THE ACUTE MITOCHONDRIAL RESPONSE TO RESISTANCE EXERCISE

CHARACTERIZING THE ACUTE MITOCHONDRIAL RESPONSE TO RESISTANCE EXERCISE IN AGING

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TITLE: Characterizing the acute mitochondrial response to resistance exercise in aging

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Abstract

Introduction: Mitochondrial dysfunction and oxidative stress increase with aging and may contribute to age-associated muscle atrophy (sarcopenia). Resistance exercise (RE) can promote the accretion of muscle mass, increase strength, and ultimately improve function in the elderly. Such beneficial effects are thought to be mitigated solely by increased muscle mass and strength; however, the contribution of the mitochondria to the beneficial effects of RE in aging have not been thoroughly characterized. While mitochondrial benefits have been established separately in both young and aged adults following chronic RE, the acute effects have not been well characterized. **Methods:** Sedentary young and aged adult males completed either an acute bout of fatiguing RE or endurance exercise (EE), and muscle biopsies were obtained at 3, 24 and 48 h post-exercise depending on the study. **Results:** Despite equivalent lean-body mass, increased age was associated with elevated mtDNA deletions, indicating potential for mitochondrial dysfunction. RE was associated with reduced mitochondrial content (transcripts, protein, and mtDNA copy number) at 48 h post-exercise, a response that did not differ with increasing age. Paradoxically, reduced mitochondrial content occurred alongside elevated total peroxisome proliferator-activated receptor γ coactivator one α (PGC-1α) mRNA; however, RE altered only the PGC-1α4 isoform post-exercise, a transcript that regulates myostatin and insulin-like growth factor one (IGF1) signaling and ultimately muscle hypertrophy and not mitochondrial adaptations. In addition, PGC-1α modulates the unfolded protein response (UPR), and RE was subsequently shown to elevate endoplasmic reticulum stress and elicit the UPR. **Conclusion:** PGC-1α mRNA increases

regardless of exercise mode; however, differential expression or regulation of alternate PGC-1α isoforms or transcriptional binding partners co-activated by PGC-1α may dictate the specific phenotypic adaptations that occur following divergent modes of exercise. Furthermore, RE acutely decreases mitochondrial content despite elevated PGC-1α mRNA, and this response is not influenced by age.

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Abbreviations and symbols

ACVR2a – activin receptor type 2a

ACVR2b – activin receptor type 2b

AMPK – adenosine monophosphate activated protein kinase

ANOVA – analysis of variance

ATF4 – activating transcription factor 4

ATF6 – activating transcription factor 6

ATG7 – Autophagy related protein 7

ATP – adenosine triphosphate

avO_2 – arterio-venous oxygen difference

BNIP3 – BCL2/adenovirus E1B 19 kDa protein-interacting protein 3

CamK – calcium/calmodulin-dependent protein kinase

CHOP – C/EBP homologous protein (DNA damage-inducible transcript 3)

COX – cytochrome *c* oxidase

COXVb – cytochrome *c* oxidase subunit 5b

CPT1 – carnitine palmitoyltransferase 1

CS – citrate Synthase

CSA – cross-sectional area

CytC – cytochrome *c*

EE – endurance exercise

eIF2α – eukaryotic initiation factor 2 alpha

eIF2B – eukaryotic initiation factor 2b

ER – endoplasmic reticulum

ERRα – estrogen-related receptor alpha

ETC – electron transport chain

FoxO3 – forkhead box O3

GADD34 – growth arrest and DNA damage-inducible protein 34

GO – gene ontology

GPX – glutathione peroxidase

GRP78 – glucose regulated protein 78

HSP70 – heat shock protein 70

IGF-1 – insulin-like growth factor 1

IGF-1 – insulin-like growth factor 2

IGFBP2 – insulin-like growth factor binding protein 2

IGFBP3 – insulin-like growth factor binding protein 3

IGFBP6 – insulin-like growth factor binding protein 6

IRE1α – inositol requiring enzyme 1 alpha

KEGG – Kyoto Encyclopedia of Genes and Genomes

LC3b – microtubule-associated protein 1 light chain 3b

MAPK – mitogen activated protein kinase

MCAD – Medium-chain acyl-CoA dehydrogenase

MEF2 – myocyte enhancer factor 2

mtCk – mitochondrial creatine kinase

MnSOD – manganese superoxide dismutase

mRNA – messenger ribonucleic acid

mtDNA – mitochondrial deoxyribonucleic acid

NADH – reduced nicotinamide adenine dinucleotide

ND1 – mitochondrial encoded NADH dehydrogenase subunit 1

ND4 – mitochondrial encoded NADH dehydrogenase subunit 4

Nix –a.k.a. BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like (BNIP3L)

Nrf-1 – nuclear respiratory factor 1

Nrf-2 – nuclear respiratory factor 2

PDGFb – Platelet-derived growth factor subunit B

PDK4 – pyruvate dehydrogenase kinase 4

PERK – protein kinase R-like ER protein kinase

PGC-1α – peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PGC-1α1 – PGC-1α isoform 1

PGC-1α4 – PGC-1α isoform 4

PGC-1β – peroxisome proliferator-activated receptor gamma coactivator 1-beta

PPARα –peroxisome proliferator-activated receptor alpha

PRC – PGC-1 related coactivator

RE – resistance exercise

ROS – reactive oxygen species

SDH – succinate Dehydrogenase

SIRT1 – Sirtuin 1

Tfam – Mitochondrial transcription factor A

Thr – threonine

tRNA – transfer ribonucleic acid

UPR – unfolded protein response

VO_2max – maximal oxygen consumption

VPS34 – vacuolar protein sorting 34

WT – wild type

XBP-1 – X-box binding protein 1

Format and organization of thesis

This thesis is prepared in the "sandwich" format as outlined in the McMaster University School of Graduate Studies' Guide for the Preparation of Theses. It includes a general introduction, three independent studies prepared in journal article format, and an overall discussion. The candidate is the first author on all manuscripts.

Statement of contribution

Effects of age and acute resistance exercise on mitochondrial transcript and protein abundance in young and old skeletal muscle (Study #1)

MAT, GP, DIO and BRM contributed to the experimental design, exercise testing and collection of tissue. JC completed the immunoprecipitation experiments. DIO and AS completed the protein analysis. DIO and MA completed the RNA and mtDNA analysis. DIO analyzed all data and wrote the manuscript. All authors were involved in the editing and final approval of the manuscript

The unfolded protein response is triggered following a single resistance-exercise bout (Study #2)

MAT, GP, DIO and BRM contributed to the experimental design, exercise testing and collection of tissue. DIO and JC completed the protein analysis. DIO and MA completed the RNA analysis. DIO analyzed all data and wrote the manuscript. All authors were involved in the editing and final approval of the manuscript

Differential effects of acute resistance and endurance exercise on specific PGC-1α isoforms (Study #3)

MAT and DIO contributed to the experimental design. MAT, GP, DIO, BM, JC performed the study and collected samples. DIO and JC completed the RNA analysis. All data was analyzed and the manuscript written by DIO. All authors were involved in editing and the final approval of the manuscript.

Introduction

Modern medical and scientific advances have increased life expectancy considerably over the last century. As a consequence of this increased lifespan, the proportion of the Canadian population over the age of 65 y (currently estimated at 16.5%) is expected to increase dramatically to 23% by the year 2050 (1). Despite these modern medical advancements, the aging process is still associated with significant deterioration of the form and function of the human body. Skeletal muscle is particularly susceptible to the aging process, and the loss of muscle mass and strength with age present the elderly with significant functional impairments. This progressive muscular atrophy, termed sarcopenia, is already a substantial burden on the healthcare system (2), a cost expected to increase with the disproportionately high use of the health care system in the expanding elderly population. Consequently, therapeutic interventions that retain or increase muscle mass and strength will allow older adults to preserve their independence and physical function while simultaneously reducing the economic cost of sarcopenia.

The etiology of sarcopenia is a complex, multifaceted phenomenon with no singular cellular process identified as a central regulator. Despite this, mitochondria have, to an increasing extent, been recognized as a key component of the aging process as a whole (3), and mitochondrial dysfunction has been implicated in muscle atrophy (4). Resistance exercise (RE) can promote increased muscle mass and strength independent of age (5); however, the effect of RE on the mitochondria in the context of aging is unclear and requires further investigation.

Aging and sarcopenia

The prevalence of sarcopenia is influenced most by age, occurring most frequently in individuals 70 to 80 years of age (6); however, variability in these estimates can also be explained by the lack of consensus on the clinical definition of sarcopenia (7). Sarcopenia is defined as the loss of muscle mass below two standard deviations from population normative data (8); however, there has been recent interest in combinations of both anthropometric and functional measurements to ascertain the quantity and quality (function) of muscle (7). The rate of occurrence of sarcopenia in elderly populations can exceed 50 % in those over the age of 80 years (6,9,10); however, rates vary significantly in the literature depending on specific characteristics of the population studied (age demographic and active-living status).

Irrespective of diagnostic semantics, skeletal muscle mass declines with age with rates of loss estimated at 0.5-1.5 % per year commencing at some point between the second to fifth decade of life (11-13). These gradual reductions result in significant loss of muscle mass over time, such that deficits in muscle cross-sectional area (CSA) in the elderly can exceed 30 % as compared to the young (14,15). In addition to the loss of mass, muscular strength and power both decline after the fourth decade of life (16), and such reductions are predictive of or associated with mobility and functional limitations (15,17). Ultimately, the gradual loss of strength results in a large disparity between young and aged individuals in the musculature of both the upper and lower body, with reductions in strength up to 30 % or greater in adults over the age of 70 years (14,15,18). This loss of strength is significant, as those with higher levels of strength are less likely to report a

functional impairment with age (19). Unfortunately, the precise intramuscular alterations that are responsible for reduced mass, strength, and power are not completely clear at this point.

In addition to the relationships between muscle size, strength, and function in the elderly, there is also evidence to suggest that the composition of the muscle, with respect to muscle fibre type, may also explain the functional deficits. Human skeletal muscle is composed of three fibre types, classified by the myosin heavy chains expressed (type I, IIa, IIx) that vary in proportion depending on the muscle group and the individual. Function varies by fibre type, such that type I fibres are highly oxidative and fatigue-resistant; whereas, type II fibres have greater shortening velocities and peak force, and have greater power production as a consequence (20). Both type I and II fibres atrophy with age (13); however, some evidence suggests that type II fibres are particularly susceptible to the aging process and atrophy at an accelerated rate as compared to type I fibres (21-24). Given the enhanced force and power producing capabilities of the type II fibre (20), their loss in either area or number holds significant functional implications for force and power production.

Despite atrophy of both the fibre types, it appears that contractile function of individual fibres is preserved since Trappe et al (25) found no detriment in single muscle fibre normalized power, force and contractile velocity regardless of fibre type. Consequently, impaired muscle function with aging may be more a quantitative than qualitative problem associated with the loss of muscle mass, and perhaps preferential type II atrophy (21-23), than an alteration to intrinsic characteristics of the fibres themselves.

The accumulation of intramuscular fat (26) and connective tissue (27) cannot be disregarded for their potential impact on the contractile function of the muscle as a whole despite the preservation single fibre contractile characteristics (25); however, human data indicates intramuscular collagen concentrations are not altered with aging (15).

The loss of strength in muscle of older adults comes with some benefit as evidenced by the enhanced fatigue resistance of aged muscle (28-31). Such changes agree with the preferential, or at least proportionally greater atrophy of type II fibres with aging (21,22), as this would attenuate force production while simultaneously enhancing fatigue resistance due to the greater relative amount of type I fibres as compared to the young. Nevertheless, sarcopenia represents a significant collective burden to the elderly population and health care system, indicating that the preservation of muscle tissue, and ultimately function, are of utmost importance to a healthy aging process.

The Mitochondrial Theory of Aging

No single explanation exists for the various deleterious alterations that occur throughout the aging process. The molecular etiology of sarcopenia is complex and likely involves multiple processes including the loss of motor units and denervation of muscle (32), impaired protein synthesis and turnover (8,33,34), altered systemic inflammation (8,35), oxidative stress (2,36) and mitochondrial dysfunction (4,37-40). Of those, mitochondrial dysfunction has been implicated as a contributor to the loss of muscle mass with age (41), and may play additional roles in the other causative factors of aging, including but not limited to telomere shortening and genomic DNA damage (42).

The free-radical theory of aging (43), proposed by Dr. Denham Harman, indicates there is a strong relationship between the accumulation of oxidative damage and cellular senescence. Harman subsequently updated the theory to acknowledge that mitochondria are the primary site of radical production (44,45), and collectively act as a *forme fruste* of a biological clock within the cell (46). The now widely known mitochondrial theory of aging dictates that cellular senescence is the result of a 'vicious cycle' of: 1) mitochondrial reactive oxygen species (ROS) production, and 2) subsequent oxidative damage to integral cellular structures that disrupts basal function. Elevated oxidative stress serves to synergistically augment reactive oxygen species production by damaging mitochondrial proteins and DNA, exacerbating reactive oxygen species production and consequent oxidative damage.

Additional cellular processes altered by aging further serve to compound the deleterious effects of elevated ROS in aging, as the 'vicious cycle' is not confined simply to the damaging effects of ROS on mitochondria. Inhibition or attenuation of the synthesis of new mitochondrial proteins occurs as there are age-associated deficits in various components of mitochondrial bioenergetic signaling including adenosine monophosphate activated protein kinase (AMPK), peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) and sirtuin 1 (SIRT1) (47,48). An inability, or at least impaired ability, to remove damaged mitochondria only adds to the vicious cycle, as proteolytic activity (47) and autophagy (49) decline with age. Consequently, the vicious cycle of the mitochondrial theory of aging may be initiated by

elevations in ROS, but impaired synthesis and degradation of mitochondria limits the ability of the cell to correct, or at least compensate for mitochondrial dysfunction (50).

Mitochondrial content and function across the lifespan

Resting and maximal oxygen consumption (VO_2max), when adjusted for lean-body mass, decrease with age (50,51). Defined by the Fick equation (52), VO_2max represents the product of cardiac output (Q) and the arterio-venous oxygen difference (avO_2). Reductions in stroke volume with age attenuates cardiac output and ultimately VO_2max (53,54); however, age-related reductions in avO_2 (55) also contribute and suggests that skeletal muscle mitochondrial content and function may decrease with age (50). The measurement of both resting and maximum VO_2 considers whole-body function and cannot directly represent the sole contribution of alterations in mitochondrial capacity, implicating the need for direct measurement of muscle mitochondrial content and function to determine the role of the mitochondria in the reduction of oxidative function with age (50).

Variation in methodologies confounds the determination of mitochondrial function and content with age (50). Given the ability of physical activity to alter mitochondrial content (56), controlling for physical activity is a concern with human mitochondrial aging studies; however, the experimental preparations (muscle homogenates, isolated mitochondria, permeabilized fibres), and the techniques (respirometry, enzyme activity, western blotting, electron microscopy, mtDNA content) used to quantify mitochondrial function and content can also contribute to the discordant

findings in the literature (50). Even the muscle group chosen for analysis can influence whether or not both muscle atrophy and mitochondrial dysfunction are detected with age (37,57). In spite of these methodological concerns, studies have documented reduced ATP production rates (50), maximal enzyme activity (51,58), mitochondrial transcript abundance (59-63), protein synthesis (51), and full-length mtDNA (36) and copy number (64). In addition, the presence of cytochrome *c* oxidase (COX) negative fibres, ragged red fibres, and mtDNA deletions and mutations increases with age (37,38,65). COX-deficient regions of muscle fibres are associated with increased mtDNA deletions and muscle atrophy, suggesting a relationship between mtDNA damage, mitochondrial dysfunction and muscle wasting (4,37,38). Ultimately, the reduction in mitochondrial function with aging comes at a consequence, increased production of free radicals (57,66), that elevate oxidative stress even in the face of compensatory antioxidant responses with age in skeletal muscle (36,57,67,68).

Oxidative stress across the lifespan

A central tenet of the mitochondrial theory of aging is that oxidative stress increases with age. Mitochondria are the primary source of reactive oxygen species in skeletal muscle, representing 0.1-0.4% of the total oxygen utilized in the electron transport chain (69). The superoxide radical, released from complex I, II or III (44,45,70) is highly reactive and reacts rapidly to form other free radicals including hydrogen peroxide (H_2O_2), the hydroxyl radical (HO^-), and peroxynitrite ($ONOO^-$). In support of the role of oxidative stress in aging, electron leakage and hydrogen peroxide release increase with age in skeletal muscle, (57,66). These reactive oxygen and nitrogen species

can be deleterious to the cell and induce damage to lipid (lipid peroxidation), proteins (protein carbonyls), and both nuclear and mitochondrial DNA (8-OH-2-deoxyguanosine).

Despite the difficulty directly assessing the production of oxygen radicals in human tissues, the resultant oxidative damage increases with age across various tissues of the body. Markers of damage to proteins and lipids have been demonstrated in skeletal muscle with advancing age (36,63,67,68,71,72). Age-related oxidative damage represents the balance between the production of ROS and the antioxidant capacity of the cell as an increase in free radicals or a decrease in antioxidant capacity could both be deleterious to the cell. The bulk of the literature supports that it is likely an elevation in free radical production that overwhelms the system rather than suppression of antioxidant defenses, as aged muscle often shows a compensatory increase in certain components of the antioxidant system (36,57,67,68). Such a response may vary by fibre type, as Picard et al (57) found elevated manganese superoxide dismutase (MnSOD), glutathione peroxidase (GPX) and catalase activity in the fast-twitch gastrocnemius of aged rats, while the soleus had no change in MnSOD and reduced activity of GPX and catalase. Ultimately, despite the overall low rate of mitochondrial ROS generation, the oxidative damage to protein and lipids accumulates with age, in spite of the variable compensatory changes to the antioxidant defense system.

In addition to oxidative damage to proteins and lipids, mitochondrial DNA (mtDNA) is particularly susceptible to oxidative stress as compared to nuclear DNA (73), and oxidative modifications to DNA increase with age (36,71,72). MtDNA is located in close proximity to the source of free radical generation but also lacks introns and

protective histone proteins characteristic of nuclear DNA that leave it exposed to potentially damaging free radicals. While our understanding of mtDNA repair mechanisms is emerging, it is also believed that the mtDNA repair is limited as compared to the nuclear genome (74,75). Consequently mtDNA deletions and mutations accumulate with age at a rate dependent on the type of tissue studied (4,36,76-83).

Damaged mtDNA coexists with normal mtDNA in aged skeletal muscle (mtDNA heteroplasmy) such that the rate of mtDNA deletions appears relatively low at approximately less than 0.1% when studied in whole muscle homogenates (84,85). The relatively low rate of mtDNA damage is a point often used to argue against the mitochondrial theory of aging (50); however this contention does not consider the heterogeneity in distribution throughout the muscle. These deletions are not randomly and equally distributed throughout the muscle, but rather accumulate in specific groupings of fibres (86). This suggests that analysis of whole muscle homogenates may not fully represent the burden of mtDNA deletions and mutations in aging (50). Despite this controversy regarding the precise relationship of mtDNA deletions and mutations to mitochondrial and muscle function, evidence suggesting the co-localization of mtDNA deletions and electron transport chain (ETC) dysfunction with atrophic regions of the muscle fibre holds important implications for the pathogenesis of sarcopenia (4).

A link between mitochondrial dysfunction and muscle atrophy

Mitochondrial dysfunction may be directly related to muscle atrophy in aging. Using three dimensional reconstructions of myofibres from aged Fisher Norway rats,

Wanagat et al (4) demonstrated that the length of segmental regions of ETC dysfunction (COX negative, succinate dehydrogenase (SDH) hyper-reactive) negatively correlated with fibre cross-sectional area, and that these abnormalities occurred more frequently in Type II fibres. The authors postulated that elevated free radicals promote mitochondrial dysfunction as per the standard mitochondrial theory of age (vicious cycle), but also provided insight into how this mitochondrial-specific damage translates into reduced muscle size. Once ROS production surpasses an undefined pathological level, these ROS eventually disrupt nuclear gene expression impairing the production of myofibrillar proteins, culminating in muscle fibre dysfunction and atrophy characteristic of sarcopenia.

The involvement of the mitochondria in sarcopenia is further supported by studies using the muscle specific over-expression of PGC-1α (87,88), a central regulator of mitochondrial biogenesis. PGC-1α overexpression attenuated the age-associated attenuation in COX-activity and mitochondrial subunit protein content, preserving mitochondrial function with age. In addition to these mitochondrial effects, PGC-1α over-expressing mice had reduced fat mass and increased lean-body mass at 22 months of age as compared to wild-type (WT) controls. Such results have been replicated in the polymerase gamma deficient mouse that displays an accelerated aging phenotype, whereby endurance exercise (EE), a potent stimulus for mitochondrial biogenesis and the endogenous elevation of PGC-1α expression (89), abrogates the deleterious changes that occur with age and promotes the retention of muscle mass in POLG1 mice (90). These results indicate that there may be a direct, mechanistic link between mitochondrial

function and muscle size (via PGC-1α), and that manipulation of PGC-1α may play an important role in the prevention or attenuation of sarcopenia.

Establishing a causal link between mitochondrial dysfunction and muscle atrophy is complicated by the fact that mitochondrial dysfunction may not directly correlate with the degree of muscle atrophy in specific muscle groups. Picard et al (57) demonstrated differential rates of atrophy across muscle groups of various fibre compositions, and that mitochondrial dysfunction differed according to fibre type. Predominately fast-twitch muscles (gastrocnemius, extensor digitorum longus) had increased abundance of electron transport chain proteins in the aged animals, with no change in state III respiratory capacity, suggesting lower mitochondrial function normalized to protein content. Conversely, predominately slow-twitch muscles had reduced mitochondrial content, citrate synthase activity, and lower state III respiration capacity, suggesting differential effects of age on both muscle atrophy and mitochondrial dysfunction. Hydrogen peroxide release was higher with age, but varied again by fibre type such that leak was higher under state III respiration in gastrocnemius (fast), and state II in the soleus (slow) (57). While this study ultimately supports the idea that aging is associated with increased mitochondrial dysfunction and muscle atrophy, it indicates that these changes are not always proportionately related to each other, and that future work should consider any observed changes in the context of specific muscle fibre-types.

While a conclusive link between mitochondrial function and age-associated muscle atrophy is yet to be determined, this data does support that mitochondria may be important to the maintenance of muscle mass (4). Furthermore, this relationship may be

easily manipulated by exercise, and such a response may be due to the activity PGC-1α following exercise (87,88,90). Further work is still required to clarify the equivocal results regarding the role of mitochondria in muscle atrophy and to reconcile the potential interactions of mitochondrial dysfunction and muscle fibre type in sarcopenia.

Resistance exercise in aged populations

Resistance exercise (RE) is an effective method to counteract the deleterious effects of sarcopenia by increasing muscle strength and size (91,92). Muscle hypertrophy is the culmination of various cellular processes that ultimately shift the balance within the muscle in favor of protein synthesis or retention (anabolism) over breakdown (catabolism) (93). RE is a potent stimulus for the induction of muscle protein synthesis, and while some data indicates impairment in protein synthesis following RE in aged muscle (anabolic resistance) (94-97), others have found that older adults show beneficial adaptations to strength training (91,92). Various studies have shown that both healthy and frail elderly adults are able to increase muscle protein synthesis acutely and alter skeletal muscle mass and strength in response to chronic training (91,92,94,98-105).

RE promotes increased muscle mass by increasing the cross sectional area in both type I and II fibres (106-108), although the effects of RE may be more pronounced on the type II fibre depending on the specific parameters of the training program (104,109). This is likely to have a substantial impact on sarcopenia, as type II fibres have been shown to atrophy at an accelerated rate with aging (21,22), and their enhanced force and power

production (20) could translate into greater restoration of function than treatments that disproportionately influence type I fibres.

Increased muscle mass and strength following RE improves quality of life and facilitates the performance of activities of daily living (110). Studies have demonstrated tangible improvements in walking speed, gait and joint mobility with even short periods of resistance exercise (8-10 weeks) (99,111-114). The benefits of strength may be greater in those with the greatest impairments in function. Chandler et al (114) demonstrated greater effects of RE on the ability to rise from a chair in older adults classified as lower functioning as compared to their higher functioning peers. This agrees with existing literature that indicates that even frail elderly adults can increase muscle mass and strength with RE (102,103), and that these adaptations translate to improved function. The fact that these exercise interventions can be completed without injury (113), suggests the cost/benefit ratio of the risk of exercise-induced injury to the beneficial gains of muscle mass, strength and function weighs favorably for RE. The precise relationship between regular RE, improved function and quality of life with morbidity and mortality has not been determined; however, as vigorous activity can decrease both (115-117), it is not an unreasonable assertion that RE could favorably impact both as well.

Strength training is a safe and effective intervention to increase muscle mass, strength and function, ultimately improving quality of life in the elderly (91,110,118). The mechanism of these functional benefits is attributed to improved muscle quality (retention of type II fibre area) and increased muscle mass and strength; however, it is unknown how RE interacts, or interferes, with the molecular mechanisms responsible for

cellular senescence (41). Despite the theorized role of the mitochondria in aging (46) and age-associated muscle loss specifically (4), few studies have directly investigated the impact of acute or chronic RE on the mitochondria in both young and older adults (119). Nevertheless, data does exist that indicates that mitochondria are influenced by RE and further work is required to clarify the interactions of RE and the mitochondria in the context of sarcopenia.

Effects of resistance exercise on the mitochondria

Resistance exercise promotes muscle hypertrophy and increases strength irrespective of age (5); however, such beneficial effects are often considered independent of the mitochondria (41). It has been proposed that RE does not directly benefit mitochondrial function in young skeletal muscle; however, few studies have investigated the mitochondrial specific adaptations to the RE. Preliminary studies indicated that younger, highly-resistance trained populations (bodybuilders, powerlifters, and Olympic weightlifters) had reduced mitochondrial content and citrate synthase (CS) enzyme activity as compared to sedentary participants and endurance athletes (120-122). This data is far from conclusive, as the cross-sectional nature of the data cannot exclude the potential for genetic variability, or that those predisposed to lower mitochondrial content and function are biased towards resistance-based activities. In addition, the training practices and consequent adaptations of elite athletes may not represent what would occur in healthy active populations, and it is difficult to control for the potential confounding effects of anabolic steroid abuse in these populations (123).

Equivocal data from longitudinal, intervention-based studies confounds our understanding of the mitochondrial adaptations to RE in the young. Both Luthi et al (124) and MacDougall et al (121,125) found reduced mitochondrial content as detected from microscopic analysis of muscle cross sections following training. This is in agreement with data obtained in trained athletes and considerable data exists that indicates that the activity of various mitochondrial enzymes are not necessarily increased with RE, but rather maintained alongside increased muscle size (126-130). Conversely Tang et al (131) demonstrated increased activity of the mitochondrial enzymes CS and β-hydroxyacyl CoA dehydrogenase, as well as the cytosolic enzyme hexokinase but not phosphofructokinase alongside increased muscle fibre cross-sectional area. Consequently, studies that support a reduction in mitochondrial content tend to rely on microscopic evaluation of areas; whereas, those that rely on enzymatic function show either no effect (maintenance) or increased function in the young following training.

Acutely, RE stimulates mitochondrial protein synthesis alongside myofibrillar protein synthesis in young, untrained skeletal muscle (132). This effect depends on the untrained state of the participants, as mitochondrial protein synthesis is unaffected by acute exercise following training. There is a discrepancy between the acute mitochondrial protein synthetic response and transcriptional response to unaccustomed RE. Gordon et al (133) found that acute RE in untrained skeletal muscle suppressed expression of various transcripts related to oxidative function and that this effect was reduced in trained muscle. In addition, expression of PGC-1α, a transcriptional co-activator that has been termed a master regulator of mitochondrial biogenesis (134), increases following acute and chronic

RE (135,136). The discordance between the mitochondrial protein synthetic and transcriptional responses in the literature necessitate further research of the acute mitochondrial response to RE in the skeletal muscle of young individuals.

It has been hypothesized that the mitochondrial effects of RE may be enhanced in elderly skeletal muscle (41), or that such beneficial effects require a certain degree of basal mitochondrial dysfunction to be present. This is supported by the simple fact that many studies that show a lack or negative effect of RE on the mitochondria were performed on young subjects (120-122,124,125,133), whose mitochondria would be relatively devoid of mutations and deletions. While specific age comparisons are sparse, Tarnopolsky et al (119) did find that elderly subjects increased citrate synthase protein content following four months of resistance exercise, while no change was detected in the younger subjects. Despite the lack of direct aged comparisons, investigations of aged muscle have shown elevated enzyme activity (137), mitochondrial creatine kinase (mtCK) protein content (137), improved creatine recovery kinetics (138) and increased mitochondrial and metabolic transcript abundance (62) following chronic RE in the elderly. Contrary to the young (120,121,125), mitochondrial volume density is increased in the elderly following RE, even alongside muscle hypertrophy (138). Of additional benefit in the context of the mitochondrial theory of aging, RE elevates antioxidant enzymes, and results in lower oxidative stress in the elderly (137,139). While VO_2max depends on factors in addition to skeletal muscle oxidative capacity, the fact that VO_2max and endurance capacity are increased in the elderly with RE indirectly supports the notion that RE may impact mitochondrial function in the elderly (140,141).

Collectively, these works suggest that RE can impact some aspects of the mitochondria, regardless of age; however, such a response may be heightened in tissues with a certain degree of mitochondrial dysfunction. Further research is required to elucidate how studies that rely on mitochondrial volume measurements tend to support a lack of effect, or negative effect relative to cell size of RE (120,121,125), whereas, those that focus on measurements of oxidative function and capacity show a net benefit of RE training.

Potential therapeutic effects of resistance exercise on the mitochondrial genome in aging

In addition to the previously observed effects of RE on mitochondrial content and function, mtDNA genome shifting has been theorized as an important therapeutic adaptation to RE in aged muscle (41). The concept of mtDNA genome shifting was first discovered in patients with mitochondrial myopathies who harbor heteroplasmic ratios of wild-type and mutant mtDNA (142). In an evaluation of the regenerative capacity of muscle derived from a patient with a specific mtDNA tRNA mutation, Shoubridge et al (142) discovered a near complete reversion of the heteroplasmy ratio in the previously biopsied region, and that cultured myoblasts were nearly devoid of the accumulation of the mutation that was evident in the originally extracted muscle tissue. Clark et al (143) produced similar results, albeit with a slightly different model, demonstrating a reduction in skeletal muscle mtDNA heteroplasmy with bupivacaine-induced muscle necrosis (and subsequent regeneration) in a patient with an mtDNA encoded tRNA mutation.

As RE is a potent stimulation for the activation of satellite cells within skeletal muscle (22,144), Taivassalo et al (145) investigated if similar results could be obtained following a more tolerable treatment. A single patient with a tRNA mutation underwent a 27-day training protocol consisting of multiple sessions per day of various eccentric and concentric contractions at 75-110% of their initial maximal voluntary contraction (MVC). At the completion of training, a shift in mtDNA was detected, evidenced by a decreased proportion of COX-negative fibres and an increase in the percentage of wild-type mtDNA. Murphy et al (146) investigated a similar phenomenon, in a more substantial group of participants with a conventional RE program. After 12 weeks of training there was an increase in the percentage of centralized nuclei, increased expression of neonatal myosin heavy chain and NCAM positive satellite cells, suggesting the bout was effective at damaging the muscle and triggering an adaptive response. Despite this there was no change in the percentage of COX-positive fibres, however, there was a reduction in COX-deficient fibres and a corresponding (but non-significant) increase in COX-intermediate fibres. No change in mitochondrial volume, as determined by CS activity occurred, and a trend towards increased complex IV activity was found. Training did not influence mtDNA copy number, and while alterations in deleted mtDNA were directionally supportive of genome shifting, no changes were statistically significant.

Two studies have addressed the concept of mtDNA genome shifting in older adults following RE training. Following four months of RE training Parise et al (137) found increased complex IV activity and mtCK protein, with no change in full-length mtDNA. All the older adults displayed deletion products, however, no change was

detected with training. In a subsequent investigation, Tarnopolsky et al (41) found increased mitochondrial enzyme activity and reduced mtDNA deletions assessed with both long-range PCR and through an RT-PCR based detection of the ratio of ND1 to ND4. This data supports the notion that RE can reduce the mutational burden in mitochondria of elderly individuals; however, further research is required to conclusively identify the satellite cell as the source of the non-deleted mtDNA.

Interactions of aging, resistance exercise and mitochondrial cellular signaling pathways

In addition to mtDNA genome shifting, the cellular signaling pathways that stimulate mitochondrial biogenesis may be preferentially activated in elderly muscle, due in part to the energetic strain exercise produces in the presence of mitochondrial dysfunction in aging. Adenosine monophosphate activated protein kinase (AMPK), calcium/calmodulin dependent protein kinase (CAMK) and mitogen activated protein kinase (MAPK) p38 are sensitive to alterations in intracellular calcium (147), elevated reactive oxygen species (148) and alterations in the AMP:ATP ratio (149,150), all consequences of contractile activity (151). These signals converge to regulate the transcription of PGC-1α, a protein that then acts as a transcriptional co-activator and the activity of other transcription factors (nuclear respiratory factors (NRF) 1 and 2, estrogen related receptor alpha (ERR α), and myocyte enhancer factor 2 (MEF2)) to promote mitochondrial biogenesis (152,153). This culminates with the PGC-1α-mediated coactivation of NRF1 to promote the transcription of mitochondrial transcription factor a (Tfam). Tfam is integral to the process of mitochondrial biogenesis acting not only as a

transcription factor for mtDNA-encoded genes, but also regulates the maintenance and replication of mtDNA (154,155).

Drummond et al (94) found that elderly participants had elevated AMPK (Thr172) phosphorylation at one and three hours following a single RE bout; whereas, AMPK was unaffected in the young. The authors did not investigate mitochondrial variables, but rather found that this AMPK response was associated with a delayed increase in the fractional protein synthetic rate (anabolic resistance), although the cumulative synthetic response was ultimately similar between age groups. While AMPK abundance and function may be attenuated in aged muscle (48,149), the fact that an equivalent bout of exercise stimulates AMPK in the elderly suggests that resistance exercise may promote mitochondrial biogenesis via AMPK/PGC-1α preferentially. This is supported by work of Tarnopolsky et al (119) who found citrate synthase protein content was preferentially elevated in elderly participants who completed a four month resistance training program, with no response in the young. Unfortunately direct comparisons of the mitochondrial response to acute and chronic resistance exercise across age groups are limited in the literature.

Alternate roles for PGC-1α in the adaptation to resistance exercise

In addition to the central role of PGC-1α in the regulation of mitochondrial adaptations to exercise (134), recent data suggest it may also coordinate other cellular signaling pathways that may be stimulated following RE. The unfolded protein response (UPR) is an intracellular signaling cascade that acts to attenuate protein synthesis,

augment chaperone protein content in the endoplasmic reticulum (ER), and process the accumulated burden of unfolded protein in response to ER stress (156). Three UPR effector proteins mediate these cellular effects: activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 alpha (IRE1α) and protein kinase R-like ER protein kinase (PERK). In the resting state, the three UPR upstream effector proteins remain bound in the ER membrane to the chaperone protein glucose-regulated protein 78 (Grp78), alternatively known as Bip (157,158). In response to the accumulation of unfolded proteins in the ER, Grp78 dissociates from the ER effector proteins to assist the folding, assembly, export and degradation of ER proteins (159). ATF6 is cleaved and translocates to the nucleus to promote UPR gene expression (158,160,161), PERK phosphorylates and inhibits eIF2α to attenuate protein translation (162), and IRE1α regulates the splicing of XBP-1, which then acts as a transcription factor to increase expression of key UPR genes (161,163). Ultimately these pathways converge to alleviate the burden of misfolded proteins in the ER, and if unable to do so promote apoptosis through the PERK/ATF4-dependent transcription of the pro-apoptotic factor CHOP (164).

Wu et al (165) recently demonstrated that PGC-1α is central to the induction of the UPR following EE, and that compromising the UPR attenuates the adaptation to exercise. Such an effect is mediated by the specific interaction of PGC-1α with ATF6, and not additional UPR transcription factors such as XBP-1. In spite of this recent finding, data regarding the stimulation of the UPR with exercise in human skeletal muscle is lacking (166), and much of the literature regarding the UPR in skeletal muscle is in various myopathies and in response to age and nutritional challenges (156). Kim et al

(166) found, after a 200 km endurance race, ER stress was elevated as evidenced by increased abundance of spliced XBP-1, and specific increases in Grp78 but not PERK and ATF4. While not a direct investigation of the UPR, Gordon et al (133) performed a microarray analysis on the acute response to RE in trained and untrained muscle and found an enrichment of genes in GO term and KEGG pathway related to the response to unfolded proteins and unfolded protein binding. Such a response was attenuated in the trained as compared to the untrained muscle. These studies provide preliminary evidence that acute exercise induces ER stress and stimulates the UPR. In addition, as RE modulates various factors that could stimulate and induce the UPR including PGC-1α expression (133,135), oxidative stress (167), and elevated protein synthesis (94,168), it is possible that RE could induce the UPR as well (156,169).

In addition to varying transcriptional partners to regulate differential cellular adaptations, recent data suggests that PGC-1α has four alternatively expressed isoforms that vary in expression according to the stimulus provided to the cell (136). PGC-1α4 is purported to regulate cellular signaling pathways that would be central to the activation of satellite cells, and contribute to the repair, regeneration and growth in skeletal muscle. Conversely, PGC-1α1 appears to regulate, or at least influence the expression of predominately mitochondrial and metabolic transcripts, consistent with its well characterized role in the adaptation to EE (134). Ruas et al (136) demonstrated these precise roles with adenovirus mediated over-expression in myotubes, but also found that this relationship held true following chronic exercise in human skeletal muscle. PGC-1α4 was only increased with pure RE training, or mixed mode RE/EE training and not EE

only, supporting the idea that PGC-1α4 regulates muscle hypertrophy and the adaptation to strength training, whereas PGC-1α1 would increase in response to EE and not RE.

Together these studies demonstrate that while a common increase in PGC-1α occurs following both RE and EE, regulation of the transcription factors co-activated by PGC-1α (165), and the specific, alternate isoforms expressed post-exercise (136) could partially explain the differential adaptations that occur depending on the mode of exercise performed.

Purpose and hypothesis

The overall purpose of this thesis is to clarify how mitochondrial content is immediately affected by RE, accounting for potential changes to transcripts, protein, and mtDNA abundance, and how this process may differ in aged skeletal muscle as compared to young. It is hypothesized that older adults will have reduced basal mitochondrial content (50), thereby limiting the energetic capacity of skeletal muscle. Ultimately, deficits in mitochondrial function will result in elevated metabolic stress following a single RE bout as compared to the young and stimulate the activity of AMPK (150). Preferential activation of AMPK in the elderly will stimulate PGC-1α activity (170) and mitochondrial biogenesis (150) to a greater degree than the young.

RE is associated with elevated PGC-1α mRNA (133,135) in the presence of reduced mitochondrial and metabolic transcripts in untrained muscle (133). This suggests that potential PGC-1α activity may serve to modulate cellular processes other than mitochondrial biogenesis, such as the UPR (165), in the period immediately following a

single RE bout. The purpose of Study #2 is to clarify the ability of RE and the following stimulation of protein synthesis to induce ER stress and stimulate the UPR. It is hypothesized that aged muscle will have indications of UPR activation at rest (171), and that RE will increase ER stress and stimulate the UPR regardless of age.

Finally, while associations of PGC-1α with various specific transcription factors could explain how a common increase in PGC-1α occurs despite ultimately divergent phenotypic adaptations to either RE or EE, it is also possible that alternately expressed isoforms could contribute to this phenomenon (136). The purpose of Study #3 is to determine whether there is differential induction of PGC-1α isoforms following acute, unaccustomed RE or EE exercise. It is hypothesized that RE will increase expression of PGC-1α4, an isoform that regulates myostatin and insulin-like growth factor one (IGF-1) signaling and EE will predominately influence PGC-1α1, which controls predominately metabolic transcripts (136).

Scope and nature of this work

Impairments in mitochondrial function are associated with muscle atrophy (4) and are proposed to contribute to the aging process (46). While RE can increase muscle size, strength and function (91,92), effectively combating the deleterious effects of sarcopenia, the role of mitochondrial adaptations in the restoration of function remain unclear (41). Given the potential role of the mitochondria in the etiology of sarcopenia and aging in general, any favorable mitochondrial effects of resistance exercise could be of substantial benefit in the promotion of healthy aging.

It is currently unclear whether the mitochondria of aged muscle are more susceptible to RE than that of the young, as evidence supports mitochondrial effects in both groups depending on the measure evaluated. In addition there is a distinct lack of evidence regarding the immediate mitochondrial response to a single bout of RE. The purpose of Study #1 was to characterize the acute mitochondrial response to a single bout of resistance exercise in sedentary but healthy younger and older men to clarify whether a preferential response occurs with age.

The results of Study #1 indicated that acute RE was associated with increased PGC-1α mRNA (indicative of PGC-1α activity (152)) with a divergent decrease in mitochondrial transcript, protein abundance and DNA copy number. These results suggest that the post-exercise elevation in PGC-1α mRNA may be indicative of cellular adaptations independent of mitochondrial function in the period acutely following RE. In order to clarify this discrepant response, Study #2 investigated whether RE induced the UPR. Recent data has demonstrated that PGC-1α acts as a transcriptional co-activator for the UPR transcription factor ATF6 (165). It is possible then, that while EE and RE have a common increase in PGC-1α mRNA acutely, the binding partners that PGC-1α co-activates to affect transcription are different depending on the exercise mode. In addition to altering PGC-1α, RE may stimulate the UPR by increasing protein synthesis and oxidative stress acutely (94,167), or through altered calcium dynamics (156,172).

Finally, Study #3 addresses recent data indicating that there are alternatively spliced isoforms of PGC-1α, and that these are differentially regulated in response to chronic resistance, endurance or mixed modes training (136). PGC-1α4 was shown to

regulate muscle hypertrophy through regulation of insulin-like growth factor 1 (IGF1) and myostatin, where PGC-1α1 was related to mitochondrial and metabolic adaptations. The acute response of these isoforms to RE or EE in human skeletal muscle has not been characterized. Given the fact that Study #1 found increased PGC-1α mRNA alongside reductions in mitochondrial content suggests that PGC-1α4, but not α1 are induced by acute resistance exercise. Consequently, Study #3 compared the acute effects of a single bout of either RE or EE on the abundance of alternatively spliced isoforms of PGC-1α and related target genes.

Paper 1: Effects of age and acute resistance exercise on mitochondrial transcript and protein abundance in young and old skeletal muscle

Effects of age and acute resistance exercise on mitochondrial transcript and protein abundance in young and old skeletal muscle

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Running head: The acute mitochondrial response to resistance exercise

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Abstract

Mitochondrial dysfunction may contribute to age-associated muscle atrophy. Previous data has shown that resistance exercise (RE) increases mitochondrial gene expression and enzyme activity in older adults; however, the acute response to RE has not been well characterized. To determine the acute mitochondrial response to RE, healthy young (21±3 y) and older (70±4 y) adults performed a unilateral RE bout for the knee extensors. Muscle biopsies were taken at rest and 3, 24 and 48 h following leg press and knee extension exercise. The expression of the mitochondrial transcriptional regulator PGC-1α mRNA was increased at 3 h post-exercise; however, all other mitochondrial variables decreased over the post-exercise period, irrespective of age. ND1, ND4 and CS mRNA were all lower at 48 h post-exercise, along with specific protein subunits of complex II, III, IV and ATP Synthase. Mitochondrial DNA copy number decreased by 48 h post-exercise, and mtDNA deletions were greater in the older adults and remained unaffected by acute exercise. Elevated mitophagy could not explain the reduction in mitochondrial proteins and DNA, as there was no increase in ubiquitinated VDAC or its association with Pink1 or Parkin, and elevated p62 and total LC3b content indicated an impairment or reduction in autophagocytic flux. In conclusion, age does not influence the acute mitochondrial response to a single bout of RE. Further work is required to clarify the divergent mitochondrial response to acute and chronic RE.

Keywords: Resistance exercise, mitochondria, autophagy, skeletal muscle

Introduction

Aging is characterized by a progressive muscular atrophy (sarcopenia) that results in significant functional impairments and reductions in quality of life for the elderly. Sarcopenia (age-associated muscle loss below two standard deviations from age-matched controls) affects up to 15% of individuals over 65 years of age (39) and is a substantial financial burden on the health care system (25). Alongside the progressive loss of muscle mass, strength decreases with age (31, 33), and this reduced strength is predictive of falls (53) and disability (23). Consequently, treatments that act to mitigate the age-associated loss of muscle mass and strength can have a profound impact on daily life in the elderly and contribute to a healthy, active aging process.

Sarcopenia has a complex etiology thought to involve multiple processes including the loss of motor units and denervation of muscle (14), impaired protein synthesis and turnover (3, 39, 67), altered systemic inflammation (39, 47), oxidative stress (19, 25) and mitochondrial dysfunction (8, 9, 31, 33, 63). Of those, mitochondrial dysfunction has been suggested as a primary contributor to the sarcopenic phenotype of skeletal muscle (53, 58) and may play a role in the additional causative factors of aging including telomere shortening and DNA damage (52). The mitochondrial theory of aging suggests that elevated free radical production over time results in the accumulation of damage to the mitochondria and its associated components (DNA). This ultimately exacerbates electron transport chain function, resulting in additional free radical production and oxidative stress, creating a vicious cycle of oxidative damage (23, 24). This theory is supported by the accumulation of oxidative damage in aged muscle (14, 16,

19, 37, 43, 44) and the gradual attenuation of mitochondrial function (3, 11, 67), protein synthesis (49) and transcript abundance (38, 65, 66) with increasing age. The occurrence of ragged red fibres, cytochrome c oxidase deficiency, and those with elevated succinate dehydrogenase activity increase with age (9), in association with mitochondrial DNA (mtDNA) deletions in aged muscle (8, 10). Relevant to the concept of sarcopenia, these regions of mitochondrial abnormalities are associated with atrophic regions of the myofibre (9, 63), suggesting a link between mitochondrial dysfunction and muscle atrophy. Resistance exercise (RE) is commonly used to promote the retention or elevation of muscle mass and strength in aging (57); however, despite the potential contribution of the mitochondria to the aged phenotype, the acute effects of RE on the mitochondria have not been thoroughly considered in the early stages of the adaptation to exercise.

It is generally thought that RE does not benefit mitochondrial function in young skeletal muscle (2, 59); however, mitochondrial protein synthesis and enzyme activities increase acutely following RE (54, 68), and peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) expression, a master regulator of mitochondrial biogenesis (71), is elevated following both acute (12) and chronic strength training (50). It has been proposed that mitochondrial adaptations to RE may be heightened in aged muscle (58). At the functional level, RE can increase VO_2max (62) in the elderly and result in improved endurance capacity (1), adaptations typically associated with endurance exercise. Melov et al (38) demonstrated that the aged transcriptome has a high number of differentially expressed mitochondrial and metabolic-related genes; however, following six months of RE the aged transcriptome more closely resembled that of young

skeletal muscle. Perhaps more reflective of actual mitochondrial function/capacity, mitochondrial creatine kinase protein content and complex IV enzyme activity are increased with RE (45). Also of relevance to the mitochondrial theory of aging, RE over four months reduced oxidative stress and increased antioxidant enzyme activity in aged skeletal muscle (46). Collectively these data indicate that mitochondria are sensitive to RE in older adults; however, the extent to which RE can promote favorable mitochondrial signaling in aged and young skeletal muscle is yet to be clearly defined.

Strength training is a safe and effective therapeutic intervention to maintain or increase muscle mass and strength in the elderly (17, 38, 60, 61); however, the role of mitochondrial adaptations in the preservation of function is not clear. Furthermore, direct comparisons of the acute mitochondrial response to RE in young and aged skeletal muscle are lacking and require further clarification. We hypothesized that older adults would have reduced mitochondrial content, thereby limiting mitochondrial function resulting in elevated metabolic stress from a relative RE bout as compared to the young. This would trigger an enhanced mitochondrial bioenergetic response to RE, the result of an elevated AMP-activated protein kinase (AMPK)/PGC-1α signaling response (26) indicative of acute exercise-induced energy crisis (15).

Materials & Methods

Subjects

Eighteen community-dwelling younger (YM; n=9, 21±3 y) and older men (OM; n=9, 70±4 y) were recruited to complete a single RE bout. Participants underwent a

routine screening prior to the study and were required to complete a health questionnaire and to have not been involved in a lower-body RE program for at least six months prior to the study. Exclusion criteria were as previously described (36); evidence of heart disease, respiratory disease, uncontrolled hypertension, renal disease, diabetes, orthopedic disabilities involving the lower limb, the use of NSAIDs or statin-related drugs and smoking. Participants were instructed to refrain from physical activity during the study period and to abstain from the consumption of alcohol and non-steroidal anti-inflammatories. All participants gave written, informed consent prior to participation. This study was approved by the Hamilton Health Sciences Human Research Ethics Board and conformed to all declarations on the use of human subjects as research participants.

Acute Exercise Protocol

Participants arrived to the clinic at 0600h and performed a unilateral RE program for the knee extensors, as previously described (15, 35, 36). Initially each subject completed an incremental, unilateral one repetition maximum (1RM) protocol for the leg press and knee extension exercises. Following determination of the 1RM, subjects completed four sets of ten repetitions of each exercise at 75%-1RM with two min rest between each set. The order of exercise was randomized such that half of the participants commenced the exercise bout with the leg press, the other half with the leg extension exercise.

Muscle Biopsies

Three hours following the exercise protocol a muscle biopsy was obtained from the unexercised and exercised leg, followed by subsequent biopsies of the exercised leg only at 24 and 48 h following the exercise bout. A percutaneous needle with manual suction was used to biopsy the *vastus lateralis*, and each biopsy was spaced in a randomized order (distal, middle, proximal) with approximately 3 cm between adjacent biopsy sites (56). Each biopsy was sectioned into smaller portions then immediately frozen in liquid nitrogen for storage at -80^{o}C for future use.

RNA Analysis

RNA was extracted from 30 mg of quadriceps tissue in 1 mL of Trizol Reagent (Invitrogen, Burlington, ON, Canada). Following electric homogenization 0.2 mL of chloroform was added per sample and the resultant clear aqueous phase was transferred to RNeasy spin columns (Qiagen, Germantown, Maryland, USA). Following these steps RNA was isolated as per the manufacturer's recommendations. To prevent contamination with genomic DNA, all RNA samples were treated with DNAase (Qiagen) while on the isolation columns. Concentration of the RNA was spectrophotometrically determined by measuring the absorbance of the solution at 260 nm (ND-1000, Nanodrop, Willmington, DE, USA).

Reverse transcription was performed on 100 ng total RNA with random hexamers as per the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with gene specific primers on the 7300 real-time PCR System

(Applied Biosystems, Foster City, CA, USA) using SYBR® Green chemistry (PerfeC$_T$a SYBR® Green Supermix with ROX, Quanta Biosciences, Gaithersburg, MD, USA). The primers used were as follows: β2-microglobulin forward: 5'-acttgtctttcagcaaggactg, reverse: 5'-ttcacacggcaggcatact, ND1 forward: 5'-aagtcaccctagccatcattctac**,** reverse: 5'-aagtcaccctagccatcattctac, ND4 forward: 5'-actgggagaactctctgtgctagt, reverse: 5'-atgtagagggagtatagggctgtg , Citrate Synthase (CS) forward: 5'-gagcagggtaaagccaagaat, reverse: 5'-cccaaacaggaccgtgtagt, PGC-1α forward: 5'-catcaaagaagcccaggtaca, reverse: 5'-ggacttgctgagttgtgcatac, PGC-1α1 forward: 5'- atggagtgacatcgagtgtgct, reverse: 5'-gagtccacccagaaagctgt, PGC-1α4 forward: 5'- tcacaccaaacccacagaga, reverse: 5'-ctggaagatatggcacat, PGC-1β forward: 5'-cctgtttatgcctccctcac, reverse: 5'-ggtgaagctgcgatccttac, PGC-1 related coactivator (PRC) forward: 5'-cctaccaaggtggaggt, reverse: 5'-agccttcatctggggacttt , mitochondrial transcription factor a (Tfam) forward: 5'-tgtgcaccggctgtgg reverse: 5'-tggacaacttgccaagacagat , nuclear respiratory factor 1 (Nrf1) forward: 5'-gagtgatgtccgcacagaacag, reverse: 5'-ttataacagtttttaactatggtcgct, Nrf2 forward: 5'-tggtccacagaccaagtcct, reverse: 5'-ccgaaatgttgagtgtggtg , Nix forward: 5'-tggaacacgtaccatcctcat, reverse: 5'tgagaactgcctctggaactac; Beclin 1 forward: 5'-caggaactcacagctccattac, reverse: 5'-tggctcctctcctgagttag; p62 forward: 5'-ggaacagatggagtcggataac, reverse: 5'-tagggactggagttcacctgta; Light Chain 3 (LC3) forward: 5'-gcagcttcctgttctggataa, reverse: 5'-gcctgattagcattgagctgta; Autophagy-related protein 7 (ATG7) forward: 5'-gtgttggagattggttcctact, reverse: 5'-acccatcaacgtcctagctaca; and BCL2 adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) forward: 5'-gcatgagtctggacggagtag, reverse: 5'-ttcagaagccctgttggtatc. All data is expressed relative to

β2-microglobulin, which did not alter expression in response to the exercise bout (data not shown) (34).

Protein Analysis

Approximately 30 mg of each muscle biopsy was homogenized with an electric tissue mincer (Pro Scientific, Oxford, CT, USA) and processed into nuclear and cytosolic fractions. Protein concentration was determined using the bicinchonic acid method as per the manufacturer's recommendations (Pierce, Rockford, IL, USA) with a spectrophotometer (Benchmark Plus, Biorad, Hercules, CA, USA). Proteins were probed using various conditions depending on the protein using the following antibodies: OXPHOS Cocktail (Abcam ab110411, Toronto, ON, Canada), ATG7 (Cell Signaling Technology #8558, Boston, MA, USA), LC3b (Sigma L7543, Saint Louis, Missouri, USA), p62 (Cell Signaling #5114), VDAC (Abcam ab15895), Ubiquitin (Santa Cruz sc-8017, Dallas, TX, USA), Pink1 (Cell Signaling # 6946), Parkin (Cell Signaling #4211), phospho-AMPKα (Thr172, Cell Signaling #2531) and total AMPKα (Cell Signaling #2532). All proteins were probed under similar conditions with primary antibodies (overnight incubation at four degrees Celsius) followed by a one-hour incubation at room temperature with anti-rabbit or anti-mouse secondary antibodies (1:10000, GE Healthcare). All blots were developed with ECL plus (GE Healthcare) and exposed to x-ray film (GE Healthcare). All films were digitized and band density was determined with ImageJ (NIH, Bethesda, Maryland, USA). Protein data is expressed relative to lactate dehydrogenase A (LDHA; Cell Signaling #2012), protein disulfide isomerase (PDI;

Abcam ab2792) protein content or Ponceau S staining (mitochondrial blot) all of which were stable with both age and exercise (data not shown).

Immunoprecipitation

For immunoprecipitation, subject samples were combined into 3 pools of 125 μg for both young and old subjects at each time point (24 total samples) ensuring that equal proteins amounts were used from each subject. Cytosolic protein lysates were initially solubilized with 1% n-dodecyl maltoside in equivalent volumes for 30 min on ice and then spun in a centrifuge at 16,000 x *g* at 4°C. Concurrently, antibody to bead coupling was performed by adding 20 μL of protein A/G plus agarose beads to spin columns (Pierce), washing twice with PBS, then additionally washing twice with 200 μL of wash buffer (PBS/0.05% n-dodecyl maltoside with protease inhibitors (Roche)). The columns were then spun to remove residual wash solution and incubated with 2 μg of anti-VDAC antibody (Abcam, ab14734) in PBS for 2 h at 4°C to conjugate the antibody to the beads. After two additional washes in wash buffer, the solubilized cytosolic lysate was added to the columns and allowed to rotate end-over-end overnight at 4°C with the beads. The following morning, the columns were spun to collect the proteins not bound to the beads (IP supernatant). After collecting the supernatant, the beads were washed twice with 200 μl of wash buffer and incubated with 50 μl of 1% SDS and spun to elute any immunoprecipitated proteins. All spins were done at 3,000 rpm for 1 min at 4°C. The immunoprecipitates were run on gels using SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed using anti-Ubiquitin (Santa-Cruz, sc-8017), anti-Parkin (Cell Signaling #4211) and anti-Pink1 (Cell Signaling # 6946) to detect the

amount of Parkin and PINK1 associated with VDAC and the amount of ubiquitinated VDAC according to the SDS-PAGE methods. No signal was present when using an IgG control antibody for immunoprecipitation.

Mitochondrial DNA

Total genomic DNA was isolated from approximately 20 mg of skeletal muscle as per the manufacturer's specifications (DNeasy®, Qiagen, Toronto, ON, Canada). The concentration and purity of the DNA were assessed using a spectrophotometer (Nanodrop-1000, Thermo Scientific, Wilmington, DE, USA). MtDNA copy number was assessed with primers designed for ND1 (forward: 5'-aagtcaccctagccatcattctac, reverse: 5'-aagtcaccctagccatcattctac) in the mitochondrial genome and considered relative to the nuclear gene β-globin (forward: 5'gggcagagccatctattgctt, reverse: 5'-gacagatccccaaaggactcaaa) using an ABI 7300 thermocycler (Invitrogen, Burlington, ON, Canada) as previously described (51). MtDNA deletions were assessed as the ratio of ND1 to ND4 DNA content (ND1 (as above); ND4 Forward: 5'-actgggagaactctctgtgctagt. reverse: 5'-atgtagagggagtatagggctgtg determined via real-time PCR.

Statistical Analysis

Statistical analysis was performed using Statistica (Statsoft, Tulsa, OK, USA). A two-way, repeated-measures ANOVA was performed (age, time) and statistical significance was accepted at $p<0.05$. Significant effects and interactions as indicated in the ANOVA were further analyzed using Tukey HSD post-hoc test. T-tests were used to

test for differences in anthropometric, functional, and mtDNA deletions between the age groups. All results are reported as mean ± SD.

Results

Subject Characteristics

Subject characteristics have been previously published for the groups used in the present study (36). While there was no difference in lean-body mass between the groups (O: 62±6.4, Y:65.9±10.8 kg), the older men (age 70±4 y) were significantly weaker, scoring lower on the leg press (O:160.8±50.1, Y: 228±93.8 lbs; $p<0.05$) and leg extension (O: 117.6±47.4, Y: 81.9±22.8 lbs; $p<0.05$) one repetition maximums, and isometric knee extension MVC (O: 228.3±45.1, Y: 324.6±85.4 Nm; $p<0.05$), than the younger participants (age 21±3 y) (36).

Mitochondrial Response to RE

Neither exercise nor age altered Thr172 phosphorylation of AMPKα (data not shown). Exercise, but not age, altered PGC-1α and PGC-1β mRNA ($f=38.68$, $p<0.001$ and $f=18.55$, $p<0.001$ respectively; Fig 1). PRC mRNA did not differ with age or exercise; however, the age effect did approach significance ($f=3.83$, $p=0.072$). PGC-1α mRNA was increased 3 h post-exercise (407% of rest sample, $p<0.001$), but returned to basal levels by 24 h. Conversely, expression of PGC-1β was below resting levels at all post-exercise time points (68% $p=0.0051$, 67% $p=0.002$, 35% $p=0.00017$ of rest at 3, 24 and 48 h). Two specific alternative isoforms of PGC-1α were differentially affected by

RE (Fig 2). There was no effect of age on either transcript; however, exercise decreased PGC-1α1 ($f=5.82$, $p=0.002$) and increased PGC-1α4 ($f=25.76$, $p<0.001$; Fig 2). PGC-1α1 was progressively decreased to 20% of resting expression at 48 h post-exercise whereas PGC-1α4 was increased at 3 h ($p<0.01$). PGC-1α4 also decreased substantially by 48 h but was not statistically significant.

Exercise but not age increased Tfam mRNA ($f=7.98$, $p=0.003$ Fig 3). Tfam mRNA increased to 124% of resting levels at 24 h post-exercise ($p<0.05$). There was an effect of age and a trend for exercise on NRF1 (age: $f=5.97$, $p=0.0296$; exercise $f=2.26$, $p=0.096$) and only exercise for NRF2 mRNA ($f=3.96$, $p=0.0137$) mRNA (Fig 3). NRF1 mRNA was 36.6% higher in the older men, and both NRF1 and 2 mRNA were reduced at 48 h post-exercise (74% n.s. and 70% $p<0.01$ respectively).

Based on the differential regulation of the mRNA for mitochondrial transcription factors, we tested to see if there was also difference in the abundance of either mitochondrial or nuclear encoded mitochondrial-related transcripts (Fig 4). Age did not influence CS mRNA (nuclear encoded); however, 48 h post-exercise (CS: $f=6.12$, $p=0.0014$) CS mRNA was 68% of resting values ($p=0.08$). ND1 and ND4 mRNA were lower with age (ND1 $f=5.07$, $p=0.04$; ND4 $f=5.92$, $p=0.02$) as the transcripts were 38% and 29% higher in the young. Exercise decreased ND1 and ND4 mRNA (ND1: $f=3$, $p=0.04$ ND4: $f=13.8$, $p<0.001$), such that both were lower relative to the resting values by 48 h post-exercise ($p<0.05$ and $p<0.001$ respectively).

Consistent with the reduced mRNA abundance, the protein content of complex IV subunit II ($f=4.67$, $p<0.01$), the 30 kDa subunit of complex II ($f=16.3$, $p<0.001$), complex III subunit core 2 ($f=21.5$, $p<0.001$) and the alpha subunit of ATP Synthase ($f=31.7$, $p<0.001$) were reduced 48 h post-exercise (76%, $p=0.071$; 80%, $p<0.001$; 78%, $p<0.001$; and 80% of rest, $p<0.001$ respectively; Fig 5), with no effect of age or exercise on complex I content (data not shown). Complex II was the only subunit affected by age ($f=4.79$, $p=0.048$), being 11% higher in young skeletal muscle ($p=0.049$, data not shown). Mitochondrial DNA deletions were higher in the older adults at rest (15% higher, $p=0.01$; Fig 6a); however, this ratio was unaffected by exercise (Fig 6b). Consistent with the mRNA and protein results, mtDNA copy number decreased over the post-exercise period ($f=3.25$, $p=0.032$; Fig 6c) to 68% of resting levels by 48 h ($p=0.03$), irrespective of age.

Autophagocytic Response to RE

We found no effect of age or exercise on the expression of ATG7, Beclin or BNIP3 while LC3b mRNA was increased ($f=3.95$, $p=0.014$ Fig 7) at 3 h post-exercise (134% of rest, $p=0.012$), with a trend towards increased expression at 24 h (127% of rest, $p=0.057$). In addition, p62 mRNA was increased ($f=12.21$, $p<0.001$) at 3 h post-exercise (133% of rest, $p=0.003$). Beclin 1 (data not shown) and VPS34 mRNA did not differ with either age or exercise. ATG7 protein content did not change with age or exercise. Total LC3b protein ($f=8.53$, $p<0.001$) trended to increase 24 h post-exercise (123% of rest, $p=0.076$) and was significantly increased at 48 h (144% of rest, $p<0.001$) independent of age (Fig 8). P62 protein did not differ by age but was affected by exercise ($f=7.856$,

$p<0.001$), increasing to 136% ($p<0.05$) and 178% ($p<0.01$) by 24 and 48 h respectively (Fig 7).

Mitochondria are targeted for autophagy through the ubiquitination of VDAC via Pink1 and Parkin and subsequent binding to LC3 via p62 or through the direct interaction of the outer mitochondrial membrane protein Nix with LC3 (29, 40, 64). Pink1 and Parkin protein content did not differ post-exercise in cytosolic fractions (data not shown), and Nix mRNA was not different from rest at any point post-exercise, ($f=4.2$, $p<0.01$); however, the mRNA content at 24 and 48 h was greater than the 3 h point (data not shown). No significant effect of age or exercise was detected for the ubiquitination of VDAC or its association with Pink1 and Parkin (Fig 9).

Discussion

Despite the positive mitochondrial and functional adaptations with long-term strength training (1, 45, 46, 50, 55, 58, 62), and the acute stimulation of mitochondrial protein synthesis (68) and enzyme activity (54), we found a distinct reduction in mitochondrial transcripts (ND1, ND4, CS, Nrf1, Nrf2), proteins (Complex II 30kDa subunit, ATP Synthase subunit alpha, Complex III subunit Core 2) and mtDNA 48 h following RE. While chronic RE is associated with the cumulative reversal of the age-related mitochondrial and metabolic transcriptional phenotype (38), our data suggests the acute mitochondrial-related transcriptional response to RE is one of reduced abundance, irrespective of age. In agreement with our data, Gordon et al (22) found an overall reduction in mitochondrial-related genes acutely following RE; however, the suppression

of oxidative genes was attenuated following 12 weeks of RE training. Collectively, these data indicate that the beneficial effects of RE on the mitochondria may require cumulative bouts of RE over periods greater than 12 weeks, and that the early response to an acute bout of exercise may not be representative or predictive of the ultimate training outcome or chronic steady state abundance of either mRNA or protein.

Despite the fact that the elderly participants had equivalent lean-body mass to the younger participants, there was evidence of reduced mitochondrial content and muscle dysfunction. We have previously characterized that the elderly individuals had reduced strength across both training exercises and maximal isometric knee extension torque (35, 36). In addition, the area of type II fibres was reduced relative to the young, despite preservation of type I fibre area. While many markers of mitochondrial content were similar across age groups, we did find reduced abundance of ND1 and ND4 mRNA, the 30kDA subunit of Complex II protein, and elevated NRf-1 mRNA and mtDNA deletions. Reduced mitochondrial content and increased mtDNA deletions are often considered hallmarks of aging process in skeletal muscle (27), and compensatory increases in Nrf-1 have been previously characterized with aging (7). While we cannot causally associate the age-related mitochondrial alterations in these individuals with type II fibre atrophy with age, evidence does suggest mitochondrial dysfunction in aging may be more prevalent in type II fibres (63). Nevertheless, the alterations in mitochondrial content with age, whether associated with muscle atrophy or not, did not appear to influence the acute mitochondrial response to RE in the elderly.

An increase in PGC-1α mRNA is often used as an indicator or predictor of consequent mitochondrial and metabolic adaptations to exercise (71); however, it appears that this is not the case for RE. Our data is in agreement with others by showing that, similar to endurance exercise (48), RE can increase PGC-1α mRNA (12). We cannot exclude the possibility that while PGC-1α mRNA is elevated, any related PGC-1α activity/function may serve to modulate other cellular processes such as the unfolded protein response (UPR) (69) and not mitochondrial biogenesis. Given the stimulatory effects of RE on skeletal muscle protein synthesis (15), it is possible that increased post-exercise protein abundance may stimulate the UPR in the endoplasmic reticulum (13), a process modulated by the interaction of PGC-1α with the UPR-transcription factor ATF6 (69). In addition, recent work has detected alternate PGC-1α isoforms that are differentially expressed dependent on the exercise modality (50). In a series of experiments Ruas et al (50) demonstrated that the PGC-1α4 isoform is specifically increased following either chronic resistance or mixed mode resistance/endurance exercise and correlates with the percentage change in the performance of the leg press exercise. This specific PGC-1α isoform does not regulate the commonly reported mitochondrial targets, but rather regulates IGF1 and ultimately represses myostatin to have a favorable affect on muscle mass. In agreement, we have established that the preferential expression of PGC-1α4 occurs with acute RE as well, and that PGC-1α1 is decreased following acute RE. In addition, we have previously shown a reduction in myostatin mRNA (36) in the same participants used in the present study. Ultimately, the fact that we found increased PGC-1α mRNA coincident with reduced mitochondrial

transcripts, protein and DNA content further suggests that differing PGC-1α isoforms are induced dependent on the mode of exercise or that PGC-1α coactivates differing transcription factors following resistance or endurance exercise (ATF6 (69) as opposed to NRF1 and NRF2 (70)). Such a relationship may be graded in nature or temporally staggered, as we did find an elevation in Tfam mRNA 24 h post-exercise, indicative of NRF1 coactivation by PGC-1α (70).

Along with the transcriptional down-regulation of mitochondrial transcripts (22), it is possible that an alternate cellular process modulated the reduction in mitochondrial protein and DNA content. Autophagocytic removal of mitochondria from the cell can occur through the bulk removal of organelles via macroautophagy but also through a targeted mechanism known as mitophagy (64). While our understanding of selective autophagy is emerging, targeting of the mitochondria to the lysosome can occur through two mechanisms (29). In the first, the mitochondrial outer membrane protein VDAC is ubiquitinated by E3-ubquitin ligase Parkin, which is targeted to dysfunctional mitochondria by the accumulation of Pink1 on the outer mitochondrial membrane (40). The poly-ubiquitin binding protein p62 then binds to ubiquitinated VDAC on the mitochondrial outer membrane and links the mitochondria to the lysosome through an interaction with LC3 (5). Alternatively, the mitochondrial outer membrane protein Nix is able to interact with LC3 directly to target mitochondria to the lysosomal membrane (29). Our immunoprecipitation data found no increase in the ubiquitination of VDAC post-exercise or its association with Pink1 and Parkin, suggesting that this pathway is not stimulated by RE, or that this occurs at a different post-exercise time-point than those

studied in the current study. In addition, we failed to detect any change in Nix mRNA indicating mitophagy is not responsible for the reduction in mitochondrial protein and DNA abundance post-exercise, although we cannot rule out a post-transcriptional role of Nix in the regulation of mitochondria following RE. Future studies will require the use of electron microscopy (the gold standard) to conclusively rule out a role for macroautophagy as an explanation for the acute reduction in mitochondrial protein abundance and mtDNA content following RE.

Few studies have investigated the effects on autophagy or mitophagy specifically and previous data is equivocal on the induction of autophagy with either acute (18, 21) or chronic RE (32) or exercise in general (30, 42). At the induction of autophagy, LC3b-I is conjugated by a complex of ATG proteins to the lipophilic phosphatidylethanolamine to generate LC3-II that can readily integrate into autophagosome membranes and therefore the ratio of LC3b-II to I is a marker of the stimulation of autophagy (4). Both Fry and Glynn et al (18, 21) have previously demonstrated either no change or a decrease in the LC3b-II/LC3b-I ratio following RE, suggesting either an inability of RE to activate or that RE inhibits autophagy. Conversely Luo et al (32) demonstrated increased ATG7, ATG5 and Beclin 1 protein with reduced p62 and a lower LC3b-II/I ratio following nine weeks of RE. These discrepant findings may be explained as differences between the acute (18, 21), and chronic response to RE (32), or the use of young adults or aged rodents.

Similar results have been obtained using acute treadmill exercise in murine and rodent skeletal muscle. Ogura et al (42) found a biphasic response of LC3b-II in cardiac

muscle, and found a strong negative correlation (r=0.79) between phosphorylation of serine 2448 of mTOR and LC3b-II post-exercise. As mTOR is a potent inhibitor of autophagy (28), and RE increases phosphorylation of serine 2448 of mTOR (15), it is possible that the acute post-exercise protein synthetic demand outweighs any stimulus for autophagy and this relationship may shift over the course of training. The control of mTOR may be central to the divergent acute and chronic autophagocytic responses, as an attenuated response of mTOR or its downstream targets has been observed with training (41) that could create a permissive autophagocytic environment in contrast to that of acute exercise. In addition, Kim et al (30) demonstrated a simultaneous reduction in multiple markers (LC3b-II, LC3b-I, ATG7, Beclin1, LAMP2a) of autophagy over the initial 12 h following a single bout of treadmill exercise. Our data is in agreement with previous work (18, 20), and indicates that acute RE either fails to activate or actively suppresses autophagy, such that accumulations in total LC3b and p62 (5, 6) are detected 48 h after RE. The results of Luo et al (32) indicate that, as suggested for the mitochondrial response, the acute autophagocytic response to RE may not be equivalent to the cumulative effect of training, and further studies are required to clarify the response of autophagy, mitophagy, and the mitochondria to chronic RE.

Conclusion

This work demonstrates that, in spite of older adults having mtDNA deletions, age does not influence the acute mitochondrial response to RE. Furthermore, while long-term strength training may promote favorable mitochondrial adaptations in aged skeletal muscle (1, 45, 46, 50, 55, 58, 62), the immediate post-exercise response indicates reduced

mitochondrial protein, transcript and mtDNA abundance, that may be related to preferential induction of specific PGC-1α isoforms (22). This suggests that the favorable long-term mitochondrial response is dependent on cumulative bouts of RE, and that the response to an isolated bout is not indicative of the ultimate adaptation to training. Further work is required in the immediate hours following RE to determine whether reductions in mitochondrial content are the result of RE-stimulated macro or mitophagy, and how PGC-1α is differentially regulated to produce the divergent adaptations associated with either resistance or endurance exercise.

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Disclosures

The authors have no conflicts to declare.

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Figure Legends

Figure 1 Expression data indicates that RE had a stimulator effect on PGC-1α 3 h following RE, whereas expression of PGC-1b was reduced at all time points. † denotes $p<0.01$ and ‡ indicates $p<0.001$.

Figure 2 RE reduced expression of the PGC-1α1 isoform by 48 h post-exercise but increased expression of PGC-1α4 at 3 h post-exercise. † denotes $p<0.01$.

Figure 3 Expression of mitochondrial transcription factors varied over the post-exercise period. Tfam mRNA was elevated at 24 h post-exercise, while Nrf-2 was significantly reduced by 48 h and Nrf-1 did not change ($p=0.096$). * denotes $p<0.05$, † $p<0.01$ and ‡ $p<0.001$.

Figure 4 Both mitochondrial and nuclear encoded mitochondrial-related transcripts were reduced following exercise. Expression of ND1 and ND4 (mitochondrial encoded) were reduced 48 h post-exercise, whereas citrate synthase approached significance ($p=0.08$). * denotes $p<0.05$, † $p<0.01$ and ‡ $p<0.001$.

Figure 5 RE reduced the abundance of specific mitochondrial protein subunits (Complex II 30kDa subunit, ATP Synthase subunit alpha, Complex III subunit Core 2) 48 h after the exercise bout. * denotes $p<0.05$, † $p<0.01$ and ‡ $p<0.001$.

Figure 6 A: The ND1/ND4 ratio is higher in older adults at rest in skeletal muscle indicating greater mtDNA deletions. B: No changes were detected in the ND1/ND4 ratio

in the post-exercise period. C: MtDNA copy number (ratio of ND1 to β2-microglobulin) was reduced 48 h following RE with no effect of age. * denotes $p<0.05$.

Figure 7 RE differentially affected autophagy-related transcripts. ATG7 did not change following exercise; however, LC3b and p62 were increased at 3 h post exercise. * denotes $p<0.05$, † $p<0.01$ and ‡ $p<0.001$.

Figure 8 RE increased the abundance of total LC3b and p62 48 h after the exercise bout while ATG7 remained unchanged. * denotes $p<0.05$, † $p<0.01$ and ‡ $p<0.001$.

Figure 9 Immunoprecipitation of VDAC found there was no change in ubiquitinated VDAC or its association with PINK1 or Parkin followed the RE bout.

Figure 1

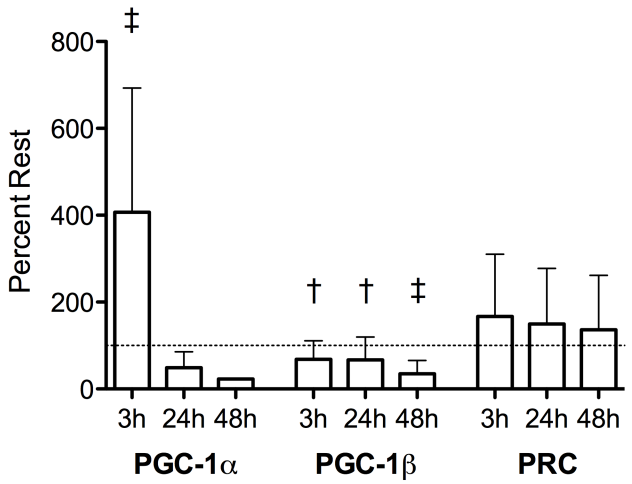
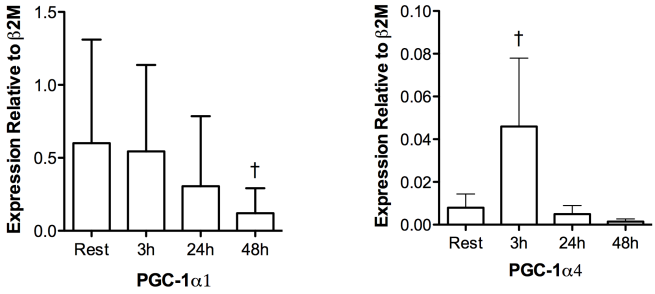


Figure 2



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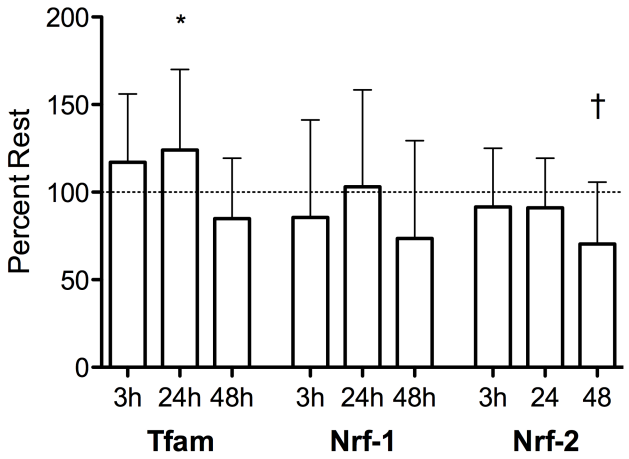


Figure 4

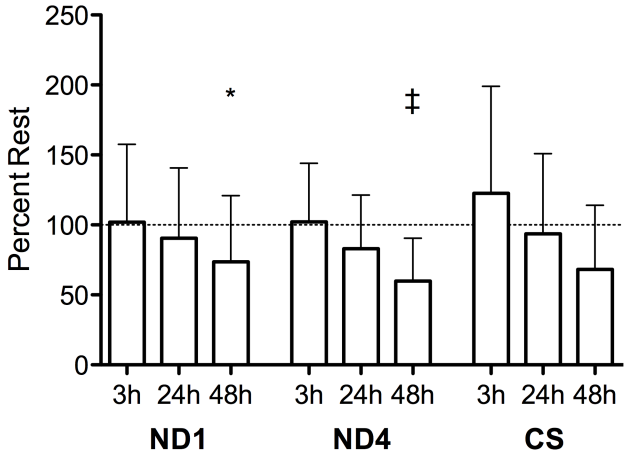


Figure 5

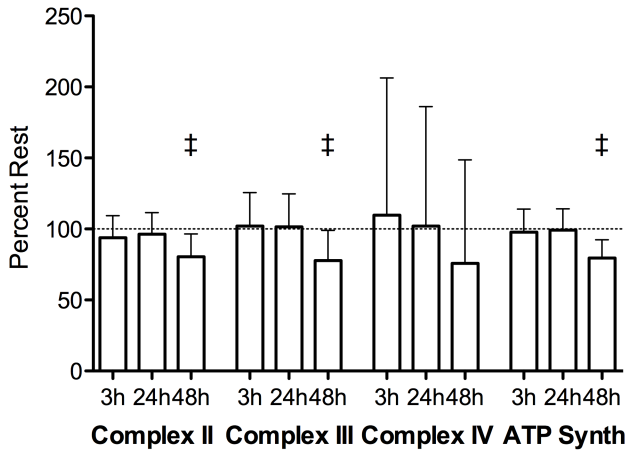


Figure 6

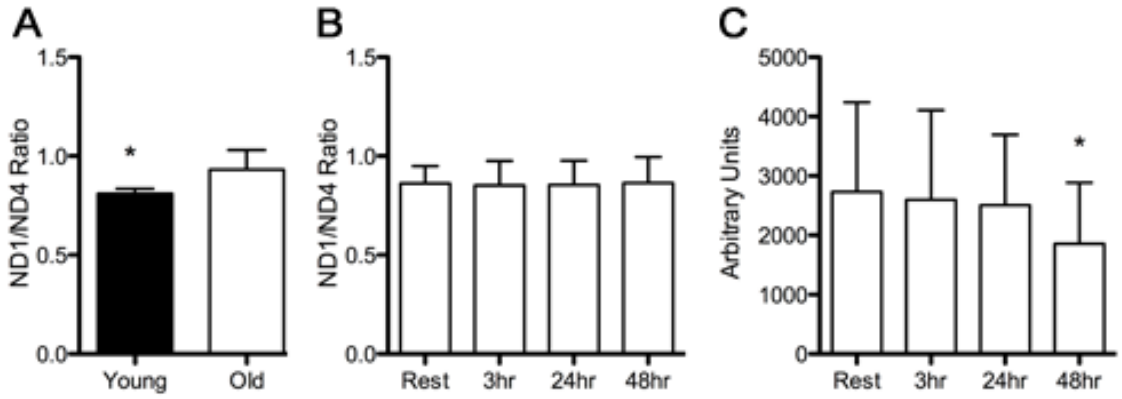


Figure 7

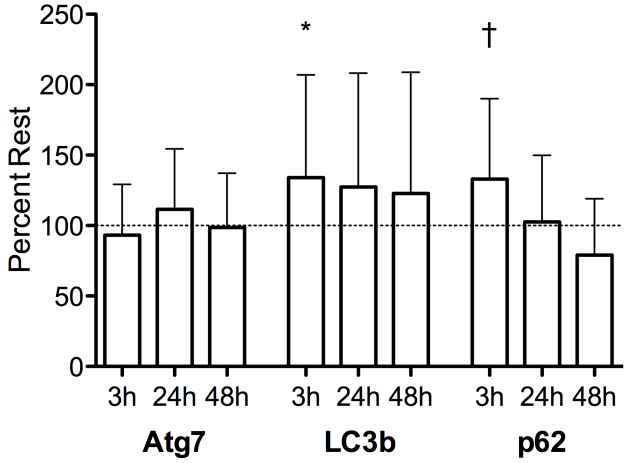


Figure 8

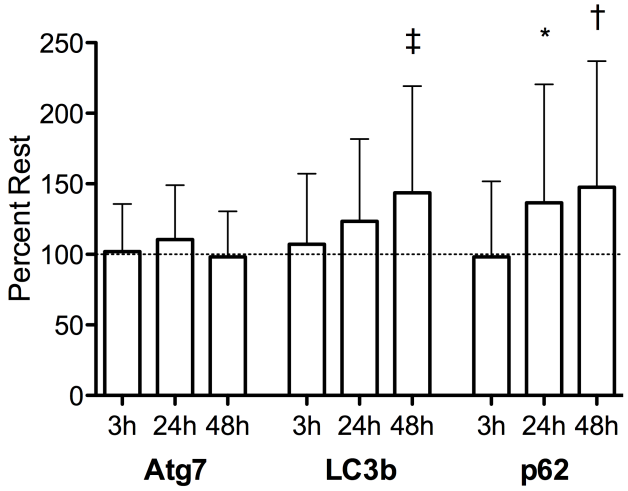
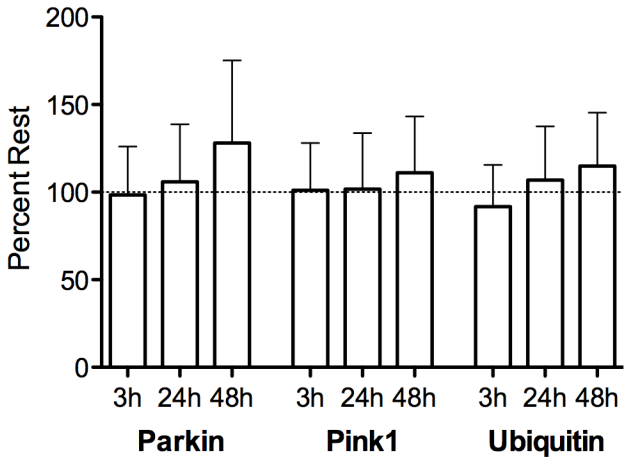


Figure 9


200
150
100
50
0
Percent Rest
3h 24h 48h
3h 24h 48h
3h 24h 48h
Parkin
Pink1
Ubiquitin

Paper 2: The unfolded protein response is triggered following a single resistance-exercise bout

The unfolded protein response is triggered following a single resistance-exercise bout

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Running Title: The unfolded protein response is activated by resistance exercise

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Abstract

Purpose: Endoplasmic reticulum (ER) stress results from an imbalance between the abundance of synthesized proteins and the protein folding capacity of the ER. In response, the cell activates the unfolded protein response (UPR), an intracellular cascade that restores ER function by attenuating protein synthesis and simultaneously inducing chaperone expression. Resistance exercise (RE) is known to acutely stimulate protein synthesis; however, an accumulation of unfolded proteins following exercise may activate the UPR. Aging may impair protein folding and the accumulation of oxidized and misfolded proteins may alter the UPR in resting or exercised aged muscle. **Methods:** Eighteen younger (Y; n=9, 21±3 y) and older (O; n=9, 70±4 y) men completed a single unilateral bout of RE using the knee extensors (4 sets of 10 repetitions at 75%-1RM on the leg press, leg extension) to determine if RE stimulates the UPR. Muscle biopsies were taken from the non-exercised and exercised vastus lateralis at 3, 24 and 48 h post-exercise. **Results:** Age did not affect any of the proteins and transcripts related to the UPR. GRP78 and PERK proteins were increased at 48 h post-exercise while IRE1α was elevated at 24 and 48 h. Despite elevated protein, GRP78 and PERK mRNA were unchanged; however, IRE1α mRNA was increased at 24 h post-exercise. ATF6 mRNA increased at 24 and 48 h while ATF4, CHOP and GADD34 mRNA were unchanged. **Conclusion:** This data suggests specific pathways of the UPR are activated by RE (ATF6, IRE1 α), while the PERK/eIF2α/CHOP pathway is not. In conclusion, acute RE results in UPR activation irrespective of age.

Keywords: Resistance exercise, unfolded protein response, aging, skeletal muscle, endoplasmic reticulum

Introduction

The endoplasmic reticulum (ER) is the intracellular organelle where various proteins are post-translationally modified and folded. Proteins that are properly folded continue transit to the Golgi for additional processing; however, under certain conditions an imbalance between the abundance of synthesized proteins and the folding capacity of the ER occurs, resulting in ER stress. In response to ER stress, the cell activates the unfolded protein response (UPR), an intracellular cascade that acts to attenuate protein synthesis while simultaneously processing accumulated misfolded proteins. The UPR operates through three effector proteins proximal to the ER that sense and respond to the presence of unfolded and misfolded proteins, activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 alpha (IRE1α) and protein kinase R-like ER protein kinase (PERK). In the inactive state, these three proteins remain bound in the ER membrane to the chaperone protein glucose-regulated protein 78 (GRP78), alternatively known as Bip (1,2). In response to the accumulation of unfolded and misfolded proteins, Grp78 dissociates from the three effector proteins to assist in the folding, assembly, export and degradation of ER resident proteins (3). Once released from Grp78, activated IRE1α splices X box-binding protein (XBP1) mRNA and the resultant transcription factor upregulates UPR target genes (4,5). ATF6 translocates to the Golgi apparatus where it is cleaved to its active form, and is shuttled to the nucleus to act as a transcription factor for UPR genes such as XBP1 and Grp78 (2,4,6). Finally, unbound from GRP78, PERK dimers phosphorylate eukaryotic initiation factor 2 alpha (eIF2α) to reduce protein translation while at the same time promoting the translation of transcripts specific to

activating transcription factor four (ATF4) (7). Ultimately, these pathways converge to alleviate the burden of unfolded proteins on the ER through a coordinate reduction in protein synthesis, increased production of chaperone proteins, the degradation of misfolded and unfolded proteins, and in the case of extreme ER stress, apoptosis (8).

Stimulation of the UPR has been observed in skeletal muscle of patients with myopathies (9,10), in mice but not humans fed a high-fat diet (11,12), in response to aging (13), and with a combination of bed rest and exogenous insulin administration (14). Little is currently known as to the extent to which the UPR contributes to normal skeletal muscle function or how it is affected by conditions of stress such as exercise. Recently Wu et al. (15) demonstrated that a single bout of running exercise in mice increased the abundance of various UPR transcripts in muscles heavily activated during treadmill exercise (quadriceps and gastrocnemius but not erector spinae). The UPR was accommodative to chronic training, as four weeks of exercise resulted in an overall differential acute response to exercise while the response of specific transcripts, particularly GRP78, was maintained despite training. Importantly, the UPR response to exercise was at least partially dependent on the association of the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α), a factor whose expression is susceptible to exercise, with the UPR transcription factor ATF6. In addition Deldicque et al. (16) found that endurance exercise superimposed over a high fat diet altered the UPR from a high fat diet alone, and that this response varied by muscle (soleus vs tibialis anterior) and tissue type (skeletal muscle, liver, and pancreas). The idea of tissue-specific differences in the UPR has been

previously demonstrated, as an UPR to exercise is absent following exercise in cardiac muscle (15,17).

UPR activation has been observed in human skeletal muscle following ultra endurance exercise (18). Following a 200 km race, spliced and full length XBP-1 mRNA were increased, indicative of ATF6 and IRE-1α activation, along with elevated Bip (GRP78) protein. This data suggests exercise can activate the UPR, and that the response may preferentially affect different signaling pathways within the UPR. However, resistance exercise is most commonly associated with net muscle protein accretion, yet data regarding the effects of resistance exercise (RE) on the UPR are lacking. Gordon et al. (19) had participants perform a unilateral exercise program for the elbow flexors for twelve weeks and obtained muscle biopsies four hours after completion of a bilateral RE session to assess the differences between the trained and untrained arm. GO term and KEGG pathway analysis found a number of transcripts related to the response to unfolded proteins and unfolded protein binding were lower in the trained arm as compared with the untrained limb. This indicates that an unaccustomed RE bout stimulates the expression of genes related to the handling of unfolded proteins (HSP70 for example); however, over time this dynamic response habituates such that increased expression of these particular genes is no longer required in the early post-exercise period.

As little data exists regarding the UPR following a single bout of RE (19) we sought to characterize the UPR following acute RE in both young and aged skeletal muscle. RE could create an intracellular environment favoring the UPR through altered calcium dynamics (20), increased oxidative stress (21), and elevated protein synthesis

(22), that could provoke the UPR (8,23). In addition, RE increases expression of PGC-1α (24,25) that is indicative of PGC-1α activity (26). As PGC-1α modulates the UPR through an association with ATF6 (15), it is possible that this mechanistic pathway is also stimulated with RE to induce the UPR. Previous data has demonstrated an elevated UPR in resting aged, rodent skeletal muscle (13) and we hypothesized that aged muscle would have a heightened, basal UPR response.

Materials & Methods

Subjects

Eighteen community-dwelling younger (Y; n=9, 21±3 y) and older (O; n=9, 70±4 y) men were recruited to complete a single RE bout as previously described (27,28). Participants underwent a routine screening prior to the study and were required to complete a health questionnaire and to have not been involved in a lower-body resistance exercise program for at least six months prior to the study. Exclusion criteria included evidence of heart disease, respiratory disease, uncontrolled hypertension, renal disease, diabetes, orthopedic disabilities involving the lower limbs, the use of NSAIDs or statin-related drugs and smoking. Participants were instructed to refrain from physical activity and abstain from the consumption of alcohol and non-steroidal anti-inflammatories during the study period. All participants gave written, informed consent prior to participation. This study was approved by the Hamilton Health Sciences Human Research Ethics Board and conformed to all declarations on the use of human subjects as research participants.

Acute Exercise Protocol

Participants arrived to the clinic at 6 AM and performed a unilateral RE program for the knee extensors as previously described (22,27,28). Each participant completed an incremental, unilateral one repetition maximum (1RM) protocol for the both leg press and knee extension exercises. Immediately following the 1RM test, subjects completed four sets of ten repetitions of each exercise at 75% of 1RM with two minutes between each set. The exercise order was randomized such that half of the participants performed the leg press initially, while the others performed the leg extension exercise.

Muscle Biopsies

A muscle biopsy was obtained three hours after RE from the unexercised and exercised *vastus lateralis*, followed by subsequent biopsies of the exercised leg only at 24 and 48 h post exercise. Each biopsy was performed with a modified Bergstrom percutaneous needle (29) and was spaced in a randomized order (distal, middle, proximal) with approximately 3 cm between adjacent biopsy sites. Each biopsy was sectioned into portions for the various techniques and frozen in liquid nitrogen for storage at -80^{o}C.

RNA Analysis

RNA was extracted from 30 mg of tissue in 1 ml of Trizol Reagent (Invitrogen, Burlington, ON, Canada). Tissue was homogenized with an electric mincer and afterwards 0.2 ml of chloroform was added per sample. The resultant aqueous phase was transferred to RNeasy spin columns (Qiagen, Germantown, Maryland, USA) and RNA

was isolated as per the manufacturer's recommendations. All RNA samples were treated with DNAase (Qiagen) while on the isolation columns to prevent contamination with genomic DNA. The purity and concentration of the RNA was determined by measuring the absorbance of the solution at 260 nm (ND-1000, Nanodrop, Willmington, DE, USA).

Total RNA (100 ng) was reverse transcribed with random hexamers as per the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR was performed with gene specific primers on the 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR® Green chemistry (PerfeC$_T$a SYBR® Green Supermix with ROX, Quanta Biosciences, Gaithersburg, MD, USA). The primers used were as follows: GRP78 forward: 5'- cgactcgaattccaaagattca reverse: 5'- cctggacagcagcaccatac, CHOP forward: 5'- agctggaagcctggtatga reverse: 5'- atttccaggaggtgaaacatag, ATF4 forward: 5'- ccctccaacaacagcaaggagga reverse: 5'- acccaacagggcatccaagtcga, ATF6 forward: 5'- cccgtattcttcagggtgctctgg reverse 5'- tagctcactccctgagttcctgct, PERK forward: 5'- gttgtcgccaatgggatagt reverse 5'- cgaggtccgacagctctaac, IRE1α forward: 5'- gcaagctgacgcccactctgta reverse: 5'- aaaggaagtgtgctgccgcg, XBP1 forward: 5'- reverse: 5'- , Spliced XBP1 forward: 5'- tgctgagtccgcagcaggtg reverse: 5'- gctggcaggctctggggaag, eIF2α forward: 5'- reverse: 5'- , and β2-microglobulin forward: 5'-acttgtctttcagcaaggactg reverse: 5'-ttcacacggcaggcatact. All data was normalized to the expression of β2-microglobulin, which was not affected by age or exercise (data not shown).

Protein Analysis

A 30 mg portion of each muscle biopsy was homogenized with an electric tissue mincer and processed into nuclear and cytosolic fractions as per the manufacturer's specifications (Pierce, Rockford, IL USA). Protease and phosphatase inhibitors (Roche, Mississauga, ON, Canada) were added to prevent degradation of proteins and preserve phosphorylation status. Protein concentrations were determined using the bichinconic acid method as per the manufacturer's recommendations (Pierce, Rockford, IL, USA) with a spectrophotometer (Benchmark Plus, Biorad, Hercules, CA, USA). Proteins were probed using various conditions depending on the protein using the following antibodies: GRP78 (Cell Signaling Technology #3177, Boston, MA, USA), IRE1α (Cell Signaling Technology #3294), and PERK (Cell Signaling Technology #3192). All blots were probed under similar conditions with primary antibodies (overnight incubation at four degrees Celsius) followed by either anti-rabbit or anti-mouse secondary antibodies (1:10000, GE Healthcare) for one hour at room temperature. Blots were developed with ECL plus (GE Healthcare) and exposed to x-ray film (GE Healthcare). All films were digitized and band density was determined with ImageJ (NIH, Bethesda, Maryland, USA). Protein data is expressed relative to protein disulfide isomerase (PDI; Abcam ab2792, Toronto, ON, Canada) that was stable across age and exercise (data not shown).

Statistical Analysis

Statistical analysis was performed with the analysis software Statistica (Statsoft, Tulsa, OK, USA). A two-way, repeated-measures ANOVA was performed (age, time)

and significance was accepted at $p<0.05$. Tukey's HSD post-hoc testing was performed when significant effects and interactions were indicated in the ANOVA. T-tests were used to test for differences in anthropometric and functional characteristics between the groups. All results are reported as mean ± SD.

Results

Subject Characteristics

Subject characteristics have been previously published for the groups used in the present study (27). There were no differences in lean-body mass with age (O: 62±6.4 kg, Y:65.9±10.8 kg) while the older men (age 70±4 y) had reduced leg strength relative to the young. Leg press (O: 160.8±50.1, Y: 228±93.8 lbs; $p<0.05$) and leg extension (O: 117.6±47.4, Y: 81.9±22.8 lbs; $p<0.05$) one repetition maximums were lower in the older adults and isometric knee extension MVC was reduced (O: 228.3±45.1, Y: 324.6±85.4 Nm; $p<0.05$) as compared to the young (age 21±3 y) (27,28).

RE stimulates the UPR

In response to ER stress, dissociation of Grp78 from IRE1α promotes the activation of IRE1α that is then free to remove a 26-nucleotide intron from XBP-1 (4). The abundance of the full length XBP-1 is regulated by the transcription factor ATF6. Consequently, spliced and full-length XBP1 are specific indicators of the induction of ER stress (5) and the UPR through the transcriptional activity of ATF6 and the splicing activity of IRE1α (4). Full length XBP-1 did not vary with age but was stimulated by

exercise (f=5.64, p=0.0023; Fig 1). Full length XBP-1 was increased to 150% of resting levels at 24 h post-exercise (p=0.052, Fig 1). A similar response was observed for spliced XBP-1 mRNA, altering expression with exercise (f=12.62, p<0.0001; Fig 1) but not age. Spliced XBP1 was elevated at 24 and 48 h (222% of rest, p<0.01 and 262% of rest, p<0.001 respectively). Despite increased abundance of both the full length and spliced transcript, the ratio of spliced to full-length transcript did increase with exercise (f=4.086, p=0.0135; Fig 1). The ratio increased to 200% of the resting values by 48 h post-exercise (p=0.012), indicative of activation of the UPR.

The UPR signaling cascade is initiated by the activity of four specific proteins: Grp78, IRE1, PERK and ATF6. Age did not influence mRNA expression of any of the four upstream UPR targets, however exercise altered expression of IRE1α (f=6.15, p=0.0013) and ATF6 (f=6.54, p<0.001) but not Grp78 and PERK (Fig 2). IRE1α mRNA increased to 151% of resting levels at 24 h post-exercise (p<0.05) while ATF6 mRNA increased at 24 and 48 h post-exercise (160% p<0.01 and 151% p<0.01 respectively). Exercise (but not age) increased Grp78 (f=34.33, p<0.0001), IRE1α (f=10.83, p<0.001), and PERK (f=5.46, p=0.003) protein content (Fig 3). Both Grp78 and PERK protein content were increased relative to resting muscle (626%%, p<0.001 and 154%, p<0.01 respectively) at 48 h post-exercise. IRE1α protein increased to 152% and 183% of resting levels at 24 and 48 h post-exercise (p<0.05 and p<0.001 respectively).

PERK functions to attenuate protein synthesis within the endoplasmic reticulum. This effect is achieved through the phosphorylation of serine 51 on eIF2α, which acts to sequester eIF2B and prevent eIF2B catalyzed guanine nucleotide exchange on eIF2α (30).

In addition, this inhibitory phosphorylation triggers the translation of proteins specific to the transcription factor ATF4 (7). There was an effect of exercise on CHOP mRNA ($f=3.4$, $p=0.027$) with expression at 48 h reduced as compared to 24 h (114% versus 73% of resting expression respectively, $p<0.05$), with no differences from resting expression. There was no effect of age or exercise GADD34 mRNA (Fig 4). Exercise did affect ATF4 mRNA ($f=4.2$, $p=0.01$); however, expression was not different from the resting condition at any time point. Rather expression at 24 h was greater than 48 h (114% and 87% of rest respectively, $p=0.052$). In addition, phosphorylation on serine 51 of eIF2α did not change with age or exercise (data not shown) while exercise ($f=17.52$, $p<0.0001$) but not age increased eIF2α mRNA at 24 and 48 h post-exercise (163%, $p<0.001$).

Discussion

Few studies have analyzed the UPR in skeletal muscle with exercise (15,16,18) and little is known of the effects of RE specifically (19). Independent of exercise, the UPR has been observed in skeletal muscle of inclusion body myositis (9), autoimmune myositis (31) and myotonic dystrophy patients (10) as well as in aged rodents (13) and those subjected to various nutritional challenges (8,11). This data extends our understanding of the UPR in skeletal muscle and indicates that while all signaling arms of the UPR may be stimulated in muscle in conditions of disease (10), the effects of acute RE, and perhaps exercise in general (18), may preferentially activate specific components of the UPR in human skeletal muscle.

The abundance of spliced XBP1 is a highly specific marker of the UPR response (5), and the full-length transcript is also indicative of the transcriptional activity of AT6 (4). We demonstrated that acute RE alters the abundance of both full length and spliced XBP-1, indicating that exercise stimulates IRE-1α and ATF6 activity. Kim et al (18) found similar results with respect to XBP-1 mRNA in skeletal muscle following an ultra-endurance race. Both the full length and spliced isoforms were increased post-exercise and similar to the response to RE observed here, the spliced isoform increased disproportionately to the full-length transcript (241% and 138% respectively). In combination, these studies indicate that unaccustomed resistance exercise or an extreme volume of endurance exercise stimulates the UPR.

The PERK pathway of the UPR acts to suppress global protein translation through phosphorylation of eIF2α that leads to the preferential translation of ATF4 mRNA due to the small upstream open reading frame in the 5'-untranslated region of ATF4 (7,32). When phosphorylated on serine 51, eIF2α binds to and inhibits eIF2B, blocking translation by preventing eIF2B catalyzed guanine nucleotide exchange on eIF2α. Our data indicates that this specific UPR pathway may not be affected by acute RE, as we failed to detect an increase in ATF4, CHOP or GADD34 mRNA and phosphorylation of eIF2α and found an increased abundance of eIF2α mRNA at 24 and 48 h post-exercise. As the UPR operates to attenuate global protein synthesis, an increase in eIF2α mRNA is contrary to what would be expected under activation of PERK. This data is in agreement with previous work (18), where a 200km ultra-endurance race stimulated other signaling

pathways of the UPR (ATF6 and IRE-1α) but failed to alter ATF4 and CHOP mRNA abundance post-exercise.

The lack of effect on ATF4 mRNA should be interpreted with caution, as it may not be directly indicative of activation of the PERK arm of the UPR pathway. Harding et al (7) demonstrated that the elevated abundance of ATF4 protein during ER stress is post-translationally controlled, as inhibition of transcription by actinomycin D treatment did not prevent the accumulation of ATF4 protein. Nevertheless, the lack of effect of RE on CHOP mRNA, whose expression is dependent on the transcriptional activity of ATF4 (7), coupled with the lack of change in the phosphorylation status of eIF2α supports the preferential activation of other, specific UPR pathways post exercise. This interpretation is confounded by the considerable overlap in the ability of the three arms of the UPR to influence transcription of CHOP mRNA, as ATF4 can bind to amino-acid-regulatory element one and two (AARE1, 2) while ATF6 and XBP-1 can promote CHOP transcription via the ER stress response elements (32). Despite considerable overlap, data from PERK deficient cells has demonstrated that the PERK/eIF2α arm of the UPR is the dominant force controlling CHOP abundance during ER stress (7); however, activation of all three UPR signaling arms is required for maximal CHOP expression (32). In addition, over-expression of Grp78 has been shown to attenuate the induction of CHOP mRNA during ER stress (33) and as we found a substantial increase in Grp78 protein post RE with no change in the primarily PERK responsive transcripts, it appears that alternate UPR pathways may dominate the early adaptive response to RE.

Recently Wu et al (15) demonstrated the pivotal role of the UPR in the adaptation to exercise. A single bout of unaccustomed treadmill exercise in C57/Bl6 mice increased Grp78 protein and mRNA abundance of all arms of the UPR, including GADD34, ATF4 and CHOP mRNA which has been shown to be unaffected in humans with exercise both here and in previous data (18). This indicates that the UPR following exercise may differ by species in skeletal muscle. This response was also specific to skeletal muscle, as cardiac muscle, whilst placed under elevated metabolic demand during exercise, did not alter UPR targets. This response was sensitive to cumulative bouts of exercise, as mice trained for four weeks had an attenuated UPR with no change in spliced XBP-1, CHOP mRNA, an attenuated ATF3 response albeit with no change in the response of Grp78 (Bip), Grp94 and ERdj4, and a chronic decrease in ATF4 mRNA as compared to untrained controls given a similar exercise challenge. This is consistent with data on human RE, as Gordon et al (19) demonstrated that untrained muscle had an enrichment of genes in the GO categories 'Response to unfolded protein', 'Response to protein stimulus', 'Unfolded protein binding' and 'protein folding' following an acute bout of RE and that expression within these categories was attenuated with training.

In subsequent experiments Wu et al (15) demonstrated that PGC-1α, a transcriptional coactivator associated with metabolic and mitochondrial adaptations to endurance exercise (34), modulates the adaptation to treadmill exercise through an interaction with the UPR transcription factor ATF6. We have previously shown an elevated abundance of PGC-1α mRNA acutely following RE in agreement with others (25,35). As this increase occurred with the concurrent reduction of mitochondrial

transcripts, proteins and DNA (25), it is possible that post-exercise elevations in PGC-1α activity may serve to modulate the UPR via ATF6 (15) post RE, as opposed to mitochondrial adaptations through coactivation of the transcription factors nuclear respiratory factor (NRF) one and two (36). Nevertheless, it is possible that other mechanisms contribute to the UPR following acute RE independent of expression of PGC-1α. Further work is required to determine how PGC-1α isoforms are differentially regulated by divergent modes of exercise and which PGC-1α binding partners serve to create phenotypically distinct adaptations despite a common increase in PGC-1α with differing modes of exercise (endurance *vs* resistance).

Contrary to our hypothesis, despite elevations in oxidized and misfolded proteins (37,38) and oxidative stress with age (39), both of which can stimulate the UPR (23), we failed to detect any effects of the aging process on the induction of the UPR in resting skeletal muscle or in the dynamic response to RE. This result is in contrast to Ogata et al (13) who demonstrated elevated Caspase-12, Grp78 and CHOP protein in the mitochondrial-free cytosolic fraction of 32 month aged rats as compared to six-month-old animals. We cannot rule out inter-species differences in the role of the UPR in skeletal muscle, as our results and others (18) differ from rodent studies with respect to both the age effect (13) and the ability of exercise to preferentially alter specific pathways within the UPR (15). It is also possible that such effects may only be present in very frail older adults and that as our older subjects were healthy, as evidenced by the similarity in lean-body mass between the age groups, basal UPR activation may not be present.

Conclusion

Resistance exercise stimulates specific pathways in the UPR response to promote the increased production of certain chaperone proteins (Grp78), likely a consequence of the increased production of various proteins during the post-exercise period. In agreement with previous data on the effects of endurance exercise (18), this work suggests that specific signaling pathways that promote the production of UPR proteins are stimulated by RE, while the pathway that attenuates protein synthesis is not (PERK). Contrary to previous data (13), age did not alter the basal abundance of UPR transcripts and proteins, and the dynamic response to RE was unaltered. Further work is required to determine whether post-exercise elevations in PGC-1α mRNA, indicative of PGC-1α activity (26), are involved in the induction of the UPR, and whether the degree to which protein synthesis is stimulated is related to or predictive of the UPR following RE.

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Disclosures

The authors have not conflicts to declare.

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Figure Legends

Figure 1 Resistance exercise increases the abundance of spliced and full length XBP1 mRNA regardless of age. Values are mean ± SD expressed as percent rest (100%). † $p<0.01$ and ‡ $p<0.001$.

Figure 2 Resistance exercise alters the mRNA abundance of specific components of the UPR. Values are mean ± SD expressed as percent rest (100%). * denotes $p<0.05$ and † $p<0.01$.

Figure 3 Resistance exercise increases the abundance GRP78, IRE1α and PERK proteins. Values are mean ± SD expressed as percent rest (100%). * denotes $p<0.05$, † $p<0.01$ and ‡ $p<0.001$.

Figure 4 Resistance exercise does not influence the expression of components of the PERK signaling arm, while increasing abundance of eIF2α mRNA. Values are mean ± SD expressed as percent rest (100%). ‡ denotes $p<0.001$.

Figures

Figure 1

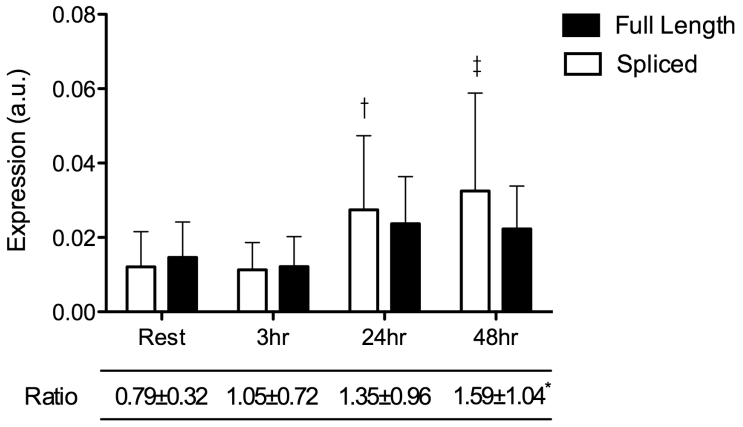


Figure 2

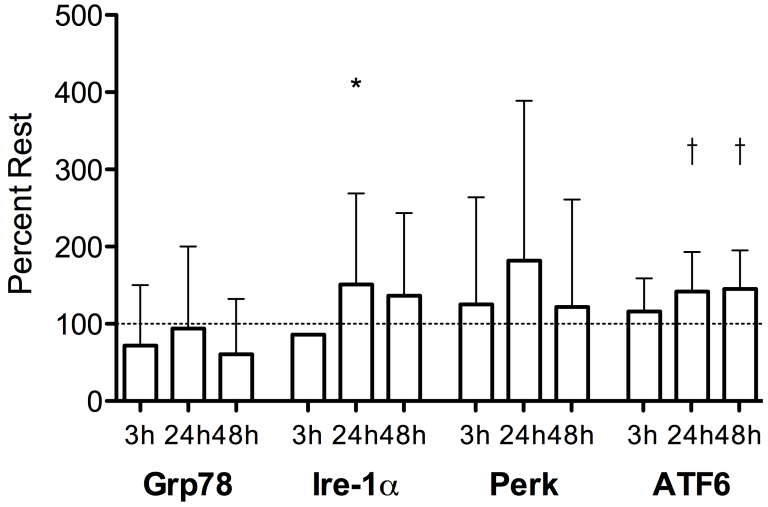


Figure 3

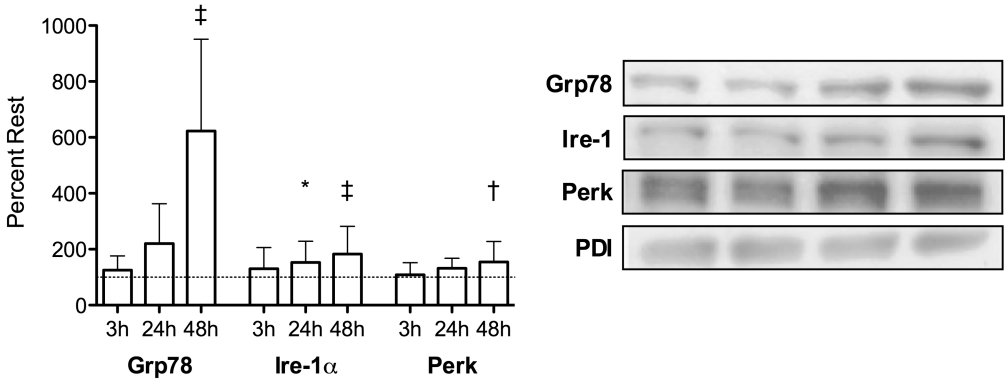
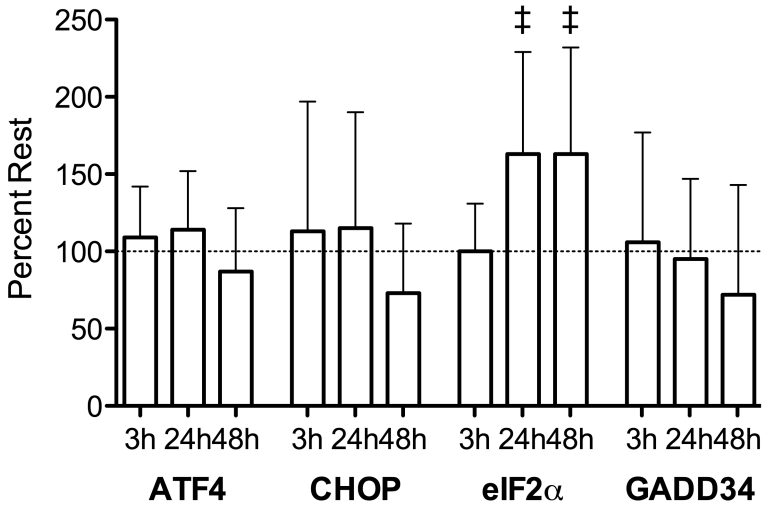


Figure 4



Paper 3: Differential regulation of PGC-1α following acute resistance or endurance exercise

Differential effects of acute resistance and endurance exercise on specific PGC-1α isoforms

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Abstract

Both resistance (RE) and endurance exercise (EE) increase peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) but result in divergent adaptations. Here we show that unaccustomed EE increases the specific α1 and α4 isoforms more so than RE and that RE preferentially affects α4. Despite this, both RE and EE affected myostatin expression to a similar extent, and a greater number of genes were altered post-exercise that were related to α4 expression than α1.

Keywords: PGC-1α, Resistance Exercise, Endurance Exercise, Skeletal muscle, Myostatin

Introduction

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a transcriptional coactivator that that is considered a master regulator of mitochondrial biogenesis (Christophe Handschin and Spiegelman 2006). As a transcriptional coactivator, PGC-1α associates with specific transcription factors including the nuclear respiratory factors (NRF1, NRF2), estrogen-related receptor alpha (ERRα), myocyte enhancer factor 2 (MEF2) and peroxisome proliferator-activated receptors (PPARs) to modulate the expression of many genes related to muscle metabolism and mitochondrial biogenesis including mitochondrial transcription factor a (TFAM) (Vega, Huss, and Kelly 2000; Z. Wu et al. 1999). In addition, PGC-1α coactivates expression of itself in an autoregulatory loop (Christoph Handschin et al. 2003). Consequently, PGC-1α is pivotal to the adaptation to endurance exercise (EE) by coordinating the expression of both nuclear and mitochondrial encoded genes central to mitochondrial biogenesis through coactivation of other transcription factors (Irrcher et al. 2003).

In addition to the well-established importance of PGC-1α to EE adaptations, PGC-1α mRNA is increased acutely following resistance exercise (RE) (Deldicque et al. 2008; Ogborn et al. 2011; Burd et al. 2012), and recent evidence suggests a role for PGC-1α (Ruas). RE is not commonly associated with mitochondrial biogenesis (Alway et al. 1988), and the fact that increased PGC-1α occurs alongside the acute reduction in mitochondrial transcripts, proteins and DNA (Ogborn et al. 2011), suggests that PGC-1α may serve other functions alternate to the well-established role in mitochondrial biogenesis following unaccustomed RE. Recently Wu et al (J. Wu et al. 2011) established

that PGC-1α modulates the unfolded protein response (UPR), and ultimately the adaptation to exercise, through an interaction with the UPR transcription factor activating transcription factor 6 (ATF6). In addition, Ruas et al (Ruas et al. 2012) demonstrated that there are alternate isoforms of PGC-1α that are differentially expressed dependent on the exercise stimulus. Increased PGC-1α isoform 4 (PGC-1α4) mRNA occurred following either chronic, pure RE, or mixed mode resistance/endurance exercise, but not EE, and was correlated with improvement in the leg press exercise. Subsequent experiments demonstrated that PGC-1α4 suppressed myostatin and increased insulin-like growth factor one (IGF1) expression resulting in myotube hypertrophy. Collectively, this data indicates that the PGC-1α1 and α4 isoforms would be differentially regulated to produce the specific adaptations following either RE or EE.

The fact that studies have shown a common increase in total PGC-1α mRNA despite divergent modes of exercise and consequent adaptations in skeletal muscle suggest that factors other than abundance of total PGC-1α control the specificity of the response by exercise mode. As previous data has shown that different PGC-1α isoforms are induced specifically dependent on the mode of chronic exercise training (Ruas et al. 2012), we sought to determine the effects of acute, fatiguing endurance and resistance exercise on PGC-1α isoform abundance. We hypothesized that RE would promote a disproportionate expression of PGC-1α4 isoform and altered expression of related transcripts (myostatin, IGF-1), while EE would promote PGC-1α1 isoform expression and the expression of related mitochondrial and metabolic genes (CPT1, Cox5b).

Materials and Methods

Subjects

The samples used in this investigation were collected as components of two separate, previously published investigations (Crane et al. 2012; McKay et al. 2012). All participants underwent a routine screening prior to the study and were required to complete a health questionnaire and to have not been involved in a lower-body resistance exercise program for at least six months prior to the study. Exclusion criteria included evidence of heart disease, respiratory disease, uncontrolled hypertension, renal disease, diabetes, orthopedic disabilities involving the lower limbs, the use of NSAIDs or statin-related drugs and smoking. Participants were instructed to refrain from physical activity and abstain from the consumption of caffeine, alcohol and non-steroidal anti-inflammatories during the study period. All participants gave written, informed consent prior to participation. This study was approved by the Hamilton Health Sciences Human Research Ethics Board and conformed to all declarations on the use of human subjects as research participants.

Resistance exercise protocol

Participants arrived to the clinic and performed a unilateral RE program for the knee extensors as previously described (McKay et al. 2012; McKay et al. 2013; Drummond et al. 2008). The participants performed an incremental, unilateral one repetition maximum (1RM) protocol for the leg press and knee extension exercises. Following the 1RM test, subjects performed four sets of ten repetitions of each exercise at

75% of 1RM with two minutes between each set. Exercise order was randomized such that half of the participants performed either the leg press or knee extension exercise initially.

Endurance exercise protocol

Participants in the EE study had a resting biopsy and VO_2max test two weeks prior to the completion of an exhaustive exercise bout. The exercise bout consisted of cycling exercise on an electrically braked ergometer (Lode Excalibur, Lode) pedaling at a workload calculated to elicit 60% of their previously determined VO_{2peak} for 30 minutes, at a cadence of 70-90 rpm. After 30 minutes, intensity was oscillated such that workload increased to 65% VO_{2peak} for 5 min, then to 60% for 5 min, 70% for 5 min, then 60% for 5 min, to a maximum of 85% VO_{2peak}. If participants reached the 85% VO_{2peak}, two minute intervals of 85% and 60% VO_{2peak} were alternated until exhaustion. As these samples were collected as a component of our previous investigation of the interactions of exercise and massage (Crane et al. 2012), it is important to note that only non-massaged samples were utilized in the present investigation.

Muscle biopsies

For the unilateral RE participants, a muscle biopsy was obtained three hours after RE from both the unexercised and exercised *vastus lateralis*. The EE participants had a resting muscle biopsy taken two weeks prior to the exhaustive exercise bout, and the final muscle biopsy was taken three hours after the EE protocol was completed. Each biopsy was performed with a modified Bergstrom percutaneous needle (Tarnopolsky et al. 2011)and was spaced in a randomized order (distal, middle, proximal) with

approximately 3 cm between adjacent biopsy sites. Each biopsy was sectioned into portions for the various techniques and frozen in liquid nitrogen for storage at -80^{o}C.

RNA Analysis

RNA was extracted from 30 mg of tissue in 1 ml of Trizol Reagent (Invitrogen, Burlington, ON, Canada). Tissue was homogenized with an electric mincer and 0.2 ml of chloroform was added per sample. The resultant aqueous phase was transferred to RNeasy spin columns (Qiagen, Germantown, Maryland, USA) and RNA was isolated as per the manufacturer's recommendations. All samples were DNAase treated (Qiagen) while on the isolation columns to prevent contamination with genomic DNA. The purity and concentration of the RNA was determined by measuring the absorbance of the solution at 260 nm (ND-1000, Nanodrop, Willmington, DE, USA).

Total RNA (100 ng) was reverse transcribed with random hexamers as per the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR was performed with gene specific primers on the 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR® Green chemistry (PerfeC$_T$a SYBR® Green Supermix with ROX, Quanta Biosciences, Gaithersburg, MD, USA). The primers sets used in this study are presented in Table 1. All data was normalized to β2-microglobulin that was not differentially expressed between groups or following exercise (data not shown).

Statistical Analysis

A repeated-measures ANOVA was used to determine the effects of RE and EE on expression of all transcripts. Tukey's HSD post-hoc testing was performed when significant effects and interactions were indicated by the ANOVA. Independent t-tests were used to test for basal differences in the subject characteristics between the groups, and for determining the percentage change or percentage of resting values between the exercise modes. All results are reported as means ± SD.

Results

Subject Characteristics

Characteristics of the participants in this investigation have been published separately previously (Crane et al. 2012; McKay et al. 2013; McKay et al. 2012), but have been combined for the current analysis and are presented in Table 2. No differences were detected for the variables between the individuals who performed EE or RE with the exception of bodyweight, which was greater in the RE group.

Effects of EE and RE on PGC-1α Isoforms and related targets at 3h post-exercise

Total PGC-1α was increased by exercise ($f=34.7$, $p<0.0001$) and there was an interaction between exercise type and time for total PGC-1α expression ($f=5.46$, $p<0.05$). The percentage of resting expression at 3 h was greater with EE than RE (1059% vs 393%, $p<0.05$, Fig 1). There was an interaction of exercise type and time regarding PGC-1α1 expression ($f=4.787$, $p=0.045$) while no time points were significantly different with

post hoc testing. However, considering the percentage of resting expression for PGC-1α1 with exercise, EE was greater than RE, which did not influence PGC-1α1 mRNA at all (212% vs 101% of rest respectively, $p<0.05$). Exercise influenced PGC-1α4 expression ($f=49.97$, $p<0.0001$), with no effect of mode. EE increased PGC-1α4 to 1387% of resting expression, while RE increased it to 710% of rest. The two fold difference between the modes failed to reach significance ($p=0.12$).

Effects of RE and EE on downstream targets of PGC-1α1 and α4

The only targets previously identified as related to the expression of PGC-1α1 and α4 (Ruas et al. 2012) that were influenced by exercise were Myostatin ($f=20.46$, $p<0.001$, Fig 2), ACVR2a ($f=12.3$, $p=0.003$, Fig 3), AVCR2b ($f=27.58$, $p<0.0001$, Fig 3), IGFBP6 ($f=4.44$, $p=0.049$, Fig 3) and PDK4 ($f=11.86$, $p=0.003$, Fig 4). There was an interaction of mode for only ACVR2b ($f=6.61$, $p<0.019$), whereby the resting expression EE was greater than all other groups (RE) and time points ($p<0.001$) however this did not affect the time course of expression by mode. The majority of transcripts were lower relative to rest, including Myostatin (44% of rest, $p<0.001$), ACVR2a (66% of rest, $p=0.003$), ACVR2b (68% of rest, $p<0.001$), IGFBP6 (78% of rest, $p=0.048$); whereas, only PDK4 increased expression post-exercise (662% of rest, $p=0.003$). The percentage of resting expression was not different between EE and RE for all transcripts with the exception of PDK4, which was greater with EE (900% vs 192% of rest respectively, $p=0.033$). Expression of cytochrome c oxidase subunit Vb (COXVb), carnitine palmitoyltransferase 1 (CPT1), cytochrome c (CytC), Follistatin, insulin-like growth factor 2 (IGF2), insulin-like growth factor binding protein 2 (IGFBP2), IGFBP3, medium-chain acyl-coenzyme A

dehydrogenase (MCAD), platelet-derived growth factor subunit B (PDGFb) were not influenced by exercise.

Discussion

This study provides additional insight into the regulation of specific PGC-1α isoforms in response to acute EE and RE. Contrary to our hypothesis, EE increased total PGC-1α and α1 and α4 isoforms to a greater extent than RE, whereas RE was expected to induce PGC-1α4 to a greater extent than EE. In agreement with Ruas et al (Ruas et al. 2012) we found no change in PGC-1α1 isoform expression with acute RE, and no change in predicted downstream target genes, with the exception of PDK4. As expected, EE potently increased PGC-1α1, and also increased PDK4, a predicted PGC-1α1 and α4 responsive gene, and to a greater extent than what occurred following RE. In spite of the lack of effect of RE on PGC-1α1, increased PDK4 mRNA was detected following RE. This is consistent with (Ruas et al. 2012) who demonstrated an overlap of PGC-1α1 and α4 target genes, with PDK4 being sensitive to both isoforms. PGC-1α is alternatively spliced (Zhang et al. 2009); however, the PGC-1α4 isoform, described recently by Ruas et al (Ruas et al. 2012) is under the control of a different promoter and has a distinct N-terminus sequence. This particular isoform was specifically related to muscle hypertrophy and changes in strength following a chronic RE program, but was not induced following EE. Conversely, our data suggests that the specificity of PGC-1α isoform modulated adaptations to differing modes of acute, unaccustomed exercise may relate to the differential induction of PGC-1α1 and not the α4 isoform. The disparity between the results presented here could be explained by the fact that Ruas et al (Ruas et al. 2012)

characterized the response to longer-term training, whereas the results presented here pertain only to the immediate 3 h post-exercise window. Further work is required to map out the temporal pattern of PGC-1α isoform expression from the initial bout of exercise to the ultimate adaptive responses to chronic training.

Despite the nearly two-fold difference in expression PGC-1α4 expression in following EE, both RE and EE had a similar reduction in myostatin mRNA 3 h post-exercise. It is well established that RE acutely decreases myostatin mRNA (Raue et al. 2006; McKay et al. 2012; Jones et al. 2004; Kim, Cross, and Bamman 2005; Hulmi et al. 2009; Kim et al. 2007), and previous studies both in humans and rodents have established that EE can reduce myostatin mRNA (Matsakas et al. 2005; Matsakas et al. 2006); however, the reduction may be greater with RE (Louis et al. 2007). Ruas et al (Ruas et al. 2012) demonstrated that chronic RE or mixed mode RE/EE training increased PGC-1α4 and decreased myostatin, while similar results were not obtained with chronic EE. Our data suggests that a single bout of unaccustomed RE and EE exercise both substantially increase PGC-1α4 and decrease myostatin, contrary to the results of Ruas et al (Ruas et al. 2012) obtained following chronic training. This indicates that the acute response may not be indicative of the ultimate adaptation to chronic training, given the fact that chronic EE did not influence either PGC-1α4 or myostatin (Ruas et al. 2012). There is also a possibility that this is either a threshold response, as both RE and EE produce similar percent changes in myostatin mRNA despite a near two-fold difference in percentage change in PGC-1α4, or that additional competing factors explain the discrepancy between EE and RE.

The potent negative regulation of muscle mass controlled by myostatin is regulated through an interaction with Activin IIb receptors. Upon myostatin binding a signaling cascade is activated involving the type I activin receptor transmembrane serine/threonine kinases that phosphorylate specific Smad proteins to modulate gene transcription and ultimately, muscle mass. In agreement with Ruas et al (Ruas et al. 2012) who found a reduction in ACVR2a and 2b in association with PGC-1α4 but not α1 over-expression, we found a reduction in ACVR2a and ACVR2b in the presence of elevated PGC-1α4 mRNA. This effect occurred independent of exercise mode, and was not sensitive to the near two-fold difference in PGC-1α4 mRNA at 3 h post-exercise between RE and EE. Unfortunately, while Ruas et al (Ruas et al. 2012) studied chronic RE, EE and mixed mode RE/EE, the specific effects of this treatment were discussed in the context of myostatin expression and specific data on the activin receptors stated. Data regarding the effects of acute RE on activin IIb is not as conclusive as for myostatin, as previous studies have demonstrated either a reduction in Activin 2b receptor mRNA acutely following RE (Hulmi et al. 2009) that may be attenuated with chronic training (Hulmi et al. 2010) or no effect of acute RE (Kim et al. 2007).

We cannot discount the possibility that there is a discrepancy between the susceptibility of genes related to metabolism and muscle growth to a single exercise stimulus. This is supported by the fact that genes identified as related to PGC-1α4 (myostatin, ACVR2a, ACVR2b, IGFBP6) outnumber those related PGC-1α1 (PDK4), four to one in the 3h post-exercise window. Wu et al (J. Wu et al. 2011) demonstrated an interaction between PGC-1α and the unfolded protein response transcription factor

activating transcription factor 6 (ATF6), with two implications: 1) the UPR is activated following a single bout of exercise and 2) increased cytochrome *c* mRNA following acute EE was seen only in animals trained for multiple weeks. The increase in cytochrome *c* mRNA occurred after acute exercise only in the trained animals, alongside an attenuated unfolded protein response. In agreement, Perry et al (Perry et al. 2010) found that, over the course of multiple high-intensity interval bouts, certain metabolic transcripts were readily increased following a single bout (CS, β-HAD), others were not (COXIV), while the expression of mitochondrial and metabolic transcription factors (PGC-1α, PGC-1β, Tfam) were increased acutely. This suggests the transcriptional abundance of transcription factors and coactivators related to metabolism increase in advance of their targets, possibly over multiple bouts of exercise, and often increase disproportionately to their intended targets (Perry et al. 2010). This provides some support for the contention that PGC-1α may serve different roles according to the trained status of the individual; however, further work is required to clarify the specificity of this response for particular genes.

This indicates that in addition to regulating the abundance of alternatively spliced isoforms, PGC-1α may also serve other cellular processes (UPR, IGF/myostatin signaling), potentially explaining the lack of effect of acute RE and EE on mitochondrial and metabolic genes following a single, acute bout of unaccustomed exercise, regardless of mode. The fact that both EE and RE resulted in muscle damage/inflammation (Crane et al. 2012; McKay et al. 2013) indicates that cellular signaling related to muscle growth and repair may dominate the initial bout of exercise (PGC-1α4, IGF/Myostatin, UPR/ATF6

signaling); however, over time as the muscle adapts signaling is shifted towards PGC-1α1 and mitochondrial biogenesis, at least for EE. Therefore, in addition to the regulation of alternatively spliced isoforms of PGC-1α, future work is required to determine the specific protein interactions of PGC-1α as different binding partners may occur in response to differing modes of exercise, and overtime as individuals transition to the trained state. Further research is also required to determine if there is a discrepancy in the acute timing of changes in mitochondrial and growth-related transcripts following either RE and EE in relation to PGC-1α isoforms outside of the 3 h post-exercise window.

Conclusion

Acute, unaccustomed EE to exhaustion results in enhanced expression of PGC-1α1, PGC-1α4 and total PGC-1α mRNA as compared to RE where each set is taken to fatigue. Despite differences in the magnitude of the PGC-1α4 response between EE and RE, a similar suppression of myostatin, ACVR2a and ACVR2b mRNA was detected between modes. Ultimately, these data suggest that genes that are sensitive to the expression of PGC-1α4 are readily altered by acute, unaccustomed exercise regardless of mode, at 3 h post-exercise. Despite elevations in PGC-1α1 mRNA with EE, purported target genes are not altered at 3 h post-exercise, with the exception of PDK4. Further work is required to clarify the role of PGC-1α4 in the regulation of skeletal muscle mass and the potential interactions with myostatin and IGFs in the response to both RE and EE.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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Figure Legends

Table 1 RT-PCR Primer Sequences

Table 2 Subject Characteristics Values are means ± SD. * denotes $p<0.05$

Figure 1 EE increased all PGC-1α isoforms and to a greater extent than RE. RE affected only total PGC-1 α and PGC-1α4 abundance. * is $p<0.05$ for the comparison of mode (RE vs EE). Graphs are means ± SD.

Figure 2 Myostatin decreased post-exercise regardless of mode. ‡ is $p<0.001$ for the overall effect of exercise, regardless of mode. Graphs are means ± SD.

Figure 3 Exercise reduced the expression of PGC-1α4 -sensitive transcripts ACVR2a, ACVR2b and IGFBP6 regardless of mode. * is $p<0.05$ for the overall effect of exercise, regardless of mode. Graphs are means ± SD.

Figure 4 Exercise increased expression of PDK4, and the percentage change was greater following EE. * is $p<0.05$ for the overall effect of exercise, regardless of mode. Graphs are means ± SD.

Figures

Table 1

Gene	Forward Sequence	Reverse Sequence
ACVR2a	GCTGTGAGGGCAATATGTGTA	AAGTGGCACCAAGGAATAGA
ACVR2b	TGGAACGAACTGTGTCATGTA	AGTCCCTGTGGGCAATAGAC
COXVb	GAAGGGACTGGACCCATACA	CGACGCTGGTATTGTCCTCTT
CPT1	CAAGTCATGGTGGGCAAGTA	CTGCCTGCACGTCTGTATTC
CytC	AGAAGGCAATGACCGCTAAA	CAGTCTGTGACCCAGGATGT
Follistatin	GGATTTCAAGGTTGGGAGAG	AGCTTCCTTCATGGCACACT
IGF2	AGTCGATGCTGGTGCTTCTC	CAGACGAACTGGAGGGTGTC
IGFBP2	CCCTCTGGAGCACCTCTACT	CTGCCCGTTCAGAGACATC
IGFBP3	TATGGTCCCTGCCGTAGA	CTTTGGAAGGGCGACACT
IGFBP6	TGTGACCATCGAGGCTTCTAC	TTCCATTGCCATCTGGAGAC
MCAD	AAAGGCATTTGCTGGAGATA	TTTGGCATCCCTCATTAGTT
Myostatin	CGTCTGGAAACAGCTCCTAAC	TCATCCCTCTGGACATCATACT
PDGFb	ATTCCCGAGGAGCTTTATGA	GGTCATGTTCAGGTCCAACTC
PDK4	CAATGGCACAAGGAATCATA	GGTTCATCAGCATCCGAGTA
PGC-1α	CAGCCTCCTTGCCCAGATCTT	TCACTGCACCACTTGAGTCCAC
PGC-1α1	ATGGAGTGACATCGAGTGTGCT	GAGTCCACCCAGAAAGCTGT
PGC-1α4	TCACACCAAACCCACAGAGA	CTGGAAGATATGGCACAT

Table 2 Subject Characteristics

	Resistance	Endurance
Age	21±3.3	22±2.8
Height	180±8.4	176±5.8
Weight	92±23.3	75±8.7*

Figure 1

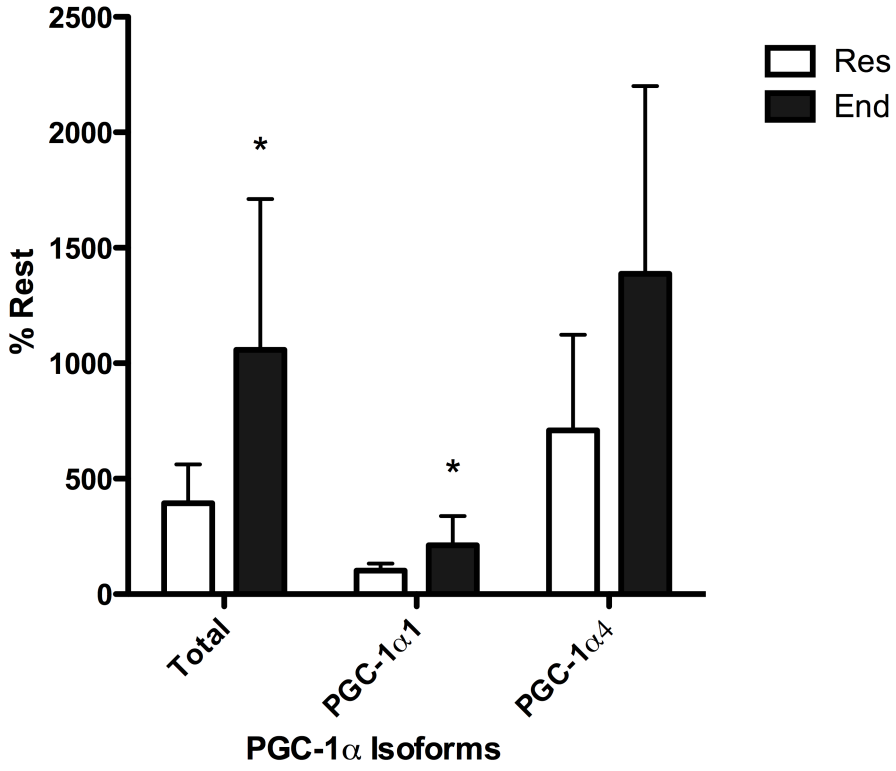


Figure 2

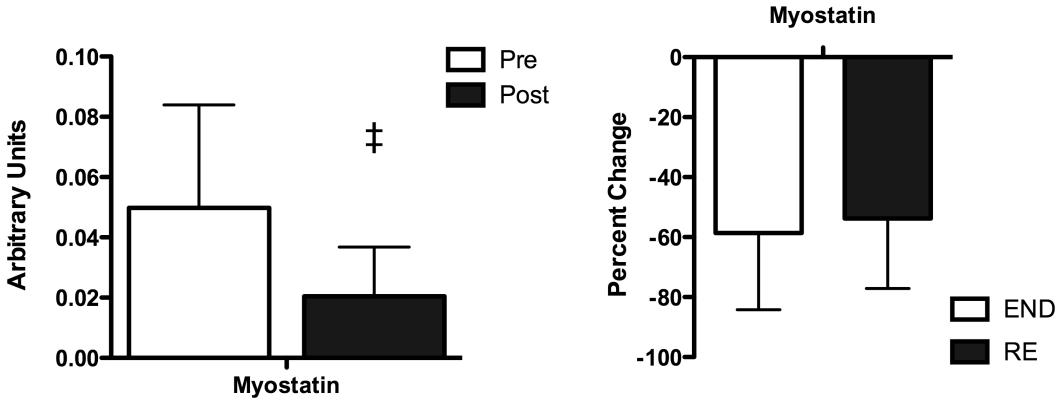


Figure 3

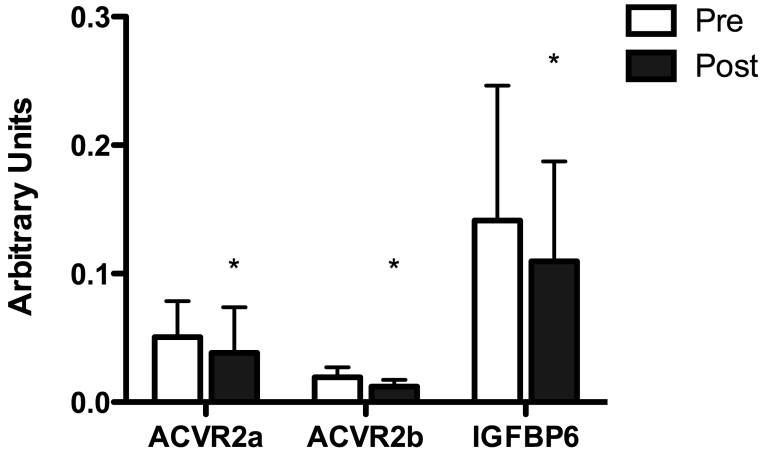
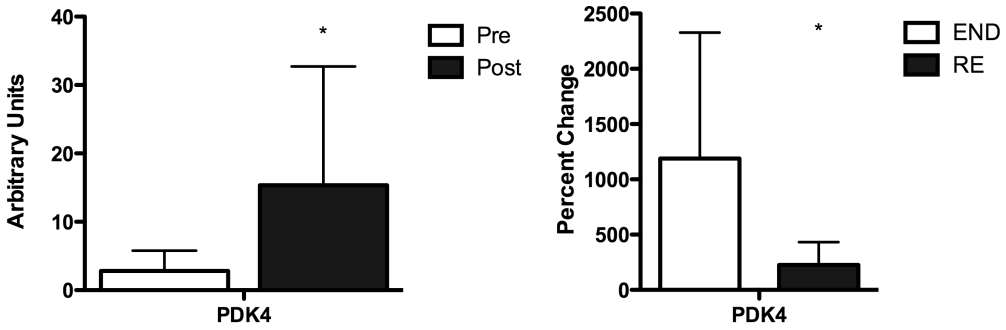


Figure 4


Arbitrary Units
40
30
20
10
0
*
Pre
Post
PDK4
Percent Change
2500
2000
1500
1000
500
0
*
END
RE
PDK4

Integrated Discussion

The maintenance of skeletal muscle mass and function is essential for a healthy, active aging process. RE is prescribed to promote the retention, or accretion of lean-body mass with age, and as a consequence, improve muscle strength and ultimately function. Despite the prominent, theorized role of the mitochondria in the aging process, relatively few studies have directly assessed the impact of RE, both acute and chronic training, on mitochondrial content and function. Given the potential links between mitochondrial dysfunction, muscle atrophy and aging, the potential for mitochondrial adaptations to contribute to improved muscle health in aging following RE are of the utmost interest.

This work specifically addressed the distinct lack of research on the acute mitochondrial effects of RE in the context of aging and holds important implications for the use of RE in the treatment of sarcopenia, the mitochondrial effects of RE, and other cellular pathways stimulated by RE that have not been well characterized in the literature. The culmination of these three, independent studies demonstrate that:

1. The acute mitochondrial response to RE does not differ with increasing age and is associated with reduced abundance of mitochondrial transcripts (ND1, ND4, CS), proteins, and DNA copy number. This reduction in mitochondrial content occurs alongside increased abundance of PGC-1α mRNA, suggesting that, in the early adaptation to RE, PGC-1α may serve cellular processes essential to the adaptation exercise independent of mitochondrial biogenesis.

2. An acute bout of RE elicits ER stress (XBP-1 splicing) regardless of age, and this stress is associated with an UPR that acts to increase the abundance of ER effector proteins (PERK, IRE-1α), transcripts (ATF6), and chaperones (Grp78).
3. Divergent modes of exercise (RE *vs.* EE) produce a common increase in total PGC-1α mRNA despite differing phenotypic adaptations. Alternatively spliced isoforms of PGC-1α are differentially regulated acutely by exercise mode such that PGC-1α1 is not induced following RE, whereas PGC-1α1 and α4 both increase following EE. Across all isoforms, EE resulted in enhanced expression of PGC-1α as compared to RE. Despite the rather large difference in exercise-induced isoform expression between modes, purported PGC-1α target genes were not differentially affected by exercise mode.

In the course of establishing these experimental findings various points of interest were uncovered relative to the existing literature on aging and the adaptations to RE, and are discussed in the subsequent sections.

Mitochondrial and muscle dysfunction persist in healthy aging

The participants used in the presented studies were active but not participating in any structured physical activity, community dwelling and were not frail. As such they would be considered relatively healthy for their age demographic. This is directly supported by the fact that lean-body mass, as determined by DEXA, was comparable between age groups in this study. Despite this there were indices of muscle and

mitochondrial dysfunction in the elderly participants. While the abundance and time-course of expression post-exercise of the majority of mitochondrial transcripts and proteins were similar between age groups, expression of ND1 and ND4 mRNA were lower, NRF1 mRNA was higher, and Complex II 30 kDa subunit protein was lower in the elderly. Despite a greater abundance of mtDNA deletions in the elderly, mtDNA copy number was not different with age. While lean-body mass was not adversely affected with age, type II muscle fibres were atrophied relative to the young, while the cross-sectional area of the type I fibres was preserved (22). In agreement with attenuated type II fibres, strength was lower by approximately 30% in all strength outcomes measures including leg press and knee extension strength, and isometric knee extension MVC. Ultimately, these data indicate that alterations in mitochondrial content occur even in healthy, aged individuals, and that lean-body mass alone does not sufficiently represent potential muscle dysfunction (type II fibre atrophy, strength loss) with age.

AMPK signaling in aging with RE

The primary hypothesis was that, as compared to young, elderly individuals would have an enhanced mitochondrial response to acute RE (41). This was based primarily on previous data suggesting preferential mitochondrial adaptations in the elderly in response to chronic RE (119), but also on the fact that an equivalent, relative exercise bout could induce elevated metabolic stress in the elderly. Even though AMPK activity may be impaired in aged muscle in response to an equivalent metabolic stress (48,149), impaired mitochondrial function and ultimately ATP production rates (50) may also accentuate metabolic stress and consequently AMPK activation (150) in the elderly following

exercise despite impaired function. This elevated metabolic stress and consequent AMPK activation may then augment PGC-1α activity (170) to promote mitochondrial biogenesis in elderly muscle to a greater extent than the young.

This hypothesis was supported by the work of Drummond et al (94) who found an increase in AMPKα Thr172 phosphorylation in the early hours following a RE bout in the elderly, a response absent in younger participants. Conversely, the data presented herein failed to demonstrate any increase in Thr172 phosphorylation on AMPK following RE regardless of age at the time point evaluated. The ability of RE to modulate AMPK abundance and activation is controversial, even when considered independent of the aging process, as equivocal data exists. Consistent with Drummond et al (94), Thomson et al (173) found enhanced AMPK phosphorylation following high frequency stimulation of the sciatic nerve in aged rats; however, two recent studies have demonstrated that AMPK is activated following acute RE in young participants as well (174,175). Consistent with the data presented here, Harber et al (176) found no change in AMPK activation following a single RE bout in young women.

The equivocal data regarding the effects of age and exercise on AMPK is a consequence of the sensitivity of this protein to a large number of factors that would require stringent control throughout the experiment. It is possible that variations in species (human, rodent), age (young, old), nutritional state (fed, fasted), post-exercise biopsy timing (1, 2 and 3h), the specific isoforms studied (α1 vs α2) and the specific parameters of the exercise bout (fatiguing, non-fatiguing; acute, chronic training) could explain the lack of congruence between multiple exercise studies regarding AMPK and

RE (48). As such, future work is required to determine the specific role of AMPK in the adaptation to RE, how specific training program variables may influence its activation, how this relates to the mitochondrial adaptations to RE, and how this may change in the context of aging.

PGC-1α, more than mitochondria

PGC-1α is regarded as a central regulator of mitochondrial biogenesis, the function of which is best understood in the adaptation to EE (177,178). As a transcriptional co-activator, PGC-1α works alongside various transcription factors including ERRα, PPARα, NRF1 and MEF2 to regulate not only expression of itself (152), but factors relevant to mitochondrial biogenesis and metabolism (153). The activity of PGC-1α is not limited to mitochondrial and metabolic domains, as various lines of evidence suggest that it can play an important role in the inhibition of protein degradation (179), the promotion of muscle hypertrophy (136), and the overall adaptation to exercise (165). Overexpression of PGC-1α can inhibit and suppress forkhead box O3 (FoxO3) function to prevent muscle atrophy (180,181) and prevent or attenuate age-associated muscle loss (87). Collectively, this indicates that PGC-1α is involved in multiple cellular processes that are involved in the maintenance of skeletal muscle health in the context of aging, and that the beneficial effects on muscle mass could exist independent of the well characterized mitochondrial effects (136). Nevertheless, the role of the mitochondria in various atrophy-producing conditions, including sarcopenia (4,36), confounds precise determination of the role of PGC-1α in alternate cellular pathways to regulate muscle mass (179), particularly with age. While this indirect reasoning may be complicated by

mitochondrial involvement, direct evidence on the interactions of PGC-1α with ATF6 to modulate the UPR (165), along with recent evidence of an alternatively spliced isoform that regulates IGF1 and myostatin (136), confirms alternate roles for PGC-1α in the regulation of muscle mass and health.

Ultimately increased PGC-1α mRNA, protein, or elevated abundance of nuclear PGC-1α acutely following exercise can no longer be used in isolation to conclude that mitochondrial adaptations will occur, particularly in the case of acute RE. In future, research into the stimulatory effects of both RE and EE on PGC-1α will need to account not only for increased abundance of PGC-1α protein and mRNA or intracellular localization, but rather take specific effort to delineate upstream signaling cascades, specific binding partners coactivated by PGC-1α and post-translational modifications to PGC-1α itself (182), as these measures may represent the level of control that determines the consequent adaptation to differing exercise modes more than simple abundance.

In addition, any efforts to determine the significance of alternate PGC-1α isoforms in the adaptation to differing modes of exercise will need to account for potential discrepancies in the acute response in both trained and untrained muscle, as well as any potential elevations in resting expression with chronic training. Contrary to the chronic training results of Ruas et al (136) who found that RE or mixed mode RE/EE but not EE induced PGC-1α4, we found that unaccustomed EE affected both α1 and α4, whereas RE only altered α4. These discrepant findings can be explained by the fact that this study utilized acute, unaccustomed RE and EE to fatigue, whereas Ruas et al (136) evaluated chronic training. The fact that both RE and EE induced a serum creatine kinase response

indicates that both modes resulted in muscle damage (22,144,183). As both RE and EE influenced transcripts related to PGC-1α4 (Myostatin, ACVR2a, ACVR2b, IGFBP6), it is possible that this is the result of stimulation of muscle repair pathways in response to damaging exercise. Consequently, as skeletal muscle habituates to the training stimulus, it is possible that EE over time results in an attenuated PGC-1α4 response, whereas RE does not. Future studies are required to determine how the transition from the trained to untrained state influences the expression of PGC-1α isoforms in response to both RE and EE.

The unfolded protein response is integral to the adaptation to exercise

Despite the fact that RE is a potent stimulus for the induction of protein synthesis (168) there is a relative dearth of investigations into how ER function, protein-folding capacity of the cell, and the UPR impacts the adaptations to RE specifically. Wu et al (165) thoroughly demonstrated the specific interactions of ATF6 and PGC-1α, and how ablation of ATF6 attenuates the beneficial adaptations to EE training; however, relatively few studies have investigated the role of the UPR in human adaptations to exercise.

Surprisingly, this data demonstrates similarities between the UPR response characterized here following RE, and the previous work of Kim et al (166) in ultra-endurance athletes having just completed a 200 km marathon. These works suggest that specific components of the UPR pathway are preferentially affected by exercise, whereas PERK may not be involved in the early adaptation to exercise. PERK functions to attenuate protein translation through phosphorylation of eIF2α, inhibiting eIF2B

catalyzed guanine nucleotide exchange. PERK-mediated phosphorylation of eIF2α also promotes the specific translation of ATF4, a UPR transcription factor that controls the expression of proteins related to antioxidant defense and the UPR in general, but also apoptosis-related transcripts (CHOP). The inability of exercise to modulate this pathway suggests that protein synthesis may be prioritized in the early adaptive period to exercise, and any associated UPR response serves to modulate chaperone abundance via ATF6 and XBP-1 to aid in protein folding following subsequent bouts of exercise. This model is also supported by Gordon et al (133) who found that untrained muscle had an elevated response of transcripts related to the unfolded protein binding as compared to trained muscle.

The acute effects of RE are not representative of chronic adaptations

The discordant findings that acute RE is associated with reduced mitochondrial transcript, protein and DNA abundance suggests that, at least from a mitochondrial perspective, the acute response is not indicative or predictive of the subsequent adaptations to training. In and of itself this is not a controversial finding, as a commonly cited limitation of exercise physiology research is that the reliance on untrained or deconditioned participants limits the applicability of experimental results to highly trained populations. There are certainly many examples in the literature where trained and untrained muscle deviate both in their structure (184,185), function (120,185-187), and in the response to acute RE (132,133,188-190). The skeletal muscle protein synthetic response (132,188,189), mitochondrial protein synthesis (132) and the overall

transcriptional response to an acute bout of RE (133) are all influenced by chronic training.

Ultimately this indicates that the cellular processes involved in the adaptive response to a single bout of RE or EE may not be indicative of pathways required for the adaptations associated with chronic training. As evidenced by the elevated serum creatine kinase response following RE and EE in Study #3 (22,183), muscle damage may explain the overlap in the transcriptional response between modes with respect to the PGC-1α isoforms and target genes; however, the fact that PGC-1α1 was unaffected by RE does indicate that the cell retains some degree of specificity acutely, suggesting these results cannot be solely explained by muscle damage alone.

It is possible that mitochondrial adaptations to RE occur in a biphasic fashion, with an acute reduction in mitochondrial content after the initial bouts, followed by subsequent increases in mitochondrial content and/or function. This is supported by the fact that various studies have shown favorable mitochondrial adaptations to chronic training (62,119,131,137,138), irrespective of age (although direct comparisons are lacking), counter to what would be expected based on the acute results presented herein. In addition Gordon et al (133) demonstrated suppression of mitochondrial and metabolic transcripts following acute RE in untrained muscle, a response absent or attenuated in trained muscle. Of particular interest in the context of these three studies, the induction of PPARGC1 was two-fold greater in trained muscle, suggesting that chronic resistance training may augment the PGC-1α mRNA response to RE. This is in agreement with Ruas et al (136) who found increased PGC-1α mRNA at rest following chronic RE and

mixed modes RE/EE training. While not RE, Wu et al (165) found that a single bout of treadmill exercise resulted in ER stress, a UPR response, and increased PGC-1α with no change in the mitochondrial transcript cytochrome *c*. Conversely, chronically trained animals, when exposed to an equivalent bout of treadmill exercise had a reduced ER stress and UPR response, a similar induction of PGC-1α, and an increase in cytochrome *c* mRNA. These data suggest that the cellular processes prioritized following acute, unaccustomed exercise may relate to muscle damage, growth and regeneration, and protein folding, whereas metabolic and mitochondrial adaptations occur following subsequent bouts. Such a process may be mediated through PGC-1α regardless of exercise mode, and the divergent adaptations that occur following either RE or EE training may be a consequence of temporally varied transcriptional co-activation.

These data suggest that mitochondrial adaptations are likely to occur following subsequent bouts of exercise (89), and that the dynamic mitochondrial response to a singular bout of RE is not representative, or predictive, of the cumulative effects of training. This agrees with existing literature that suggests that the acute response to a single bout of exercise may not hold predictive value, however basal expression of certain subsets of genes or specific genotypic differences may in fact be more predictive of adaptive potential, at least as far as strength and hypertrophy are concerned (191-194). Future research is required to clearly delineate how, over multiple bouts of exercise, different signaling pathways are affected as muscle transitions from the untrained to trained state, and the predictive value of the acute fluctuations in specific genes can predict or represent the adaptation to training.

Future Directions

The results of these three studies provide various directions for future work not only in the context of aging and mitochondrial function but also in the adaptive process to RE in general. The fact that the acute mitochondrial response is not consistent with the chronic adaptations (41,119,131) necessitates a study that characterizes the acute response and correlates these immediate alterations with the ultimate adaptations to training. Taking multiple biopsy samples following multiple bouts of exercise over the course of training is required to determine when the transition from the untrained and trained state occurs (transition from reduced to increased mitochondrial content). Such a study should account for the fact that there are two populations of elderly individuals: those who are sarcopenic and considered frail, and those similar to the participants in these studies who had comparable lean-body mass to the young. In addition, strict controls for the physical activity of the participants over multiple years, characterizing both the fed and fasted state, and addressing the role of fatigue and non-fatiguing contractions will produce a clearer picture of how RE affects AMPK activation and how this relates to subsequent mitochondrial bioenergetic signaling.

PGC-1α appears to be involved in the adaptation to RE and EE but may orchestrate different transcriptional responses depending on the mode of exercise to modulate the differential phenotypic adaptations to exercise (134,136,195). This work demonstrates, in agreement with Ruas et al (136), that variations in alternate PGC-1α isoform expression may at least partially explain this phenomenon. There are many additional upstream and downstream factors that can influence PGC-1α activity (182),

that could also contribute to the adaptations to RE and EE. A study that addresses the acute, nuclear translocation of PGC-1α and the specific interactions with various transcription factors with co-immunoprecipitation may lead to new insights into how a common increase in PGC-1α following RE and EE results in distinct phenotypic adaptations. It may be that these specific interactions provide greater insight into the adaptation to exercise than simply measuring mRNA and protein abundance, especially when a common increase occurs in PGC-1α despite differing exercise treatments.

In addition to the mitochondrial adaptations, the fact that an UPR occurs following RE requires further investigation as such a response is not well characterized in the literature with exercise in general (166), and particularly RE (133). Alterations in UPR targets occurred late in the post-exercise period in general, and perhaps it is not exercise directly that stimulates the UPR, but rather the consequent protein synthetic response following RE. Future studies that quantify both the immediate protein synthetic response, followed by the induction of ER stress (XBP1 splicing) and the UPR would explain how these pathways are interrelated, and perhaps how the UPR acts in the control of protein synthesis following RE.

Conclusion

The maintenance of skeletal muscle mass, function, and ultimately health, is an essential component of a healthy aging process. Sarcopenia places the elderly at risk for additional comorbidities associated with a sedentary lifestyle, and represents both a substantial barrier to the maintenance of an independent lifestyle and economic cost to society (2). A clear understanding of the molecular response of aged muscle to both acute

and chronic RE is essential to the formation of effective exercise prescription guidelines for the promotion of skeletal muscle health in aging. It is clearly established that RE is a safe and effective treatment for age-associated muscle dysfunction that promotes increased muscle mass and strength (91,92); however, it is not known precisely how RE interferes with the molecular pathologies associated with aging (196). It is not reasonable to assume exercise prescriptions based on young populations are suitable for the elderly or clinical populations (5), and evidence certainly exists that aging can impact satellite cell activity (22,144), the protein synthetic and signaling response to RE (94-97), that may act to attenuate the hypertrophic response (197,198). While the ultimate mitochondrial adaptations to RE training may be enhanced in the elderly (41,119), the acute response is not congruent with the adaptations to chronic training, and the dynamic mitochondrial response following RE is not impaired with age.

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