protein synthesis using stable isotopic tracers. Similarly, ribosome biogenesis is usually assessed through measurements of cell signaling, mRNA expression, protein levels, total RNA and/or rRNA levels, variables that are at best only indirect measures of biogenesis. Tracer methodologies have also been developed and used to measure de novo ribosomal biogenesis (Brook et al., 2017). However, considering the fact that only few exercise training studies have used direct measures of ribosome and/or mitochondrial biogenesis, studies using indirect measures will be included and discussed in the current review. Readers are strongly encouraged to consider whether direct or indirect measures were used when interpreting the results of the studies presented herein.

Resistance and endurance training increase skeletal muscle ribosome biogenesis and mitochondrial biogenesis, respectively. Mitochondrial biogenesis increases aerobic capacity (Costill et al., 1976; Burgomaster et al., 2008; Yeo et al., 2008; Murias et al., 2011; Cochran et al., 2014; Vigelso et al., 2014), and ribosome biogenesis has been associated with skeletal muscle hypertrophy [reviewed in (Chaillou et al., 2014; Wen et al., 2016; Mcglory et al., 2017; Bamman et al., 2018; Roberts et al., 2018a; Figueiredo and Mccarthy, 2019; Kim et al., 2019)]. It is generally believed that skeletal muscle adaptations to exercise are highly specific. Increased ribosome biogenesis with resistance training is seemingly prioritized over mitochondrial biogenesis (Wilkinson et al., 2008; Figueiredo et al., 2021), while there is evidence to suggest increased mitochondrial biogenesis with endurance training is prioritized over ribosome biogenesis (Morrison et al., 1989; Gibala et al., 2009). In addition, an interference effect may occur if both endurance and resistance exercise are included in the same training session or program (i.e., concurrent training). For example, published reports show that endurance training compromises muscle hypertrophy response to resistance training (Kraemer et al., 1995; Jones et

al., 2013) and, although researchers have tried to unveil the mechanisms underlying the interplay between resistance and endurance training, the molecular underpinnings of these observations are still unclear. However, evidence suggests that both processes can occur simultaneously (Tang et al., 2006; Fyfe et al., 2016, 2018). Therefore, the purpose of this review is to discuss whether this curious competition between mitochondrial and ribosome biogenesis exists during different exercise training programs (i.e., only resistance training, only endurance training, or concurrent training) and show the available evidence both in favor and against it. We also discuss whether both processes can concomitantly increase with certain exercise training paradigms and provide future research avenues in this area of exercise physiology.

AMPK AND mTOR SIGNALING HELP REGULATE MITOCHONDRIAL AND RIBOSOME BIOGENESIS, RESPECTIVELY

Two critical signaling proteins that facilitate the adaptive responses to exercise training include the 5' AMP-activated protein kinase (AMPK) and the mechanistic target of rapamycin (mTOR). As an important regulator of cellular energy homeostasis, AMPK is a hetero-trimeric cytosolic enzyme with a catalytic α-subunit and regulatory β and γ subunits. The α-subunit phosphorylates cytoplasmic and nuclear proteins to affect the expression of various mRNAs. High adenosine monophosphate (AMP) concentrations during exercise (as a result of high ATP turnover) lead to increased binding of AMP with AMPK (Richter and Ruderman, 2009), but it has been shown that ADP could also activate AMPK (Oakhill et al., 2011). In addition, glycogen interacts with the β-subunit of AMPK, and muscle glycogen depletion during exercise results in the loss of the interaction between these molecules, which increases AMPK activity (Steinberg et al., 2006). Stress-responsive proteins, such as serine/threonine kinase 11 and

calcium/calmodulin-dependent protein kinase 2, can also act to phosphorylate AMPK at the Thr172 residue and increase its activity (Richter and Ruderman, 2009). Evidence in multiple cell lines and tissues suggests that increased AMPK signaling facilitates mitochondrial gene expression to provide for mitochondrial biogenesis (Reznick et al., 2007; Yan et al., 2013; Marin et al., 2017). In this regard, endurance exercise studies with rodents and humans have shown AMPK signaling and mRNAs involved in mitochondrial biogenesis increase hours following exercise (Fujii et al., 2000; Atherton et al., 2005; Jorgensen et al., 2005). Additionally, researchers have used the muscle-specific double knockout AMPK β1 and β2 mouse model (β1β2M-KO) to demonstrate functional AMPK is critical in maintaining muscle mitochondrial content (O'neill et al., 2011).

The mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway is widely recognized as a regulatory hub for overload-induced skeletal muscle hypertrophy (Goodman, 2019). mTORC1 is a multi-subunit complex that consists of the mTOR protein as well as Raptor and mTOR associated protein LST8 homolog (mLST8; Saxton and Sabatini, 2017). Like AMPK, active mTORC1 complexes possess kinase activity to phosphorylate downstream proteins that facilitate the assembly and initiation of translation-competent ribosomes. Bodine et al. (2001) were the first to demonstrate mTOR signaling was required for muscle hypertrophy. Specifically, the authors administered rapamycin (an mTOR inhibitor) to mice, and observed synergist ablation-induced hypertrophy was completely abrogated in the plantaris muscle. Human studies have since shown that phosphorylation of mTOR and its downstream substrates (i.e., p70s6k, 4EBP1) are critically involved in facilitating post-exercise increases in muscle protein synthesis (Drummond et al., 2009; Gundermann et al., 2014). Further, acute increases in mTOR signaling markers following one bout of resistance exercise are associated with muscle hypertrophy

following weeks of resistance training (Terzis et al., 2008; Hulmi et al., 2009; Mayhew et al., 2009; Mitchell et al., 2013). Aside from upregulating muscle protein synthesis, more recent evidence suggests mTOR signaling regulates ribosome biogenesis across multiple cell lines [reviewed in (Mayer and Grummt, 2006)]. Notably, Nader et al. (2005) were the first to demonstrate this mechanism occurs in skeletal muscle cells in vitro. Von Walden et al. (2016) later demonstrated that mTOR signaling enhances ribosome biogenesis in skeletal muscle cells in vitro by modifying chromatin at the rDNA promoter. For further information on this topic, readers are encouraged to refer to other excellent reviews (Kim et al., 2019; Von Walden, 2019).

WHAT EVIDENCE IS THERE SUGGESTING MITOCHONDRIAL AND RIBOSOME BIOGENESIS MAY COMPETE?

Several lines of evidence exist suggesting skeletal muscle mitochondrial and ribosome biogenesis may compete at the molecular level in response to different modes of exercise training. For instance, we have reported that Otsuka Long-Evans Tokushima Fatty rats exposed to 12weeks of treadmill training demonstrated ~60% lower total RNA per mg wet tissue (a surrogate of skeletal muscle ribosome density) compared to untrained animals (Romero et al., 2017), and data from these same animals showed skeletal muscle citrate synthase activity (a surrogate of mitochondrial volume) was ~16% higher in trained vs. untrained animals (Martin et al., 2012). While we did not assess markers of AMPK activation it is notable that others have shown treadmill running results in acute increases in markers of AMPK activity following exercise (Ruderman et al., 2003). Other rodent studies partially agree with our findings. For instance, Morrison et al. (1989) reported that rats that underwent 2weeks of treadmill training had greater hindlimb citrate synthase activity (~40%, $p<0.05$) compared to untrained rats, while

18S rRNA (a surrogate of ribosome density) was similar between groups. Hayase and Yokogoshi (1992) reported that rats that underwent 7days of treadmill exercise had non-significantly lower levels of total RNA/mg protein in the mixed gastrocnemius muscle (−5.6%, p=0.060) and soleus muscle (−4.7%, p=0.111) compared to untrained rats.

Regarding human studies, transcriptomic results from the 20-week HERITAGE cardiovascular training study indicated that certain ribosomal mRNAs in the vastus lateralis were downregulated from pre-to post-training (Teran-Garcia et al., 2005). Additionally, Wilkinson et al. (2008) used a 10-week unilateral leg training protocol to demonstrate the differential molecular adaptations to resistance vs. endurance training. Specifically, 10 healthy men with minimal training > 8months prior to the initiation of the study trained one leg using the knee extensor exercise (2–3days per week) and the other leg using a cycle ergometer (2–3days per week). The authors reported that basal myofibrillar protein synthesis rates increased from pre- to post-intervention within the resistance-trained leg only (~0.08%/h at POST vs. ~0.06%/h at PRE). Myofibrillar protein synthesis rates were also greater at the 10-week time point in the resistance vs. endurance-trained leg. While markers of ribosome biogenesis were not assessed, these data suggest resistance training may have increased ribosome density via biogenesis given the ~30% increase in basal myofibrillar protein synthesis rates. These data additionally suggest ribosome biogenesis was likely unaffected with endurance training. A recent study conducted by Figueiredo et al., (2021) supports the competition between mitochondrial and ribosome biogenesis theory. The authors investigated the genetic and epigenetic regulation of ribosome biogenesis with either endurance or resistance exercise and found that markers of ribosome biogenesis were increased with resistance exercise but decreased with endurance exercise (30min post-exercise). In addition, the authors reported that, in general, resistance exercise activated the

mTOR pathway while endurance exercise activated the AMPK pathway. Collectively, these studies suggest endurance training does not alter ribosome biogenesis or may interfere with certain aspects of the process. However, more human endurance training studies are needed before definitive conclusions can be drawn.

Despite sparse evidence linking endurance training to unaltered or decreased ribosome biogenesis, several human studies have shown that resistance training increases ribosome density (as measured by total RNA per mg tissue; Kadi et al., 2004; Figueiredo et al., 2015; Stec et al., 2016; Brook et al., 2017; Reidy et al., 2017; Mobley et al., 2018; Hammarstrom et al., 2020). Separate reports have also shown that resistance training does not alter or decreases mitochondrial volume (as measured by citrate synthase activity assays or transmission electron microscopy; Macdougall et al., 1982; Luthi et al., 1986; Tesch et al., 1987; Parise et al., 2005; Porter et al., 2015). It is uncommon for the same study to report both variables. However, two human studies from our laboratory have examined changes in markers of skeletal muscle ribosome density and mitochondrial volume in response to resistance training. In one study, untrained young men participated in 12weeks (3days per week) of full-body resistance training (Roberts et al., 2018b), and following training, total RNA per mg tissue (vastus lateralis) increased by 23% ($p<0.05$), while vastus lateralis citrate synthase activity non-significantly decreased by 11% ($p=0.064$). Similar to these findings, we reported 6weeks of unaccustomed high volume resistance training in previously-trained young men increased vastus lateralis total RNA per mg tissue by 28% ($p<0.05$). In contrast, vastus lateralis citrate synthase activity decreased by 12% ($p<0.05$; Haun et al., 2019). Critically, both studies suggest ribosome biogenesis occurred with unaccustomed resistance training, whereas mitochondrial biogenesis either did not occur or was delayed relative to increases in myofiber hypertrophy. In addition,

Hanson et al. (2019) found that performing a bout of endurance exercise before resistance exercise led to an acute decrease in markers of ribosome biogenesis compared to resistance exercise alone. However, it is important to note that markers of ribosome biogenesis were restored 3h post-exercise.

To summarize, several human studies suggest that unaccustomed resistance training increases ribosome density (likely through increased ribosome biogenesis), whereas mitochondrial density remains constant or decreases. Whether or not decrements in citrate synthase activity in these studies resulted from "mitochondrial dilution" via skeletal muscle hypertrophy rather than a decrease in mitochondrial biogenesis and/or a loss in mitochondria is debatable and is discussed elsewhere (Groennebaek and Vissing, 2017). Notably, most studies only used measures of ribosome and/or mitochondrial content or other indirect measures of biogenesis and their results should be interpreted with caution. Given the overall lack of data in this area, more research is needed to interpret the relevance of these findings.

WHY WOULD MITOCHONDRIAL AND RIBOSOME BIOGENESIS COMPETE WITH ONE ANOTHER IN RESPONSE TO EXERCISE TRAINING?

Ample molecular evidence exists to explain why mitochondrial and ribosome biogenesis may compete with one another during periods of exercise training. First, AMPK mechanistically blocks mTORC1 signaling through direct phosphorylation of the complex (Shaw, 2009) as well as through the phosphorylation and activation of the hamartin-tuberin (TSC1/2) complex [reviewed in (Shaw, 2009)], which is an upstream inhibitor of mTORC1 signaling. Given the proposed role mTORC1 signaling has on skeletal muscle ribosome biogenesis, it seems plausible that this process is impaired during situations of heightened AMPK signaling. In support of this

hypothesis, we have reported that treating C2C12-derived myotubes with 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR, a stimulator of AMPK activity) for 6hours reduced 47S pre-rRNA levels by 16% compared to vehicle-treated cells (Mobley et al., 2016); notably, while a one-way ANOVA with multiple cell culture treatments indicated no difference between the groups in our publication, a direct comparison between AICAR and vehicle-treated cells indicated $p<0.05$ between these two conditions. Researchers have also reported similar phenomena in other cell lines. For instance, several AMPK activators (e.g., phenformin, resveratrol, and AICAR) have been shown to disrupt nucleolar organization and inhibit ribosomal RNA synthesis in LLC-PK1 kidney proximal tubule epithelial cells (Kodiha et al., 2014). In HEK293T cells, glucose deprivation-induced AMPK activation has been reported to lead to increased phosphorylation of the RNA polymerase I-associated transcription factor TIF-IA at Ser635 (Hoppe et al., 2009). This phosphorylation event reduced the interaction of TIF-IA with other transcription factors and ultimately reduced the assembly of functional transcription initiation complexes at the rDNA promoter. Others have also shown a reduction in ribosome biogenesis in COS7 and HEK293 cells and transgenic mice overexpressing γ2-AMPK (Cao et al., 2017). Thus, it is apparent that a conserved outcome of AMPK activation in several cell types involves inhibition of ribosome biogenesis.

Evidence also exists suggesting mTORC1 signaling may reduce certain aspects of mitochondrial biogenesis. For instance, (Deepa et al., 2013) reported mRNAs involved with mitochondrial biogenesis (i.e., Ppargc1a, Nrf1, and Esrra) increased in the white adipose tissue of female db/db mice administered rapamycin (an mTOR inhibitor) for 6months. Chiao et al. (2016) reported that cardiac muscle mitochondrial biogenesis markers (i.e., PPARGC1A and TFAM protein levels) increased during the first 2weeks of a 10-week rapamycin feeding

experiment in mice. There are also data showing mTORC1 signaling may disrupt autophagy, which in turn may affect mitochondrial remodeling (Choi et al., 2012). This is relevant to the competition paradigm given that autophagy is critical for mitochondrial remodeling and function in skeletal muscle cells in vitro (Sin et al., 2016). Furthermore, Johnson et al. (2014) presented evidence of reciprocal regulation of protein synthesis in the cytosol and the mitochondria of human embryonic kidney cells. The authors found that amino acid starvation led to an inhibition of mTORC1 and a decrease in cytosolic protein synthesis, whereas there was an increase in active AMPK, mitochondria density (i.e., increased citrate synthase activity), mitochondrial translation and function. Collectively, several lines of evidence support the notion that AMPK activation impairs ribosome biogenesis, and some evidence suggests that mTORC1 signaling may negatively affect certain aspects of mitochondrial biogenesis. However, the latter data are not as conclusive.

Aside from the aforementioned AMPK and mTORC1 data, sequencing data from human blood cells show ribosomal DNA (rDNA) and mitochondrial copy number (or "dose"), both of which can vary between individuals, are inversely correlated between one another (Gibbons et al., 2014). In explaining these findings, the authors suggested a tight regulatory relationship exists between rDNA abundance, the mRNA expression of ribosomal proteins, and mitochondrial DNA (mtDNA) abundance. While these data are provocative in making the case for ribosome and mitochondria competition, determining whether this relationship exists in skeletal muscle remains unknown.

Finally, ribosome biogenesis and mitochondrial biogenesis require cellular energy that is greater than metabolic homeostasis. In general, transcription and translation are ATP-consuming processes (Lynch and Marinov, 2015). The 80S ribosome contains 79 proteins and four rRNAs,

and there are ~1,500 mitochondrial proteins (Boengler et al., 2011). Thus, the transcription of these components requires ATP, and the translation of mRNAs into protein requires additional ATP. It has also been suggested that ribosome assembly in eukaryotes is an energy-consuming process given that the nuclear export and assembly of the ribosome subunits involves various nucleotide-hydrolyzing enzymes (Strunk and Karbstein, 2009). Rodent studies have reported that muscle ribosomes and mitochondria exhibit rapid decay rates in response to unloading schemes (Steffen and Musacchia, 1984; Wagatsuma et al., 2011). These findings also support the notion that maintaining ribosome and mitochondrial densities are an energetic burden to muscle cells. Therefore, aside from the aforementioned mechanisms, which may contribute to the competition between ribosome and mitochondrial biogenesis, these latter points call into question as to whether or not muscle cells have the "energy bandwidth" to simultaneously promote both processes.

WHAT EVIDENCE IS THERE SUGGESTING MITOCHONDRIAL AND RIBOSOME BIOGENESIS DO NOT COMPETE?

To this point, we have provided evidence in favor of the biogenesis competition paradigm, in which ribosome biogenesis is prioritized with resistance training while mitochondrial biogenesis is prioritized with endurance training, or an interference effect is observed when both modes of exercise are performed concurrently. However, there is also evidence available suggesting that both processes can occur simultaneously. Tang et al. (2006), for example, reported increased muscle fiber hypertrophy and mitochondrial density (i.e., citrate synthase activity) in young males after 12weeks of resistance training, although markers of ribosomal or mitochondrial biogenesis were not assessed. Our laboratory has also reported

increased citrate synthase activity after resistance training in a cohort of older participants, concomitantly with an increase in hypertrophy (Lamb et al., 2020). The same cohort of participants showed an increase in protein content of the mitochondrial electron transport chain complexes and markers of mitochondrial remodeling (Mesquita et al., 2020). However, there was no change in PGC-1a and TFAM protein content, and the activation of these signaling pathways were not interrogated. Nonetheless, our studies highlight the possibility that age and/or fitness status may play a role in the interaction between the biogenesis processes.

Notably, studies using tracer methodology to directly measure ribosome and mitochondrial biogenesis have shown that resistance training is capable of increasing both ribosome (Sieljacks et al., 2019) and mitochondrial biogenesis (Groennebaek et al., 2018). Regarding changes in the signaling pathway involved in mitochondrial adaptations, resistance training increased ACC (Ser79) and p38-MAPK phosphorylation, but AMPK phosphorylation remained unchanged (Groennebaek et al., 2018). Importantly, the authors reported no significant correlation between mitochondrial protein synthesis and changes in citrate synthase activity. Similarly, even though total RNA content also increased in Sieljack et al. (2019) study, the authors found no significant difference between total RNA content and RNA synthesis rate. The results of both studies suggest that resistance exercise can lead to both ribosome and mitochondrial biogenesis but reinforce the need to be careful when using measures of organelle content (i.e., citrate synthase activity and total RNA content) as an indicative of biogenesis.

In addition, concurrent training, which involves simultaneously engaging in resistance and endurance training, is a prime candidate for increasing ribosome and mitochondrial biogenesis. A landmark study by Hickson (1980) showed that concurrent training interfered with strength and hypertrophy adaptations when compared with resistance training alone. However, a

comprehensive review by Fyfe et al. (2014) challenges the notion as to whether concurrent training interferes with resistance training adaptations. Moreover, a series of meta-analyses (Denadai et al., 2017; Murlasits et al., 2018; Sabag et al., 2018) suggest the interference effect elicited through endurance training is contextual and depends on factors such as endurance training modality (e.g., run training vs. cycle training) as well as endurance training frequency and duration. A number of other variables can be manipulated and potentially affect the outcome, including the training timing, which mode of training is done first, the time between the two bouts (hours or days), and whether nutritional support is given between bouts.

Furthermore, studies show that concurrent training increases maximal aerobic capacity as well as strength and hypertrophy (Mccarthy et al., 1995; Balabinis et al., 2003; Sillanpaa et al., 2008; Lundberg et al., 2014). These studies did not determine if phenotypic changes coincided with increased mitochondrial and ribosome biogenesis. However, Fyfe and colleagues have published two reports suggesting concurrent resistance training and high-intensity interval training may increase both processes. The first study (Fyfe et al., 2016) showed that compared with resistance exercise only, high-intensity interval training and resistance exercise enhanced ACC phosphorylation (Ser79; a readout of AMPK activity), PPARGC1A mRNA expression (suggestive of increased mitochondrial biogenesis), and mTOR phosphorylation [Ser2448; which may indicate enhanced mTOR activity, although this has been debated (Figueiredo et al., 2017)]. The second study by Fyfe et al. (2018) involved three groups of participants who undertook resistance training only, high-intensity interval training + resistance training, or moderate-intensity continuous training + resistance training for 8weeks. Following the training intervention, basal 45S pre-rRNA, 28S rRNA, and 5.8S rRNA expression were greater in the two groups that incorporated high-intensity interval training or moderate-intensity continuous

training vs. resistance training alone. Total RNA per mg tissue also increased in the high-intensity interval training + resistance training, or moderate-intensity continuous training + resistance groups by ~20–30%, albeit these increases were not statistically significant. Lundberg et al. (2014) have also reported that 5weeks of concurrent training increases quadriceps hypertrophy (+6%), endurance performance (+22%), and muscle citrate synthase activity (+18%).

Moreover, the order of exercise (resistance exercise followed by endurance exercise or the opposite) is an important variable in concurrent studies. Wang et al. (2011) showed that performing a bout of resistance exercise after endurance exercise enhanced the signaling cascade for mitochondrial biogenesis. The authors found a concomitant activation of AMPK and mTOR and an increased expression of PGC-1a and PGC-1-related coactivator (PRC). However, markers of ribosome biogenesis were not examined making it difficult to determine if mitochondrial and ribosome biogenesis coincided. Apró et al. (2013), on the other hand, investigated the effects of performing endurance exercise after resistance exercise on mTORC1 and AMPK signaling pathways. Activation of the mTORC1 by resistance exercise was not impaired by subsequent concurrent endurance exercise. However, the authors found that phosphorylation of AMPK was decreased 3h after both resistance exercise-only and concurrent exercise, suggesting that prior activation of mTORC may suppress AMPK activation.

Beyond concurrent training, it is possible that other types of training, such as low-load blood flow restricted or low-load/high-volume resistance training to failure may simultaneously enhance mitochondrial and ribosome biogenesis. The studies of Groeenebaek et al. and Sieljacks et al. cited previously found that low-load blood flow restricted resistance training increased both mitochondrial (Groennebaek et al., 2018) and ribosome biogenesis (Sieljacks et al., 2019), with

no difference when compared to a high-load resistance training. Furthermore, low-load/high-volume resistance training paradigm can assume several forms, but the most studied paradigm involves participants performing sets at 30% 1RM to failure (30FAIL; Mitchell et al., 2012; Jenkins et al., 2016, 2017; Morton et al., 2016, 2019; Haun et al., 2017). Lim et al. (2019) recently published a study which compared three groups of participants who trained for 10weeks (3days/week) with either 80FAIL, 30FAIL, or 30% 1RM loads, which were volume-matched to the 80FAIL group. While the authors did not report significant changes in mitochondrial volume markers (i.e., cytochrome C and COX IV protein levels), robust alterations in these markers occurred in the 30FAIL group. Markers of mitochondrial remodeling (i.e., PARKIN, OPA1, and FIS1 protein levels) also increased only in the 30FAIL group. Indeed, this evidence suggests mitochondrial biogenesis may have increased in the 30FAIL group, albeit markers of ribosome biogenesis were not assessed. Nonetheless, muscle hypertrophy did occur in the 30FAIL group. Thus, considering these studies, it seems plausible that 30% 1RM resistance training to failure may enhance mitochondrial and ribosome biogenesis.

OTHER CONSIDERATIONS TO THE COMPETITION PARADIGM

A major limitation to the biogenesis competition paradigm is that AMPK and mTORC1 is primarily responsible for said competition. If this is indeed the case, then the paradigm would likely have to operate through an AMPK-mTORC1 signaling "switch" in response to each form of training. This switch has been proposed to occur in the skeletal muscle of rats following prolonged low-frequency stimulation vs. short bursts of high-frequency stimulation (Atherton et al., 2005). However, the acute post-exercise time course data regarding AMPK and mTORC1 activity in humans are more nuanced. For instance, Dreyer et al. (2006) reported that one bout of

unaccustomed resistance exercise concomitantly increases AMPK activity and mTORC1 signaling markers 2hours post-exercise. Likewise, Mascher et al. (2011) reported that a 60-min cycling bout concomitantly increases AMPK activity and mTORC1 signaling markers 2hours post-exercise. The study by Wilkinson et al. (2008) similarly demonstrated that a bout of unilateral resistance and endurance training increased the phosphorylation of AMPK (Thr172) immediately following exercise. According to the competition paradigm, these findings suggest that resistance and endurance exercise should initiate mitochondrial and ribosomal biogenesis.

Furthermore, Coffey et al. (2006) explored the effects of training status and accustomization to different exercise training modes (resistance vs. endurance exercise) and their data illustrate the complexity of the signaling AMPK and mTORC pathways response to exercise. The authors had a group of endurance trained and a group of resistance trained individuals perform one bout of endurance exercise and one bout of resistance exercise on different sessions. Their results suggest that untrained individuals might present a more generic response to exercise, with increases in both signaling pathways with either endurance or resistance exercise. However, as one becomes more accustomed to an exercise mode through training, the signaling responses to exercise seem to be attenuated. Moreover, AMPK signaling seems to be less specific, being activated with both endurance and resistance exercises, while mTORC is preferentially activated in response to resistance exercise (Vissing et al., 2013). In addition, considering that the response to exercise in untrained subjects seems to be fairly generic, performing concurrent training instead of resistance-only or endurance-only exercise could have an additive instead of an interference effect. This is supported by the work of Wang et al. (2011), which showed that performing resistance exercise after cycling enhanced markers of mitochondrial adaptations compared to cycling-only. However, this effect is likely dependent

on a myriad of other factors, such as interval between exercise bouts and the volume of each differentiated exercise mode. More studies specifically designed to answer that question are warranted. The illustration in Figure 1 summarizes how AMPK and mTORC1 crosstalk during and following bouts of endurance and resistance exercise facilitates mitochondrial and ribosome biogenesis, respectively. It should be noted, however, that endurance exercise does not exclusively activate AMPK and inhibit mTORC1 signaling. Likewise, resistance exercise does not exclusively activate mTORC1 signaling and de-activate AMPK.

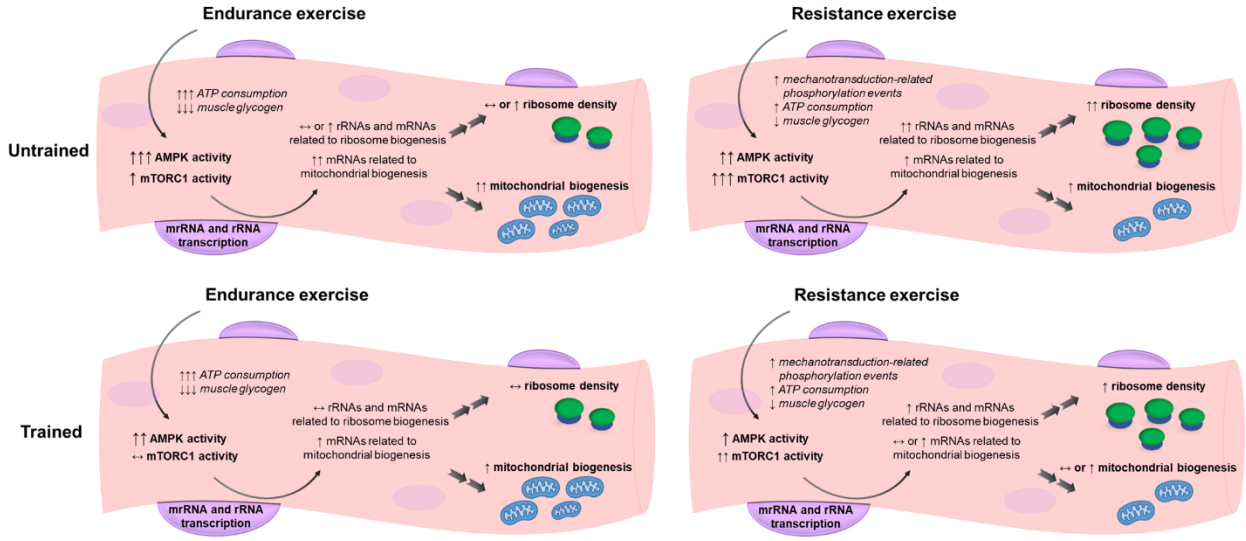

Figure 1. Hypothetical representation of ribosome and mitochondrial biogenesis and the respective signaling responses to endurance and resistance exercise in untrained and trained states. Untrained individuals present a more generic response to exercise, with increases in both 5' AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin complex 1 (mTORC1) signaling with either endurance or resistance exercise, which may ultimately lead to a concomitant increase in ribosome and mitochondrial biogenesis. Upon training, the signaling responses to exercise seem to be attenuated and mitochondrial biogenesis is prioritized with endurance training, while ribosome biogenesis is prioritized with resistance training. Note that even in trained subjects, AMPK exhibits a more generic response to exercise compared to mTORC1.

Moreover, the timing of skeletal muscle biopsies and therefore of the measurements of AMPK/mTORC1 activation is commonly referred as a limitation and a possible source of

inconsistencies found between different studies (Gibala et al., 2009; Figueiredo et al., 2015; Stec et al., 2015). Besides establishing the time-course of activation, the exact timing of measurements can give important information regarding the interplay between AMPK and mTORC signaling pathways. There is evidence to suggest that even though both mTORC1 and AMPK can be activated in response to resistance exercise, mTORC1 is activated once AMPK signaling subsides (Vissing et al., 2013). Similar findings have been reported with an ex-vivo endurance exercise model (Jakobsgaard et al., 2021). In addition, Vissing et al. (2013) results showed that mTORC1 peak activation was at 5h post-exercise and remained upregulated until 22h pos-exercise. Therefore, if studies do not collect muscle tissue in several time-points (e.g., only perform biopsies 1h and/or 3h post-exercise), this important information about the signaling response to exercise could be missed. However, it is important to note that as previously mentioned, other studies have shown that AMPK and mTORC1 can be concomitantly activated (Wang et al., 2011; Fyfe et al., 2016).

Data from both animal and in vitro models also challenge the AMPK-mTORC1 switch theory. Drake et al. (2013) demonstrated a null effect of mTOR inhibition on mitochondrial biogenesis markers in mice fed a rapamycin-supplemented diet for 12weeks. There are also in vitro data suggesting mTOR signaling enhances mitochondrial biogenesis (Morita et al., 2013). Likewise, a review by Morita et al. (2015) provides several lines of evidence to suggest mTORC1 enhances mitochondrial function through the increased translation of transcription factors that regulate the expression of nuclear-encoded mitochondrial genes. An elegant study performed by Cunningham et al. (2007) demonstrated through a series of experiments that mTOR is necessary for proper mitochondrial oxidative function and biogenesis. The authors found that inhibition of mTOR by rapamycin decreased the expression of important

mitochondrial transcription factors, gene targets of PGC-1α, and mitochondrial respiration in C2C12 myotubes. In addition, mice exposed to the same treatment also experienced similar effects. The authors proceeded with additional experiments to show that mTOR-dependent regulation of mitochondrial biogenesis and function is achieved through direct modulation of YY1-PGC-1α.

Furthermore, it is notable that mTOR complex 2 (mTORC2) is also involved with mitochondrial physiology. The differences between the mTORC1 and mTORC2 complexes are subtle; specifically, mTORC2 contains the mTOR, Rictor, LST8 and SIN1 proteins (Loewith et al., 2002). Whereas mTORC1 functions as a nutrient/amino acid sensing complex, mTORC2 receives intracellular signals from extracellular growth factor binding (Jhanwar-Uniyal et al., 2019). Interestingly, data suggest that mTORC2 stimulates mitochondrial biogenesis in liver (Betz et al., 2013) and myeloid dendritic cells (Watson et al., 2019), although equivocal data exist in macrophages from Rictor-knockout mice (Oh et al., 2017). Studies examining mTORC2 activity responses to exercise bouts or training are sparse relative to studies examining mTORC1 responses. However, evidence suggests that mTORC2 activity increases in response to an endurance bout in rodents (Kleinert et al., 2017). In contrast, skeletal muscle mTORC2 activity seems unresponsive to a bout of resistance training in humans based upon the localization of the complex not being altered following a bout of resistance training (Hodson et al., 2017). These data add to the proposed competition paradigm in that mTOR may be involved in both biogenesis processes depending upon whether mTORC1 or mTORC2 is stimulated. However, again, more studies are needed before definitive conclusions can be drawn.

Additionally, we have previously made the case that both ribosome and mitochondrial biogenesis are metabolically demanding processes and that maintaining a high density of both

organelles would place an energetic demand on the cells. However, as mitochondria are the main energy-producing organelles in the cell, it could also be argued that it is counterintuitive to decrease its density when the cell is facing an increased energy demand, such as during increased ribosome biogenesis and cytosolic protein synthesis after resistance exercise. Moreover, several proteins needed for mitochondrial biogenesis are encoded in nuclear DNA and synthesized by cytosolic ribosomes before they can be imported into the mitochondria (Jornayvaz and Shulman, 2010; Perry and Hawley, 2018). Again, it would be counterintuitive to decrease ribosome density when there is an increased demand for nuclear-encoded proteins needed for mitochondrial biogenesis. Therefore, ribosome and mitochondrial biogenesis would be expected to be closely related processes.

CONCLUSION

There is compelling evidence suggesting that competition between ribosomal and mitochondrial biogenesis does not exist. Several studies have shown results suggesting that both processes can occur simultaneously in response to different types of exercise (Dreyer et al., 2006; Tang et al., 2006; Mascher et al., 2011; Fyfe et al., 2016; Lamb et al., 2020). Evidence from studies using tracer methodology especially indicate that resistance training is capable of inducing both mitochondrial (Groennebaek et al., 2018) and ribosome (Sieljacks et al., 2019) biogenesis. Further, evidence for mTOR regulation of mitochondrial biogenesis (Cunningham et al., 2007) highlights that the interaction between AMPK and mTORC signaling pathways is more complex than initially thought. Therefore, it is likely that instead of a competition between the two processes, what happens is an exercise mode-specific response, where endurance exercise stimulates AMPK signaling more so than mTORC1 signaling during periods of

recovery between exercise bouts, and resistance exercise stimulates the opposite phenomena. There is also the possibility that other unidentified signaling mediators increase following bouts of resistance and endurance exercise that interfere with mitochondrial and ribosome biogenesis, respectively. In this regard, −omics-based investigations may be fruitful in uncovering these potential targets if said targets exist.

Given the evidence cited in this review, it is pragmatic for individuals who seek to enhance muscle hypertrophy and aerobic capacity to engage in concurrent training. However, whether concurrent training accomplishes these adaptations through increased mitochondrial and ribosome biogenesis remains to be fully elucidated. The available data suggesting 30FAIL and blood flow restricted resistance training can enhance both processes is also compelling, and future studies examining this possibility are warranted. Importantly, more studies utilizing tracer methodology to directly assess both mitochondrial and ribosome biogenesis are needed. Research examining the interplay between the mitochondrial and ribosome biogenesis responses to exercise training will ultimately augment our understanding of skeletal muscle physiology. Critically, such research will be fruitful for individuals seeking to apply this knowledge in applied settings.

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Chapter 2 - Effects of Aging and Long-term Physical activity on Mitochondrial Physiology and Redox State of the Cortex and Cerebellum of Female Rats

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INTRODUCTION

Aging is characterized by a progressive decline in physiological function that is observed in several tissues, including the brain (Mattson & Arumugam, 2018). The impairment of mitochondrial function has been considered one of the major players in brain aging and has been linked to the development of many neurodegenerative diseases (Grimm & Eckert, 2017; Mattson & Arumugam, 2018). Indeed, age-related decreases in different measures of mitochondrial function in the brain, including basal respiration (Lores-Arnaiz et al., 2016), state 3 respiration (Lores-Arnaiz & Bustamante, 2011; Thomsen et al., 2018) and the activity of the electron transport system complexes (I-III, IV, and V) (Long et al., 2009; Navarro et al., 2002, 2004), have been repeatedly reported in the literature. In addition, disrupted mitochondrial dynamics has been recognized as a critical factor in the aging process and neurodegeneration (Marques-Aleixo et al., 2012). Substantial sex disparities also exist with regards to age-related metabolic changes in the brain (Grimm & Eckert, 2017; Zhao et al., 2016). Females have been shown to undergo changes in brain mitochondrial physiology, such as decreased expression of genes related oxidative phosphorylation, mitochondrial biogenesis and dynamics, at earlier stages than males (Zhao et al., 2016), and to be disproportionately affected by neurodegenerative diseases, such as Alzheimer's disease (Zhu et al., 2021).

Another important aspect of brain aging and mitochondrial dysfunction is a disruption of the balance between reduction–oxidation reactions (redox state). An increase in the production of reactive oxygen species (ROS) and a concomitant decrease in the antioxidant defense system lead to a state of chronic oxidative stress, causing damage to protein, lipids, and DNA (Sas et al., 2018). Although ROS are produced as a result of different enzymatic reactions, mitochondria are believed to be the main source of ROS (Grimm & Eckert, 2017), and are especially susceptible to oxidative damage. Therefore, mitochondrial dysfunction can lead to a vicious cycle of increased ROS production and damage to the organelles.

Physical exercise has been regarded as a therapeutic approach to improve mitochondrial function and the redox state of the brain, possibly preventing or delaying the development of neurodegenerative diseases (Marques-Aleixo et al., 2012). For example, Marques-Aleixo et al. (2015) reported that 12 weeks of exercise favorably altered different mitochondrial biomarkers in the cortex and cerebellum of young male rats. However, studies investigating the effects of both aging and long-term physical activity on mitochondrial physiology, especially with regards to mitochondrial dynamics, and on the redox state of the brain of females are scarce. Therefore, the purpose of this study was to investigate the effects of aging and long-term voluntary physical activity on markers of mitochondrial function and remodeling, oxidative damage, and the antioxidant defense system in the cerebral cortex and cerebellum of female rats.

MATERIALS AND METHODS

Animals

Female Lewis rats were obtained from a commercial vendor (Envigo) at 3 months of age. Rats were caged individually and maintained under controlled conditions with water and food ad

libitum until 6 months of age was reached, when they were separated into three groups: (1) control group (YNG, n = 10); (2) sedentary (MA-SED, n = 12); and physical activity (MA-PA, n = 12). The YNG group was euthanized at 6 months and used as young reference group, while MA-SED and MA-PA groups were aged to 15 months. The MA-PA group had 24-h access to a running wheel and were allowed to run voluntarily for 9 months. The average weekly run distance and total run distance were recorded for each rat in the MA-PA group. Running wheels of the exercise group were locked 20 h before euthanasia to avoid interference of acute effects of exercise. All experimental procedures were approved by the local Animal Care and Use Committee (protocol #: 2020–3647).

Animal euthanasia and tissue collection

Rats were euthanized by CO2 gas followed by cervical dislocation after a minimum of a 4 h-fasting. After decapitation, brain cortex and cerebellum were rapidly removed, flash-frozen in liquid nitrogen, and stored at −80°C for later molecular analyses.

Western blotting

Approximately 25 mg of each brain cortex and cerebellum were homogenized in cell lysis buffer (Cell Signaling) using tight-fitting plastic pestles. Protein concentrations were determined using a BCA assay (Thermo Fisher Scientific). Samples were prepared for Western Blotting at 1 µg/µl, 10 µl loaded onto a 4%–15% SDS-polyacrylamide gel and transferred to preactivated PDF membranes. Membranes were stained for Ponceau S, blocked with 5% nonfat milk powder, and incubated overnight at 4°C with the following primary antibodies at 1:2000 dilution: total OXPHOS rodent (Abcam Cat# ab110413, RRID:AB_2629281), PGC-1α

(GeneTex Cat# GTX37356, RRID:AB_11175466), NRF1 (GeneTex Cat# GTX103179, RRID:AB_11168915), TFAM (Abnova Corporation Cat# H00007019-D01P, RRID:AB_1715621), Mfn2 (BioVision Cat# 3882–100, RRID:AB_2142625), Opa1 (Cell Signaling Technology Cat# 67589, RRID:AB_2799728), Fis1 (Abcam Cat# ab71498, RRID:AB_1271360), Drp1 (Novus Cat# NB110-55288SS, RRID:AB_921147), Pink1 (Cell Signaling Technology Cat# 6946, RRID:AB_11179069), Parkin (Cell Signaling Technology Cat# 2132, RRID:AB_10693040), SOD1 (GeneTex Cat# GTX100554, RRID:AB_10618670), SOD2 (GeneTex Cat# GTX116093, RRID:AB_10624558), CAT (GeneTex Cat# GTX110704, RRID:AB_1949848), and GPX-1 (GeneTex Cat# GTX116040, RRID:AB_2037097). Membranes were washed in TBST and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology Cat# 7074, RRID:AB_2099233) or anti-mouse IgG (Cell Signaling Technology Cat# 7076, RRID:AB_330924) secondaries. Membranes were digitally imaged using a gel documentation system (Bio-Rad). Raw target band densities were obtained and normalized by Ponceau values. Finally, band values were normalized by the mean of the YNG group to obtain percent change in comparison to the YNG group.

Oxidative damage markers

Lipid peroxidation and protein oxidation were assessed as markers of oxidative damage to both cortex and cerebellum. Lipid peroxidation levels were assessed by determining 4-hydroxynonenal (4HNE) (Abcam Cat# ab46545, RRID:AB_722490) levels via Western Blotting as described above. Additionally, protein carbonyl levels were assessed using the Oxyblot protein oxidation detection kit (Millipore; #S7150) following manufacturer's instructions.

Citrate synthase and mitochondrial complexes activities

The activity of the enzyme citrate synthase and of the mitochondrial complexes I–III, II, and IV were determined spectrophotometrically at 37°C using cortex and cerebellum homogenates. Briefly, approximately 50 mg of each tissue were homogenized in a sucrose homogenization buffer using a glass dounce homogenizer according to Spinazzi et al. (2012). Samples were subjected to a series of freeze–thaw cycles before the assessment of enzymatic activities. Citrate synthase activity was determined by monitoring the increase in absorbance at 412 nm from the reduction of 5,5′-dithiobis (2- nitrobenzoic acid) coupled to the reduction of Acetyl-CoA (Trounce et al., 1996). The activity of the NADH cytochrome c oxireductase (complexes I-III) was determined by the increase in absorbance at 550 nm after the addition of NADH (10 mM) (Spinazzi et al., 2012). Succinate dehydrogenase (complex II) activity assay was adapted from Spinazzi et al. (2012) and Trounce et al. (1996). In short, complex II activity was measured as a function of the decrease in absorbance at 600 nm from 2,6-dichloroindophenol reduction in the presence of Antimycin A, KCN, and rotenone. Lastly, cytochrome c oxidase (complex IV) activity was measured by following the oxidation of reduced cytochrome c at 550 nm according to Spinazzi et al. (2012). The activity of the mitochondrial complexes was normalized by citrate synthase activity to account for changes in mitochondrial content and further normalized by the YNG group to be expressed as percent change from the YNG group.

Statistics

Normality of the dependent variables was assessed using Shapiro–Wilk tests. For normally distributed data, one-way ANOVAs were utilized to test for differences between

groups with Tukey's HSD post hoc tests when appropriate. Kruskal-Wallis tests were used for variables that were not normally distributed. Welch One-Way tests were used for normally distributed variables that presented heterogeneity of variances, followed by Games-Howell tests when appropriate. Correlations between run distance and dependent variables were analyzed with Pearson or Spearman correlation tests. Statistical significance was established at $\alpha \leq 0.050$. Data are presented as mean ± standard deviation and ±95% confidence intervals (CI) are presented for statistically significant differences between groups. Statistics were performed using RStudio Version 2022.2.3.492 for Windows.

RESULTS

Running distance

Rats in the MA-PA group ran on average 45.44 ± 25.49 km weekly and a total of 1579 ± 56 km throughout the entire duration of the study. There was no significant correlation between either average weekly or total distance run and any of the dependent variables ($p > 0.05$ for all correlations). A plot with the correlations for both cortex and cerebellum can be seen in Figure S1 (https://figshare.com/s/64114d9298ac4361ad27) and S2 (https://figshare.com/s/8ed6467d5643741d390e). A detailed description of the weekly running distance, as well as phenotype of the rats of the present study, including body weight, food intake, and weight of different tissues, can be found in Osburn et al. (2022).

Mitochondrial content

There were no significant differences in citrate synthase activity between groups for either cortex ($p = 0.245$) or cerebellum ($p = 0.558$) (Figure 2-1a,b, respectively). No significant

differences were detected for the protein levels of any of the mitochondrial complexes in either cortex; (CI: p = 0.690; CII: p = 0.240; CIII: p = 0.443; CIV: p = 0.949; CV: p = 0.948; Figure 2-1c) or cerebellum (CI: p = 0.436; CII: p = 0.101; CIII: p = 0.144; CIV: p = 0.502; CV: p = 0.754; Figure 2-1d).

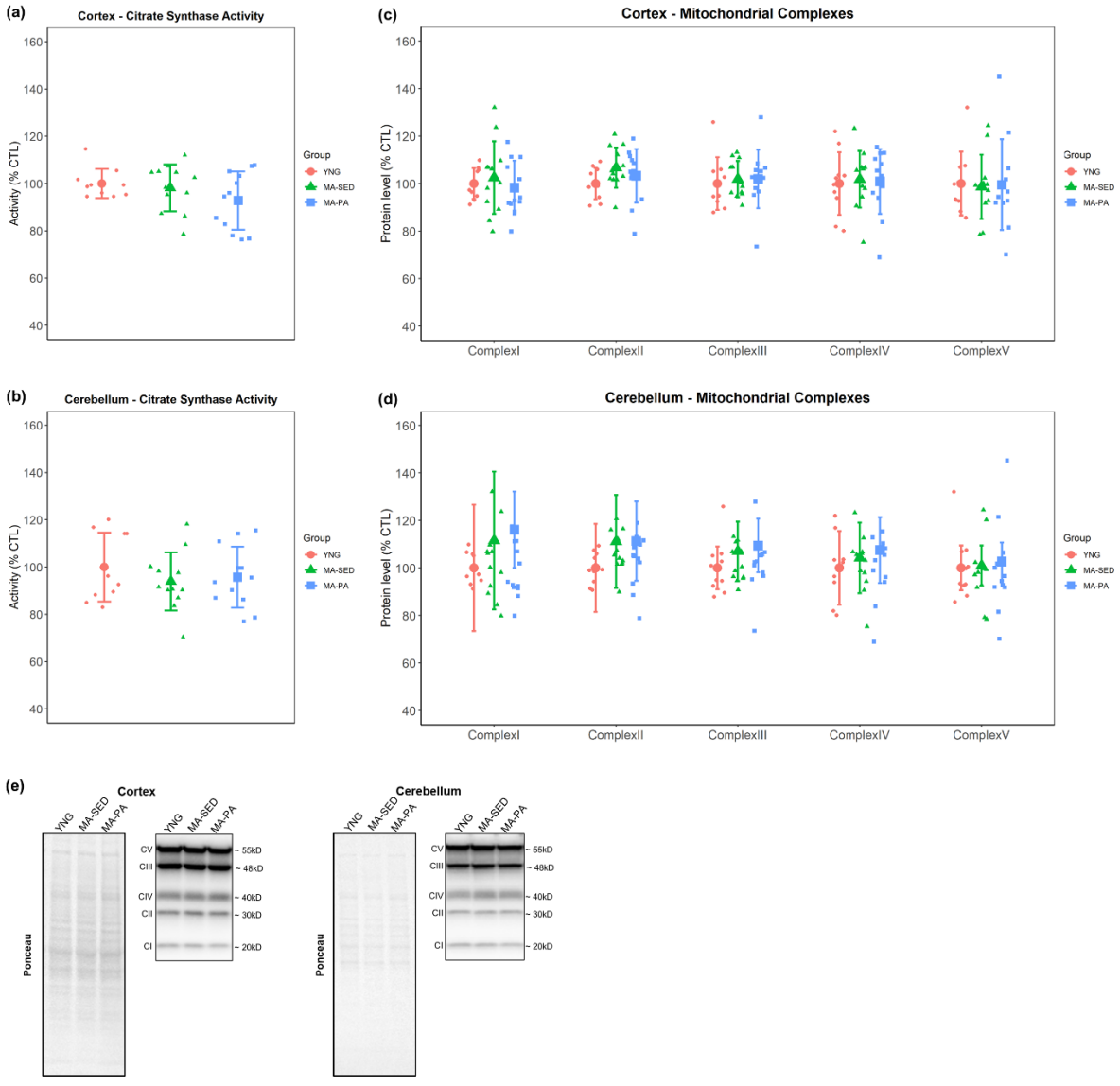

Figure 2-1. Markers of mitochondrial content. Citrate synthase activity (a, b), protein content of mitochondrial complexes I–V (c, d), and representative western blot images (e). No significant differences between groups were detected for markers of mitochondrial content in any of the tissues. Data are presented as mean ± SD and individual data points. YNG, young (n = 10); MA-SED, middle-aged sedentary (n = 12); MA-PA, middle-aged physical activity (n = 12).

Mitochondrial complexes activities

No significant differences were detected for the activity of complex I + III ($p = 0.275$) or complex II ($p = 0.334$) in the cortex. However, complex IV activity was 85.3% (±95% CI [69.2%]) higher in the MA-PA group compared to YNG ($p = 0.016$) and 82.30% (±95% CI [51.6%]) higher compared to MA-SED ($p = 0.023$). In the cerebellum, no significant differences between groups were found for complex I + III ($p = 0.520$), complex II ($p = 0.845$), or complex IV ($p = 0.284$). Mitochondrial complexes activities for both cortex and cerebellum can be seen in Figure 2-2a–f.

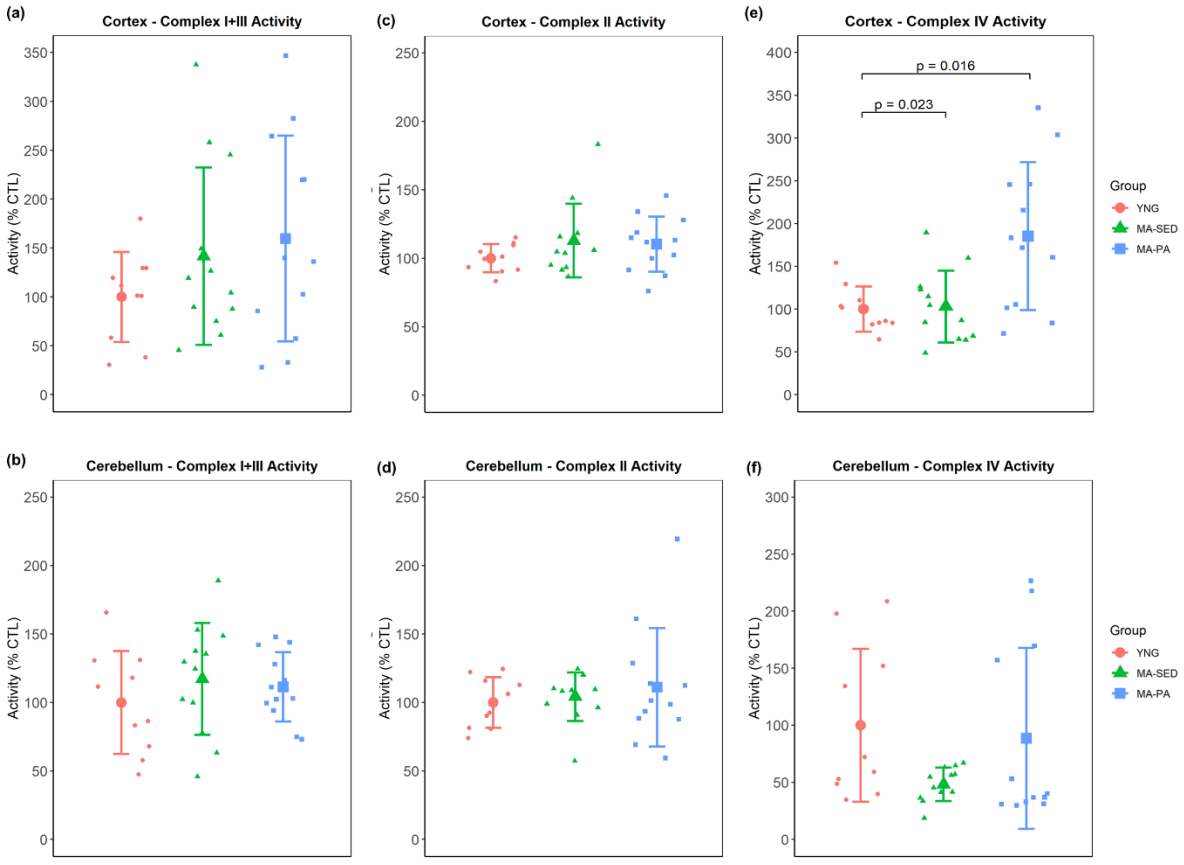

Figure 2-2. Enzymatic activity of mitochondrial complexes. Enzymatic activity of complex I + III (a, b), complex II (c, d), and complex IV (e, f). One-way ANOVA revealed higher complex IV activity in MA-PA compared to YNG and MA-SED. Data are presented as mean ± SD and individual data points. YNG, young (n = 10); MA-SED, middle-aged sedentary (n = 12); MA-PA, middle-aged physical activity (n = 12).

Mitochondrial biogenesis

PGC-1α, NRF-1, and TFAM protein levels were analyzed as markers of mitochondrial biogenesis. There were no significant differences between groups in the levels of PGC-1α ($p = 0.754$), NRF-1 ($p = 0.374$), or TFAM ($p = 0.834$) in the cortex (Figure 2-3a). Similarly, no significant differences were detected in the cerebellum (PGC-1α: $p = 0.994$; NRF-1: $p = 0.396$; TFAM: $p = 0.094$; Figure 2-3b). TFAM levels in the cerebellum of the MA-SED group showed a tendency to be higher than both YNG (9.50%, ±95% CI [13.35%]) and MA-PA (10.82%, ±95% CI [12.73%]).

Mitophagy

PINK1 and Parkin were used as markers of mitophagy. There were no significant differences in the protein levels of PINK1 ($p = 0.708$) or Parkin ($p = 0.408$) in the cortex (Figure 2-3c). In the cerebellum, there was no significant difference between groups for PINK1 ($p = 0.785$; Figure 2-3d). Parkin was not detected in the cerebellum samples.

Mitochondrial fusion

There were no significant differences in the protein levels of markers of mitochondrial fusion in the cortex (Mfn2: $p = 0.727$; OPA1: $p = 0.981$; Figure 2-4a) or cerebellum (Mfn2: $p = 0.718$; OPA1: $p = 0.865$; Figure 2-4b). Long (L-, top band) and short (S-, bottom band) isoforms of OPA1 were further analyzed to identify any specific group-differences. There were no significant differences in L-OPA1 ($p = 0.989$) or S-OPA1 ($p = 0.961$) in the cortex. Similarly, no differences were detected in L-OPA1 ($p = 0.854$) or S-OPA1 ($p = 0.881$) in the cerebellum.

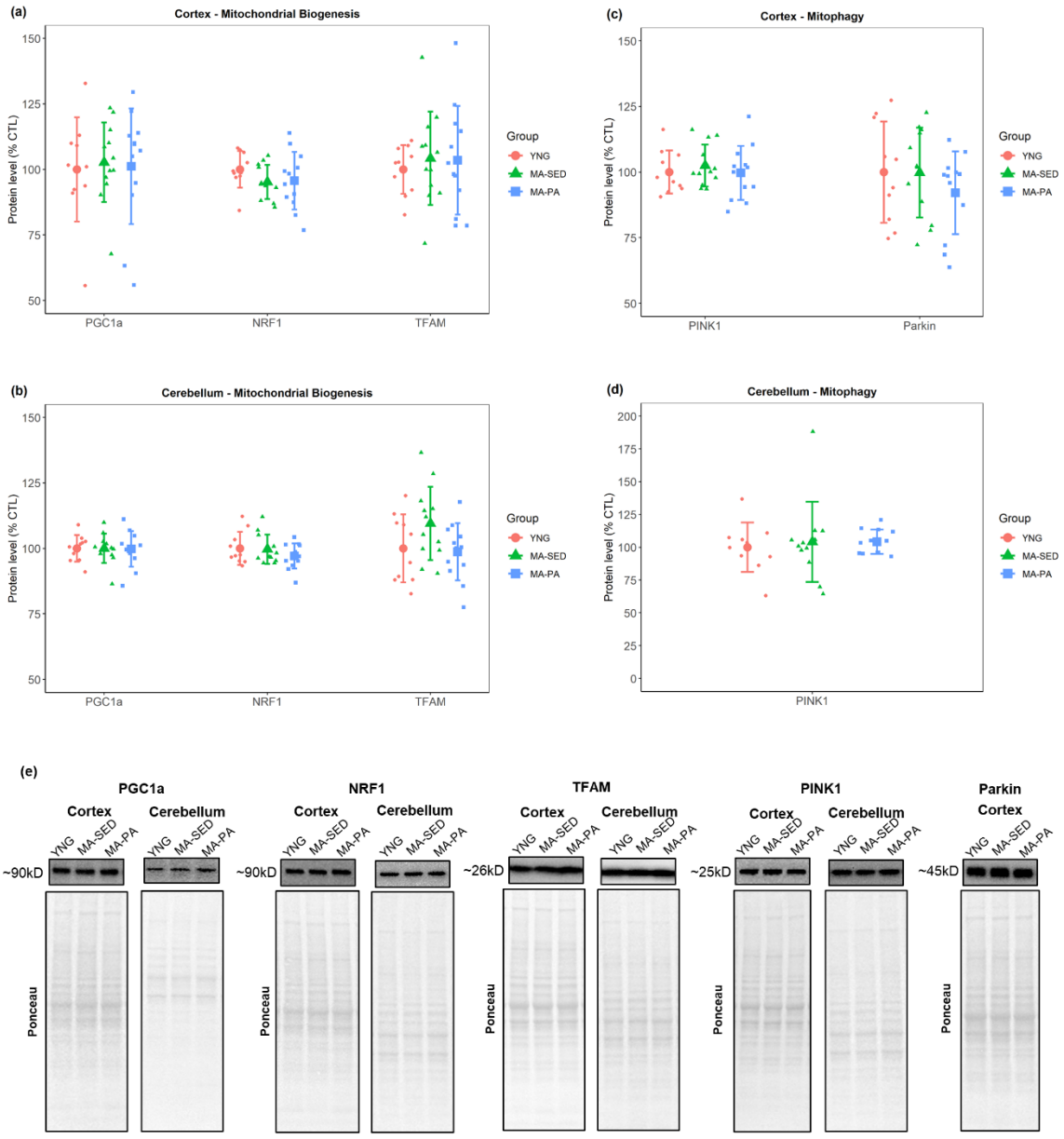

Figure 2-3. Markers of mitochondrial biogenesis and mitophagy. Markers of mitochondrial biogenesis (a, b) and mitophagy (c, d), with representative western blot images (e). No significant differences were detected for markers of mitochondrial biogenesis or mitophagy in the cortex and cerebellum. Data are presented as mean ± SD and individual data points. YNG, young (n = 10); MA-SED, middle-aged sedentary (n = 12); MA-PA, middle-aged physical activity (n = 12). Note that the following proteins share the same ponceau: Cortex: PGC-1a and SOD2; TFAM and OPA1; Parkin, Mfn2, and Fis1; Cerebellum: PGC-1a and SOD2; NRF1 and PINK1; TFAM and OPA1.

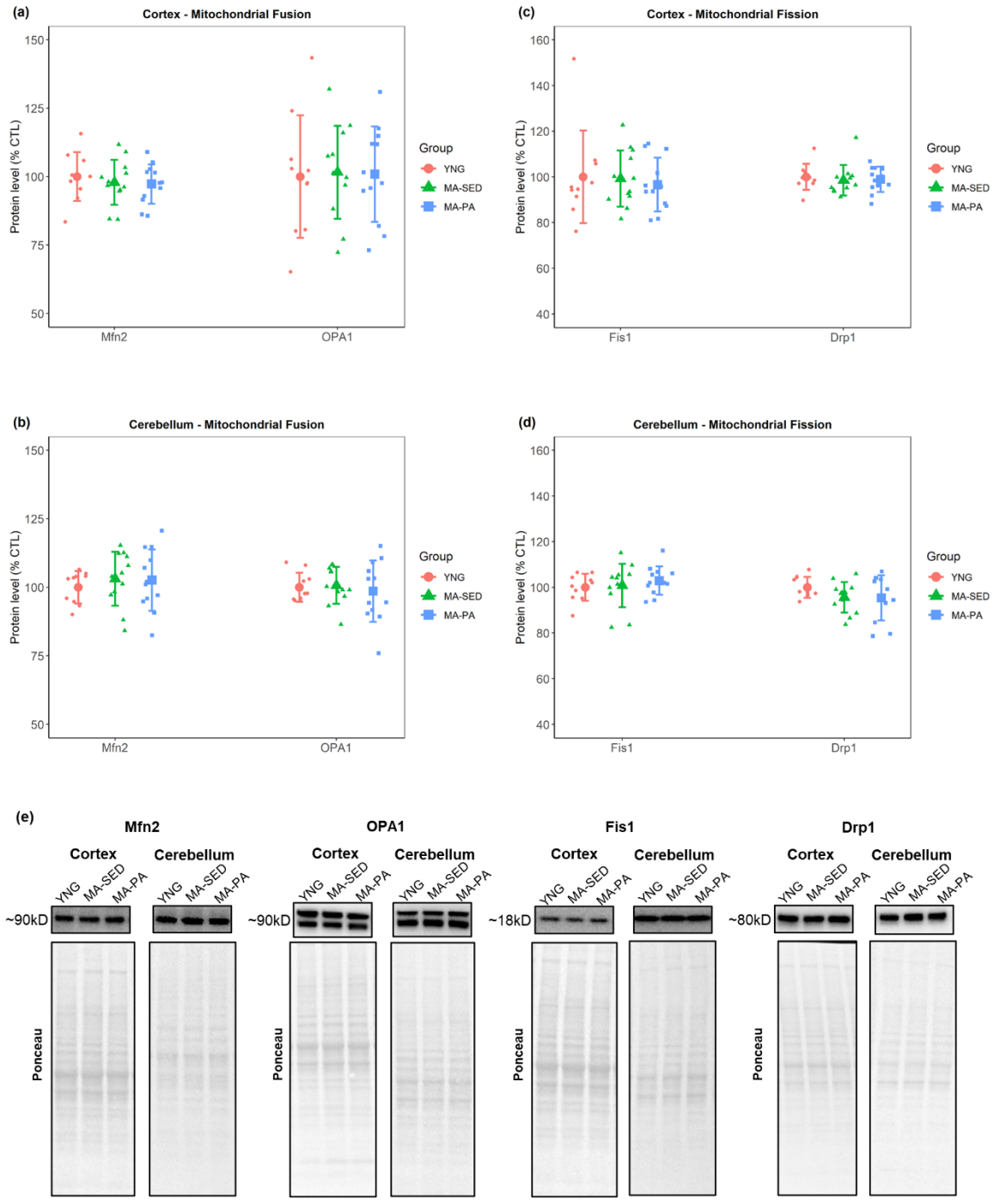

Figure 2-4. Markers of mitochondrial fusion and fission. Markers of mitochondrial fusion (a, b) and fission (c, d), with representative western blot images (e). No significant differences were detected for markers of mitochondrial fusion or fission in the cortex and cerebellum. Data are presented as mean ± SD and individual data points. YNG, young (n = 10); MA-SED, middle-aged sedentary (n = 12); MA-PA, middle-aged physical activity (n = 12). Note that the following proteins share the same ponceau: Cortex: Drp1, CAT, and GPX1; Cerebellum: Drp1, CAT, GPX1, and SOD1. Importantly, cerebellum GPX1 membrane was stripped after the image was captured and reincubated with SOD1.

Mitochondrial fission

The protein levels of markers of mitochondrial fission were not significantly different between groups in the cortex (Fis1: $p = 0.962$; Drp1: $p = 0.471$; Figure 2-4c) or cerebellum (Fis1: $p = 0.643$; Drp1: $p = 0.299$; Figure 2-4d).

Antioxidants

In the cortex, there were no significant differences between groups in the levels of SOD1 ($p = 0.785$), CAT ($p = 0.904$), and GPX1 ($p = 0.429$). However, SOD2 was 6.53% (±95% CI [5.23%], $p = 0.012$) higher in the MA-SED group compared to YNG. In the cerebellum, no significant differences were detected for any of the antioxidants investigated (SOD1: $p = 0.254$; SOD2: $p = 0.055$; CAT: $p = 0.920$; GPX1: $p = 0.771$). SOD2 exhibited a tendency to be higher in the MA-SED group compared to YNG (22.11%, ±95% CI [21.52%]). Protein levels of antioxidants can be seen in Figure 2-5a,b.

Oxidative damage

In the cortex, there was no significant difference between groups in the levels of 4HNE ($p = 0.864$), while protein carbonyls were 12.49% (±95% CI [11.68%], $p = 0.034$) lower in the MA-PA group compared to YNG (Figure 2-6a). No significant differences between groups were detected in 4HNE ($p = 0.775$) or protein carbonyls ($p = 0.203$) in the cerebellum (Figure 2-6b).

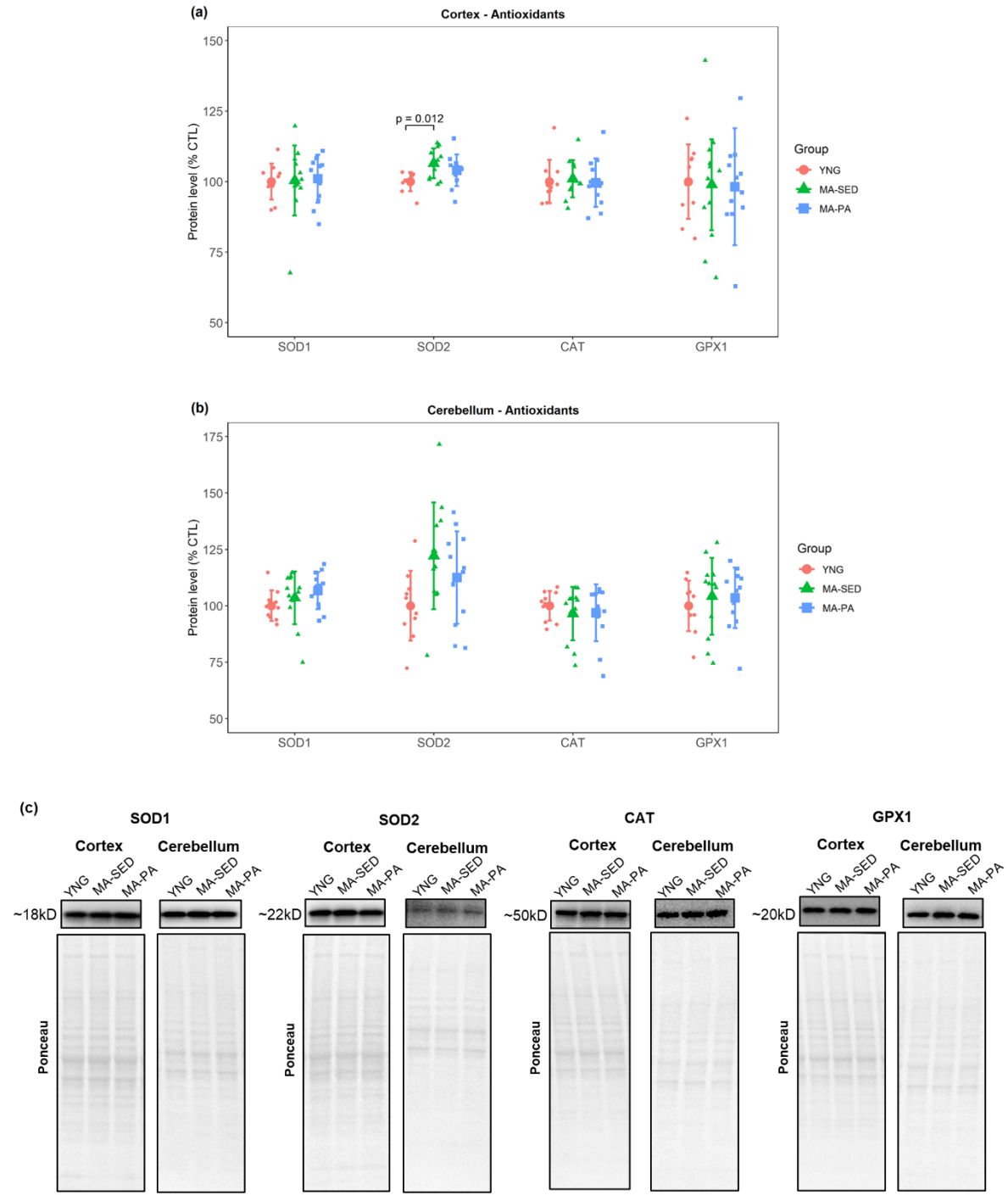

Figure 2-5. Endogenous antioxidant enzymes. Protein levels of antioxidants in the cortex (a) and cerebellum (b), with representative western blot images (c). The only significant difference detected was higher levels of SOD2 in the cortex of MA-SED compared to YNG. Data are presented as mean ± SD and individual data points. YNG, young (n = 10); MA-SED, middle-aged sedentary (n = 12); MA-PA, middle-aged physical activity (n = 12). Note that proteins that share the same ponceau have been reported in previous images.

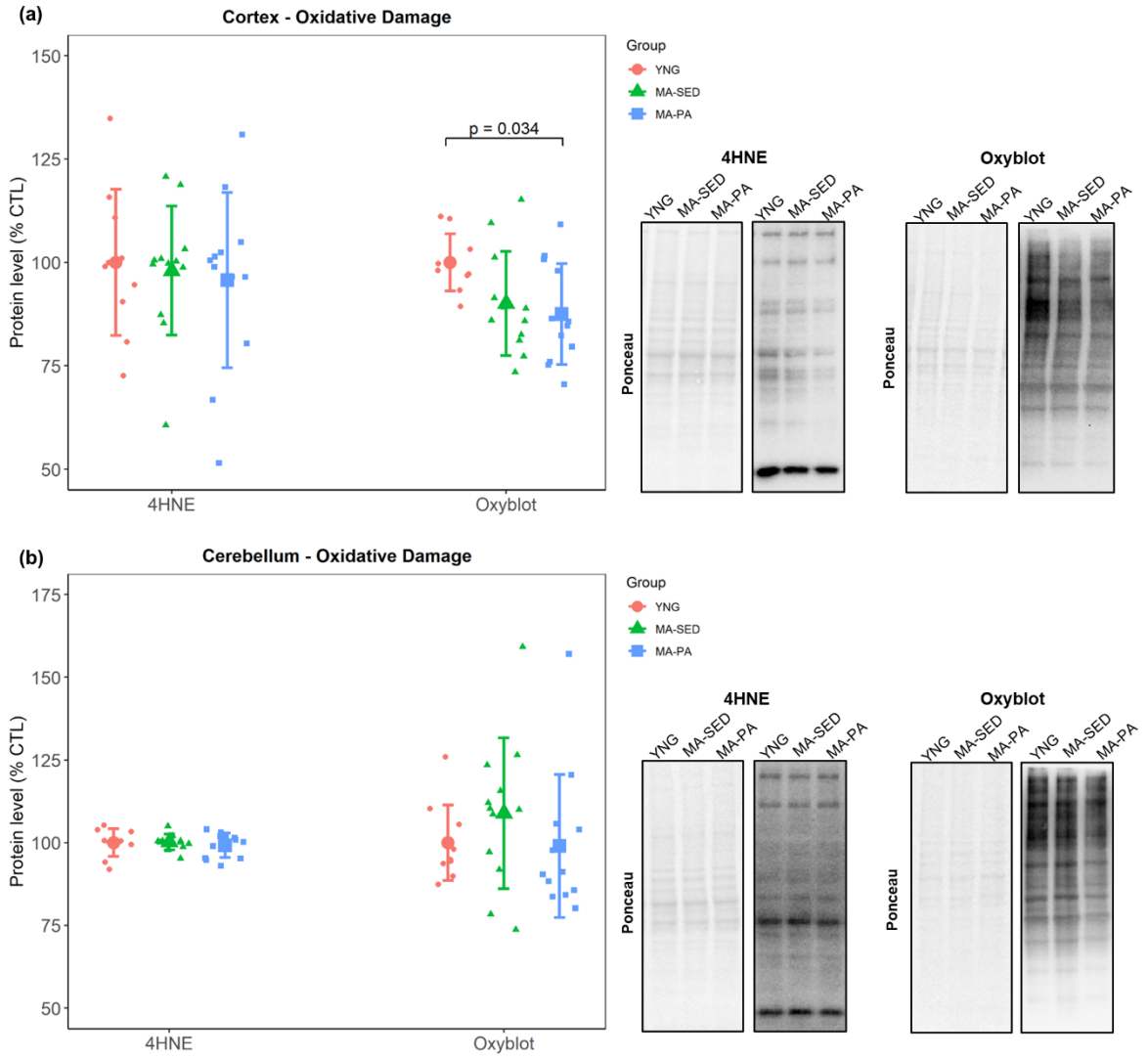

Figure 2-6. Markers of oxidative damage. Markers of oxidative damage in the cortex (a) and cerebellum (b), with representative western blot images. Levels of protein carbonyls were lower in the cortex of MA-PA compared to YNG, while there were no significant differences in markers of oxidative damage in the cerebellum. Data are presented as mean ± SD and individual data points. YNG, young (n = 10); MA-SED, middle-aged sedentary (n = 12); MA-PA, middle-aged physical activity (n = 12).

DISCUSSION

A disruption of mitochondrial function and the redox homeostasis of the cells have been considered underlying mechanisms in brain aging and in the development of several age-related neurodegenerative diseases. Furthermore, substantial sex disparities have been shown for age-

related metabolic changes that occur in the brain, with females experiencing earlier declines in IGF signaling, glycolysis, mitochondrial function and remodeling, and redox balance (Zhao et al., 2016). Physical exercise has been considered an important therapeutic approach to counteract such age-related changes in the brain and possibly prevent the development of neurodegenerative diseases (Bernardo et al., 2016; Marques-Aleixo et al., 2012). In the current study, we investigated the effects of both aging and long-term voluntary wheel running on mitochondrial physiology and redox state of the cortex and cerebellum of female rats. Overall, our results showed only minimal differences due to aging or voluntary wheel running in the variables interrogated.

While mitochondria are believed to have a major role in the aging process, the effects of aging on brain mitochondrial content are controversial. Our results showed no changes in citrate synthase activity or the protein content of the mitochondrial complexes in either cortex or cerebellum. These results are in agreement with previous studies showing no change in citrate synthase activity (Radak et al., 2001) or in the content of mitochondrial complexes I–V (Long et al., 2009) in the brains of male rats. Navarro et al. (2002), on the other hand, showed that while citrate synthase activity remained unchanged in the brains of middle-aged (52 week-old) female mice, this surrogate of mitochondrial content decreased in older (72 week-old) mice, with a similar response in males, suggesting that mitochondrial content might only decrease at late stages of aging. Additionally, our results showed no significant effects of voluntary wheel running on mitochondrial content. Different studies have shown an increase in markers of mitochondrial content in response to exercise in different brain regions, including the cortex and cerebellum (Marques-Aleixo et al., 2015; Steiner et al., 1985). However, it is important to note that both studies investigated the effects of short-term exercise (8–12 weeks) in a sample of

young male rodents, which may partially explain the discrepancies between studies. Even though it is possible that the beneficial effects of physical activity/exercise seen in young individuals are lost as they age, more studies investigating the effects of long-term voluntary physical activity and/or controlled exercise on brain mitochondrial content are needed.

A decline in mitochondrial function has been considered one of the hallmarks of aging in several tissues (Lopez-Otin et al., 2013), including the brain (Mattson & Arumugam, 2018). Several studies have revealed an age-related decline in the activity of the electron transport system complexes in the brains of both male (Long et al., 2009; Navarro et al., 2004; Pollard et al., 2016) and female (Navarro et al., 2002, 2004) rodents. However, the effects of aging on mitochondrial respiration are less conclusive. Gusdon et al. (2017), for example, found that older mice exhibited decreased activity of complexes I + III but increased mitochondrial respiration driven by malate+glutamate and by succinate. In the current study, there were no significant effects of aging on the activity of complexes I + III, complex II, or complex IV in either cortex or cerebellum. Voluntary wheel running, however, did increase the activity of complex IV in the cortex. Exercise has been shown to have neuroprotective effects, enhancing cognitive function and delaying the development of neurodegenerative diseases (Marques-Aleixo et al., 2012). Furthermore, exercise has been reported to increase the activity of mitochondrial complexes I (Marques-Aleixo et al., 2015) and I + III (Gusdon et al., 2017) in the brains of both young and old rodents, respectively, while complex II activity was unaffected. Our results partially agree with the current literature, suggesting that complex II may be less responsive to exercise (Gusdon et al., 2017; Marques-Aleixo et al., 2015; Navarro et al., 2004).

Besides the function of the electron transport system complexes, proper mitochondrial dynamics has been recognized as an important aspect of mitochondrial and cell health (Chen &

Chan, 2009; Wai & Langer, 2016). The remodeling of the mitochondrial network depends on a fine-tuned balance between mitochondrial biogenesis and mitophagy, and between mitochondrial fusion and fission. While mitochondrial biogenesis contributes to the increase, or the prevention of an age-related decline, of mitochondrial content, mitophagy ensures that damaged portions of the mitochondrion are degraded, helping on the maintenance of a healthy mitochondrial population (Wang et al., 2021; Yoo & Jung, 2018). In alignment with the lack of changes in markers of mitochondrial content, we did not observe changes in markers of mitochondrial biogenesis or mitophagy with aging or voluntary wheel running. Marques-Aleixo et al. (2015) and Steiner et al. (1985) found that short-term exercise significantly increased markers of mitochondrial biogenesis in cortex but not in the cerebellum of young rodents. On the other hand, Gusdon et al. (2017) showed that 3 weeks of treadmill running did not change protein levels of PGC-1α and TFAM in the cortex of young and old mice. However, to the best of our knowledge, there are no studies available that investigated the effects of long-term voluntary physical activity and aging on brain mitochondrial biogenesis and/or mitophagy.

While a disruption of mitochondrial dynamics is implicated in the development of several neurodegenerative diseases (Chen & Chan, 2009; Gao et al., 2017), studies investigating changes in mitochondrial dynamics with healthy aging are scarce. In one of the few studies available, Thomsen et al. (2018) showed using both transmission electron microscopy and gene expression analysis, that aging led to increased fusion in the cortex, but increased fission in the hippocampus of middle-aged male mice, highlighting that the changes are region-specific. Our results showed no significant changes in markers of mitochondrial fusion or fission in the brain regions investigated with aging or physical activity. Twelve weeks of exercise has been shown to improve mitochondrial dynamics in the brains of young healthy (Marques-Aleixo et al., 2015)

and also in a Alzheimer's disease mouse model (Li et al., 2019; Yan et al., 2019). In older animals, Gusdon et al. (2017) found increased fission and no change in fusion markers in the cortex in response to 3 weeks of exercise. Regarding fusion, different OPA1 isoforms appear to have different roles, in which the long isoforms support mitochondrial fusion while the short isoforms appear to have a more energetic-related role (Del Dotto et al., 2017). However, there were no isoform-specific changes with either aging or physical activity. Again, no study to date has investigated the effects of long-term physical activity/exercise on brain mitochondrial remodeling, which makes it difficult to draw assertive conclusions from our data.

Besides a decline in mitochondrial function, brain aging is accompanied by a disruption of the redox status. A decrease in the antioxidant defense system with a concomitant increase in oxidative damage is considered a strong driver of the aging process (Harman, 1956; Salmon et al., 2010; Sas et al., 2018). Several studies report an age-related decline in enzymatic activity of various antioxidants (Bayliak et al., 2021; Navarro et al., 2004). Our results showed that overall, there was no change in the antioxidants investigated with either aging or voluntary wheel running. Even though it is possible that the aged rats herein were simply not old enough to exhibit the age-related decrease in antioxidants commonly reported in the literature, the study of Bayliak et al. (2021) highlights that most changes (e.g., increased lipid peroxidation, decreased total antioxidant capacity and reduced glutathione) are already evident at middle age. Therefore, possible age-related changes in the variables investigated herein should already be evident at MA. In addition, Meng et al. (2007) also found no change in the protein levels of SOD1, SOD2, CAT, and GPX with aging in the brains of rats. Therefore, it is possible that the age-related decline in the antioxidant defense system comes from post-translational modifications instead of changes in their content. In terms of oxidative damage markers, we did not observe significant

changes in lipid peroxidation or protein oxidation with aging. However, long-term voluntary wheel running decreased protein oxidation in the cortex. These results agree with Radak et al. (2001), who found that 9 weeks of swimming decreased protein carbonyls but not lipid peroxidation in the brain of middle-aged rats.

Limitations of the present study include the fact that we did not have a group of male individuals to directly distinguish sex effects. In addition, we cannot rule out the possibility that changes in the variables investigated herein would be detected at later stages of aging or with more controlled exercise regimens.

CONCLUSIONS

In summary, the results of the current study showed minimal changes in several markers of mitochondrial content, function, and dynamics in the cortex and cerebellum of female rats in response to both aging and long-term physical activity. Furthermore, the redox status of the tissues investigated remained overall unaltered. Our findings suggest that the brain mitochondrial physiology and redox homeostasis of females may be more resilient to the aging process than initially thought. In addition, the lack of effects of voluntary wheel running indicates that neurological benefits of exercise may be dependent on more controlled intensity and duration. It is important to note that most studies in the field used male rodents, making it difficult to compare the results of the current study and highlights the need for more studies using females. In addition, high variability in the literature with regards to rodents' strains, sex, length of aging, exercise regimens, and brain areas, makes it difficult to draw assertive conclusions about the subject.

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Chapter 3 - Acute and chronic effects of resistance training on skeletal muscle markers of mitochondrial remodeling in older adults

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INTRODUCTION

Aging is a process characterized by a progressive decline in skeletal muscle health, which is associated with decreased quality of life and increased mortality in the elderly population (Berger & Doherty, 2010; Visser & Schaap, 2011). Besides decreases in muscle mass and strength (Berger & Doherty, 2010), termed sarcopenia, an age-related reduction in skeletal muscle oxidative capacity (Short et al., 2005) and in overall cardiorespiratory fitness (Gonzalez-Freire et al., 2018) has also been reported. However, researchers have postulated that the decreased physical activity and fitness commonly reported for older adults, and not aging per se, might be the cause of a reduced skeletal muscle mitochondrial function (Coen, Musci, Hinkley, & Miller, 2019). Although the causes of sarcopenia and reduced skeletal muscle function are multifactorial, mitochondria have been considered to play an important role in these processes (Jang, Blum, Liu, & Finkel, 2018; Marzetti et al., 2014; Sun, Youle, & Finkel, 2016).

In skeletal muscle, mitochondria exist as a highly dynamic network that responds to the energy demands of the cell. Mitochondria undergo constant remodeling through the generation of new mitochondria (biogenesis), joining (fusion), splitting (fission), and degradation of

dysfunctional portions (mitophagy) (Youle & Bliek, 2012). Adequate remodeling processes are essential for maintaining functional mitochondria (Tilokani, Nagashima, Paupe, & Prudent, 2018; Youle & Bliek, 2012). Fusion of the outer mitochondrial membrane is mediated by the Mitofusins 1 and 2 (Mfn1 and Mfn2), while Optic Atrophy 1 (Opa1) mediates the fusion of the inner mitochondrial membrane (Pernas & Scorrano, 2016). The fission process is carried out by Dynamin related protein 1 (Drp1), which is recruited from the cytosol by Fission protein 1 (Fis1) (Yoon, Krueger, Oswald, & McNiven, 2003). Lastly, mitophagy occurs mainly through the PTEN-induced putative kinase 1 (Pink1)/Parkin pathway (Youle & Narendra, 2011). Pink1 accumulates in the outer mitochondrial membrane of damaged mitochondria and recruits Parkin, which promotes their elimination (Youle & Narendra, 2011).

Exercise is considered an important strategy to prevent the decline in muscle function during aging and to improve or maintain mitochondrial function. Resistance training (RT) is a well-known method to increase muscle mass and strength at any age (Folland & Williams, 2007), while endurance training promotes mitochondrial adaptations (Holloszy, 1967; Konopka, Suer, Wolff, & Harber, 2014). However, there is a growing body of literature investigating the mitochondrial adaptations to RT in both younger (Alvehus, Boman, Söderlund, Svensson, & Burén, 2014; Lim et al., 2019; Porter, Reidy, Bhattarai, Sidossis, & Rasmussen, 2015) and older (Flack et al., 2016; Manfredi et al., 2013; Miller et al., 2020; Zampieri et al., 2016) participants, with mixed results. As pointed out by Parry, Roberts, & Kavazis (2020), RT may impose a sufficient energetic stimulus to promote mitochondrial adaptations in older participants. Although there is a growing interest in the effects of RT on mitochondrial biogenesis in older participants, there is a paucity of research investigating its effects on markers of mitochondrial remodeling, which is essential for quantity and quality control of the mitochondrial network.

Therefore, the aim of this study was to investigate the acute and chronic effects of RT on markers of mitochondrial content and remodeling in older, untrained individuals.

METHODS

Ethics approval

The current investigation was a secondary analysis of a study that aimed to explore the effects of RT with peanut protein supplementation on skeletal muscle hypertrophy in older untrained subjects (Lamb et al., 2020). Notably, participants either consumed ~72 g per day of a peanut supplement providing ~30 g protein (PBfit; Betterbody Foods, Lindon, UT, USA) or no supplement in an unblinded fashion (NCT04015479). The study was approved by the Institutional Review Board at Auburn University (Protocol # 19–249 MR 1907) and was carried out in compliance with the Declaration of Helsinki. Two-way repeated measures analysis of variance (ANOVA) showed no effects of peanut protein supplementation on any of the variables investigated in this study ($p > .05$ for all variables). Therefore, the supplemented and non-supplemented groups were combined for analysis. All participants were informed about the procedures and possible risks of the study and provided written consent prior to participation.

Participants

Sixteen older (n = 6 males, n = 10 females; age = 59 ± 4 years) participants without recent RT experience were recruited to participate in the study. Inclusion criteria required participants to be 55–80 years old, have a body mass index of less than 35, have not been participating in RT for more than 2 days/week, and to abstain from nutritional supplementation one month prior to enrollment. Participants also had to be free of overt cardio-metabolic diseases

(e.g., type II diabetes, severe hypertension, heart failure) or conditions that precluded the collection of a skeletal muscle biopsy. Notably, none of the participants had reported structured RT over the past year.

Study design

Participants visited the laboratory on three different occasions, during which a biopsy of the vastus lateralis (VL) was taken for later molecular analyses. All visits occurred at the same time of the day, and biopsies occurred in the morning hours following an overnight fast. Three participants, however, reported to the laboratory during evening hours following a ~4–5 hr fast. The first visit (Pre) occurred ~2–5 days prior to the first day of RT. Participants then started the RT program (details below) and reported to the lab for the second biopsy 24 hr following the first training session (Acute). The training program continued for 10 weeks and participants had the third biopsy taken 72 hr after the last training session (Chronic). Additional testing to evaluate the effects of the RT program on body composition, muscle thickness of the VL, and peak torque for right leg extensors were also performed during each visit and have been described and published previously (Lamb et al., 2020).

Resistance training program

A complete description of the RT program can be found in Lamb et al. (2020). Briefly, the training program was supervised, lasted 10 weeks in duration, and consisted of a whole-body workout performed twice weekly. During each session, participants performed three sets of 10–12 repetitions of leg press, leg extensions, leg curls, barbell bench press, and cable pull downs, with at least 1 min of rest between sets. At the end of each set, the level of difficulty was rated by

participants (0 = easy, 10 = hard). If the participant rated the set difficulty below 7, weight was modestly adjusted to increase resistance. If the participant rated the set difficulty at 10, or the participant was not able to complete the set because of difficulty, weight was removed. Participants were consistently instructed by staff members to be as truthful as possible when assessing the difficulty of sets. Our goal was to ensure participants gauged sets between a 7 and 9 rating.

Muscle biopsies

Muscle biopsies were taken before (Pre), 24 hr following the first training session (Acute), and 72 hr following the last training session (Chronic). A 5-gauge needle was used to obtain biopsies of the right leg VL as previously described by our laboratory (Kephart et al., 2015). Following biopsies, tissue was rapidly teased of blood and connective tissue, flash frozen using liquid nitrogen, and subsequently stored at −80°C for future use.

Western blotting

Muscle samples were removed from −80°C and crushed on a liquid nitrogen-cooled ceramic mortar using a ceramic pestle. Approximately 20 mg of tissue was placed in 200 µl of lysis buffer (25 mM Tris, pH 7.2, 0.5% Triton X-100, 1x protease inhibitors) and homogenized using tight-fitting plastic pestles. Samples were centrifuged at 1,500 g for 10 min at 4°C. Supernatants were then collected and used to determine protein concentrations using a commercially available bicinchoninic acid kit (Thermo Fisher Scientific; Walthan, MA, USA). Afterwards, supernatants were prepared for Western blotting using 4x Laemmli buffer and distilled water (diH2O).

Samples (12 µl) were pipetted onto gradient sodium dodecyl sulfate-polyacrylamide gels (4%–15% Criterion TGX Stain-free gels; Bio-Rad Laboratories; Hercules, CA, USA), and proteins were separated by electrophoresis (200 V for approximately 45 min). After electrophoresis, proteins were transferred to preactivated polyvinylidene difluoride membranes (Bio-Rad Laboratories) for 2 hr at 200 mA. Gels were then Ponceau stained for 3 min, washed with diH2O for 30 s, quickly dried, and digitally imaged with a gel documentation system (UVP, LLC, Upland, CA, USA). Following Ponceau imaging, membranes were reactivated in methanol, blocked with nonfat milk for 1 hr (5% w/v diluted in Tri-buffered saline with 0.1% Tween 20, or TBST (Tris-buffered saline with Tween 20)), washed three times in TBST only (Berg et al., 2020), and incubated for 1 hr with primary antibodies (1:2000 v/v dilution in TBST with 5% BSA). Primary antibodies were used to detect: Total OXPHOS Human Cocktail (Abcam Cat# ab110411, RRID:AB_2756818), COX IV (Cell Signaling Technology Cat# 4850, RRID:AB_2085424), PGC-1α (GeneTex Cat# GTX37356, RRID:AB_11175466), NRF1 (GeneTex Cat# GTX103179, RRID:AB_11168915), TFAM (Abnova Corporation Cat# H00007019-D01P, RRID:AB_1715621), Mfn1 (Cell Signaling Technology Cat# 14739, RRID:AB_2744531), Mfn2 (BioVision Cat# 3882-100, RRID:AB_2142625), Opa1 (Cell Signaling Technology Cat# 67589, RRID:AB_2799728), Fis1 (Abcam Cat# ab71498, RRID:AB_1271360), Drp1 (Novus Cat# NB110-55288SS, RRID:AB_921147), Pink1 (Cell Signaling Technology Cat# 6946, RRID:AB_11179069), and Parkin (Cell Signaling Technology Cat# 2132, RRID:AB_10693040). Due to the protocol utilized, we were unable to detect complex IV in Total OXPHOS Human Cocktail, which has been previously reported (Herbst et al., 2014; Miotto, McGlory, Holloway, Phillips, & Holloway, 2018). Therefore, we interrogated

COX IV as an individual target. Validation of the antibodies used has been previously reported (Balan et al., 2019; Campbell, To, & Spector, 2019; Liu, Peyton, & Durante, 2013; Ordureau et al., 2018; Parry et al., 2019; Pillon et al., 2020; Radde et al., 2016; Tarpey et al., 2019; Yao et al., 2019; Zhang et al., 2017; Zhong et al., 2019). Following primary antibody incubations, membranes were washed three times in TBST only for 5 min, and incubated for 1 hr with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology Cat# 7074, RRID:AB_2099233) or anti-mouse IgG (Cell Signaling Technology Cat# 7076, RRID:AB_330924). Membranes were then washed in TBST only (3x5 min), developed using chemiluminescent substrate (Millipore; Burlington, MA, USA), and digitally imaged in a gel documentation system (UVP, LLC, Upland, CA, USA). Raw target band densities were obtained using imaging software ImageJ (NIH, Bethesda, MD, USA), and the values were normalized to Ponceau staining. These values were then divided by the mean of baseline values (Pre) to obtain fold-difference values.

Statistics

All statistical analyses were performed using SPSS v21.0 (IBM Corp, Armonk, NY, USA). Data are expressed as mean ± standard deviation (SD). Repeated measures ANOVAs were performed to examine the effects of RT on individual targets. The sphericity assumption on all dependent variables was tested using the Mauchly's test, and the Greenhouse–Geisser correction was used when the sphericity assumption was violated. Post hoc Bonferroni tests were used when appropriate. Statistical significance was established at $p < .050$.

RESULTS

Participant characteristics and training adaptations

Although participant characteristics and certain training adaptations were reported in Lamb et al. (2020), they are listed here for convenience to the reader. The participant cohort was made up of n = 6 males and 10 females. The average age of participants prior to training was 59 ± 4 years of age. Participants had a body mass index of 31.7 ± 5.6 kg/m2, a fat-free mass index (FFMi; DXA FFM in kg divided by height in m2) of 18.0 ± 2.9 kg/m2, and a body fat percentage of 39.3 ± 6.3%; the latter two variables being determined by dual energy X-ray absorptiometry (DXA).

Regarding training adaptations, the participants experienced an increase in DXA FFM (±1.0 ± 1.9 kg), albeit this trended towards significance ($p = .061$). However, VL muscle thickness (assessed using an ultrasound) increased from 1.88 ± 0.45 cm to 2.02 ± 0.37 cm with training, and knee extensor peak torque at 60°/s increased from 115 ± 43 to 127 ± 40 N m with training. Both of these increases were significant ($p < .05$).

Mitochondrial content

Acute RT did not significantly affect the skeletal muscle protein levels of any of the electron transport chain complexes analyzed (CI: +13% [$p = 1.000$], CII: +1% [$p = 1.000$], CIII: +8% [$p = 1.000$], CIV: +2% [$p = 1.000$], CV: +3% [$p = 1.000$]). However, at the end of 10 weeks of training, all five complexes had increased protein levels compared to baseline values (CI: +180% [$p < .001$], CII: +39% [$p = .020$], CIII: +89% [$p < .001$], CIV: +43% [$p < .001$], CV: +78% [$p < .001$]) (Figure 1).

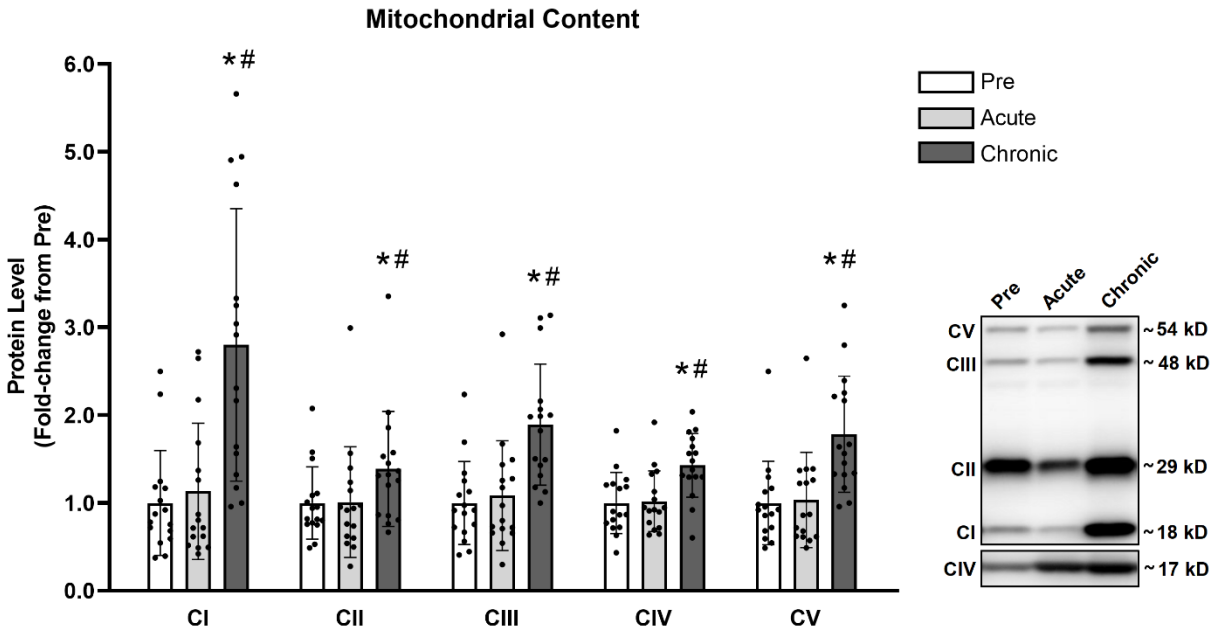

Figure 3-1. Chronic RT significantly increased protein levels of complexes I to V. Legend: CI to V, Complex I to V; Pre, protein levels at baseline; Acute, protein levels at 24 hr after the first training session; Chronic, protein levels at 72 hr after the last training session; *, significantly different from Pre ($p < .050$); #, significantly different from Acute ($p < .050$). Data are presented as means ± SD, n = 16 (6 men, 10 women)

Mitochondrial biogenesis

No significant differences ($p > .050$) were detected for skeletal muscle PGC-1α or TFAM protein levels following acute or chronic RT. NRF1 protein levels were elevated following acute RT (+98%, $p = .019$) and albeit not statistically significant, trended to increase following chronic RT (+116%, $p = .082$) (Figure 2).

Mitochondrial fusion

Mfn1 and Mfn2 protein levels of skeletal muscle did not significantly change following acute RT (Mfn1: +16% ($p = .430$), Mfn2: +20%, ($p = .207$)) but increased following chronic RT (Mfn1: +90% ($p = .003$), Mfn2: +110% ($p < .001$)). Opa1 protein levels, on the other hand, increased following acute (+115%, $p = .011$) and chronic RT (+261%, $p = .004$) (Figure 3).

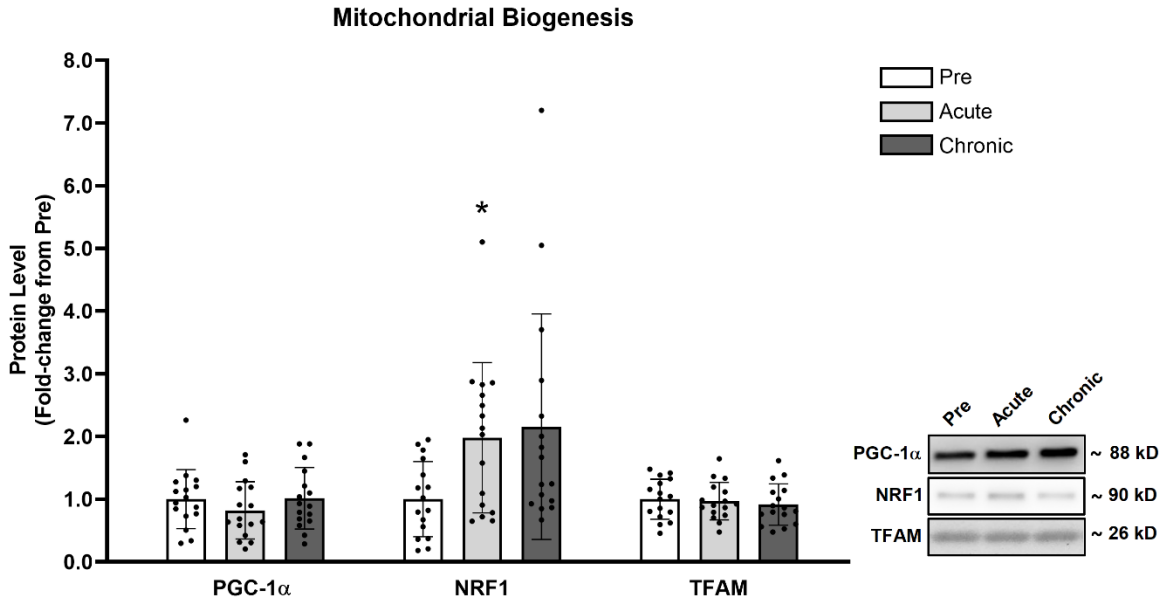

Figure 3-2. RT acutely increased NRF1 but did not alter PGC-1α or TFAM protein levels. Legend: Pre, protein levels at baseline; Acute, protein levels at 24 hr after the first training session; Chronic, protein levels at 72 hr after the last training session; *, significantly different from Pre ($p < .050$). Data are presented as means ± SD, n = 16 (6 men, 10 women)

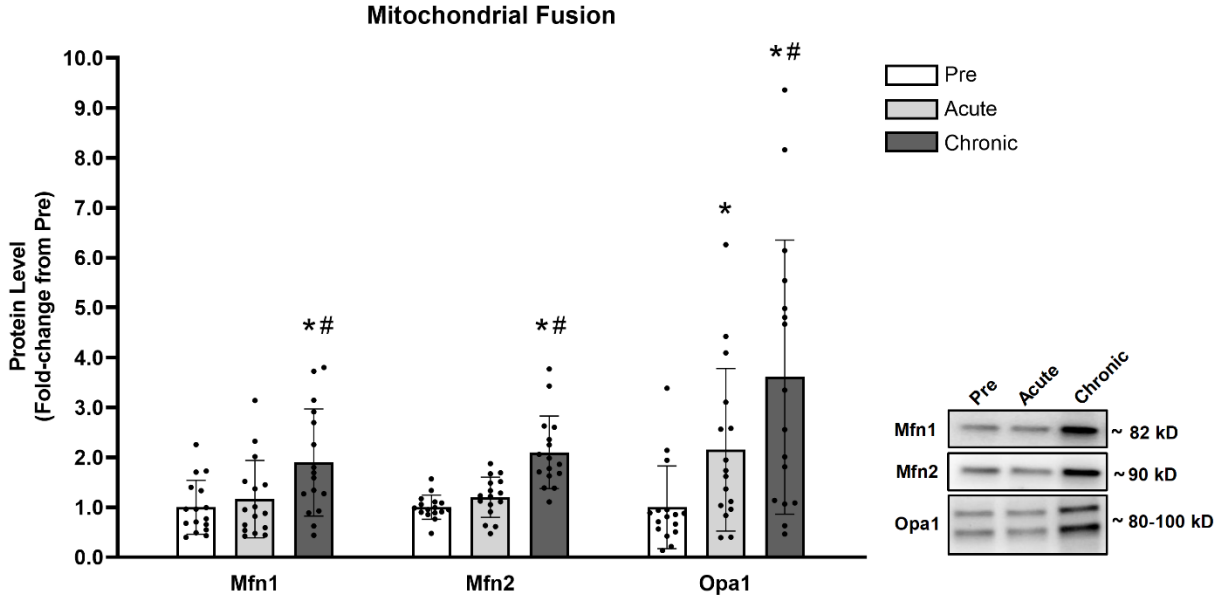

Figure 3-3. Chronic RT significantly increased markers of mitochondrial fusion. Legend: Pre, protein levels at baseline; Acute, protein levels at 24 hr after the first training session; Chronic, proteins level at 72 hr after the last training session; *, significantly different from Pre ($p < .050$); #, significantly different from Acute ($p < .050$). Data are presented as means ± SD, n = 16 (6 men, 10 women)

Mitochondrial fission

Drp1 protein levels did not significantly change following acute RT (+38%, p = .443), but increased following chronic RT (+134%, p = .038). Fis1 protein levels did not present any significant changes in response to acute (−1%, p = .584) or chronic (−5%, p = .584) RT (Figure 4).

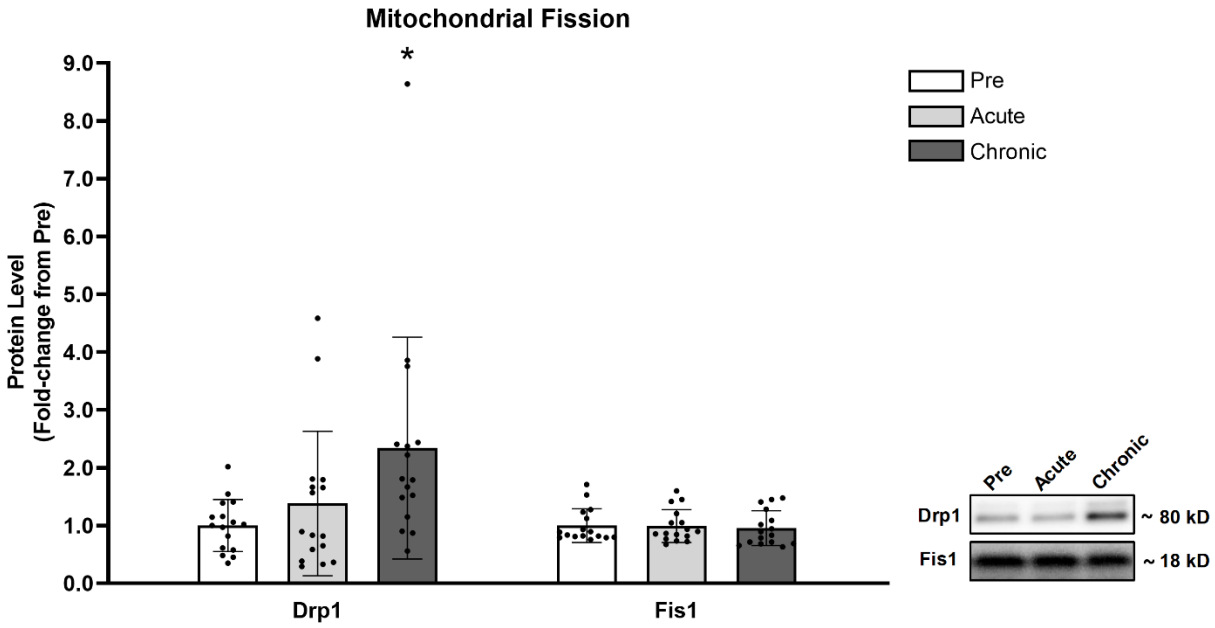

Figure 3-4. Drp1 protein levels significantly increased following chronic RT, while Fis1 remained unchanged. Legend: Pre, protein levels at baseline; Acute, protein levels at 24 hr after the first training session; Chronic, protein levels at 72 hr after the last training session; *, significantly different from Pre ($p < .050$). Data are presented as means ± SD, n = 16 (6 men, 10 women)

Mitophagy

No significant differences ($p > .050$) were detected for Pink1 or Parkin protein levels following acute or chronic RT (Figure 5).

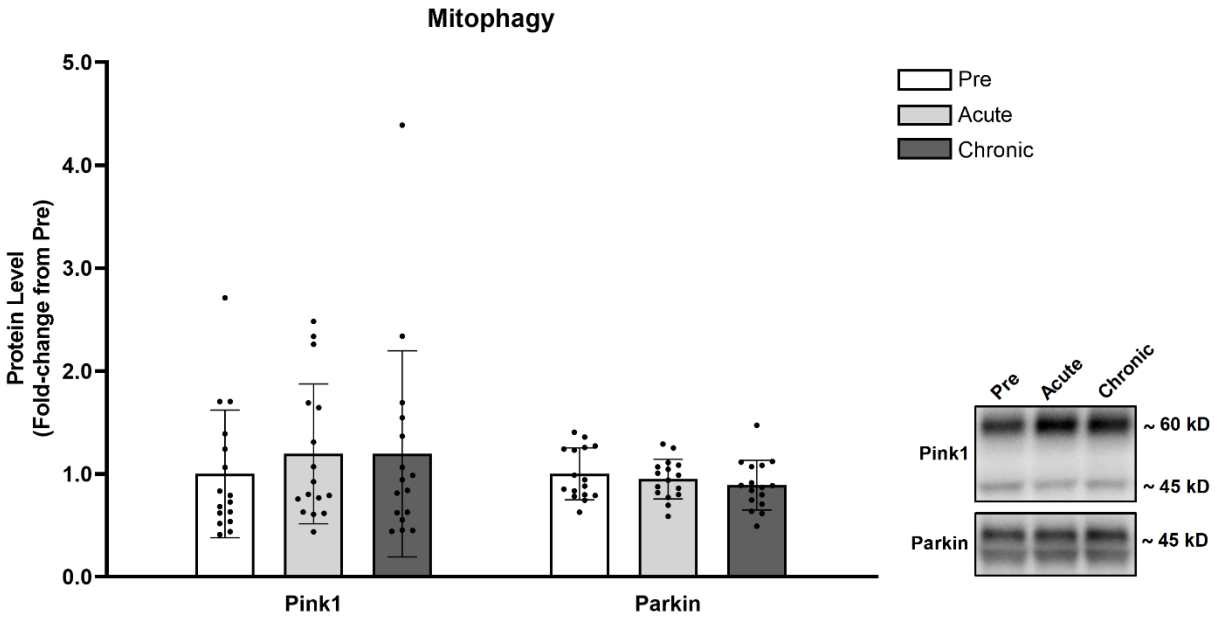

Figure 3-5. Mitophagy was not significantly altered in response to RT. Legend: Pre, protein levels at baseline; Acute, protein levels at 24 hr after the first training session; Chronic, protein levels at 72 hr after the last training session. Data are presented as means ± SD, n = 16 (6 men, 10 women)

DISCUSSION

Resistance training has been well recognized as an effective method to increase muscle hypertrophy and strength (Folland & Williams, 2007). However, whether or not RT induces mitochondrial adaptations remains equivocal and poorly investigated, especially in older individuals (Parry et al., 2020). We investigated the acute and chronic effects of RT on markers of mitochondrial content and remodeling in older individuals. Our findings demonstrated that 10 weeks of RT led to increases in mitochondrial OXPHOS protein content and dynamics, although only mild acute stimulus for mitochondrial biogenesis was detected.

A previous study conducted by our group with the same cohort investigated herein showed that the RT protocol led to significantly increased strength, localized hypertrophy, and several markers of skeletal muscle mitochondrial metabolism (Lamb et al., 2020). In addition, we previously reported that a robust increase in citrate synthase activity was observed,

suggesting increased mitochondrial content in response to RT. Therefore, the increased protein levels of complexes I to V observed in this study is in agreement with the previous findings. Our results are also in agreement with other studies that found increased mitochondrial content in older subjects in response to RT (Jubrias, Esselman, Price, Cress, and Conley, 2001; Manfredi et al., 2013; Robinson et al., 2017). Robinson et al. (2017), for example, showed via proteomics that 12 weeks of RT increased mitochondrial protein abundance in older participants, albeit there was no improvement in mitochondrial function. However, it is important to note that no significant changes in mitochondrial markers have also been reported with RT in younger (Porter et al., (2015)) and older populations (Flack et al., 2016; Irving et al., 2019; Parise, Brose, & Tarnopolsky, 2005). A recent study conducted by Berger and Doherty (2010) showed a somewhat surprising decrease in mitochondrial function but no change in markers of mitochondrial content following 8 weeks of maximal strength training in older adults. While reasons for these equivocal findings are difficult to reconcile, we posit that this may be related to both the characteristics of the training protocol and to the fitness and physical activity levels of the participants. Training protocols that elicit greater metabolic demands seem to be more beneficial to mitochondrial adaptations (Lim et al., 2019). Furthermore, physical activity levels and fitness are possible confounding factors in the relationship between mitochondria and the aging process, in a way that a decline in physical activity, and not aging per se, is what causes a decrease in mitochondrial content or function (Coen et al., 2019). Therefore, RT might only be beneficial to individuals who are poorly conditioned prior to intervention, acting to restore a "healthy" mitochondrial status. The RT protocol adopted in this study was probably enough of a stimulus to elicit oxidative adaptations given that these participants were poorly conditioned

prior to RT. In this regard, future studies are needed to determine how preexisting physical activity levels in older participants affect mitochondrial adaptations to RT.

Given the increased mitochondrial protein content in response to chronic RT, levels of proteins involved in the transcriptional control of mitochondrial biogenesis were also expected to be increased. Mitochondrial biogenesis requires a coordinated regulation of transcription of both nuclear and mitochondrial genes. Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) is considered to be a key regulator of mitochondrial biogenesis (Handschin & Spiegelman, 2006; Jornayvaz & Shulman, 2010). PGC-1α interacts with and activates NRF1, increasing nuclear transcription of mitochondrial genes (Hood, 2009). In addition, PGC-1α also controls the expression of the mitochondrial transcriptional factor TFAM, a key regulator of mitochondrial DNA expression (Wu et al., 1999). Despite the clear roles of PGC-1α, NRF1, and TFAM on mitochondrial biogenesis, the only significant change detected in this study was the acute increase in NRF1 protein levels. Ogborn et al. (2015) investigated the response of PGC-1α, NRF1, and TFAM mRNA levels following one bout of resistance exercise in older subjects. The authors observed no change in NRF1 mRNA, and initial increases in PGC-1α and TFAM (at 3 and 24 hr postexercise, respectively) followed by a return to baseline levels 48 hr following exercise. Another study also reported that chronic RT did not alter PGC-1α or TFAM mRNA levels in older participants (Flack et al., 2016). However, as highlighted by Robinson et al. (2017), adaptations at the transcriptional level do not necessarily imply proteomic adaptations. Thus, care should be taken when comparing the results of studies that used different measures. Irving et al. (2019) found an increase in the protein levels of PGC-1α but no change in TFAM after 8 weeks of RT. Similar contradictory results showing increased mitochondrial content but no change in PGC-1α, NRF1, or TFAM have been reported in young individuals in response to

RT (Lim et al., 2019) and in older subjects in response to endurance training (Balan et al., 2019). Although there is no clear explanation for the overall lack of change in biogenesis markers despite the substantial increase in mitochondrial content found in this study, the contradictory results might be related to the timing of biopsy. Furthermore, researchers have recently reevaluated the role of PGC-1α on mitochondrial biogenesis and showed that other proteins, such as PPARβ, may also be important in regulating mitochondrial biogenesis (Islam & Bonafiglia, 2019; Islam, Hood, & Gurd, 2020). Future studies should investigate how RT may be affecting other regulators of mitochondrial biogenesis in both younger and older individuals.

Besides mitochondrial biogenesis, the control of mitochondrial quantity and quality depends on the integration of coordinated events such as fusion, fission, and mitophagy. Studies have shown that proper mitochondrial dynamics are impaired during aging (Marzetti et al., 2014) and that exercise is an important strategy for counteracting such effects (Marzetti et al., 2014; Ziaaldini, Hosseini, & Fathi, 2017). In this study, RT did not acutely change any of the proteins related to mitochondrial dynamics. However, Mfn1, Mfn2, Opa1, and Drp1 were increased following 10 weeks of RT. The observed changes point to a scenario of increased mitochondrial fusion. Our results are in agreement with a study that investigated the effects of RT on mitochondrial dynamics in rats (Kitaoka, Ogasawara, Tamura, Fujita, & Hatta, 2015). The authors showed that chronic RT led to increased fusion but did not change fission. Four months of endurance training has also been shown to induce the same adaptations in older participants (Arribat et al., 2019). Lim et al. (2019), in turn, showed that lower-load higher-volume RT, believed to impose a greater metabolic demand, increased Fis1, Drp1, and Opa1 protein levels from biopsies obtained in young participants, but did not change Mfn2 protein levels. The differences in response between younger and older individuals might be related to an already

unbalanced dynamics in favor of fission with aging (Carter, Chen, & Hood, 2019). This increased fission leads to a fragmented mitochondrial network, which in turn is linked to mitochondrial dysfunction (Marzetti et al., 2014; Zemirli, Morel, & Molino, 2018). Therefore, the increased fusion observed in this study could be a positive adaptation to counteract age-related increased fission. Further, increased mitochondrial damage is reported with aging (Johnston, De, & Parise, 2008; Tarnopolsky, 2009) and fusion could have a role in mixing the contents of two mitochondria and possibly diluting damaged material. Thus, our data support the fact that RT positively affects mitochondrial fusion markers, and this adaptation may have led to an enhancement in mitochondrial function.

As previously stated, mitophagy is another essential process for mitochondrial quality control, as it removes dysfunctional portions of mitochondria. Mitophagy is believed to be impaired with aging, and an age-related impairment in mitophagy could be linked to the accumulation of damaged mitochondria with aging (Jang et al., 2018). Exercise has been considered a strategy to increase mitophagy and therefore counteract such processes (Carter et al., 2019; Drake, Wilson, & Yan, 2019). Our results showed no change in Pink1 and Parkin protein levels in response to acute or chronic RT, which is not surprising considering that fission precedes mitophagy and a more robust increase in markers of mitochondrial fusion compared to fission was detected. Another study has shown that Parkin protein levels in rat skeletal muscle are not altered in response to RT (Kitaoka et al., 2015). Moreover, protein levels of Pink1 and Parkin have been shown to not be altered in older human participants following RT (Ogborn et al., 2015). On the other hand, Lim et al. (2019) reported increases in mitophagy protein markers in younger participants in response to RT, especially in the group performing lower-load higher-volume RT. It is possible that the discrepancy in the results is related to the different training

protocols used and that a greater metabolic stress is needed to stimulate mitophagy. It is important to note that our results do not necessarily mean unaltered mitophagy. Pink1 and Parkin are subjected to phosphorylation events that impact their activity (Zhuang, Li, Chen, & Wang, 2016) and the levels of these phosphorylated proteins were not analyzed in this study. In addition, we cannot rule out the possibility that mitophagy was increased through Pink1/Parkin-independent pathways. Notwithstanding, these preliminary data suggest that RT does not alter mitophagy markers in older individuals.

Experimental considerations

Limitations of this study include the fact that we did not have a control group of younger participants, and therefore, the changes observed herein might not be age-specific. Furthermore, mitochondrial function can differ between males and females (Ferreira, 2018; Miotto et al., 2018), but our sample size did not enable such comparisons to be made in this study. Another limitation is that the acute responses to RT were determined at single time point (24 hr following the first training session). Therefore, our data regarding the acute responses must be interpreted with caution, as we may have missed important changes in the markers analyzed. In addition, even though changes in proteins related to mitochondrial content and dynamics were detected, measures of oxidative phosphorylation were not conducted due to tissue limitations. It is important to note, however, that studies have linked increased protein content to the observed increased mitochondrial function in response to RT before (Holloway et al., 2018; Porter et al., 2015). Even though such observations regarding markers of mitochondrial remodeling are scarce, studies have also demonstrated that markers of mitochondrial fusion (e.g., Mfn1 and Mfn2) can directly impact mitochondrial function (Bach et al., 2003; Chen, Chomyn, & Chan,

2005). As stated prior, mitochondrial dysfunction is intricately linked with muscle aging (Jang et al., 2018; Marzetti et al., 2014), and such measures could provide insightful information about the potential benefits of RT.

CONCLUSIONS

In conclusion, the results of this study showed that 10 weeks of RT increased mitochondrial protein content and markers of mitochondrial dynamics, although no changes in these markers were detected following the first training bout. The results suggest that the acute response may not be representative of the chronic effects of RT, and that repeated bouts are necessary to achieve mitochondrial benefits of RT in older populations. Critically, besides the known improvements in muscle mass and strength, RT could be a viable approach to improve the levels of proteins involved in oxidative phosphorylation and in mitochondrial dynamics.

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Chapter 4 - Effects of Resistance Training on the Redox Status of Skeletal Muscle in Older Adults

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INTRODUCTION

Advancing age is characterized by a decline in skeletal muscle mass and function (Berger and Doherty, 2010) and is associated with the development of several chronic diseases (Niccoli and Partridge, 2012). Different hallmarks of aging have been identified including genomic instability, loss of proteostasis, and mitochondrial dysfunction (López-Otín et al., 2013). A disruption of the redox homeostasis (i.e., balance between the production and removal of reactive species) can lead to a state of oxidative stress, and has been considered one of the cellular mechanisms driving the aging process (Harman, 1956; Salmon et al., 2010; Jang and Van Remmen, 2011; Dai et al., 2014).

Several studies have reported increased oxidative stress with aging (Pansarasa et al., 2000; Gianni et al., 2004; Ryan et al., 2008). Although reactive species are now recognized as important regulators of several signaling pathways (Powers et al., 2010), their overproduction can cause damage to different cellular components. Essential in preventing such damage and maintaining the redox homeostasis in skeletal muscle are the antioxidant enzymes superoxide dismutase 1 (SOD1) and 2 (SOD2), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GSR) (Powers et al., 2011). Together, these endogenous antioxidants

constitute a defense and repair system against the potential harmful effects of reactive species. In addition, a family of stress induced proteins called heat-shock proteins (HSPs) have been shown to be responsive to oxidative stress and to have a role in the skeletal muscle defense system (Morton et al., 2009; Cobley et al., 2015; Dimauro et al., 2016).

Exercise has been considered a therapeutic tool to reduce chronic oxidative stress. Indeed, a review conducted by de Sousa et al. (de Sousa et al., 2017) suggested that exercise training promotes increased antioxidant capacity and decreased oxidative damage, regardless of the type of exercise, volume and intensity. Nevertheless, studies investigating the effects of resistance training (RT) on antioxidant and oxidative stress parameters are scarce. A recent systematic review and meta-analysis conducted by Cuyul-Vásquez et al. (Cuyul-Vásquez et al., 2020) sought to elucidate the effects of RT on redox homeostasis in older individuals. While their results suggested that RT has no beneficial effects on various biomarkers, the authors emphasize that the results should be interpreted with caution because of the limited number and low methodological quality of studies available. In addition, Cuyul-Vásquez et al. (Cuyul-Vásquez et al., 2020) highlight the fact that most studies interrogated plasma, which may not reflect the redox signaling in skeletal muscle (Parker et al., 2018) and may be inappropriate for determining antioxidant enzymatic activity (Cobley et al., 2017).

To the best of our knowledge, no study to date has performed a thorough investigation of the effects of RT on antioxidants and oxidative damage in skeletal muscle of older humans using a transcriptional, post-transcriptional and post-translational approach. Therefore, the purpose of this study was to investigate the effects of RT on redox status of skeletal muscle of older adults. Specifically, we examined the effects of 6 weeks of RT on mRNA, protein, and enzymatic activity of several endogenous antioxidants and markers indicative of oxidative stress. Moreover,

we measured protein levels of certain HSPs given their association with oxidative stress. For referencing purposes, we also compared the assayed parameters to a group of college-aged individuals in order to examine if RT was capable of restoring the assayed biomarkers to "youth-like" levels. Our study demonstrated that 6 weeks of RT significantly decreased lipid peroxidation and increased antioxidant enzymatic activities. Further, RT increased mRNA levels of all assayed antioxidant genes, and either did not change or decreased protein levels of antioxidants.

MATERIALS AND METHODS

Ethical approval

This study was a secondary analysis of two previous studies approved by the Institutional Review Board at Auburn University. The first study (Protocol # 19-249 MR 1907) investigated the effects of peanut protein supplementation and RT on muscle mass and strength of older participants. The second study (Protocol # 19-245 MR 1907) aimed to explore the effects of high-load low-volume and high-volume low-load RT paradigms on muscle hypertrophy in college-aged males. All participants provided written consent prior to participation and both studies conformed to standards set by the Declaration of Helsinki. Two-way repeated measures analysis of variance (ANOVA) was conducted to analyze the effects of peanut protein supplementation on the variables investigated in the current study. The results showed no effects of supplementation ($p > 0.050$) for most variables, and therefore, the supplemented and non-supplemented groups were combined for analysis. The variables that were affected by supplementation ($p < 0.050$; TAC and GSR) will be highlighted and discussed in the results and discussion sections.

Participants

Thirteen older males (age = 64 ± 9 years) without recent RT experience were recruited to participate in this study. Inclusion criteria required participants to be 50-80 years old, have not been participating in structured RT for at least three months prior, and to abstain from nutritional supplementation one month prior to enrollment. Participants also had to be free of overt cardio-metabolic diseases (e.g., type II diabetes, severe hypertension, heart failure) or conditions that precluded the collection of a skeletal muscle biopsy. Data from ten recreationally resistance-trained college-aged males (age = 23 ± 3 years, average training experience = 7 years) were also obtained and are presented in the graphs and Tables 1 and 2 below.

Table 4-1. PCR primer sequences for mRNA analysis of antioxidant genes

Gene	Primer sequences	Amplicon length	Position	NCBI Ref. Seq.
SOD1	**FP (5' → 3'):** TGTGGCCGATGTGTCTATTGAA **RP (5' → 3'):** CACCTTTGCCCAAGTCATCTG	109 bp	**FP:** 430-451 **RP:** 519-539	NM_000454
SOD2	**FP (5' → 3'):** GTTGGGGTTGGCTTGGTTTC **RP (5' → 3'):** GCCTGTTGTTCCTTGCAGTG	89 bp	**FP:** 511-530 **RP:** 580-599	NM_000636
CAT	**FP (5' → 3'):** CTGACTACGGGAGCCACATC **RP (5' → 3'):** AGATCCGGACTGCACAAAGG	92 bp	**FP:** 1540-1559 **RP:** 1612-1631	NM_001752
GPx-1	**FP (5' → 3'):** ACGAGGGAGGAACACCTGAT **RP (5' → 3'):** TCTGGCAGAGACTGGGATCA	80 bp	**FP:** 775-794 **RP:** 835-854	NM_000581
GSR	**FP (5' → 3'):** AAAAAGTACACCGCCCCACA **RP (5' → 3'):** ATCTGGCTCTCATGAGGGGT	71 bp	**FP:** 576-595 **RP:** 1612-1631	NM_000637
GAPDH	**FP (5' → 3'):** AACCTGCCAAATATGATGAC **RP (5' → 3'):** TCATACCAGGAAATGAGCTT	193 bp	**FP:** 828-847 **RP:** 1001-1020	NM_002046

FP, forward primer; RP, reverse primer; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; CAT, catalase; GPx-1, glutathione peroxidase 1; GSR, glutathione reductase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table 4-2. Participant characteristics and training adaptations

	Old-Pre	Old-Post	Young
Body mass (kg)	89.8 ± 11.7	90.1 ± 11.5	89.7 ± 11.7
FFM (kg)	59.2 ± 6.1	59.9 ± 6.4 *	69.3 ± 8.0 *#
FM (kg)	27.4 ± 7.5	27.0 ± 7.2	17.2 ± 7.2 *#
VL thickness (cm)	2.18 ± 0.4	2.31 ± 0.3 *	2.8 ± 0.3 *#
mCSA (cm^2)	148.9 ± 21.4	151.1 ± 21.7	201.3 ± 28.9 *#
Knee ext – 60º/s (N.m)	154 ± 55	173 ± 41 *	229 ± 46 *#
Knee ext – 120º/s (N.m)	125 ± 41	142 ± 34 *	191 ± 36 *#

Data are presented as mean ± standard deviation; FFM, fat-free mass; FM, fat mass; VL, vastus lateralis, mCSA, muscle cross-sectional area; ext, extension. *, different from Old-Pre ($p < 0.050$); #, different from Old-Post ($p < 0.050$).

Study design

Participants visited the laboratory on two different occasions prior to beginning the RT program. The first visit consisted of a battery of assessments including height, body mass, full body dual energy x-ray absorptiometry (DXA), ultrasound of the right leg vastus lateralis (VL), and right leg strength assessment using an isokinetic dynamometer. On the second visit, a biopsy of the VL was taken from the right leg for posterior molecular analyses. Participants then completed the 6-week RT program and reported back to laboratory for post-training assessments 72 hours following their last training bout.

Testing sessions

A complete description of testing procedures can be found in Lamb et al. (Lamb et al., 2020b). Briefly, participants reported to the laboratory following an overnight fast, body mass and height were assessed, and images were captured from the right leg VL using real-time B-mode ultrasonography (LOGIQ S7 Expert, GE Healthcare, USA). Thereafter, participants underwent full body DXA scan (Lunar Prodigy; GE Corporation, Fairfield, CT, USA) for

determination of fat free mass (FFM) and fat mass (FM). Following DXA scan, participants performed maximal isokinetic right leg extensions at 60°/sec and 120°/sec.

Muscle biopsies

Muscle biopsies were taken from the right leg VL before the RT program (Old-Pre) and 72 hours following the last training session (Old-Post) for the older participants and in the basal state for the college-aged group (Young). A 5-gauge needle was used to obtain biopsies of the right leg VL as previously described by our laboratory (Kephart et al., 2015). Following biopsies, tissue was rapidly teased of blood and connective tissue, flash frozen using liquid nitrogen, and subsequently stored at -80°C for future use.

Resistance training (RT) program

The RT program consisted of a whole-body workout performed twice weekly for six weeks. Each session was composed of the following exercises: 1) leg press, 2) leg extensions, 3) leg curls, 4) barbell bench press, 5) cable pull downs. Participants per-formed 3 sets of 10-12 repetitions for each exercise with 1 minute of rest between sets. Participants were asked to rate the level of difficulty for each set (0 = easy, 10 = hard), and weight was adjusted accordingly to ensure a 7-9 rating. All exercises and training sessions were recorded, and all participants increased weight lifted throughout the pro-gram. More in-depth details about training volume and progression can be found in Lamb et al. (Lamb et al., 2020b).

mRNA analysis

A subset of ten older and eight younger participants was used for mRNA analysis. Approximately 20 mg of muscle were placed in 500 µl of Ribozol (Ameresco, Solon, OH) and RNA isolation proceeded following manufacturer's instructions. RNA concentrations were determined in duplicate using a NanoDrop Lite (Thermo Fisher Scientific) and cDNA (2 µg) was synthesized using a commercial qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). RT-qPCR was performed in a RT-PCR thermal cycler (Bio-Rad) using SYBR-green-based methods with gene-specific primers designed with primer designer software (Primer3Plus, Cambridge, MA). For all primer sets, pilot qPCR reactions and melt data indicated that only one amplicon was present. The forward and reverse primer sequences for all genes are listed in Table 1. Fold change values were performed using the 2ΔΔCq method where 2ΔCq = 2^[housekeeping gene (HKG) Cq – gene of interest Cq] and 2ΔΔCq (or fold change) = [2ΔCq value/2ΔCq average of Old-Pre]. Glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene.

Western Blotting

Approximately 20 mg of muscle tissue was homogenized in lysis buffer (25 mM Tris, pH 7.2, 0.5% Triton X-100, 1x protease inhibitors) using tight-fitting plastic pestles. Samples were then centrifuged at 500_ g for 5 min at 4 _C. Supernatants were placed in new cryotubes, and protein concentrations were determined from supernatants using a commercially available BCA kit (Thermo Fisher Scientific, Walthan, MA, USA). Supernatants were then prepared for Western blotting using 4x Laemmli buffer and distilled water (diH2O).

Twelve microliters (12 _L) of Western blot preps were loaded on gradient SDS polyacrylamide gels (4–15% Criterion TGX Stain-free gels; Bio-Rad Laboratories; Hercules, CA, USA), and proteins were separated by electrophoresis for approximately 45 min at 200 V. Subsequently, proteins were transferred to pre-activated PVDF membranes (Bio-Rad Laboratories) for 2 h at 200 mA. Membranes were then Ponceau stained, quickly washed with diH2O, dried, and digitally imaged with a gel documentation system (UVP, LLC, Upland, CA, USA).). Following Ponceau imaging, methanol was used to re-activate membranes, which were then blocked with nonfat milk (5% w/v diluted in Tri-buffered saline with 0.1% Tween 20, or TBST) for 1 hour, washed three times in TBST (5 minutes per wash), and incubated for 1 hour with primary antibodies (1:2000 v/v dilution in TBST with 5% BSA). Primary antibodies included: SOD1 (GeneTex Cat# GTX100554, RRID:AB_10618670), SOD2 (GeneTex Cat# GTX116093, RRID:AB_10624558), CAT (GeneTex Cat# GTX110704, RRID:AB_1949848), GPx-1 (GeneTex Cat# GTX116040, RRID:AB_2037097), HSP60 (Abcam Cat# ab46798, RRID:AB_881444), HSP70 (Abcam Cat# ab79852, RRID:AB_1603786), HSP90 (Abcam Cat# ab13495, RRID:AB_1269122), and 4-hydroxynonenal (4HNE) (Abcam Cat# ab46545, RRID:AB_722490). Protein carbonyl levels were assessed using the Oxyblot protein oxidation detection kit (Millipore, Billerica, MA; #S7150). Validation of the antibodies has been previously reported (Apostolopoulos et al., 2018; Basso et al., 2018; Wang et al., 2018; Parry et al., 2019; Tarpey et al., 2019). Afterwards, membranes were washed in TBST and incubated with anti-rabbit secondary antibody (Cell Signaling Technology Cat# 7074, RRID:AB_2099233) for 1 hour. Membranes were washed in TBST again, developed using chemiluminescent substrate (Millipore; Burlington, MA, USA), and digitally imaged in a gel documentation system (UVP, LLC, Upland, CA, USA). ImageJ software (NIH, Bethesda, MD, USA) was used to obtain the

raw density of target bands. The most prominent band (~45kD) in Ponceau stains were used to normalize the target bands. The values were then divided by the mean of Old-Pre group to obtain fold-change values. 4HNE blots were analyzed from approximately 100kD to 17kD, while the whole lane (>245kD to approximately 20kD) was analyzed for protein carbonyl blots.

Enzymatic activities

Muscle lysates were used to determine total antioxidant capacity (TAC) (Cayman, MI, USA, Cat# 709001), and the enzymatic activities of catalase (Cayman, MI, USA, Cat# 707002), glutathione peroxidase (Cayman, MI, USA, Cat# 703102), and glutathione reductase (Cayman, MI, USA, Cat# 703202) according to the manufacturer's instructions for each assay kit. Enzymatic activity was normalized by the amount of protein (mg) used in each essay. Superoxide dismutase activity was also assessed using a colorimetric assay kit (Cayman, MI, USA, Cat# 706002) but interference of reagents used in the homogenization of muscle samples prevented the acquisition of reliable data. Therefore, SOD activity will not be reported herein.

Statistics

Data are expressed as mean ± standard deviation (SD) values, and 95% confidence intervals are presented for differences between time-points and between groups. Shapiro-Wilks test were used to assess the distribution of data for each dependent variable. Dependent samples t-tests were used to analyze pre x post data for the older group. In addition, independent samples t-tests were used to compare Old-Pre and Old-Post to Young group. Variables that did not present a normal distribution were analyzed using Wilcoxon Signed Rank or Mann-Whitney U tests for dependent and independent variables, respectively. Statistical significance was

established at $p < 0.050$. All statistical analyses were performed using SPSS v21.0 (IBM Corp, Armonk, NY, USA).

RESULTS

Participant characteristics and training adaptations

Participant characteristics and training adaptations can be found in Table 2. Notably, the RT program increased the following variables in older participants: FFM ($p < 0.001$, 95% CI [0.27, 1.24]), VL thickness ($p = 0.040$, 95% CI [0.01, 0.25]), and knee extension torque at 60º/s ($p = 0.033$, 95% CI [1.77, 36.28]) and 120º/s ($p = 0.022$, 95% CI [2.89, 31.00]). In addition, there was a trend to decrease FM ($p = 0.081$, 95% CI [-1.02, 0.07]).

mRNA expression

Muscle mRNA expression is presented in Figure 1. Resistance training significantly increased the mRNA expression of all assayed antioxidants (SOD1: $p = 0.018$, 95% CI [0.22, 1.82]; SOD2: $p = 0.027$, 95% CI [0.10, 1.28]; CAT: $p = 0.032$, 95% CI [0.07, 1.13]; GPx-1: $p = 0.022$, 95% CI [0.10, 1.59]) except for GSR, which presented a trend to increase but did not reach statistical significance ($p = 0.092$, 95% CI [-0.11, 1.12]). Notably, the reference gene (GAPDH) was not affected from Pre to Post in the older participants ($p=0.523$).

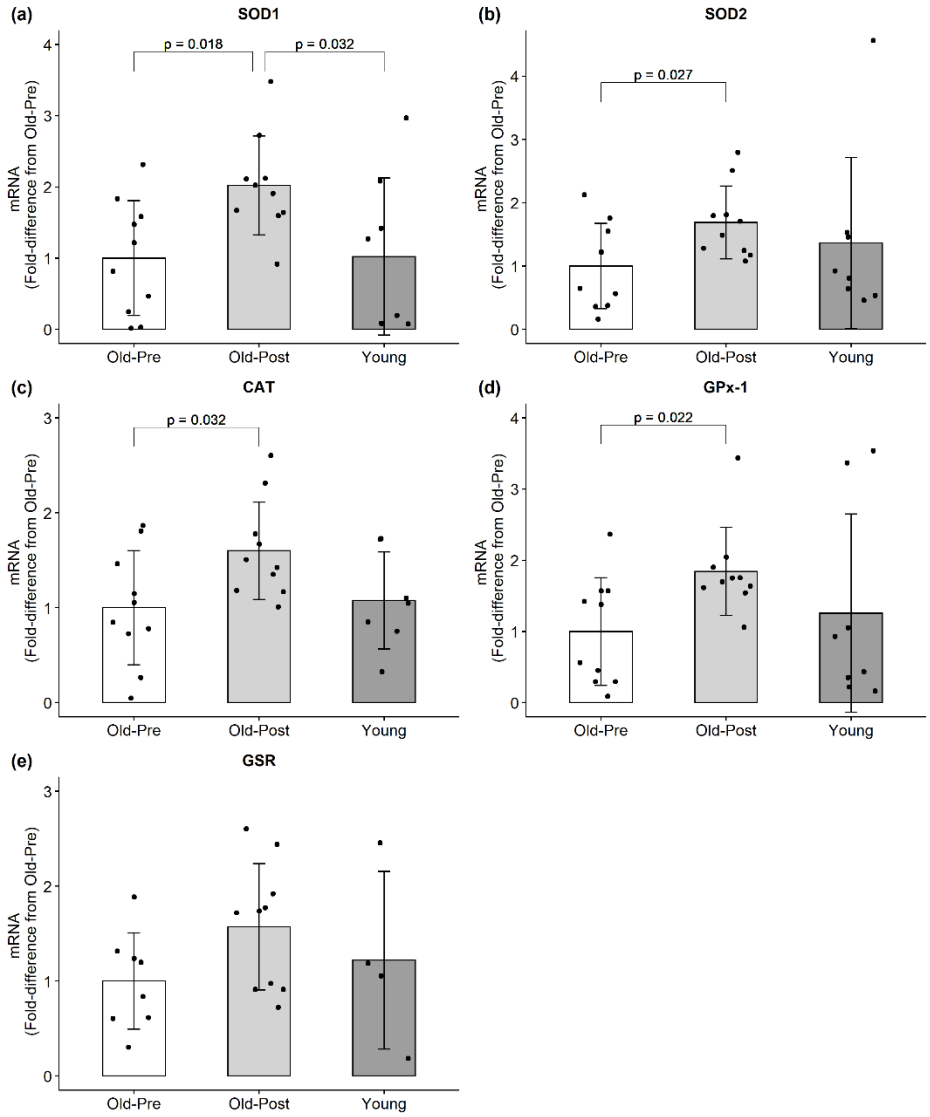

Figure 4-1. mRNA expression of endogenous antioxidants. All data are presented as mean ± SD values, and individual data points representing each participant is superimposed on each bar graph. (a) SOD1; (b) SOD2; (c) CAT; (d) GPx-1; (e) GSR.

Protein Levels

Protein Levels

In the older group, resistance training significantly decreased CAT ($p = 0.013$, 95% CI (−0.02, −0.12)) (Figure 2) and increased HSP60 protein levels ($p = 0.001$, 95% CI (0.22, 0.68)) (Figure 3). Regarding markers of oxidative damage, 4HNE decreased following RT ($p = 0.042$, 95% CI (0.01, 0.15)), but protein carbonyl levels remained unchanged ($p = 0.852$) (Figure 4).

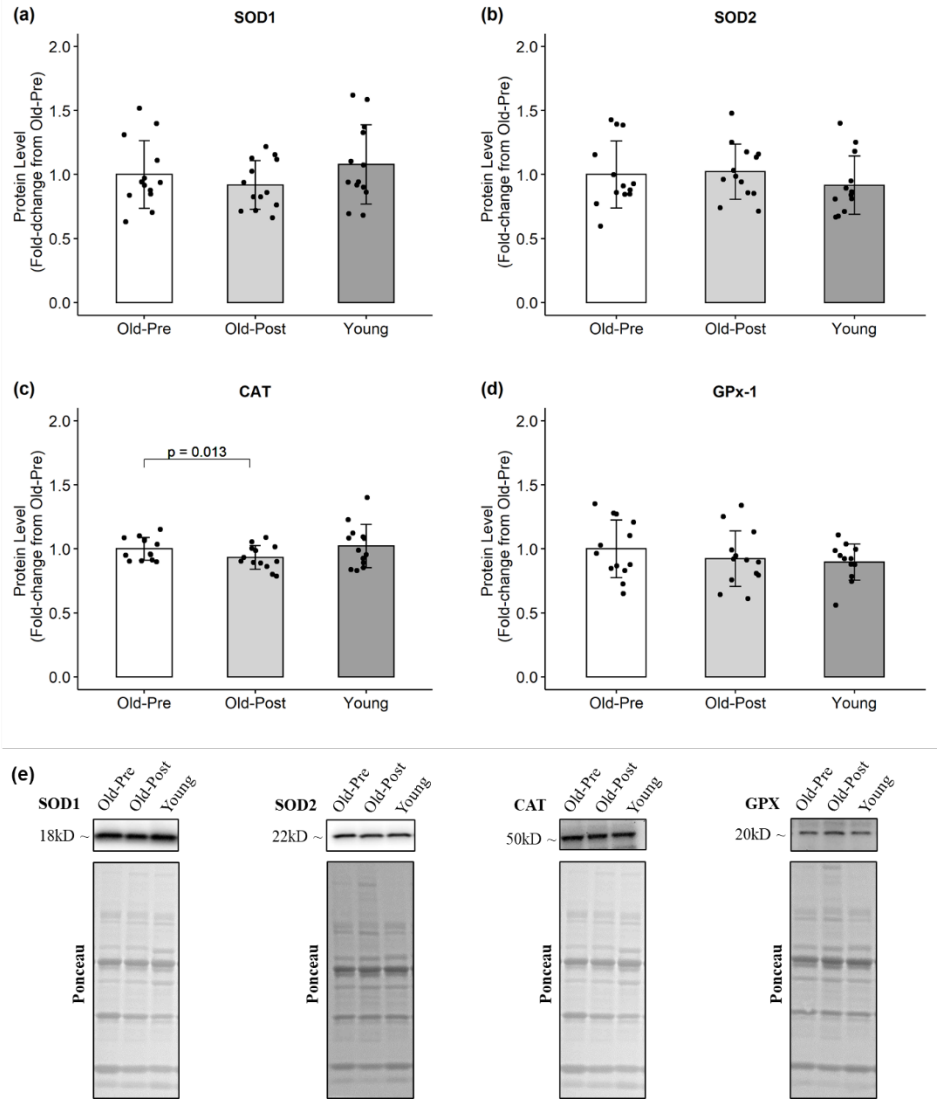

Figure 4-2. Protein levels of endogenous antioxidants. All data are presented as mean ± SD values, and individual data points representing each participant are superimposed on each bar graph. (a) SOD1; (b) SOD2; (c) CAT; (d) GPx-1; (e) representative Western blots.

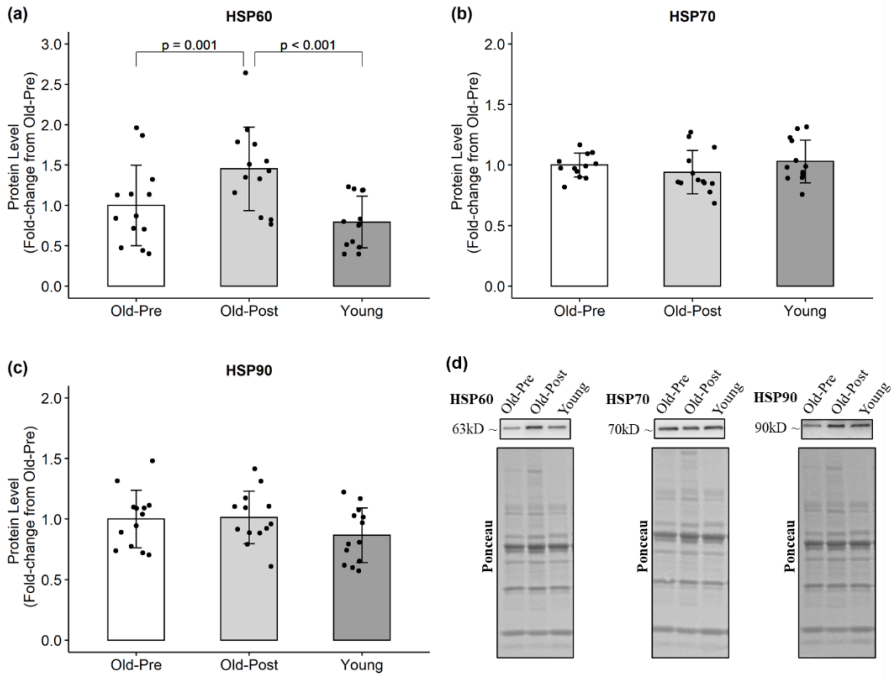

Figure 4-3. Protein levels of HSPs. All data are presented as mean ± SD values, and individual data points representing each participant are superimposed on each bar graph. (a) HSP60; (b) HSP70; (c) HSP90; (d) representative Western blots.

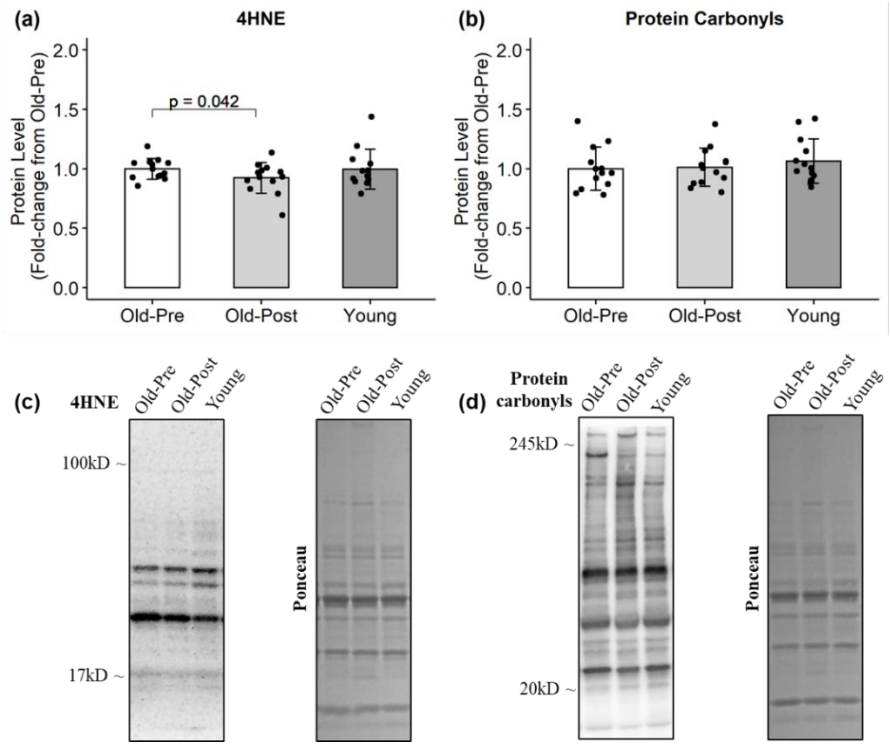

Figure 4-4. Protein levels of oxidative damage markers. All data are presented as mean ± SD values, and individual data points representing each participant are superimposed on each bar graph. (a) 4HNE; (b) protein carbonyls; (c, d) representative Western blots.

Enzymatic Activity

RT increased the enzymatic activity of CAT (p = 0.032, 95% CI (2.28, 41.56)). On the other hand, RT decreased GPX activity (p = 0.026, 95% CI (−0.80, −10.35)) (Figure 5). TAC and GSR activities exhibited an interaction effect in response to the peanut protein supplementation and RT (p = 0.037 and p = 0.005, respectively) where the peanut protein supplemented group had a lower increase in TAC and GSR activities than the placebo. However, when data were analyzed collectively, RT increased TAC (p = 0.008, 95% CI (1.60, 8.73)) and GSR (p = 0.001, 95% CI (1.24, 3.61)) (Figure 5).

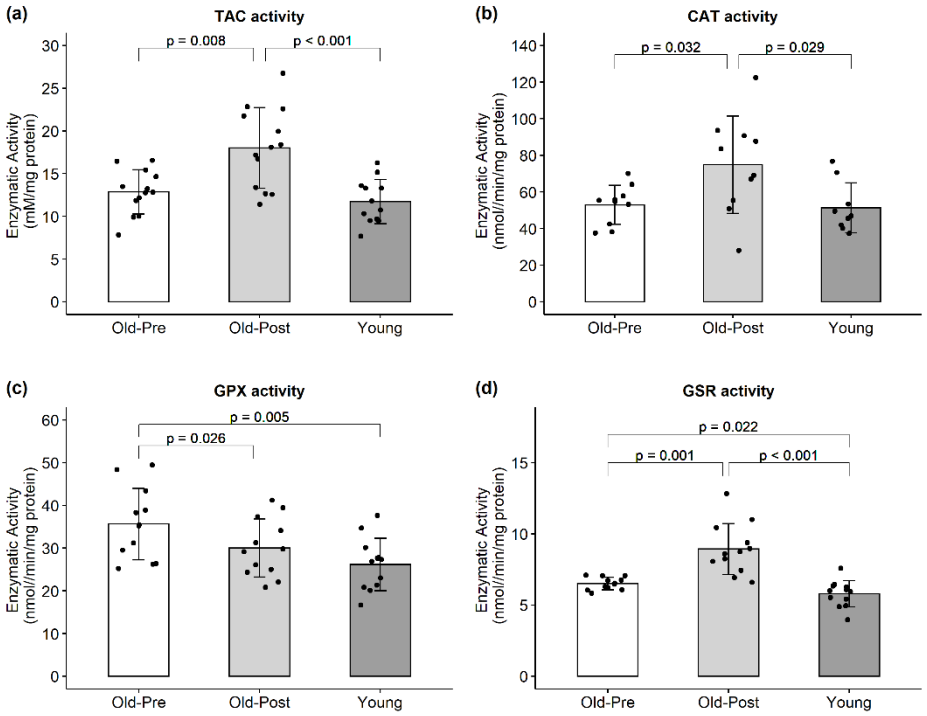

Figure 4-5. Enzymatic activity of endogenous antioxidants. All data are presented as mean ± SD values, and individual data points representing each participant are superimposed on each bar graph. (a) Total antioxidant capacity; (b) CAT activity; (c) GPX activity; (d) GSR activity.

DISCUSSION

The aim of this study was to investigate the effects of RT on the redox status of skeletal muscle in older adults. Previous studies by our group in a separate cohort of older individuals showed that 10 weeks of RT led to improvement in several markers of muscle metabolism (Lamb et al., 2020a) and mitochondrial remodeling (Mesquita et al., 2020). In line with these findings, 6 weeks of RT promoted positive adaptations in strength, body composition and in the redox status of skeletal muscle. Specifically, our results showed that RT increased mRNA expression, decreased (e.g., CAT) or did not alter protein content, and increased enzymatic activity of the antioxidants investigated. In addition, RT increased protein levels of HSP60 and decreased lipid peroxidation.

Our results align with prior literature that indicates that the mRNA levels of antioxidants seem to be mainly unaltered in skeletal muscle with aging (Oh-ishi et al., 1995; Ji, 2007, 2008, 2015). However, data regarding the effects of exercise training on mRNA levels of antioxidants are limited and equivocal, especially related to RT adaptations. Ryan et al. (Ryan et al., 2008, 2010), for example, found unaltered mRNA ex-pression of antioxidants in the skeletal muscle of young and aged rats exposed to chronic repetitive loading. The significant increase in mRNA expression observed in the present study is in agreement with a previous study conducted by García-López et al. (García-López et al., 2007). The authors found increased mRNA expression of SOD1, SOD2, CAT, and GPx in peripheral blood mononuclear cells of middle-aged men after 21 weeks of RT. Therefore, RT seems to promote cellular stress of sufficient magnitude to activate signaling pathways and increase antioxidants transcript levels in older adults, and this may occur over multiple tissues.

Although we observed significant increases in mRNA levels of the antioxidants analyzed, protein levels were either unaltered or decreased following RT. Although surprising at first, several authors have reported mRNA levels to be a poor indicator of protein content or enzymatic activity of antioxidants (García-López et al., 2007; Lambertucci et al., 2007; Cobley et al., 2017). García-Lopez et al. (García-López et al., 2007), for example, found increased mRNA but unaltered protein levels of most of the antioxidants investigated. The authors hypothesized that the discrepancies observed could be related, at least in part, to differences in mRNA stability or translational efficiency. Most studies available to date have found no change in antioxidants protein levels in older subjects in response to RT. For instance, Parise et al. (Parise et al., 2005a) found no change in SOD1, SOD2, and CAT protein content in the skeletal muscle of older participants after 14 weeks of RT. The protein levels of SOD1, SOD2, CAT, and GPx-1 were also unaltered in the skeletal muscle of rats exposed to chronic repetitive loading (Ryan et al., 2008, 2010). There are also reports of decreased SOD1 (Murlasits et al., 2006) and a trend for CAT protein levels to decrease (Holloway et al., 2018) in response to RT. Although difficult to reconcile the reasons for the decrease in protein content of antioxidants, especially with increased mRNA levels, we speculate that this may be related to the increased antioxidant enzymatic activities, which may increase antioxidant efficiency, coupled with the decreased lipid peroxidation observed in the present study. Specifically, the increased antioxidants efficiency could have decreased oxidative stress, which in turn reduced the need for high protein levels of antioxidants. Another explanation is that an initial decrease in protein content may have initiated a negative feedback signal, increasing both gene expression and enzymatic activities of various antioxidants. While it remains uncertain as to why these paradoxical observations were made,

these findings clearly demonstrate a complex interrelationship exists between antioxidant mRNA, protein, and enzyme activity levels that should be further investigated.

It is well-documented that aging is associated with an increase in oxidative damage to lipid, proteins, and DNA (Mecocci et al., 1999; Pansarasa et al., 2000; Ryan et al., 2008; Cobley et al., 2015). However, our results did not show any significant differences in lipid peroxidation or protein carbonyl levels between older and younger participants. This discrepancy could be related to the age of the participants included in the present study. Although our subjects were of advanced age (mean = 64 y), previous studies have shown that oxidative damage was significantly higher only in subjects considerably older (>66-70y) (Mecocci et al., 1999; Pansarasa et al., 2000). Even though the older group did not display greater lipid peroxidation at baseline compared to the younger group, 6 weeks of RT significantly decreased 4HNE levels. Parise and colleagues have previously shown that RT is capable of decreasing oxidative damage to DNA (Parise et al., 2005b) but not to proteins (Parise et al., 2005c) in older participants. Resistance training has also been shown to decrease lipid peroxidation levels in the plasma of young participants (Azizbeigi et al., 2014) and in the skeletal muscle of aged rats (Ryan et al., 2008, 2010).

Despite reports that aged skeletal muscle fails to upregulate HSPs following acute exercise, possibly due to heightened basal expression, we found increased HSP60 protein levels after 6 weeks of RT. HSPs help protect against the potential harmful effects of ROS production (Cobley et al., 2014; Dimauro et al., 2016), and have been shown to in-crease in response to RT (Gjøvaag and Dahl, 2006; Murlasits et al., 2006; Paulsen et al., 2012). Therefore, the increase in HSP60 could have been an adaptation to the transient increases in ROS production, helping to protect the skeletal muscle cells from subsequent bouts of oxidative stress. In addition, it has

been suggested before that high levels of oxidative stress may impair the exercise induced increase in HSPs (Murlasits et al., 2006). Therefore, the increased HSP levels observed in the present study could also be a consequence of diminished oxidative stress.

The enzymatic activity of antioxidants in skeletal muscle increases with aging (Gianni et al., 2004; Ji, 2015), supposedly as an adaptation to increased chronic oxidative stress. In the present study, GPX and GSR activities were higher in the Old-Pre versus Young participants, despite no significant difference in markers of oxidative damage between these two groups. Our results showed that 6 weeks of RT increased total antioxidant capacity in the skeletal muscle of older subjects, as well as CAT and GSR activities. Our data agrees with a previous study conducted by Parise et al. (Parise et al., 2005c), which also showed increased antioxidant enzyme activity in older males after RT. Because acute RT has been shown to promote a transient increase in oxidative stress (Bloomer et al., 2005, 2007; Güzel et al., 2007; Çakır-Atabek et al., 2015), the increase in antioxidant activities observed could be an adaptation to repeated exposure to elevated oxidative stress. Surprisingly, GPX activity was decreased following RT. Ryan et al. (Ryan et al., 2008) also observed the same response in the skeletal muscle of rats after chronic loading, and speculated that either GPX activity was suppressed by high levels of H2O2 or the increased CAT activity was sufficient to counteract the increased H2O2 production.

In conclusion, we showed that 6 weeks of RT promoted beneficial adaptations in the redox status of the skeletal muscle in older adults. RT significantly decreased lipid peroxidation and increased antioxidant enzymatic activities. Therefore, RT may be a viable approach to counteract a possible age-related disruption of skeletal muscle redox homeostasis in older adults. Furthermore, our findings suggest a multilevel control of the antioxidant system response to RT, involving transcriptional, post-transcriptional and post-translational controls. Thus, researchers

should exercise caution when investigating and interpreting results from only one of the levels of control. It should be noted that the duration of the RT program adopted in the present study was considerably short, and the long-term redox adaptations might differ from the ones observed herein. Moreover, future studies should investigate the effects of different RT protocols, such as low-load high-volume training, which have been suggested to promote greater metabolic adaptations (Parry et al., 2020; Roberts et al., 2020).

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Chapter 5 – Resistance Training Diminishes Mitochondrial Adaptations to Subsequent Endurance Training

INTRODUCTION

Endurance performance is determined by a complex interaction of physiological, biomechanical, and neuromuscular factors. Maximal oxygen consumption (VO_2max), lactate threshold, and running economy are widely considered the main limiting factors of endurance performance (Bassett and Howley, 2000; Joyner and Coyle, 2008). Skeletal muscle oxidative phosphorylation capacity, which in turn is determined by mitochondrial volume density and function, is also considered a strong predictor of endurance performance (Jacobs et al., 2011).

A variety of training paradigms may be used to improve endurance performance including moderate intensity continuous training (MICT) and high-intensity interval training (HIIT) (Burgomaster et al., 2008; Daussin et al., 2008; MacInnis and Gibala, 2017). Resistance training (RT), on the other hand, has long been underappreciated in regard to enhancing endurance performance and is often not a part of the training program of high-level athletes (Beattie et al., 2014). However, several studies have shown a beneficial effect of RT on endurance performance, which is usually linked to an improvement of running economy through neuromuscular adaptations (Balsalobre-Fernandez et al., 2016; Blagrove et al., 2018; Paavolainen et al., 1999).

The enhancement of endurance performance through RT may affect attributes other than running economy. Different studies have shown that RT may also lead to positive mitochondrial adaptations (Groennebaek et al., 2018; Lim et al., 2019; Mesquita et al., 2020; Ruple et al., 2021a). However, endurance performance was not investigated in these studies. Interestingly, a

study conducted by Lee et al. (Lee et al., 2018) in rats found that RT promoted enhanced mitochondrial adaptations to a subsequent block of RT, which seemed to be related to increased myonuclear number per myofiber achieved in the first block of training. While these data are promising, it is currently unknown whether RT enhances mitochondrial adaptations to subsequent endurance training (ET) in humans and whether the enhanced mitochondrial adaptations would lead to better endurance performance. In addition, while several studies have investigated the effects of concurrent training, when both RT and ET are combined within the same training session or program, no study to date has employed a design that investigated RT-only followed by ET-only. This is especially relevant considering a recent study reported that prior ET facilitated adaptations to a subsequent period of RT (Thomas et al., 2022).

Therefore, the purpose of this study was to investigate the effects of prior RT on the molecular and performance adaptations to subsequent ET in humans. We hypothesized that RT prior to ET would augment skeletal muscle mitochondrial adaptations to ET, ultimately leading to improved endurance performance.

MATERIALS AND METHODS

Ethical Approval

The current study was reviewed and approved by the Institutional Review Board at Auburn University (Protocol # 21-390 FB) and conformed to the standards of the Declarations of Helsinki, except that it was not registered as a clinical trial.

Participants

Twenty-five healthy young male participants (baseline characteristics in Table 1) were recruited to participate in this study. Participants should not have participated in structured (more than once weekly for at least two months) RT over the last three years or ET over the last six months prior to joining the current study. All participants were informed of the procedures and risks of the current study before providing written consent.

Table 5-1. Participant characteristics obtained during the familiarization session

Characteristic	Overall (n=25)	RT+ET (n=13)	ET-only (n=12)
Age (years)	23 ± 4	23 ± 4	24 ± 4
Body mass (kg)	84.8 ± 18.0	81.2 ± 16.8	88.6 ± 19.2
Height (cm)	181 ± 8	180 ± 9	181 ± 9
BMI (kg/m^2)	25.9 ± 4.6	25.2 ± 5	26.7 ± 4.2
VO_2peak (ml/kg/min)	39.7 ± 7.6	40.4 ± 8.9	38.9 ± 6.2

Abbreviations: RT+ET, group that performed 7 weeks of resistance training followed by 7 weeks of endurance training; ET-only, group that performed 7 weeks of endurance training only; BMI, body mass index; VO_2peak, peak aerobic capacity

Familiarization Session

Participants visited the laboratory to become familiarized with the exercises and tests used in the study. First, participants performed a maximal cardiorespiratory test on a motorized treadmill. The incremental treadmill test was composed of several two-minute stages and started with a fast-paced walk (6.4 km/h, 0% inclination) as a warm-up for three minutes. After that, the speed of the treadmill increased by 1 km/h and inclination by 1% after every stage until the participant reached volitional exhaustion. There was a 30-second break after each stage, during which participants stopped running and reported their ratings of perceived exertion (RPE) according to Borg's CR10 scale (Borg, 1982). Peak oxygen consumption (VO_{2peak}) was

determined by the highest 30-second average value using a metabolic cart (True Max 2400, ParvoMedics, Salt Lake City, UT, USA). After the maximal cardiorespiratory test, participants were taught how to properly perform the leg press, bench press, leg extension, cable-bar pull-down, and leg curl exercises. Participants were allowed to perform a few sets and repetitions until they demonstrated proper lifting technique.

Experimental Design

VO_{2peak} values obtained during the familiarization visit were used to assign participants to each group in a balanced manner, RT+ET (n=13, VO_2peak = 40.4 ± 8.9 ml/kg/min) and ET-only (n=12, VO_2peak = 38.9 ± 6.2 ml/kg/min). Importantly, there was no significant difference between groups (p=0.634).

Participants in the RT+ET group completed seven weeks of RT followed by seven weeks of ET. Participants in the ET-only group performed seven weeks of ET. During each of the three testing sessions (details provided below), participants underwent a battery of assessments in two visits. The first visit included height, body mass, full-body dual-energy X-ray absorptiometry (DEXA), ultrasound of the right vastus lateralis (VL), and a biopsy from the right VL. In the second visit, participants performed a maximal cardiorespiratory test and 3-repetition maximum strength tests. The order of the tests was the same for all time-points. The first and second visits of the testing sessions occurred within 48 to 96 hours and within one week after the last training session, respectively. The experimental design is depicted in Fig. 5-1.

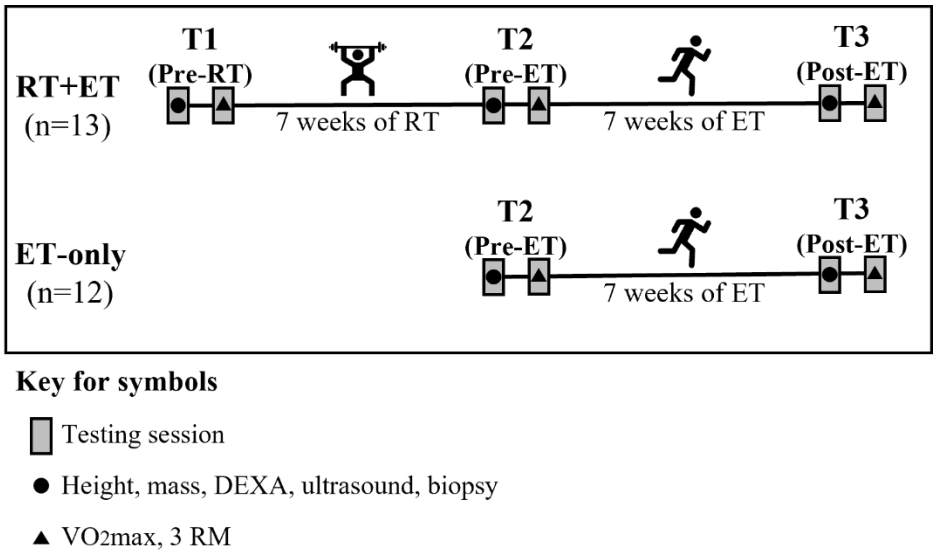


Figure 5-1. Experimental Design. Abbreviations: RT+ET, group that performed 7 weeks of resistance training followed by 7 weeks of endurance training; ET-only, group that performed 7 weeks of endurance training only; RT, Resistance Training; ET, Endurance Training

Testing Sessions (T1, T2, and T3)

Testing sessions were composed of two visits on different days. During the first day, participants reported to the laboratory following at least four hours of food deprivation. Body mass and height were assessed with a digital scale (Seca 769; Hanover, MD, USA). DEXA (Lunar Prodigy; GE Corporation, Fairfield, CT, USA) was performed to determine lean body mass, fat mass, and body fat percentage. Following the DEXA scan, real-time B-mode ultrasonography (NextGen LOGIQe R8, GE Healthcare, Chicago, IL, USA) was used to determine the thickness of the VL of the right leg as previously described by our laboratory (Ruple et al., 2022a; Ruple et al., 2022b). Measurements were taken at the midway point between the iliac crest and proximal patella. After the ultrasound scans, skeletal muscle biopsy samples were collected from the right VL at the same location of the ultrasound imaging using a 5-gauge Bergstrom needle. Briefly, participants laid in the supine position on an athletic training table and the upper thigh was shaven and cleaned with 70% isopropanol before receiving a 0.8

mL injection of 1% lidocaine. Participants rested for 5-10 minutes for the lidocaine to take effect before the area was cleaned with chlorhexidine and a pilot incision through the dermis was made with a sterile No. 11 surgical blade (AD Surgical; Sunnyvale CA, USA). Approximately 50-100 mg of skeletal muscle tissue was collected, immediately teased of blood and connective tissue, and separated for histological and biochemical analysis. Mounting for histology in optimal cutting temperature (OCT) media occurred as previously described by our laboratory (Ruple et al., 2021a). A separate ~20-40 mg tissue sample was placed in pre-labelled foil and flash-frozen in liquid nitrogen for Western blotting and biochemical analyses described below. Finally, ~10 mg of muscle was fixed in 4% paraformaldehyde for 48 hours at room temperature for single fiber analysis and is further described below. Notably, removal of tissue and all tissue processing occurred within a 5-minute period. Furthermore, OCT and flash frozen foil samples were removed from liquid nitrogen throughout the day during muscle collections and stored at -80ºC for later analyses.

During the second day of testing, participants performed a maximal cardiorespiratory test as previously described in the familiarization session. For the testing session, blood was also collected from the participants' fingertips at rest, after completion of each stage, and at the end of the test. A handheld lactate analyzer device (Lactate Plus, Nova Biomedical) was used to obtain blood lactate concentration values. Blood lactate values were used to determine the speed and inclination corresponding to the onset of blood lactate accumulation (OBLA, i.e., 4 mmol/L) using the Lactater package in RStudio. In addition, a validation step was conducted at the end of the test. After completing the test, participants rested for 10 minutes, were connected to the metabolic cart again and ran for as long they could at a speed and inclination corresponding to the stage following the stage they stopped during the test. This step was included as a

verification method to ensure that participants reached maximal oxygen consumption (VO_2max). The highest 30-second average oxygen consumption value obtained during the test was considered the participants' VO_2max. After the VO_2max test, participants completed three-repetition maximum (3RM) strength tests for the leg press, bench press, and leg extension exercises. Participants performed two sets for warm-up and had up to five trials per exercise to reach 3RM values with three to five minutes of rest between trials. Proper range of motion was assessed for each exercise during the warm-up with the aid of a measurement tape, and repetitions were considered valid if participants reached appropriate ranges of motion.

Resistance Training

Resistance training was performed twice weekly by the RT+ET group only, and each training session included leg press, bench press, leg extension, cable pull-down, and leg curls. Sets of six repetitions were performed for the exercises targeting quadriceps muscles (i.e., leg press and leg extension), while three sets of ten repetitions were performed for the other exercises (i.e., bench press, cable pull-down, leg curls). Volume and load for the quadriceps were progressively increased throughout the seven weeks and can be seen in Table 5-2. Although the load increment was pre-planned as shown, participant feedback was taken into consideration for load adjustments. After each set for each exercise, participants reported their repetitions in reserve (RIR) by answering how many more repetitions they think they could have done (Zourdos et al., 2016). If RIR > 2, the load was increased by approximately 5-10 lbs for upper-body exercises and 10-20 lbs for lower-body exercises. If participants failed to perform the programmed number of repetitions, load was decreased in a similar fashion. Participants rested

for two to three minutes between sets of exercises. Appropriate range of motion was ensured using the range of motion recorded at T1-testing.

Table 5-2. Strength training volume and load progression

Week	Day	Sets x reps*	1RM
1	1	6 x 6	70%
	2	6 x 6	70%
2	3	7 x 6	75%
	4	7 x 6	75%
3	5	8 x 6	80%
	6	8 x 6	80%
4	7	9 x 6	85%
	8	8 x 6	85%
5	9	9 x 6	88%
	10	9 x 6	88%
6	11	10 x 6	91%
	12	9 x 6	91%
7	13	10 x 6	95%
	14	10 x 6	95%

Abbreviation: 1RM, one-repetition maximum. Symbol: *Total number of sets and reps per session for exercises targeting quadriceps (e.g., 6 x 6 = 3 sets of 6 repetitions for the leg press and 3 sets of 6 repetitions for the leg extension; 9 x 6 = 5 sets of 6 repetitions for the leg press and 4 sets of 6 repetitions for the leg extension).

Endurance Training

All participants performed seven weeks of a high-intensity interval training (HIIT)-based ET on a motorized treadmill, and in RT+ET participants, this training occurred the week immediately following their seven week-RT period. A HIIT-based training protocol was chosen because HIIT induces mitochondrial and cardiovascular adaptations in a time-efficient manner (Daussin et al., 2008; Gibala, 2021). For each training session, participants started with a 3-minute warm-up, followed by 5-10 sets (5 sets in the 1st week; 8 sets in the 2nd week; 9 sets in the 3rd week; 10 sets for the remaining weeks) of 1 minute running at a high intensity interspersed by 1.5 to 3 minutes running at a low intensity. The intensity of the “sprints” and the

recovery was determined using the speed and inclination values achieved in the VO_2max test (Table 3). Similar to the RT program, participant feedback was taken into consideration to adjust the intensity of training. At the end of each "sprint" bout, participants rated their perceived exertion (RPE) using the CR-10 Borg Scale. In the first week, if participant's final RPE was lower than 5 ("strong"), the intensity of the "sprint" bout was increased in the next training session by 5%. From the second week onward, if participant's final RPE was lower than 7 ("very strong"), intensity (i.e., treadmill speed) was also increased by 5%. If participants were not able to complete the programmed number of "sprints", intensity was decreased by 5%. The ET program can be seen in Table 5-3:

Table 5-3. Endurance Training program

Week	Frequency (times/week)	Sets	Effort dur (min)	Effort int (VO_2max)	Recovery dur (min)	Recovery int (VO_2max)
1	2	5	1	80%	3	60%
2	3	8	1	85%	1.5	60%
3	3	9	1	90%	1.5	60%
4	3	10	1	90%	1.5	60%
5	3	10	1	90%	1.5	60%
6	3	10	1	95%	1.5	60%
7	3	10	1	100%	1.5	60%

Abbreviations: Min, minutes; dur, duration; int, intensity

Biochemical Assays

Approximately 30 mg of muscle tissue that was flash-frozen in foil was retrieved from -80ºC, weighed using an analytical scale, and homogenized in a sucrose homogenization buffer using a glass Dounce homogenizer according to Spinazzi et al. (Spinazzi et al., 2012). Samples were centrifuged at 600 × g for 10 minutes at 4ºC. Protein concentrations from the resulting supernatants were determined using a commercially available BCA kit (Thermo Fisher

Scientific, Waltham, MA, USA). Supernatants were then used for citrate synthase (CS) activity and western blotting.

Western Blotting

Muscle supernatants were prepared for Western blotting using 4x Laemmli buffer and deionized water (diH_2O) at equal protein concentration. Ten microliters of sample were pipetted onto SDS gels (4%–15% Criterion TGX Stain-free gels; Bio-Rad Laboratories; Hercules, CA, USA), and proteins were separated by electrophoresis (200 V for approximately 40 minutes). Proteins were then transferred to preactivated PVDF membranes (Bio-Rad Laboratories) for 2 hours at 200 mA. Gels were then Ponceau stained for 10 min, washed with diH_2O for 30 seconds, dried, and digitally imaged (ChemiDoc Touch, Bio-Rad). Following Ponceau imaging, membranes were reactivated in methanol, blocked with nonfat milk for 1 hour, washed three times in Tris-buffered saline with Tween 20 (TBST) and incubated with primary antibodies overnight (1:2000 v/v dilution in TBST with 5% BSA). Primary antibodies were used to detect the following: total OXPHOS rodent (Abcam Cat# ab110413, RRID:AB_2629281), PGC-1α (GeneTex Cat# GTX37356, RRID:AB_11175466), NRF1 (GeneTex Cat# GTX103179, RRID:AB_11168915), TFAM (Abnova Corporation Cat# H00007019- D01P, RRID:AB_1715621), MFN1 (Cell Signaling Technology Cat# 14739, RRID:AB_2744531), MFN2 (BioVision Cat# 3882– 100, RRID:AB_2142625), DRP1 (Novus Cat# NB110- 55288SS, RRID:AB_921147), PINK1 (Cell Signaling Technology Cat# 6946, RRID:AB_11179069), and PARKIN (Cell Signaling Technology Cat# 2132, RRID:AB_10693040). Following primary antibody incubations, membranes were washed three times in TBST for 5 minutes and incubated for 1 hour with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology

Cat# 7074, RRID:AB_2099233) or anti-mouse IgG (Cell Signaling Technology Cat# 7076, RRID:AB_330924). Membranes were then washed in TBST, developed using chemiluminescent substrate (Millipore; Burlington, MA, USA), and digitally imaged. Raw target band densities were obtained and normalized by Ponceau densitometry values.

RNA Isolation and cDNA Synthesis for qPCR Analysis

Approximately 10 mg of muscle tissue that was flash-frozen in foil was retrieved from -80ºC, weighed using an analytical scale, homogenized in Ribozol (Ameresco, Solon, OH, USA), and RNA was isolated according to manufacturer's instructions. RNA concentrations were determined in duplicate using a NanoDrop Lite (Thermo Fisher Scientific, Walthan, MA, USA), and total RNA was determined by normalizing the RNA values to muscle mass homogenized (i.e., µg/mg wet tissue). In an attempt to account for muscle size changes, "absolute" RNA content was estimated by multiplying relative total RNA by mixed fiber cross-sectional area (fCSA) determined by immunohistochemistry (described later) and by vastus lateralis thickness.

For gene expression analyses, 2 µg of cDNA was synthesized using a commercial qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). RT-qPCR was performed in an RT-PCR thermal cycler (Bio-Rad) using SYBR green-based methods with gene-specific primers designed with primer designer software (Primer3Plus, Cambridge, MA, USA). For all primer sets, pilot qPCR reactions and melt data indicated that only one amplicon was present. The forward and reverse primer sequences of all genes are listed in Table 5-4. Fold-change values were determined using the $2^{\Delta\Delta Cq}$ method, where $2^{\Delta Cq}$ = 2^(housekeeping gene (HKG) Cq - gene of interest Cq) and $2^{\Delta\Delta Cq}$ (or fold change) = ($2^{\Delta Cq}$ value/$2^{\Delta Cq}$ average of baseline values). The

geometric mean of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and valosin-containing protein (VCP) was used as the HKG normalizer.

Table 5-4. qPCR primer sequences

Gene	Primer Sequences
GAPDH	FP (5' → 3'): AACCTGCCAAATATGATGAC
	RP (5' → 3'): TCATACCAGGAAATGAGCTT
VCP	FP (5' → 3'): TGGCATGACTCCCTCCAAAG
	RP (5' → 3'): CAGCTCAFFACCCTTGATCG
45S pre-rRNA	FP (5' → 3'): GAACGGTGGTGTGTCGTT
	RP (5' → 3'): GCGTCTCGTCTCGTCTCACT
18S rRNA	FP (5' → 3'): GCCGCTAGAGGTGAAATTCT
	RP (5' → 3'): TCGGAACTACGACGGTATCT
5.8S rRNA	FP (5' → 3'): GTGGATCACTCGGCTCGTG
	RP (5' → 3'): CGCAAGTGCGTTCGAAGTG

Abbreviations: FP, forward primer; RP, reverse primer, GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VCP, valosin-containing protein.

Citrate Synthase Activity

Citrate synthase activity was determined by monitoring the increase in absorbance at 412 nm from the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) coupled to the reduction of acetyl-CoA (Trounce et al., 1996). Similar to what was listed above in regard to estimating absolute RNA content changes, "absolute" CS activity was calculated by multiplying maximal CS activity (relative) by mixed fCSA and by vastus lateralis thickness.

Immunohistochemistry (IHC)

A portion of skeletal muscle samples preserved in OCT were sectioned at 7 μm thickness using a cryotome (Leica Biosystems; Buffalo Grove, IL, United States) and adhered to positively

charged histology slides. Slides were then stored at -80°C until batch processing. Slides were mounted in a manner that all time-points for each participant were analyzed concomitantly to avoid batch-to-batch variation.

For fiber type-specific fCSA and myonuclei number quantification, slides were air-dried for 90-120 minutes prior to a 5-minute acetone incubation at -20ºC. Slides were washed 3x5 minutes in 1x phosphate buffered saline (PBS) and incubated with 3% H_2O_2 for 10 minutes at room temperature. After washing, slides were incubated with autofluorescence quenching reagent for 1 min (TrueBlack; Biotium, Fremont, CA, USA). Slides were washed again in PBS and blocked with a 5% goat serum and 2.5% horse serum solution for 1 hour at room temperature. After blocking, slides were incubated overnight at 4ºC with a primary antibody cocktail containing 1:20 Mandra (dystrophin) (Developmental Studies Hybridoma Bank; Iowa City, IA, USA) + 1:100 BA-D5 (Myosin Heavy Chain I) (Developmental Studies Hybridoma Bank; Iowa City, IA, USA) + 2.5% horse serum in PBS. The following day, sections were incubated for 1 hour with a secondary antibody cocktail containing 1:250 anti-mouse IgG1 AF594 (Thermo Fisher Scientific; Waltham, MA, USA; cat. no. A-21125) + anti-mouse IgG2b AF488 (Thermo Fisher Scientific; Waltham, MA, USA; cat. no. A-21141) in PBS. Slides were then washed and stained with 1:10,000 DAPI (4=,6-diamidino-2-phenylindole, Thermo Fisher Scientific; catalog #: D3571) for 15 minutes at room temperature before coverslips were applied using PBS + glycerol as mounting medium.

For fiber type-specific satellite cell content quantification, a similar protocol was used. However, additional steps were performed to amplify satellite cells. After blocking slides with 5% goat serum and 2.5% horse serum, slides were blocked with streptavidin and biotin solutions at room temperature for 15 minutes each. Thereafter, slides were incubated overnight at 4ºC with

primary antibody cocktail containing 1:20 Mandra (dystrophin) (Developmental Studies Hybridoma Bank; Iowa City, IA, USA) + 1:100 BA-D5 (Myosin Heavy Chain I) (Developmental Studies Hybridoma Bank; Iowa City, IA, USA) + 1:20 PAX7 (Developmental Studies Hybridoma Bank; Iowa City, IA, USA) + 2.5% horse serum in PBS. The following day, slides were incubated for 90 minutes in secondary 1:1000 biotin solution (anti-mouse IgG1, Jackson ImmunoResearch; West Grove, PA, USA), followed by a 60-minute incubation with secondary 1:500 streptavidin (SA-HRP, Thermo Fisher Scientific; catalog #: S-911), and a 20-minute incubation with 1:200 tyramide AF555 (Thermo Fisher Scientific, catalog #: B-40957).

For fiber type-specific mitochondrial content, the translocase of outer mitochondrial membrane 20 (TOMM20) protein was stained as previously described and validated (Ruple et al., 2021a) using serial sections. The protocol used for mitochondrial staining was similar to those used for fCSA, myonuclei, and satellite cell determination, although the blocking solution included 0.1% Triton X. The primary antibody cocktail included 1:20 Mandy s8 (dystrophin) (Developmental Studies Hybridoma Bank; Iowa City, IA, USA) and 1:200 TOMM20 (Abcam; Cambridge, MA, USA, ab186735) in 5% bovine serum albumin. The following day, slides were incubated for 1 hour with a secondary antibody cocktail: 1:250 anti-rabbit IgG Texas Red 594 (Vector Labs, Newark, CA, USA; TI-1000) + anti-mouse IgG2b AF488 (Thermo Fisher Scientific; Waltham, MA, USA; cat. no. A-21141) in PBS. Slides were then washed in PBS and coverslips were applied using PBS + glycerol as mounting medium.

Single fiber analyses were also performed to quantify myonuclei content. As stated above, muscle tissue (~10 mg) was fixed in 4% paraformaldehyde for 48 hours at room temperature following biopsies. Tissue was then washed in PBS and stored at 4ºC until batch processing. Tissue was subsequently incubated in 40% NaOH in slow rotation for approximately

2 hours to facilitate extracellular matrix digestion and myofiber disaggregation. Tissue was then washed in PBS through a 40 μm cell strainer and transferred to PBS. Small myofiber bundles were mechanically teased apart under a light microscope, placed in PBS, and centrifuged at 13,000 rpm. PBS was removed and myofibers were stained with DAPI for 15 minutes. Individual myofibers were mounted with PBS-glycerol solution on positively charged slides. The number of fibers analyzed were as follows (mean ± SD): RT+ET group, T1: 19±3, T2: 20±1, T3: 20±1; ET-only group, T2: 20±1, T3: 20±1.

Following mounting, digital images for each analysis were captured with a fluorescence microscope (Nikon Instruments) using the 20x objective. Fiber type-specific fCSA and myonuclear number were analyzed using the open-sourced software MyoVision (Wen et al., 2018). Satellite cells were manually quantified using NIKON NIS Elements software (Nikon Instruments, Melville, NY, USA) and are reported as PAX7 positive per 100 fibers. Mitochondrial content was determined in serial sections using ImageJ (NIH) as previously described (Ruple et al., 2021a) and reported as percentage change from baseline. In short, the red channel of TOMM20 images was converted to grayscale and a threshold function was applied. Fibers were then manually traced and mitochondrial area was determined as a percentage of the fiber area. Absolute mitochondrial content via TOMM20 was estimated by multiplying the percentage of TOMM20 by mixed, type I, and type II fiber cross-sectional areas. For single fiber nuclei content, a brightfield image was taken to determine fiber border. Thereafter, three images of the DAPI filter were taken at different depths to capture the maximum number of nuclei. Single fiber and nuclei measurements were made by a blinded investigator using ImageJ (NIH). Myonuclei content is expressed as number of nuclei per 100 μm. Single fiber myonuclear

domain (MND) was calculated by dividing the fiber segment volume (μm^3) by the total number of myonuclei (Moro et al., 2020).

Statistical Analysis

Statistics were performed using RStudio Version 2022.12.0. Shapiro-Wilk tests were used to assess the distribution of data for each dependent variable. Two separate analyses were conducted. First, dependent variable responses to resistance training (T1 x T2) in the RT+ET group were analyzed using dependent samples t-tests (for normally distributed data) or Wilcoxon signed rank tests (for non-normally distributed data). Adaptations to endurance training (T2 x T3) in both groups were analyzed using two-way analysis of variance (ANOVA) tests, followed by Tukey post-hoc tests when appropriate. Associations between select variables were also conducted using Pearson's or Spearman's correlations. Statistical significance was established at $p < 0.05$. All data are expressed as mean ± standard deviation (SD) values, and 95% confidence intervals are presented for statistically significant differences.

RESULTS

Strength and Body Composition Changes

Participants in the RT+ET group significantly increased 3RM values in leg press (T1: 173 ± 54 kg, T2: 259 ± 56 kg, ± 95% CI [25], $p<0.001$), bench press (T1: 55 ± 13 kg, T2: 64 ± 14 kg, ± 95% CI [3], $p<0.001$) and leg extension (T1: 97 ± 25 kg, T2: 128 ± 20 kg, ± 95% CI [8], $p<0.001$) in response to RT. Further, participants significantly increased lean body mass (+1.4 kg, ± 95% CI [0.9], $p=0.005$) and VL thickness (+0.22 cm, ± 95% CI [0.08], $p<0.001$), and

decreased body fat percentage (-0.9% ± 95% CI [0.7], p=0.028) in response to RT, whereas no significant changes in body mass (p=0.052) and fat mass (p=0.288) occurred.

In response to ET in both groups, there were no significant effects of group (G), time (T), or interaction (GxT) for body mass (G, p=0.400; T, p=0.111; GxT, p=0.870; Fig. 5-2A), lean body mass (G, p=0.797; T, p=0.242; GxT, p=0.324; Fig. 5-2B), or fat mass (G, p=0.118; T, p=0.199; GxT, p=0.818; Fig. 5-2C). A significant effect of G (p=0.048) and T (p<0.001), but no GxT (p=0.087), was evident for body fat percentage (Fig. 5-2D). Body fat percentage was higher in the ET-only group (6.4% ± 95% CI [5.9]) and decreased over time (0.9% ± 95% CI [0.5]). No significant main effects of G (p=0.685) or T (p=0.053) were evident for VL thickness (Fig. 5-2E), but there was a significant GxT (p=0.003). Notably, a decrease in VL thickness occurred in the RT+ET group from T2 to T3 (-0.12 cm ± 95% CI [0.06], p=0.007), but not in the ET group (p=0.805).

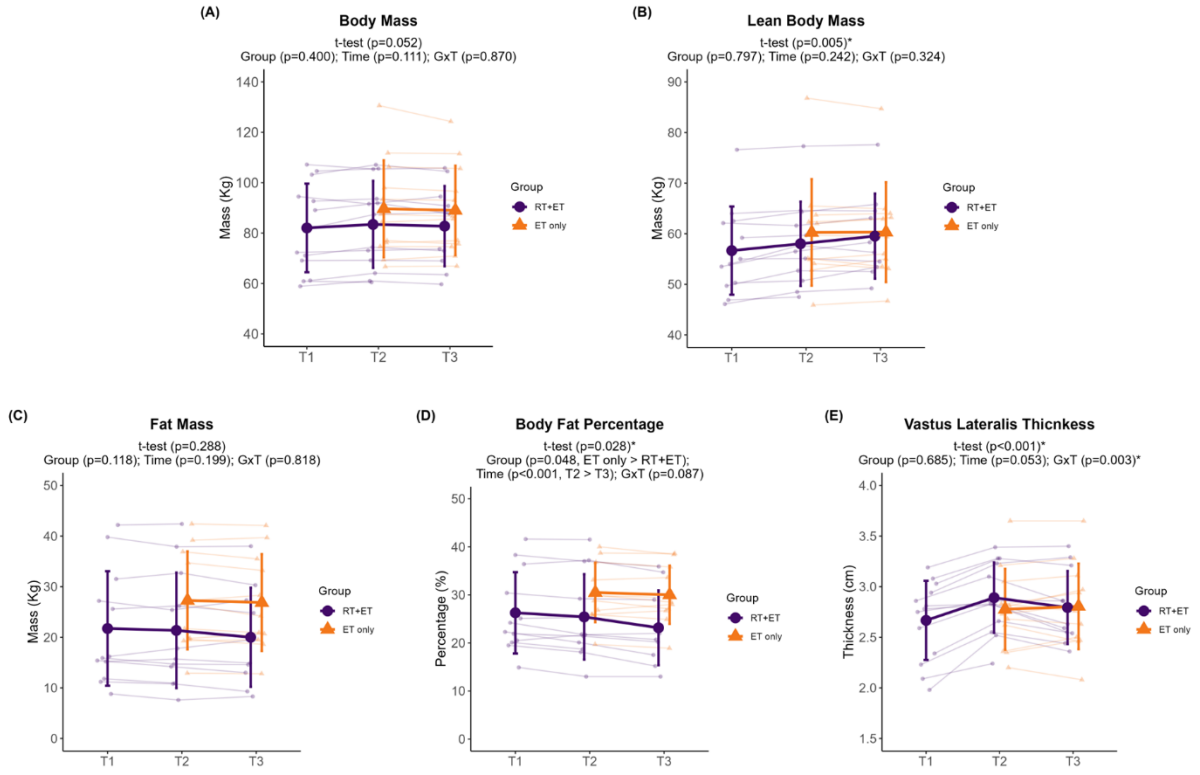


Figure 5-2. Body composition variables response to RT and ET. (A) Body mass. (B) Lean body mass. (C) Fat mass. (D) Body fat percentage. (E) Vastus lateralis thickness. T1 = Pre-RT; T2 = Pre-ET; T3 = Post-ET. Data are expressed as mean ± SD, and individual respondent values are also depicted. Abbreviations: RT+ET, group that performed 7 weeks of resistance training followed by 7 weeks of endurance training; ET-only, group that performed 7 weeks of endurance training only; GxT, group x time interaction. Notes: t-test p-values are for the RT period in the RT+ET group, and the two-way ANOVA main effect and interaction p-values are for the ET period in both groups.

Endurance Performance

In response to RT, RT+ET participants presented no significant changes in any of the aerobic performance variables (absolute VO_2max, p=0.058; relative VO_2max, p=0.794; time to exhaustion, p=0.624; OBLA, p=0.929).

In response to ET in both groups, there was a significant increase over time in all endurance performance variables. Absolute VO_2max increased 0.38 L/min (± 95% CI [0.07], p<0.001), with no significant main effect of G (p=0.674) or GxT (p=0.154) (Fig. 5-3A). Relative VO_2max increased 4.74 ml/kg/min (± 95% CI [0.83], p<0.001), with no significant main effect of G (p=0.108) or GxT (p=0.082) (Fig. 5-3B). Time to exhaustion during the VO_2max treadmill test increased 86 seconds (± 95% CI [23], p<0.001), with no significant main effect of G (p=0.430) or GxT (p=0.257) (Fig. 5-3C). Speed at OBLA increased 0.8 km/h (± 95% CI [0.3], p<0.001), with no significant main effect of G (p=0.354) or GxT (p=0.315) (Fig. 5-3D).

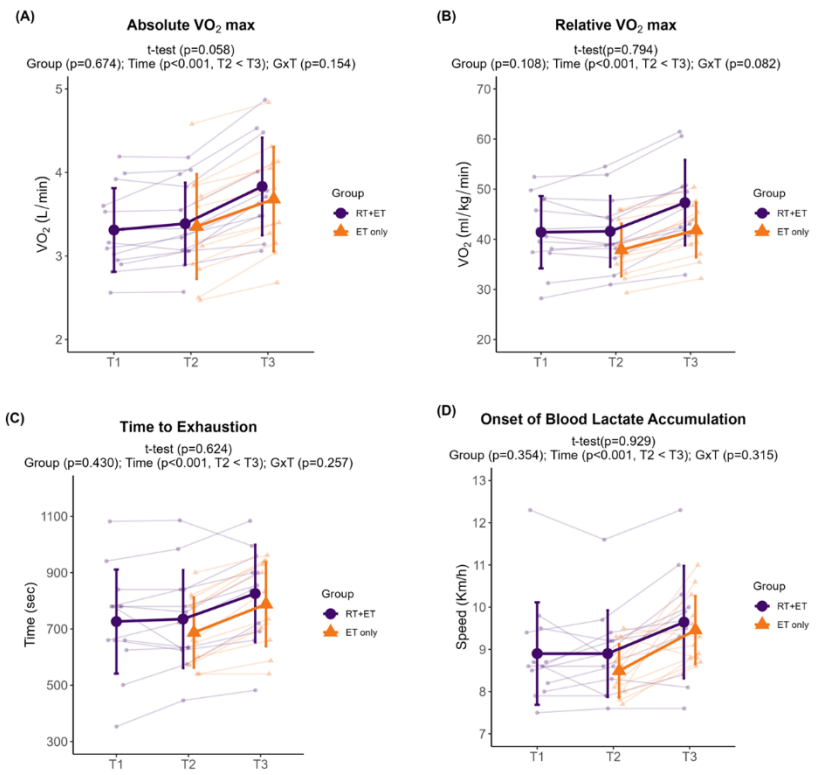


Figure 5-3. Endurance performance variables response to RT and ET. (A) Absolute VO_2max. (B) Relative VO_2max. (C) Time to exhaustion. (D) Onset of blood lactate accumulation. T1 = Pre-RT; T2 = Pre-ET; T3 = Post-ET. Data are expressed as mean ± SD, and individual respondent values are also depicted. Abbreviations: RT+ET, group that performed 7 weeks of resistance training followed by 7 weeks of endurance training; ET-only, group that performed 7 weeks of endurance training only; GxT, group x time interaction. Notes: t-test p-values are for the RT period in the RT+ET group, and the two-way ANOVA main effect and interaction p-values are for the ET period in both groups.

Mitochondrial remodeling

In response to RT, RT+ET participants exhibited no significant increases in the mitochondrial protein complexes (CI, p=0.659; CII, p=0.110; CIII, p=0.408; CIV, p=0.803; CV, p=0.103). Regarding markers of mitochondrial biogenesis, PGC-1α remained unaltered (p=0.455), NRF-1 increased (0.22 a.u., ± 95% CI [0.10], p<0.001), and TFAM decreased (0.39 a.u., ± 95% CI [0.26], p=0.006). The mitophagy marker PINK1 remained unaltered (p=0.143), while PARKIN significantly decreased (0.36 a.u., ± 95% CI [0.22], p=0.004). Markers of

mitochondrial fusion did not change (MFN1, p=0.703; MFN2, p=0.187), but DRP1, a marker of mitochondrial fission, significantly increased (0.19 a.u., ± 95% CI [0.13], p=0.006).

In response to ET in both groups, there were significant increases in mitochondrial protein complexes I-IV from T2 to T3 (CI: 0.23 ± 95% CI [0.15], p=0.006; CII: 0.25 ± 95% CI [0.15], p=0.004; CIII: 0.42 ± 95% CI [0.34], p=0.026; CIV: 0.32 ± 95% CI [0.23], p=0.012) (Fig. 5-4A). There was no significant main effect of G or GxT for complexes I-III (CI: G, p=0.917 GxT, p=0.062; CII: G, p=0.507, GxT, p=0.177; CIII: G, p=0.987, GxT, p=0.173). For complex IV, there was no significant main effect of G (p=0.878), but a significant GxT (p=0.039), where ET-only was higher at T3 compared to T2 (0.58 a.u., ± 95% CI [0.32], p=0.011). No significant main effects of G (p=0.398), T (p=0.173), or GxT (p=0.164) were evident for complex V. Regarding mitophagy markers (Fig. 5-4B), no significant main effects of G (p=0.840) or T (p=0.778), or GxT (p=0.140) were evident for PINK1 protein levels. PARKIN levels significantly decreased from T2 to T3 (0.23 a.u. ± 95% CI [0.16], p=0.010), but there was no significant main effect of G (p=0.175) or GxT (p=0.389). Regarding mitochondrial biogenesis markers (Fig. 5-4C), there were no main effects of G (p=0.214) or T (p=0.926) for PGC-1α, but there was a significant GxT (p=0.001), where ET-only was higher at T3 compared to RT+ET at T3 (0.26 ± 95% CI [0.17], p=0.041). There were no significant main effects of G, T, or GxT for NRF1 (G, p=0.192; T, p=0.912; GxT, p=0.099) or TFAM (G, p=0.062; T, p=0.078; GxT, p=0.458). Regarding mitochondrial dynamics markers (Fig. 5-4D), no significant main effects of G, T, or GxT were evident for MFN1 (G, p=0.555; T, p=0.079; GxT, p=0.717) or DRP1 (G, p=0.857; T, p=0.122; GxT, p=0.096). MFN2 significantly increased from T2 to T3 (0.03 ± 95% CI [0.02], p=0.030), but there was no significant main effect of G (p=0.066) or GxT (p=0.161).

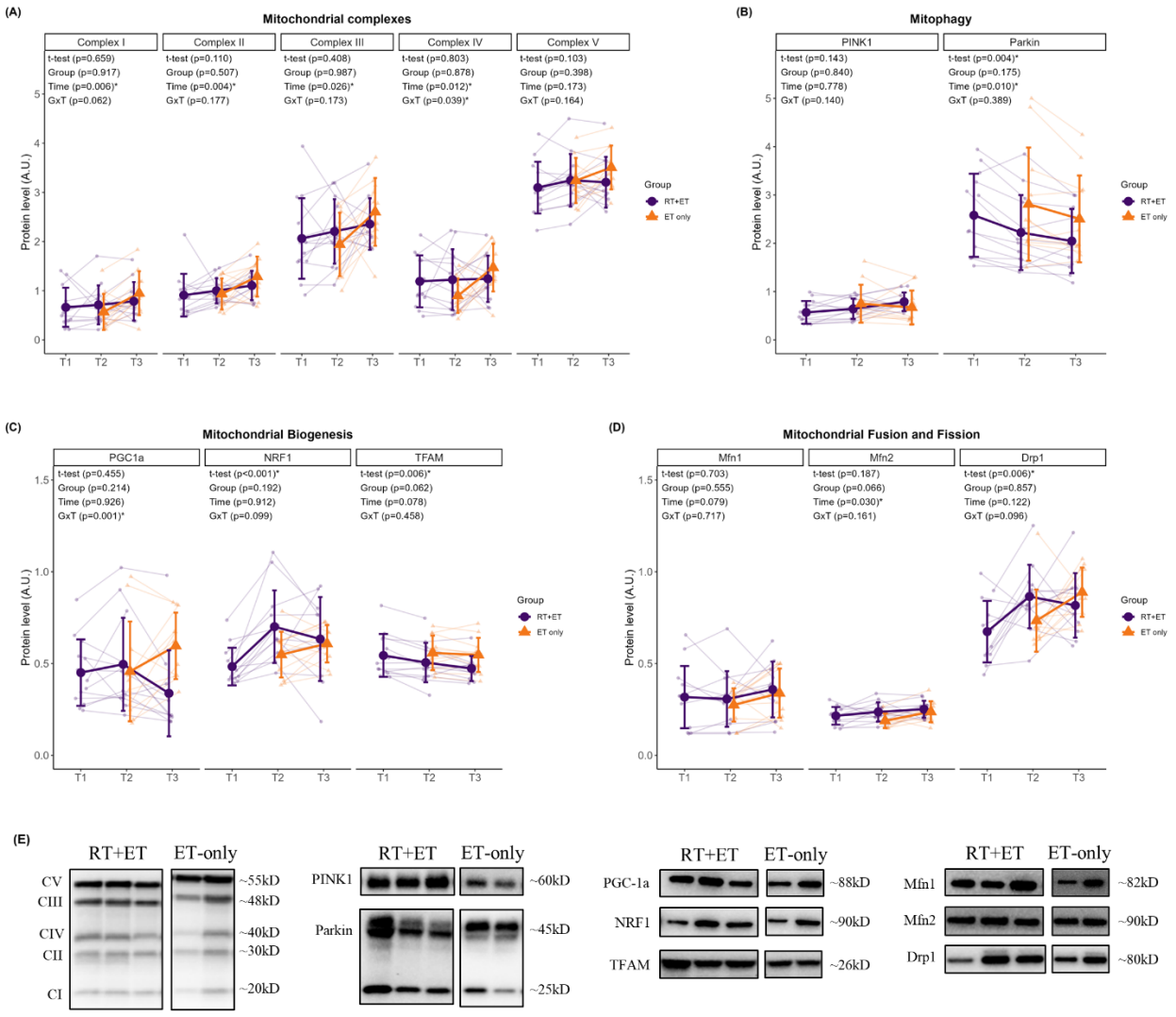


Figure 5-4. Markers of mitochondrial remodeling response to RT and ET. (A) Mitochondrial complexes. (B) Mitophagy. (C) Mitochondrial biogenesis. (D) Mitochondrial fusion and fission. (E) Representative Western blots. T1 = Pre-RT; T2 = Pre-ET; T3 = Post-ET. Data are expressed as mean ± SD, and individual respondent values are also depicted. Abbreviations: RT+ET, group that performed 7 weeks of resistance training followed by 7 weeks of endurance training; ET-only, group that performed 7 weeks of endurance training only; GxT, group x time interaction. Notes: t-test p-values are for the RT period in the RT+ET group, and the two-way ANOVA main effect and interaction p-values are for the ET period in both groups.

Ribosome content

In response to RT, there were no significant increases in relative total RNA levels in the RT+ET group (p=0.443). However, when accounting for muscle size, there was a significant

increase in absolute RNA estimated by fCSA (944,387 a.u. ± 95% CI [866,900], p=0.035), although there was no increase in absolute RNA estimated by VL thickness (p=0.175). 45S pre-rRNA remained unaltered (p=0.124), while 18S rRNA (0.46 ± 95% CI [0.40], p=0.006) and 5.8S rRNA (0.43 ± 95% CI [0.42], p=0.048) significantly increased with RT.

In response to ET in both groups, there were no significant effects of G or T for relative total RNA (G, p=0.690; T, p=0.779), absolute RNA by fCSA (G, p=0.740; T, p=0.451), or absolute RNA by VL thickness (G, p=0.815; T, p=0.896) (Fig. 5-5A-C). However, significant GxT were evident for all variables. Relative total RNA was higher in RT+ET group at T2 compared to ET-only group at T2 (181 µg ± 95% CI [118.4], p=0.004), absolute RNA by fCSA post-hoc tests were p>0.050 for all comparisons, and absolute RNA by VL thickness was higher in RT+ET group at T2 compared to ET-only group at T2 (321.4 µg.cm ± 95% CI [321.4], p=0.010) . For rRNA transcript levels (Fig. 5-5D), there were no significant main effects of G or T, and no significant GxT (45S pre-rRNA: G, p=0.794; T, p=0.461; GxT, p=0.873; 18S rRNA: G, p=0.501; T, p=0.661; GxT, p=0.292; 5.8S rRNA: G, p=0.223; T, p=0.485; GxT, p=0.095).

Immunohistochemistry

Fiber cross-sectional area. In response to RT, there were significant increases in mixed (757 μm^2 ± 95% CI [455], p=0.002) and type II (949 μm^2 ± 95% CI [527] p<0.001) fCSA in the RT+ET group. However, there were no significant changes in type I fCSA (p=0.129). In response to ET in both groups, there were no significant main effects of G, T, or GxT in mixed (G, p=0.772; T, p=0.087; GxT, p=0.413), type I (G, p=0.668; T, p=0.396; GxT, p=0.992), or type II (G, p=0.497; T, p=0.088; GxT, p=0.340) fCSA (Fig. 5-6A).

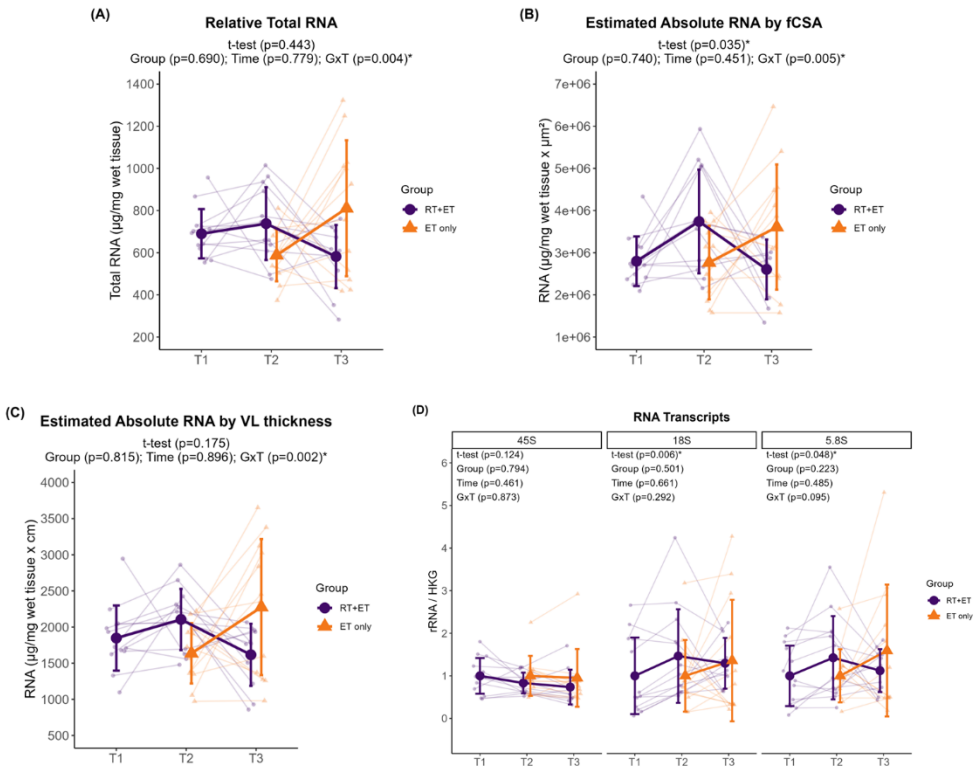


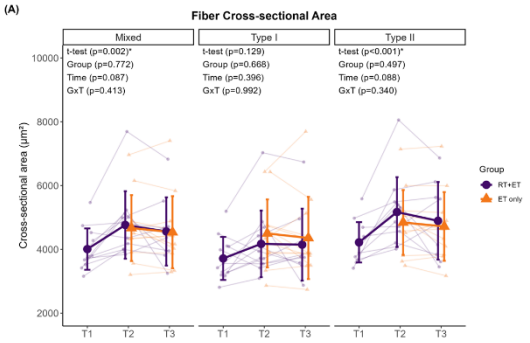
Figure 5-5. Markers of ribosome content response to RT and ET. (A) Total RNA concentrations. (B) Estimated absolute RNA content (adjusted for mixed fiber cross-sectional area values). (C) Estimated absolute RNA content (adjusted for VL thickness values) (D) Ribosomal RNA transcripts. T1 = Pre-RT; T2 = Pre-ET; T3 = Post-ET. Data are expressed as mean ± SD, and individual respondent values are also depicted. Abbreviations: RT+ET, group that performed 7 weeks of resistance training followed by 7 weeks of endurance training; ET-only, group that performed 7 weeks of endurance training only; GxT, group x time interaction. Notes: t-test p-values are for the RT period in the RT+ET group, and the two-way ANOVA main effect and interaction p-values are for the ET period in both groups.

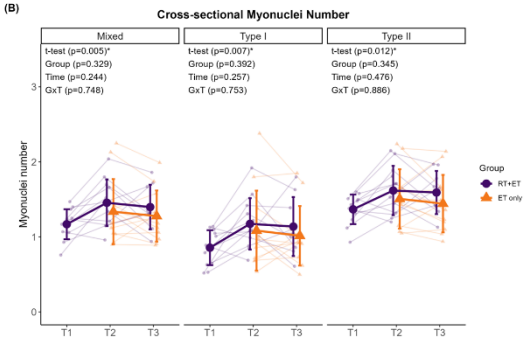
Myonuclear number. In response to RT, myonuclear number in cross-section increased in mixed (0.3 ± 95% CI [0.2], p=0.005), type I (0.3 ± 95% CI [0.2], p=0.007), and type II (0.3 ± 95% CI [0.2], p=0.012) in the RT+ET group. Myonuclear number as quantified through single fiber analysis, however, did not reach statistical significance (p=0.081). In response to ET in both groups, there were no significant main effects of G, T, or GxT for mixed (G, p=0.329; T, p=0.244; GxT, p=0.748), type I (G, p=0.392; T, p=0.257; GxT, p=0.753), or type II (G, p=0.345; T, 0.476; GxT, p=0.886) myonuclear number in cross-section (Fig. 5-6B). Single fiber

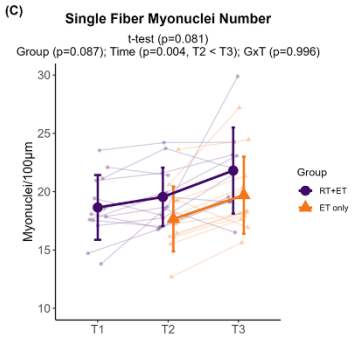
myonuclei number increased from T2 to T3 (2.0 ± 95% CI [1.2], p=0.004), with no main effect of G (p=0.087) or GxT (p=0.996) (Fig. 5-6C).

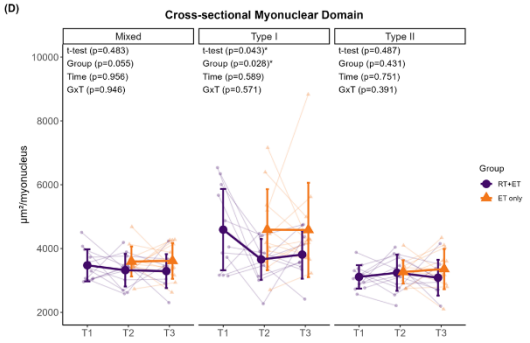
Myonuclear domain (MND). In response to RT, there was a decrease in cross-sectional MND in type I fibers (934 μm^2/myonucleus ± 95% CI [900], p=0.043) in the RT+ET group, but not in mixed (p=0.483) or type II fibers (p=0.487). However, MND values assessed through single fiber analysis exhibited no significant change (p=0.176). In response to ET in both groups, ET-only exhibited greater cross-sectional MND values in type I fibers compared to RT+ET group (G effect: 941 μm^2/myonucleus ± 95% CI [741], p=0.028). There was no main effect of T (p=0.589) or GxT (p=0.571) for type I MND (Fig. 5-6D). In addition, there were no significant main effects of G, or T, or GxT in mixed (G, p=0.055; T, p=0.956; GxT, p=0.946) or type II (G, p=0.431; T, p=0.751; GxT, p=0.391) fibers. For single fiber MND, there were no significant effects of G (p=0.090), T (p=0.894), or GxT (p=0.608) (Fig. 5-6E).

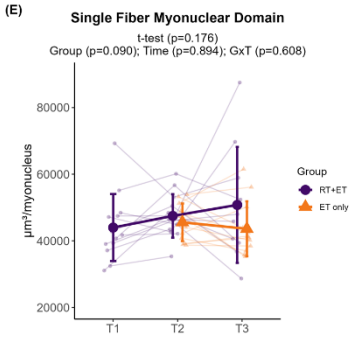
Satellite Cells. In response to RT, there was a significant increase in satellite cell content in mixed (5.2 ± 95% CI [2.7], p=0.002), type I (5.4 ± 95% CI [4.9], p=0.014), and type II (5.5 ± 95% CI [3.1], p=0.004) fibers in the RT+ET group. In response to ET in both groups, there were no significant main effects of G or T, or GxT for mixed (G, p=0.378; T, p=0.114; GxT, p=0.109), type I (G, p=0.179; T, p=0.169; GxT, p=0.606), or type II (G, p=0.747; T, p=0.144; GxT, p=0.079) fibers (Fig. 5-6F).

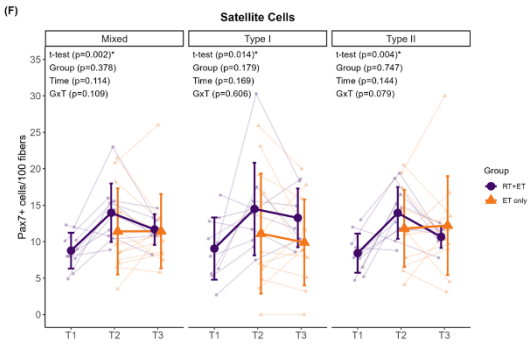

(A)
Fiber Cross-sectional Area
Mixed
Type I
Type II
t-test (p=0.002)*
Group (p=0.772)
Time (p=0.087)
GxT (p=0.413)
t-test (p=0.129)
Group (p=0.668)
Time (p=0.396)
GxT (p=0.992)
t-test (p<0.001)*
Group (p=0.497)
Time (p=0.088)
GxT (p=0.340)
Cross-sectional area (µm²)
Group
RT+ET
ET only
T1
T2
T3

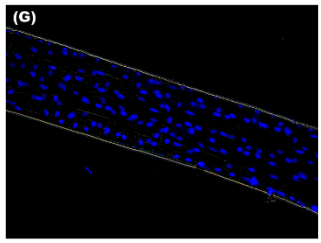

(B)
Cross-sectional Myonuclei Number
Mixed
Type I
Type II
t-test (p=0.005)*
Group (p=0.329)
Time (p=0.244)
GxT (p=0.748)
t-test (p=0.007)*
Group (p=0.392)
Time (p=0.257)
GxT (p=0.753)
t-test (p=0.012)*
Group (p=0.345)
Time (p=0.476)
GxT (p=0.886)
Myonuclei number
Group
RT+ET
ET only
T1
T2
T3

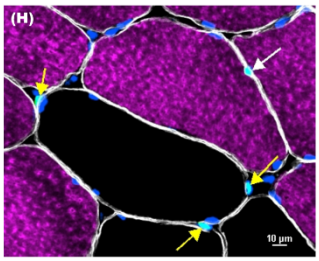

(C)
Single Fiber Myonuclei Number
t-test (p=0.081)
Group (p=0.087); Time (p=0.004, T2 < T3); GxT (p=0.996)
Myonuclei/100µm
Group
RT+ET
ET only
T1
T2
T3

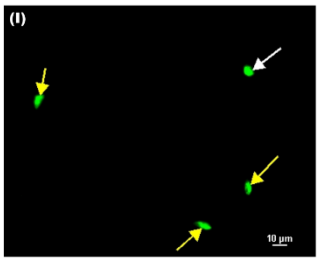

(D)
Cross-sectional Myonuclear Domain
Mixed
Type I
Type II
t-test (p=0.483)
Group (p=0.055)
Time (p=0.956)
GxT (p=0.946)
t-test (p=0.043)*
Group (p=0.028)*
Time (p=0.589)
GxT (p=0.571)
t-test (p=0.487)
Group (p=0.431)
Time (p=0.751)
GxT (p=0.391)
µm²/myonucleus
Group
RT+ET
ET only
T1
T2
T3


(E)
Single Fiber Myonuclear Domain
t-test (p=0.176)
Group (p=0.090); Time (p=0.894); GxT (p=0.608)
µm²/myonucleus
Group
RT+ET
ET only
T1
T2
T3


(F)
Satellite Cells
Mixed
Type I
Type II
t-test (p=0.002)*
Group (p=0.378)
Time (p=0.114)
GxT (p=0.109)
t-test (p=0.014)*
Group (p=0.179)
Time (p=0.169)
GxT (p=0.606)
t-test (p=0.004)*
Group (p=0.747)
Time (p=0.144)
GxT (p=0.079)
Pax7+ cells/100 fibers
Group
RT+ET
ET only
T1
T2
T3


(G)


(H)
10 µm


(I)
10 µm

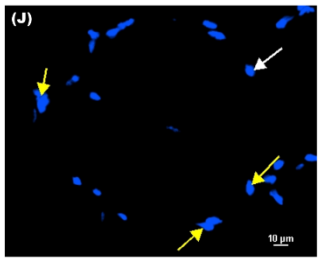

(J)
10 µm

Figure 5-6. Fiber cross-sectional area, myonuclei and satellite cells number, and myonuclear domain responses to RT and ET. (A) Fiber cross-sectional area. (B) Cross-sectional myonuclei number. (C) Single fiber myonuclei number. (D) Cross-sectional myonuclear domain. (E) Single fiber myonuclear domain. (F) Satellite cells content. (G) Single fiber representative image. (H-J) Representative images of cross-sectional staining. (H) Dystrophin (white), MHCI (magenta), DAPI (blue), Pax7 (green). (I) Pax7. (J) Pax7 + DAPI. T1 = Pre-RT; T2 = Pre-ET; T3 = Post-ET. Data are expressed as mean ± SD, and individual respondent values are also depicted. Abbreviations: RT+ET, group that performed 7 weeks of resistance training followed by 7 weeks of endurance training; ET-only, group that performed 7 weeks of endurance training only; GxT, group x time interaction. Notes: t-test p-values are for the RT period in the RT+ET group, and the two-way ANOVA main effect and interaction p-values are for the ET period in both groups.

Mitochondrial content assessed using CS activity and TOMM20 IHC

In response to RT, relative CS activity remained unaltered (p=0.152), but absolute CS activity significantly estimated by fCSA (481,561 nmol/min/mg.μm^2 ± 95% CI [335,161], p=0.009) and by VL thickness (152 nmol/min/mg.cm ± 95% CI [117]) increased in response to RT. There were no significant changes in relative mitochondrial content as assessed through TOMM20 IHC in mixed (p=0.296), type I (p=0.982), or type II (p=0.070) fibers in the RT+ET group. Regarding the estimation of total mitochondrial content, which accounts for fCSA changes, there was a significant increase in type II fiber mitochondrial content (45% ± 95% CI [38], p=0.026) but not in mixed (p=0.113) or type I (p=0.476) fibers.

In response to ET in both groups, mitochondrial content assessed through relative CS activity was higher in the ET-only group compared to RT+ET group (main effect of G: 92.7 mmol/min/mg ± 95% CI [81.3], p=0.036) (Fig. 5-7A). However, there was no significant main effect of T (p=0.461) or GxT (p=0.162). Additionally, there were no significant effects of G, T, or GxT in total mitochondrial content estimated by mixed fCSA (G, p=0.170; T, p=0.117; GxT, p=0.132) or by VL thickness (G, p=0.074; T, p=0.257; GxT, p=0.080) in response to ET (Fig. 5-7B-C).

There were significant GxT for mitochondrial content as assessed through TOMM20 IHC in mixed (p=0.043) and type II fibers (p=0.012). Post-hoc tests for mixed fibers returned p-values > 0.050 for all comparisons. Mitochondrial content in type II fibers was greater in the RT+ET group at T2 compared to ET-only group at T2 (33.8% ± 95% CI [20.2], p=0.036), but there were no significant main effects (for mixed: G, p=0.853 and T, p=0.935; for type II G p=0.463 and T, p=0.801) (Fig. 7D). Type I fibers exhibited no significant main effects of G (p=0.609) or T (p=0.474), or GxT (p=0.158). Similar responses were observed for total mitochondrial content estimations (Fig. 7E). A significant GxT was observed in type II fibers (p=0.018), where mitochondrial content was higher in the RT+ET group at T2 compared to ET-only group at T2 (52.2% ± 95% CI [30.6], p=0.032). No significant main effects of G (p=0.162) or T (p=0.519) were detected for type II fibers. In addition, no significant effects of G, T, or GxT were found in mixed (G, p=0.348; T, p=0.385; GxT, p=0.054) or type I (G, p=0.916; T, p=0.812; GxT, p=0.158).

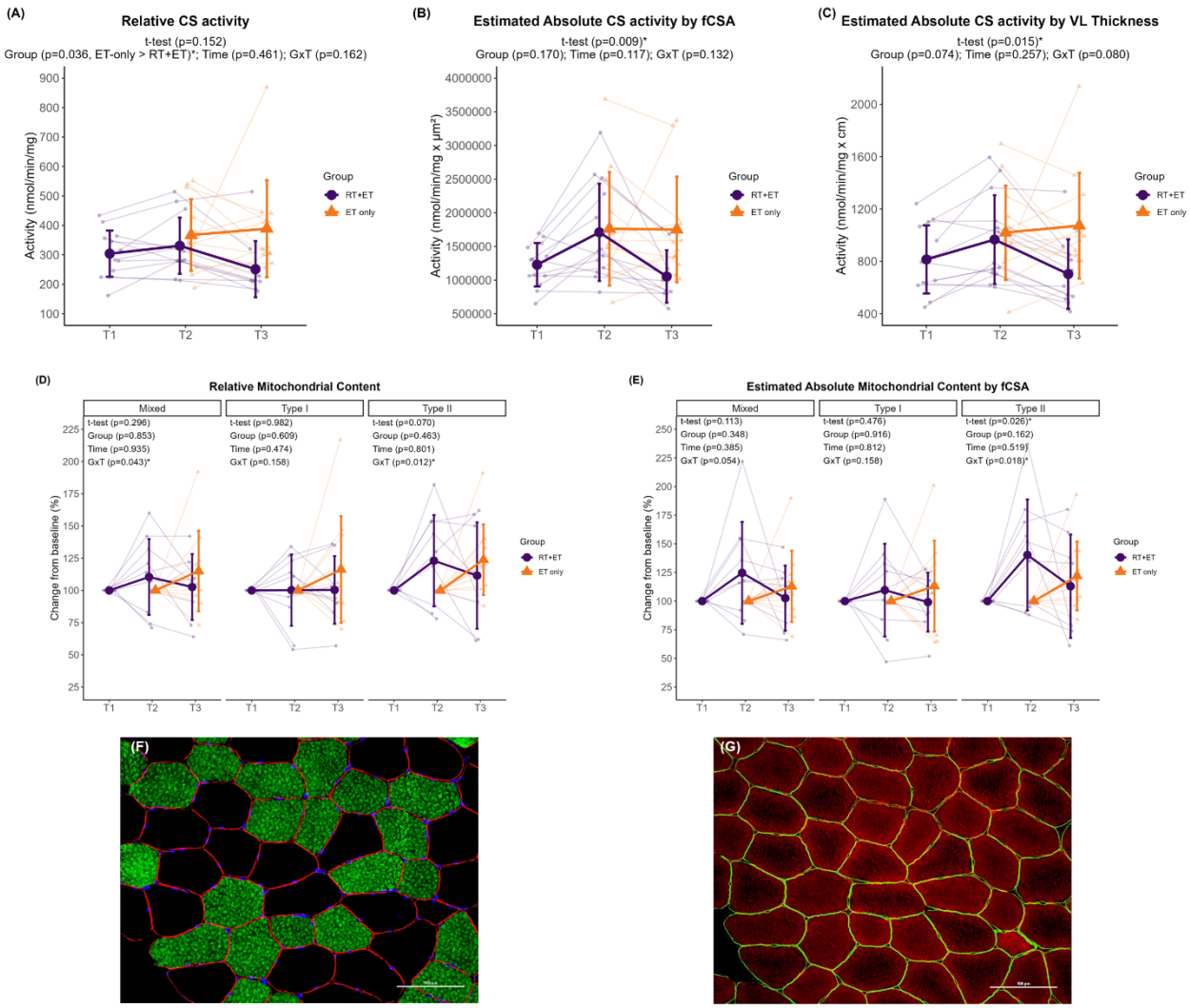


Figure 5-7. Mitochondrial content responses to RT and ET. (A) Relative maximal CS activity. (B) Total mitochondrial content estimation (via maximal CS activity and mixed fCSA values). (C) Total mitochondrial content estimation (via maximal CS activity and VL thickness). (D) Relative mitochondrial content (via TOMM20 IHC). (E) Total mitochondrial content estimation (via TOMM20 IHC and mixed fCSA values). (F-G) Representative images of serial cross-sectional staining. (F) Dystrophin (red), MHCI (green), DAPI (blue). (G) Dystrophin (green), TOMM20 (red). T1 = Pre-RT; T2 = Pre-ET; T3 = Post-ET. Data are expressed as mean ± SD, and individual respondent values are also depicted. Abbreviations: RT+ET, group that performed 7 weeks of resistance training followed by 7 weeks of endurance training; ET-only, group that performed 7 weeks of endurance training only; GxT, group x time interaction. Notes: t-test p-values are for the RT period in the RT+ET group, and the two-way ANOVA main effect and interaction p-values are for the ET period in both groups.

Correlations

Correlation between the values at T2 from select variables (e.g., relative total RNA, fCSA, nuclei, SCs) and the percent change of mitochondrial content variables (i.e., relative CS activity, mixed fibers, type I and type II mitochondrial content (TOMM20)) in response to ET were analyzed in a group-specific manner due to the distinctive responses of each group to ET. The only significant correlation found was between relative CS activity and mixed fCSA in the ET-only group ($r = 0.64$, $p=0.028$). The correlation between all other variables can be found in Supplementary Figure 3.

DISCUSSION

Resistance training has long been appreciated for increasing muscle mass and strength, and emerging evidence highlights that RT may also promote positive mitochondrial adaptations. Most studies investigating the differences and interplay between RT and ET adaptations have compared concurrent training to single-mode training, using various experimental designs. To the best of our knowledge, this is the first study to investigate the effects of performing a period of RT-only on the adaptations to a subsequent period of ET-only. Our main findings demonstrate that RT performed prior to ET had no additional benefits to ET adaptations. Moreover, even though both groups improved endurance performance similarly, prior RT seemed to impair most mitochondrial adaptations to subsequent ET.

In the current study, seven weeks of RT elicited adaptations commonly reported in the literature, which demonstrates the effectiveness of the RT protocol adopted herein. Participants in the RT+ET group improved body composition and strength, and increased VL thickness, mixed and type II fCSA, myonuclear number, markers of ribosome content, and satellite cell

number. Various methods can be implemented to increase endurance performance, with the most common being MICT and HIIT. High-intensity interval training protocols have been shown to improve endurance performance in as little as two weeks (Gillen and Gibala, 2014). VO_2max and the speed at lactate threshold are considered key determinants of endurance performance (Bassett and Howley, 2000; Joyner and Coyle, 2008). In the current study, relative VO_2max improved 13.4% in the RT+ET group and 10.6% in the ET-only, which is within the range reported in the literature (Daussin et al., 2008; Milanovic et al., 2015). In addition, the speed at OBLA increased 7% in the RT+ET group and 12% in ET-only group. However, performing a block of RT before initiating ET did not significantly enhance these adaptations to ET. Additionally, much of our molecular data suggest that seven weeks of RT performed prior to seven weeks of ET may interfere with mitochondrial adaptations, and this will be the crux of the remainder of the discussion.

The mitochondrial adaptations to RT are not well defined based on prior literature. For instance, while it is commonly believed that RT is not an effective method to achieve positive mitochondrial adaptations, different researchers have reported increases in markers of mitochondrial content and function in younger (Groennebaek et al., 2018; Lim et al., 2019; Tang et al., 2006) and older (Lamb et al., 2020; Mesquita et al., 2020; Robinson et al., 2017; Ruple et al., 2021b) individuals. In the current study, there were no significant changes in mitochondrial protein complex concentrations or markers of mitochondrial content (TOMM20 and CS activity) with RT. However, type II fiber total mitochondrial content (as estimated by considering changes in fCSA) increased. This increase in total, but not relative mitochondrial content, suggests that the expansion of the mitochondrial network occurred in line with type II myofiber size increases. Alternatively stated, we speculate that the metabolic demands of RT did not facilitate

mitochondrial expansion per se, but that the expansion of the mitochondrial network occurred in proportion to myofiber size to optimize a mitochondrial-to-myofiber volume ratio.

Satellite cells and myonuclear accretion have been extensively studied in the context of RT and skeletal muscle hypertrophy. Whether or not these events are required for hypertrophy is still a topic of debate (McCarthy et al., 2017; Murach et al., 2021), albeit satellite cells and myonuclei content are commonly reported to increase with RT (Mobley et al., 2017; Murach et al., 2018; Petrella et al., 2006). However, the effects of ET on satellite cells and myonuclear counts have received less attention in the literature. In line with our expectations, seven weeks of RT in the RT+ET group increased mixed, type I and type II myofiber myonuclear number and satellite cell number. However, in both RT+ET and ET-only groups, HIIT training did not elicit significant changes in fCSA, cross-sectional myonuclei or satellite cells number. Our results agree with previous studies that showed no changes in type I and II satellite cell or myonuclear number after different forms of ET (Joanisse et al., 2013; Joanisse et al., 2015), and continue to support that RT (but not ET) acts as a stimulus to affect these variables. As with the sparse research examining how ET affects satellite cell number, studies that have examined the effects of ET on ribosome biogenesis markers are also limited. There is a common dichotomous viewpoint whereby RT promotes ribosome biogenesis and ET promotes mitochondrial biogenesis, with an interference effect between the two processes if RT and ET are performed concurrently (Coffey and Hawley, 2017; Mesquita et al., 2021). However, it is possible that untrained individuals can present a generic response to exercise training whereby ribosome and mitochondrial biogenesis can occur in response to both RT and ET (Mesquita et al., 2021). In response to the HIIT period in the current study, the RT+ET group presented decreases in ribosome content while the ET-only group presented paradoxical increases in these variables.

While these events are difficult to reconcile, the RT+ET response may be related to the cessation of RT and not a response to ET per se, as ribosome content has been previously shown to decrease rapidly upon RT cessation. Hammarström et al. (Hammarstrom et al., 2022), for example, showed a similar decrease in total RNA concentrations (19.3%) after eight days of detraining in humans. Furthermore, Figueiredo et al. (Figueiredo et al., 2021) found that the decrease in ribosome content during muscle disuse was correlated with the decrease in muscle CSA. Therefore, it is possible that ET did not provide sufficient stimulus for ribosome maintenance, as has been shown by Romero et al. (Romero et al., 2017) when providing treadmill ET in rats over a 12-week period. Indeed, this hypothesis is speculative given that we do not have time coursed biopsies to examine markers of ribosome degradation in the RT+ET group, and more research is needed in this regard. The increase in ribosome content in the ET-only group is novel and equally as intriguing. Prior rodent work from our laboratory suggests that 12 weeks of HIIT-style treadmill ET increases ribosome biogenesis markers in lieu of decreasing skeletal muscle ribosome content (Romero et al., 2017). Subsequent work from Figueiredo and collaborators (Figueiredo et al., 2021) indicated that a bout of resistance exercise upregulates several markers in skeletal muscle indicative of increased ribosome biogenesis, whereas this does not occur in response to a steady-state bout of cycling. Others have also shown that weeks of concurrent training enhances ribosome biogenesis relative to resistance training alone (Fyfe et al., 2018). Hence, these prior and our current data suggest that the mode of exercise (e.g., HIIT versus steady state) and (perhaps) species differences may affect the ribosome biogenesis response to ET.

The majority of studies investigating molecular adaptations in response to ET have focused on mitochondrial variables due to their importance in oxidative metabolism. Several

studies have shown increased mitochondrial content and function in response to various forms of ET (Granata et al., 2018; Jacobs et al., 2013; MacInnis and Gibala, 2017). Considering that approximately 98% of the proteins that make up mitochondria are encoded by the nuclear genome (Ryan and Hoogenraad, 2007), we hypothesized that RT-mediated increases in myonuclei and ribosomes would increase both the transcriptional and translational capacity of myofibers, allowing for enhanced mitochondrial adaptations. In fact, Lee and collaborators (Lee et al., 2018) reported that prior RT facilitated mitochondrial adaptations to a subsequent block of RT in rats. Using both rodent and cell models, these authors also demonstrated that higher myonuclear number was related to a greater expression of mitochondrial genes and proteins in response to exercise. However, even though RT led to increased myonuclei and ribosome content in the current study, most mitochondrial adaptations to subsequent ET were blunted. For example, the protein levels of mitochondrial complexes I-IV in the ET-only group showed increases from 32% to 66%, while the RT+ET group only increased from 1% to 11%. Moreover, mixed fiber relative mitochondrial content increased 15% in the ET-only group but decreased 13% in the RT+ET group. Once more, the reasons for such distinctive responses to ET are difficult to reconcile. However, given that the RT+ET group also exhibited decreases in several other variables (e.g., VL thickness, fCSA, and RNA levels), we speculate that these participants existed in an enhanced catabolic/proteolytic state during the duration of the seven week ET period. In support of this hypothesis are certain lines of evidence that have used stable isotopes to ascertain mixed and fractional synthetic protein turnover rates. It is well-known that resistance exercise acutely stimulates increases in both muscle protein synthesis and breakdown, albeit with chronic training, increases in muscle protein synthesis generally exceed increases in muscle protein breakdown (Kumar et al., 2009; Phillips et al., 1997; Reidy et al., 2017). These events

promote a longer-term net positive in protein balance and result in myofiber and whole-tissue skeletal muscle hypertrophy. On the other hand, while ET increases muscle protein synthesis and breakdown (Carraro et al., 1990; Kumar et al., 2009), the increases in muscle protein synthesis may be specific to mitochondrial (rather than myofibrillar) protein synthesis (Wilkinson et al., 2008). In addition, ET has been shown to increase several proteolytic markers in skeletal muscle (Pasiakos and Carbone, 2014; Stefanetti et al., 2015). When considering these prior data and our current observations, it remains possible that the transition from RT to ET in the RT+ET group promoted a sustained elevation in muscle protein breakdown mechanisms while diminishing the protein synthetic response. An ultimate consequence of this shift may have included myofiber atrophy accompanied by a decrease in cellular mitochondrial and ribosome content. While this is an attractive hypothesis to explain several of the RT+ET observations herein, it is speculative and further investigation is needed to confirm this hypothesis.

Experimental Considerations

There are limitations to the current study. First, the n-sizes and biopsy time points were limited in scope. Moreover, only younger adult men were examined herein. Hence, these data should be viewed with these limitations in mind. Additionally, it is important to note that we did not ascertain muscle protein synthesis or breakdown rates, and proteolytic markers were not assayed. As such, much of our speculations regarding the RT+ET adaptations require further inquiry. Markers of mitochondrial function were also not measured in the current study, and it is possible that mitochondrial function improved in response to RT and/or ET. Finally, the inclusion of a control group with a detraining period after RT is lacking, and the inclusion of such a group would have helped distinguish the effects of RT cessation from ET adaptations.

Conclusions

In conclusion, the results of the present study showed that prior RT had no additional benefits on performance adaptations to ET. Additionally, several mitochondrial adaptations to ET (as well as other molecular outcomes) were blunted in the RT+ET group following the ET period. Whether these maladaptive responses at the molecular level have longer-term functional consequences remains to be determined.

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Concluding Remarks

Mitochondria are organelles essential for life due to their roles in energy provision and a myriad of cellular processes. Mitochondria form a complex network that responds to the energetic demands of the cell. Mitochondria are essential for the maintenance of health and a disruption of their function has been implicated in the aging process and in the development of several diseases. Physical exercise has been considered a therapeutic intervention to improve mitochondrial function and counteract mitochondrial decay with aging and in pathological conditions. As such this dissertation investigated the effects of aging and different exercise modalities on different aspects of mitochondrial physiology.

It is generally believed that skeletal muscle adaptations to exercise are highly specific. Historically, there is a common distinction between resistance training (RT) and endurance training (ET), in which RT promotes increases in hypertrophy and ribosome biogenesis, and ET promotes cardiovascular and mitochondrial biogenesis. Previous studies have suggested the existence of a competition between ribosome and mitochondrial biogenesis, in which the first adaptation is prioritized with RT while the latter is prioritized with ET. In addition, reports have shown an interference effect when both exercise modes are performed concurrently. However, there is compelling evidence suggesting that a competition between ribosomal and mitochondrial biogenesis does not exist. Several studies have shown results suggesting that both processes can occur simultaneously in response to different types of exercise. Therefore, it is likely that instead of a competition between the two processes, what happens is an exercise mode-specific response, where ET stimulates mitochondrial biogenesis more so than ribosome biogenesis, and RT stimulates the opposite phenomena.

Aging is characterized by a progressive decline in physiological function that is observed in several tissues, including the skeletal muscle and the brain. The impairment of mitochondrial function has been considered one of the major players in aging and has been linked to the development of many diseases. Another important aspect of aging and mitochondrial dysfunction is a disruption of the balance between reduction–oxidation reactions (redox state). An increase in the production of reactive oxygen species (ROS) and a concomitant decrease in the antioxidant defense system lead to a state of chronic oxidative stress, causing damage to protein, lipids, and DNA. However, we found minimal changes in several markers of mitochondrial content, function, and dynamics in the cortex and cerebellum of female rats in response to both aging and long-term physical activity. Furthermore, the redox status of the tissues investigated remained overall unaltered. The brain mitochondrial physiology and redox homeostasis of females may be more resilient to the aging process than initially thought. In addition, the lack of effects of voluntary wheel running indicates that neurological benefits of exercise may be dependent on more controlled intensity and duration.

Little is known about the effects of RT on mitochondrial physiology. However, recent reports highlight that RT may be able to promote positive mitochondrial adaptations, especially in older adults. Ten weeks of RT increased mitochondrial protein content and improved mitochondrial remodeling, although no changes in these markers were detected following the first training bout. The results suggest that the acute response may not be representative of the chronic effects of RT, and that repeated bouts are necessary to achieve mitochondrial benefits of RT in older populations. Furthermore, short term RT also promoted beneficial adaptations in the redox status of the skeletal muscle in older adults. RT significantly decreased lipid peroxidation and increased antioxidant enzymatic activities. Therefore, RT may be a viable approach to

counteract a possible age-related disruption of skeletal muscle redox homeostasis in older adults. Furthermore, the antioxidant system response to RT is a multilevel-controlled process, involving transcriptional, post-transcriptional and post-translational controls.

Lastly, although RT has long been appreciated for increasing muscle mass and strength, it is often overlooked when it comes to endurance performance. In addition, emerging evidence highlights that RT may also promote positive mitochondrial adaptations. However, RT performed prior to a subsequent block of ET had no additional benefits on adaptations to ET. Additionally, most mitochondrial adaptations to ET were blunted in the RT+ET group, but such impairments seem to be related to the cessation of RT.

The field of mitochondrial physiology is an exciting field with much room for growth as we continue to unveil their relationship with other organelles and their involvement in different cellular processes. The studies contained in this dissertation contribute to the understanding of mitochondrial adaptations in different tissues in response to different conditions, such as aging and exercise. Building on existing literature, the results of this dissertation can help to design interventions, both behavioral and pharmacological, to improve mitochondrial physiology and ultimately the health of the general population.

Appendices

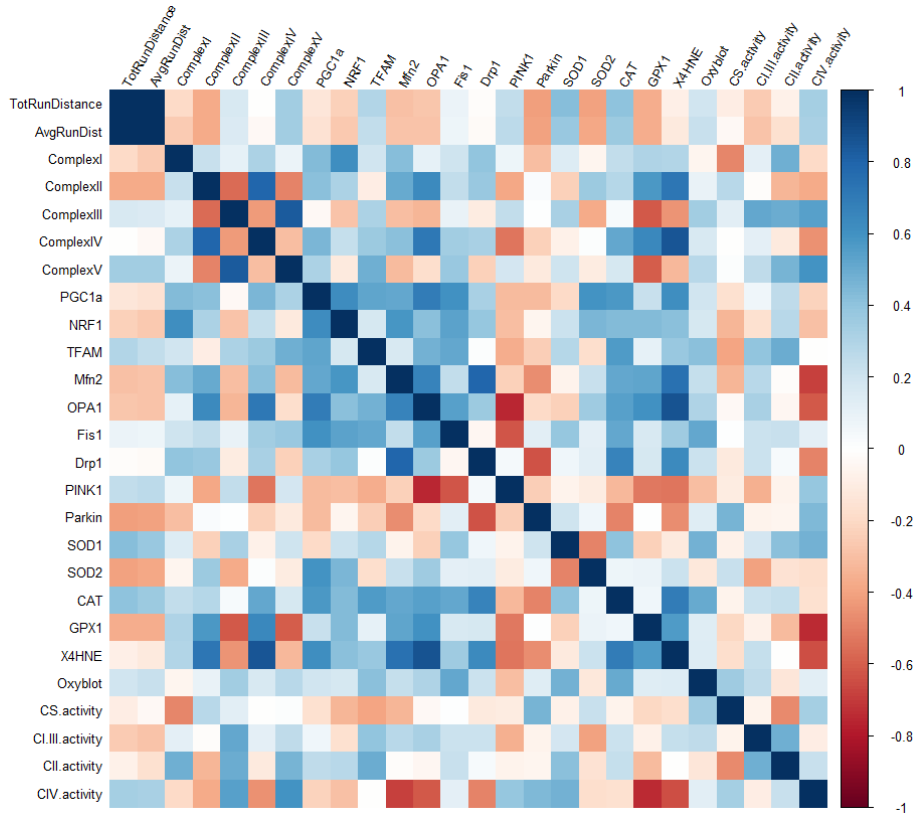

Figure S1. CTX correlations

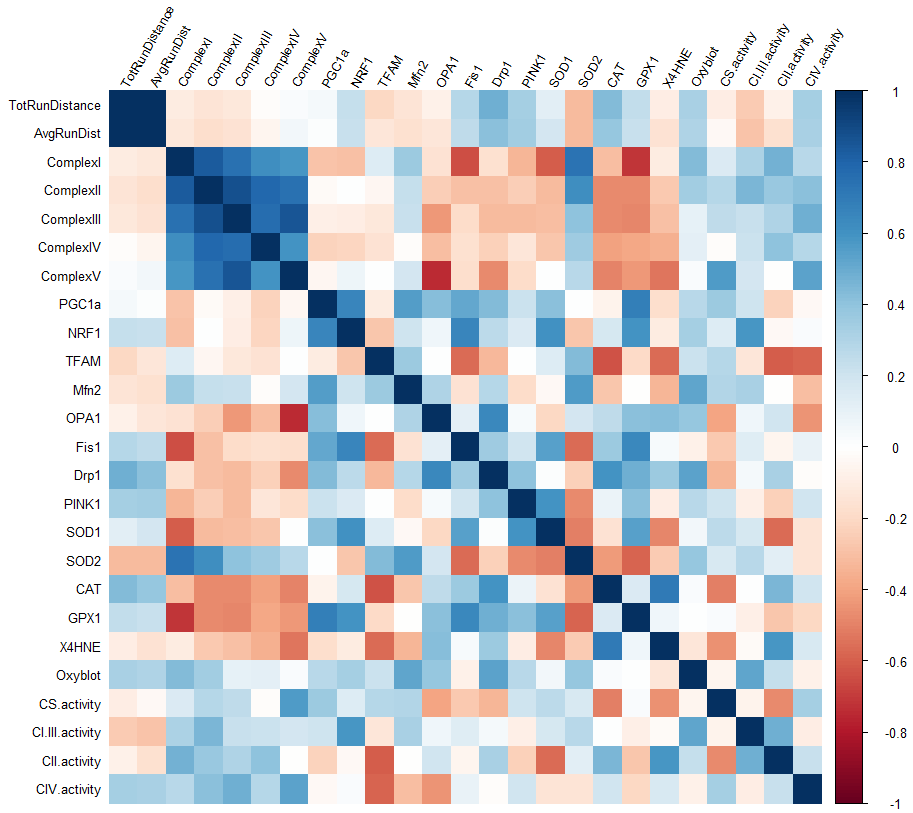

Figure S2. CRB correlations

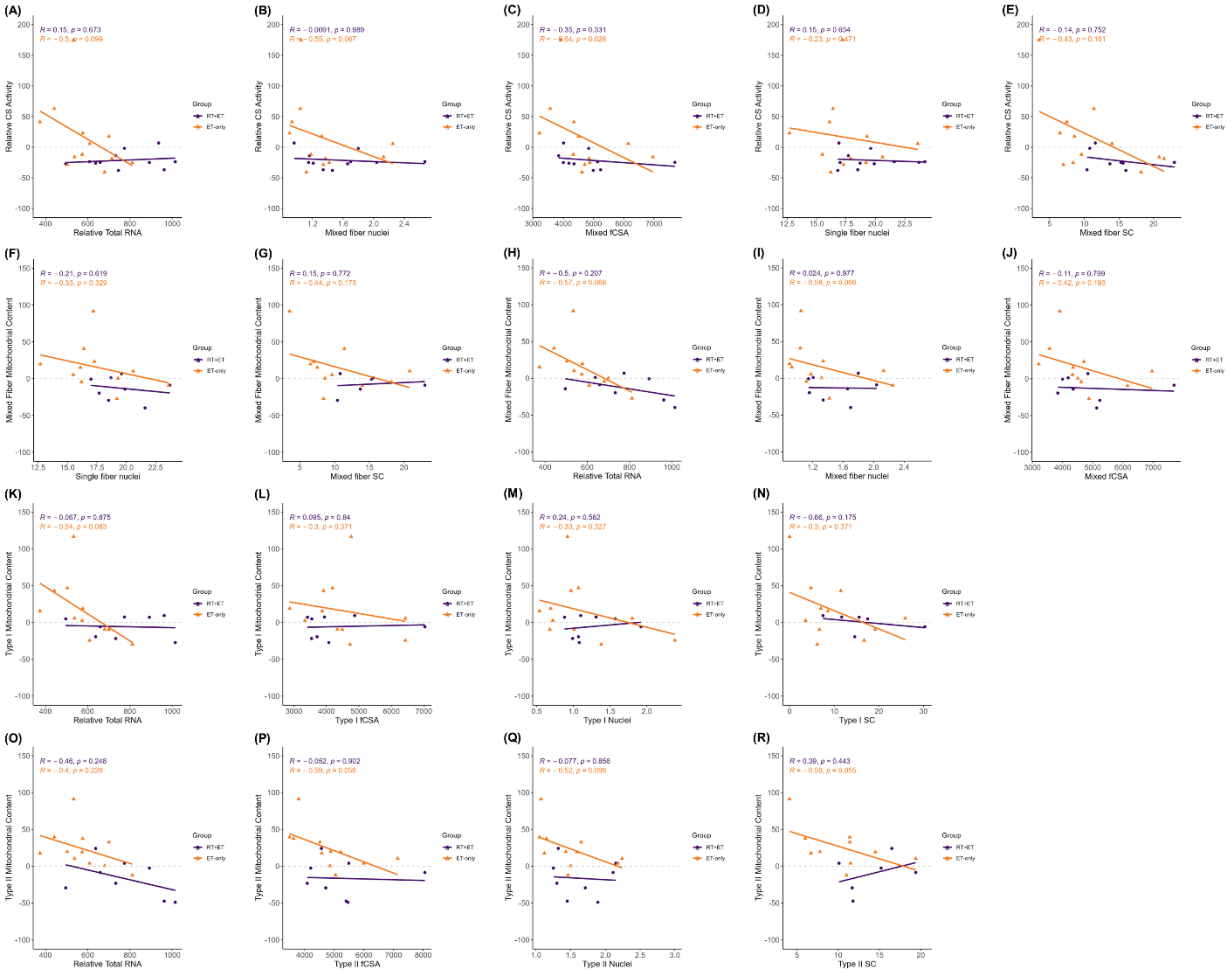

Figure S3. Correlations between mitochondrial content and select variables