# IS EXERCISE-INDUCED REDOX SIGNALING AFFECTED BY AGING?

By Ethan L. Ostrom

A Dissertation

Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

in Biological Sciences

Northern Arizona University

April 2021

Approved:

Tinna Traustadóttir, Ph.D., Chair

Kiisa Nishikawa, Ph.D.

Archana Varadaraj, Ph.D.

Derek Sonderegger, Ph.D.

## ABSTRACT

### IS EXERCISE-INDUCED REDOX SIGNALING AFFECTED BY AGING?

Ethan L. Ostrom

Redox signaling dysfunction is a key characteristic of aging cells and tissues and has wide ranging cellular and physiological effects. Exercise is one of the best tools available to promote healthy aging and reduce disease burden, however some evidence suggests exercise is not as effective in older populations. This is likely driven by redox signaling dysfunction because of the critical involvement of redox signaling mechanisms in exercise adaptations. In this work, I have used several studies in humans and mice with multiple methods to elucidate the mechanisms of Nrf2-mediated redox stress response to exercise and the effects of aging on this response. I demonstrate that exercise induced Nrf2 activation is impaired in older compared to younger adults, and that exercise training only partially reverses these effects. Redox stress responses to a non-exercise stressor, a forearm ischemia-reperfusion challenge, is not different between ages, but exercise training does improve the response compared to controls in humans. Finally, acute contractile stimulation of skeletal muscle increases Nrf2 signaling within the muscle, and high intensity stimulation activates Nrf2 in contralateral unstimulated muscle, but low intensity stimulation does not. These effects appear to occur through inhibiting the negative regulator, Keap1. Taken together, these data indicate that exercise is a powerful inducer of the redox stress response transcription factor, Nrf2, but that aging impairs our ability to respond to an acute exercise bout. Future work should aim to discover and utilize therapeutics that act synergistically with exercise to restore redox homeostasis and redox signaling function.

## ACKNOWLEDGEMENTS

I would like to thank my advisor, Tinna Traustadóttir, for her continued support throughout this process, and my committee members, Kiisa Nishikawa, Archana Varadaraj, and Derek Sonderegger, for their expertise and guidance. Without all of you, it would have not been completed. Thank you. I would also like to thank our collaborators for engaging discussions and suggestions through this process. These have been instrumental to my development as a scientist and collaborations that I hope continue into the future. Specifically, I would like to thank the late Nate Nieto, Victor Jimenez, Fernando Monroy, Aimee Eggler, James Cobley, Ana Valencia, and Dave Marcinek, all of whom have shaped my work in various important ways. Thank you.

To my family and friends, thank you for always supporting me and my passions. I know you may not understand exactly what I do, but that hasn't stopped you from helping when you can and being there for me when I need you. Words cannot express my gratitude for such a wonderful support system. Thank you.

Lastly, to my wife, Nikki, your love and support have always meant the most to me, and through this process, despite my ups and downs, despite the long nights and lonely weekends, you have always made me feel loved and supported. For that I will always be grateful. Thank you.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## DEDICATION

This work is dedicated to my wife, Nikki, my dog Ziggy, and my family, Mom, Dad, Marleigh, John, Haisely, and Henry. I love you, thank you for everything.

## PREFACE

Three chapters in this dissertation are formatted for submission as original research articles in peer reviewed journals (chapters 2-4), and one chapter is formatted for publication as an invited book chapter (chapter 5). Each of the original research articles is composed in the following format: Title, abstract, introduction, methods, results, discussion and conclusions, acknowledgements, references. Tables and figures are embedded within the text next to relevant sections. My research interests are in aging, redox biology, and stress response signaling. My motivation for studying the redox biology of aging and exercise are to understand and improve healthy aging in the world. My dissertation work has focused on using various intensities of acute exercise to measure redox stress responses in human Peripheral Blood Mononuclear Cells (PBMCs) as well as mouse skeletal muscle. I have also used forearm ischemia-reperfusion as models of acute stress in young and older humans to investigate redox stress resilience in aging. Exercise training was used as an adaptive stimulus to change the dynamic response of redox signaling and improve stress resilience in aging humans. My focus has been on the inducible redox stress response transcription factor, Nrf2, and its responses to acute exercise and exercise training. Previously, our lab showed that Nrf2 signaling is blunted in older individuals after acute exercise. My dissertation work is corroborating these findings, showing that even after exercise training, older adults have an impaired signaling response to acute exercise compared to their younger counterparts. This impaired response has been shown by others *in response to different stimuli*, indicating a

compromised redox signaling mechanism instead of a lack of sensitivity to a given stimulus.

Chapter 1 is a comprehensive literature review of Nrf2 signaling in aging and response to exercise. In this chapter, I discuss the overarching themes and concepts as they relate to Nrf2 signaling and responses to exercise in aging, using literature from cell culture work, animal models, and humans. Chapter 2 is the first manuscript published in my dissertation work, focusing on the time course responses of Nrf2 and its downstream targets in response to acute exercise and after exercise training compared to a control intervention. In this study, we show that Nrf2 signaling is blunted in aging PBMCs but that training reverses some of this dysfunction. One interesting finding here is that the magnitude of improvement with training in younger individuals ~~is~~ being significantly better than older adults. Together, these results suggest that training improves responsiveness in older adults, but not at a level that is comparable to young individuals. it is still not able to improve to the same degree as young individuals.

Chapter 3 discusses the effect of exercise training on a non-exercise redox stressor, the ischemia / reperfusion trial (I/R Trial). We show that training improves the responses to an I/R trial regardless of age or sex. This suggests that older adults retain some of their ability to withstand a non-exercise redox stressor even though other redox signaling mechanisms like Nrf2 signaling are compromised, and that these two measures, although broadly redox specific, may not necessarily overlap in their cellular or molecular mechanisms.

Chapter 4 investigates the effects of high and low intensity stimulation on redox stress responses in mouse skeletal muscle. We show divergent signaling responses in high versus low intensity contractile stimulus on Nrf2 and Keap1, the Nrf2 negative regulator. High-intensity stimulation elicits a systemic effect, where low-intensity stimulation does not. High-intensity stimulation activated Nrf2-ARE binding and decreased Keap1 in both stimulated and unstimulated limbs where low-intensity stimulation only activated Nrf2-ARE binding and decreased Keap1 in the stimulated limb.

The final chapter 5 is the culmination of this body of work, providing an updated working model of exercise-induced redox signaling, based on the newest and most cutting-edge research in the field and the results from my dissertation work incorporated into the model. This dissertation adds to the field of redox biology, exercise, and aging through the combination of a quality randomized controlled trial, and the introduction of several novel redox signaling concepts in the field, detailed in chapter 2, 4, and 5.

## CHAPTER 1 – Literature Review

According to the CDC, the number of people above the age of 65 will double by the year 2050 totaling approximately 89 million Americans [1]. Advanced age is associated with reduced physical and mental functioning, as well as a plethora of chronic diseases including cardiovascular disease (CVD), type 2 diabetes (T2D), dementia, and cancer [2, 3]. Individuals who have one or more of these diseases have a reduced quality of life and many struggle with basic activities of daily living. Furthermore, the economic and social burdens of age-associated diseases are very high [3]. An estimated 14 million people die of a chronic disease every year; CVD, cancer, and diabetic complications make up 34% of these deaths [4]. These statistics underscore the importance of understanding the underlying mechanisms of aging to provide efficacious therapies to enhance successful aging.

Biological aging is multifaceted and a highly complex process. It is characterized by several cellular and physiological changes, including: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication [5]. The reason redox dysfunction is so important in aging and gerontology is because redox biology mediates all of the biological aging mechanisms listed above, so molecular targets that control redox regulation would likely be a viable therapeutic option to promote healthy aging at a mechanistic level [6-12]. Utilizing a lifestyle intervention that is effective in promoting and maintaining redox balance would be ideal, as lifestyle interventions are the cheapest, yet most effective when considering the cellular mechanisms of aging [13, 14].

Exercise is a powerful physiologic stressor, and potentially the best intervention for promoting healthy aging and reducing the risk of developing age-associated diseases [13, 15, 16]. It is well established that physical inactivity is a key driver in the development of these common diseases [13]. Conservative estimates indicate that physical inactivity costs 53.8 billion dollars worldwide due to CVD, diabetes, cancer and dementia [17]. Increasing physical activity and exercise would not only improve healthy aging and quality of life, but also reduce healthcare costs. It is unequivocal that exercise provides pleiotropic personal health and societal benefits. Accumulating evidence suggests that the beneficial effects of exercise act in part through redox-dependent mechanisms [18-20]. However, the mechanisms of adaptation in the antioxidant defense system in response to exercise is still not well understood and is a crucial area in redox biology and aging research.

**The Free Radical Theory of Aging**

For decades, aging researchers have tried to understand the underlying mechanisms driving aging, disability, and dysfunction. One of the fundamental components of aging and disease is oxidative stress. Denham Harman first attributed the damaging effects of free radicals and reactive oxygen species (ROS) in his seminal paper in 1956 as a molecular mechanism that led to aging [21]. The free radical theory of aging proposed that ROS damage cellular constituents, making it harder for cells to carry out necessary functions and to recycle macromolecules. The damaged proteins, lipids, and DNA accumulate with age, causing dysfunction and disease. This theory explicitly implied that all free radicals are harmful and need to be extinguished before they can exert their damaging effects on cellular machinery. As a result, investigators tried supplementing

small molecular antioxidants like vitamin C and E to counter the “damaging” effects of ROS and oxidative stress. Many of these studies did not produce significant effects on mortality or improve function in healthy individuals [22]. In fact, a meta-analysis has shown that vitamin C and E supplementation have detrimental effects on mortality risk [23].

These findings do not seem to fit with the free radical theory of aging; however, it should be noted that there is a key mechanistic difference between small non-enzymatic antioxidants like vitamin C and E and endogenous enzymatic antioxidants. Vitamin C and E do not employ any specificity when reducing free radicals, they reduce any free radicals they contact. However, the endogenous system has specific enzymes that reduce certain free radicals. This is important because the cell has the ability to modulate enzyme activity by post-translational modifications, especially enzymes with a long half-life. This allows enzymes to operate at just the right activity level for the specific cellular level of ROS, providing enough control to minimize imbalances and damaging effects of ROS while allowing appropriate redox-sensitive cell signaling to endure. There is no such control mechanism with vitamins C and E in the cellular milieu.

With these findings in mind, researchers set out to find which antioxidant enzymes were essential for improving health and longevity. Perez and colleagues used transgenic manipulations to test the effects of the antioxidant defense system on longevity of animal models. Interestingly, out of 18 genetic manipulations in the antioxidant defense system, only one (knockout of superoxide dismutase, SOD) showed a significantly decreased lifespan compared to control mice [24]. While this does suggest SOD is a critical enzyme for cellular health and longevity, these findings do not seem to support the free radical

theory of aging. We would expect most manipulations of antioxidant enzyme gene expression to have a significant effect on lifespan (positive effect-overexpression or negative effect-knockout). These findings forced researchers in the field to re-evaluate the free radical theory of aging.

It could be argued however, that only assessing the lifespan of an organism misses the point of aging research. Perez & colleagues did not address whether the transgenic manipulations increased health span and quality of life in these rodents. Perhaps a hard question to answer, but surely there are objective ways of measuring quality of life or physical function in animals. The point is, no one lives forever; so, should we allocate time and resources to finding eternal youth, or should we focus on research that promotes living well? We would argue the underlying biology of health span, physical function, cognitive function, and overall quality of life should be the primary focus of research and therapeutic interventions. A successful intervention is one that compresses the years of life spent in a disease state. It is not a given that an intervention that increases lifespan will also increase health span [25]. So, focusing solely on interventions that increase lifespan seems naïve, especially when considering the calamitous economic consequences of increasing lifespan without concomitantly increasing health span [25].

Since these findings by Perez and colleagues (2009), there has been a perspective-shift in redox biology. More studies have focused on the antioxidant defense system and redox homeostasis as a framework for interrogating aging and age-associated diseases. While free radicals and ROS are damaging to macromolecular structures, this does not necessarily lead to aging or death, and these chemicals are a healthy part of cell signaling [26]. Furthermore, the series of investigations from Perez and colleagues were conducted

on enzymes that directly reduce free radicals and ROS in our bodies. It is possible that these enzymes are not the most important or critical enzymes in the system.

It is unlikely that transgenic or knock out mice in any single antioxidant gene is sufficient to produce a significant change in longevity because there are redundancies in the system that can compensate for any experimentally induced deficiencies. Perhaps a needed follow up experiment to Perez et al. (2009) is to experimentally compromise the whole endogenous antioxidant defense system using several knockout genes. This may tell us more about the importance of redox balance with respect to aging and disease. Despite the limitations of the study by Perez and colleagues, this research was a key step in shifting the way aging research looked at ROS and the antioxidant defense system.

In an attempt to reconcile these findings, other research groups have updated the aging hypothesis, termed the redox stress hypothesis of aging. This hypothesis suggests that as we get older, our cells slowly shift to a more oxidized state, affecting protein thiols as well as redox sensitive molecular switches and cellular signaling [26, 27]. Proteins are sensitive to redox perturbations through cysteine sulfhydryl residues [26]. These sulfhydryls act as allosteric modification sites that are reversible, much like the phosphorylation/dephosphorylation of proteins to activate or inactive them. These cysteine “switches” change the conformation of a protein, thereby modulating its activity and function [26]. Many redox-sensitive proteins and signaling systems have been shown to be altered in aging and disease states which are independent of ROS production [28]. Furthermore, there is evidence to show redox couples like GSH:GSSG and Cys:CySS shift to a more oxidized state and are important indicators of a pathologic cellular milieu found in aging and disease states [27-29].

The molecular underpinnings of this hypothesis by Sohal and Orr (2012) led to a more comprehensive and nuanced theory of aging: the “cell signaling disruption theory of aging” proposed by Gomez-Cabrera and others [12]. The authors developed this theory because the free radical theory of aging has been disproven by multiple studies including the work by Perez and colleagues. Mainly two concepts disprove Harman’s (year) initial free radical theory. First, exceptionally long-lived animals such as the naked mole rat show elevated levels of oxidative stress throughout life [30], and second, antioxidant supplementation does not improve longevity or health span in other animals [12]. If the transient increases in oxidative stress or redox stress can be handled by the cell’s antioxidant defense system, then the increased ROS will result in improved cellular function. However, if oxidative stress is above and beyond the cell’s defenses, it will result in deleterious effects. It would then follow that supplementing with antioxidants would not allow the signaling to occur that is mediated by ROS. This theoretical framework fits all the data available for a modified free radical theory of aging, where ROS and oxidative stress can be adaptive or pathological.

**Redox Imbalance and Aging**

The slow but steady increase in redox imbalances with age has been linked to impaired Nrf2 signaling [31]. Nrf2, also known as Nuclear factor (erythroid derived 2)-like 2) or NFE2L2 is an inducible transcription factor that responds to electrophilic or oxidative stressors. Paraquat, a mitochondrial toxin that induces an oxidative stress, was used as a model to test the redox stress resilience of older and younger *C. elegans* and human fibroblast cells [11]. Older worms and fibroblasts showed increased ROS compared to the young at baseline. Interestingly, when treated with paraquat, the older worms and

fibroblasts showed no significant changes in ROS levels. This was accompanied by no significant changes in redox regulated stress responses: phosphorylated-AKT, phosphorylated-Adenosine Monophosphate Kinase (p-AMPK), phosphorylated-Extracellular signal Related Kinase 1/2 (p-ERK1/2), Nrf2 induction, or downstream targets. Younger cells and worms responded robustly to the stress in each of the measured redox-sensitive signaling molecules. Furthermore, younger cells and worms showed increases in proteasome activity and increased mRNA of chaperone proteins compared to older cells and worms. The ability to respond to redox stressors and imbalances, bringing the organism back to homeostasis is important for being able to maintain homeostasis and respond beneficially to future stressors. This response is compromised in aging. This concept adds to the redox theory of aging, suggesting that the response to a stressor and the ability to adapt is highly dependent on basal expression of stress response proteins and that these factors are compromised with aging.

Human and Rat Lens Epithelial Cells (LECs) also show age-related deficits in Nrf2 induction. Older cells have a reduced Nrf2 binding capacity to the promoter of the antioxidant enzyme Peroxiredoxin 6 (Prdx6). The impaired DNA binding capacity in older cells was ameliorated with sulforaphane (SFN) treatment. SFN, a potent Nrf2 inducer, showed a robust dose-dependent increase in DNA binding in younger and older cells compared to baseline. Interestingly, there is still a clear and statistically significant difference between the amount of Nrf2 activation between younger and older cells after SFN treatment. This was directly related to the amount of Nrf2 bound to its site-specific enhancer region of the DNA called the antioxidant response element (ARE) found in all promoter regions of the genes it regulates [32].

These results are corroborated by recent work on younger and older Human Bronchial Epithelial Cells (HBECs). HBECs were also treated with sulforaphane followed by measurements of Nrf2, Bach1, and c-myc [33]. The results showed that older cells had an impaired ability to respond to the SFN stress, indicated by lower levels of Nrf2 signaling and downstream mRNA transcripts [33].

The effect of age on Nrf2 signaling is also shown in experimentally induced Nrf2 knockouts using murine models [34]. Older $Nrf2^{-/-}$ mice showed impaired antioxidant proteins in skeletal muscle, which would be expected. Interestingly though, young $Nrf2^{-/-}$ mice showed normal levels of antioxidant enzymes aside from significantly lower expression of one enzyme, NQO1 [34]. So even in the complete absence of Nrf2, there are minimal effects until later in life. Taken together, impaired redox homeostasis is a key characteristic of aging cells but not young cells, even in experimentally induced deficiencies such as $Nrf2^{-/-}$ mice [34]. Furthermore, these age-associated deficiencies manifest as the inability to respond to a stressor because of blunted Nrf2 inducibility.

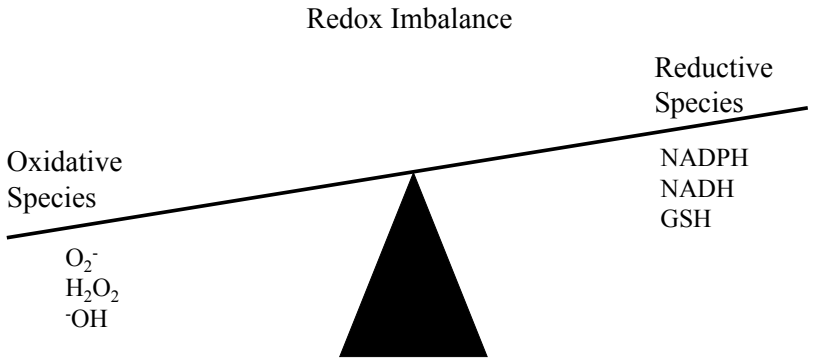



**Figure 1.** Redox imbalances caused by high amounts of ROS or low amounts of reducing agents. Note: The balance can shift to a state of reductive stress if the reductive species overwhelm the oxidative species in the cell.

An important note is that all of these studies, aside from Miller et al. [34], used a stressor to trigger Nrf2 activation. Together, these data suggest that basal levels of redox balance may not necessarily be different between young and older and highlight the importance of experimentally challenging the system. The cellular response to a stressor gives much more relevant information about the functional capacity of the organism. Furthermore, the *time* it takes a cell or organism to respond to a stressor can be another useful indication of an individual's ability to respond to redox perturbations. Studies from our lab suggest that older individuals do not respond as quickly to a redox insult like forearm ischemia reperfusion as younger individuals [35, 36]. This is not to say that older adults are always in a state of redox imbalance, rather the *rate* at which they respond is compromised. Our findings in humans are also supported by recent cell culture studies [11, 33, 37].

The underlying issues seen with the decay of redox homeostasis is that redox-sensitive pathways are somehow compromised with age, leading to decreased cellular protection

and inability of the cell to adequately respond to stressors [10, 38]. These redox imbalances are evident before pathology of a disease or age-associated dysfunction is readily detectable. The cellular milieu shifts to a more oxidized state with age and may be the driving factor in these diseases [7, 28]. These imbalances resulting in increased levels of oxidative stress are seen in CVD and heart failure [39, 40], diabetes [41] cancer [42, 43] and dementia [44]. These diseases are some of the most prominent diseases with the highest mortality rates in our society today [4, 13, 45-47]. In fact, the *common soil hypothesis* implicates redox perturbations as the shared driving mechanism in the progression of type 2 diabetes and cardiovascular disease [46]. These data taken together suggest redox imbalances with aging are important and possibly causal factors for disease prevalence. It also underlines the importance of therapies that target redox dysfunction with a particular focus on therapies that can restore redox homeostasis in aging and diseased populations.

While most researchers have primarily focused on redox imbalances in the context of oxidative stress, it is important to understand the pendulum can swing in the other direction. Recently, a mouse model of constitutive Nrf2 activation has been developed [48]. These mice display hyper-activation of Nrf2-ARE transcripts, and as a result suffer from reductive stress; the redox imbalance resulting from an over-production of reductive species versus oxidative stress (Figure 1), which also has pathological traits in cardiac tissue remodeling. This is the first example of an experimental model of reductive stress and, taken with the evidence cited above, provides good basis for the concept of redox balance [48]. Furthermore, this indicates that promoting redox balance (not just mitigating

oxidative stress but also reductive stress) might be a strong therapeutic target for aging and disease states.

The idea of redox imbalance or dysfunction has been increasingly accepted as our understanding of the importance of ROS as a primary messenger in cellular signaling pathways has advanced. Redox imbalance more aptly describes imbalances stemming from the interactions between ROS and the antioxidant defense system on cellular signaling. Redox imbalances could be in the context of oxidized/reduced protein thiol residues or oxidized/reduced lipids. Both varieties of molecules can lead to altered cellular signaling without any indication of increased oxidative or reductive stress measured by traditional markers and methods [29]. While oxidative stress has been studied extensively at this point, both in experimental models and observed in clinical populations, there are few research articles on reductive stress [49, 50]. Further work in this area needs to be done to elucidate the reductive stress concept in clinical populations if it exists. Generally, shifts in the redox state of the cell have effects on any and every protein that can be oxidized or reduced, changing cellular signaling that could lead to a pathological state [28, 29].

**Nrf2 Transcriptional Activation**

***Nrf2 Activation Mechanisms***- Nrf2 is an inducible redox stress response transcription factor that is ubiquitously expressed basic leucine zipper transcription factor with a cap ‘n collar structure [51]. Moi and colleagues (1994) first described Nrf2 while studying Nuclear Factor Erythroid 2, a different transcription factor that has a high degree of homology with Nrf2, thus the name: Nrf2 [51]. It has since been demonstrated that the

biologically active form of Nrf2 is 90-110kDa and it has six evolutionarily conserved protein domains called Neh domains [52]. In unstimulated conditions, Nrf2 is bound to its cytosolic repressor Keap1 (Kelch-like ECH-Associated Protein 1) whereby Keap1 ubiquinates Nrf2 and targets Nrf2 for degradation via the proteasome [53]. When Nrf2 is activated, it dissociates from Keap1 and translocates to the nucleus where it can bind to Antioxidant Response Elements (ARE's) in promoter regions of target genes [53]. This binding of Nrf2 to the enhancer regions increases the probability of the transcription machinery forming a complex to transcribe mRNA of the specific gene downstream of the ARE. Nrf2 regulates hundreds of different genes, many of which are involved in the antioxidant defense system, drug metabolism, and cytoprotection [54, 55].

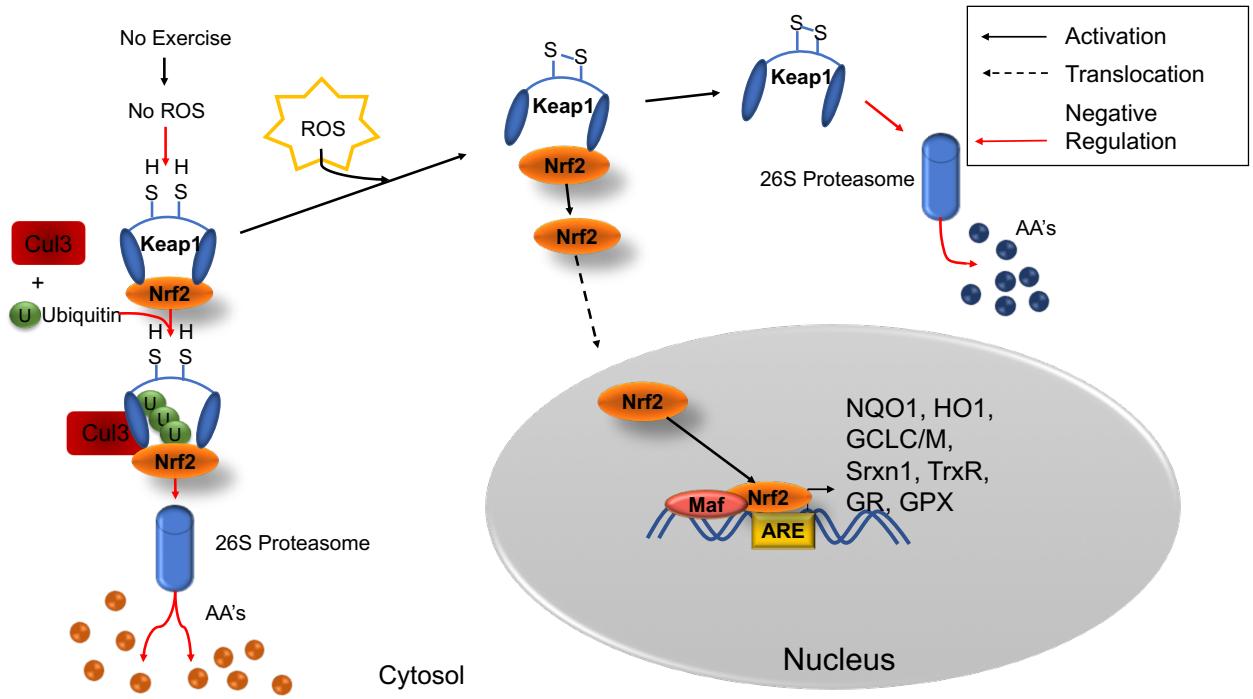



**Figure 2.** Nrf2 signaling mechanisms. During unstressed conditions, Nrf2 is targeted for degradation in a ubiquitin-CUL3 dependent process. Elevated reactive species in the cell react with Keap1 cysteine residues, causing a conformational change and release of Nrf2 from Keap1 and proteasomal degradation. Nrf2 translocates to the nucleus where it heterodimerizes with small Maf proteins and increases gene expression of antioxidant target genes by binding to the ARE/EpRE.

***Kelch like ECH-Associated Protein 1 (Keap1)-*** Keap1 (Fig. 2) is the primary negative regulator of Nrf2 and binds to Nrf2 as a homodimer [56]. Keap1 is an adapter protein for the E3 ubiquitin ligase, cullin 3. This interaction allows Cul3 to transfer ubiquitins onto Nrf2, targeting Nrf2 for degradation by the 26S proteasome under normal unstressed conditions. During an electrophilic or oxidative stress, cysteine residues on Keap1 are oxidized causing a conformational change in the Keap1 protein, not allowing Nrf2 to be ubiquinated. Nrf2 is then able to dissociate from Keap1 and translocate into the nucleus where it can bind to ARE sequences and increase transcription. Increases in reactive oxygen/nitrogen species (RONS) within the cell oxidize specific sulfhydryl side chains on cysteine residues in Keap1 that cause a conformational change and subsequent

dissociation between Keap1 and Nrf2 [56-58]. These conformational changes can result from intra- or inter-molecular disulfide bridges, which are facilitated by small molecular oxidants like hydrogen peroxide ($H_2O_2$), nitric oxide (NO), alkenals, and other oxidizing agents [59, 60]. Furthermore, Keap1 cysteines can also be modified by electrophilic attack with small phytonutrient chemicals like sulforaphane [61-63]. Each of these different types of stressors that act on Keap1 cysteines allows Nrf2 to accumulate into the nucleus where it heterodimerizes with small Maf proteins to bind to the ARE and increase downstream transcription [64, 65].

After the dissociation of Nrf2 from Keap1, Nrf2 is phosphorylated on Serine 40 via the serine threonine kinase PKC [66-69], and acetylated via CBP/p300 [70]. The involvement of MAP Kinases on Nrf2 activation mechanisms have been equivocal [71-75]. Therefore, further research is needed in cell culture and animal model studies to understand the breadth of Nrf2 activation mechanisms.

Additionally, there are signaling mechanisms that suppress the activity of the negative regulators of Nrf2. It seems that these negative regulators are equally important when considering both the total Nrf2 cellular content as well as the active form of the molecule that is allowed to translocate to the nucleus and induce transcription. Xue and colleagues (2014) showed that Nrf2 undergoes a cyclical nuclear translocation pattern, and increased transcripts of downstream targets are dependent upon increasing the frequency of Nrf2 cycling into and out of the nucleus [76]. This was the first study to show that increasing Nrf2 translocation frequency is an important factor for increasing downstream gene expression. Increasing the nuclear import is not enough to cause sustained activation of Nrf2-ARE signaling, but that negative regulators and nuclear

export are just as important for the increases in ARE transcripts. Glycogen Synthase Kinase 3β is one molecule that acts as a negative regulator of Nrf2. GSK3β activates Fyn, a kinase that phosphorylates Nrf2 on Tyrosine 568 causing nuclear export and inhibition of ARE transcription [77]. Thus, it would seem that also targeting Nrf2 negative regulators that impair nuclear import or induce nuclear export without degradation may increase Nrf2-ARE transcripts by increasing the frequency of Nrf2 cycling into and out of the nucleus.

***Short and Long-lived Models: Effects of Nrf2 Signaling and Redox Balance -*** Hutchinson-Gilford Progeria Syndrome (HGPS) is a disease caused by a mutation in LMNA gene that codes for the nuclear envelope protein Lamin A [78]. This disease is characterized by premature aging, cellular senescence, and hardening of the arteries, which increases the risk for CVD and stroke. Antioxidant defense system dysfunction contributes to the progression of this disease [78]. The sequestration of Nrf2 by pre-lamin protein in the nucleus prevents the Nrf2-mediated ARE binding causing significant reductions in antioxidant enzyme expression [78]. Treatment with sulforaphane, an Nrf2 activator found in cruciferous vegetables, improves proteasomal activity in HGPS fibroblasts [79]. The proteasome system is regulated through redox sensitive cysteines via Nrf2-ARE signaling [80-82]. These results suggest Nrf2 is a key regulator of disease progression in the accelerated-aging disease HGPS.

Given the increased levels of oxidative stress in aging and disease progression, it seems logical that impairments in Nrf2-ARE signaling would be present. This has been explored with respect to diabetes, CVD, neurodegenerative diseases, and cancer [83-87]. Evidence for the importance of Nrf2 comes from genetic manipulations of the Nrf2 gene

as well as observations of Nrf2 activity in long lived model organisms. Genetic knockout of Nrf2 homologs in *C. elegans* and *Drosophila* show elevated oxidative stress, and shorter lifespans than their wild type counterparts [88, 89]. Over expression of SKN-1 (the Nrf2 homolog) in *C. elegans* provides protection against oxidative stress and results in significantly greater lifespan. Additionally, overexpression of Nrf2 homolog CncC in *Drosophila* provides increased protection against oxidative stress and increased survival rates. Increased resistance to oxidative stress and survival rates are also shown in Keap1 knock out animals. Flies heterozygous for Keap1 (+/-) showed 8-10% increase in lifespan compared to their wildtype counterpart due to increased CncC activity [89]. These results are corroborated in the naked mole rat, which has lower amounts of Keap1 and β-transducin repeat-containing protein (βTrCP), which also targets Nrf2 for degradation [90]. In summary, animals who either have overexpression of Nrf2 and their homologs, or mild impairments in the negative regulator Keap1 and βTrCP show greater protection against oxidative stress and redox imbalance, and significantly increased lifespan.

Long lived animal models show remarkably similar phenotypic traits to models with experimentally induced increases in Nrf2 signaling [90, 91]. Conversely, models of aging pathology, and chronic disease have similar characteristics as models of Nrf2 knockout, and inhibition of Nrf2 [92, 93]. Interventions used to activate Nrf2 have been successful in improving redox balance and reducing oxidative stress levels in aging models and long-lived animals [40, 91]. Taken together, the evidence is mounting for Nrf2 activation as a viable therapeutic target for increased lifespan and health span [30, 40, 90].

***Antioxidant Response Element (ARE) -*** Nrf2 targets hundreds of different metabolic, antioxidant, xenobiotic and detoxicant enzymes [30, 54, 94] through binding the

antioxidant response element (ARE) sequence found in promoter regions of Nrf2 target genes. The ARE sequence seems to be conserved across many species, however there are still many ambiguities about its exact sequence [95]. The modeling approach performed by Wang and colleagues used 57 experimentally derived functional ARE sequences to create a database for functional polymorphisms in the ARE sequence. A position weight matrix was used to determine the importance of each nucleotide for binding Nrf2 in the 21-base sequence [95]. The data suggests that the ARE sequence with the strongest binding of Nrf2 is: TGACTCAGCA [95]. These researchers also show that the CC allele in the ARE sequence upstream of hemoglobin epsilon 1 (*HBE1*) gene has a higher expression level compared to the CT, TT, GC, and GG alleles. Other investigations indicate Nrf2 polymorphisms also affect the interaction and binding capacity of Nrf2 to the ARE. These may be functionally relevant SNPs in disease risk and outcomes for patients [96]. Functional polymorphisms provide a mechanistic explanation for individuals who may be more or less prone to inducible expression of beneficial target gene transcription through Nrf2-ARE signaling. This is an area of research that needs further attention and has great potential for application to health and disease.

Another key mechanistic factor in ARE activation is the heterodimerization of small Maf proteins. Igarashi and colleagues were the first to show that the family of small Maf proteins forms heterodimers with Nrf2 to allow ARE binding and gene transcription [64]. It was later shown that the heterodimerization was required for transcription of target genes, indicating the necessity of the heterodimerization of Maf and Nrf2 for proper functioning [97]. These small Maf proteins are ubiquitously expressed in mammals, while different isoforms are expressed differentially between tissues. It is not known whether

exercise has effects on the expression and activity of Maf proteins.

Overall, we are beginning to understand the molecular mechanisms of Nrf2-ARE activation. A more complete understanding will translate to a more precise prescription of lifestyle interventions to maximize the activity and health benefits this evolutionary conserved system brings. While Nrf2 plays a role in transcribing hundreds of different genes, several key enzymes have emerged from the literature as being highly Nrf2 dependent and key rate limiting steps in synthesis of compounds in the antioxidant defense system. These enzymes will be discussed in greater detail below.

***NADPH Quinone Oxoreductase 1 (NQO1)***- NQO1 is a flavoenzyme that uses NADPH to reduce quinones and other aromatic compounds. It reduces single electron transfers that create semiquinones and lead to ROS production. This enzyme is especially important for ischemia-reperfusion (I/R) injury. Reperfusion following an ischemic event increases semiquinone production in the electron transport chain. Semiquinones readily release their electrons directly onto molecular oxygen causing increased superoxide generation characteristic of reperfusion injury. The increase in free radical generation propagates throughout the cell via semiquinone oxidation causing damage and oxidative stress. NQO1 directly reduces these semiquinones extinguishing the free radical propagation before they can generate superfluous amounts of superoxide radicals. Activation of NQO1 significantly reduced oxidative stress and reperfusion damage in a mouse model of renal I/R, while NQO1 knockout exacerbated the injury [98].

Activation or genetic knockout of Nrf2 show similar effects as overexpression or inactivation of NQO1 in I/R induced injury in mouse liver [99]. These data suggest that

even if there is an intact NQO1 gene and protein in the cell, the inducible expression of NQO1 gene is important for responding to such stressors [99]. Furthermore, it is apparent that NQO1 expression is heavily dependent on proper Nrf2 induction and signaling [100, 101]. It is apparent that NQO1 is a key cytoprotective enzyme, and that Nrf2 is a necessary part of NQO1 mediated cytoprotection to cellular stressors like ischemia reperfusion.

Aging cells have shown higher levels of NQO1 mRNA expression but lower protein content than young cells. Furthermore, older cells had an impaired ability to upregulate gene expression in response to a Nrf2 activator compared to younger cells [33]. Acute exercise has been shown to increase NQO1 gene expression in the myocardium of young mice [102] and furthermore NQO1 protein content was increased in mice after 6-weeks of moderate exercise training [18]. NQO1 is an important cellular defense protein regulated by Nrf2 induction. Increasing NQO1 expression is an important step to improve cellular redox status and reduce oxidative stress insults.

***Heme Oxygenase 1 (HO-1)***- Heme metabolism is very important for the turnover of heme containing proteins like hemoglobin, and cytochrome p450, and mitigating injury from heme toxicity, or ischemia-reperfusion [103]. HO-1 catalyzes the metabolism of heme using molecular oxygen and oxidation of NADPH to create biliverdin, an iron ion, carbon monoxide, and a water molecule [104, 105]:

$$\text{Heme} + O_2 + \text{NADPH} + H^+ + \text{HO-1} \rightarrow \text{biliverdin} + Fe^{2+} + \text{CO} + \text{NADP}^+ + H_2O \qquad (1)$$

Carbon monoxide has a vasodilatory effect in vascular tissue and the reduction of an iron ion mitigates the transfer of electrons in redox couples with heme [105]. HO-1 induction or overexpression has several different health benefits including improved vascular function, greater resistance to oxidative stressors, and increased vascular growth [105].

Nrf2 induces HO-1 gene expression in several cell lines and in coordination with other inducible transcription factors [33, 106-108]. Interestingly, aged organisms show an increased basal expression of HO-1, while the inducibility of the HO-1 gene transcript is abrogated [31]. The impaired responsiveness in older individuals may be more important for progression of redox imbalance and disease progression than the overall basal expression of HO-1. Several investigations have shown increased inducible expression of HO-1 in humans and mice in response to exercise [18-20]. As with NQO1, the ability of HO-1 to respond to stressors is compromised with aging, but regular physical activity improves the ability to maintain the function of these enzymes [18, 36].

***Glutathione (GSH)***- Glutathione is the most abundant endogenous antioxidant in the cellular milieu [28]. It provides reducing agents for enzymatic and non-enzymatic reduction of free radicals and oxidized macromolecules. GSH is usually reported as a ratio of reduced to oxidized glutathione (GSH:GSSG) and is found in the millimolar range at the cellular level indicating this redox couple is one of the most important cellular antioxidants if only by sheer number [109].

Aging contributes to the decline of total GSH content of the cell and a shift towards a more oxidized glutathione ratio (GSH:GSSG) because of overall increased oxidative burden

with age. This results in an increased activity of enzymes that use GSH, such as Glutathione Peroxidase (GPx) and Glutathione-S-Transferase (GST) [31, 110]. GSH content shows tissue specific declines with age in rat liver, rat brain, and human lymphocytes [111, 112] and a more oxidized ratio of GSH:GSSG in mouse myocardium [18]. Additionally, there is a steady linear decrease in GSH content of lymphocytes in humans every decade starting from age 11 up to age 60 [112]. Furthermore, long lived species show a greater GSH:GSSG ratio indicating greater potential to withstand oxidative stress and redox perturbations [90]. Conversely, other investigations have shown no change in mouse liver GSH content with age, and increased GSH content with age in rat skeletal muscle [113-115]. These results indicate a compensatory mechanism for maintaining or increasing GSH content as organisms age. This area needs more research and robust methodology to make the relationship between aging and GSH content clear, but currently it seems the changes are tissue specific.

Recent preliminary data show that acute exercise decreases GSH content in human brain, as acute exercise promotes a shift to a more oxidized cellular environment causing more GSH to be consumed than normal resting conditions [116]. These results are in agreement with a study in mouse skeletal muscle where mice ran on a treadmill for either one hour or six hours and GSH ratio and other markers of Nrf2 signaling were measured [117]. Acute exercise, regardless of duration, induced a greater oxidized-to-reduced ratio of glutathione compared to non-exercising control mice. These results are consistent with the findings that exercise is an oxidative stressor and would oxidize more GSH to maintain redox homeostasis. It is less clear how the glutathione system responds to exercise training ,especially in older individuals.

There are two potential ways to increase antioxidant capacity and improve redox homeostasis in the GSH:GSSG system. The first is to increase recycling of GSSG to GSH through Glutathione Reductase (GSR). This can be achieved by increasing the activity (allosteric activation) of GSR. The cell can also increase the amount of GSR (through increases in transcription and/or translation) so that more GSR can cycle through oxidized GSSG even without changing allosteric activity of GSR. The second way to increase GSH is to increase *de novo* synthesis of new glutathione via activating Glutamate Cysteine Ligase Catalytic Subunit (GCLc). GCLc is the rate limiting step in glutathione synthesis [118]. This would require the cell to either increase allosteric activation of GCLc to increase enzyme kinetics, or to increase the total amount of GCLc by increasing transcription and/or translation of GCLc gene or mRNA respectively. Perhaps the most efficacious approach is a combination of increasing the amount and activity of both GSR and GCLc to provide increased recycling of GSSG to GSH and adding to the overall pool of cellular GSH.

***Glutathione Reductase (GSR)***- Glutathione reductase (GSR) is an NADPH-dependent enzyme that reduces oxidized glutathione, contributing to the cycling of oxidized to reduced GSH ratio. Aging plays an important role in the activity of GSR as demonstrated by impaired GSR activity in response to 14 days of hind limb unloading in older rats compared to their younger counterparts [115]. This suggests loading and muscle activation is partially responsible for GSR expression and activity. Since GSR is responsive to the oxidative milieu of the cell and muscle stimulation increases ROS and oxidative stress, it is likely that part of the stimulus for GSR expression depends on maintaining an oxidative load in the muscle.

$$GSSG + NADPH + GSR \rightarrow 2\ GSH + NADP^{+} \qquad (2)$$

In agreement with this research, our lab has shown that GSR activity in PBMCs increases significantly in response to an acute bout of high intensity interval exercise, and less so in response to moderate intensity continuous exercise [119]. This increased activity results in improved GSH:GSSG cycling and maintenance of redox balance.

In contrast to acute exercise, adaptation to exercise training may elicit a different response, such as increasing the total pool of GSH instead of just increasing the activity of GSR. Furthermore, it is not likely that we would see any significant increases in the total GSR pool in response to *one* acute exercise bout [19]. However, GSR accretion may occur after the build-up of several exercise bouts or perhaps weeks after regular exercise training.

***Glutamate Cysteine Ligase Catalytic Subunit (GCLc)***- GCLc is the catalytic subunit responsible for the synthesis of glutathione [118]. The ability to withstand redox perturbations largely depends on GSH levels, making GCLc an important enzyme in the system to synthesize new GSH for the cellular antioxidant pool. Aging has been shown to affect GCLc expression, likely contributing to impaired redox capacity. Chen et al. (2010) showed that aging rat skeletal muscle had severely compromised GCLc activity and expression after two weeks of hind-limb unloading compared to young adult rats [115]. More recently, an investigation showed that GCL expression was significantly lower in older mouse liver homogenates compared to their younger counterparts even without any stress stimulus [113]. Taken together, GCLc activity is compromised in aging animals, however this needs to be corroborated in humans.

The two-step reaction mechanism for *de novo* GSH synthesis from its constituent amino acids is shown below: Glutamate, Cysteine, and Glycine, catalyzed by GCLc (eq 3) and Glutathione Synthetase (GS) (eq 4), respectively [118]. The GCLc/m holoenzyme is the rate limiting reaction in the de novo synthesis of GSH ([109, 115]). Bringing the holoenzyme together GCLc/m decreases the $k_m$ (binding affinity) of ATP approximately 6-fold, leading to a 4-fold increase in the reaction rate and product formation ($\gamma$GC)

$$\text{Glu} + \text{Cys} + \text{ATP} + \text{GCLc} \rightarrow \text{L-Glutamyl-L-Cysteine } (\gamma\text{GC}) \qquad \text{(eq 3)}$$

$$\gamma\text{GC} + \text{Gly} + \text{ATP} + \text{GS} \rightarrow \text{GSH} \qquad \text{(eq 4)}$$

Because the increased quantity of GSH is constrained by the rate limiting step in glutathione biosynthesis, it follows that GCLc should be one of the primary targets for increasing GSH in the cell. Studies have shown increases in GCLc content in response to exercise training in mouse myocardium and thoroughbred horses, however it is not known how GCLc responds to an acute exercise bout or exercise training in humans [18, 120]. The glutathione antioxidant system has many other enzymes including GST, GPx, and others that rely on GSH for reducing equivalents. The reason we have focused on GSH:GSSG, GSR, and GCLc is because these are the three essential components of the system on which all other enzymes rely. Therefore, if any of these three are compromised in any way, the other enzymes in the system will also be affected.

Each of the enzymes listed above (NQO1, HO-1, and GCLc) is a well-known Nrf2 targets. Together, these enzymes help maintain redox homeostasis and mitigate oxidative stress. An acute exercise bout is an oxidative stressor [121], and when applied in regular manner through exercise training leads to beneficial adaptations of the antioxidant defenses [121].

However, the molecular mechanisms of how exercise training has beneficial antioxidant effects are not well understood. Furthermore, the time course responses of changes in redox balance to an acute bout of exercise or exercise training have not been well elucidated in humans.

**Nrf2-Dependent Acute Exercise Responses**

Acute exercise is well established as an oxidative stressor and a non-pharmacological approach to activate Nrf2 and promote redox balance [102, 122, 123]. The activation of Nrf2 through exercise is thought to be primarily driven by reactive species production. The primary enzymes that contribute to ROS production in response to exercise are the NADPH oxidases (NOX) family of enzymes, and xanthine oxidase [123, 124]. Skeletal muscle NOX2 inhibition shows impaired ROS-sensitive signaling in response to one acute exercise bout [125], indicating that NOX2 is the primary driver of ROS-sensitive exercise signaling responses. While complex I and complex III of the Electron Transport Chain (ETC) have been shown to produce superoxide radicals, the likelihood of this being the primary site of ROS production during exercise is low because the increased efficiency of the ETC during exercise (State 3 respiration) actually reduces ROS production compared to basal respiration [126].

Rodents that ran for 120 or 150 minutes had higher levels of Ref1 and Nrf2 mRNA and protein content than rodents that ran for either 45 or 90 minutes. Longer exercise durations resulted in higher intracellular levels of $H_2O_2$, resulting in higher activation of Nrf2 and downstream targets [127]. Furthermore, treatment with N-Acetylcysteine (NAC), a ROS quenching antioxidant, or L-NAME, a NO synthetase inhibitor, resulted in inhibition

of exercise-induced Nrf2, GST, and GSR mRNA [128]. Additionally, chronic treatment with L-NAME or NAC ameliorates the effects of exercise training as well [128]. Taken together these results demonstrate that oxidative stress accumulates linearly with increased exercise duration and that the two main oxidants produced by exercise, $H_2O_2$ and NO, are required for the inducible expression of Nrf2 and its targets. These *in vivo* results corroborate cell culture studies showing that $H_2O_2$ and NO activate Nrf2 [59, 60]. These findings highlight the importance of exercise duration on the activation of Nrf2 and downstream targets.

Antioxidant enzyme activity increases in response to exercise in many different tissues [129]. The magnitude of activation is positively correlated to the amount of oxidative stress induced by the acute exercise. The exercise type, intensity, and duration affect the magnitude of oxidative stress and thus the activity of the antioxidant defense system [130]. Larger increases in exercise-induced oxidative stress are seen with short duration high intensity exercise, and long duration low intensity exercise (greater than 1 hour) [130]. Similar to the positive relationship between exercise-induced oxidative stress and antioxidant enzyme activity, the amount of oxidative stress also drives Nrf2 activation. Evidence to support this hypothesis comes from rodent models, and some human studies. Rats that ran on a treadmill for six hours showed increases in Nrf2-ARE binding activity, Nrf2 protein expression, and down-stream Nrf2 mRNA transcripts. However, rats that exercised for one hour showed no differences from resting animals [117]. This study illustrates the possibility of a duration threshold for activation of Nrf2 activity, although one could argue against using a six-hour exercise session in humans for pragmatic reasons.

Another way to bolster the exercise stimulus is to increase intensity. One bout of high intensity interval exercise shows transiently increased activity in the primary antioxidants SOD, Catalase, and GPx enzymes [121]. Enzyme activity falls back to near baseline levels within three hours after completion of the exercise bout. These results are in line with studies from our lab comparing high intensity interval exercise (HIT) and constant workload (CW) exercise. Thirty minutes of high intensity exercise showed greater increases in GSR activity compared to thirty minutes of constant workload exercise. Significant increases in plasma $F_2$-isoprostanes, which are a product of lipid peroxidation and the gold standard measurement of oxidative stress, were also seen immediately after both exercise bouts. However, there was a larger increase in $F_2$-isoprostanes after the high intensity protocol than the constant workload. Interestingly, both HIIT and CW exercise bouts elicited similar increases in Nrf2 nuclear localization in human PBMCs. This study indicates that, with duration held constant, higher intensity exercise elicits greater transient oxidative stress and GSR activity than constant workload exercise, without any significant differences in Nrf2 signaling [119]. Taken together with the studies investigating the effect of exercise duration, these results suggest that the overall duration of the bout may be more important for increasing Nrf2 protein expression and activation of downstream targets than intensity. Further research is needed to compare the responses from muscle and PBMCs.

Basal expression of Nrf2 does not seem to affect skeletal muscle oxidative stress in young animals, but the effects are more readily seen in older animals. Young Nrf2 knockout mice have similar oxidative stress levels and antioxidant enzyme expression compared to age-matched wild type mice, aside from NQO1 mRNA and protein expression [34]. Older Nrf2

knockout mice show higher levels of oxidative stress and suppressed antioxidant enzyme expression in skeletal muscle. These findings indicate a minimal effect of basal expression of antioxidant enzyme expression *in young mice,* but detriments are seen more readily in aged Nrf2 knockout mice [34]. Introducing an acute exercise stress in Nrf2 knockout rodents causes a large increase in oxidative stress markers of young mouse hearts compared to their wild type counterparts [102].The wild type mice showed increased Nrf2-ARE activation in response to the exercise bout compared to resting conditions but not in the Nrf2 knockout mice, indicating that an intact Nrf2-ARE signaling axis is necessary for exercise-induced oxidative stress responses in mouse cardiac tissue.

The effects of age on the Nrf2-ARE activation have been corroborated in humans. An acute exercise stress shows increased Nrf2 nuclear localization, and increased gene expression of HO-1 and NQO1 in PBMCs of young individuals, however this response was lost in older individuals [19]. Interestingly, there was a significant increase in Nrf2 accumulation in the whole cell fraction in older individuals, but there was no increase in nuclear accumulation. These findings indicate that nuclear translocation is impaired in older individuals. It is unclear what mechanism drives this impairment, but it is possible that regular exercise training or nutritional interventions may provide a stimulus to re-establish proper signaling, function, and redox homeostasis.

Taken together these results indicate that an intact Nrf2 system is required for responding to acute exercise-induced oxidative stress, and that signaling impairments are seen in genetic knockout animals, and older rodents and humans. Rescuing the age associated Nrf2 signaling impairments is vital for improving redox balance. Comparisons between

the relevant studies show the duration of the acute exercise bout may be more important for Nrf2 induction than the intensity of the exercise [117, 119, 127]. This is likely related to a linear accumulation of reactive species, specifically $H_2O_2$ and NO, in response to longer bouts of exercise [127, 128]. The larger the oxidative stress produced by exercise, the greater the magnitude of Nrf2 activation and downstream gene products. This explains why the treatment with antioxidants actually blunts the effects of an exercise stimulus [131, 132]. The effects of exercise training on Nrf2 signaling in younger and older individuals have yet to be elucidated, but some preliminary studies suggest that redox homeostasis can be restored with regular exercise. Further research is needed to understand the underlying mechanisms and thus inform adequate exercise prescription.

**Nrf2-Dependent Exercise Training Adaptations**

Several investigations have looked at the effects of exercise training on Nrf2 signaling in aging in several different tissues and model systems. While these studies provide a good foundation for basic questions on exercise, redox homeostasis, and healthy aging, the adaptive process is still unclear. Furthermore, the responsiveness to an acute exercise bout does not necessarily dictate the response over a longer period of training time, (weeks, months, or years).

A cross sectional study in humans compared basal Nrf2 activity and oxidative stress in skeletal muscles of young active, older active, and sedentary older individuals. Older active individuals showed higher Nrf2 content and Nrf2:Keap1 ratio compared to young and sedentary older individuals [133]. Young individuals did not have high levels of reactive species , which would explain lower Nrf2 levels. The older individuals had higher

markers of oxidative stress compared to young, but there were no differences in markers of oxidative stress between the older active group and older sedentary group. The observation that the older active group showed much higher basal levels of Nrf2 than the sedentary group. This suggests an activity-dependent increase in basal Nrf2 expression, which may not necessarily change the basal levels of oxidative stress markers but would allow the older active individuals to respond to an oxidative stimulus better than their sedentary counterparts because of the increased antioxidant capacity provided by the increased Nrf2 levels.

In line with these observations, older mice show higher levels of oxidative stress than young after an acute exercise stress, and the ability to induce Nrf2-ARE signaling in response to the acute exercise is blunted in older mice [18]. However, after six weeks of aerobic exercise training, older mice recovered their ability to respond to an acute exercise stress comparable to the young mice, indicating that exercise training provides an adaptive stimulus that leads to improved ability to upregulate the antioxidant defense system in response to subsequent stressors [18].

As has already been discussed, redox fluctuations associated with acute exercise depend on the duration and intensity of the bout and the age of the organism. High intensity exercise training on antioxidant defenses was tested in cardiac tissue of WT and Nrf2 null mice [134]. Older age-matched wild type and Nrf2 null mice were subjected to high intensity interval exercise for six weeks. Differences in antioxidant gene expression in basal resting conditions and redox state of the Nrf2 null and wild type mice were minimal. However, in exercising conditions there was a significant decrease in Nrf2 regulated genes and antioxidant capacity in Nrf2 null mice compared to controls. Furthermore,

markers of atrial hypertrophy were significantly higher in Nrf2 null mice compared to wild type mice along with a morphological shift in myocytes indicating atrial hypertrophy in response to the exercise [134]. The reduced ability of Nrf2 null mouse myocardium to mitigate oxidative stressors associated with high intensity training shows that Nrf2 is important for protection against the repeated training stresses in cardiac tissue.

More recently chronic endurance exercise was used to test the effects of Nrf2 knockout on cardiac function and oxidative stress in older mice [135]. Age-matched WT and Nrf2 null mice were subjected to six weeks of moderate endurance exercise. Under resting conditions, Nrf2 knockouts showed reduced antioxidant gene and protein expression compared to wild type controls. These effects were further exacerbated in chronic endurance exercise for NQO1, GCLC, GSR, and GST-$\alpha$ genes. Expression of other antioxidant genes was unaltered in response to the exercise and gene manipulation, suggesting alternative inducible mechanisms of transcription for G6PD, GPX1, GST-μ, SOD1, SOD2, and CAT. Interestingly, ventricular remodeling seen in Nrf2 null mice also occurred in healthy wild type mice after exercise training, however only the maladaptive morphological characteristics like increased myocardial hypertrophy and impaired ejection fraction were seen in Nrf2 null mice [135].

Several well-established signaling pathways converge on Nrf2 to increase nuclear localization that results in increased stress protection (Fig. 2). One study used either coronary artery ligation (CAL), sham control surgery, or aerobic interval training plus CAL to elicit ischemia reperfusion-like injury in rats [136]. CAL was used as an experimental method to induce myocardial infarction in the rodents. CAL rats showed decreased levels of antioxidant enzymes, nuclear levels of Nrf2 protein, phosphorylated AMPK, reduced

PGC1α, and PI3K–Akt signaling. Aerobic exercise ameliorated these results, showing improved markers of mitochondrial biogenesis, ARE stress response genes, and PI3K–Akt signaling. Furthermore, the increases in Nrf2 and antioxidant genes were partially blocked with a PI3K–Akt signaling inhibitor (LY294002), indicating that PI3K–Akt signaling axis is at least partially responsible for Nrf2 activation. These findings demonstrate the protective effects of exercise on Nrf2 and the convergent signaling pathways important for mitigating the negative effects of events like myocardial infarction.

Taken together, these data demonstrate that the Nrf2-ARE signaling axis does not have much of an effect in basal expression of antioxidant enzyme activity in skeletal or cardiac tissue of either young wild type or Nrf2 null rodents. However, when you expose Nrf2 null animals to an exercise stress, severe detriments are seen in the tissues' ability to respond to the oxidative stress load. Furthermore, the exercise stress is even greater in an aging cohort than in young Nrf2 null animals. Without the Nrf2 system, older animals have elevated levels of basal oxidative stress, and exercise stress further exacerbates these issues. Exercise training or moderate physical activity in older individuals results in improved Nrf2 signaling and antioxidant defense system [18, 133]. Nrf2 also improves stress resistance in both high intensity and moderate intensity exercise training, mitigating pathological remodeling of cardiac tissue and experimental models of myocardial infarction [134-136]. These studies highlight the importance of Nrf2 signaling for maintenance of redox homeostasis in cardiac tissue. Furthermore, regular exercise can promote healthy cardiac remodeling to prevent age-related cardiomyopathies through Nrf2 signaling and redox homeostasis.

## The Hormetic Effect of Reactive Species and Exercise Adaptations

It is clear that Nrf2 signaling is imperative for cellular protection against stressful events, either acute exercise or ischemia-reperfusion injury in cardiac tissue. However, reactive species are important primary messengers for the adaptive process. The primary reactive species that are produced in response to exercise are superoxide radical and nitric oxide. Superoxide radical can dismutate spontaneously or through enzymatic processes to $H_2O_2$. Data strongly support the physiological relevance and importance of $H_2O_2$ and nitric oxide (NO) because they are small non-polar molecules that are weak oxidants compared to true radical species (single electron in outer valence shell). This means longer half-lives for a more sustained signal and allows both molecules to permeate membranes [124]. $H_2O_2$ and nitric oxide (NO) are two of the primary messengers in response to exercise that provides a stimulus for increasing mitochondrial biogenesis in skeletal muscle, improve vascular function, and initiate cell signaling cascades to increase cellular functional capacity [124, 137]. This is the paradox of reactive species and health, because some reactive species generation is necessary for proper cell signaling and function, but elevation past a certain threshold becomes detrimental. This paradox is explained quite well using the hormesis model first proposed in the context of exercise and reactive species generation by Radak et al., [138]. This discussion that follows is an extension of that concept with inclusion of Nrf2 signaling and the antioxidant defense system.

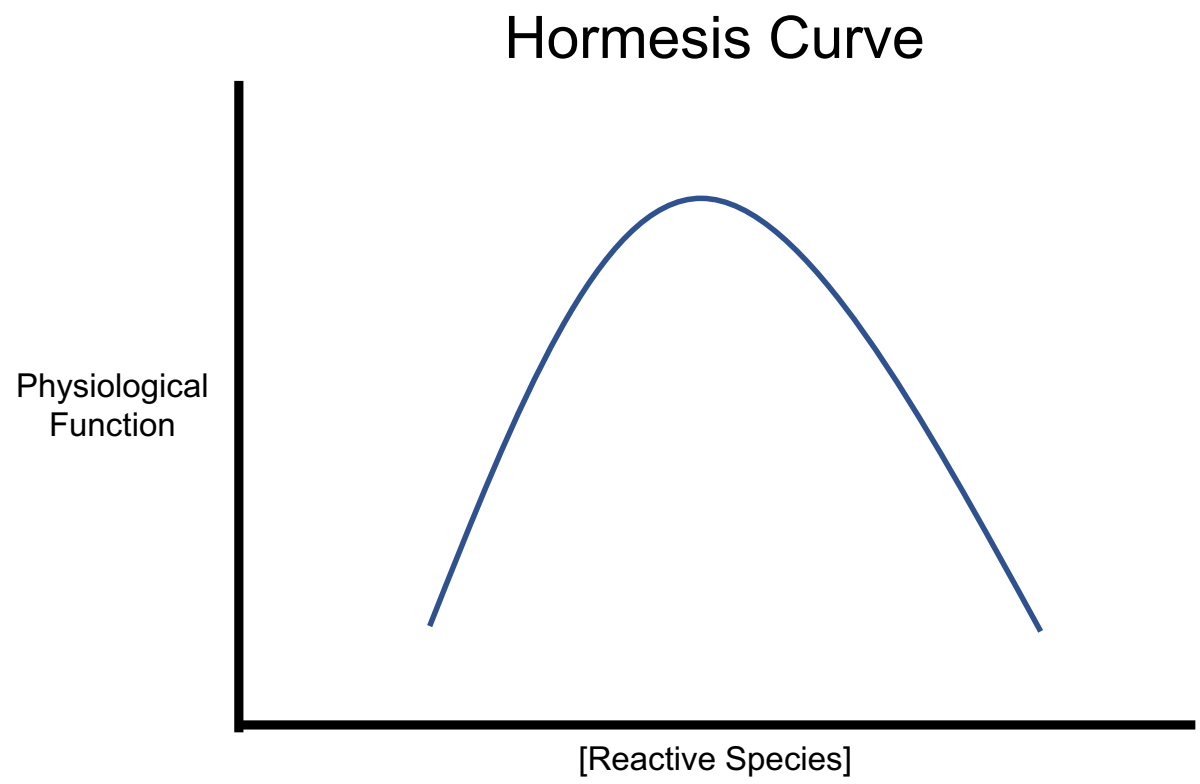



**Figure 3.** Hormetic effects of reactive species on physiological function. The moderate range of reactive species results in the highest physiological function while either extreme results in lower physiological function.

Preservation of skeletal muscle health and function is an important component of healthy aging, and mitochondria are perhaps the most important organelle for maintaining metabolic and contractile function in cardiac and skeletal muscle in aging [139]. It is well established that the energy sensing kinase, AMPK, and the master regulator of mitochondrial biogenesis, PGC1$\alpha$, are sensitive to redox perturbations. Both AMPK and PGC1$\alpha$ are central to endurance training adaptations and improved metabolic health and performance. Oxidizing these proteins with treatment of $H_2O_2$ leads to increased mitochondrial content and aerobic capacity leading to overall increases in fitness and health [139-141]. The signaling effects from reactive species generated by exercise are

hypothesized to follow a dose response bell curve or hormesis curve (Figure 3). As the reactive species concentration increases in the cell, we see an increase in adaptive signaling, but too high of a reactive species concentration and the physiological function and adaptive process are impaired [138, 142, 143]. Nrf2 signaling and the antioxidant defense system act to counter reactive species production and balance these redox perturbations during and after exercise. Based on previous studies discussed, when Nrf2 is functioning and responsive, the cell or organ system can maintain physiological function while adapting appropriately to exercise-induced reactive species stimuli, otherwise, dysfunction and damage ensue.

Several lines of evidence indicate that reactive species have hormetic effects in exercising rodents and humans. To experimentally test the effects of reactive species on aerobic exercise adaptations several studies have given exogenous antioxidants Vitamin C and E to rodents or humans before and during an exercise training intervention and measuring the effects in markers of adaptation. Results show almost complete ablation of markers of adaptation in animals or humans given antioxidants compared to placebo. This is confirmed with significantly blunted insulin sensitivity, glucose tolerance, $VO_2$ max, and endurance capacity compared to exercise only groups [132, 144].

Modulating reactive species production during exercise with small molecular antioxidant treatment to scavenge ROS (N-Acetyl Cysteine), treatment with NO synthase inhibitors (L – NAME), or NOX2 inhibitors (apocynin) that produce NO and superoxide radical respectively, lead to blunted responses to exercise training [125, 128]. This evidence strongly suggests there is a requisite level of reactive species for adaptive responses to exercise. Conversely, when Nrf2 is knocked out in animals there is also a blunted acute

exercise response in markers of mitochondrial biogenesis, and blunted training responses resulting in significantly lower exercise capacity, lower mitochondrial mass, and energy expenditure in knockout mice compared to wild type mice [128]. These data indicate that i) experimentally inhibiting exercise-induced reactive species production by quenching reactive species or inhibiting reactive species producing enzymes causes a blunted adaptive response because the individual is on the low end of the hormesis bell curve and ii) inhibiting the antioxidant defense system also causes detriments in signaling events and exercise adaptations because the individuals are operating at the other extreme of the hormesis curve.

In humans, natural variations of oxidative stress in response to an acute exercise bout predicts responsiveness to exercise training. Individuals with higher levels of oxidative stress show greater improvements in aerobic capacity after a six-week exercise intervention compared to individuals with lower levels of oxidative stress [145]. In fact, there was a linear dose response relationship- low moderate and high levels of oxidative stress resulted in low, moderate, and high responsiveness to training respectively [145]. Researchers can also induce damage and impair physiological function in normal healthy animals if the stimulus is extreme enough. Exhaustive exercise in rodents increases reactive species beyond levels the endogenous antioxidant defense system can handle, causing damage and impaired physiological function [146, 147]. Interestingly, if animals are pre-treated with an Nrf2 activator like sulforaphane prior to an acute exhaustive exercise bout, their responses are improved, and they show less damage than control or placebo treatments [146, 147]. The pre-treatment with SFN provides a preconditioning effect in the antioxidant defense system by potentially increasing protein amount and

activity before the exhaustive exercise bout, mitigating some of the potentially damaging reactive species that accumulate from exhaustive exercise. Increasing the antioxidant defense system activity acts to keep the transient increase in reactive species from being overwhelming and allows the cell to positively adapt to the exercise stimulus instead of spiraling into oxidative damage and concomitant dysfunction.

The complete relationship between Nrf2-ARE dependent responses to exercise and reactive species hormesis are not fully elucidated. Recently, data comparing Nrf2 knockout mice with wild type mice show similar adaptations in markers of mitochondrial biogenesis in response to six weeks of free wheel running in skeletal muscle [148]. These data disagree with previous findings from Merry & Ristow who showed an adaptive impairment in Nrf2 KO mice [128]. However, it is important to note differences in methods and study design that could potentially explain these discrepancies. Firstly, the exercise modality between studies was different. Merry & Ristow employed treadmill exercise while Crilly et al. used freewheel running. These differences in modality could potentially result in divergent adaptations if the intensity was different. This is speculative as the two are not directly comparable from an intensity perspective, but an important point, nonetheless. Second, Crilly and colleagues showed that prior to exercise training, Nrf2 knockout rodents had lower basal and state 3 respiration efficiencies and increased ROS production from isolated skeletal muscle mitochondria. After training, the Nrf2 knockout animals had improved both state 4 and state 3 efficiencies and showed lower ROS production compared to pre-training and similar ROS production compared to their wild type counterparts. This indicates that the improvements in mitochondrial health and

efficiency with training lead to lower ROS production independent of Nrf2 signaling [148].

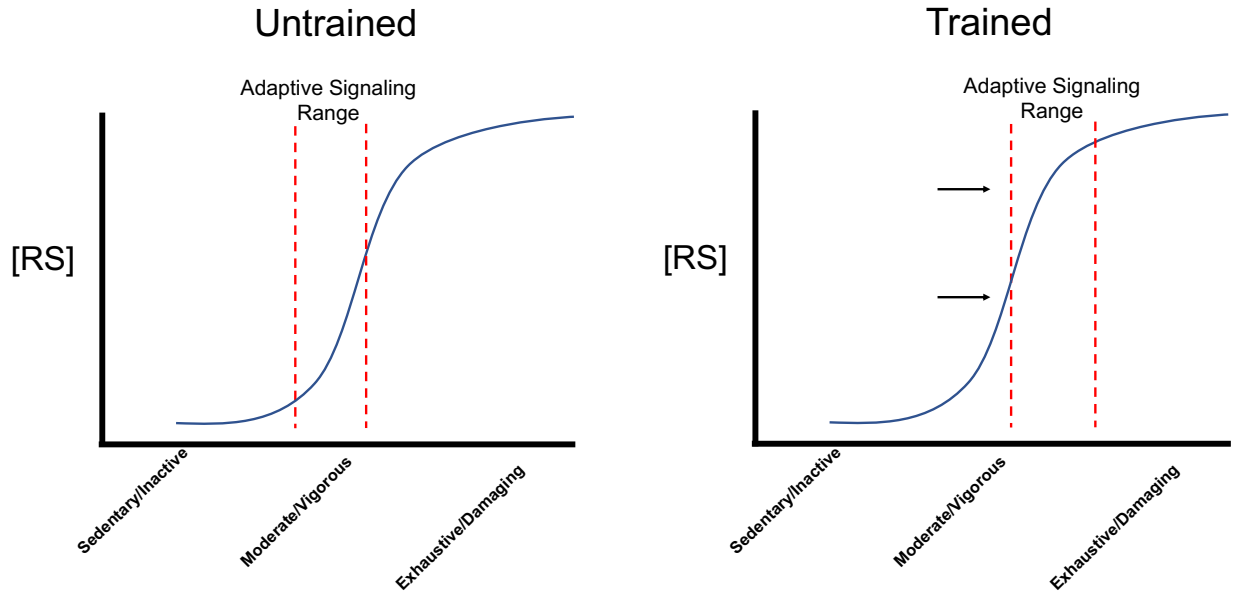



**Figure 4.** Hypothesized shift in reactive species' hormetic effects with training. Untrained individuals require less reactive species generation for aerobic adaptations while more trained individuals require larger doses of reactive species resulting from longer or more intense exercise bouts.

These Nrf2 knockout mice were not showing any significant detriments in physiological function or mitochondrial biogenesis markers because they were still operating within the adaptive range of the reactive species relative to their redox capacity. It seems this was driven by the improvements in mitochondrial efficiency and lower ROS production from respiring mitochondria despite the lack of Nrf2. Perhaps another explanation is that as animals or humans move from an untrained or sedentary state to a more trained state the hormesis curve shifts up and to the right, improving redox capacity, but also requiring a larger stimulus for exercise-induced adaptations [143]. This could also explain the higher exercise dose

needed to induce reactive species for the same relative amplitude of adaptive signaling in a trained state. In other words, a trained individual can handle a greater oxidative stress load without it becoming detrimental to physiological function [143]. This would also explain the plateau in training response as individuals become more fit, and additional adaptations require disproportionate levels of training and effort to continue to improve.

In summary, based on these findings discussed above, the hormesis model explains the effects of acute exercise responses with respect to reactive species production [143, 149]. Small amounts of reactive species do not provide enough of a stimulus for a response, but high levels from exhaustive exercise or overtraining and the cellular constituents become overwhelmed resulting in physiological dysfunction. The hormesis concept explains the data we have seen from studies discussed about the genetic ablation of Nrf2 or the treatment with Nrf2 activating compounds and their effects on aerobic capacity in rodents. Furthermore, the training state of the individual or animal is important because it shifts the hormesis curve, requiring a lower or higher exercise stimulus to produce adequate cell signaling cascades for an adaptive response (Figure 4). Therefore, the "optimal dosage" of exercise should be prescribed whenever possible. The challenging question then becomes, what is the optimal dosage? Further research is needed to determine the optimal dosage, but is related to the individuals training history, dietary factors, genetic makeup, as well as training variables like frequency, intensity, and duration of each exercise session as well as the total weekly volume of training.

**Limitations in Exercise, Aging, & Nrf2 Signaling Research**

Most laboratories that study Nrf2 signaling, aging, and exercise focus on skeletal or

cardiac muscle. However, it is important to consider the potential differences and varying importance of the Nrf2 antioxidant defense system in different tissues. For example, a robust antioxidant response may be more important in tissues whose primary function is to filter or mitigate environmental toxins like the liver, kidneys, lungs, and epithelial tissue. Tissue-specific protein and RNA expression levels of Nrf2 support this idea. The highest expression levels of Nrf2 are found in kidneys, intestines, skin, lungs, and ovaries, with relatively low expression levels in cardiac and skeletal muscle tissue (Human Protein Atlas). This also highlights the importance between using an environmental stressor (exposure to radiation, chemicals, or food born toxins) versus a physiological stressor such as exercise.

The discrepancies in Nrf2 signaling are even apparent with different types of muscle tissue. The studies in cardiac muscle clearly show that Nrf2 knockout is a significant detriment to the structure and function of the heart and that this effect is exacerbated by exercise [18]. It is possible the differences observed between skeletal and cardiac muscle are due to the divergent demands of the tissues. Cardiac tissue is constantly contracting and working, while skeletal muscle spends considerably less time contracting. When we consider the physiological adaptations to exercise, the underlying metabolic properties of heart muscle do not change drastically because the tissue is already acting in a maximal fashion. The primary adaptations that occur in the heart in response to exercise training are structural and morphological changes (i.e., ventricles have a larger chamber volume and the muscle pumps harder resulting in more blood per beat, improved ejection fraction, and stroke volume). Because the heart is always pumping, the added stress of ischemia, oxidative stress, or metabolic stress from exercise in Nrf2 null mice does not act as a

hormetic stimulus to adapt by increasing mitochondrial content and metabolic capacity. The more likely scenario is the oxidative stress causes damage that cannot be easily repaired. As a result, the stresses may be too much for the heart to handle and so the need for a robust antioxidant defense system is high.

On the contrary, in skeletal muscle the primary adaptation in response to aerobic exercise training is to increase metabolic capacity, specifically oxidative phosphorylation that occurs in mitochondria of skeletal muscle. Mitochondria of sedentary people and animals have poor efficiency, resulting in greater levels of ROS production in an untrained state, whether the mitochondria are in state 3 or state 4 respiration (resting or exercise) [148]. Furthermore, there is variation in antioxidant adaptations in different types of skeletal muscle with more slow twitch fibers and muscles having greater adaptation than fast fibers and muscles [150]. As discussed previously, the reactive species produced in response to exercise act as a stimulus to increase mitochondrial content thus increasing metabolic capacity. The proper balance between reactive species production that lead to adaptations and antioxidant responses that mitigate the stimulus has to be struck for the individual to adapt to exercise and improve physiological function and health. These tissue-specific discrepancies should be addressed in future studies comparing Nrf2 expression in multiple tissues.

Finally, an often-overlooked limitation of many studies is how they employ the exercise and the conclusions they draw from those findings. In many studies, exercise is used a stressor to induce damage. Because Nrf2 is a stress response system it will respond to a greater extent from exhaustive exercise than a normal exercise training bout, so it is unclear whether or not Nrf2 is driving any physiological adaptations or if it is just called in

to play when the exercise becomes increasingly stressful. Whether the studies in rodents using exercise as a stressor are translatable to human exercise is still unclear. Humans typically do not exercise to complete exhaustion as a part of a normal training routine, nor do they regularly exercise for 6 hours at a time [117] with the exception of competition in ultramathons or Triathlons. It would be more applicable to use an intensity and duration of exercise that is within a normal exercise intensity and duration – one that can be repeated day after day. Many researchers use the exhaustive exercise bout as a maximal physiologic stressor not as a training stimulus to induce a beneficial adaptation. While the exhaustive exercise bout provides additional evidence to support the hormesis concept and is interesting, the pragmatism and translational component seems lost. It is clear that Nrf2 is necessary for an organism to respond appropriately and recover from a maximal exercise bout or exhaustive exercise session, but because of the way researchers are using the exercise stimulus it is still debatable whether Nrf2 is responsible for driving exercise induced adaptations or Nrf2 is just responding to the massive stress induced by exhaustive exercise. Future studies in murine models should address these limitations.

**Conclusions**

Research in redox biology, aging, and exercise has come a long way in the last decade, however some effects and interactions between exercise, aging, and the antioxidant defense system are still unclear. Cell culture and *in vitro* data indicate Nrf2 is crucial for cell survival and adaptive responses to stress, largely because of data showing aging cells cannot respond to redox stressors as well as young cells. Exercise physiology is beginning to tell us more about the molecular effects of exercise on redox signaling and aging. Small physiological challenges from acute exercise bouts are compounded over

time with training and the individual is better able to withstand the exercise stress. What role Nrf2 and the antioxidant defense system has to play in this arena is still in question. Further research is needed to elucidate these relationships and bridge the gap between redox biology of aging and the molecular mechanisms of exercise induced adaptations to determine the most important factors responsible for driving health span and lifespan.

The work presented in the following chapters has addressed some of these questions outlined in this literature review. Mainly this work demonstrates that exercise induced Nrf2 signaling is in fact impaired in older adults, but exercise training can partially reverse these effects. Responses to a non-exercise redox stressor, a forearm ischemia reperfusion challenge, is improved with training regardless of age or sex. And finally, that high intensity muscle stimulation elicits a systemic redox signaling effect where Nrf2 is activated in unstimulated muscle by stimulation in the contralateral limb. This work is continuing to enhance our understanding from both a clinical and basic perspective and I am excited to continue this work.

## REFERENCES

[1] CDC, The State of Aging and Health in America, 2013.

[2] R. Holliday, The close relationship between biological aging and age-associated pathologies in humans, J Gerontol A Biol Sci Med Sci 59(6) (2004) B543-6.

[3] L. Fontana, Modulating human aging and age-associated diseases, Biochimica et biophysica acta 1790(10) (2009) 1133-8.

[4] WHO, Noncommunicable Diseases Country Profiles, 2014.

[5] C. Lopez-Otin, M.A. Blasco, L. Partridge, M. Serrano, G. Kroemer, The hallmarks of aging, Cell 153(6) (2013) 1194-217.

[6] H. Cui, Y. Kong, H. Zhang, Oxidative stress, mitochondrial dysfunction, and aging, Journal of signal transduction 2012 (2012) 646354.

[7] A. Chandrasekaran, M.d.P.S. Idelchik, J.A. Melendez, Redox control of senescence and age-related disease, Redox Biology 11 (2017) 91-102.

[8] S. Davinelli, D.C. Willcox, G. Scapagnini, Extending healthy ageing: nutrient sensitive pathway and centenarian population, Immunity & Ageing 9(1) (2012) 9.

[9] M. El Assar, J. Angulo, J.A. Carnicero, S. Walter, F.J. Garcia-Garcia, E. Lopez-Hernandez, J.M. Sanchez-Puelles, L. Rodriguez-Manas, Frailty Is Associated With Lower Expression of Genes Involved in Cellular Response to Stress: Results From the Toledo Study for Healthy Aging, Journal of the American Medical Directors Association 18(8) (2017) 734.e1-734.e7.

[10] T. Finkel, N.J. Holbrook, Oxidants, oxidative stress and the biology of ageing, Nature 408(6809) (2000) 239-47.

[11] J. Meng, Z. Lv, X. Qiao, X. Li, Y. Li, Y. Zhang, C. Chen, The decay of Redox-stress Response Capacity is a substantive characteristic of aging: Revising the redox theory of aging, Redox Biol 11 (2017) 365-374.

[12] J. Viña, C. Borras, K.M. Abdelaziz, R. Garcia-Valles, M.C. Gomez-Cabrera, The Free Radical Theory of Aging Revisited: The Cell Signaling Disruption Theory of Aging, Antioxidants & Redox Signaling 19(8) (2013) 779-787.

[13] N. Garatachea, H. Pareja-Galeano, F. Sanchis-Gomar, A. Santos-Lozano, C. Fiuza-Luces, M. Moran, E. Emanuele, M.J. Joyner, A. Lucia, Exercise attenuates the major hallmarks of aging, Rejuvenation research 18(1) (2015) 57-89.

[14] N. Garatachea, A. Santos-Lozano, D.C. Hughes, #xf3, A. mez-Cabello, I. Ara, Physical Exercise as an Effective Antiaging Intervention, BioMed Research International 2017 (2017) 2.

[15] D.E. Warburton, C.W. Nicol, S.S. Bredin, Health benefits of physical activity: the evidence, Cmaj 174(6) (2006) 801-9.

[16] H. Pareja-Galeano, N. Garatachea, A. Lucia, Exercise as a Polypill for Chronic Diseases, Prog Mol Biol Transl Sci 135 (2015) 497-526.

[17] D. Ding, K.D. Lawson, T.L. Kolbe-Alexander, E.A. Finkelstein, P.T. Katzmarzyk, W. van Mechelen, M. Pratt, The economic burden of physical inactivity: a global analysis of major non-communicable diseases, The Lancet 388(10051) (2016) 1311-1324.

[18] S.S. Gounder, S. Kannan, D. Devadoss, C.J. Miller, K.J. Whitehead, S.J. Odelberg, M.A. Firpo, R. Paine, 3rd, J.R. Hoidal, E.D. Abel, N.S. Rajasekaran, Impaired transcriptional activity of Nrf2 in age-related myocardial oxidative stress is reversible by moderate exercise training, PLoS One 7(9) (2012) e45697.

[19] A.J. Done, M.J. Gage, N.C. Nieto, T. Traustadottir, Exercise-induced Nrf2-signaling is impaired in aging, Free radical biology & medicine 96 (2016) 130-8.

[20] A.J. Done, T. Traustadottir, Nrf2 mediates redox adaptations to exercise, Redox Biol 10 (2016) 191-199.

[21] D. Harman, Aging: A Theory Based on Free Radical and Radiation Chemistry, Journal of Gerontology 11(3) (1956) 298-300.

[22] A. Soare, E.P. Weiss, J.O. Holloszy, L. Fontana, Multiple dietary supplements do not affect metabolic and cardio-vascular health, Aging (Albany NY) 6(2) (2014) 149-57.

[23] G. Bjelakovic, D. Nikolova, L.L. Gluud, R.G. Simonetti, C. Gluud, Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis, Jama 297(8) (2007) 842-57.

[24] V.I. Perez, A. Bokov, H. Van Remmen, J. Mele, Q. Ran, Y. Ikeno, A. Richardson, Is the oxidative stress theory of aging dead?, Biochimica et biophysica acta 1790(10) (2009) 1005-14.

[25] M. Jafari, Healthspan Pharmacology, Rejuvenation research 18(6) (2015) 573-80.

[26] M. Schieber, N.S. Chandel, ROS function in redox signaling and oxidative stress, Current biology : CB 24(10) (2014) R453-62.

[27] R.S. Sohal, W.C. Orr, The redox stress hypothesis of aging, Free radical biology & medicine 52(3) (2012) 539-55.

[28] D.P. Jones, Extracellular redox state: refining the definition of oxidative stress in aging, Rejuvenation research 9(2) (2006) 169-81.

[29] Y.M. Go, D.P. Jones, Cysteine/cystine redox signaling in cardiovascular disease, Free radical biology & medicine 50(4) (2011) 495-509.

[30] K.N. Lewis, J. Mele, J.D. Hayes, R. Buffenstein, Nrf2, a guardian of healthspan and gatekeeper of species longevity, Integr Comp Biol 50(5) (2010) 829-43.

[31] H. Zhang, K.J.A. Davies, H.J. Forman, Oxidative stress response and Nrf2 signaling in aging, Free radical biology & medicine 88(Pt B) (2015) 314-336.

[32] E. Kubo, B. Chhunchha, P. Singh, H. Sasaki, D.P. Singh, Sulforaphane reactivates cellular antioxidant defense by inducing Nrf2/ARE/Prdx6 activity during aging and oxidative stress, Sci Rep 7(1) (2017) 14130.

[33] L. Zhou, H. Zhang, K.J.A. Davies, H.J. Forman, Aging-related decline in the induction of Nrf2-regulated antioxidant genes in human bronchial epithelial cells, Redox Biology 14 (2018) 35-40.

[34] C.J. Miller, S.S. Gounder, S. Kannan, K. Goutam, V.R. Muthusamy, M.A. Firpo, J.D. Symons, R. Paine, 3rd, J.R. Hoidal, N.S. Rajasekaran, Disruption of Nrf2/ARE signaling impairs antioxidant mechanisms and promotes cell degradation pathways in aged skeletal muscle, Biochimica et biophysica acta 1822(6) (2012) 1038-50.

[35] S.S. Davies, T. Traustadottir, A.A. Stock, F. Ye, Y. Shyr, S.M. Harman, L.J. Roberts, 2nd, Ischemia/reperfusion unveils impaired capacity of older adults to restrain oxidative insult, Free radical biology & medicine 47(7) (2009) 1014-8.

[36] T. Traustadottir, S.S. Davies, Y. Su, L. Choi, H.M. Brown-Borg, L.J. Roberts, 2nd, S.M. Harman, Oxidative stress in older adults: effects of physical fitness, Age (Dordrecht, Netherlands) 34(4) (2012) 969-82.

[37] C.D. Pomatto Laura, J.A. Davies Kelvin, The role of declining adaptive homeostasis in ageing, The Journal of Physiology 595(24) (2017) 7275-7309.

[38] M.E. Carlson, H.S. Silva, I.M. Conboy, Aging of signal transduction pathways, and pathology, Experimental cell research 314(9) (2008) 1951-61.

[39] S.V. Lakshmi, G. Padmaja, P. Kuppusamy, V.K. Kutala, Oxidative stress in cardiovascular disease, Indian journal of biochemistry & biophysics 46(6) (2009) 421-40.

[40] M. Narasimhan, N.S. Rajasekaran, Exercise, Nrf2 and Antioxidant Signaling in Cardiac Aging, Front Physiol 7 (2016) 241.

[41] H. Kaneto, N. Katakami, D. Kawamori, T. Miyatsuka, K. Sakamoto, T.A. Matsuoka, M. Matsuhisa, Y. Yamasaki, Involvement of oxidative stress in the pathogenesis of diabetes, Antioxid Redox Signal 9(3) (2007) 355-66.

[42] B.N. Ames, L.S. Gold, Endogenous mutagens and the causes of aging and cancer, Mutation research 250(1-2) (1991) 3-16.

[43] E. Panieri, M.M. Santoro, ROS homeostasis and metabolism: a dangerous liason in cancer cells, Cell death & disease 7(6) (2016) e2253.

[44] W.R. Markesbery, Oxidative stress hypothesis in Alzheimer's disease, Free radical biology & medicine 23(1) (1997) 134-47.

[45] O.I. Aruoma, Free radicals, antioxidants and international nutrition, Asia Pac J Clin Nutr 8(1) (1999) 53-63.

[46] A. Ceriello, E. Motz, Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited, Arterioscler Thromb Vasc Biol 24(5) (2004) 816-23.

[47] N.R. Madamanchi, A. Vendrov, M.S. Runge, Oxidative stress and vascular disease, Arterioscler Thromb Vasc Biol 25(1) (2005) 29-38.

[48] G. Shanmugam, M. Narasimhan, S. Tamowski, V. Darley-Usmar, N.S. Rajasekaran, Constitutive activation of Nrf2 induces a stable reductive state in the mouse myocardium, Redox Biology 12 (2017) 937-945.

[49] N.S. Rajasekaran, P. Connell, E.S. Christians, L.J. Yan, R.P. Taylor, A. Orosz, X.Q. Zhang, T.J. Stevenson, R.M. Peshock, J.A. Leopold, W.H. Barry, J. Loscalzo, S.J.

Odelberg, I.J. Benjamin, Human alpha B-crystallin mutation causes oxido-reductive stress and protein aggregation cardiomyopathy in mice, Cell 130(3) (2007) 427-39.

[50] F. Singh, A.L. Charles, A.I. Schlagowski, J. Bouitbir, A. Bonifacio, F. Piquard, S. Krahenbuhl, B. Geny, J. Zoll, Reductive stress impairs myoblasts mitochondrial function and triggers mitochondrial hormesis, Biochimica et biophysica acta 1853(7) (2015) 1574-85.

[51] P. Moi, K. Chan, I. Asunis, A. Cao, Y.W. Kan, Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region, Proc Natl Acad Sci U S A 91(21) (1994) 9926-30.

[52] A. Lau, W. Tian, S.A. Whitman, D.D. Zhang, The Predicted Molecular Weight of Nrf2: It Is What It Is Not, Antioxidants & Redox Signaling 18(1) (2013) 91-93.

[53] L. Baird, M. Yamamoto, The Molecular Mechanisms Regulating the KEAP1-NRF2 Pathway, Mol Cell Biol 40(13) (2020).

[54] J.D. Hayes, A.T. Dinkova-Kostova, The Nrf2 regulatory network provides an interface between redox and intermediary metabolism, Trends in biochemical sciences 39(4) (2014) 199-218.

[55] A.T. Dinkova-Kostova, J.W. Fahey, R.V. Kostov, T.W. Kensler, KEAP1 and done? Targeting the NRF2 pathway with sulforaphane, Trends in Food Science & Technology 69 (2017) 257-269.

[56] K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J.D. Engel, M. Yamamoto, Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain, Genes Dev 13(1) (1999) 76-86.

[57] K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, T. O'Connor, M. Yamamoto, Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles, Genes to cells : devoted to molecular & cellular mechanisms 8(4) (2003) 379-91.

[58] E. Kansanen, S.M. Kuosmanen, H. Leinonen, A.L. Levonen, The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer, Redox Biol 1 (2013) 45-9.

[59] S. Fourquet, R. Guerois, D. Biard, M.B. Toledano, Activation of NRF2 by nitrosative agents and H2O2 involves KEAP1 disulfide formation, The Journal of biological chemistry 285(11) (2010) 8463-71.

[60] M. McMahon, D.J. Lamont, K.A. Beattie, J.D. Hayes, Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals, Proceedings of the National Academy of Sciences of the United States of America 107(44) (2010) 18838-43.

[61] M. Kobayashi, L. Li, N. Iwamoto, Y. Nakajima-Takagi, H. Kaneko, Y. Nakayama, M. Eguchi, Y. Wada, Y. Kumagai, M. Yamamoto, The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds, Molecular and cellular biology 29(2) (2009) 493-502.

[62] C. Hu, A.L. Eggler, A.D. Mesecar, R.B. van Breemen, Modification of keap1 cysteine residues by sulforaphane, Chem Res Toxicol 24(4) (2011) 515-21.

[63] D.D. Zhang, M. Hannink, Distinct Cysteine Residues in Keap1 Are Required for Keap1-Dependent Ubiquitination of Nrf2 and for Stabilization of Nrf2 by Chemopreventive Agents and Oxidative Stress, Molecular and cellular biology 23(22) (2003) 8137-8151.

[64] K. Igarashi, K. Kataoka, K. Itoh, N. Hayashi, M. Nishizawa, M. Yamamoto, Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins, Nature 367(6463) (1994) 568-72.

[65] T. Ishii, K. Itoh, S. Takahashi, H. Sato, T. Yanagawa, Y. Katoh, S. Bannai, M. Yamamoto, Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages, The Journal of biological chemistry 275(21) (2000) 16023-9.

[66] H.C. Huang, T. Nguyen, C.B. Pickett, Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2, Proceedings of the National Academy of Sciences of the United States of America 97(23) (2000) 12475-80.

[67] H.C. Huang, T. Nguyen, C.B. Pickett, Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription, The Journal of biological chemistry 277(45) (2002) 42769-74.

[68] S. Numazawa, M. Ishikawa, A. Yoshida, S. Tanaka, T. Yoshida, Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress, American journal of physiology. Cell physiology 285(2) (2003) C334-42.

[69] D.A. Bloom, A.K. Jaiswal, Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression, The Journal of biological chemistry 278(45) (2003) 44675-82.

[70] Y. Katoh, K. Itoh, E. Yoshida, M. Miyagishi, A. Fukamizu, M. Yamamoto, Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription, Genes to Cells 6(10) (2002) 857-868.

[71] Y.S. Keum, S. Yu, P.P. Chang, X. Yuan, J.H. Kim, C. Xu, J. Han, A. Agarwal, A.N. Kong, Mechanism of action of sulforaphane: inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element-mediated heme oxygenase-1 in human hepatoma HepG2 cells, Cancer research 66(17) (2006) 8804-13.

[72] S. Naidu, V. Vijayan, S. Santoso, T. Kietzmann, S. Immenschuh, Inhibition and Genetic Deficiency of p38 MAPK Up-Regulates Heme Oxygenase-1 Gene Expression via Nrf2, The Journal of Immunology 182(11) (2009) 7048.

[73] Z. Sun, Z. Huang, D.D. Zhang, Phosphorylation of Nrf2 at Multiple Sites by MAP Kinases Has a Limited Contribution in Modulating the Nrf2-Dependent Antioxidant Response, PLoS ONE 4(8) (2009) e6588.

[74] R. Yu, C. Chen, Y.Y. Mo, V. Hebbar, E.D. Owuor, T.H. Tan, A.N. Kong, Activation of mitogen-activated protein kinase pathways induces antioxidant response element-mediated gene expression via a Nrf2-dependent mechanism, The Journal of biological chemistry 275(51) (2000) 39907-13.

[75] L.M. Zipper, R.T. Mulcahy, Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes, Biochemical and biophysical research communications 278(2) (2000) 484-92.

[76] M. Xue, H. Momiji, N. Rabbani, G. Barker, T. Bretschneider, A. Shmygol, D.A. Rand, P.J. Thornalley, Frequency Modulated Translocational Oscillations of Nrf2 Mediate the

Antioxidant Response Element Cytoprotective Transcriptional Response, Antioxid Redox Signal 23(7) (2015) 613-29.

[77] A.K. Jain, A.K. Jaiswal, GSK-3beta acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2, The Journal of biological chemistry 282(22) (2007) 16502-10.

[78] N. Kubben, W. Zhang, L. Wang, T.C. Voss, J. Yang, J. Qu, G.H. Liu, T. Misteli, Repression of the Antioxidant NRF2 Pathway in Premature Aging, Cell 165(6) (2016) 1361-1374.

[79] D. Gabriel, D. Roedl, L.B. Gordon, K. Djabali, Sulforaphane enhances progerin clearance in Hutchinson-Gilford progeria fibroblasts, Aging cell 14(1) (2015) 78-91.

[80] M.-K. Kwak, N. Wakabayashi, J.L. Greenlaw, M. Yamamoto, T.W. Kensler, Antioxidants Enhance Mammalian Proteasome Expression through the Keap1-Nrf2 Signaling Pathway, Molecular and cellular biology 23(23) (2003) 8786-8794.

[81] M. Dodson, M. Redmann, N.S. Rajasekaran, V. Darley-Usmar, J. Zhang, KEAP1-NRF2 signalling and autophagy in protection against oxidative and reductive proteotoxicity, The Biochemical journal 469(3) (2015) 347-55.

[82] A. Arlt, I. Bauer, C. Schafmayer, J. Tepel, S.S. Muerkoster, M. Brosch, C. Roder, H. Kalthoff, J. Hampe, M.P. Moyer, U.R. Folsch, H. Schafer, Increased proteasome subunit protein expression and proteasome activity in colon cancer relate to an enhanced activation of nuclear factor E2-related factor 2 (Nrf2), Oncogene 28(45) (2009) 3983-96.

[83] B.P. Dieter, Dysregulation of Nrf2 Signaling in Diabetes: An Opportunity for a Multitarget Approach, J Diabetes Metab 6(1) (2015).

[84] R. Howden, Nrf2 and Cardiovascular Defense, Oxid Med Cell Longev (2013).

[85] M. Narasimhan, N.S. Rajasekaran, Cardiac Aging - Benefits of Exercise, Nrf2 Activation and Antioxidant Signaling, Adv Exp Med Biol 999 (2017) 231-255.

[86] C.P. Ramsey, C.A. Glass, M.B. Montgomery, K.A. Lindl, G.P. Ritson, L.A. Chia, R.L. Hamilton, C.T. Chu, K.L. Jordan-Sciutto, Expression of Nrf2 in neurodegenerative diseases, J Neuropathol Exp Neurol 66(1) (2007) 75-85.

[87] M.B. Sporn, K.T. Liby, NRF2 and cancer: the good, the bad and the importance of context, Nat Rev Cancer 12(8) (2012) 564-571.

[88] J.H. An, K. Vranas, M. Lucke, H. Inoue, N. Hisamoto, K. Matsumoto, T.K. Blackwell, Regulation of the Caenorhabditis elegans oxidative stress defense protein SKN-1 by glycogen synthase kinase-3, Proceedings of the National Academy of Sciences of the United States of America 102(45) (2005) 16275-80.

[89] G.P. Sykiotis, D. Bohmann, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila, Dev Cell 14(1) (2008) 76-85.

[90] K.N. Lewis, E. Wason, Y.H. Edrey, D.M. Kristan, E. Nevo, R. Buffenstein, Regulation of Nrf2 signaling and longevity in naturally long-lived rodents, Proceedings of the National Academy of Sciences of the United States of America 112(12) (2015) 3722-7.

[91] D.R. Bruns, J.C. Drake, L.M. Biela, F.F. Peelor, 3rd, B.F. Miller, K.L. Hamilton, Nrf2 Signaling and the Slowed Aging Phenotype: Evidence from Long-Lived Models, Oxid Med Cell Longev 2015 (2015) 732596.

[92] A. Uruno, Y. Furusawa, Y. Yagishita, T. Fukutomi, H. Muramatsu, T. Negishi, A. Sugawara, T.W. Kensler, M. Yamamoto, The Keap1-Nrf2 System Prevents Onset of Diabetes Mellitus, Molecular and cellular biology 33(15) (2013) 2996-3010.

[93] T. Hosoya, A. Maruyama, M.I. Kang, Y. Kawatani, T. Shibata, K. Uchida, E. Warabi, N. Noguchi, K. Itoh, M. Yamamoto, Differential responses of the Nrf2-Keap1 system to laminar and oscillatory shear stresses in endothelial cells, The Journal of biological chemistry 280(29) (2005) 27244-50.

[94] R.K. Thimmulappa, K.H. Mai, S. Srisuma, T.W. Kensler, M. Yamamoto, S. Biswal, Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray, Cancer research 62(18) (2002) 5196-203.

[95] X. Wang, D.J. Tomso, B.N. Chorley, H.Y. Cho, V.G. Cheung, S.R. Kleeberger, D.A. Bell, Identification of polymorphic antioxidant response elements in the human genome, Human molecular genetics 16(10) (2007) 1188-200.

[96] J.M. Marzec, J.D. Christie, S.P. Reddy, A.E. Jedlicka, H. Vuong, P.N. Lanken, R. Aplenc, T. Yamamoto, M. Yamamoto, H.Y. Cho, S.R. Kleeberger, Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury, FASEB J 21(9) (2007) 2237-46.

[97] T. Toki, J. Itoh, J.i. Kitazawa, K. Arai, K. Hatakeyama, J.-i. Akasaka, K. Igarashi, N. Nomura, M. Yokoyama, M. Yamamoto, E. Ito, Human small Maf proteins form heterodimers with CNC family transcription factors and recognize the NF-E2 motif, Oncogene 14 (1997) 1901.

[98] G.T. Gang, J.H. Hwang, Y.H. Kim, J.R. Noh, K.S. Kim, J.Y. Jeong, D.E. Choi, K.W. Lee, J.Y. Jung, M. Shong, C.H. Lee, Protection of NAD(P)H:quinone oxidoreductase 1 against renal ischemia/reperfusion injury in mice, Free radical biology & medicine 67 (2014) 139-49.

[99] K. Kudoh, H. Uchinami, M. Yoshioka, E. Seki, Y. Yamamoto, Nrf2 Activation Protects the Liver From Ischemia/Reperfusion Injury in Mice, Ann Surg 260(1) (2014) 118-127.

[100] X.H. Lin, H. Yang, L.C. Zhou, Z.M. Guo, Nrf2-dependent induction of NQO1 in mouse aortic endothelial cells overexpressing catalase, Free Radical Bio Med 51(1) (2011) 97-106.

[101] L. Wang, X. He, G.D. Szklarz, Y. Bi, Y. Rojanasakul, Q. Ma, The aryl hydrocarbon receptor interacts with nuclear factor erythroid 2-related factor 2 to mediate induction of NAD(P)H:quinoneoxidoreductase 1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin, Archives of biochemistry and biophysics 537(1) (2013) 31-8.

[102] V.R. Muthusamy, S. Kannan, K. Sadhaasivam, S.S. Gounder, C.J. Davidson, C. Boeheme, J.R. Hoidal, L. Wang, N.S. Rajasekaran, Acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium, Free radical biology & medicine 52(2) (2012) 366-76.

[103] S. Kumar, U. Bandyopadhyay, Free heme toxicity and its detoxification systems in human, Toxicol Lett 157(3) (2005) 175-88.

[104] J.C. Docherty, B.A. Schacter, G.D. Firneisz, S.B. Brown, Mechanism of Action of Heme Oxygenase - a Study of Heme Degradation to Bile Pigment by O-18 Labeling, Journal of Biological Chemistry 259(21) (1984) 3066-3069.

[105] A. Loboda, M. Damulewicz, E. Pyza, A. Jozkowicz, J. Dulak, Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism, Cell Mol Life Sci 73(17) (2016) 3221-47.

[106] J. Alam, J.L. Cook, How many transcription factors does it take to turn on the heme oxygenase-1 gene?, American journal of respiratory cell and molecular biology 36(2) (2007) 166-74.

[107] J. Alam, D. Stewart, C. Touchard, S. Boinapally, A.M. Choi, J.L. Cook, Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene, The Journal of biological chemistry 274(37) (1999) 26071-8.

[108] J. Alam, C. Wicks, D. Stewart, P. Gong, C. Touchard, S. Otterbein, A.M. Choi, M.E. Burow, J. Tou, Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells. Role of p38 kinase and Nrf2 transcription factor, The Journal of biological chemistry 275(36) (2000) 27694-702.

[109] Y. Chen, H.G. Shertzer, S.N. Schneider, D.W. Nebert, T.P. Dalton, Glutamate cysteine ligase catalysis: dependence on ATP and modifier subunit for regulation of tissue glutathione levels, J Biol Chem 280(40) (2005) 33766-74.

[110] Y. Zhu, P.M. Carvey, Z. Ling, Age-related changes in glutathione and glutathione-related enzymes in rat brain, Brain research 1090(1) (2006) 35-44.

[111] A.F. Aydin, C. Kucukgergin, G. Ozdemirler-Erata, N. Kocak-Toker, M. Uysal, The effect of carnosine treatment on prooxidant-antioxidant balance in liver, heart and brain tissues of male aged rats, Biogerontology 11(1) (2010) 103-9.

[112] S. Samarghandian, M. Azimi-Nezhad, F. Samini, Preventive effect of safranal against oxidative damage in aged male rat brain, Experimental animals 64(1) (2015) 65-71.

[113] P. Jiang, Y. Sheng, L. Ji, The age-related change of glutathione antioxidant system in mice liver, Toxicology mechanisms and methods 23(6) (2013) 396-401.

[114] D. Toroser, Rajindar S. Sohal, Age-associated perturbations in glutathione synthesis in mouse liver, The Biochemical journal 405(Pt 3) (2007) 583-589.

[115] C.-n. Chen, H.M. Brown-Borg, S.G. Rakoczy, D.A. Ferrington, L.V. Thompson, Aging Impairs the Expression of the Catalytic Subunit of Glutamate Cysteine Ligase in Soleus Muscle Under Stress, The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 65A(2) (2010) 129-137.

[116] S. Rai, A. Chowdhury, R. Reniers, S.J. Wood, S.J.E. Lucas, S. Aldred, A pilot study to assess the effect of acute exercise on brain glutathione, Free radical research 52(1) (2018) 57-69.

[117] T. Li, S. He, S. Liu, Z. Kong, J. Wang, Y. Zhang, Effects of different exercise durations on Keap1-Nrf2-ARE pathway activation in mouse skeletal muscle, Free radical research 49(10) (2015) 1269-74.

[118] S.C. Lu, Glutathione synthesis, Biochim Biophys Acta 1830(5) (2013) 3143-53.

[119] A.J. Done, M.J. Newell, T. Traustadottir, Effect of exercise intensity on Nrf2 signalling in young men, Free radical research 51(6) (2017) 646-655.

[120] J.-W. Park, J.-Y. Choi, S.A. Hong, N.Y. Kim, K.-T. Do, K.-D. Song, B.-W. Cho, Exercise induced upregulation of glutamate-cysteine ligase catalytic subunit and glutamate-cysteine ligase modifier subunit gene expression in Thoroughbred horses, Asian-Australasian Journal of Animal Sciences 30(5) (2017) 728-735.

[121] G. Fisher, D.D. Schwartz, J. Quindry, M.D. Barberio, E.B. Foster, K.W. Jones, D.D. Pascoe, Lymphocyte enzymatic antioxidant responses to oxidative stress following high-intensity interval exercise, J Appl Physiol (1985) 110(3) (2011) 730-7.

[122] K.J. Davies, A.T. Quintanilha, G.A. Brooks, L. Packer, Free radicals and tissue damage produced by exercise, Biochemical and biophysical research communications 107(4) (1982) 1198-205.

[123] M.J. Jackson, A. Vasilaki, A. McArdle, Cellular mechanisms underlying oxidative stress in human exercise, Free radical biology & medicine 98 (2016) 13-17.

[124] S.K. Powers, E.E. Talbert, P.J. Adhihetty, Reactive oxygen and nitrogen species as intracellular signals in skeletal muscle, J Physiol 589(Pt 9) (2011) 2129-38.

[125] C. Henriquez-Olguin, A. Diaz-Vegas, Y. Utreras-Mendoza, C. Campos, M. Arias-Calderon, P. Llanos, A. Contreras-Ferrat, A. Espinosa, F. Altamirano, E. Jaimovich, D.M. Valladares, NOX2 Inhibition Impairs Early Muscle Gene Expression Induced by a Single Exercise Bout, Front Physiol 7 (2016) 282.

[126] G.K. Sakellariou, M.J. Jackson, A. Vasilaki, Redefining the major contributors to superoxide production in contracting skeletal muscle. The role of NAD(P)H oxidases, Free radical research 48(1) (2014) 12-29.

[127] P. Wang, C.G. Li, Z. Qi, D. Cui, S. Ding, Acute exercise stress promotes Ref1/Nrf2 signalling and increases mitochondrial antioxidant activity in skeletal muscle, Exp Physiol 101(3) (2016) 410-20.

[128] T.L. Merry, M. Ristow, Nuclear factor erythroid-derived 2-like 2 (NFE2L2, Nrf2) mediates exercise-induced mitochondrial biogenesis and the anti-oxidant response in mice, J Physiol 594(18) (2016) 5195-207.

[129] L.L. Ji, Antioxidants and oxidative stress in exercise, Proc Soc Exp Biol Med 222(3) (1999) 283-92.

[130] S.K. Powers, Z. Radak, L.L. Ji, Exercise-induced oxidative stress: past, present and future, J Physiol 594(18) (2016) 5081-92.

[131] M.C. Gomez-Cabrera, C. Borras, F.V. Pallardo, J. Sastre, L.L. Ji, J. Vina, Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats, J Physiol 567(Pt 1) (2005) 113-20.

[132] M.C. Gomez-Cabrera, E. Domenech, M. Romagnoli, A. Arduini, C. Borras, F.V. Pallardo, J. Sastre, J. Vina, Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance, Am J Clin Nutr 87(1) (2008) 142-9.

[133] A. Safdar, J. deBeer, M.A. Tarnopolsky, Dysfunctional Nrf2-Keap1 redox signaling in skeletal muscle of the sedentary old, Free radical biology & medicine 49(10) (2010) 1487-93.

[134] R.R. Kumar, M. Narasimhan, G. Shanmugam, J. Hong, A. Devarajan, S. Palaniappan, J. Zhang, G.V. Halade, V.M. Darley-Usmar, J.R. Hoidal, N.S. Rajasekaran, Abrogation of Nrf2 impairs antioxidant signaling and promotes atrial hypertrophy in response to high-intensity exercise stress, J Transl Med 14 (2016) 86.

[135] G. Shanmugam, M. Narasimhan, R.L. Conley, T. Sairam, A. Kumar, R.P. Mason, R. Sankaran, J.R. Hoidal, N.S. Rajasekaran, Chronic Endurance Exercise Impairs Cardiac Structure and Function in Middle-Aged Mice with Impaired Nrf2 Signaling, Front Physiol 8 (2017) 268.

[136] H.K. Jiang, Y. Miao, Y.H. Wang, M. Zhao, Z.H. Feng, X.J. Yu, J.K. Liu, W.J. Zang, Aerobic interval training protects against myocardial infarction-induced oxidative injury by

enhancing antioxidase system and mitochondrial biosynthesis, Clin Exp Pharmacol Physiol 41(3) (2014) 192-201.

[137] C. Goto, K. Nishioka, T. Umemura, D. Jitsuiki, A. Sakagutchi, M. Kawamura, K. Chayama, M. Yoshizumi, Y. Higashi, Acute moderate-intensity exercise induces vasodilation through an increase in nitric oxide bioavailiability in humans, Am J Hypertens 20(8) (2007) 825-30.

[138] Z. Radak, H.Y. Chung, S. Goto, Exercise and hormesis: oxidative stress-related adaptation for successful aging, Biogerontology 6(1) (2005) 71-5.

[139] D.F. Dai, Y.A. Chiao, D.J. Marcinek, H.H. Szeto, P.S. Rabinovitch, Mitochondrial oxidative stress in aging and healthspan, Longevity & healthspan 3 (2014) 6.

[140] Z. Wu, P. Puigserver, U. Andersson, C. Zhang, G. Adelmant, V. Mootha, A. Troy, S. Cinti, B. Lowell, R.C. Scarpulla, B.M. Spiegelman, Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1, Cell 98(1) (1999) 115-24.

[141] I. Irrcher, V. Ljubicic, D.A. Hood, Interactions between ROS and AMP kinase activity in the regulation of PGC-1α transcription in skeletal muscle cells, American Journal of Physiology-Cell Physiology 296(1) (2009) C116-C123.

[142] Z. Radak, H.Y. Chung, E. Koltai, A.W. Taylor, S. Goto, Exercise, oxidative stress and hormesis, Ageing Res Rev 7(1) (2008) 34-42.

[143] Z. Radak, K. Ishihara, E. Tekus, C. Varga, A. Posa, L. Balogh, I. Boldogh, E. Koltai, Exercise, oxidants, and antioxidants change the shape of the bell-shaped hormesis curve, Redox Biol 12 (2017) 285-290.

[144] M. Ristow, K. Zarse, A. Oberbach, N. Kloting, M. Birringer, M. Kiehntopf, M. Stumvoll, C.R. Kahn, M. Bluher, Antioxidants prevent health-promoting effects of physical exercise in humans, Proceedings of the National Academy of Sciences of the United States of America 106(21) (2009) 8665-70.

[145] N.V. Margaritelis, A.A. Theodorou, V. Paschalis, A.S. Veskoukis, K. Dipla, A. Zafeiridis, G. Panayiotou, I.S. Vrabas, A. Kyparos, M.G. Nikolaidis, Adaptations to endurance training depend on exercise-induced oxidative stress: exploiting redox interindividual variability, Acta physiologica (Oxford, England) 222(2) (2018).

[146] M. Malaguti, C. Angeloni, N. Garatachea, M. Baldini, E. Leoncini, P.S. Collado, G. Teti, M. Falconi, J. Gonzalez-Gallego, S. Hrelia, Sulforaphane treatment protects skeletal muscle against damage induced by exhaustive exercise in rats, Journal of applied physiology (Bethesda, Md. : 1985) 107(4) (2009) 1028-36.

[147] S. Oh, S. Komine, E. Warabi, K. Akiyama, A. Ishii, K. Ishige, Y. Mizokami, K. Kuga, M. Horie, Y. Miwa, T. Iwawaki, M. Yamamoto, J. Shoda, Nuclear factor (erythroid derived 2)-like 2 activation increases exercise endurance capacity via redox modulation in skeletal muscles, Sci Rep 7(1) (2017) 12902.

[148] M.J. Crilly, L.D. Tryon, A.T. Erlich, D.A. Hood, The role of Nrf2 in skeletal muscle contractile and mitochondrial function, Journal of applied physiology (Bethesda, Md. : 1985) 121(3) (2016) 730-40.

[149] M.G. Nikolaidis, A. Kyparos, C. Spanou, V. Paschalis, A.A. Theodorou, I.S. Vrabas, Redox biology of exercise: an integrative and comparative consideration of some overlooked issues, J Exp Biol 215(Pt 10) (2012) 1615-25.

[150] S.K. Powers, D. Criswell, J. Lawler, L.L. Ji, D. Martin, R.A. Herb, G. Dudley, Influence of exercise and fiber type on antioxidant enzyme activity in rat skeletal muscle, The American journal of physiology 266(2 Pt 2) (1994) R375-80.

# CHAPTER 2 – Aerobic Exercise Training Partially Reverses the Impairment of Nrf2 Activation in Older Humans

Ethan L. Ostrom[1], and Tinna Traustadóttir[1*]

[1]Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona

[*]To Whom Correspondence Should Be Addressed:

Tinna Traustadóttir, Ph.D.

Department of Biological Sciences

Northern Arizona University

PO Box 5640

Flagstaff, AZ 86011-5640

Phone: 928-523-7291

Fax : 928-523-7500

E-mail: tinna.traustadottir@nau.edu

## ABSTRACT

Nuclear factor erythroid-2-related factor 2 (Nrf2), is an inducible transcription factor that improves redox balance through stimulating antioxidant gene expression. In older humans the Nrf2 response to a single bout of acute exercise is blunted compared to young indicating impaired redox signaling. The purpose of this randomized controlled trial was to investigate if the signaling impairment could be reversed with exercise training in older men and women, while also comparing to young. Young (18-28y, n=21) and older (≥60y, n=19) men and women were randomized to 8-week aerobic exercise training (ET; 3d/wk, 45min/d) or a non-exercise control group (CON). Nrf2 nuclear localization, gene expression for *NQO1*, *HO1*, and *GCLC*, and GCLC protein were measured in PBMCs in response to acute exercise trial (AET; 30-min cycling at 70% $VO_2$ peak pre- and post-intervention at 7 timepoints (Pre, +10m, +30m, +1h, +4h, +8h, +24h). Young had greater Nrf2 signaling response compared to older at pre-intervention ($p$=0.05), whereas the older had significantly higher basal Nrf2 levels ($p$=0.004). ET decreased basal Nrf2 expression compared to CON ($p$=0.032) and improved the Nrf2 signaling response in both young and older ($p<0.05$). The degree of restoration in Nrf2 signaling response was related to the degree of change in basal Nrf2 ($p$=0.039), which was driven by older adults ($p$=0.014). Lower basal nuclear Nrf2 levels were associated with changes seen in AET responses for Nrf2 and GCLC protein, as well as NQO1 and GCLC mRNA. Together these data demonstrate that exercise training improves Nrf2 signaling and downstream gene expression and that lower basal Nrf2 levels are associated with a more dynamic acute response. Our results provide evidence that the impaired Nrf2 signaling in sedentary older

adults can be restored to a degree with moderate exercise training, albeit not to the level seen in young.

**ClinicalTrials.gov ID: NCT03419988**

**Keywords: Redox Homeostasis, Exercise, Aging, Nrf2 Signaling, NQO1, GCLC**

## 1. Introduction

Aging is associated with reductions in physiological function and overall decreased stress resilience [1-3]. This contributes to an increased risk for developing non-communicable diseases including cardiovascular disease, diabetes, cancer and dementia [4]. One major thread connecting these diseases is dysregulated redox homeostasis due to oxidative stress imbalances [5-8]. The redox stress hypothesis of aging highlights the increasingly oxidative milieu in older organisms contributing to the lack of ability to respond to a redox stressor [9]. One of the predictions that can be made from this hypothesis is the declining ability of the cell or tissue to respond in a dynamic manner to redox stressors such as acute exercise or ischemia/reperfusion [10-12]. Cells accomplish appropriate responses to redox stressors primarily through redox stress response proteins like the inducible transcription factor nuclear factor erythroid-2-related factor 2 (Nrf2). Nrf2 is responsible for activating a host of gene targets including antioxidant, metabolic, and xenobiotic gene products. Under unstimulated conditions, Nrf2 is bound to its negative regulator and redox sensor, Keap1, in the cytosol. Oxidative or electrophilic stress activates Nrf2 by modifying Keap1 cysteine residues preventing Nrf2 degradation, increasing Nrf2 nuclear

accumulation, and leading to increased mRNA transcripts of downstream targets [13-16]. The ability of a cell or organism to mount a successful response to redox stressors is dependent on an intact Nrf2 system to provide dynamic adjustments to the redox environment [17-19].

Loss of responsiveness in the Keap1-Nrf2 signaling system is a characteristic of older organisms and cells in response to various redox stressors [20]. Bronchial epithelial cells derived from older humans showed impaired Nrf2 responses when treated with the phytonutrient sulforaphane, compared to cells from young donors [21]. Similarly, impairments were found in redox sensitive cell signaling pathways, chaperone protein responses, and ability to degrade oxidatively damaged proteins via the proteasome in human fibroblasts and *C. elegans* when challenged with several different oxidative stress treatments [22]. These data indicate that the redox stress response is impaired in aging organisms independent of the type of redox stress.

Regular exercise is one of the most successful interventions to prevent or delay age-associated diseases. Aerobic (non-damaging) exercise induces a series of biochemical signaling cascades that result in changes in cell physiology, many of which are mediated by redox mechanisms [23-26]. Transient redox stress elicited by acute exercise increases Nrf2 activation in young animals and humans, thus improving cellular resistance to subsequent redox stressors [10, 27-29]. However, animal studies demonstrate that Nrf2 activation in response to acute exercise is impaired with aging [30], and we have previously shown that older men have an impaired Nrf2 signaling response to an acute exercise bout [10]. Taken together, these data highlight an important problem that has yet to be addressed in aging humans: the delineation between acute responses and

adaptations to regular exercise in redox stress response systems. The purpose of this randomized controlled trial was to investigate whether an eight-week exercise training intervention could improve or reverse the age-related decline in redox stress response signaling in older men and women, with young adults serving as additional controls. This study tested the hypothesis that the divergent pattern of responses to acute exercise seen between age groups would be attenuated after exercise training, due to an improved response in older individuals.

## 2. Methods

*2.1 Subjects* – Twenty-six young (18-28y) and 29 older (60-77y) men and women from the surrounding community and campus completed screening for study participation. Subjects were eligible to participate if they were currently inactive as defined by no regular exercise for at least 6 months prior to study admission, as per self-report. Subjects were generally healthy, not overly obese (BMI ≤ 33.0 kg/m$^2$), non-smokers, and not taking antioxidant supplements in excess of a multi-vitamin. Multivitamins were not excluded because the dosages of antioxidants are within the range of normal dietary intake, and we are unaware of any confounding effects in redox measures at moderate doses. Any condition that would contraindicate maximal exercise testing or participation in exercise training including clinically significant electrocardiogram (EKG) abnormalities at rest or during the maximal exercise test, elevated blood pressure at rest or musculoskeletal problems excluded subjects from participating in the study. Women who were taking estrogen replacement therapy or birth control were excluded from participation as research has previously shown that estrogen has a significant effect on markers of

oxidative stress [12]. All participants signed a written informed consent approved by the Northern Arizona University Institutional Review Board.

*2.2 Study Design* – The study was an 8-week randomized controlled trial where young and older individuals were randomly assigned to either an exercise training intervention (ET) or a non-exercise control group (CON). Subjects completed pre-screening over the phone to determine eligibility for study participation. If there were no obvious exclusions, the subject was scheduled for a screening visit in the lab followed by a maximal graded exercise test on a cycle ergometer to determine baseline aerobic capacity ($VO_2$ peak). In a separate visit subjects completed an acute exercise trial (30 minutes of cycling at 70% $VO_2$ peak) to measure redox signaling capacity responses from blood samples taken at baseline and six additional time points as described below (see figure 1). After baseline testing was complete, subjects were randomized to ET or CON groups. Subjects randomized to CON were asked not to change their activity levels during the study and were offered the opportunity to receive the supervised exercise training program after completing the control arm of the study as a way to minimize attrition. Subjects randomized to ET performed supervised aerobic exercise training three times per week for eight weeks (24 sessions). After the 8 weeks of either ET or CON, subjects returned to the lab and completed the same testing as performed before the intervention. To control for any potential confounding effects of diet on the acute exercise trial, subjects completed a 2-day food log prior to the baseline test and then were asked to consume the same diet for the two days prior to the post-intervention trial. Study design is shown in Figure 1.

**Figure 1.**

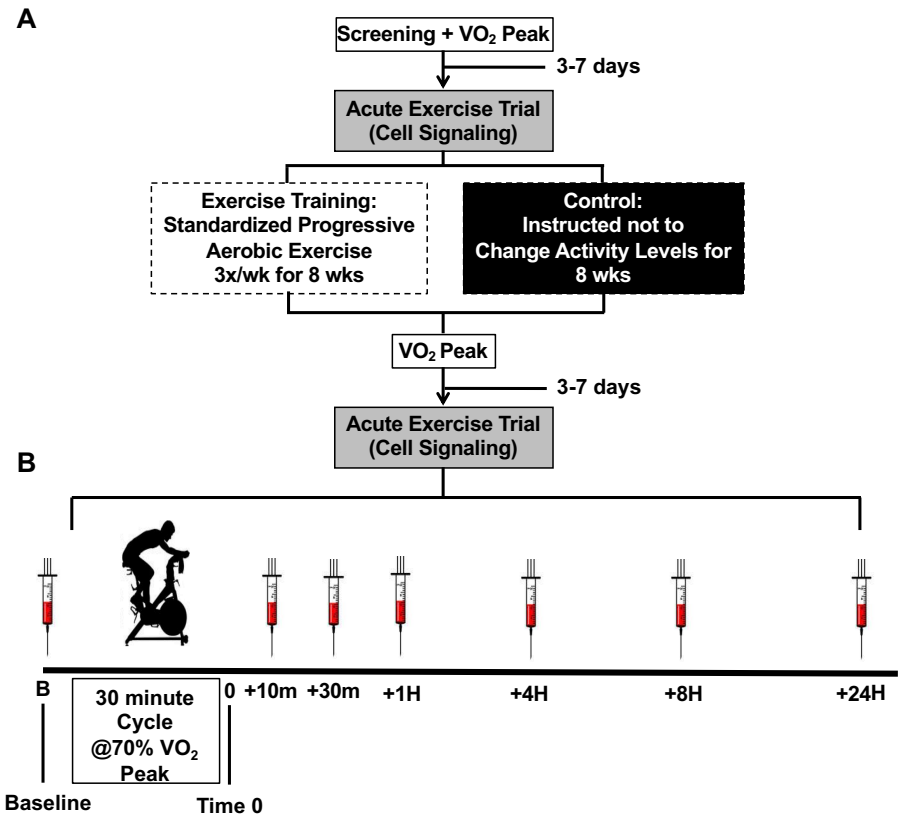



**Figure 1. Study design**. (**A**) Subjects were randomized to the eight-week exercise training intervention (ET) or non-exercise control (CON). $VO_2$ peak testing and acute exercise trial were completed pre- and post-intervention. (**B**) Acute Exercise Trial: After a baseline blood draw, subjects cycled on a stationary cycle ergometer for 30 minutes at 70% $VO_2$ peak. Following the acute exercise trial (Time 0), blood was drawn at 10-min, 30-min, 1-hr, 4-hrs, 8-hrs and 24-hrs post-exercise. For time-course comparisons all time points were normalized to the individuals' baseline blood draw. Acute exercise trial time course responses were tested before and after the intervention.

*2.3 Screening visit* – After participants signed the informed consent they completed a health history questionnaire and the modified Historical Leisure Physical Activity questionnaire for assessment of lifetime physical activity [31, 32]. Height, weight, waist circumference and resting blood pressure were measured and a 12-lead supine resting

EKG was obtained to rule out any cardiac abnormalities that would preclude participants from performing a maximal aerobic capacity test. Body composition was measured using seven-site skinfold using a Lange skinfold caliper. Body density was calculated using normalized equations for men and women specified by age. The body fat percent equation by Siri et al. was used for final body fat calculations [33]. All skinfold measures were done by the same researcher to prevent intertester variability.

*2.4 Peak Oxygen Consumption Test* – $VO_2$ peak was measured with a graded exercise test (GXT) performed on a cycle ergometer as previously described [34]. The starting workload was selected based on the predicted maximal workload for each individual and was increased every minute until volitional exhaustion. Participants were instructed to maintain a pedaling rate of 60-70 rpm throughout the test. Oxygen uptake was measured via indirect calorimetry using a metabolic measurement cart (CareFusion, Yorba Linda, CA). Heart function was monitored with continuous 12-lead EKG. $VO_2$ peak was considered achieved if two of the following three criteria were met: 1) a plateau in $VO_2$ with an increase in workload, 2) a respiratory exchange ratio (RER) ≥ 1.10, or 3) heart rate within 10 beats of the age-predicted maximal heart rate [35]. Standard contraindications to exercise testing, as well as termination criteria, outlined by the American College of Sports Medicine were followed at all times [36].

*2.5 Exercise Intervention* – Subjects randomized to the exercise intervention performed supervised aerobic exercise training three times per week for eight weeks (24 sessions

in total) at the NAU Student Recreation Center. The intervention was progressive in duration and intensity and included a mix of moderate intensity continuous training and high intensity interval training. Every session, however, was standardized so that each subject performed the same duration and relative intensity at a given session. Intensity was monitored by heart rate monitors (Polar, Inc., Finland) and Rate of Perceived Exertion [37]. Relative intensity was normalized to the individual's maximal heart rate measured during the maximal exercise test. The intervention was progressive in duration and intensity and included a mix of moderate intensity continuous training and high intensity interval training. Durations started with 30-minute constant workload sessions, and progressed to 50-minute constant workload by the end of the intervention, while also progressing the intensity through increasing steady state heart rate range each week. High intensity interval sessions started modestly and progressed in intensity and duration each week. A mix of work:rest ratios were used to provide variation for subjects engagement as well as ensuring adequate range of intensity / duration combinations that meet ACSM weekly exercise prescription recommendations [38]. Target RPE and HR range were provided for each individual every exercise session.

*2.6 Acute Exercise Trial* – Subjects reported to the laboratory pre- and post-intervention to complete the acute exercise trial. The trial started between 6:00 and 9:00 am and subjects were instructed to refrain from any exercise or physical exertion for 48-hours prior. An intravenous (IV) catheter was inserted into the antecubital vein and a baseline blood sample was collected. The subject then performed the acute exercise trial consisting of 30-minutes of cycling at 70% $VO_2$ peak, maintaining a pedaling rate of 60-

70 rpm throughout. We have previously shown that this protocol elicits Nrf2 activation, as well as significant increase in antioxidant enzyme activity [10, 29]. Oxygen consumption and heart rate were measured throughout the trial using the same system as described above. After completion of the exercise, blood was drawn again at the following time points: 10-min, 30-min, 1-hr, 4-hrs, 8-hrs, and 24-hrs. The blood was drawn via the IV catheter through the 1-hr time point and thereafter with venipuncture using a 21-G butterfly needle (Becton Dickinson, Franklin Lakes, NJ). Subjects were allowed to leave the lab after the +1-hour blood draw and return for the last three draws. The acute exercise session was designed as a physiological stimulus to induce signaling cascades in the redox stress response system that are associated with physiological adaptations. We have previously shown that Nrf2 content does not change over similar sampling time points without exercise [11]. The intensity of the acute exercise session was normalized to the individuals $VO_2$ peak to standardize the stimulus regardless of differences in maximal aerobic capacity in order to make valid comparisons in molecular measurements across age groups.

*2.7 PBMC Isolation and Cell Counting* – Whole blood was collected into EDTA vacutainers to prevent coagulation, diluted 1:1 with phosphate buffered saline (PBS) and layered onto Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Samples were spun at 900xg for 30 minutes at room temperature. Following centrifugation, the lymphocyte rich layer was collected and washed twice in PBS with phosphatase inhibitors (Halt Phosphatase Cocktail, ThermoFisher, Waltham, MA) at 400xg for 10 mins at 4°C. The supernatant was discarded, and the cell pellet was re-suspended in 1.5 mL second wash

buffer. Cells were counted using Countess Automated Cell Counter (Invitrogen, Carlsbad, CA). Aliquots of $2x10^6$ PBMC cell lysate were suspended with 100 μL of RNAlater (Ambion Biotechnology, Austin, TX) and stored at -80°C to preserve RNA stability until gene expression analyses. Additional volume of $1x10^6$ and $3x10^6$ cells per mL sample were aliquoted for whole cell and nuclear fractionation, respectively, for western blot measures.

*2.8 Cell Fractionation and Preparation for Western Blotting* – Cells were fractionated into cytosolic and nuclear fractions using a modified version of the REAP protocol [39]. Briefly, $3x10^6$ cells were lysed in the presence of phosphatase and protease inhibitors in 0.1% Triton PBS solution by pipet mixing five times followed by a 10 second pop-spin in a microcentrifuge. Supernatant was aliquoted as the cytosolic fraction and mixed with 4x Laemmli sample buffer in a 3:1 ratio. The pellet was resuspended in 1000μL 0.1% Triton PBS solution followed by a 10 second pop-spin. Following centrifugation, the supernatant was discarded, and the nuclear fraction was resuspended in 1x Laemmli sample buffer. For whole cell lysates, $1x10^6$ cells were loaded into microcentrifuge tubes and lysed using 0.1% Triton PBS and protease-phosphatase inhibitor cocktails, followed by mixing with 4X Laemmli sample buffer. Nuclear fractions and whole cell lysates were sonicated by microprobe (Sonifier 150, Branson Ultrasonics, Danby, Connecticut) on ice at level 2, three times for 5 seconds, interspersed by two minutes of cooling on ice. All samples were heated at 100°C for 5 minutes followed immediately by freezing at -80°C for storage until analysis.

*2.9 Western Blot Analyses* – Samples were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. Nuclear fractions were incubated with rabbit polyclonal primary antibody for Nrf2 (H-300, Santa Cruz Biotechnology, Dallas TX, 1:500), while the whole cell fraction was incubated with GCLC primary antibody (EP13475, Abcam, Cambridge MA, 1:1000), followed by incubation with their respective anti-rabbit and anti-mouse secondary antibodies (925-32211, and 926-68070, Li-Cor Biosciences, Lincoln NE,1:20,000). Mouse monoclonal antibodies were used for internal control proteins β-actin and Lamin-B1 (sc-4778, and sc-365214, Santa Cruz Biotechnology, Dallas TX, 1:1000). β-actin and Lamin-B1 served as loading controls in whole cell and nuclear fractions, respectively. All samples were multiplexed by co-incubation with rabbit and mouse monoclonal primary antibodies respectively, followed by co-incubation with anti-rabbit and anti-mouse secondary antibodies designed to appear in the 800 and 700 channels respectively. Membranes were imaged using Li-Cor Odyssey Fc Infrared Imaging System (Li-Cor Biosciences, Lincoln NE). Band intensity was measured by densitometry using Image Studio Lite (Li-Cor Biosciences, Lincoln NE) and changes in protein levels were determined by comparing band intensity from post-intervention samples to levels from pre-intervention samples. For all acute exercise trial analyses, proteins of interest were normalized to 1 at baseline within each individual to analyze patterns and magnitudes of responses by age and training group. Basal protein expression was compared across age and training group by normalizing to internal control proteins and expressed as a ratio in arbitrary units.

*2.10 RT-qPCR – Reverse transcriptase quantitative polymerase chain reaction* – RNA

was extracted using the Qiagen RNeasy Plus Mini Kit (Qiagen, Gaithersberg, MD) following the manufacturers' instructions. Briefly, Qiagen RNA extraction kits were used to isolate purified RNA from participants at baseline, 1-hr, 4-hrs, 8-hrs, and 24-hrs after completion of the acute exercise trial. RNA quality was assessed with a NanoDrop (ThermoFisher), using RNA amount (ng/μL) and purity ($A_{260/280}$). Once an acceptable RNA quantity was measured the RNA was converted into cDNA via iScript cDNA conversion kit (BioRad, Hercules, CA). RT-qPCR utilized paired primers for NADPH Quinone Oxidoreductase 1 (NQO1), Heme Oxygenase 1 (HO1), and Glutamate Cysteine Ligase Catalytic subunit (GCLC) with Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) as an internal reference gene for normalization. The assays were run in triplicate on a CFX96-Touch (Bio-Rad, Hercules, CA) and each run included negative controls, and non-template controls. Differences in quantification cycles ($C_q$) were recorded after adjusting for the amount of mRNA in a sample and normalized to the internal reference gene (GAPDH). GAPDH was selected as the housekeeping gene as previous studies have shown it to be a reliable, stably expressed gene in response to aerobic exercise in humans [40, 41]. Experimental design, quality control, and minimum information for reporting qPCR steps from the MIQE guidelines were followed [42]. The gene-specific primer pairs are outlined in Table 1.

Table 1. Forward and reverse primers for RT – qPCR

| Primers | Forward 5' to 3' | Reverse 5' to 3' |
|---|---|---|
| **NQO1** | GGATTGGACCGAGCTGGAA | AATTGCAGTGAAGATGAAGGCAAC |
| **HO-1** | AAGAGGCCAAGACTGCGTTC | GGTGTCATGGGTCAGCAGC |
| **GCLC** | GCTGTCTTGCAGGGAATGTT | ACACACCTTCCTTCCCATTG |
| **GAPDH** | GAAGGTGAAGGTCGGAGTC | GAAGATGGTGATGGGATTTC |

*2.11 Statistical Analyses* – Subject characteristics and baseline data were analyzed by 2-way ANOVA (Age x Intervention Group). Significant differences in subject characteristics and baseline values were added as covariates to the statistical models to control for any confounding variables. Each marker measured for gene expression, and protein content was analyzed across time points (repeated measures), as well as between age groups, and by sex. Baseline acute exercise responses for gene expression data were analyzed using (2 x 5) repeated measures ANOVA (Age by Time) with significant differences in baseline anthropometric data placed in the model as covariates (sex, body mass, BMI, waist circumference, body fat percent). Similar analyses were run for protein expression responses but using 2 x 7 repeated measures ANOVA (Age x Time). All analyses on the effects of training or control intervention were 2 x 2 x 5 (training group x intervention time x sampling time point for gene expression) or 2 x 2 x 7 (training group x intervention time x sampling time point for protein expression) repeated measures ANOVA with covariates age, sex, and any other significantly different physical or physiological measurements entered into the model. Statistical significance was set at $p < 0.05$ and the analyses were performed using statistical programs SPSS and R. Data are reported as mean ± SEM unless otherwise noted.

## 3. Results

Fifty-five subjects were screened and of those 9 were disqualified or lost prior to pre-testing ($VO_2$ max too high n=1, declined to participate n=5, unable to contact n=3). A total of 46 individuals completed pre-testing and started the intervention. Two young individuals dropped out citing time commitment, two subjects were withdrawn due to issues with venipuncture (one young, one older), and two older individuals were withdrawn by study personnel due to medical issues (blood pressure dysregulation, arrhythmia at post-testing). All six subjects that were withdrawn during testing or intervention were in the ET group, there were no dropouts in the CON group. The data presented are from 40 individuals who completed the intervention; 21 young and 19 older, with equal representation between sexes. Baseline subject characteristics are shown in Table 2. As expected, there were age-related differences at baseline where the young individuals had significantly lower body mass, body mass index (BMI), and body fat percentage (BF%), as well as smaller waist circumference. Similarly, age-related differences in the response to maximal exercise testing showed expected results where young individuals had significantly higher $VO_2$ peak values compared to older adults (38.5 vs. 25.8 mL/kg/min, respectively, $p<0.001$), higher maximal heartrate (189 vs 148 bpm, respectively, $p<0.001$), and higher maximal workload (210 vs. 155 watts, respectively, $p<0.001$). Importantly, none of the baseline characteristics differed between those randomized to the ET intervention versus those randomized to the control group (see Table 2).

The goal for the acute exercise trial was an average intensity of 70% $VO_2$ peak. The attained mean $VO_2$ peak for the acute exercise trial across the whole cohort was 70.5%

of $VO_2$ peak. Young individuals averaged 69.8% of $VO_2$ peak while older individuals averaged 71.2% of $VO_2$ peak (Table 2). The exercise intensity did not differ statistically between young and older individuals or between ET and CON.

Table 2. Baseline physical characteristics

| Physical Characteristics & Exercise Testing | Young | | Older | | *p* Value | | |
|---|---|---|---|---|---|---|---|
| | CON (n=10) | ET (n=11) | CON (n=9) | ET (n=10) | Age | Training Grp | Age x Training Grp |
| **Men / Women Ratio (n)** | 5 / 5 | 5 / 6 | 5 / 4 | 5 / 5 | - | - | - |
| **Age** | 21.9 (± 3) | 21.6 (± 3) | 65.1 (± 3) | 68.3 (± 5) | <0.001 | NS | NS |
| **Height(cm)** | 177 (± 11) | 171 (± 10) | 172 (± 13) | 170 (± 8.9) | NS | NS | NS |
| **Mass(Kg)** | 68.3 (± 12.3) | 66.0 (± 8.4) | 87.3 (± 14.8) | 76.8 (± 16.5) | 0.002 | NS | NS |
| **BMI (Kg/m^2)** | 21.6 (± 2.5) | 22.5 (± 3.9) | 29.3 (± 2.3) | 26.3 (± 3.8) | <0.001 | NS | NS |
| **Body Fat %** | 15 (± 7.5) | 17 (± 10.3) | 29.9 (± 3.9) | 25.2 (± 4.2) | <0.001 | NS | NS |
| **Waist Circ. (cm)** | 76.7 (± 6.7) | 76.6 (± 6.4) | 102 (± 10.2) | 93.5 (± 11) | <0.001 | NS | NS |
| **HLPA** | 2.83 (± 0.3) | 2.95 (± 0.6) | 2.86 (± 0.9) | 2.36 (± 0.47) | NS | NS | NS |
| **$VO_2$ Peak** | | | | | | | |
| **Absolute VO2 (mL/min)** | 2621 (± 724) | 2563 (± 554) | 2161 (± 570) | 2067 (± 510) | 0.01 | NS | NS |
| **Relative VO2 (mL/Kg/min)** | 38.0 (± 6.6) | 38.9 (± 7.4) | 24.6 (± 3.5) | 26.9 (± 3.1) | <0.001 | NS | NS |
| **Max Heart Rate (bpm)** | 189 (± 9) | 188 (± 8) | 145 (± 21) | 150 (± 18) | <0.001 | NS | NS |
| **RER** | 1.21 (± 0.06) | 1.28 (± 0.1) | 1.31 (± 0.05) | 1.28 (± 0.08) | 0.04 | NS | NS |
| **Workload (Watts)** | 212 (± 62) | 208 (± 42) | 158 (± 39) | 153 (± 41) | 0.001 | NS | NS |
| **Acute Exercise Trial** | | | | | | | |
| **% of $VO_2$ Peak** | 69.0 (± 2.4) | 70.5 (± 2.4) | 71.4 (± 3.9) | 71 (± 2.7) | NS | NS | NS |
| **% of HR Max** | 81 (± 6) | 81 (± 6) | 79 (± 8) | 82 (± 7) | NS | NS | NS |

BMI – Body Mass Index, HLPA – Historical Leisure Physical Activity Questionnaire, RER – Respiratory Exchange Ratio. Data are shown as mean (± SD)

### *3.1 Effects of the exercise intervention on cardiorespiratory fitness*

Figure 2 shows the changes in peak oxygen consumption in response to the ET or CON intervention. Aerobic capacity increased significantly in ET (mean change: 4.5 mL/kg/min, +14.7%) and did not change in CON (mean change: 0.2 mL/kg/min, +1%), indicated by main effect of group, $p = 0.004$, and intervention by group interaction, $p = 0.013$. When including covariates in the model, main effects of training group (ET vs CON), age, sex, and change in BMI (ΔBMI) were also associated with $VO_2$ peak measurements (RM

ANCOVA, ET vs CON $p < 0.001$, Age $p < 0.001$, Sex $p < 0.001$, $\Delta$BMI $p = 0.022$). Within the ET group, there were no differences in the magnitude of improvement across age or sex (Repeated Measures ANCOVA $\Delta VO_2$ x Age $p = 0.405$ and $\Delta VO_2$ x Sex, $p = 0.4$, Figure 2C-F) indicating that the individuals who underwent the ET intervention responded to the same degree regardless of age or sex.

There were no significant changes in resting blood pressure, maximal heart rate, and RER in either ET or CON. There were no significant changes in overall body mass in ET or CON, but there was a significant decrease in body fat mass, and waist circumference in ET, with no change in CON (one-way ANOVA $\Delta$Body Fat %, $p = 0.023$, $\Delta$waist circumference, $p = 0.001$).

**Figure 2.**

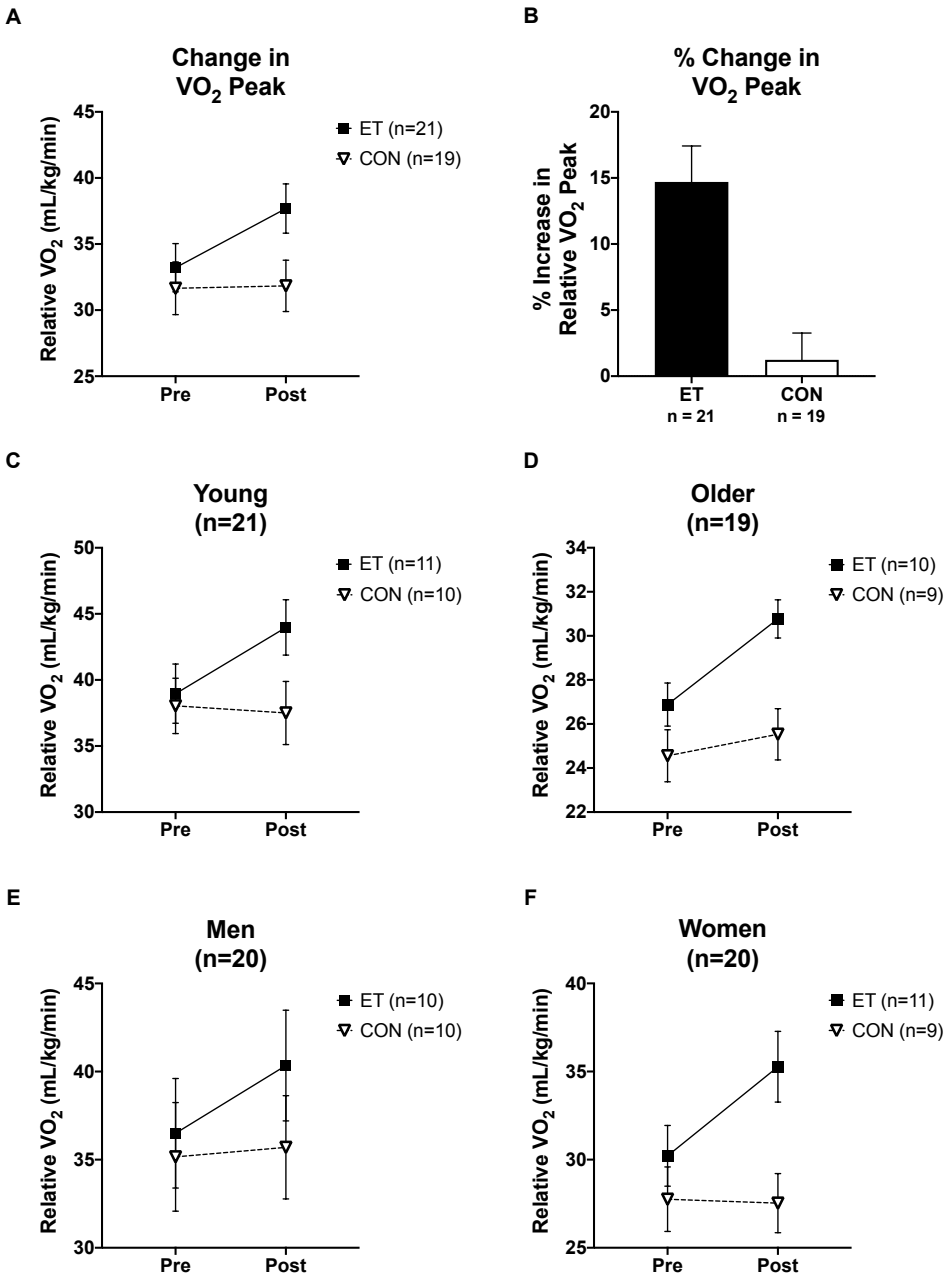



**Figure 2. $VO_2$ peak changes in response to the intervention.** (**A&B**) Relative $VO_2$ peak (ml/kg/min) improved in trained subjects but not control subjects, regardless of age or sex (Intervention x Training group interaction p = 0.013). (**C-F**) Changes in $VO_2$ peak by age and sex. Within the exercise trained group, there were no differences in the degree of fitness improvement by age or sex indicating the exercise training intervention had the

same effect across age and sex (Compare all ET group changes in panels C-F). (Δ indicates the difference between Pre and Post Intervention for a given measurement). Black squares indicate ET group, open triangles indicate CON group. Data shown as Mean ± SEM.

### *3.2 Exercise-induced Nrf2 signaling: Pre-Intervention*

Age-group differences in Nrf2 nuclear localization in response to the acute exercise trial were not significant when analyzed without covariates (RM ANOVA, main effect of age, $p = 0.118$) but approached significance when entering measures of fitness and body composition into the model (RM ANCOVA, main effect of age, $p = 0.05$). When the data were examined further, unexpected sex differences emerged. Figure 3 shows the pre-intervention responses in men (A) and women (B). Young men showed an increase in Nrf2 nuclear localization with a peak at thirty minutes after a single acute exercise session, while older men did not respond (within sex main effect of age, $p < 0.07$, with or without covariates), in agreement with our earlier findings in men [10]. In contrast, there were no significant differences between young and older women in the responses (within sex main effect of age, $p = 0.714$, with covariates $p = 0.279$).

**Figure 3.**

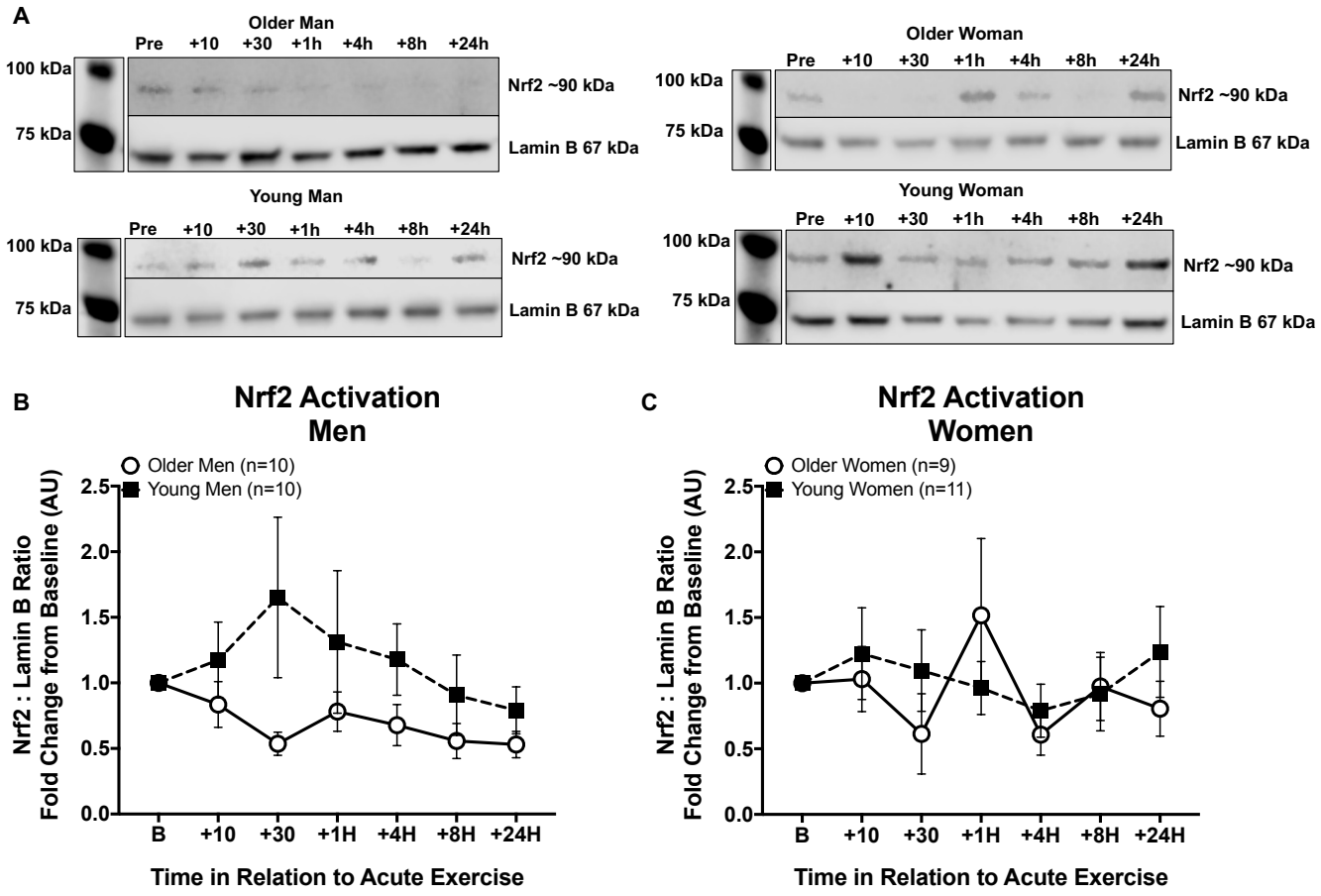



**Figure 3. Nrf2 signaling responses to a single acute exercise session by age and sex**. All responses are normalized to the individual's baseline draw (B) and set equal to 1 in order to control for any differences in absolute expression levels across individuals. (**A**) Representative Nrf2 western blots with Lamin B used as internal control, for an older man, older woman, young man, and young woman pre-Intervention. (**B**) Nrf2 signaling responses in young and older men. Black squares indicate response in young men and open circles indicate response in older men across time. (**C**) Young women (black squares) and older women (open circles) showed minimal Nrf2 signaling responsiveness to an acute exercise bout and were not significantly different from each other. Analyzed together with fitness and body composition covariates in the model, there was a significant main effect of age with young individuals showing a higher response in Nrf2 activation than older individuals (RM ANCOVA Age $p$ = 0.05). Data shown as Mean ± SEM.

### *3.3 Molecular Signaling Responses to Acute Exercise: Effect of Exercise Training*

Nrf2 signaling responses after exercise training were significantly higher in ET whereas the response did not change in CON (intervention by group interaction $p = 0.012$, effect of age $p = 0.014$, and effect of ET versus CON, $p = 0.034$, Figure 4A & B). This response was partially explained by the degree of improvement of $VO_2$ peak in the training group, ($p = 0.019$), the change in nuclear baseline levels of Nrf2 ($p = 0.039$), and the interaction of time by age ($p = 0.007$). Both young and older subjects significantly improved their Nrf2 signaling responses post-ET, however, when comparing only the ET group pre-post intervention the main effect of age was greater than the age differences at baseline ($p = 0.04$, Figure 4 C versus E), while the inactive controls did not show any significant change from pre-intervention (Figure 4 D&F).

The acute exercise trial induced a significant increase in HO1 mRNA in the cohort as a whole (Figure 5, main effect of time, $p = 0.039$). However, there were no significant differences between the groups or in response to the intervention. Additionally, there were no effects of age, sex, or any other covariate on HO1 mRNA response.

NQO1 mRNA response showed a significant interaction between intervention and age ($p = 0.045$, Figure 6), however when including the change in baseline Nrf2 expression ($\Delta$ Baseline Nrf2) the interaction was no longer significant. There was a significant interaction between the NQO1 mRNA response and measures of body composition and improvements in fitness (Time x $\Delta$Waist circumference, $p = 0.004$, Time x $\Delta$ Body Fat %,

$p$ = 0.008, Time x $\Delta VO_2$ peak $p$ = 0.017). There was a significant three-way interaction between intervention, time, and training group (Figure 6, $p$ = 0.028). The large standard error in the young control group was due to the response of one individual post-intervention. Analyzing the data without this outlier did not change the results. Additionally, there were six individuals who did not show any NQO1 amplification, hence the lower number of subjects for this gene set than the others. Interestingly, each of the individuals who did not show NQO1 amplification were of Native American, Asian, or Middle Eastern ethnicity.

For changes in GCLC mRNA responses to acute exercise, there was a significant main effect of time ($p$ = 0.005, Figure 7) There was also a three-way interaction between intervention by time by training group ($p$ = 0.05, Figure 7).There was a significant interaction between the intervention and change in baseline nuclear Nrf2 protein content (Intervention x $\Delta$ Baseline Nrf2 $p$ = 0.006). There was a significant interaction between the change in baseline Nrf2 levels and GCLC mRNA response to acute exercise (Time x $\Delta$ Baseline Nrf2 $p$ < 0.001). There was also a significant three-way interaction between the intervention, GCLC response, and the change in baseline Nrf2 protein expression (Intervention x Time x $\Delta$ Baseline Nrf2 $p$ < 0.001).

For GCLC protein responses there was a main effect of the change in peak $VO_2$ ($\Delta VO_2$ peak) on GCLC protein responses to ET ($p$ = 0.044). There was also an intervention by time interaction (p = 0.024), and a three-way interaction intervention by time by $\Delta VO_2$ (p = 0.031, Figure 8). There was a significant interaction between GCLC protein response

and the change in baseline GCLC protein content (Time x Δ Baseline GCLC protein, $p$ = 0.05). The new steady state protein concentrations after training were primarily being driven by men, although there were no significant differences in GCLC protein response by age or sex.

**Figure 4.**

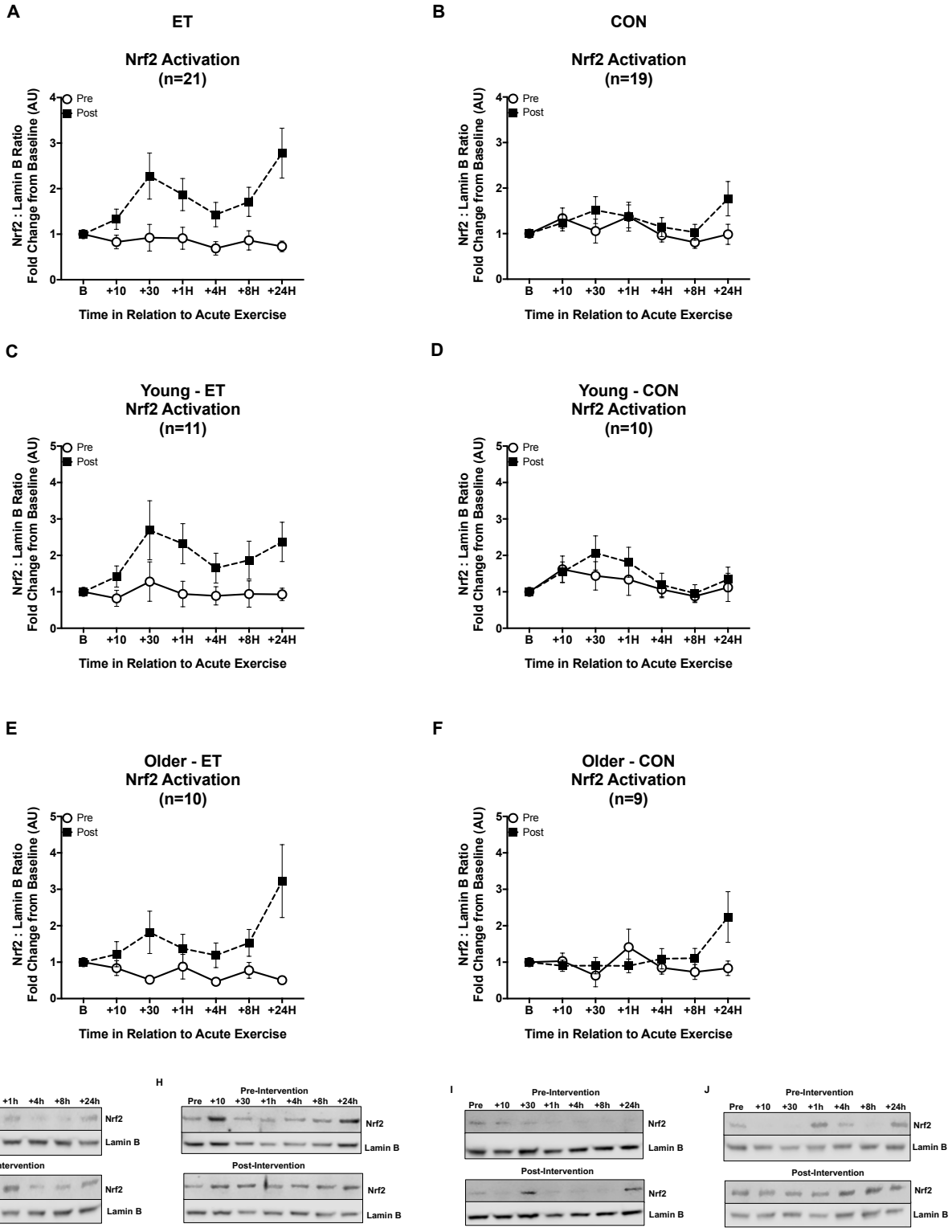

A
ET
Nrf2 Activation
(n=21)
B
CON
Nrf2 Activation
(n=19)
C
Young - ET
Nrf2 Activation
(n=11)
D
Young - CON
Nrf2 Activation
(n=10)
E
Older - ET
Nrf2 Activation
(n=10)
F
Older - CON
Nrf2 Activation
(n=9)
Pre
Post
Nrf2 : Lamin B Ratio
Fold Change from Baseline (AU)
B +10 +30 +1H +4H +8H +24H
Time in Relation to Acute Exercise
G
H
I
J
Pre +10 +30 +1h +4h +8h +24h
Nrf2
Lamin B
Post Intervention
Pre-Intervention
Post-Intervention

**Figure 4. Nrf2 signaling responses to acute exercise before and after exercise training or inactive control intervention by age**. Open circles indicate Nrf2 activation pre-intervention, and black squares indicate Nrf2 activation post-intervention. Panels A, C, & E are ET subjects while panels B, D, & F are CON subjects. (**A&B**) Eight weeks of aerobic exercise training increased the Nrf2 signaling response to an acute exercise bout in the whole cohort, while the Nrf2 signaling in the control group did not change. (**C&E**) The increase in Nrf2 signaling after training was similar in pattern for young and older adults, although young improved their responses to a greater degree than older ET subjects (main effect of age, $p = 0.014$). (**D&F**) Nrf2 signaling did not change from pre-intervention in older or young control groups. Representative western blots for (G) Young-ET, (H) Young-CON, (I) Older-ET, and (J) Older-CON. Data shown as Mean $\pm$ SEM.

**Figure 5.**

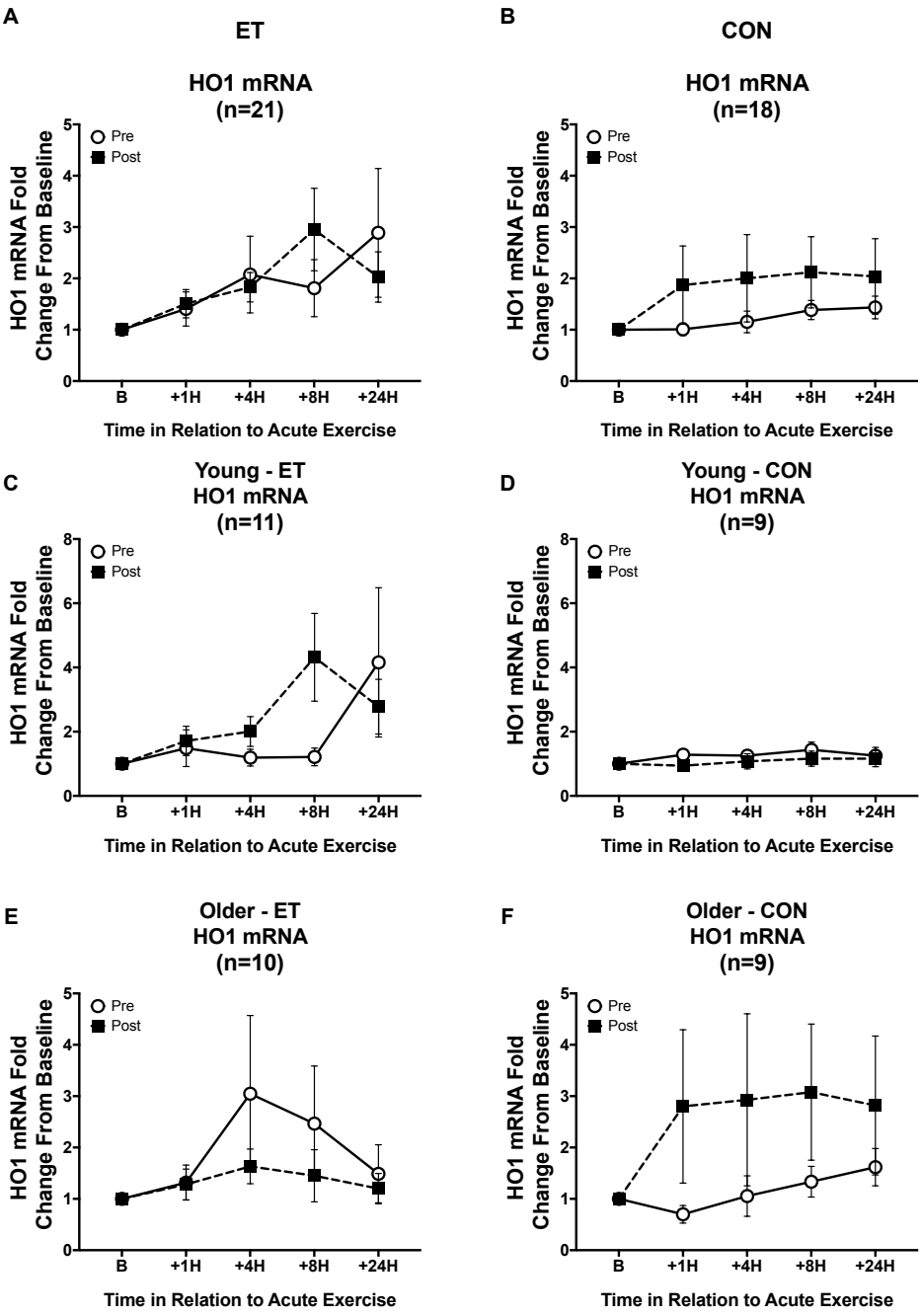



**Figure 5. HO1 mRNA signaling responses to an acute exercise bout before and after the intervention**. Open circles indicate HO1 mRNA responses pre-intervention, while black squares indicate responses post-intervention. (**A&B**) There was a significant

response to an acute exercise bout across the whole cohort ($p$ = 0.039), but there were no differences in HO1 mRNA responses after training or control intervention. (**C-F**) Additionally, there were no significant differences across age or sex. The pattern of responses differs across ET and CON within age groups, but these were not statistically significant. Data shown as Mean ± SEM.

**Figure 6.**

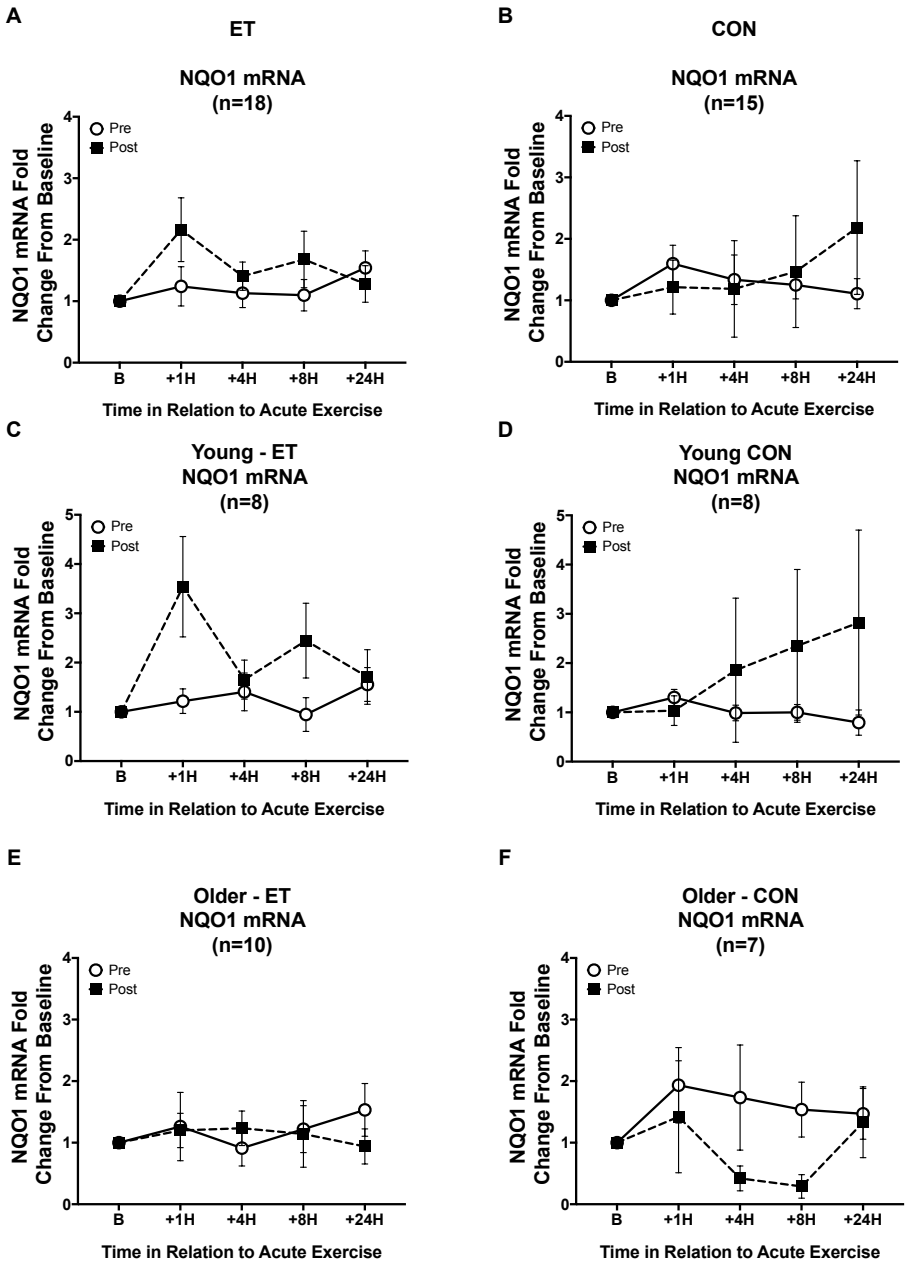



**Figure 6. NQO1 mRNA signaling responses to an acute exercise bout before and after the intervention.** Open circles indicate NQO1 mRNA responses to the acute exercise trial pre-intervention, while black squares indicate responses post-intervention. (**A&B**) In the whole cohort, there was a slight increase in NQO1 mRNA response after training, which was dependent on baseline Nrf2 expression changes (Δ Baseline Nrf2),

but no difference in response after control intervention. (**C&E**) This increase in NQO1 mRNA response after ET appears to be driven by younger individuals, not older. (**D&F**) Post-control intervention older individuals show a decreased response while younger controls show a delayed increase in NQO1 mRNA driven by one outlier in the post-intervention CON response. Panels A, C, & E are ET subjects, while B, D, & F are CON subjects. Data shown as Mean ± SEM.

**Figure 7.**

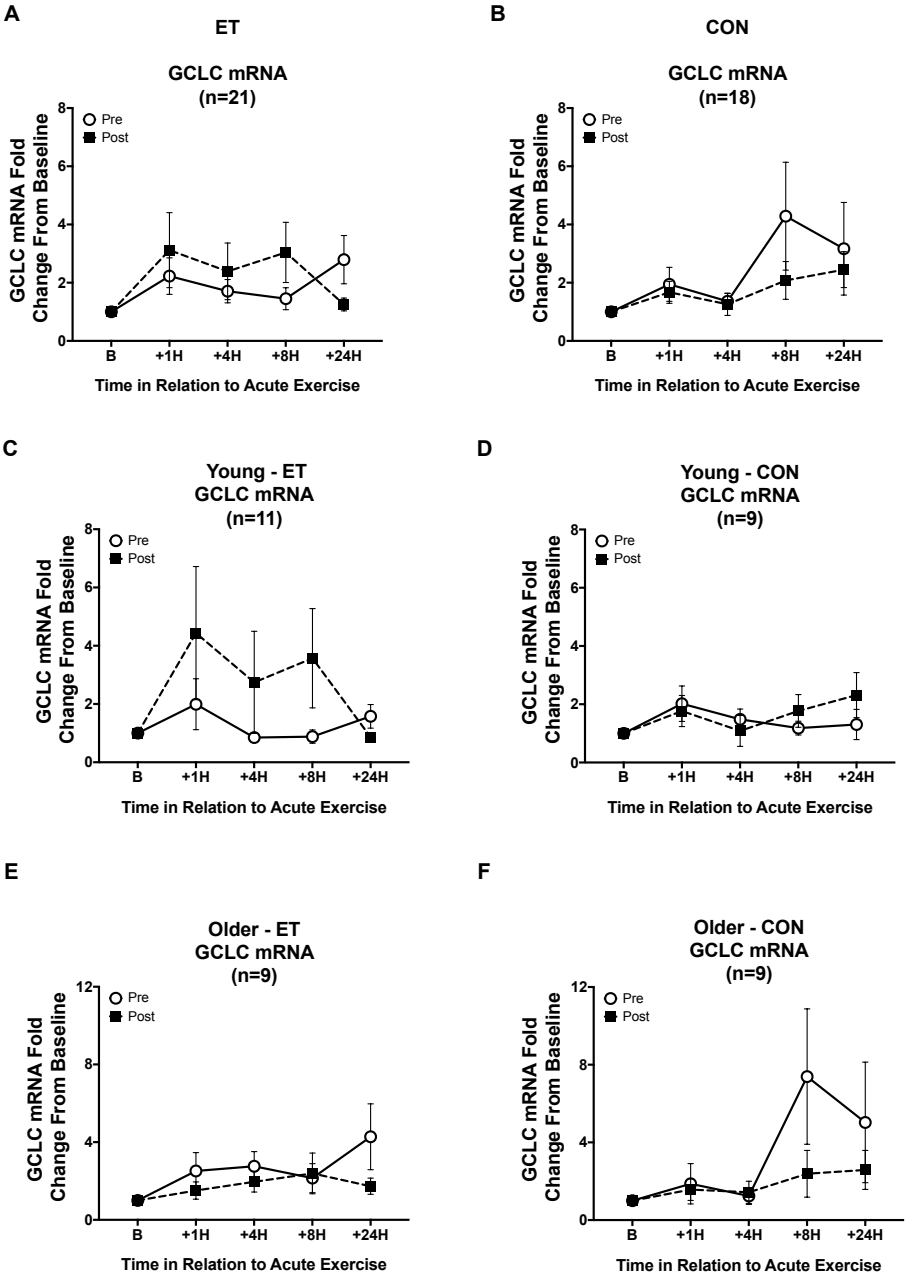



**Figure 7. GCLC mRNA signaling responses to an acute exercise bout before and after the intervention**. Open circles are pre-intervention GCLC mRNA responses and black squares are post-intervention responses. Panels A, C, & E are ET subjects and B,

D, & F are CON subjects. (**A&B**) Overall comparison of intervention, time, and training group responses to the acute exercise trial. There were significant interactions between intervention, time, and training group in GCLC mRNA response ($p$ = 0.05) as well as an intervention by time by ΔBaseline GCLC protein interaction ($p$ = 0.001). (**C-F**) Show acute exercise trial responses by age and training group. Data shown as Mean ± SEM.

**Figure 8.**

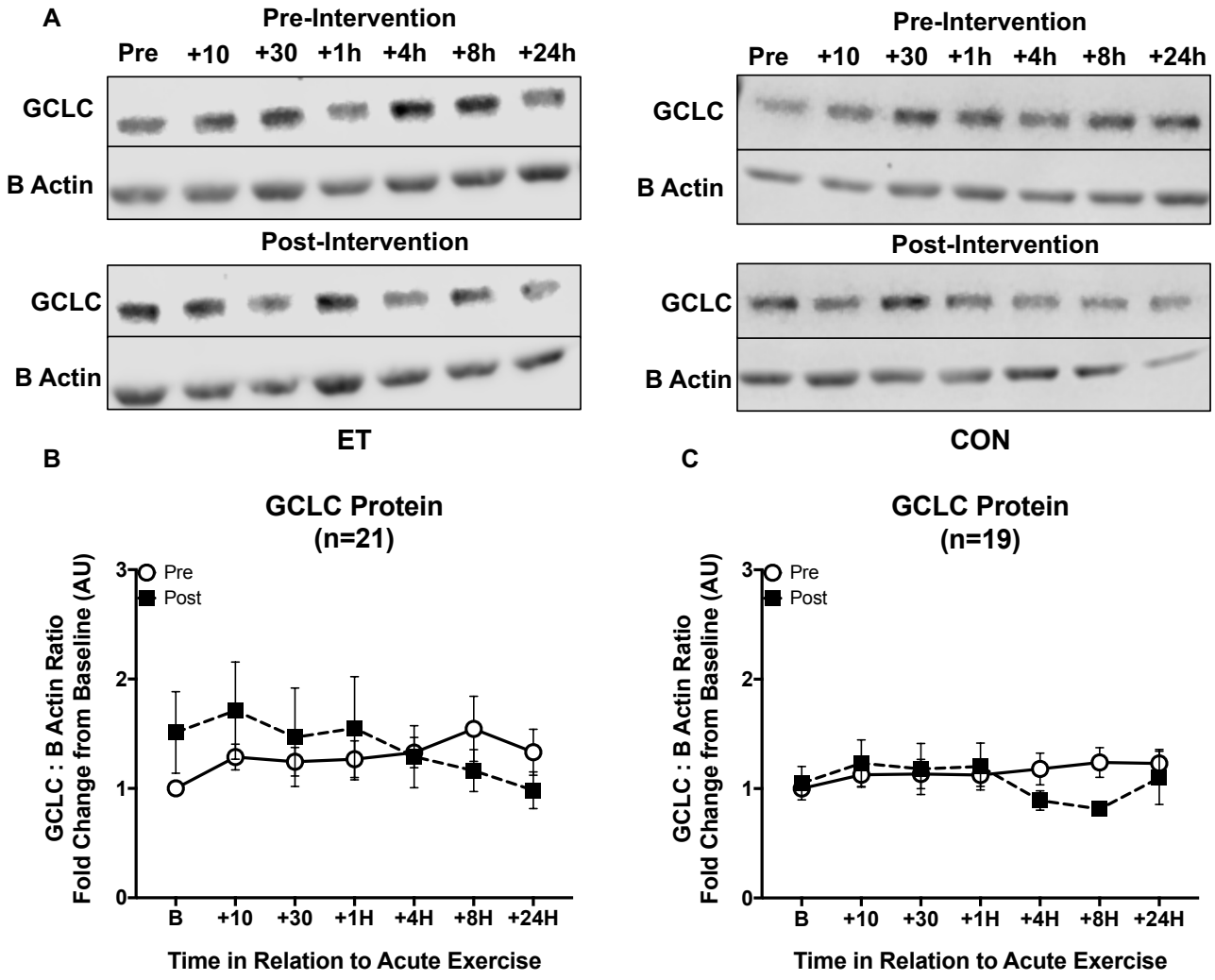



**Figure 8. GCLC protein responses to an acute exercise bout before and after eight weeks of ET or CON**. Open circles indicate pre-intervention GCLC protein responses to acute exercise, while black squares indicate post-intervention responses. (**A**) Representative blots of GCLC with β-Actin used as an internal control for ET and CON groups pre- and post-intervention respectively. (**B**) GCLC shows an increase in the steady state concentrations after exercise training compared to pre-intervention in trained subjects. (**C**) GCLC protein responses were not different after the inactive control intervention. There was a significant interaction between the GCLC protein response and

the change in baseline GCLC protein content ($p$ = 0.016). There were no significant differences between young and older individuals or by sex in response to the training. Data shown as Mean ± SEM.

### *3.4 Aerobic Exercise Training Changes Basal Steady State Levels of Nrf2 but not GCLC*

The Nrf2 and GCLC protein analysis for acute exercise responses was normalized to 1 for each of the individual's pre-intervention baseline values in order to make comparisons across individuals. This does not allow for analysis of basal expression levels for each protein across age and sex. Therefore, to test whether there were basal expression differences in Nrf2 or GCLC across age, sex, or in response to the intervention, we analyzed each individual's baseline samples pre and post intervention. There was a main effect of age on basal expression levels of Nrf2 before the intervention where older adults had significantly higher levels of Nrf2 at rest compared to young (independent sample t test, $p$ = 0.004, Figure 9A). Basal Nrf2 levels decreased in older adults after the ET but increased in CON (Intervention by Training group interaction $p$ = 0.034). Young individuals did not change significantly from pre to post intervention in either ET or CON groups. There was no statistically significant difference in in GCLC protein content across age or sex at pre-intervention (independent sample t test $p$ = 0.428, Figure 9B). After exercise training GCLC protein was elevated at baseline driven by the increases shown in older adults, although this did not reach statistical significance (Figure 9B). There were no sex differences in response to exercise training or control intervention for basal GCLC protein expression.

**Figure 9.**

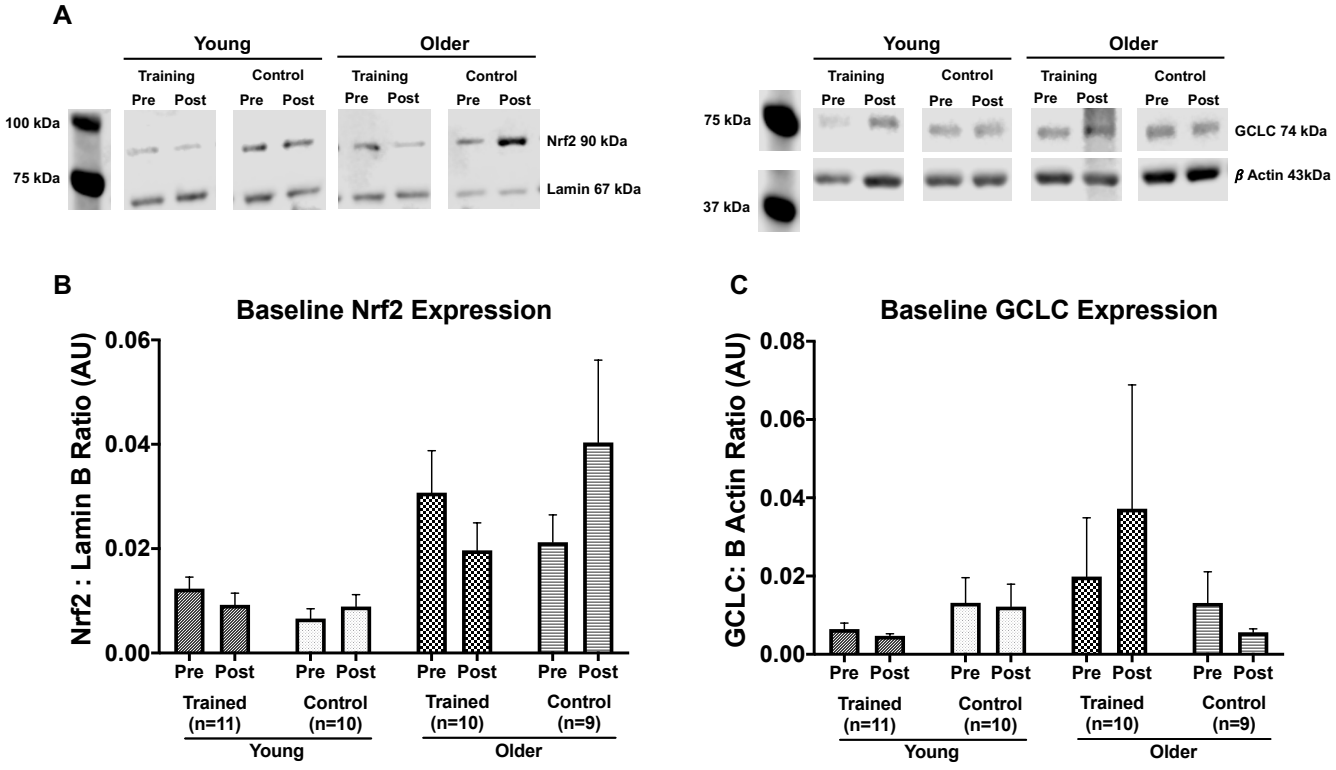



**Figure 9. Baseline expression of Nrf2 and GCLC before and after ET or CON**. (**A**) Representative blot images for young and older ET and CON individuals for Nrf2 and GCLC protein expression respectively. (**B**) Baseline Nrf2 protein expression was higher in older adults before the exercise intervention compared to young (independent sample t test, p = 0.004) but decreased with ET closer to young Nrf2 expression levels. Older controls showed the opposite pattern, increasing baseline Nrf2 expression after eight weeks of CON. Repeated measures ANCOVA shows a significant interaction between training group and pre-post intervention ($p = 0.032$). The majority of these differences are explained by age differences ($p < 0.001$) (**C**) There was no change in GCLC protein in young trained or control subjects. In older subjects there was no significant difference in basal GCLC protein after training or control interventions. Data shown as Mean ± SEM.

## 4. Discussion

Exercise is one of the most powerful tools available for improving health span [43-45]. Many of the beneficial effects rely on redox-dependent cell signaling mechanisms to induce exercise adaptations [46]. ROS and other electrophiles generated by exercise are

critical signaling molecules that lead to improved physiological function and healthy adaptations [47-53]. However, we and others have shown that redox dependent activation of Nrf2 through acute exercise is impaired in aging populations [10, 27].

This investigation tested the hypothesis that exercise training (repeated stimulus of acute exercise) would restore Nrf2 signaling effects in older adults, with an additional comparison to the responses of young. To our knowledge, this is the first randomized control trial to measure responses to acute exercise as well as effects of exercise training on Nrf2 time course responses in young and older men and women. Notably, the pre-intervention results replicate previous findings from our lab which was the first study to show that Nrf2 signaling in response to acute exercise was impaired with age in humans [10]. The current data, which in contrast to our earlier study included both sexes, demonstrate an effect of age on Nrf2 activation in response to the acute exercise trial when accounting for baseline levels of body composition and fitness. However, this effect of age was no longer significantly different when including baseline levels of Nrf2 expression suggesting that the variation in Nrf2 responses to exercise are dependent on basal Nrf2 expression levels. Additionally, there were clearly divergent responses between men and women. These sex differences were not anticipated, and by design the study was not powered to detect sex differences once the data were parsed by age and training group.

It is well established that exercise training changes the molecular signaling patterns that manifest from a single acute exercise bout, however this has never been tested in the

Nrf2 signaling pathway and aging human populations. The decrease in basal Nrf2 expression observed in the older group after exercise training may seem counterintuitive. However, since Nrf2 is a stress response transcription factor, it would be expected to be lowly expressed under resting unstressed conditions. Elevated Nrf2 levels at rest in older adults suggests that there are higher levels of redox stress compared to young and is in line with findings in skeletal muscle in mice where older mice have higher activation of redox-sensitive transcription factors at rest but fail to increase the activation in response to an acute stressor [54]. Lowering the basal redox stress levels would therefore be advantageous. The decrease in baseline Nrf2 levels in older adults after training is an indicator that training reduces redox stress under resting conditions. This subsequently improved the signaling capacity to respond to stressful events like exercise with the appropriate magnitude and timing necessary to adapt to the stimulus similar to that observed in young. A recent investigation that compared individuals that were stratified based on their acute exercise oxidative stress response, measured as urinary $F_{2}$-isoprostanes, demonstrated that higher oxidative stress responses to acute exercise resulted in greater performance adaptations to endurance training than individuals with lower oxidative stress response to acute exercise [55]. The study also found that the oxidative stress response was correlated to resting levels of oxidative stress. The same group more recently demonstrated that targeted antioxidant supplementation to restore a deficiency was beneficial for exercise performance, whereas supplementation in non-deficient individuals had no effect on exercise performance [56]. In agreement with our data, young and older mice subjected to exercise training showed decreases in Nrf2 content after training [57]. This general pattern of response in redox signaling systems

has been hypothesized and predicted by others in the field [58], where accumulating oxidative stress in aging cells leads to an inability to respond further to a redox stressor. As far as we are aware this pattern of response has not been demonstrated in humans until now.

Nrf2 activation response to acute exercise improved significantly in response to the exercise intervention (Figure 4). The older individuals in the ET group showed significant improvement compared to pre intervention levels. Studies in mouse myocardium show similar improvements in Nrf2 signaling and redox status after exercise training [27]. Contrary to our hypothesis, there was greater improvement in Nrf2 signaling in young individuals compared to older in response to the training. Comparing the magnitude of peak improvement (+30-min time point) in young (+1.41-fold) versus older individuals (+1.30-fold), the relative improvements in Nrf2 signaling after ET were greater in young individuals than in older individuals ($p$ = 0.04) suggesting that the improvements elicited from exercise training do not completely reverse biological aging. Similar differences have been found in lifelong trained versus untrained older adults [59]. Their data showed that redox signaling changes that occur with aging are not completely reversed even with lifelong training, again pointing to an underlying biological aging process independent of training status [59, 60]. Our results are in agreement with these findings, and show that redox signaling decline in older adults is an inherent biological aging process independent of training status. Whether the response can be further augmented with greater exercise volume (intensity, duration) or through synergistic effects between exercise and phytonutrient Nrf2 activators remains to be elucidated.

Both NQO1 and GCLC mRNA showed significant responses to the acute exercise bouts as well as age related differences in signaling post intervention. The three-way interaction of intervention, time, and training group for NQO1 mRNA response indicates that only the ET group improved signaling responses after training. The change in basal Nrf2 levels explains the age-related differences in signaling post-ET. GCLC mRNA responded to the acute exercise bout and the interaction of intervention by time by training group indicates ET improved GCLC mRNA responses after training. Again, the responses to acute exercise after training were explained by the changes in baseline Nrf2 levels. Taken together these gene expression data support the conclusions drawn by the Nrf2 western blotting data.

While HO1 mRNA did respond to a single acute bout across the entire cohort, there were no age or sex differences (Figure 5). Furthermore, there were no significant effects of changing Nrf2 levels on HO1 mRNA responses to acute exercise before or after exercise training. Other literature has shown that HO1 is transcriptionally regulated by a number of other transcription factors [61, 62], therefore we speculate that Nrf2 has less of a contribution to HO1 responses to acute exercise in human PBMCs. The divergent responses pre-post intervention for older adults by training group (Figure 5, E & F) are puzzling. One possible explanation is that redox stress decreased with training, and the increase in Nrf2 signaling is maintaining higher steady state levels of mRNA, so it appears that the response to acute exercise did not change. Conversely, the older controls had

lower amounts after the intervention and needed to respond to a greater degree in order to maintain redox homeostasis. However, these responses were not significantly different.

It is expected that time course changes in steady state proteins such as GCLC will likely take longer duration to show any significant accumulation. This was the rationale for performing the 4-, 8-, and 24-hour blood draws and the notion that any increase in protein content would be seen after peaks in gene expression occurred. Changes in steady state protein concentration is dependent on multiple inputs including protein turnover, localization, and post-translational modifications, providing a healthy debate over just how important mRNA accretion is for protein accumulation in response to exercise [63]. However, the peak of GCLC protein accumulation occurred at 8 hours after the acute exercise bout at pre-intervention, which is in line with the time course of expected changes where Nrf2 peaked at 30 minutes, and GCLC mRNA peaked at 1 hour for young and 8 hours in older subjects. Together these time course data provide rationale for using mRNA as an outcome measure, particularly when the primary outcome is transcription factor activity. The GCLC protein changes post-ET were directly related to the degree of improvement in $VO_2$ peak. Additionally, the degree of responsiveness to an acute exercise bout after ET was dependent on the degree of change in baseline GCLC protein.

Our overall findings suggest that exercise training reduces baseline levels of Nrf2 in order to allow the system to respond more robustly to subsequent redox stressors. The lack of responsiveness to stressors is a general phenomenon in aging populations that leads to overall impaired stress resilience. While older adults did improve Nrf2 nuclear localization

in response to acute exercise after ET, this did not necessarily translate to improved mRNA accretion. The increases in mRNA levels in GCLC were modest in older adults, and the changes in baseline GCLC protein seem to be driven by older adults, but they were not significant. These non-significant changes could be due to sex differences confounding the results because the changes in GCLC levels after ET seemed to be primarily driven by older men. Transcriptomic approach may be better able to detect changes across the host of genes regulated by Nrf2.

The free radical theory of aging described by Denham Harman in the late 1950's was and still is an influential theoretical framework in redox biology and aging fields [64, 65]. Although the macromolecular damage hypothesis has been discarded, it has been replaced with more refined and nuanced versions of the hypothesis [9, 26]. The redox stress hypothesis of aging and the cell signaling disruption theory of aging both posit that the observed increases in oxidative stress in aging do not necessarily lead to damaged macromolecules but impair redox homeostasis and the ability to elicit a molecular signaling response to restore redox homeostasis. Based on these hypotheses, one would predict that older cells would show attenuated redox signaling responses, such as the Keap1-Nrf2 signaling axis, to stimuli compared to younger cells. This was previously shown in an eloquent series of experiments by Meng et al. [22] who tested the redox stress response capacity hypothesis using five different redox stressors in a cell culture model and in *C. Elegans*. Older cells and organisms showed lower ROS production, impaired redox cell signaling, and impaired redox homeostasis in response to redox stressors compared to young [22]. Similarly to our human data, their results show that

redox stress associated with aging leads to further impaired redox signaling responses resulting in a decreased ability to restore redox homeostasis.

Experimental manipulation of baseline oxidative stress has been shown to alter the response to acute exercise in young men [66]. The subjects underwent a 12-day intervention of either a pro-oxidant stimulus of passive smoking (elevated baseline) or an antioxidant stimulus of vitamin C supplementation (lowered baseline). The subsequent oxidative stress response to acute exercise was lower in the passive smoking group than in the group that had been treated with an antioxidant. It is now widely accepted that exercise-induced ROS signaling is key to long-term adaptations and therefore the impaired response in the passive smoking group would be considered mal-adaptive. Our results show the older individuals who underwent exercise training demonstrated lower baseline Nrf2 levels and augmented responses to acute exercise, which is consistent with the findings of Theodorou et al. [66].

*Strengths and Limitations*

The RCT design of this study along with the extremely tight data in the control groups strengthens the results and data interpretation. The adherence to the exercise intervention and low drop out (none in the control groups) is both a testament to the dedication of the participants and the study personnel. The main limitation of the study was that it was underpowered to adequately detect sex differences. Rather than limiting the study cohort to older individuals, we thought it was important to be able to compare the response to the exercise intervention to those seen in young and there were no

available data indicating sex differences in the response. Recently published data from Drosophila show sexually dimorphic responses to oxidative stress [67]. Future studies should include an adequate subject number to analyze sex differences in redox signaling responses in humans. We make the assumption that higher baseline levels of Nrf2 pre-intervention were due to elevated or aberrant production of ROS or other electrophilic species, and that exercise training helped reduce baseline production of those reactive species. However, this is somewhat speculative since ROS or any reactive species adducts were not measured.

## 5. Conclusions

The major finding of the current investigation was that the observed age-related impairment in stimulated Nrf2 activation was improved through exercise training, demonstrating that the pathway retains plasticity to respond. The second novel finding was that basal nuclear Nrf2 levels were elevated in older adults, which may explain the inability to respond to an acute stimulus. The exercise intervention lowered the basal nuclear Nrf2 content and the pre-post change in the levels was a significant predictor of the improvement in the stimulated response. The effect of the Nrf2 activation on the downstream response was less robust in the older individuals and may have been affected by the unexpected sex differences. Future studies should add a transcriptomic approach because of the number of gene targets regulated by Nrf2. Furthermore, the age-related differences in signaling responses for both Nrf2 and downstream target genes NQO1 and GCLC after ET prevailed after the intervention, which suggests that despite the improvement in older adults, there remains an element of biological aging not affected

by exercise training or that a greater stimulus is needed. Our findings support the redox stress hypothesis of aging and the decay of redox stress response capacity, and add additional evidence for exercise hormesis theoretical frameworks [9, 26, 50]. Future work should investigate the degree to which these signaling responses are apparent in other tissues in aging models, and model systems of disease where oxidative stress is a key clinical manifestation of the particular disease.

## REFERENCES

[1] B.F. Miller, D.R. Seals, K.L. Hamilton, A viewpoint on considering physiological principles to study stress resistance and resilience with aging, Ageing Res Rev 38 (2017) 1-5.

[2] L.C.D. Pomatto, K.J.A. Davies, The role of declining adaptive homeostasis in ageing, J Physiol 595(24) (2017) 7275-7309.

[3] L.C.D. Pomatto, P.Y. Sun, K.J.A. Davies, To Adapt or not to Adapt: Consequences of Declining Adaptive Homeostasis and Proteostasis with Age, Mech Ageing Dev (2018).

[4] B.K. Kennedy, S.L. Berger, A. Brunet, J. Campisi, A.M. Cuervo, E.S. Epel, C. Franceschi, G.J. Lithgow, R.I. Morimoto, J.E. Pessin, T.A. Rando, A. Richardson, E.E. Schadt, T. Wyss-Coray, F. Sierra, Geroscience: linking aging to chronic disease, Cell 159(4) (2014) 709-13.

[5] S.V. Lakshmi, G. Padmaja, P. Kuppusamy, V.K. Kutala, Oxidative stress in cardiovascular disease, Indian journal of biochemistry & biophysics 46(6) (2009) 421-40.

[6] H. Kaneto, N. Katakami, D. Kawamori, T. Miyatsuka, K. Sakamoto, T.A. Matsuoka, M. Matsuhisa, Y. Yamasaki, Involvement of oxidative stress in the pathogenesis of diabetes, Antioxid Redox Signal 9(3) (2007) 355-66.

[7] W.R. Markesbery, Oxidative stress hypothesis in Alzheimer's disease, Free radical biology & medicine 23(1) (1997) 134-47.

[8] E. Panieri, M.M. Santoro, ROS homeostasis and metabolism: a dangerous liason in cancer cells, Cell death & disease 7(6) (2016) e2253.

[9] R.S. Sohal, W.C. Orr, The redox stress hypothesis of aging, Free radical biology & medicine 52(3) (2012) 539-55.

[10] A.J. Done, M.J. Gage, N.C. Nieto, T. Traustadóttir, Exercise-induced Nrf2-signaling is impaired in aging, Free Radic Biol Med 96 (2016) 130-8.

[11] A.J. Done, M.J. Newell, T. Traustadóttir, Effect of exercise intensity on Nrf2 signalling in young men, Free Radic Res 51(6) (2017) 646-655.

[12] S.S. Davies, T. Traustadóttir, A.A. Stock, F. Ye, Y. Shyr, S.M. Harman, L.J. Roberts, 2nd, Ischemia/reperfusion unveils impaired capacity of older adults to restrain oxidative insult, Free Radic Biol Med 47(7) (2009) 1014-8.

[13] K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J.D. Engel, M. Yamamoto, Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain, Genes Dev 13(1) (1999) 76-86.

[14] T. Ishii, K. Itoh, S. Takahashi, H. Sato, T. Yanagawa, Y. Katoh, S. Bannai, M. Yamamoto, Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages, The Journal of biological chemistry 275(21) (2000) 16023-9.

[15] K.I. Tong, B. Padmanabhan, A. Kobayashi, C. Shang, Y. Hirotsu, S. Yokoyama, M. Yamamoto, Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response, Molecular and cellular biology 27(21) (2007) 7511-21.

[16] E. Kansanen, S.M. Kuosmanen, H. Leinonen, A.L. Levonen, The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer, Redox Biol 1 (2013) 45-9.

[17] C.J. Miller, S.S. Gounder, S. Kannan, K. Goutam, V.R. Muthusamy, M.A. Firpo, J.D. Symons, R. Paine, 3rd, J.R. Hoidal, N.S. Rajasekaran, Disruption of Nrf2/ARE signaling impairs antioxidant mechanisms and promotes cell degradation pathways in aged skeletal muscle, Biochimica et biophysica acta 1822(6) (2012) 1038-50.

[18] V.R. Muthusamy, S. Kannan, K. Sadhaasivam, S.S. Gounder, C.J. Davidson, C. Boeheme, J.R. Hoidal, L. Wang, N.S. Rajasekaran, Acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium, Free Radic Biol Med 52(2) (2012) 366-76.

[19] M. Narasimhan, J. Hong, N. Atieno, V.R. Muthusamy, C.J. Davidson, N. Abu-Rmaileh, R.S. Richardson, A.V. Gomes, J.R. Hoidal, N.S. Rajasekaran, Nrf2 deficiency promotes apoptosis and impairs PAX7/MyoD expression in aging skeletal muscle cells, Free Radic Biol Med 71 (2014) 402-14.

[20] H. Zhang, K.J.A. Davies, H.J. Forman, Oxidative stress response and Nrf2 signaling in aging, Free Radic Biol Med 88(Pt B) (2015) 314-336.

[21] L. Zhou, H. Zhang, K.J.A. Davies, H.J. Forman, Aging-related decline in the induction of Nrf2-regulated antioxidant genes in human bronchial epithelial cells, Redox Biology 14 (2018) 35-40.

[22] J. Meng, Z. Lv, X. Qiao, X. Li, Y. Li, Y. Zhang, C. Chen, The decay of Redox-stress Response Capacity is a substantive characteristic of aging: Revising the redox theory of aging, Redox Biol 11 (2017) 365-374.

[23] C. Henriquez-Olguin, A. Diaz-Vegas, Y. Utreras-Mendoza, C. Campos, M. Arias-Calderon, P. Llanos, A. Contreras-Ferrat, A. Espinosa, F. Altamirano, E. Jaimovich, D.M. Valladares, NOX2 Inhibition Impairs Early Muscle Gene Expression Induced by a Single Exercise Bout, Frontiers in physiology 7 (2016) 282.

[24] C. Henriquez-Olguin, J.R. Knudsen, S.H. Raun, Z. Li, E. Dalbram, J.T. Treebak, L. Sylow, R. Holmdahl, E.A. Richter, E. Jaimovich, T.E. Jensen, Cytosolic ROS production by NADPH oxidase 2 regulates muscle glucose uptake during exercise, Nat Commun 10(1) (2019) 4623.

[25] C. Henriquez-Olguin, L.B. Renani, L. Arab-Ceschia, S.H. Raun, A. Bhatia, Z. Li, J.R. Knudsen, R. Holmdahl, T.E. Jensen, Adaptations to high-intensity interval training in skeletal muscle require NADPH oxidase 2, Redox Biol 24 (2019) 101188.

[26] J. Viña, C. Borras, K.M. Abdelaziz, R. Garcia-Valles, M.C. Gomez-Cabrera, The Free Radical Theory of Aging Revisited: The Cell Signaling Disruption Theory of Aging, Antioxidants & Redox Signaling 19(8) (2013) 779-787.

[27] S.S. Gounder, S. Kannan, D. Devadoss, C.J. Miller, K.J. Whitehead, S.J. Odelberg, M.A. Firpo, R. Paine, 3rd, J.R. Hoidal, E.D. Abel, N.S. Rajasekaran, Impaired transcriptional activity of Nrf2 in age-related myocardial oxidative stress is reversible by moderate exercise training, PloS one 7(9) (2012) e45697.

[28] A.J. Done, T. Traustadóttir, Nrf2 mediates redox adaptations to exercise, Redox Biol 10 (2016) 191-199.

[29] T.C. Nordin, A.J. Done, T. Traustadottir, Acute exercise increases resistance to oxidative stress in young but not older adults, Age (Dordr) 36(6) (2014) 9727.

[30] S.S. Gounder, S. Kannan, D. Devadoss, C.J. Miller, K.S. Whitehead, S.J. Odelberg, M.A. Firpo, R. Paine, 3rd, J.R. Hoidal, E.D. Abel, N.S. Rajasekaran, Impaired

transcriptional activity of Nrf2 in age-related myocardial oxidative stress is reversible by moderate exercise training, PloS one 7(9) (2012) e45697.

[31] C.M. Cottreau, R.B. Ness, A.M. Kriska, Physical activity and reduced risk of ovarian cancer, Obstet Gynecol 96(4) (2000) 609-14.

[32] C.S. Winters-Hart, J.S. Brach, K.L. Storti, J.M. Trauth, A.M. Kriska, Validity of a questionnaire to assess historical physical activity in older women, Med Sci Sports Exerc 36(12) (2004) 2082-7.

[33] W.E. Siri, Body composition from fluid spaces and density: analysis of methods. 1961, Nutrition 9(5) (1993) 480-91; discussion 480, 492.

[34] T. Traustadóttir, A.A. Stock, S.M. Harman, High-dose statin use does not impair aerobic capacity or skeletal muscle function in older adults, Age (Dordr) 30(4) (2008) 283-91.

[35] W.M. Kohrt, M.T. Malley, A.R. Coggan, R.J. Spina, T. Ogawa, A.A. Ehsani, R.E. Bourey, W.H. Martin, 3rd, J.O. Holloszy, Effects of gender, age, and fitness level on response of VO2max to training in 60-71 yr olds, Journal of applied physiology (Bethesda, Md. : 1985) 71(5) (1991) 2004-11.

[36] M. American College of Sports, ACSM's guidelines for exercise testing and prescription, Sixth edition. Philadelphia : Lippincott Williams & Wilkins, [2000] ©20002000.

[37] U. Wisloff, A. Stoylen, J.P. Loennechen, M. Bruvold, O. Rognmo, P.M. Haram, A.E. Tjonna, J. Helgerud, S.A. Slordahl, S.J. Lee, V. Videm, A. Bye, G.L. Smith, S.M. Najjar, O. Ellingsen, T. Skjaerpe, Superior cardiovascular effect of aerobic interval training versus moderate continuous training in heart failure patients: a randomized study, Circulation 115(24) (2007) 3086-94.

[38] C.E. Garber, B. Blissmer, M.R. Deschenes, B.A. Franklin, M.J. Lamonte, I.M. Lee, D.C. Nieman, D.P. Swain, M. American College of Sports, American College of Sports Medicine position stand. Quantity and quality of exercise for developing and maintaining cardiorespiratory, musculoskeletal, and neuromotor fitness in apparently healthy adults: guidance for prescribing exercise, Med Sci Sports Exerc 43(7) (2011) 1334-59.

[39] K. Suzuki, P. Bose, R.Y. Leong-Quong, D.J. Fujita, K. Riabowol, REAP: A two minute cell fractionation method, BMC research notes 3 (2010) 294.

[40] D.J. Mahoney, K. Carey, M.H. Fu, R. Snow, D. Cameron-Smith, G. Parise, M.A. Tarnopolsky, Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise, Physiological genomics 18(2) (2004) 226-31.

[41] M. Catoire, M. Mensink, M.V. Boekschoten, R. Hangelbroek, M. Muller, P. Schrauwen, S. Kersten, Pronounced effects of acute endurance exercise on gene expression in resting and exercising human skeletal muscle, PloS one 7(11) (2012) e51066.

[42] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines:

minimum information for publication of quantitative real-time PCR experiments, Clin Chem 55(4) (2009) 611-22.

[43] H. Pareja-Galeano, N. Garatachea, A. Lucia, Exercise as a Polypill for Chronic Diseases, Prog Mol Biol Transl Sci 135 (2015) 497-526.

[44] N. Garatachea, H. Pareja-Galeano, F. Sanchis-Gomar, A. Santos-Lozano, C. Fiuza-Luces, M. Moran, E. Emanuele, M.J. Joyner, A. Lucia, Exercise attenuates the major hallmarks of aging, Rejuvenation Res 18(1) (2015) 57-89.

[45] N. Garatachea, A. Santos-Lozano, D.C. Hughes, #xf3, A. mez-Cabello, I. Ara, Physical Exercise as an Effective Antiaging Intervention, BioMed Research International 2017 (2017) 2.

[46] T.L. Merry, M. Ristow, Nuclear factor erythroid-derived 2-like 2 (NFE2L2, Nrf2) mediates exercise-induced mitochondrial biogenesis and the anti-oxidant response in mice, J Physiol 594(18) (2016) 5195-207.

[47] S. Goto, H. Naito, T. Kaneko, H.Y. Chung, Z. Radak, Hormetic effects of regular exercise in aging: correlation with oxidative stress, Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme 32(5) (2007) 948-53.

[48] Z. Radak, H.Y. Chung, S. Goto, Exercise and hormesis: oxidative stress-related adaptation for successful aging, Biogerontology 6(1) (2005) 71-5.

[49] Z. Radak, H.Y. Chung, E. Koltai, A.W. Taylor, S. Goto, Exercise, oxidative stress and hormesis, Ageing research reviews 7(1) (2008) 34-42.

[50] Z. Radak, K. Ishihara, E. Tekus, C. Varga, A. Posa, L. Balogh, I. Boldogh, E. Koltai, Exercise, oxidants, and antioxidants change the shape of the bell-shaped hormesis curve, Redox Biol 12 (2017) 285-290.

[51] Z. Radak, Z. Zhao, E. Koltai, H. Ohno, M. Atalay, Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling, Antioxid Redox Signal 18(10) (2013) 1208-46.

[52] M.C. Gomez-Cabrera, C. Borras, F.V. Pallardo, J. Sastre, L.L. Ji, J. Vina, Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats, J Physiol 567(Pt 1) (2005) 113-20.

[53] M.C. Gomez-Cabrera, E. Domenech, M. Romagnoli, A. Arduini, C. Borras, F.V. Pallardo, J. Sastre, J. Vina, Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance, The American journal of clinical nutrition 87(1) (2008) 142-9.

[54] A. Vasilaki, F. McArdle, L.M. Iwanejko, A. McArdle, Adaptive responses of mouse skeletal muscle to contractile activity: The effect of age, Mech Ageing Dev 127(11) (2006) 830-9.

[55] N.V. Margaritelis, A.A. Theodorou, V. Paschalis, A.S. Veskoukis, K. Dipla, A. Zafeiridis, G. Panayiotou, I.S. Vrabas, A. Kyparos, M.G. Nikolaidis, Adaptations to endurance training depend on exercise-induced oxidative stress: exploiting redox interindividual variability, Acta Physiol (Oxf) 222(2) (2018).

[56] N.V. Margaritelis, V. Paschalis, A.A. Theodorou, A. Kyparos, M.G. Nikolaidis, Antioxidant supplementation, redox deficiencies and exercise performance: A falsification design, Free Radic Biol Med 158 (2020) 44-52.

[57] M.J. Crilly, L.D. Tryon, A.T. Erlich, D.A. Hood, The role of Nrf2 in skeletal muscle contractile and mitochondrial function, Journal of applied physiology 121(3) (2016) 730-40.

[58] R.V. Musci, K.L. Hamilton, M.A. Linden, Exercise-Induced Mitohormesis for the Maintenance of Skeletal Muscle and Healthspan Extension, Sports (Basel, Switzerland) 7(7) (2019).

[59] J.N. Cobley, G.K. Sakellariou, D.J. Owens, S. Murray, S. Waldron, W. Gregson, W.D. Fraser, J.G. Burniston, L.A. Iwanejko, A. McArdle, J.P. Morton, M.J. Jackson, G.L. Close, Lifelong training preserves some redox-regulated adaptive responses after an acute exercise stimulus in aged human skeletal muscle, Free Radic Biol Med 70 (2014) 23-32.

[60] J.N. Cobley, P.R. Moult, J.G. Burniston, J.P. Morton, G.L. Close, Exercise improves mitochondrial and redox-regulated stress responses in the elderly: better late than never!, Biogerontology 16(2) (2015) 249-64.

[61] J. Alam, J.L. Cook, How many transcription factors does it take to turn on the heme oxygenase-1 gene?, Am J Respir Cell Mol Biol 36(2) (2007) 166-74.

[62] S. Inouye, Y. Hatori, T. Kubo, S. Saito, H. Kitamura, R. Akagi, NRF2 and HSF1 coordinately regulate heme oxygenase-1 expression, Biochem Biophys Res Commun 506(1) (2018) 7-11.

[63] B.F. Miller, A.R. Konopka, K.L. Hamilton, Last Word on Viewpont: On the rigorous study of exercise adaptations: why mRNA might not be enough?, Journal of Applied Physiology 121(2) (2016) 601-601.

[64] D. Harman, Aging: A Theory Based on Free Radical and Radiation Chemistry, Journal of Gerontology 11(3) (1956) 298-300.

[65] D. Harman, The Biologic Clock: The Mitochondria?, Journal of the American Geriatrics Society 20(4) (1972) 145-147.

[66] A.A. Theodorou, V. Paschalis, A. Kyparos, G. Panayiotou, M.G. Nikolaidis, Passive smoking reduces and vitamin C increases exercise-induced oxidative stress: does this make passive smoking an anti-oxidant and vitamin C a pro-oxidant stimulus?, Biochem Biophys Res Commun 454(1) (2014) 131-6.

[67] L.C.D. Pomatto, J. Tower, K.J.A. Davies, Sexual Dimorphism and Aging Differentially Regulate Adaptive Homeostasis, The journals of gerontology. Series A, Biological sciences and medical sciences 73(2) (2018) 141-149.

# CHAPTER 3 – Effects of Exercise Training on Redox Stress Resilience in Young and Older Adults

Ethan L. Ostrom[1], Savannah R. Berry[1], and Tinna Traustadóttir[1*]

[1]Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona

[*]To Whom Correspondence Should Be Addressed:

Tinna Traustadóttir, Ph.D.

Department of Biological Sciences

Northern Arizona University

PO Box 5640

Flagstaff, AZ 86011-5640

Phone: 928-523-7291

Fax : 928-523-7500

E-mail: tinna.traustadottir@nau.edu

## ABSTRACT

***Purpose.*** Perturbations in redox homeostasis can lead to physiological dysfunction and impaired stress resilience. This randomized controlled trial investigated whether aerobic exercise training could improve redox stress resilience measured by the response to a non-exercise redox stressor (forearm ischemia/reperfusion; I/R trial) in young and older men and women. We hypothesized that older adults would have impaired responses to redox perturbations, but that exercise training would reverse the dysfunction. ***Methods.*** Young (18-28yo, n=21) and older (60-77yo, n=19) men and women were randomized to 8-week exercise training (ET; 3 d/wk, 45 min/day) or a non-exercise control group (CON). Aerobic capacity was measured by $VO_2$ peak test on a cycle ergometer. Plasma $F_2$-isoprostane responses to the I/R trial were measured across 7 time points: pre-trial, and 15-, 30-, 60-, 120-, 180-, and 240-minutes post-trial. The I/R trial was completed before and after the 8-week exercise intervention or control arm. ***Results.*** There were no significant differences in I/R trial responses across age, sex, or groups randomized to training or control. Exercise training significantly improved I/R trial response compared to controls ($p$<0.01). This improvement was directly related to the degree of improvement in $VO_2$ peak (Pearson correlation r = -0.464, $p$=0.003). ***Conclusions.*** These data demonstrate that the beneficial effects of moderate exercise in previously inactive adults elicit increased redox capacity that translates to an improved response to a non-exercise redox stressor regardless of age or sex. Furthermore, greater improvements in aerobic fitness resulted in greater resilience to the I/R-induced redox stress.

**ClinicalTrials.gov ID: NCT03419988**

**Key Words:** $VO_2$ max; $F_2$-isoprostanes; Ischemia/Reperfusion; Aging

## INTRODUCTION

Exercise is one of the most powerful pleiotropic interventions available to reduce the risk of developing many chronic conditions [1-3]. Redox signaling is one of several critical mechanisms involved in adaptations to exercise [4-6]. These adaptations may also improve redox homeostasis and redox stress resilience to non-exercise stressors. However, investigations using randomized control trials combined with a dynamic redox perturbation and gold standard redox stress outcome measures are lacking.

We have previously used forearm ischemia reperfusion trial as a dynamic non-exercise stressor to elicit oxidative stress measured by plasma $F_2$-isoprostanes across multiple time points. Ischemia or hypoxia followed by reperfusion/reoxygenation is known to elicit formation of reactive oxygen species, from diverse sources including mitochondria and enzymes (see [7] for a detailed review of mechanisms). $F_2$-isoprostanes are a reliable marker of lipid peroxidation, formed by nonenzymatic peroxidation of arachidonic acid [8]. Higher plasma $F_2$-isoprostane levels are associated with increased risk for cardiovascular disease and atherosclerosis, lower cognitive performance in healthy older adults, and poorer clinical outcomes in elderly patients [9-12]. Our previous work has shown a significant relationship between an individual's aerobic fitness level and the plasma $F_2$-isoprostane response to an ischemia reperfusion trial (I/R trial) in older adults [13], and that aerobic exercise training in a small pilot study of middle-aged adults improved their

response to the I/R trial [14]. We also demonstrated a preconditioning effect of a single acute exercise bout 16 hours prior to an I/R trial in young individuals, but this protective preconditioning effect was absent in older adults [15]. Together these data suggest older adults have impaired redox stress resilience, and that a single bout of exercise does not elicit a preconditioning effect, but aerobic exercise training may improve the response. However, the previous exercise intervention study did not employ a control group or young adults as a comparison, so whether or not exercise training improves the dynamic response to a non-exercise redox stressor requires confirmation. Therefore, the aim of this study was to investigate the effect of exercise training on $F_2$-isoprostane response to a forearm ischemia reperfusion challenge using a randomized control trial design and including young and older men and women. We hypothesized that older adults would show elevated I/R trial responses compared to young adults before training, but that exercise training would improve I/R trial responses in older adults while young would improve but to a lesser degree than older adults. This study was part of a larger investigation examining the relationship between acute exercise signaling and regular exercise training in redox signaling systems, which have been reported elsewhere [16].

## MATERIALS AND METHODS

**Subjects**. Twenty-six young (18-28y) and 29 older (60-77y) men and women from the surrounding community and campus completed screening for study participation. Subjects were eligible to participate if they were currently inactive as defined by no regular exercise for at least 6 months prior to study admission, as per self-report. Subjects were

generally healthy, not overly obese (BMI ≤ 33.0 kg/m$^2$), non-smokers, and not taking antioxidant supplements in excess of a multi-vitamin. Any condition that would contraindicate maximal exercise testing or participation in exercise training including clinically significant electrocardiogram (EKG) abnormalities at rest or during the maximal exercise test, elevated blood pressure at rest or musculoskeletal problems excluded subjects from participating in the study. Women who were taking estrogen replacement therapy or birth control were excluded from participation as research has previously shown that estrogen has a significant effect on markers of oxidative stress [17]. All participants signed a written informed consent approved by the Northern Arizona University Institutional Review Board.

**Study Design**. The study was an 8-week randomized controlled trial where young and older individuals were randomly assigned to either an exercise training intervention (ET) or a non-exercise control group (CON). Subjects completed pre-screening over the phone to determine eligibility for study participation. If there were no obvious exclusions, the subject was scheduled for a screening visit in the lab followed by a maximal graded exercise test on a cycle ergometer to determine baseline aerobic capacity ($VO_2$ peak). In a separate visit, subjects completed the I/R trial to measure $F_2$-isoprostane responses from blood samples taken at baseline and six additional time points as described below (figure 1). After baseline testing was complete, subjects were randomized to ET or CON groups. Subjects randomized to CON were asked not to change their activity levels during the study and were offered the opportunity to receive the supervised exercise training

program after completing the control arm of the study as a way to minimize attrition. Subjects randomized to ET performed supervised aerobic exercise training three times per week for eight weeks (24 sessions). After the 8 weeks of either ET or CON, subjects returned to the lab and completed the same testing as performed before the intervention. To control for any potential confounding effects of diet on the I/R trial, subjects completed a 2-day food log prior to the baseline test and then were asked to consume the same diet for the two days prior to the post-intervention trial.

**Figure 1.**

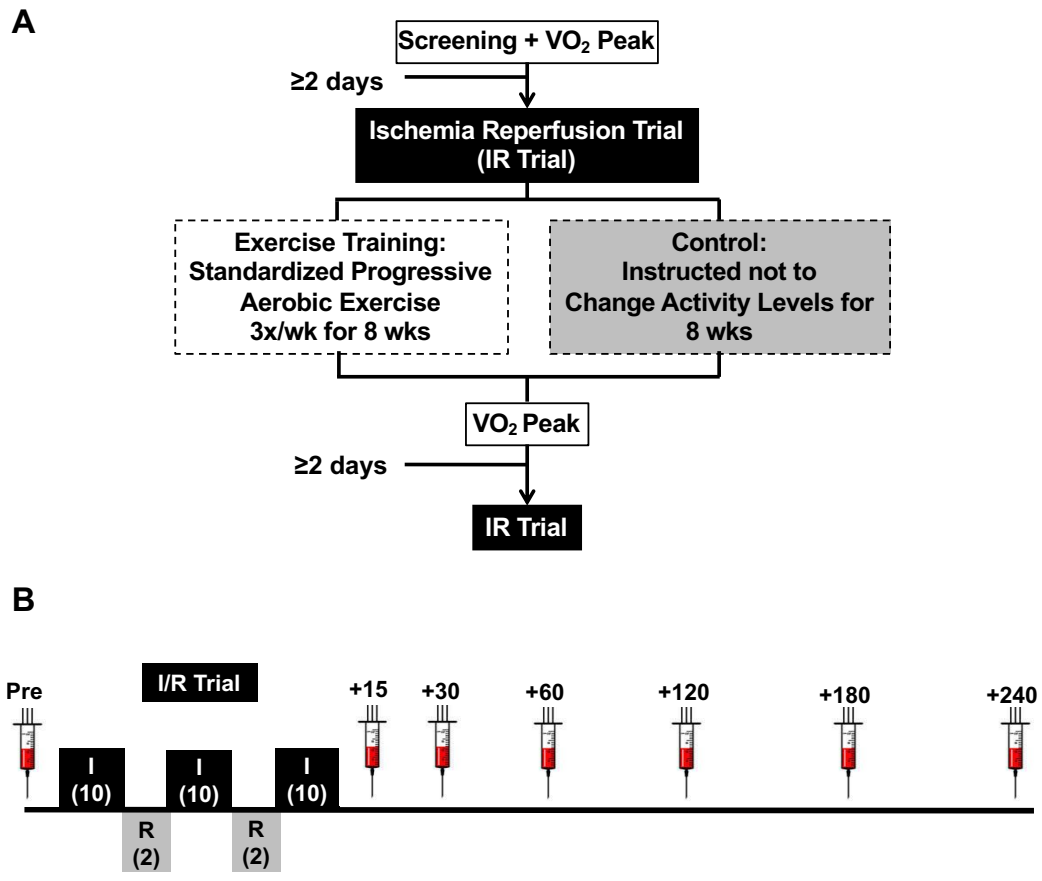



**Figure 1. Study design**. (**A**) Subjects were randomized to the eight-week exercise training intervention (ET) or non-exercise control (CON). $VO_2$ peak testing and forearm

ischemia/reperfusion trial were completed pre- and post-intervention. (**B**) Forearm Ischemia/Reperfusion (I/R) Trial: After a baseline blood draw (Pre), a blood pressure cuff was placed on the arm and inflated to 200mmHg for 10 minutes (Ischemia = "I" in black boxes) followed by deflation for 2 minutes (Reperfusion = "R" in grey boxes). This was repeated for a total of three ten-minute ischemic periods interspersed by 2 minutes of reperfusion each. After final cuff deflation, blood was drawn at 15-min, 30-min, 60-min, 120-min, 180-min and 240-min post-I/R trial.

**Screening visit**. After participants signed the informed consent, they completed a health history questionnaire and the modified Historical Leisure Physical Activity questionnaire for assessment of lifetime physical activity [18, 19]. Height, weight, waist circumference and resting blood pressure were measured and a 12-lead supine resting EKG was obtained to rule out any cardiac abnormalities that would preclude participants from performing a maximal aerobic capacity test. Body composition was measured using a Lange skinfold caliper for the seven-site skinfold method. Body density was calculated using normalized equations for men and women specified by age. The body fat percent equation by Siri et al. was used for final body fat calculations [20]. All skinfold measures were done by the same researcher to prevent intertester variability.

**Peak Oxygen Consumption Test.** $VO_2$ peak was measured with a graded exercise test (GXT) performed on a cycle ergometer as previously described [21]. The starting workload was selected based on the predicted maximal workload for each individual and was increased every minute until volitional exhaustion. Participants were instructed to maintain a pedaling rate of 60-70 rpm throughout the test. Oxygen uptake was measured via indirect calorimetry using a metabolic measurement cart (CareFusion, Yorba Linda,

CA). Heart rate and myocardial electrical activity were monitored with continuous 12-lead EKG. $VO_2$ peak was considered achieved if two of the following three criteria were met: 1) a plateau in $VO_2$ with an increase in workload, 2) a respiratory exchange ratio (RER) ≥ 1.10, or 3) heart rate within 10 beats of the age-predicted maximal heart rate [22]. Standard contraindications to exercise testing, as well as termination criteria, outlined by the American College of Sports Medicine were followed at all times [23].

**Exercise Intervention**. Subjects randomized to the exercise intervention performed supervised aerobic exercise training three times per week for eight weeks (24 sessions in total) at the Student Recreation Center. The intervention was progressive in duration and intensity and included a mix of moderate intensity continuous and high intensity interval aerobic training. All sessions were performed on either cycle ergometer, treadmill, or elliptical depending on subject's preference and musculoskeletal limitations. Each session for the 8-week intervention was planned *a priori* and the intensity was based on heart rate relative to each individual. Thus, each participant (whether young or older) exercised at the same relative intensity and did the exact same workout for a given session. Durations started with 30-minute constant workload sessions and progressed to 50-minute constant workload by the end of the intervention, while also progressing the intensity through increasing steady state heart rate range each week. High intensity interval sessions started modestly and progressed in intensity and duration each week. A mix of work:rest ratios were used to provide variation for subjects engagement as well as ensuring adequate range of intensity/duration combinations that meet ACSM weekly

exercise prescription recommendations [24]. Target RPE and HR range were provided for each individual every exercise session.

**Forearm Ischemia/Reperfusion (I/R) Trial.** The I/R trial is a non-exercise stressor used to determine the subjects redox stress resilience and systemic cytoprotective mechanisms like the antioxidant defense system [13, 17]. The challenge consists of three bouts of ten-minute periods of ischemia using a blood pressure cuff on the arm inflated to 200mmHg separated by two minutes of reperfusion. Plasma $F_2$-isoprostanes were measured from blood draws taken at baseline, and 15, 30, 60, 120, 180, and 240 minutes after the last cuff deflation (Figure 1B). At each time point, blood was drawn into 10mL SST tubes, inverted 5 times, and allowed to clot for 30 minutes. Tubes were then centrifuged at 3000rpm for 15 minutes at 4ºC. After centrifugation, 1800µL of plasma was aliquoted into 2mL microcentrifuge tubes and stored at -80ºC until further analysis. $F_2$-isoprostanes were analyzed as previously described [17]. Briefly, free $F_2$-isoprostanes in plasma were quantified, after purification and derivatization, using gas chromatography/negative ion chemical ionization – mass spectrometry with [$^2H_4$] 15- $F_{2t}$-isoprostane as an internal standard [25]. Compounds were analyzed as pentafluorobenzyl ester, trimethylsilyl ether derivatives by monitoring mass-to-charge ratios of 569 and 573 for endogenous $F_2$-isoprostanes and the [$^2H_4$] 15-$F_{2t}$-isoprostane internal standard, respectively. These analyses were performed at the Vanderbilt University Eicosanoid Core Center.

**Statistical Analyses.** Baseline physical characteristics and demographics were analyzed across groups by 2 x 2 (age group x training group) ANOVA. Pre-intervention $F_2$-isoprostane response to the I/R trial was analyzed by repeated measures ANCOVA 2 x 7 (age x time) with sex, body mass (kg), body mass index (BMI), body fat percent (BF%), and waist circumference (WC) measurements placed in the model as covariates to control for any confounding effects of these measures on the I/R trial response. The integrated $F_2$-isoprostane response was calculated for each individual and each trial as area under the curve (AUC) and area under the response curve (AURC) by the method of the trapezoidal rule [26]. For analyses in response to exercise training or control interventions, $F_2$-isoprostanes were analyzed by 2 x 2 x 7 repeated measures ANCOVA (training group x intervention x time point). The change in AUC and AURC ($\Delta$AUC and $\Delta$AURC respectively) were calculated by subtracting Pre from Post (Post AUC – Pre AUC = $\Delta$AUC) to determine the overall change in I/R trial responsiveness after training or control interventions. Since our previous work showed significant correlations between the I/R trial response and aerobic fitness, a Pearson correlation was used to assess the relationship between the degree of fitness improvement in response to training ($\Delta$ relative $VO_2$ peak) and the $\Delta$AUC or $\Delta$AURC of $F_2$-isoprostanes in response to the I/R trial. Statistical significance was set at $p < 0.05$ and all analyses were performed using SPSS 27.0 (IBM Corp., Armonk, NY). Data are reported as mean ± SEM unless otherwise noted.

## RESULTS

**Subject characteristics.** Twenty-nine older subjects and 26 young subjects were screened and of those 9 were disqualified due to exclusion criteria or declined to

participate before testing began ($VO_2$ max too high n=1, declined to participate n=5, unable to contact n=3). A total of 46 individuals completed pre-testing and started the intervention. Four subjects were withdrawn by study personnel: two due to issues with blood draws and two due to medical issues (blood pressure dysregulation, arrhythmia at post-testing). Additionally, two young individuals dropped out citing time commitment. The data presented are from 21 young and 19 older individuals who completed the intervention, with equal representation between sexes (men n=20, women n=20). Baseline subject characteristics are shown in Table 1. As expected, there were age-related differences at baseline where the young individuals had significantly lower body mass, body mass index (BMI), and body fat percentage (BF%), as well as smaller waist circumference. Similarly, age-related differences in the response to maximal exercise testing showed expected results where young individuals had significantly higher $VO_2$ peak values compared to older adults (38.5 vs. 25.8 mL/kg/min, respectively, $p<0.001$), higher maximal heartrate (189 vs 148 bpm, respectively, $p<0.001$), and higher maximal workload (210 vs. 155 watts, respectively, $p<0.001$). Importantly, none of the baseline characteristics differed between those randomized to the ET intervention versus those randomized to the control group (see Table 1).

**Table 1. Baseline physical characteristics**

| | Young | | Older | | *p* value | | |
|---|---|---|---|---|---|---|---|
| | CON (n=10) | ET (n=11) | CON (n=9) | ET (n=10) | Age | Training Group | Age x Training Grp |
| **Men / Women ratio (n)** | 5 / 5 | 5 / 6 | 5 / 4 | 5 / 5 | - | - | - |
| **Age** | 21.9 (± 3) | 21.6 (± 3) | 65.1 (± 3) | 68.3 (± 5) | <0.001 | NS | NS |
| **Height(cm)** | 177 (± 11) | 171 (± 10) | 172 (± 13) | 170 (± 8.9) | NS | NS | NS |
| **Mass(Kg)** | 68.3 (± 12.3) | 66.0 (± 8.4) | 87.3 (± 14.8) | 76.8 (± 16.5) | 0.002 | NS | NS |
| **BMI (Kg/m^2)** | 21.6 (± 2.5) | 22.5 (± 3.9) | 29.3 (± 2.3) | 26.3 (± 3.8) | <0.001 | NS | NS |
| **Body Fat %** | 15 (± 7.5) | 17 (± 10.3) | 29.9 (± 3.9) | 25.2 (± 4.2) | <0.001 | NS | NS |
| **Waist Circumference (cm)** | 76.7 (± 6.7) | 76.6 (± 6.4) | 102 (± 10.2) | 93.5 (± 11) | <0.001 | NS | NS |
| **HLPA** | 2.83 (± 0.3) | 2.95 (± 0.6) | 2.86 (± 0.9) | 2.36 (± 0.47) | NS | NS | NS |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Blood Pressure (SBP/DBP)** | 112/72 | 112/74 | 124/76 | 120/76 | NS | NS | NS |
| **$VO_2$ Peak Testing** | | | | | | | |
| **$VO_2$ Peak (mL/min)** | 2621 (± 724) | 2563 (± 554) | 2161 (± 570) | 2067 (± 510) | 0.01 | NS | NS |
| **$VO_2$ Peak (mL/Kg/min)** | 38.0 (± 6.6) | 38.9 (± 7.4) | 24.6 (± 3.5) | 26.9 (± 3.1) | <0.001 | NS | NS |
| **Max Heart Rate (bpm)** | 189 (± 9) | 188 (± 8) | 145 (± 21) | 150 (± 18) | <0.001 | NS | NS |
| **RER** | 1.21 (± 0.06) | 1.28 (± 0.1) | 1.31 (± 0.05) | 1.28 (± 0.08) | 0.04 | NS | NS |
| **$WL_{Max}$ (Watts)** | 212 (± 62) | 208 (± 42) | 158 (± 39) | 153 (± 41) | 0.001 | NS | NS |

Data are mean (± SD). BMI – Body Mass Index, HLPA – Historical Leisure Physical Activity Questionnaire, RER– Respiratory Exchange Ratio, $WL_{Max}$ – Maximal workload

**Effects of age and sex on $F_2$-isoprostane response to ischemia/reperfusion stress**

The I/R trial elicited a significant response across time in the whole cohort (p = 0.001). There was no significant effect of age in the baseline IR trial response. In fact, older individuals showed a slightly lower $F_2$-isoprostane response to the IR trial at each time point compared to young (Figure 2A). There was a sex by time interaction (p = 0.022) shown by an earlier increase in plasma $F_2$-isoprostanes in women compared to a more delayed response in men (Figure 2B). Interestingly when parsed by sex, the lower responses in older individuals were being driven by men (Panel C – young vs older men p = 0.009), while the opposite was true in women. Older women showed higher $F_2$-

isoprostane responses to the I/R Trial than younger women, although this was not significant (Panel D – young vs older women $p = 0.4$). There were no significant confounding effects of body mass, BMI, body fat percent, or waist circumference on $F_2$-isoprostane response to the I/R trial.

**Figure 2.**

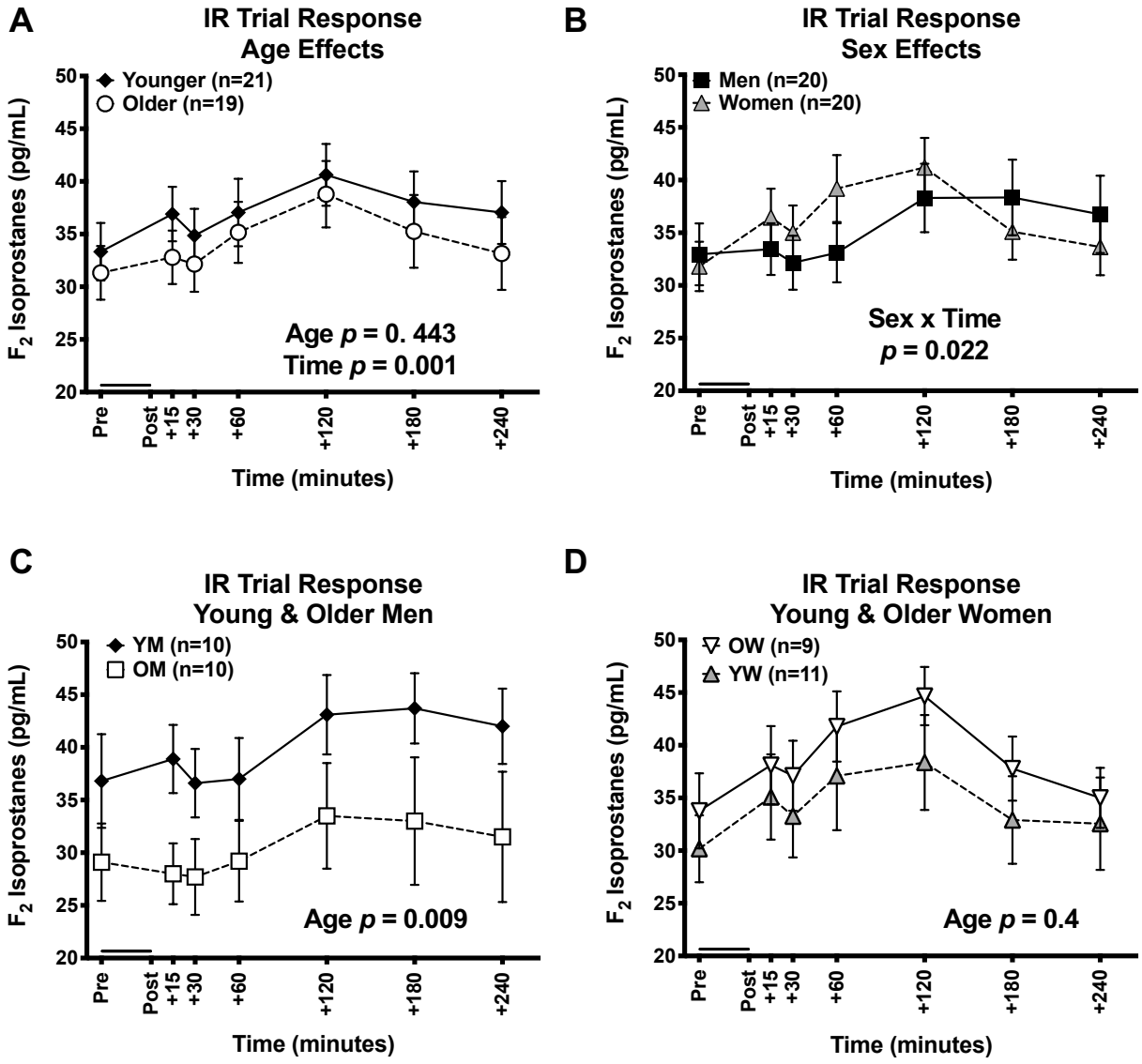



**Figure 2.** Pre-intervention $F_2$-isoprostane response to the I/R trial by age and sex. The I/R trial elicited a significant response in $F_2$-isoprostanes across the whole cohort (p = 0.001). A) There were no significant differences across age (Young – black diamonds, Older – open circles). B) There was a significant sex x time interaction (Women grey triangles, Men – black squares, p = 0.022). This is illustrated by the faster rise in $F_2$-isoprostanes in women compared to men. Young men (YM – Black diamonds in panel C) were significantly higher than older men (OM –Open squares in panel C - age effect in men p = 0.009), while the opposite was true in women, where older women (OW – Open triangles in panel D) showed higher responses than younger women (YW – Grey triangles in panel D) although this was not significant.

**Effects of the exercise intervention on cardiorespiratory fitness**

The effects of the intervention on changes in cardiorespiratory fitness and body composition were previously reported [16] but are included here as well for reference. Aerobic capacity increased significantly in ET (mean change: 4.5 mL/kg/min, +14.7%) and did not change in CON (mean change: 0.2 mL/kg/min, +1%), indicated by intervention by group interaction, $p = 0.013$ and main effect of group, $p = 0.004$. Within the training group, there were no differences in the magnitude of improvement across age or sex indicating that the individuals who underwent training responded to the same degree regardless of age or sex. There were no significant changes in resting blood pressure, maximal heart rate, and RER in either ET or CON. There were no significant changes in overall body mass in ET or CON, but there was a significant decrease in body fat mass, and waist circumference in ET, with no change in CON (one-way ANOVA $\Delta$Body Fat %, $p = 0.023$, $\Delta$waist circumference, $p = 0.001$).

**Exercise Training Improves I/R Trial Response Compared to Inactive Controls**

There were no differences in $F_2$-isoprostane response to the I/R trial between exercise and control groups before the intervention. Exercise training significantly decreased absolute values of $F_2$-isoprostanes response to the I/R trial after exercise training (Figure 3A pre vs post) with no significant changes in the control group response (Figure 3B pre vs post) (pre-post intervention x training group interaction $p = 0.008$). The improvement seen in the trained group occurred in young and older individuals, with no significant differences in improvements across age (Figure 3 pre vs post in panel C and E). There was an interaction of Time x $\Delta$ Relative $VO_2$ Peak ($p = 0.034$), indicating a relationship

between changes in $VO_2$ peak and the pattern of the I/R trial response. The sex by time interaction that was present pre-intervention was no longer present after the intervention. Figure 3 illustrates the training group versus controls as a whole cohort (Panels A & B), young (Panels C & D), older (Panels E & F), Men (Panels G & H) and Women (Panels I & J). In each case, the interaction of pre-post intervention by training group was significant (intervention x group $p = 0.008$), and there were no differences in the degree of improvement between age groups or sex. The change in the overall response (AUC) was significantly decreased in response to exercise training but not in controls (training group, $p = 0.009$), with no significant differences between age or sex for the degree of improvement in the AUC. Pearson correlation showed a significant relationship between the improvements in relative $VO_2$ peak ($\Delta VO_2$ Peak) and the improvements in AUC for $F_2$-isoprostanes response to the I/R trial (Figure 4, $r = -0.464$, $p = 0.003$).

**Figure 3.**

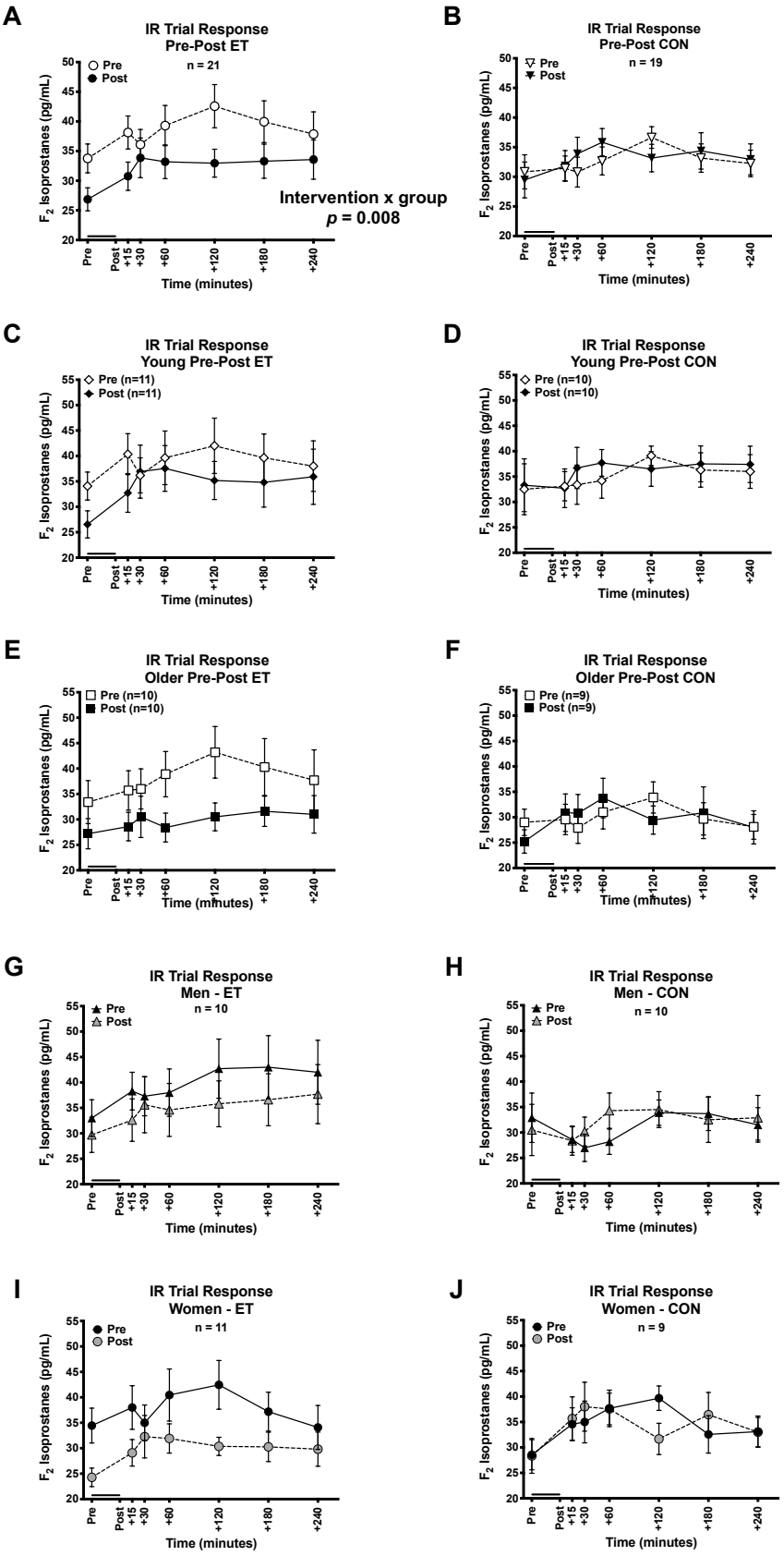

A
IR Trial Response
Pre-Post ET
n = 21
Pre
Post
Intervention x group
p = 0.008
B
IR Trial Response
Pre-Post CON
n = 19
C
IR Trial Response
Young Pre-Post ET
Pre (n=11)
Post (n=11)
D
IR Trial Response
Young Pre-Post CON
Pre (n=10)
Post (n=10)
E
IR Trial Response
Older Pre-Post ET
Pre (n=10)
Post (n=10)
F
IR Trial Response
Older Pre-Post CON
Pre (n=9)
Post (n=9)
G
IR Trial Response
Men - ET
n = 10
H
IR Trial Response
Men - CON
n = 10
I
IR Trial Response
Women - ET
n = 11
J
IR Trial Response
Women - CON
n = 9
F2 Isoprostanes (pg/mL)
Time (minutes)
Pre
Post
+15
+30
+60
+120
+180
+240

**Figure 3.** Exercise training and control intervention responses to the I/R trial in young and older adults. (A and B) ET and CON groups pre and post training or control arm. Panels C and D show young ET and young CON groups respectively. Panels E and F show older ET and older CON groups respectively. Panels G and H show men ET and men CON groups respectively. Panels I and J show women ET and women CON groups respectively. Both age groups and sexes showed improvements in I/R trial response after exercise training, while CON groups did not change. The I/R trial response to training or control intervention was not different between men and women.

**Figure 4.**

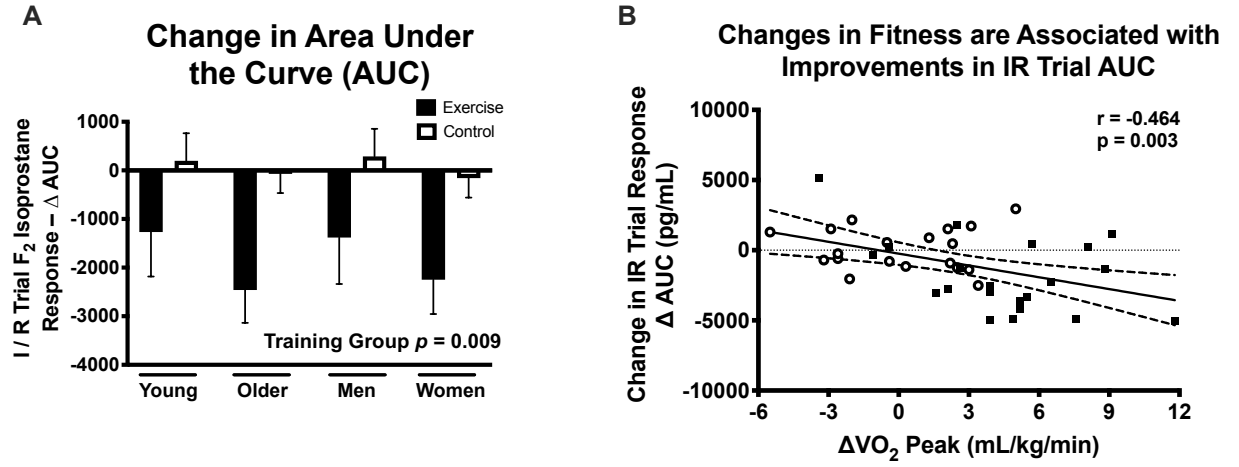



**Figure 4.** A) Changes in overall response (AUC) in response to exercise training versus control by age, and sex. There were no significant differences by age or sex, only a main effect of exercise training on reduction the $F_2$-isoprostane response to the I/R Trial. B) Pearson correlation between the change in relative $VO_2$ peak and the change in AUC for the I/R trial $F_2$-isoprostane response for all subjects (r = -0.464, p = 0.003). Open circles (**O**) indicate CON group, closed squares (■) indicate ET group.

## DISCUSSION

Many studies measure steady state redox stress (i.e. basal protein carbonyls, 8-oxoG, or isoprostanes) without attempting to elucidate a dynamic time course response to transient stressors. This is a significant shortfall of many study designs because most redox

signaling events and fluctuations in redox stress are highly dynamic, occurring at microscopic (nanoseconds – e.g., half-life of hydroxyl radical) and macroscopic timescales (hours/days – e.g., steady state changes in antioxidant enzyme protein content). Understanding the dynamic nature of redox stressors provides more valuable information than a single snapshot at one time point.

The main finding of this randomized controlled trial was that an 8-week exercise training intervention improved the ability to respond to a redox challenge compared to a control group. These results are in agreement with our earlier studies demonstrating improved responses in middle-aged individuals in response to an exercise intervention and a cross-sectional comparison between fit and unfit adults [13, 14]. Other studies have demonstrated lower $F_2$-isoprostane levels in response to a 2-year calorie restriction in healthy adults or a combination of exercise and calorie restriction in patients with moderate to severe kidney disease [27, 28].

These findings refute our initial hypothesis that older adults have higher levels of $F_2$-isoprostanes. The lack of age effects are puzzling given our labs previous findings [17] and other research showing increased oxidative stress with age. In the parallel set of experiments with this cohort, our results indicated that Nrf2 protein was significantly elevated in older adult PBMCs compared to young adults and the resulting dynamic Nrf2 signaling response to an acute exercise trial was impaired because of this aberrant elevation in basal levels of Nrf2. Exercise training partially reversed this effect in older adults, however not to the same degree as young adults [16]. The divergent results in the current $F_2$-isoprostane data suggest that alterations in protein thiol based redox

signaling (Nrf2-ARE signaling) do not necessarily correlate with lipid peroxidation products. One potential mechanism to explain these discrepancies is compartmentalization of redox signaling [29]. Therefore, it is possible that the location of $F_2$-isoprostane generation is not in PBMCs and the mechanisms leading to increases in Nrf2 signaling in older adults, does not interact or affect $F_2$-isoprostane generation and appearance in the plasma in response to a non-exercising stressor. Future work should attempt to delineate where $F_2$-isoprostane generation occurs in response to the I/R trial and the relationship between lipid peroxidation and protein thiol redox status.

Overall, across the cohorts, the degree of improvement in aerobic capacity ($VO_2$ peak) correlated with the improvements in the $F_2$-isoprostane response to the I/R trial (AUC). Previous research has shown that redox inter-individuality predicts the degree of improvement in fitness in response to an exercise intervention [30]. Other studies have reported that significantly lower levels of urinary $F_2$-isoprostane levels in response to a year-long exercise intervention in postmenopausal women were only seen in those who had greater than 15% improvement in maximal oxygen consumption [31] while in young women an exercise intervention only lowered plasma $F_2$-isoprostane levels in those who had the highest levels at baseline [32]. Whether or not $F_2$-isoprostanes drive adaptive responses to exercise or exercise causes a decrease in $F_2$-isoprostanes cannot be determined by our study design or previous work, however an intriguing concept nonetheless [30]. Regardless of mechanism, our data indicate that the biologically relevant and meaningful changes in fitness ($VO_2$ peak) translate to changes redox stress outcome measures in healthy adults, and support existing data for the role of exercise in reducing risk for cardiovascular diseases through regulation of redox status [33].

The current study employed an exercise intervention that prescribed the minimum recommended weekly volume for adults by the American College of Sports Medicine (ACSM), however, it is possible that some individuals in this cohort might benefit from a higher volume based on previous research [34].

It is important to recognize that basal redox stress levels do not provide information about the ability of the system to respond to a challenge, especially relating to the very dynamic nature of the redox response. This highlights a particular strength of the current investigation, using forearm ischemia/reperfusion to induce an oxidative stimulus. Our primary outcome measure, $F_2$-isoprostanes, are considered the gold standard for measuring oxidative stress *in vivo* [35], however future studies should incorporate additional measures of redox balance in response to the I/R trial to further understand the response and potentially apply these results in the clinical setting. Furthermore, the formation and bioactivity of $F_2$-isoprostanes should be elucidated further with mechanistic investigations in response to the I/R trial.

**Limitations**

One limitation is that physical activity outside the exercise sessions or in the controls was not monitored. However, it seems unlikely that this played a role in our results because there were minimal changes in the control groups $VO_2$ peak and $F_2$-isoprostane outcome measures. Another potential limitation is the minimal dietary control in this study. Information on potential group differences in diet composition could be valuable and help shed additional light on individual differences in responses. Finally, the comparisons made pre-intervention across sex were adequately powered, however once parsed by

age and training group, this study was no longer adequately powered to detect sex differences. This study was designed to investigate the effects of aging on the response to the exercise intervention versus controls. Including a young group as a comparison to the older group is a strength but larger studies are needed to tease apart sex differences in redox responses to the I/R trial.

## Conclusions

To our knowledge, this is the first randomized controlled trial to show that exercise training improves response to a non-exercise redox stressor, forearm ischemia/reperfusion challenge, in young and older men and women. The mean improvement of approximately 15% in aerobic capacity in response to the eight-week exercise intervention is similar or slightly better than reported in other exercise intervention studies [36]. The significant correlation between changes in aerobic capacity and changes in the I/R trial response corroborate previous findings in our lab [14] and suggest that exercise or physical activity recommendations should focus on increasing peak aerobic capacity in order to maximize changes in redox capacity.

## Acknowledgements

We would like to thank undergraduate and graduate students in the Traustadóttir Lab that helped with testing and training subjects. We would also like to thank our participants for

their hard work and adherence. The results of the study are presently clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.


## Funding

This work was supported by National Institutes of Health grant R15 AG055077.


## Conflict of Interest

The authors report no conflict of interest.

## REFERENCES


[1] H. Pareja-Galeano, N. Garatachea, A. Lucia, Exercise as a Polypill for Chronic Diseases, Prog Mol Biol Transl Sci 135 (2015) 497-526.

[2] N. Garatachea, H. Pareja-Galeano, F. Sanchis-Gomar, A. Santos-Lozano, C. Fiuza-Luces, M. Moran, E. Emanuele, M.J. Joyner, A. Lucia, Exercise attenuates the major hallmarks of aging, Rejuvenation Res 18(1) (2015) 57-89.

[3] N. Garatachea, A. Santos-Lozano, D.C. Hughes, #xf3, A. mez-Cabello, I. Ara, Physical Exercise as an Effective Antiaging Intervention, BioMed Research International 2017 (2017) 2.

[4] T.L. Merry, M. Ristow, Nuclear factor erythroid-derived 2-like 2 (NFE2L2, Nrf2) mediates exercise-induced mitochondrial biogenesis and the anti-oxidant response in mice, J Physiol 594(18) (2016) 5195-207.

[5] S.S. Gounder, S. Kannan, D. Devadoss, C.J. Miller, K.J. Whitehead, S.J. Odelberg, M.A. Firpo, R. Paine, 3rd, J.R. Hoidal, E.D. Abel, N.S. Rajasekaran, Impaired transcriptional activity of Nrf2 in age-related myocardial oxidative stress is reversible by moderate exercise training, PloS one 7(9) (2012) e45697.

[6] C. Henriquez-Olguin, L.B. Renani, L. Arab-Ceschia, S.H. Raun, A. Bhatia, Z. Li, J.R. Knudsen, R. Holmdahl, T.E. Jensen, Adaptations to high-intensity interval training in skeletal muscle require NADPH oxidase 2, Redox Biol 24 (2019) 101188.

[7] C. Li, R.M. Jackson, Reactive species mechanisms of cellular hypoxia-reoxygenation injury, Am J Physiol Cell Physiol 282(2) (2002) C227-41.

[8] K.P. Moore, V. Darley-Usmar, J. Morrow, L.J. Roberts, 2nd, Formation of F2-isoprostanes during oxidation of human low-density lipoprotein and plasma by peroxynitrite, Circ Res 77(2) (1995) 335-41.

[9] J.S. Powers, L.J. Roberts, 2nd, E. Tarvin, N. Hongu, L. Choi, M. Buchowski, Oxidative stress and multi-organ failure in hospitalized elderly people, Journal of the American Geriatrics Society 56(6) (2008) 1150-2.

[10] Z.J. Zhang, Systematic review on the association between F2-isoprostanes and cardiovascular disease, Ann Clin Biochem 50(Pt 2) (2013) 108-14.

[11] D.A. Camfield, K. Nolidin, K. Savage, J. Timmer, K. Croft, M. Tangestani Fard, T. Simpson, L. Downey, A. Scholey, A. Pipingas, S. Deleuil, C. Stough, Higher plasma levels

of F2-isoprostanes are associated with slower psychomotor speed in healthy older adults, Free Radic Res 53(4) (2019) 377-386.

[12] S.S. Davies, L.J. Roberts, 2nd, F2-isoprostanes as an indicator and risk factor for coronary heart disease, Free Radic Biol Med 50(5) (2011) 559-66.

[13] T. Traustadóttir, S.S. Davies, Y. Su, L. Choi, H.M. Brown-Borg, L.J. Roberts, 2nd, S.M. Harman, Oxidative stress in older adults: effects of physical fitness, Age (Dordr) 34(4) (2012) 969-82.

[14] A.J. Done, T. Traustadóttir, Aerobic exercise increases resistance to oxidative stress in sedentary older middle-aged adults. A pilot study, Age (Dordr) 38(5-6) (2016) 505-512.

[15] T.C. Nordin, A.J. Done, T. Traustadottir, Acute exercise increases resistance to oxidative stress in young but not older adults, Age (Dordr) 36(6) (2014) 9727.

[16] E.L. Ostrom, T. Traustadóttir, Aerobic exercise training partially reverses the impairment of Nrf2 activation in older humans, Free Radical Biology and Medicine 160 (2020) 418-432.

[17] S.S. Davies, T. Traustadóttir, A.A. Stock, F. Ye, Y. Shyr, S.M. Harman, L.J. Roberts, 2nd, Ischemia/reperfusion unveils impaired capacity of older adults to restrain oxidative insult, Free Radic Biol Med 47(7) (2009) 1014-8.

[18] C.M. Cottreau, R.B. Ness, A.M. Kriska, Physical activity and reduced risk of ovarian cancer, Obstet Gynecol 96(4) (2000) 609-14.

[19] C.S. Winters-Hart, J.S. Brach, K.L. Storti, J.M. Trauth, A.M. Kriska, Validity of a questionnaire to assess historical physical activity in older women, Med Sci Sports Exerc 36(12) (2004) 2082-7.

[20] W.E. Siri, Body composition from fluid spaces and density: analysis of methods. 1961, Nutrition 9(5) (1993) 480-91; discussion 480, 492.

[21] T. Traustadóttir, A.A. Stock, S.M. Harman, High-dose statin use does not impair aerobic capacity or skeletal muscle function in older adults, Age (Dordr) 30(4) (2008) 283-91.

[22] W.M. Kohrt, M.T. Malley, A.R. Coggan, R.J. Spina, T. Ogawa, A.A. Ehsani, R.E. Bourey, W.H. Martin, 3rd, J.O. Holloszy, Effects of gender, age, and fitness level on response of VO2max to training in 60-71 yr olds, Journal of applied physiology (Bethesda, Md. : 1985) 71(5) (1991) 2004-11.

[23] American College of Sports Medicine, D. Riebe, J.K. Ehrman, G. Liguori, M. Magal, ACSM's guidelines for exercise testing and prescription, Tenth edition. ed., Wolters Kluwer, Philadelphia, 2018.

[24] C.E. Garber, B. Blissmer, M.R. Deschenes, B.A. Franklin, M.J. Lamonte, I.M. Lee, D.C. Nieman, D.P. Swain, M. American College of Sports, American College of Sports Medicine position stand. Quantity and quality of exercise for developing and maintaining cardiorespiratory, musculoskeletal, and neuromotor fitness in apparently healthy adults: guidance for prescribing exercise, Med Sci Sports Exerc 43(7) (2011) 1334-59.

[25] J.D. Morrow, L.J. Roberts, 2nd, Mass spectrometric quantification of F2-isoprostanes in biological fluids and tissues as measure of oxidant stress, Methods in enzymology 300 (1999) 3-12.

[26] J.C. Pruessner, C. Kirschbaum, G. Meinlschmid, D.H. Hellhammer, Two formulas for computation of the area under the curve represent measures of total hormone

concentration versus time-dependent change, Psychoneuroendocrinology 28(7) (2003) 916-31.

[27] T.A. Ikizler, C. Robinson-Cohen, C. Ellis, S.A.E. Headley, K. Tuttle, R.J. Wood, E.E. Evans, C.M. Milch, K.A. Moody, M. Germain, C. Limkunakul, A. Bian, T.G. Stewart, J. Himmelfarb, Metabolic Effects of Diet and Exercise in Patients with Moderate to Severe CKD: A Randomized Clinical Trial, J Am Soc Nephrol 29(1) (2018) 250-259.

[28] L.M. Redman, S.R. Smith, J.H. Burton, C.K. Martin, D. Il'yasova, E. Ravussin, Metabolic Slowing and Reduced Oxidative Damage with Sustained Caloric Restriction Support the Rate of Living and Oxidative Damage Theories of Aging, Cell Metab 27(4) (2018) 805-815 e4.

[29] C. Henriquez-Olguin, R. Meneses-Valdes, T.E. Jensen, Compartmentalized muscle redox signals controlling exercise metabolism – Current state, future challenges, Redox Biology 35 (2020) 101473.

[30] N.V. Margaritelis, A.A. Theodorou, V. Paschalis, A.S. Veskoukis, K. Dipla, A. Zafeiridis, G. Panayiotou, I.S. Vrabas, A. Kyparos, M.G. Nikolaidis, Adaptations to endurance training depend on exercise-induced oxidative stress: exploiting redox interindividual variability, Acta Physiol (Oxf) 222(2) (2018).

[31] P.T. Campbell, M.D. Gross, J.D. Potter, K.H. Schmitz, C. Duggan, A. McTiernan, C.M. Ulrich, Effect of exercise on oxidative stress: a 12-month randomized, controlled trial, Med Sci Sports Exerc 42(8) (2010) 1448-53.

[32] A.Y. Arikawa, W. Thomas, M. Gross, A. Smith, W.R. Phipps, M.S. Kurzer, K.H. Schmitz, Aerobic training reduces systemic oxidative stress in young women with elevated levels of F2-isoprostanes, Contemporary clinical trials 34(2) (2013) 212-7.

[33] T. Tofas, D. Draganidis, C.K. Deli, K. Georgakouli, I.G. Fatouros, A.Z. Jamurtas, Exercise-Induced Regulation of Redox Status in Cardiovascular Diseases: The Role of Exercise Training and Detraining, Antioxidants (Basel) 9(1) (2019).

[34] D. Montero, C. Lundby, Refuting the myth of non-response to exercise training: 'non-responders' do respond to higher dose of training, J Physiol 595(11) (2017) 3377-3387.

[35] M.B. Kadiiska, B.C. Gladen, D.D. Baird, D. Germolec, L.B. Graham, C.E. Parker, A. Nyska, J.T. Wachsman, B.N. Ames, S. Basu, N. Brot, G.A. Fitzgerald, R.A. Floyd, M. George, J.W. Heinecke, G.E. Hatch, K. Hensley, J.A. Lawson, L.J. Marnett, J.D. Morrow, D.M. Murray, J. Plastaras, L.J. Roberts, 2nd, J. Rokach, M.K. Shigenaga, R.S. Sohal, J. Sun, R.R. Tice, D.H. Van Thiel, D. Wellner, P.B. Walter, K.B. Tomer, R.P. Mason, J.C. Barrett, Biomarkers of oxidative stress study II: are oxidation products of lipids, proteins, and DNA markers of CCl4 poisoning?, Free Radic Biol Med 38(6) (2005) 698-710.

[36] G. Huang, C.A. Gibson, Z.V. Tran, W.H. Osness, Controlled endurance exercise training and VO2max changes in older adults: a meta-analysis, Prev Cardiol 8(4) (2005) 217-25.

# CHAPTER 4 – High intensity muscle stimulation activates a systemic Nrf2-mediated redox stress response

Ethan L. Ostrom[1], Ana P. Valencia[2], David J. Marcinek[2,3], & Tinna Traustadóttir[1*]

[1] Department of Biological Sciences, Northern Arizona University

[2] Department of Radiology, [3] Institute for Stem Cell and Regenerative Medicine, University of Washington School of Medicine

To Whom Correspondence Should Be Addressed:

Tinna Traustadóttir, Ph.D.

Department of Biological Sciences

Northern Arizona University

PO Box 5640

Flagstaff, AZ 86011-5640

Phone: 928-523-7291

Fax : 928-523-7500

E-mail: tinna.traustadottir@nau.edu

## ABSTRACT

**Introduction** High intensity exercise is an increasingly popular mode of exercise to elicit similar or greater adaptive responses compared to traditional moderate intensity continuous exercise. However, the molecular mechanisms underlying these adaptive responses are still unclear. The purpose of this pilot study was to compare high and low intensity contractile stimulus on the Nrf2-mediated redox stress response in mouse skeletal muscle. **Methods** An intra-animal design was used to control for variations in individual responses to muscle stimulation by using a stimulated limb (STIM) and comparing to the contralateral unstimulated control limb (CON). High Intensity (HI – 100Hz), Low Intensity (LI – 50Hz), and Naïve Control (NC – Mock stimulation vs CON) groups were used to compare these effects on Nrf2-ARE binding, Keap1 protein content, and downstream gene and protein expression of Nrf2 target genes. **Results** Muscle stimulation significantly increased Nrf2-ARE binding in LI-STIM compared to LI-CON ($p = 0.0098$), while Nrf2-ARE binding was elevated in both HI-CON and HI-STIM compared to NC ($p = 0.0007$). The Nrf2-ARE results were mirrored in the downregulation of Keap1, where Keap1 expression in HI-CON and HI-STIM were both significantly lower than NC ($p = 0.008$) and decreased in LI-STIM compared to LI-CON ($p = 0.015$). In addition, stimulation increased NQO1 protein compared to contralateral control regardless of stimulation intensity ($p = 0.019$). **Conclusions** Taken together, these data suggest a systemic redox signaling exerkine is activating Nrf2-ARE binding and is intensity gated, where Nrf2-ARE activation in contralateral control limbs were only seen in the HI group. Other research in exercise induced Nrf2 signaling support the general finding that Nrf2 is activated in peripheral tissues in response to exercise, however the specific exerkine

responsible for the systemic signaling effects is not known. Future work should aim to delineate these redox sensitive systemic signaling mechanisms.

**Key Words: High Intensity Exercise, Redox signaling, Nrf2-Keap1, Muscle Contraction**

## INTRODUCTION

Exercise induces beneficial adaptations through redox signaling cascades that are mediated by the redox stress response transcription factor nuclear erythroid related factor 2 (Nrf2) [1-3]. The redox stress response system is a critical cytoprotective mechanism that protects both the cell from endogenous and environmental redox stressors and contributes to adaptive processes to exercise [4, 5]. The redox signal transduction cascade is a highly complex and coordinated system involving the generation of reactive oxygen species, the oxidation of redox-relay molecules or direct oxidation of sensor molecules with thiol switches like Kelch-like ECH-associated protein 1 (Keap1), and effector molecules that change activity, localization, protein-protein interactions, or protein turnover in response to the redox signaling cascade [6, 7]. The overall physiological adaptation resulting from redox signaling cascades depends on the rate of accumulation of these reactive species, and the steady-state levels of enzymatic and non-enzymatic antioxidants [4], as well as the basal oxidation state of proteins with thiol-based redox switches [7-9].

Reactive oxygen species (ROS) generation is required for appropriate skeletal muscle adaptations to exercise such as mitochondrial biogenesis [10]. Inhibiting exercise-induced ROS signaling with antioxidants impairs markers of mitochondrial biogenesis including AMPK and PGC1$\alpha$ activation [11-13], Nrf2 activation [2], and downstream gene expression [14]. Furthermore, there is a linear relationship between exercise-induced ROS accumulation and Nrf2 activation [15], which is dependent on the duration of the exercise bout [15, 16]. Nrf2 can also be activated in human skeletal muscle in response to supramaximal exercise of shorter duration [17], suggesting that longer durations are not required to induce Nrf2 activity if the intensity of the bout is high enough. High intensity interval training is a popular training method to obtain improvements in cardiorespiratory fitness quickly and efficiently, partly through increases in mitochondrial biogenesis – processes that are redox signaling dependent [10, 18-20].

The molecular pattern of redox signaling responses can differ depending on the intensity of the exercise which leads to different adaptations and resulting phenotypes [21-23]. However only two studies to date have directly compared moderate intensity and high intensity exercise and Nrf2 activation, one in human peripheral blood mononuclear cells (PBMCs), and one in human skeletal muscle [24, 25]. There were no differences in Nrf2 signaling between exercise intensities in either human PBMCs or human muscle [24, 25]. However other studies comparing different exercise intensities have shown divergent redox signaling effects in other redox proteins, highlighting some interesting caveats in the field [26]. Therefore, more detailed and well controlled studies comparing differing intensity stimuli on Nrf2 redox stress response signaling are needed.

One issue regarding the redox stress response to exercise, is the considerable variability in the adaptive signaling responses across individuals [27]. This makes it difficult to predict the hormetic response, and thus the overall adaptation to regular exercise, and impacts the ability to effectively prescribe exercise to clinical and non-clinical populations. Therefore, the purpose of this pilot study was to test high and low muscle stimulation intensities on redox stress responses in mouse skeletal muscle using a within-animal study design to control for any intra-animal variation in responses to the contractile stimulus, as well as a between-animal control using a mock stimulation condition. We hypothesized that high intensity stimulation would lead to greater adaptations and activation of Nrf2 signaling compared to low intensity exercise. Here we show a novel systemic Nrf2-ARE activation mechanism gated by high intensity stimulation but not low intensity stimulation that increases Nrf2-ARE binding by acting through inhibition of the negative regulator Keap1.

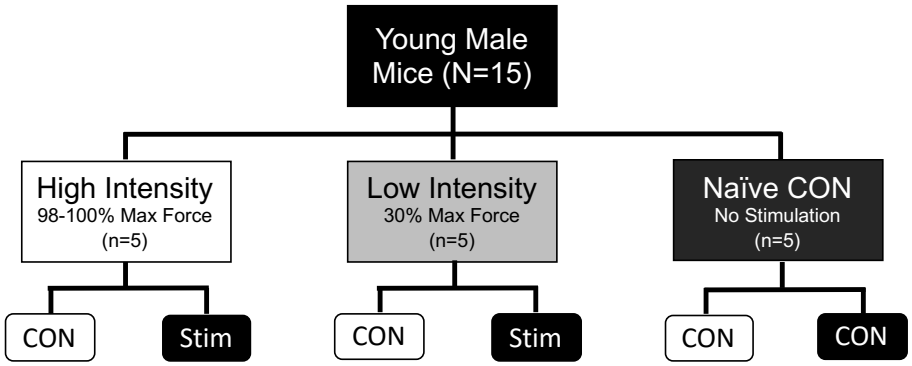
## METHODS

*Animals* – Young (6mo) C57BL/6 male mice were received from the Jackson Labs. All mice were maintained at 21°C on a 14/10 light/dark cycle and given standard mouse chow and water *ad libitum*. The study was approved by the University of Washington Institutional Animal Care and Use Committee (IACUC) and the tissue transfer was approved by the Northern Arizona University IACUC.

*Study Design* – There were three experimental groups for this study (Figure 1): High intensity stimulus (HI, n=5), low intensity stimulus (LI, n=5), and naïve unstimulated

control (NC, n=5). An intra-animal study design was used for HI and LI groups to control for within animal variation, where the stimulated (STIM) limb was used as the exercise condition and contralateral unstimulated (CON) limb was used as the non-exercise control. The Naïve Controls (NC) were used as the inter-animal controls and were anesthetized for the same amount of time as the other two groups before both hind limbs were harvested, but no muscle stimulation occurred in either limb. Naïve controls were used to control for systemic effects that could affect both limbs in the HI and LI groups. Nrf2-mediated redox stress response signaling was compared between each group (HI, LI, & NC) as well as paired comparisons within animals unstimulated control and stimulated (exercised) limbs (Figure 1).

**Figure 1.** Study Design




*In Vivo Muscle Stimulation* – Muscle stimulation was performed on the right leg of anesthetized mice (1-2% isofluorane) resting on a heated plate at 37°C as previously described [28]. The knee was secured, and foot taped to a footplate perpendicular to the tibia. The footplate was connected to a force transducer (Aurora Scientific, ON, Canada). The tibial nerve was stimulated using Grass Stimulator (S88X, Astro Med, Inc.) at optimal

voltage (1-4V) that was selected by measuring maximal isometric torque of plantarflexion during isometric contractions (200ms train, 0.1ms pulse, 100Hz). Following optimization, the plantarflexors underwent a fatigue protocol with isometric contractions (200ms train, 0.1ms pulse) induced by a high (100Hz) or low (50Hz) stimulation frequency every fourth second for 30 minutes. Following the fatigue procedure, the electrodes were removed and the was leg released from the instrument. While on anesthesia, mouse remained on the heated plate until muscle harvest. The gastrocnemius and soleus muscle from the stimulated and unstimulated legs (contralateral control) were removed 30 minutes after the end of muscle stimulation and flash frozen in liquid nitrogen and stored at -80˚C. Animals were euthanized through cervical dislocation.

*Gene Expression* – Soleus muscles were homogenized in RLT buffer (+ 1% BME). RNA extraction was done using RNeasy Plus Mini Kit following Proteinase K digestion and elimination of DNases using RNase-free DNase kit (all reagents from Qiagen). Isolated RNA was then converted to cDNA using Bio-Rad iScript kit and RT-qPCR using Bio-Rad SYBR Sso Advanced. Samples were analyzed using the $\Delta\Delta$Ct method. A panel of six housekeeping genes were used to determine which were the best three genes to use for internal controls (Table 1). The geometric mean for the three most stable housekeeping genes were used to quantify changes in target gene mRNA expression in response to stimulation [29]. All primer sequences are listed in Table 1.

**Table 1. Primer Sequences for RT - qPCR**

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| RPL41 | GCCATGAGAGCGAAGTGG | CTCCTGCAGGCGTCGTAG |
| RPL27 | AAGCCGTCATCGTGAAGAACA | CTTGATCTTGGATCGCTTGGC |
| RPL7L1 | ACGGTGGAGCCTTATGTGAC | TCCGTCAGAGGGACTGTCTT |
| RER1 | GCCTTGGGAATTTACCACCT | CTTCGAATGAAGGGACGAAA |
| ACTB | CCTCGCCTTTGCCGA | TGGTGCCTGGGGCG |
| PPIA | CCCACCTGTTTCTTCGACAT | CCATGTCTCAGAGCACGAAA |
| HMOX1 | CCTCACTG GCAGGAAATCATC | CCTCGTGGAGACGCTTTACATA |
| NQO1 | GGGTCGTCTTGGCAACCA | CAGATGTTGAGGGAGGATCGTAA |
| GCLC | GCTGTCTTGCAGGGAATGTT | ACACACCTTCCTTCCCATTG |
| GSTP1 | GCTCTTACCACGTGCAGCTT | GGCTGGGAAGAGGAAATGGA |
| GSR | GCTATGCAACATTCGCAGATG | AGCGGTAAACTTTTTCCCATTG |
| NFE2L2 | CGAGATATACGCAGGAGAGGTAAGA | GCTCGACAATGTTCTCCAGCTT |

*Glutathione Content* – Intracellular glutathione levels were measured as previously described [30] using a spectrophotometric assay based on the affinity of 2,3-naphthalenedicarboxaldehyde (NDA) for γ-glutamylcysteinylglycine (GSH). Skeletal muscle (10-20mg) was homogenized in ice-cold Locke's buffer (10 mM HEPES, 5.5 mM KCl, 10 mM glucose, 5 mM NaHCO3, and 130 mM NaCl). A portion of the homogenate

was set aside for protein quantification using a Bradford assay. An equal volume of 200mM 5-sulfosalicylic acid dehydrate (SSA) was mixed in. After resting on ice for 15 minutes the samples were centrifuged at 12,000 x g for 3 minutes at 4 °C. The supernatant was plated into a 96 well plate in duplicate. Standards with known GSH concentrations (0-50μM) were also plated in duplicate. 0.2 M NEM/0.02 M KOH were added to each well followed by 10 mM TCEP. After a 20-minute incubation at room temperature, 0.5 N NaOH was added followed by 10mM NDA. After a 30-minute incubation the plate was read at fluorescence intensity using 472 (excitation) and 528 (emission). GSH levels were assessed using the standard curve and normalized to protein concentration.

*Western Blotting* – Gastrocnemius muscles were homogenized on ice for 5 minutes in the presence of 25μl of Cell Lytic MT cell lysis buffer (Sigma-Aldrich) per mg of tissue. Lysis buffer was supplemented with 0.1% protease inhibitors (Sigma-Aldrich) and 0.2% phosphatase inhibitor (ThermoFisher). Following homogenization, aliquots were taken from original samples for Bradford assays to determine protein concentrations. Next, sample buffer was added to the original sample then boiled and stored at -80˚C. Thirty micrograms of protein were separated by gel electrophoresis in each well followed by wet transfer on to nitrocellulose membranes and one hour blocking in TBS + 5% non-fat dry milk. Blots were incubated with Keap1 (EPR22664-26, Abcam, Cambridge MA) GCLC (EP12345, Abcam), GSR (Santa Cruz Biotech, Dallas TX), HO1 (Cell Signaling Technology, Danvers MA), or NQO1 (Cell Signaling Technology, Danvers MA)

monoclonal antibodies to detect proteins with Ponceau S, used for loading controls (Cell Signaling Technology, Danvers MA).

*Nrf2-ARE Binding Assay* – Gastrocnemius muscles were homogenized, lysed, and nuclear fractions extracted per manufacturer's instructions (Nrf2-ARE binding kit, TransAM, Carlsbad CA). Briefly, samples were homogenized in a 1:30 ratio (mg tissue:µl buffer) followed by centrifugation, isolation, and lysis of the nuclear pellet. After Bradford assays were run to determine protein content in nuclear fractions, nuclear lysates were incubated in a 96 well plate coated with ARE consensus oligonucleotides for Nrf2 Binding. Following three washes, primary Anti-Nrf2 antibodies were incubated (1:1000) in each well used to detect Nrf2 followed by secondary antibody incubation and colorimetric development. Absorbance was read at 450nm on a Synergy HT plate reader (Bio-Tek, Winooski, VT).

*Statistical Analysis* – For all measures, a three by two repeated measures ANOVA (Intensity group by Stimulation condition) was run for analysis of intensity group differences, stimulation differences and their interaction. Tukey's post hoc analysis was used for CON vs STIM pairwise comparisons within group. All relevant p values reported are adjusted p values using Tukey's multiple comparisons post hoc analysis. Statistical analysis was performed using SPSS (IBM, version 27) and GraphPad Prism software (San Diego, CA).

## RESULTS

*Muscle stimulation*

The results from the muscle stimulation protocol in HI and LI are shown in figure 2. The force output of a tetanic contraction at 100Hz was not different between groups (12.1 mN-m ± 0.9 and 11.9 mN-m ± 1.7 for LI-STIM and HI-STIM respectively). The fatigue protocol at HI-STIM led to a final force output that was 27.7% ± 5.6 of initial 100Hz tetanus, and LI-STIM led to a final force output that was 12.8% ± 5.6 of a 100Hz tetanus and 37.3% ± 2.8 of the initial 50Hz contraction.

**Figure 2.** Muscle force output and fatiguing stimulation.

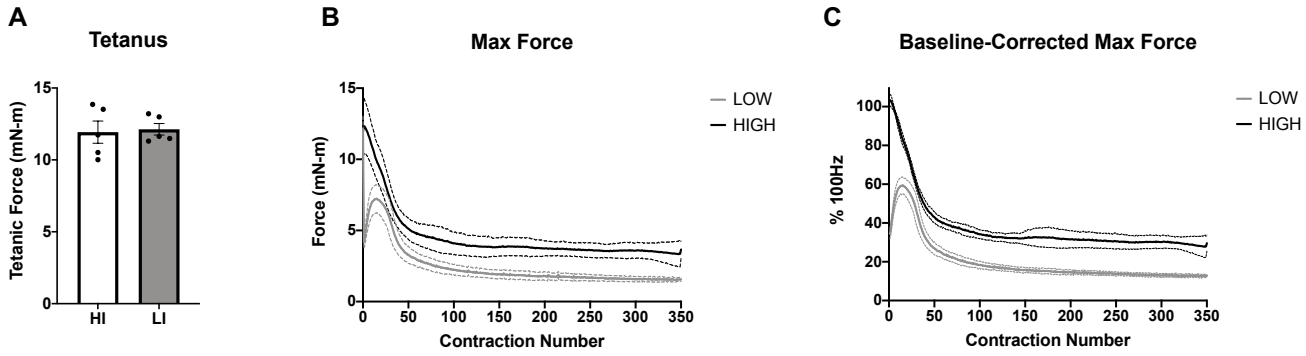



**Figure 2.** A) Both low and high intensity stimulation groups had similar maximal tetanic force at 100Hz. B) The absolute force (mN-m) over time in response to either High (100Hz) or Low (50Hz) intensity stimulation frequency. C) The normalized force to maximal tetanus. Data are Mean ± SD.

*Stimulation Intensity Reveals Divergent Effects on Nrf2-ARE Binding and Keap1 Protein Dynamics in Skeletal Muscle*

Nrf2-ARE binding was low under basal unstimulated conditions (Figure 3A, Naïve Control group and LI-CON condition). There was a significant interaction between stimulation condition by intensity (RM ANOVA Group x Stimulation condition, p = 0.005), where Nrf2

ARE binding significantly increased in LI-STIM condition compared to LI-CON (Figure 3A, ** p = 0.047), while NC and HI were not significantly different between conditions (Figure 3A, HI-CON vs HI-STIM, or both NC-CON conditions). Nrf2-ARE binding was significantly higher in the HI group under both conditions compared to NC group (Figure 3A, $^{\#\#}$ p = 0.007). Keap1 showed a significant main effect of intensity indicated by the difference between high intensity and naïve controls (Figure 3B, $^{\#\#}$p = 0.008). There was also an interaction of stimulation by intensity (p = 0.035) and a main effect of stimulation (p = 0.016). The interaction of stimulation by intensity was driven by a significant decrease in Keap1 protein in LI-STIM condition compared to LI-CON (Figure 3B, *p = 0.015) with no changes between CON vs STIM conditions in HI or differences between the two NC-CON. There were no differences in total glutathione content across groups, or in response to stimulation within groups (Figure 3D).

**Figure 3.** Nrf2-ARE Binding and Keap1 Protein Expression in Response to Skeletal Muscle Stimulation

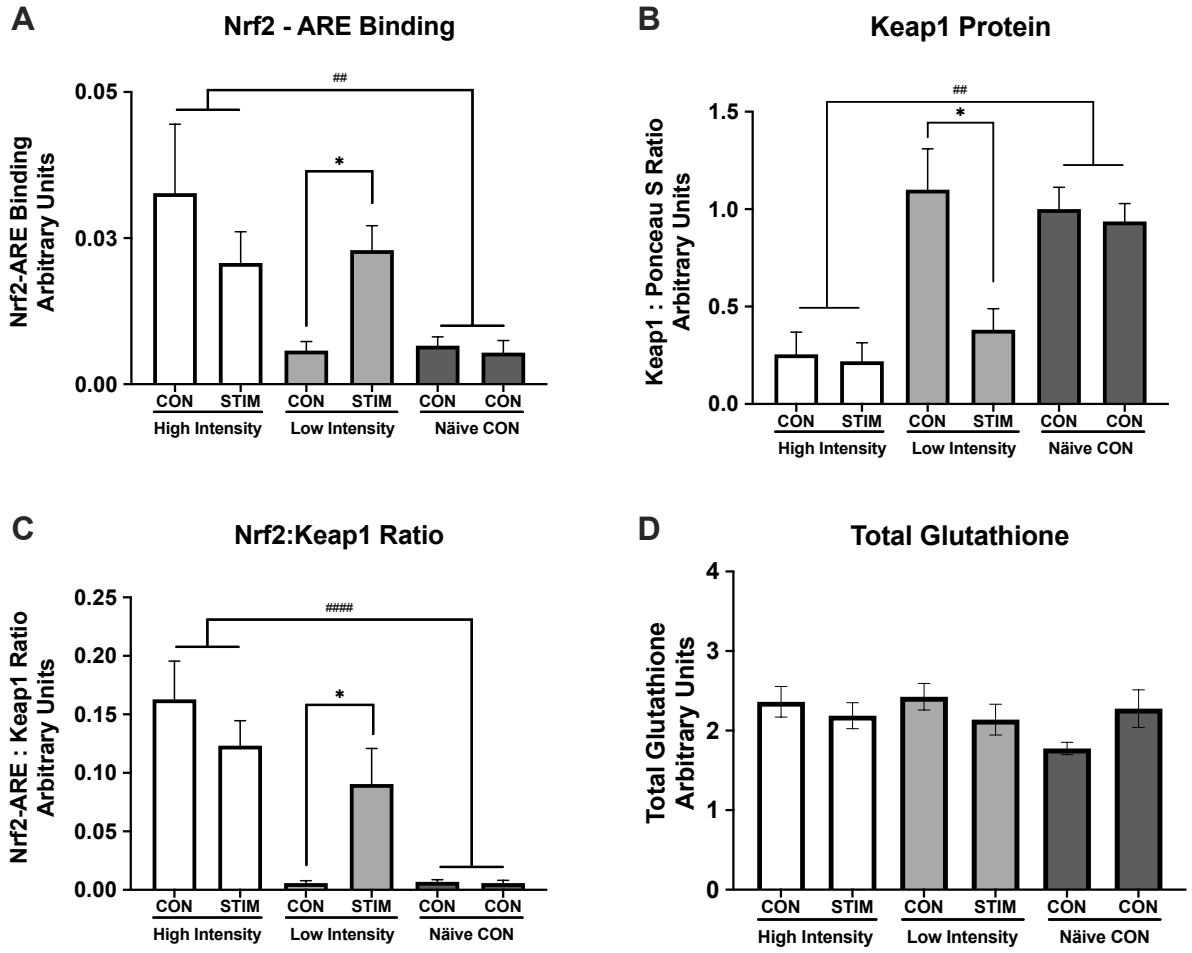



**Figure 3.** Keap1-Nrf2-ARE signaling response to high and low intensity stimulation and total glutathione content. A) Nrf2-ARE binding is low under basal unstimulated conditions (Naïve Control group and LI CON condition) but increases in response to low intensity stimulation (LI-CON vs LI-STIM, * p = 0.047). Nrf2-ARE binding is significantly higher in the HI group compared to NC group ($^{\#\#}$ p = 0.007) . B) Keap1 content is unchanged between limbs in the Naïve Control group or the LI-CON, however there is a significant decrease in Keap1 content in the LI-STIM limb (*p = 0.015). Keap1 content is significantly lower in the HI group under both conditions compared to NC ($^{\#\#}$p = 0.008). C) Nrf2:Keap1 ratio illustrates the same pattern as either Nrf2-ARE binding or Keap1 protein content alone (HI vs NC, $^{\#\#\#\#}$ p < 0.0001). D) Total glutathione content was not different across groups and did not change significantly in response to stimulation. Representative western blot image of Keap1 protein is shown in Figure 5B. Keap1 was normalized to Left hind limb of the NC group. Data are presented as mean ± SEM.

*Gene expression responses to skeletal muscle stimulation*

A panel of six housekeeping genes were used to assess their stability in response to skeletal muscle stimulation (Figure 4A and 4B). Variability from CON to STIM conditions was assessed for each housekeeping gene (Figure 4A) as well as variation by intensity group (Figure 4B). RPL41, RPL27, and RPL7l1 were selected as the best genes to use for analysis because their within animal variation was lowest across groups (∆Cq in response to stimulation was minimized). Target gene fold change was calculated using the geometric mean of the 3 housekeeping genes with the ∆∆Cq method. Target gene responses to muscle stimulation were not significantly different from CON or across intensities, although there was a trend for an increase in GCLC mRNA expression in response to stimulation (Figure 4C, p = 0.06).

**Figure 4.** Gene Expression of Redox Stress Response Genes to Skeletal Muscle Stimulation

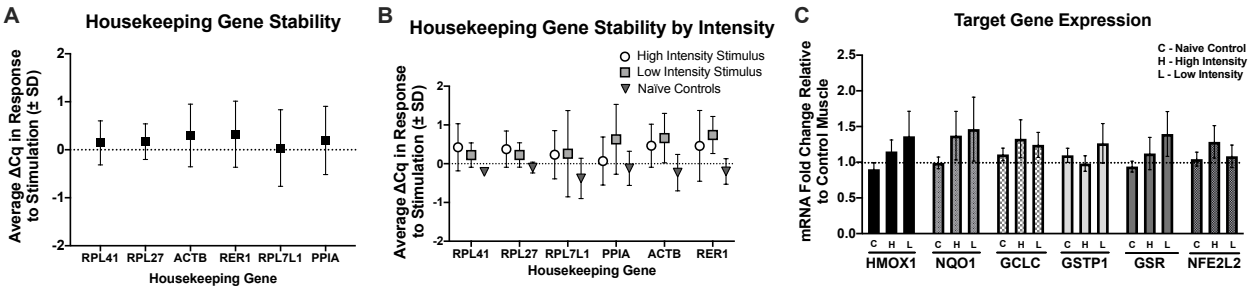



**Figure 4.** A) Housekeeping gene average Cq change (∆ Cq) in response to skeletal muscle stimulation. B) Housekeeping gene average Cq change (∆ Cq) by group. Based on these results RPL41, RPL27, and RPL7l1 were selected for housekeeping genes. C) Effects of high intensity (H), low intensity (L) muscle stimulations, and control (C) for target genes HMOX1, NQO1, GCLC, GSR, and NFE2L2 fold change from unstimulated muscle (dotted line). In Naïve Controls the mock stimulated muscle (right limb) was compared to the unstimulated control (left limb in all cases). There were no statistically significant increases in gene expression in response to stimulation in either group, although GCLC showed trends for increases regardless of intensity groups. RPL41, RPL27, and RPL7l1 were used for analysis of target gene fold change. H = High intensity stimulation group, L = Low intensity stimulation group, C = Naïve control group.

*Changes in redox stress response proteins in response to skeletal muscle stimulation*

There was a main effect of intensity for HO1 Protein expression ($p = 0.01$) with HO1 significantly elevated in the high intensity group compared to the Naïve controls (Figure 5C, $p = 0.003$). There was a significant effect of stimulation condition on NQO1 protein (Figure 5D, $p = 0.019$) with no differences between high and low intensity stimulus and no differences between stimulation groups. There was a significant main effect of stimulation on GCLC protein content where the stimulation condition decreased GCLC protein slightly (Figure 5E, $p = 0.03$) with no differences between groups. There were no significant differences between groups or in response to muscle stimulation for GR protein (Figure 5F).

**Figure 5.** Redox stress protein response to muscle stimulation.

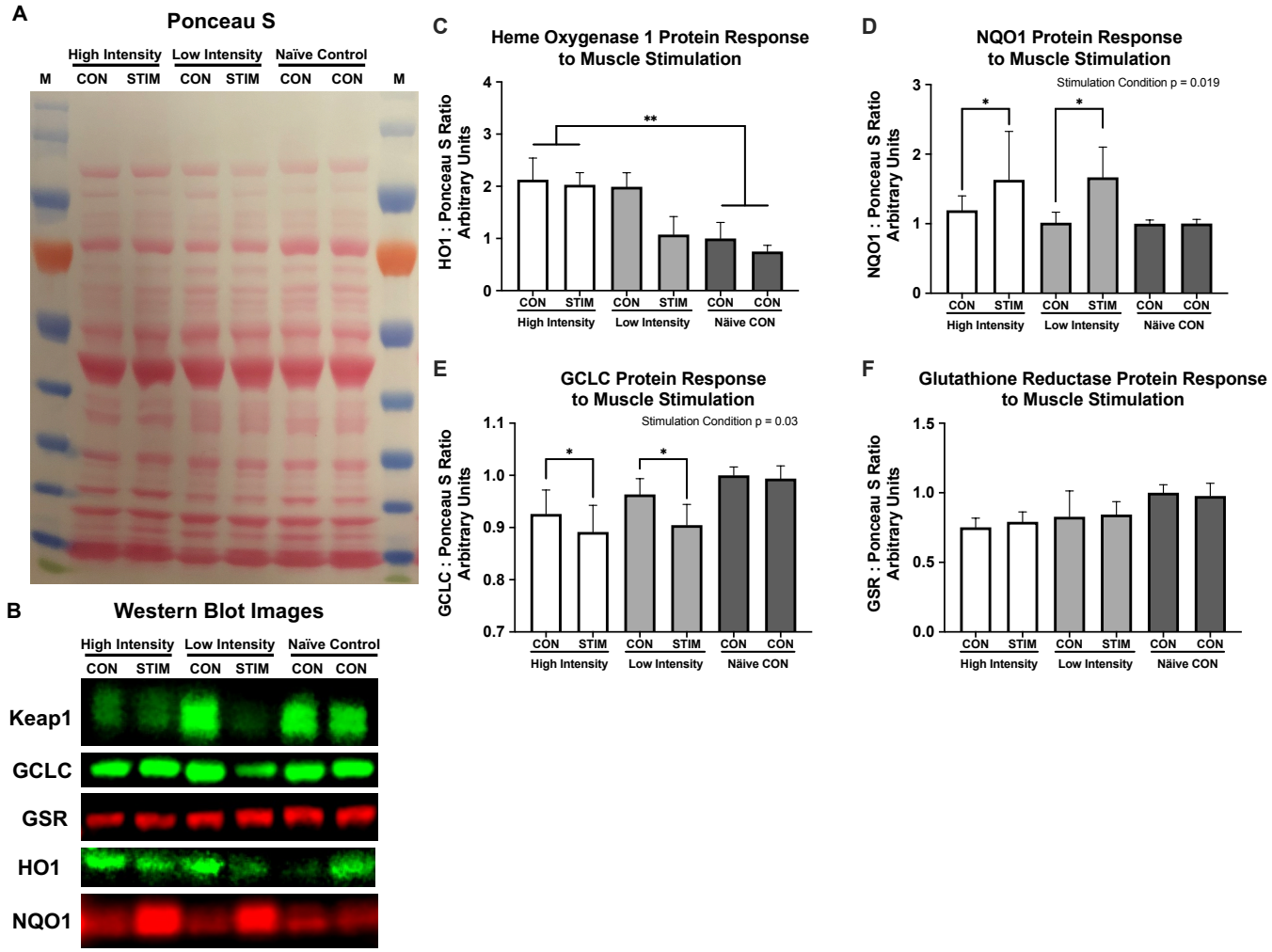



**Figure 5.** Redox stress protein response to muscle stimulation. A) Representative Ponceau S stain, B) Representative western blot images. C) Heme Oxygenase 1 protein is significantly elevated in both conditions of the high intensity group compared to the Naïve control group ($p = 0.002$). D) NQO1 protein increased significantly in response to stimulation (Stimulation condition $p = 0.019$), with no differences between intensities, or across groups. E) GCLC decreases slightly in stimulation conditions regardless of intensity (Stimulation condition $p = 0.03$) with no differences between groups. Glutathione reductase is unchanged in response to muscle stimulation or across intensity groups. All values are normalized to left limb of the NC group and set equal to 1 for western blot graphs.

## DISCUSSION

Exercise is one of the most powerful pleiotropic interventions to improve health and fitness. These health benefits are mediated in part by redox signaling responses to an exercise bout, including activation of the inducible redox stress response transcription factor Nrf2. Nrf2 mediates hundreds of different cytoprotective and metabolic genes increasing metabolic and redox capacity with repeated transient stressors like exercise training. Recently there has been an increased interest in using high intensity interval exercise as a more time efficient way to elicit beneficial adaptations [18]. How the molecular signature from high intensity exercise differs from moderate intensity exercise is still unclear. The aim of this pilot study was to investigate the effects of high intensity and low intensity muscle stimulation on redox stress response markers in mouse skeletal muscle.

Previous studies on Nrf2 signaling responses to exercise have used whole animal treadmill exercise, measuring Nrf2 in several different tissues [2, 15, 16, 31-37], or whole-body exercise in humans [1, 17, 24, 25, 38-40]. We have recently shown that basal levels of Nrf2 affect its inducibility to an acute exercise bout in humans [39]. Therefore, in order to control for intra-animal variations in basal redox homeostasis and basal Nrf2 activation, we elected to use an intra-animal design, where the right limb of the animals were stimulated with either high intensity or low intensity muscle stimulation, while the contralateral unstimulated limb served as the internal control. Unexpectedly, we found that both stimulated and contralateral unstimulated limb of the high intensity group

showed increased Nrf2-ARE binding activity. This is in contrast to the low intensity group where Nrf2 was low in the unstimulated contralateral control limb, but significantly increased in the stimulated limb. The low levels of Nrf2-ARE binding in both Naïve control limbs confirm that Nrf2 activity is low in resting healthy tissue.

Western blots of Keap1, the negative regulator of Nrf2, show opposite effects, where Keap1 protein is highly expressed in both naïve control limbs, and the low intensity control limb, as predicted with the low levels of Nrf2-ARE binding. Keap1 decreases in response to low intensity stimulation, releasing the inhibition on Nrf2 and resulting in increase in Nrf2 activation and ARE binding in low intensity stimulation. Keap1 is lowly expressed in both control and stimulated limbs of the high intensity group, in line with the increases in Nrf2 activation in both stimulated and contralateral control limbs. The ratio of Nrf2-ARE binding to Keap1 content also illustrates the relationship between Keap1 protein and Nrf2-ARE binding. A recently published paper reported significant decreases in Keap1 content after acute exhaustive exercise in human skeletal muscle and concomitant increases in Nrf2 [17], our data here are in agreement with these results. Together these data demonstrate i) Basal Nrf2 activation is low under resting or unstressed conditions in young adult skeletal muscle; ii) Nrf2 activity is inducible in response to muscle stimulation; iii) high intensity, but not low intensity muscle contraction, activates Nrf2 even in unstimulated muscles; and iv) Keap1 protein content mirrors these findings, indicating these inducible Nrf2 responses are acting through canonical Keap1 inhibition in response to skeletal muscle stimulation.

We measured gene expression 30 minutes after the muscle stimulation was finished. The timing may have been a limitation in detecting peak changes in gene expression of these redox stress response genes because there were no statistically significant increases. We and others have shown Nrf2 regulated genes to be induced between 1-4 hours after completion of an exercise bout [38, 39]. However, these results were in human PBMCs, and given that NQO1 protein content increased in response to stimulation in the current investigation, the gene expression may occur earlier in skeletal muscle than in PBMCs. Therefore, future research will utilize different timepoints to assess gene expression changes.

Our results show minimal changes to GSR, and slight but significant decreases in GCLC protein in response to muscle stimulation. The decrease in GCLC here is likely a temporal effect, where the acute stimulation increases proteasomal activity causing decreases in constitutively expressed proteins like GCLC, and the increase in protein likely occurs after the increase in mRNA expression. We have shown that GCLC protein increases significantly eight hours after a single bout of exercise, which is dependent on the increases in mRNA at 1 and 4 hours after completion of the exercise bout [39]. Therefore, increasing the time of skeletal muscle harvest would likely capture greater GCLC protein accretion in response to the exercise bout.

In contrast to GCLC protein expression, HO1 and NQO1 protein increased, albeit in different patterns, in response to muscle contraction. The elevated HO1 expression in both limbs of the high intensity group mirrors the activation of Nrf2 and suggests that HO1

protein expression is rapidly increased and intensity dependent. HO1 mRNA induction has also been shown to be intensity or dose dependent in human skeletal muscle, supporting these findings [41]. NQO1 protein was inducible in response to muscle stimulation in both high and low intensity muscle stimulation, with no significant differences between groups. NQO1 induction in response to exercise has been demonstrated in other reports [42] and is highly dependent on Nrf2 induction [43]. The fact that NQO1 mRNA was not statistically significant but the protein was, suggests that the time course for NQO1 mRNA accretion and protein is shifted closer to the exercise bout than GCLC mRNA. This poses an interesting paradox where some redox stress response genes are early response genes, while others may be late response genes. This may be due to other inducible transcription factors and inhibitors differentially regulating phase II antioxidant genes. For example, NF-kB is known to also regulate GCLC/M gene transcripts [44] which could be responsible for the discrepancies between gene transcripts and protein accumulation in our results.

Taken together, these data suggest that there is an intensity gated redox sensitive exerkine released from high intensity contraction, but not low intensity contraction, that is driving these divergent Nrf2 signaling events in response to high and low intensity exercise, respectively. Recent work has shown that pH gated release of succinate is a myokine involved in adaptive responses to exercise [45]. Others have shown that peroxiredoxins and thioredoxins are released into the blood plasma compartment in an intensity and time dependent manner in response to high intensity, but not moderate intensity exercise in humans [26]. While we cannot ascertain what exerkine is involved

with our current results, the literature suggest that intensity gating mechanisms may explain the divergent effects seen in molecular responses to exercise, and that these mechanisms may be redox dependent [24, 26, 46]. This intensity gated systemic redox signaling model is illustrated in Figure 6, where the signal is released into the blood stream in response to the high intensity exercise and activates Nrf2 signaling in the contralateral unstimulated muscle, while low intensity muscle stimulation does not meet the threshold to propagate a systemic redox signaling response.

**Figure 6.** Model of systemic redox exerkine signaling is gated by muscle stimulation intensity

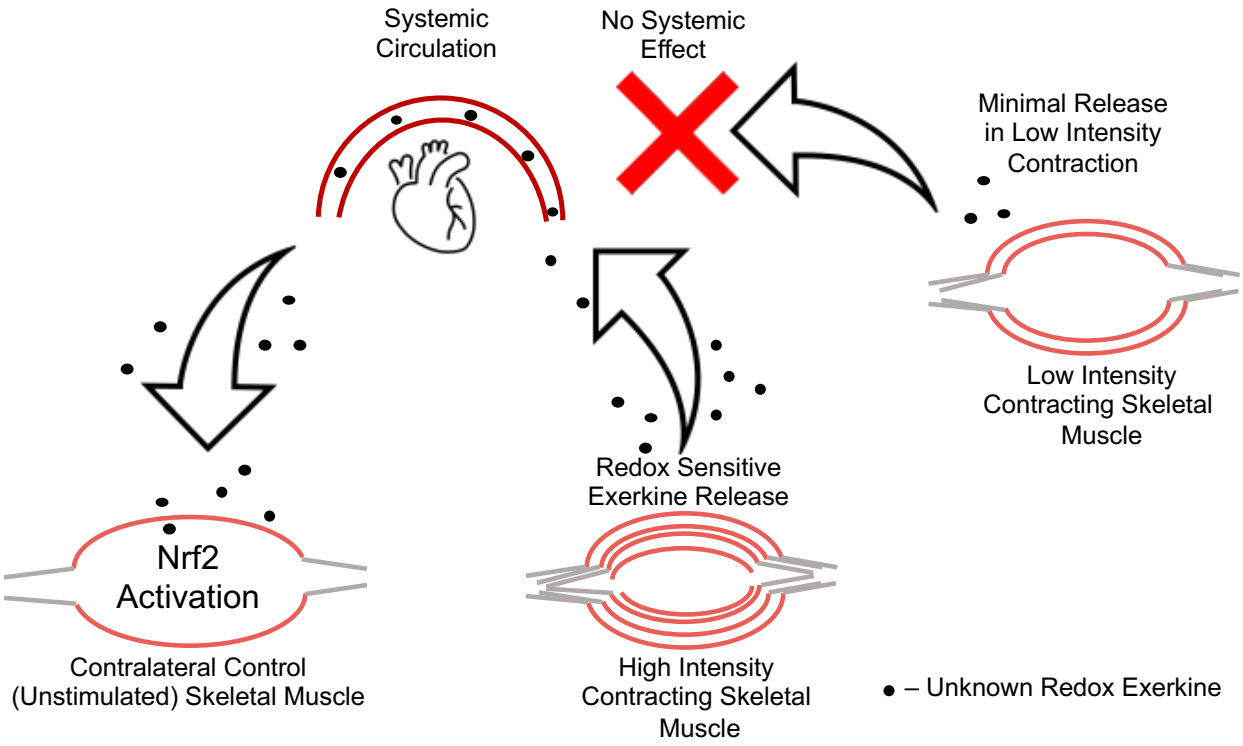



**Figure 6.** High intensity muscle contraction induces release of a redox active exerkine that travels through the blood stream and acts on unstimulated skeletal muscle. This effect is not seen in low intensity contralateral control muscle, suggesting the redox exerkine release from skeletal muscle is intensity gated. In other words, this redox exerkine is only released upon stimuli above a certain stress threshold or is released at lower intensities but not at sufficient concentrations to cause signaling effects in other tissues.

*Strengths, Limitations, and Future Directions*

The strength of the current study is the utilization of multiple controls to assess skeletal muscle redox signaling: Internal contralateral unstimulated control limbs compared to stimulated limbs, as well as Naïve Control animals with control limbs and “mock”

stimulated control limbs. However, one limitation is the redox balance measures in this cohort. Total glutathione was measured, but after partitioning the sample for each assay there was not enough left to perform any additional measures of $NAD^+$/NADH ratio, ROS production assays, or other redox balance measures. While additional redox balance markers would provide a more complete picture, perhaps more relevant to the current study is thiol redox status in target proteins. Future work should investigate protein thiol redox state in key signaling proteins like Keap1 in response to exercise. It is well known that Keap1 thiols are oxidized in response to oxidative stress, and that these modifications activate Nrf2. However, we are unaware of any study that has directly investigated the redox status of Keap1 protein thiols in response to exercise specifically. Furthermore, in studies measuring Nrf2 in tissues other than muscle tissue, it is still unclear how redox signaling mechanisms are being propagated.

Future investigations should aim to identify the redox exerkine responsible for systemic signaling effects in the inducible redox stress response signaling system. In these studies that demonstrate exercise induced Nrf2 activation in peripheral tissues like nervous tissue, PBMCs, and lung tissue, and the current investigation, it is unlikely that superoxide and hydrogen peroxide are viable secondary messenger signaling candidates given their half-life and concentration of extracellular enzymes capable of quenching these signals. The prerequisite for this specific redox myokine is that it is stable enough to travel through the bloodstream and affect other peripheral tissues, including skeletal muscle. It is also possible that there is a relay of some kind, where the exerkine can bind to a surface receptor on a neighboring cell that initiates an intracellular signaling cascade which elicits

ROS production. The sheer number of possible candidate-myokines including proteins, lipids, RNA, exosomes, and microRNAs that could be responsible for the peripheral redox signaling makes this a difficult task. These findings are consistent with previous literature on high intensity exercise eliciting different molecular signaling cascades than low intensity exercise and illustrate an interesting dichotomy between low intensity and high intensity exercise. However, this is the first report that we are aware of demonstrating Nrf2 activation in response to high intensity but not low intensity exercise in a contralateral non-exercised muscle. These findings provide exciting future directions to unveil the novel signaling mechanisms highlighted here.

*Conclusions*

This pilot study set out to test the effects of muscle contraction intensity on Nrf2-mediated redox signaling. The current study design demonstrates a muscle contraction induced systemic redox signaling that appears to be intensity gated; where contralateral unstimulated muscles are responding to high intensity, but not low intensity stimulation. These effects are exerted in part on the Keap1-Nrf2-ARE signaling axis, as well as downstream gene and protein expression for the major Nrf2 targets: NQO1 and HO1 protein. While these data are still preliminary, follow-up experiments are being designed to confirm these findings, including discovering the type of molecule or set of molecules responsible for these systemic redox signaling effects.

**Funding**

This project was funded by the NIH P01 AG001751 to DJM and R15 AG055077 to TT.

## Declaration of Competing Interest

The authors report no conflict of interest.

## REFERENCES

[1] A.J. Done, T. Traustadóttir, Nrf2 mediates redox adaptations to exercise, Redox Biol 10 (2016) 191-199.

[2] T.L. Merry, M. Ristow, Nuclear factor erythroid-derived 2-like 2 (NFE2L2, Nrf2) mediates exercise-induced mitochondrial biogenesis and the anti-oxidant response in mice, J Physiol 594(18) (2016) 5195-207.

[3] T.L. Merry, C. MacRae, T. Pham, C.P. Hedges, M. Ristow, Deficiency in ROS-sensing nuclear factor erythroid 2-like 2 causes altered glucose and lipid homeostasis following exercise training, American Journal of Physiology-Cell Physiology 318(2) (2019) C337-C345.

[4] M.G. Nikolaidis, N.V. Margaritelis, A. Matsakas, Quantitative Redox Biology of Exercise, International journal of sports medicine (2020).

[5] P.A. Kramer, J. Duan, W.-J. Qian, D.J. Marcinek, The Measurement of Reversible Redox Dependent Post-translational Modifications and Their Regulation of Mitochondrial and Skeletal Muscle Function, Frontiers in physiology 6 (2015) 347-347.

[6] J.N. Cobley, H. Husi, Immunological Techniques to Assess Protein Thiol Redox State: Opportunities, Challenges and Solutions, Antioxidants (Basel, Switzerland) 9(4) (2020) 315.

[7] C. Stretton, J.N. Pugh, B. McDonagh, A. McArdle, G.L. Close, M.J. Jackson, 2-Cys Peroxiredoxin oxidation in response to Hydrogen Peroxide and contractile activity in skeletal muscle: A novel insight into exercise-induced redox signalling?, Free Radical Bio Med (2020).

[8] M.D. Campbell, J. Duan, A.T. Samuelson, M.J. Gaffrey, G.E. Merrihew, J.D. Egertson, L. Wang, T.K. Bammler, R.J. Moore, C.C. White, T.J. Kavanagh, J.G. Voss, H.H. Szeto, P.S. Rabinovitch, M.J. MacCoss, W.J. Qian, D.J. Marcinek, Improving mitochondrial function with SS-31 reverses age-related redox stress and improves exercise tolerance in aged mice, Free radical biology & medicine 134 (2019) 268-281.

[9] B. McDonagh, G.K. Sakellariou, N.T. Smith, P. Brownridge, M.J. Jackson, Differential Cysteine Labeling and Global Label-Free Proteomics Reveals an Altered Metabolic State in Skeletal Muscle Aging, Journal of Proteome Research 13(11) (2014) 5008-5021.

[10] C.A. Piantadosi, H.B. Suliman, Redox regulation of mitochondrial biogenesis, Free Radic Biol Med 53(11) (2012) 2043-53.

[11] I. Irrcher, V. Ljubicic, D.A. Hood, Interactions between ROS and AMP kinase activity in the regulation of PGC-1α transcription in skeletal muscle cells, American Journal of Physiology-Cell Physiology 296(1) (2009) C116-C123.

[12] M.C. Gomez-Cabrera, C. Borras, F.V. Pallardo, J. Sastre, L.L. Ji, J. Vina, Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats, J Physiol 567(Pt 1) (2005) 113-20.

[13] M.C. Gomez-Cabrera, E. Domenech, M. Romagnoli, A. Arduini, C. Borras, F.V. Pallardo, J. Sastre, J. Vina, Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance, Am J Clin Nutr 87(1) (2008) 142-9.

[14] M. Ristow, K. Zarse, A. Oberbach, N. Kloting, M. Birringer, M. Kiehntopf, M. Stumvoll, C.R. Kahn, M. Bluher, Antioxidants prevent health-promoting effects of physical exercise in humans, Proc Natl Acad Sci U S A 106(21) (2009) 8665-70.

[15] P. Wang, C.G. Li, Z. Qi, D. Cui, S. Ding, Acute exercise stress promotes Ref1/Nrf2 signalling and increases mitochondrial antioxidant activity in skeletal muscle, Exp Physiol 101(3) (2016) 410-20.

[16] T. Li, S. He, S. Liu, Z. Kong, J. Wang, Y. Zhang, Effects of different exercise durations on Keap1-Nrf2-ARE pathway activation in mouse skeletal muscle, Free Radic Res 49(10) (2015) 1269-74.

[17] A. Gallego-Selles, M. Martin-Rincon, M. Martinez-Canton, M. Perez-Valera, S. Martín-Rodríguez, M. Gelabert-Rebato, A. Santana, D. Morales-Alamo, C. Dorado, J.A.L. Calbet, Regulation of Nrf2/Keap1 signalling in human skeletal muscle during exercise to exhaustion in normoxia, severe acute hypoxia and post-exercise ischaemia: Influence of metabolite accumulation and oxygenation, Redox Biology 36 (2020) 101627.

[18] K.A. Burgomaster, K.R. Howarth, S.M. Phillips, M. Rakobowchuk, M.J. MacDonald, S.L. McGee, M.J. Gibala, Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans, The Journal of Physiology 586(1) (2008) 151-160.

[19] M.J. MacInnis, M.J. Gibala, Physiological adaptations to interval training and the role of exercise intensity, J Physiol 595(9) (2017) 2915-2930.

[20] M.J. Gibala, J.A. Hawley, Sprinting Toward Fitness, Cell Metab 25(5) (2017) 988-990.

[21] V. Chavanelle, N. Boisseau, Y.F. Otero, L. Combaret, D. Dardevet, C. Montaurier, G. Delcros, S.L. Peltier, P. Sirvent, Effects of high-intensity interval training and moderate-intensity

continuous training on glycaemic control and skeletal muscle mitochondrial function in db/db mice, Scientific Reports 7(1) (2017) 204.

[22] J.S. Ramos, L.C. Dalleck, A.E. Tjonna, K.S. Beetham, J.S. Coombes, The Impact of High-Intensity Interval Training Versus Moderate-Intensity Continuous Training on Vascular Function: a Systematic Review and Meta-Analysis, Sports Medicine 45(5) (2015) 679-692.

[23] J.S. Guseh, T.W. Churchill, A. Yeri, C. Lo, M. Brown, N.E. Houstis, K.G. Aragam, D.E. Lieberman, A. Rosenzweig, A.L. Baggish, An expanded repertoire of intensity-dependent exercise-responsive plasma proteins tied to loci of human disease risk, Scientific Reports 10(1) (2020) 10831.

[24] A.J. Done, M.J. Newell, T. Traustadóttir, Effect of exercise intensity on Nrf2 signalling in young men, Free Radic Res 51(6) (2017) 646-655.

[25] H. Islam, J.T. Bonafiglia, P.C. Turnbull, C.A. Simpson, C.G.R. Perry, B.J. Gurd, The impact of acute and chronic exercise on Nrf2 expression in relation to markers of mitochondrial biogenesis in human skeletal muscle, Eur J Appl Physiol 120(1) (2020) 149-160.

[26] A.J. Wadley, G. Keane, T. Cullen, L. James, J. Vautrinot, M. Davies, B. Hussey, D.J. Hunter, S. Mastana, A. Holliday, S.V. Petersen, N.C. Bishop, M.R. Lindley, S.J. Coles, Characterization of extracellular redox enzyme concentrations in response to exercise in humans, J Appl Physiol (1985) 127(3) (2019) 858-866.

[27] N.V. Margaritelis, A.A. Theodorou, V. Paschalis, A.S. Veskoukis, K. Dipla, A. Zafeiridis, G. Panayiotou, I.S. Vrabas, A. Kyparos, M.G. Nikolaidis, Adaptations to endurance training depend on exercise-induced oxidative stress: exploiting redox interindividual variability, Acta physiologica (Oxford, England) 222(2) (2018).

[28] P.A. Kramer, J. Duan, M.J. Gaffrey, A.K. Shukla, L. Wang, T.K. Bammler, W.J. Qian, D.J. Marcinek, Fatiguing contractions increase protein S-glutathionylation occupancy in mouse skeletal muscle, Redox Biol 17 (2018) 367-376.

[29] K.C. Thomas, X.F. Zheng, F. Garces Suarez, J.M. Raftery, K.G.R. Quinlan, N. Yang, K.N. North, P.J. Houweling, Evidence Based Selection of Commonly Used RT-qPCR Reference Genes for the Analysis of Mouse Skeletal Muscle, PLOS ONE 9(2) (2014) e88653.

[30] G. Giordano, C.C. White, L.G. Costa, Assessment of glutathione homeostasis, Methods Mol Biol 758 (2011) 205-14.

[31] S.S. Gounder, S. Kannan, D. Devadoss, C.J. Miller, K.J. Whitehead, S.J. Odelberg, M.A. Firpo, R. Paine, 3rd, J.R. Hoidal, E.D. Abel, N.S. Rajasekaran, Impaired transcriptional activity of Nrf2 in age-related myocardial oxidative stress is reversible by moderate exercise training, PloS one 7(9) (2012) e45697.

[32] C.J. Miller, S.S. Gounder, S. Kannan, K. Goutam, V.R. Muthusamy, M.A. Firpo, J.D. Symons, R. Paine, 3rd, J.R. Hoidal, N.S. Rajasekaran, Disruption of Nrf2/ARE signaling impairs antioxidant mechanisms and promotes cell degradation pathways in aged skeletal muscle, Biochim Biophys Acta 1822(6) (2012) 1038-50.

[33] V.R. Muthusamy, S. Kannan, K. Sadhaasivam, S.S. Gounder, C.J. Davidson, C. Boeheme, J.R. Hoidal, L. Wang, N.S. Rajasekaran, Acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium, Free Radic Biol Med 52(2) (2012) 366-76.

[34] Y.H. Tsou, C.T. Shih, C.H. Ching, J.Y. Huang, C.J. Jen, L. Yu, Y.M. Kuo, F.S. Wu, J.I. Chuang, Treadmill exercise activates Nrf2 antioxidant system to protect the nigrostriatal dopaminergic neurons from MPP+ toxicity, Exp Neurol 263 (2015) 50-62.

[35] M. Yamada, M. Iwata, E. Warabi, H. Oishi, V.A. Lira, M. Okutsu, p62/SQSTM1 and Nrf2 are essential for exercise-mediated enhancement of antioxidant protein expression in oxidative muscle, Faseb j 33(7) (2019) 8022-8032.

[36] A.S. Aguiar, Jr., M. Duzzioni, A.P. Remor, F.S. Tristao, F.C. Matheus, R. Raisman-Vozari, A. Latini, R.D. Prediger, Moderate-Intensity Physical Exercise Protects Against Experimental 6-Hydroxydopamine-Induced Hemiparkinsonism Through Nrf2-Antioxidant Response Element Pathway, Neurochem Res 41(1-2) (2016) 64-72.

[37] M.J. Crilly, L.D. Tryon, A.T. Erlich, D.A. Hood, The role of Nrf2 in skeletal muscle contractile and mitochondrial function, Journal of applied physiology 121(3) (2016) 730-40.

[38] A.J. Done, M.J. Gage, N.C. Nieto, T. Traustadóttir, Exercise-induced Nrf2-signaling is impaired in aging, Free Radic Biol Med 96 (2016) 130-8.

[39] E.L. Ostrom, T. Traustadóttir, Aerobic exercise training partially reverses the impairment of Nrf2 activation in older humans, Free Radical Biology and Medicine 160 (2020) 418-432.

[40] A. Safdar, J. deBeer, M.A. Tarnopolsky, Dysfunctional Nrf2-Keap1 redox signaling in skeletal muscle of the sedentary old, Free Radic Biol Med 49(10) (2010) 1487-93.

[41] H. Pilegaard, G.A. Ordway, B. Saltin, P.D. Neufer, Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise, American Journal of Physiology-Endocrinology and Metabolism 279(4) (2000) E806-E814.

[42] M. Horie, E. Warabi, S. Komine, S. Oh, J. Shoda, Cytoprotective Role of Nrf2 in Electrical Pulse Stimulated C2C12 Myotube, PloS one 10(12) (2015) e0144835.

[43] S. Dhakshinamoorthy, A.K. Jaiswal, Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene, Oncogene 20(29) (2001) 3906-3917.

[44] L. Zhou, H.J. Forman, H. Zhang, Aging of the antioxidant/inflammatory axis in human lung epithelial cells in vitro mimics aging in animal studies, The FASEB Journal 30(S1) (2016) 1296.3-1296.3.

[45] A. Reddy, L.H.M. Bozi, O.K. Yaghi, E.L. Mills, H. Xiao, H.E. Nicholson, M. Paschini, J.A. Paulo, R. Garrity, D. Laznik-Bogoslavski, J.C.B. Ferreira, C.S. Carl, K.A. Sjøberg, J.F.P. Wojtaszewski, J.F. Jeppesen, B. Kiens, S.P. Gygi, E.A. Richter, D. Mathis, E.T. Chouchani, pH-Gated Succinate Secretion Regulates Muscle Remodeling in Response to Exercise, Cell (2020).

[46] A.J. Wadley, S. Aldred, S.J. Coles, An unexplored role for Peroxiredoxin in exercise-induced redox signalling?, Redox Biol 8 (2016) 51-8.

# CHAPTER 5 – Basal Redox Status Influences the Adaptive Redox Response to Regular Exercise

Oxidative Eustress in Exercise Physiology

Ethan L. Ostrom[1*] and Tinna Traustadóttir[1]

[1]Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011, USA

Correspondence: Ethan Ostrom (elo42@nau.edu)

## ABSTRACT

Reactive oxygen species (ROS) produced during acute exercise can act as second messengers to drive improvements in metabolic capacity and redox stress response proteins. The transient nature of the ROS signal during and immediately after exercise makes this beneficial to the organism. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an inducible redox stress response transcription factor responsible for transcribing hundreds of different antioxidant and metabolic enzymes that regulate the adaptive response to exercise. Nrf2 increases activity in response to acute exercise, leading to increased gene transcription and protein content. Habitual oxidative distress induced by inactivity, chronic overfeeding, and age-related mitochondrial dysfunction also increase Nrf2 under basal conditions. While this is initially protective, the increase in Nrf2 drives a ceiling effect that prevents Nrf2 from responding further to additional transient stimuli like acute exercise. Exercise training improves Nrf2 signaling responses in young healthy, and to a degree in aged humans. The increase in Nrf2 signaling magnitude after training in young healthy humans cannot be explained by traditional views of the antioxidant defense system solely as ROS quenchers. Recent studies have shown that enzymes like peroxiredoxins and glutathione S-transferases actively participate in the redox signaling space as redox relay intermediates during acute oxidative eustress. These data provide a more complete explanation of the observed Nrf2 signaling effects seen in response to exercise, although need to be experimentally confirmed. Thus, basal redox balance affects acute responses to oxidative stimuli and the interaction between acute and chronic stimuli determine, in part, whether the stimuli is a eustress or a distress. Furthermore,

the redox relay mechanisms played by traditional antioxidant enzymes are novel paradigm shift with major implications in exercise physiology. This is an exciting time in the redox biology and exercise field, but these models require further investigation.

**Key Words**: Nrf2 signaling, Redox Stress Response, Exercise Adaptation,

***Introduction***

Eustress is defined as moderate or normal physiological or environmental stress that is beneficial to the organism. Distress occurs when physiological or environmental stress is of sufficient duration or intensity to overwhelm the system and cause damage or dysfunction to the cell, tissue, organ, or organism. Acute exercise is a powerful physiologic eustress altering metabolic flux to meet the demands of the contracting skeletal muscle. Repeated application of this physiological stimulus, as in regular exercise training, leads to steady state changes in protein content and beneficial health effects. These favorable adaptions are regulated in part by reactive oxygen species (ROS) production during the exercise bout – oxidative eustress. ROS accumulation initiates redox reactions with protein thiols that act as redox switches, altering protein localization, turnover, interacting partners, or enzyme activity [1, 2]. Thus, an acute exercise bout can temporarily alter the vicinal milieu within a subcellular compartment to a more oxidized microenvironment leading to constructive changes in redox status, and subsequently resulting in beneficial long-term adaptations through changes in gene expression and protein content [3-8] (Figure 1).

**Figure 1.**

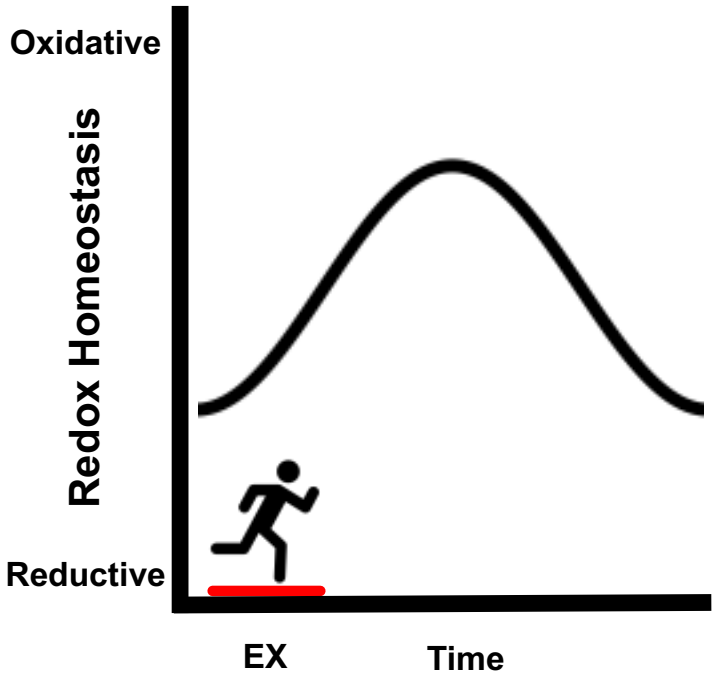



**Figure 1.** Acute exercise (EX) acts as a transient stimulus to produce ROS. This alters the redox microenvironment within the cell, activating redox signaling responses and adaptive changes in cellular capacity and stress resilience.

ROS act beneficially under physiological concentrations. However, past a certain threshold, ROS can cause hyperoxidation of protein thiols, formation of DNA adducts and lipid peroxidation products, potentially leading to accumulation of macromolecular damage and resulting dysfunction [9, 10]. Oxidative distress can occur in response to acute exercise if the exercise bout is sufficiently intense [11]. However, the increases in markers of DNA damage in response to high intensity exercise are often temporary and may therefore not be associated with any permanent dysfunction [11]. Chronic oxidative distress is more commonly seen with aging, sedentary behavior, and overfeeding, driven by elevated basal levels of mitochondrial ROS production [12-16]. This increase in basal

ROS results in site-specific occupancy of oxidized protein thiols leading to aberrant redox signaling and dysfunctional responses to subsequent redox stressors [13]. The key aspect of exercise-induced oxidative signaling is that the signal is constrained by the duration and intensity of the bout – in other words the oxidative stress is transient. This is in contrast to chronic low grade oxidative stress seen in aging and overfeeding diseases like type 2 diabetes where the oxidative distress is constant [13-18]. However, effects of aging may also be due to other mechanisms. Recent proteomic data in a mouse model has demonstrated that aging is associated with reprogramming of redox signaling that is distinct from that of young resulting in the loss of redox networks involved in tissue regulation [19]. The differences between acute and chronic ROS production may be relevant for describing the threshold between what constitutes an oxidative eustress and oxidative distress signal. This threshold is intrinsic to the hormesis model of exercise induced ROS production [20], however where this threshold lies is difficult to quantitatively define and will likely vary depending on reactive species [1] and is therefore difficult to assess where the signal stops being an adaptive eustress.

***Sites of ROS generation and chronic oxidative distress***

ROS can be generated from many different enzymatic and non-enzymatic sites in the cell, including mitochondria, NADPH oxidases (NOXs), xanthine oxidase [21], and lipoxygenases (LOX) [22]. The compartmentalization of these enzymes constrains the ROS products and can create oxidative or reductive hotspots in certain regions or compartments of the cell [23]. *In vitro*, *ex vivo*, and *in vivo* work all suggest that under

resting conditions in skeletal muscle, the mitochondria are the main source of ROS generation, which contribute to the basal tone of redox signaling [22, 24-26]. During exercise (or conditions mimicking exercise *in vitro*) the sources of ROS generation shift away from mitochondria and towards NOX and XO enzymes [22, 26]. This occurs because energy demand increases, providing ADP stimulated state 3 respiration in mitochondria, and a decline in overall superoxide ($O_2^-$) leak through decreasing membrane potential and proton motive pressure release [18]. The concomitant recruitment of NOX subunits to the plasma membrane during exercise activate the complex and increase production of superoxide [4]. During resting conditions, chronic overfeeding with high fat diets increases mitochondrial superoxide leak because of the basal state 4 respiration plus the high driving pressure created by the buildup of NADH from intermediate metabolism [18]. Together this creates a high backpressure through the mitochondrial membrane potential, and $O_2^-$ / $H_2O_2$ leak are one release valve mechanism when calorie consumption is high and ATP demand is low [18]. The chronic levels of mitochondrial oxidative distress from aging, sedentary behavior, and overfeeding stimuli all likely contribute to the increasing dysfunctional redox signaling seen with aging and diseased populations, and consequently in dysfunctional redox responses to exercise [27]. Together these data suggest the chronic oxidative distress in aging and disease populations are being driven by increased ROS production in the mitochondria and leading to redox signaling dysfunction. Treating with mitochondrial antioxidants reverses the age-related elevation in the oxidized thiol proteome [13]. Some evidence suggests that the mitochondrial antioxidant MitoQ decreases exercise-induced mitochondrial DNA damage [10]. Together these data indicate that targeting antioxidants to mitochondria

may be successful in preventing chronic oxidative distress whether it is from increasing age-related mitochondrial dysfunction or protecting the cell from overexertion/overtraining induced acute oxidative distress. If true, these mitochondrial targeted antioxidants may promote transient oxidative eustress through exercise, where other general antioxidants have failed [28-32]. Clearly this is an area where further research is warranted.

***Mechanisms of adaptive responses to exercise: Nrf2***

A critical mechanism involved in the adaptive response to exercise is activating transcription factors that increase mRNA content, driving increases in protein content and exercise capacity [33, 34]. Many different transcription factors have some level of redox regulation, including NF-kB, AP-1, HIF1, HSF1, SP1, Notch, CREB, and Nrf2 [35]. Here we focus on Nrf2 because canonical Nrf2 activation is dependent on oxidative modification of Keap1, Nrf2 directly regulates gene expression for several hundred antioxidant and metabolic enzymes [36], and Nrf2 is a critical component of exercise induced adaptations [37].

Nrf2 is an inducible redox stress response transcription factor activated by increases in environmental or endogenous oxidative species [38]. Figure 2 shows a general schematic of the Nrf2 signaling pathway. Under unstressed conditions Nrf2 is bound by its negative regulator Keap1, which targets Nrf2 for degradation via the 26S proteasome. Nrf2 would be expected to be lowly expressed under those conditions because the majority will be degraded by the proteasome. During an oxidative stress stimulus, solvent exposed

protein thiols on the backbone of Keap1 become oxidized, impairing Keap1's ability to target Nrf2 for degradation [39-42]. This allows Nrf2 to accumulate in the nucleus, where it heterodimerizes with small Maf proteins, binds to the Antioxidant Response Element (ARE) and increases gene expression responsible for increasing metabolic and redox stress capacity. To our knowledge, direct oxidation of Keap1 in response to acute exercise has not been experimentally verified. However, Nrf2 activation occurs in a duration- and intensity-dependent manner which is associated with the accumulation of reactive species generation [43, 44] suggesting that Keap1 is likely oxidatively modified and degraded in response to exercise. Indeed, we have recently found Keap1 to be degraded in an intensity dependent manner in response to muscle stimulation in mice (unpublished data). In addition, a recent study demonstrated decreased Keap1 in human skeletal muscle in response to a maximal exercise test, with a concomitant increase in Nrf2 [45].

Others have shown increases in Nrf2 signaling in response to acute exercise in mouse heart [46, 47], brain [48, 49], and lung tissue in response to exercise [50]. Increases in Nrf2 signaling in response to acute exercise have also been shown in human skeletal muscle [45] and human PBMCs [51-53]. These changes lead to improvements in antioxidant protein content and greater redox stress capacity. Together these data suggest that Nrf2 mediates the redox dependent adaptations to exercise through generation of ROS during the exercise bout [37]. It should be noted that it has yet to be determined how Nrf2 becomes activated in peripheral tissues other than skeletal muscle. Nrf2 activation in tissues like the brain, lungs, and PBMCs are unlikely to be driven by

direct activation of ROS derived from skeletal muscle because: i) the half-life of reactive species is low, ii) the concentration of intracellular and extracellular antioxidant enzymes is very high, and iii) simple diffusion ($H_2O_2$) across multiple cellular and organellar membranes make the probability of ROS acting as a second messenger over long timescales and distances very low. Unraveling the “Redox Exerkine Signaling” dilemma provides an interesting and challenging future direction for the redox biology and exercise physiology field.

**Figure 2.**

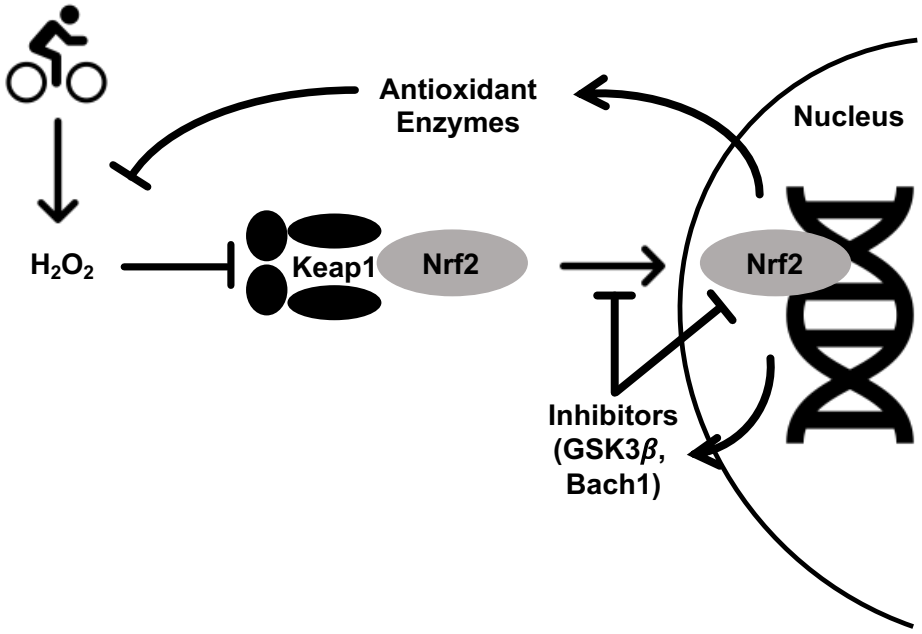



**Figure 2.** ROS-induced Nrf2 activation. An endogenous or environmental signal like exercise increases ROS production which interacts with solvent exposed cysteine residues on Keap1. Cysteine oxidation activates Nrf2 allowing it to translocate to the nucleus and bind to ARE sequences. This increases gene expression and protein content of antioxidant enzymes, as well as other proteins that negatively regulate Nrf2 like Bach1, which is a competitive inhibitor of ARE binding. Together these antioxidant enzymes and competitive inhibitors quench the signal, completing the negative feedback loop.

***Nrf2 Response to Exercise Training***

We have recently shown that basal levels of Nrf2 nuclear protein after training are unchanged in young healthy individuals, while older individuals who had higher basal levels prior to the intervention, showed a decrease in Nrf2 nuclear content [53]. This decline in Nrf2 protein in response to training has also been shown in mouse skeletal muscle [54]. The decline of basal nuclear Nrf2 in response to training in older adults improved their acute response to a single exercise bout, although the improvement was

still not as robust as young adults [53]. Together, these data suggest that the important health metric in Nrf2 signaling is the magnitude of the response to a transient oxidative eustress, as opposed to the basal resting levels [53]. Other research using Nrf2 knockout animals has shown that the adaptive response to training requires Nrf2 activation to stimulate mitochondrial biogenesis and increase redox stress capacity [30]. Furthermore, treating mice with the antioxidants N-Acetylcysteine or L-NAME impairs exercise-induced activation of Nrf2 and results in impaired markers of mitochondrial biogenesis [30]. Together these findings demonstrate that Nrf2 signaling is required for mitochondrial biogenesis in response to training, and that Nrf2 levels mirror the oxidative stress levels in the cell. It would therefore be predicted to observe elevated basal nuclear Nrf2 levels in aging, sedentary individuals, and chronic overfeeding. This increase in basal Nrf2 is initially adaptive, however, a side effect of this increasing chronic oxidative distress is a ceiling effect on Nrf2 inducibility. Any added transient stimulus – such as acute exercise – on top of the chronic oxidative distress results in diminished signaling effects and adaptive responses [55-57]. Despite the declining adaptive responses in aging populations, exercise training still elicits responsiveness in some redox regulated signaling networks [7].

***Role of basal redox status in the adaptive response to exercise***

The data above suggest that the antioxidant enzymes and proteins thiols that respond to acute exercise are important for exercise training adaptations and are dependent on basal redox status [58]. Figure 3 shows the relationship between basal redox status and acute oxidative eustress induced by exercise in healthy redox homeostasis (left), and in a chronic state of oxidative distress (right). In support of this concept, classifying individuals based on oxidative markers in response to acute exercise significantly predicted an individual's adaptive response to exercise training. Individuals with moderate and high increases in oxidative stress markers in response to acute exercise showed significantly greater adaptations to a six-week aerobic exercise training protocol compared to individuals with a low acute oxidative response to exercise [58, 59]. Additionally, if researchers randomized healthy people to either vitamin C supplementation (antioxidant stimulus) or passive smoking (pro-oxidant stimulus), the $F_2$-isoprostane and glutathione response to acute exercise was altered. Vitamin C supplementation lowered basal oxidative stress levels, and as a result those individuals showed appropriate responses to acute exercise. Conversely, if chronic oxidative distress is experimentally elevated using passive cigarette smoking, the redox responses to acute exercise were blunted [60]. In a later study, the same research group performed a targeted antioxidant supplementation of either vitamin C or N-Acetylcysteine after assessing basal blood measures. This allowed the researchers to tailor the intervention to reverse a deficiency in either vitamin C levels or glutathione. This targeted approach improved $VO_2$ peak greater than a non-targeted antioxidant supplementation approach [61]. Together these

data suggest that the threshold between ROS induced oxidative eustress and oxidative distress is malleable [20] and that there is an optimal basal redox status. Furthermore, if that optimal basal redox steady state is not achieved, responses to acute exercise will be altered, potentially impairing exercise adaptations.

An alternative hypothesis has recently been presented, around the concept that the adaptive redox response to exercise is dysfunctional with aging due to a more reduced state of protein thiols in the cytosol, rather than oxidized [62]. The rationale behind this hypothesis is that skeletal muscle denervation that increases with age, induces a burst of mitochondrial ROS production that initially stimulates axonal sprouting. This burst in ROS production also increases antioxidant proteins in the cytosol through redox signaling adaptations and over time contribute to an overall shift to a more reduced cytosolic milieu. The more reduced cytosolic environment prevents significant protein thiol oxidation, which results in impaired redox signaling and overall declining adaptations to exercise with increasing age. It is important to note that these two hypotheses are not mutually exclusive. Redox states may differ between cellular compartments or tissues and therefore it is possible that there is a shift to a more reduced state in some compartments and a shift to a more oxidized state in others [63]. Furthermore, the global protein thiol oxidation state is dynamic and therefore thiol oxidation states are likely dependent on the timeframe of the tissue sample collection, which may explain some of the differences seen in the field [13, 14, 62, 64]. Regardless of the discrepancies, collectively the data suggest that the redox homeostatic set point changes with a combination of age, sedentary behavior, and overfeeding, which alters redox specific adaptations to exercise.

**Figure 3.**

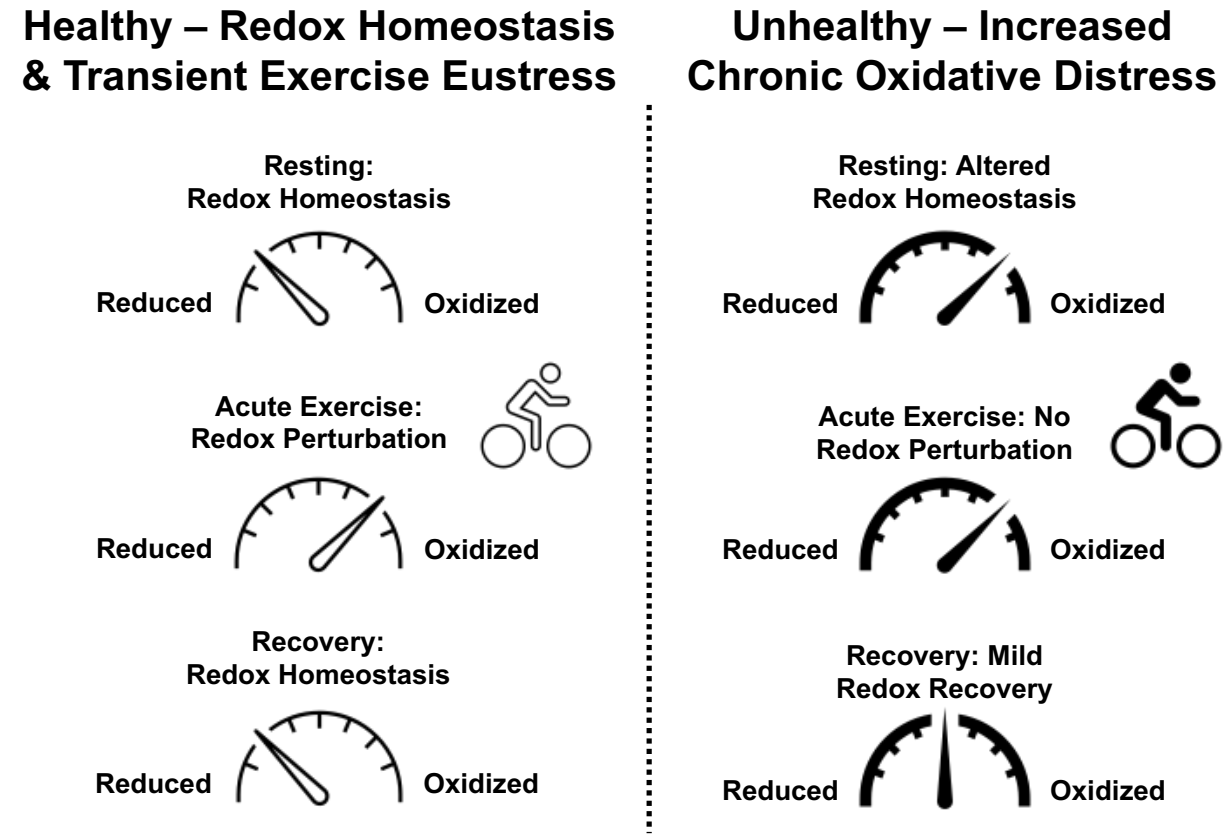



**Figure 3.** The relationship between basal redox state and acute redox responses. Under normal healthy circumstances (Left side) redox homeostasis favors the reduced state. During an acute exercise bout, the state shifts to a more oxidized condition, followed by recovery to resting redox homeostasis after completion of the exercise. In unhealthy conditions there is a shift in redox homeostasis to favor an oxidizing environment creating a chronic oxidative distress. This impairs redox signaling mechanisms in response to acute exercise, preventing the dynamic nature of transient signaling to occur. This is followed by a mild recovery after completion of the exercise bout.

***A paradigm shift in the relationship between antioxidant enzymes and redox signaling***

The generally accepted idea of redox signaling and adaptive responses to exercise is that acute increase in ROS will stimulate Nrf2 activation, which consequently leads to

upregulation of antioxidant gene transcription. With regular temporal Nrf2 activation, the adaptive response will increase the antioxidant enzyme capacity, resulting in the oxidative stimulus being quenched quicker (Figure 2), given the same ROS production.

In this model, one would predict that the redox stress response capacity would increase after exercise training such that the same oxidative stimulus would be quenched quicker and more efficiently upon subsequent stressors of the same magnitude. In other words, at the same given oxidative stimulus intensity, the signal transduction response would be diminished. However, we have recently shown that Nrf2 signaling response to acute exercise increases in magnitude after exercise training [53], suggesting the oxidant-induced negative feedback mechanism does not provide a complete picture of exercise induced redox signaling (figure 4). While it is certainly possible that exercise training increases the ability to produce ROS at the same relative exercise intensity compared to pre-training, it is also likely that our implicit assumptions about the relationship between the antioxidant defense system and redox signaling mechanisms are flawed.

**Figure 4.**

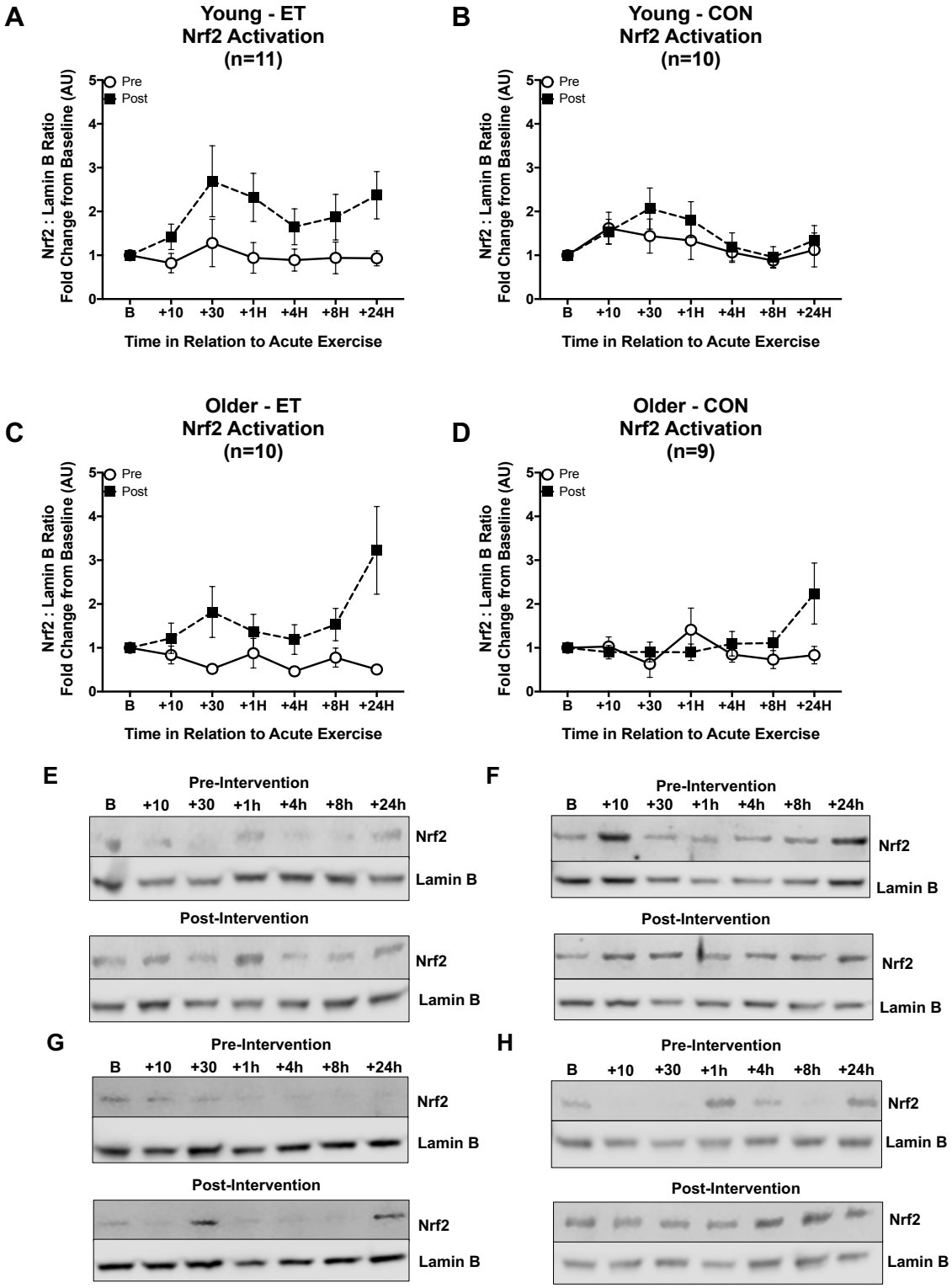

A
Young - ET
Nrf2 Activation
(n=11)
B
Young - CON
Nrf2 Activation
(n=10)
C
Older - ET
Nrf2 Activation
(n=10)
D
Older - CON
Nrf2 Activation
(n=9)
Pre
Post
Nrf2 : Lamin B Ratio
Fold Change from Baseline (AU)
B +10 +30 +1H +4H +8H +24H
Time in Relation to Acute Exercise
E
F
G
H
Pre-Intervention
Post-Intervention
B +10 +30 +1h +4h +8h +24h
Nrf2
Lamin B

**Figure 4. Nrf2 signaling responses to acute exercise before and after exercise training or inactive control intervention by age**. Open circles indicate Nrf2 activation pre-intervention, and black squares indicate Nrf2 activation post-intervention. Panels A & C are ET subjects while panels B & D are CON subjects. (**A&B**) Eight weeks of aerobic exercise training increased the Nrf2 signaling response to an acute exercise bout in the young-trained cohort improved, while the Nrf2 signaling in the control group did not change. (**C&D**) Aerobic training in the older group showed improvements, while the older control group showed no changes. The increase in Nrf2 signaling after training was similar in pattern for young and older adults, although young improved their responses to a greater degree than older ET subjects (A vs D post-intervention, main effect of age, $p$ = 0.014). Representative western blots for (E) Young-ET, (F) Young-CON, (G) Older-ET, and (H) Older-CON. ET – Endurance Trained, CON – Control intervention. Data shown as Mean ± SEM. Data reprinted with permission from [50].

Hydrogen peroxide ($H_2O_2$) has been viewed as the main signaling molecule in redox signal transduction, where it was assumed that $H_2O_2$ reacts directly with protein thiols to initiate a signaling cascade. However, there are several problems with this assumption. Peroxiredoxins (Prxs) are some of the most abundantly and ubiquitously expressed antioxidant proteins in mammalian cells [65], and they react with $H_2O_2$ on orders of magnitude faster than even the most reactive solvent exposed protein thiols. Therefore, it would appear that Prxs are able to quench the oxidative stress signal from endogenous and exogenous signals before it can initiate a signal transduction cascade through other protein thiols [65]. Thus, Prxs likely outcompete most if not all protein thiols in the cell that utilize redox signaling mechanisms. The floodgate hypothesis proposes that $H_2O_2$ signaling persists by first causing hyperoxidation-induced inhibition of Prx enzymes, which then allows $H_2O_2$ to accumulate and diffuse throughout the cell to react with other protein thiols to initiate a redox signaling cascade. This model, if true, would circumvent the problems with kinetic efficiencies between $H_2O_2$ and Prxs versus $H_2O_2$ and protein thiols [66]. While this may occur [67], the floodgate hypothesis still does not explain redox signaling specificity. How does $H_2O_2$ react with some protein thiols and not others? More

recently it has been shown that Prxs have much more diverse functions than just peroxide quenchers.

Recently, Prxs have also been shown to form mixed disulfide intermediates with well-known stress response signaling cascades. One study used an eloquent series of experiments to demonstrate that Prx2 can take oxidizing equivalents from hydrogen peroxide and transfer those oxidizing equivalents to the transcription factor STAT3, causing STAT3 dimerization and tetramerization, which impairs STAT3 transcriptional activity [68]. These data indicate that Prx2 is acting as a redox middleman by taking oxidizing equivalents from $H_2O_2$ and transferring them to other redox sensitive proteins [68]. Prx1 has also been shown to act as a $H_2O_2$ signaling intermediate by forming mixed disulfide links with the protein ASK1 [69]. ASK1 is required for phosphorylation and activation of p38 MAPK. ASK1 oxidation through Prx1 mediated oxidation promoted p38 phosphorylation and activation, while knockdown of Prx1 impaired p38 phosphorylation and activation [69]. MAPK p38 signaling is an important redox sensitive mediator of the adaptive responses to exercise [4, 70]. Prx mediated oxidation of ASK1 is likely a viable redox-mediated adaptive signaling process in exercise adaptations, although this has yet to be empirically determined. In addition to these findings, previous studies have demonstrated increased levels of oxidized Prx dimers in isolated PBMCs following acute exercise and increased abundance of Prx2 following an exercise intervention [71]. The mechanism described by the ‘redox relay’ alleviates the inconsistencies with the floodgate model by relying on the superior kinetic efficiency of Prxs, as well as providing a

mechanism for specificity through steric hindrances in protein-protein interactions between Prxs and their target redox sensitive proteins.

Together these data provide evidence that Prxs are involved in gene transcriptional regulation and protein chaperone behavior in addition to their traditional antioxidant capacities. Based on these findings it would be reasonable to predict that the increase in antioxidant enzymes in response to exercise training would not only prevent unwanted hyperoxidation and oxidative distress but also increase redox signaling flux through specific signaling pathways in response to oxidative eustress stimuli. In this way these antioxidant enzymes are simultaneously acting as true antioxidants (quenching the signal), and as redox relay intermediates. We suspect that the increases in Nrf2 signaling after exercise training in our recent work may be mediated by these redox relay mechanisms. The negative feedback loop model (Figure 2) would not explain an increase in Nrf2 signaling after training unless the increase in antioxidant enzymes also worked to direct the oxidative signal through these signaling pathways, however this still need to be confirmed. For a more complete review of Prx mediated redox signaling in exercise we refer the reader to this excellent review by Wadley et al. [72].

Another redox signaling mechanism is protein glutathionylation. This is where glutathione is added to a cysteine residue creating a mixed disulfide (–SSG) modification which can also alter protein function. This reaction is catalyzed by the family of glutathione synthetase transferases (GSTs) while glutaredoxins are responsible for removing glutathione from protein thiols. Glutathionylation occupancy increases in mouse skeletal

muscle subjected to fatiguing contractions [73]. This increase is likely a protective mechanism to desensitize the thiol proteome to hyperoxidation induced by overexertion and prevent further oxidation through inhibiting metabolic pathways that generate more ROS [74, 75]. Additionally, glutathione modification of Keap1 cysteines have been shown to induce Nrf2 activation [76]. This suggests that ROS-induced increases in protein glutathionylation is a positive feed-forward loop that enhances Nrf2 signaling in addition to minimizing ROS production through inhibiting enzymes that generate ROS and quench the signal through GPx mediated reduction of $H_2O_2$. Taken together it seems likely that the traditional view that antioxidant enzymes are pure antioxidants, (i.e., they quench ROS signals) needs to be refined. A more nuanced model about the relationship between the antioxidant defense system and Nrf2 activation in response to exercise is shown in figure 5, along with the chronic oxidative distress stemming from sedentary behavior and overfeeding induced mitochondrial dysfunction seen in aging populations. However, more research is needed to know if this redox relay signaling mechanism occurs in response to acute exercise-induced oxidative eustress.

**Figure 5.**

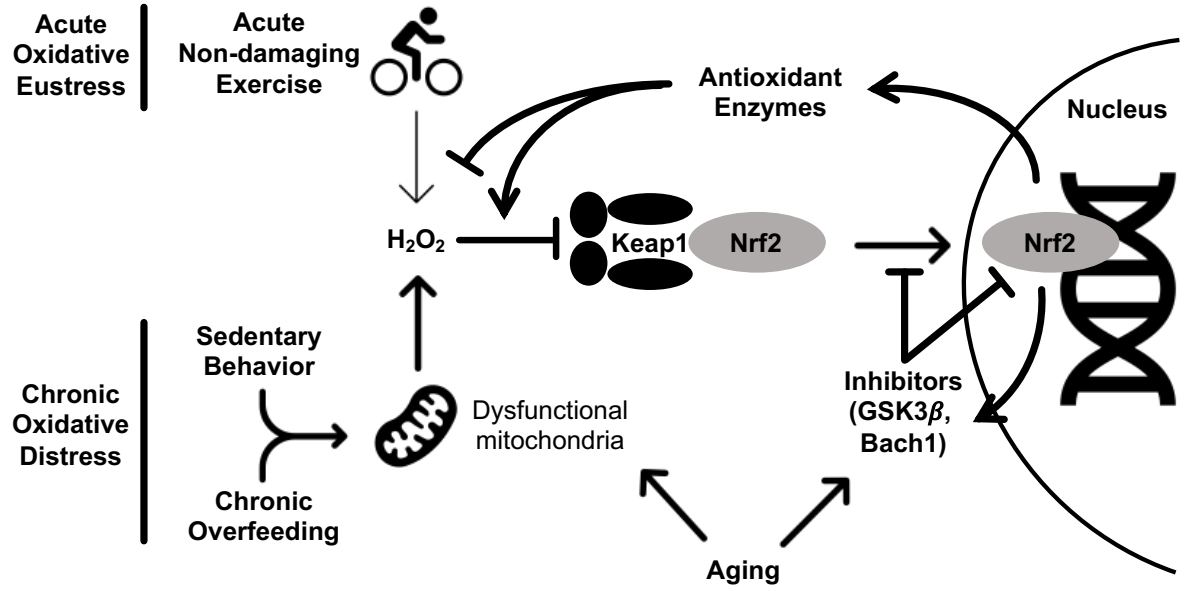



**Figure 5.** Effects of acute and chronic oxidative stimuli on adaptive redox signaling responses mediated by Nrf2. Chronic oxidative distress from sedentary behavior, overfeeding, or aging lead to increases in chronic low levels of ROS production through mitochondria. This activates Nrf2, increasing antioxidant enzyme capacity, but also increasing Nrf2 negative regulators Bach1 and GSK3β. Acute exercise generates ROS, but in a system that is already saturated with increased protein thiol oxidation and high levels of negative regulators, the ROS signal from exercise becomes less effective (depicted by thin arrow). Regular exercise training can restore some of the signaling homeostasis but not to the same degree as young individuals. The traditional view that antioxidant enzymes like PRDXs and GSTs act to quench the ROS signal (negative feedback loop) is not supported entirely by new literature in the field. Rather these enzymes may be acting as redox signaling mediators (positive feedforward loop) transferring oxidizing equivalents from ROS to protein thiols and using glutathionylation as a signaling mediator or protective mechanism in response to over production of ROS. This relationship is depicted by antioxidant enzymes enhancing the effect of hydrogen peroxide on Keap1 ( → ) in addition to quenching the signal ( ⟞ ).

***Conclusions and Future Directions***

Oxidative eustress initiates adaptive processes in response to exercise that stem from multiple enzymatic sources in the cell. These processes initiate cell signaling cascades through protein thiol oxidation / reduction reactions that improve cellular metabolism and increase redox stress response capacity. These increases in mitochondrial biogenesis and the antioxidant defense system are governed partly by the inducible redox stress response transcription factor, Nrf2. Increases in Nrf2 activation and downstream target genes occur in response to acute exercise through a combination of ROS sources. The chronic increases in basal or resting levels of mitochondrial ROS from sedentary behavior, overfeeding, and age-related mitochondrial dysfunction increase basal levels of Nrf2, which likely impairs the Nrf2 signaling system from responding further to an added transient oxidative eustress like exercise. This is exacerbated by the feedforward redox signaling mechanisms elicited by the initial adaptive response to increasing basal ROS production, simultaneously protecting the cell from its own dysfunctional redox circuitry and making it harder for transient oxidative eustress signals like exercise to be effective.

Overall, these concepts – particularly the redox relay signaling mechanisms of the antioxidant defense system – should be tested further in young healthy, aging, and diseased populations, as well as in appropriate model systems. This is an exciting area of research, with many questions still unanswered. The progression of several new assays and measurement techniques to delineate these challenging questions, including immunological techniques to assess protein thiol redox status [77-79] are exciting. These methods provide promising orthogonal approaches to

redox proteomic techniques and will likely advance the exercise physiology field dramatically when applied with appropriate study designs.

## REFERENCES


[1] M.G. Nikolaidis, N.V. Margaritelis, A. Matsakas, Quantitative Redox Biology of Exercise, Int J Sports Med (2020).

[2] P.A. Kramer, J. Duan, W.-J. Qian, D.J. Marcinek, The Measurement of Reversible Redox Dependent Post-translational Modifications and Their Regulation of Mitochondrial and Skeletal Muscle Function, Frontiers in physiology 6 (2015) 347-347.

[3] C.A. Piantadosi, H.B. Suliman, Mitochondrial transcription factor A induction by redox activation of nuclear respiratory factor 1, J Biol Chem 281(1) (2006) 324-33.

[4] C. Henriquez-Olguin, A. Diaz-Vegas, Y. Utreras-Mendoza, C. Campos, M. Arias-Calderon, P. Llanos, A. Contreras-Ferrat, A. Espinosa, F. Altamirano, E. Jaimovich, D.M. Valladares, NOX2 Inhibition Impairs Early Muscle Gene Expression Induced by a Single Exercise Bout, Front Physiol 7 (2016) 282.

[5] C. Henriquez-Olguin, L.B. Renani, L. Arab-Ceschia, S.H. Raun, A. Bhatia, Z. Li, J.R. Knudsen, R. Holmdahl, T.E. Jensen, Adaptations to high-intensity interval training in skeletal muscle require NADPH oxidase 2, Redox Biol 24 (2019) 101188.

[6] C. Henriquez-Olguin, J.R. Knudsen, S.H. Raun, Z. Li, E. Dalbram, J.T. Treebak, L. Sylow, R. Holmdahl, E.A. Richter, E. Jaimovich, T.E. Jensen, Cytosolic ROS production by NADPH oxidase 2 regulates muscle glucose uptake during exercise, Nat Commun 10(1) (2019) 4623.

[7] J.N. Cobley, G.K. Sakellariou, D.J. Owens, S. Murray, S. Waldron, W. Gregson, W.D. Fraser, J.G. Burniston, L.A. Iwanejko, A. McArdle, J.P. Morton, M.J. Jackson, G.L. Close, Lifelong training preserves some redox-regulated adaptive responses after an acute exercise stimulus in aged human skeletal muscle, Free Radical Biology and Medicine 70 (2014) 23-32.

[8] J.N. Cobley, P.R. Moult, J.G. Burniston, J.P. Morton, G.L. Close, Exercise improves mitochondrial and redox-regulated stress responses in the elderly: better late than never!, Biogerontology 16(2) (2015) 249-264.

[9] A.J. Wadley, Y.W. Chen, S.J. Bennett, G.Y. Lip, J.E. Turner, J.P. Fisher, S. Aldred, Monitoring changes in thioredoxin and over-oxidised peroxiredoxin in response to exercise in humans, Free Radic Res 49(3) (2015) 290-8.

[10] J. Williamson, C.M. Hughes, J.N. Cobley, G.W. Davison, The mitochondria-targeted antioxidant MitoQ, attenuates exercise-induced mitochondrial DNA damage, Redox Biol 36 (2020) 101673.

[11] D.V. Tryfidou, C. McClean, M.G. Nikolaidis, G.W. Davison, DNA Damage Following Acute Aerobic Exercise: A Systematic Review and Meta-analysis, Sports Med 50(1) (2020) 103-127.

[12] D.F. Dai, Y.A. Chiao, D.J. Marcinek, H.H. Szeto, P.S. Rabinovitch, Mitochondrial oxidative stress in aging and healthspan, Longev Healthspan 3 (2014) 6.

[13] M.D. Campbell, J. Duan, A.T. Samuelson, M.J. Gaffrey, G.E. Merrihew, J.D. Egertson, L. Wang, T.K. Bammler, R.J. Moore, C.C. White, T.J. Kavanagh, J.G. Voss, H.H. Szeto, P.S. Rabinovitch, M.J. MacCoss, W.J. Qian, D.J. Marcinek, Improving

mitochondrial function with SS-31 reverses age-related redox stress and improves exercise tolerance in aged mice, Free Radic Biol Med 134 (2019) 268-281.

[14] B. McDonagh, G.K. Sakellariou, N.T. Smith, P. Brownridge, M.J. Jackson, Differential Cysteine Labeling and Global Label-Free Proteomics Reveals an Altered Metabolic State in Skeletal Muscle Aging, Journal of Proteome Research 13(11) (2014) 5008-5021.

[15] A. Mansouri, F.L. Muller, Y. Liu, R. Ng, J. Faulkner, M. Hamilton, A. Richardson, T.-T. Huang, C.J. Epstein, H. Van Remmen, Alterations in mitochondrial function, hydrogen peroxide release and oxidative damage in mouse hind-limb skeletal muscle during aging, Mechanisms of Ageing and Development 127(3) (2006) 298-306.

[16] A. Vasilaki, A. Mansouri, H. Van Remmen, J.H. Van Der Meulen, L. Larkin, A.G. Richardson, A. McArdle, J.A. Faulkner, M.J. Jackson, Free radical generation by skeletal muscle of adult and old mice: effect of contractile activity, Aging Cell 5(2) (2006) 109-117.

[17] E.J. Anderson, M.E. Lustig, K.E. Boyle, T.L. Woodlief, D.A. Kane, C.T. Lin, J.W. Price, 3rd, L. Kang, P.S. Rabinovitch, H.H. Szeto, J.A. Houmard, R.N. Cortright, D.H. Wasserman, P.D. Neufer, Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans, J Clin Invest 119(3) (2009) 573-81.

[18] K.H. Fisher-Wellman, T.M. Weber, B.L. Cathey, P.M. Brophy, L.A. Gilliam, C.L. Kane, J.M. Maples, T.P. Gavin, J.A. Houmard, P.D. Neufer, Mitochondrial respiratory capacity and content are normal in young insulin-resistant obese humans, Diabetes 63(1) (2014) 132-41.

[19] H. Xiao, M.P. Jedrychowski, D.K. Schweppe, E.L. Huttlin, Q. Yu, D.E. Heppner, J. Li, J. Long, E.L. Mills, J. Szpyt, Z. He, G. Du, R. Garrity, A. Reddy, L.P. Vaites, J.A. Paulo,

T. Zhang, N.S. Gray, S.P. Gygi, E.T. Chouchani, A Quantitative Tissue-Specific Landscape of Protein Redox Regulation during Aging, Cell 180(5) (2020) 968-983 e24.

[20] Z. Radak, K. Ishihara, E. Tekus, C. Varga, A. Posa, L. Balogh, I. Boldogh, E. Koltai, Exercise, oxidants, and antioxidants change the shape of the bell-shaped hormesis curve, Redox Biol 12 (2017) 285-290.

[21] J.H. Suh, S.V. Shenvi, B.M. Dixon, H. Liu, A.K. Jaiswal, R.M. Liu, T.M. Hagen, Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid, Proc Natl Acad Sci U S A 101(10) (2004) 3381-6.

[22] G.K. Sakellariou, M.J. Jackson, A. Vasilaki, Redefining the major contributors to superoxide production in contracting skeletal muscle. The role of NAD(P)H oxidases, Free Radic Res 48(1) (2014) 12-29.

[23] A.G. Manford, F. Rodriguez-Perez, K.Y. Shih, Z. Shi, C.A. Berdan, M. Choe, D.V. Titov, D.K. Nomura, M. Rape, A Cellular Mechanism to Detect and Alleviate Reductive Stress, Cell 183(1) (2020) 46-61 e21.

[24] R.L. Goncalves, C.L. Quinlan, I.V. Perevoshchikova, M. Hey-Mogensen, M.D. Brand, Sites of superoxide and hydrogen peroxide production by muscle mitochondria assessed ex vivo under conditions mimicking rest and exercise, J Biol Chem 290(1) (2015) 209-27.

[25] R.L.S. Goncalves, M.A. Watson, H.-S. Wong, A.L. Orr, M.D. Brand, The use of site-specific suppressors to measure the relative contributions of different mitochondrial sites to skeletal muscle superoxide and hydrogen peroxide production, Redox Biology (2019) 101341.

[26] C. Henriquez-Olguin, R. Meneses-Valdes, T.E. Jensen, Compartmentalized muscle redox signals controlling exercise metabolism – Current state, future challenges, Redox Biology 35 (2020) 101473.

[27] J. Viña, C. Borras, K.M. Abdelaziz, R. Garcia-Valles, M.C. Gomez-Cabrera, The Free Radical Theory of Aging Revisited: The Cell Signaling Disruption Theory of Aging, Antioxidants & Redox Signaling 19(8) (2013) 779-787.

[28] M.C. Gomez-Cabrera, C. Borras, F.V. Pallardo, J. Sastre, L.L. Ji, J. Vina, Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats, J Physiol 567(Pt 1) (2005) 113-20.

[29] M.C. Gomez-Cabrera, E. Domenech, M. Romagnoli, A. Arduini, C. Borras, F.V. Pallardo, J. Sastre, J. Vina, Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance, Am J Clin Nutr 87(1) (2008) 142-9.

[30] T.L. Merry, M. Ristow, Nuclear factor erythroid-derived 2-like 2 (NFE2L2, Nrf2) mediates exercise-induced mitochondrial biogenesis and the anti-oxidant response in mice, J Physiol 594(18) (2016) 5195-207.

[31] M. Ristow, K. Zarse, A. Oberbach, N. Kloting, M. Birringer, M. Kiehntopf, M. Stumvoll, C.R. Kahn, M. Bluher, Antioxidants prevent health-promoting effects of physical exercise in humans, Proc Natl Acad Sci U S A 106(21) (2009) 8665-70.

[32] S.C. Broome, J.S.T. Woodhead, T.L. Merry, Mitochondria-Targeted Antioxidants and Skeletal Muscle Function, Antioxidants (Basel, Switzerland) 7(8) (2018) 107.

[33] I. Irrcher, V. Ljubicic, D.A. Hood, Interactions between ROS and AMP kinase activity in the regulation of PGC-1α transcription in skeletal muscle cells, American Journal of Physiology-Cell Physiology 296(1) (2009) C116-C123.

[34] C.G. Perry, J. Lally, G.P. Holloway, G.J. Heigenhauser, A. Bonen, L.L. Spriet, Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle, J Physiol 588(Pt 23) (2010) 4795-810.

[35] H.S. Marinho, C. Real, L. Cyrne, H. Soares, F. Antunes, Hydrogen peroxide sensing, signaling and regulation of transcription factors, Redox Biology 2 (2014) 535-562.

[36] J.D. Hayes, A.T. Dinkova-Kostova, The Nrf2 regulatory network provides an interface between redox and intermediary metabolism, Trends Biochem Sci 39(4) (2014) 199-218.

[37] A.J. Done, T. Traustadóttir, Nrf2 mediates redox adaptations to exercise, Redox Biol 10 (2016) 191-199.

[38] L.E. Tebay, H. Robertson, S.T. Durant, S.R. Vitale, T.M. Penning, A.T. Dinkova-Kostova, J.D. Hayes, Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it attenuates degenerative disease, Free Radic Biol Med 88(Pt B) (2015) 108-146.

[39] S. Fourquet, R. Guerois, D. Biard, M.B. Toledano, Activation of NRF2 by nitrosative agents and H2O2 involves KEAP1 disulfide formation, J Biol Chem 285(11) (2010) 8463-71.

[40] Aimee L. Eggler, E. Small, M. Hannink, Andrew D. Mesecar, Cul3-mediated Nrf2 ubiquitination and antioxidant response element (ARE) activation are dependent on the partial molar volume at position 151 of Keap1, Biochemical Journal 422(1) (2009) 171-180.

[41] C. Hu, A.L. Eggler, A.D. Mesecar, R.B. van Breemen, Modification of keap1 cysteine residues by sulforaphane, Chem Res Toxicol 24(4) (2011) 515-21.

[42] D.D. Zhang, M. Hannink, Distinct Cysteine Residues in Keap1 Are Required for Keap1-Dependent Ubiquitination of Nrf2 and for Stabilization of Nrf2 by Chemopreventive Agents and Oxidative Stress, Molecular and Cellular Biology 23(22) (2003) 8137-8151.

[43] P. Wang, C.G. Li, Z. Qi, D. Cui, S. Ding, Acute Exercise Induced Mitochondrial H2O2 Production in Mouse Skeletal Muscle: Association with p66Shc and FOXO3a Signaling and Antioxidant Enzymes, Oxidative Medicine and Cellular Longevity 2015 (2015) 10.

[44] T. Li, S. He, S. Liu, Z. Kong, J. Wang, Y. Zhang, Effects of different exercise durations on Keap1-Nrf2-ARE pathway activation in mouse skeletal muscle, Free Radic Res 49(10) (2015) 1269-74.

[45] A. Gallego-Selles, M. Martin-Rincon, M. Martinez-Canton, M. Perez-Valera, S. Martin-Rodriguez, M. Gelabert-Rebato, A. Santana, D. Morales-Alamo, C. Dorado, J.A.L. Calbet, Regulation of Nrf2/Keap1 signalling in human skeletal muscle during exercise to exhaustion in normoxia, severe acute hypoxia and post-exercise ischaemia: Influence of metabolite accumulation and oxygenation, Redox Biol 36 (2020) 101627.

[46] S.S. Gounder, S. Kannan, D. Devadoss, C.J. Miller, K.J. Whitehead, S.J. Odelberg, M.A. Firpo, R. Paine, 3rd, J.R. Hoidal, E.D. Abel, N.S. Rajasekaran, Impaired transcriptional activity of Nrf2 in age-related myocardial oxidative stress is reversible by moderate exercise training, PloS one 7(9) (2012) e45697.

[47] V.R. Muthusamy, S. Kannan, K. Sadhaasivam, S.S. Gounder, C.J. Davidson, C. Boeheme, J.R. Hoidal, L. Wang, N.S. Rajasekaran, Acute exercise stress activates

Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium, Free Radic Biol Med 52(2) (2012) 366-76.

[48] Y.H. Tsou, C.T. Shih, C.H. Ching, J.Y. Huang, C.J. Jen, L. Yu, Y.M. Kuo, F.S. Wu, J.I. Chuang, Treadmill exercise activates Nrf2 antioxidant system to protect the nigrostriatal dopaminergic neurons from MPP+ toxicity, Exp Neurol 263 (2015) 50-62.

[49] A.S. Aguiar, Jr., M. Duzzioni, A.P. Remor, F.S. Tristao, F.C. Matheus, R. Raisman-Vozari, A. Latini, R.D. Prediger, Moderate-Intensity Physical Exercise Protects Against Experimental 6-Hydroxydopamine-Induced Hemiparkinsonism Through Nrf2-Antioxidant Response Element Pathway, Neurochem Res 41(1-2) (2016) 64-72.

[50] H. Kubo, K. Asai, K. Kojima, A. Sugitani, Y. Kyomoto, A. Okamoto, K. Yamada, N. Ijiri, T. Watanabe, K. Hirata, T. Kawaguchi, Exercise Ameliorates Emphysema Of Cigarette Smoke-Induced COPD In Mice Through The Exercise-Irisin-Nrf2 Axis, Int J Chron Obstruct Pulmon Dis 14 (2019) 2507-2516.

[51] A.J. Done, M.J. Gage, N.C. Nieto, T. Traustadóttir, Exercise-induced Nrf2-signaling is impaired in aging, Free Radic Biol Med 96 (2016) 130-8.

[52] A.J. Done, M.J. Newell, T. Traustadóttir, Effect of exercise intensity on Nrf2 signalling in young men, Free Radic Res 51(6) (2017) 646-655.

[53] E.L. Ostrom, T. Traustadóttir, Aerobic exercise training partially reverses the impairment of Nrf2 activation in older humans, Free Radical Biology and Medicine 160 (2020) 418-432.

[54] M.J. Crilly, L.D. Tryon, A.T. Erlich, D.A. Hood, The role of Nrf2 in skeletal muscle contractile and mitochondrial function, Journal of applied physiology 121(3) (2016) 730-40.

[55] R.V. Musci, K.L. Hamilton, M.A. Linden, Exercise-Induced Mitohormesis for the Maintenance of Skeletal Muscle and Healthspan Extension, Sports (Basel) 7(7) (2019).

[56] L.C.D. Pomatto, K.J.A. Davies, The role of declining adaptive homeostasis in ageing, The Journal of Physiology 595(24) (2017) 7275-7309.

[57] L.C.D. Pomatto, K.J.A. Davies, Adaptive homeostasis and the free radical theory of ageing, Free Radical Biology and Medicine 124 (2018) 420-430.

[58] N.V. Margaritelis, A. Kyparos, V. Paschalis, A.A. Theodorou, G. Panayiotou, A. Zafeiridis, K. Dipla, M.G. Nikolaidis, I.S. Vrabas, Reductive stress after exercise: The issue of redox individuality, Redox biology 2 (2014) 520-528.

[59] N.V. Margaritelis, A.A. Theodorou, V. Paschalis, A.S. Veskoukis, K. Dipla, A. Zafeiridis, G. Panayiotou, I.S. Vrabas, A. Kyparos, M.G. Nikolaidis, Adaptations to endurance training depend on exercise-induced oxidative stress: exploiting redox interindividual variability, Acta Physiol (Oxf) 222(2) (2018).

[60] A.A. Theodorou, V. Paschalis, A. Kyparos, G. Panayiotou, M.G. Nikolaidis, Passive smoking reduces and vitamin C increases exercise-induced oxidative stress: does this make passive smoking an anti-oxidant and vitamin C a pro-oxidant stimulus?, Biochem Biophys Res Commun 454(1) (2014) 131-6.

[61] N.V. Margaritelis, V. Paschalis, A.A. Theodorou, A. Kyparos, M.G. Nikolaidis, Antioxidant supplementation, redox deficiencies and exercise performance: A falsification design, Free Radic Biol Med (2020).

[62] M.J. Jackson, On the mechanisms underlying attenuated redox responses to exercise in older individuals: A hypothesis, Free Radic Biol Med 161 (2020) 326-338.

[63] J. Duan, T. Zhang, M.J. Gaffrey, K.K. Weitz, R.J. Moore, X. Li, M. Xian, B.D. Thrall, W.-J. Qian, Stochiometric Quantification of the Thiol Redox Proteome of Macrophages Reveals Subcellular Compartmentalization and Susceptibility to Oxidative Perturbations, Redox Biology (2020) 101649.

[64] Y. Michailidis, A.Z. Jamurtas, M.G. Nikolaidis, I.G. Fatouros, Y. Koutedakis, I. Papassotiriou, D. Kouretas, Sampling time is crucial for measurement of aerobic exercise-induced oxidative stress, Med Sci Sports Exerc 39(7) (2007) 1107-13.

[65] S. Stocker, K. Van Laer, A. Mijuskovic, T.P. Dick, The Conundrum of Hydrogen Peroxide Signaling and the Emerging Role of Peroxiredoxins as Redox Relay Hubs, Antioxid Redox Signal 28(7) (2018) 558-573.

[66] Z.A. Wood, L.B. Poole, P.A. Karplus, Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling, Science 300(5619) (2003) 650-3.

[67] S. Hanzen, K. Vielfort, J. Yang, F. Roger, V. Andersson, S. Zamarbide-Fores, R. Andersson, L. Malm, G. Palais, B. Biteau, B. Liu, M.B. Toledano, M. Molin, T. Nystrom, Lifespan Control by Redox-Dependent Recruitment of Chaperones to Misfolded Proteins, Cell 166(1) (2016) 140-51.

[68] M.C. Sobotta, W. Liou, S. Stöcker, D. Talwar, M. Oehler, T. Ruppert, A.N. Scharf, T.P. Dick, Peroxiredoxin-2 and STAT3 form a redox relay for H2O2 signaling, Nat Chem Biol 11(1) (2015) 64-70.

[69] R.M. Jarvis, S.M. Hughes, E.C. Ledgerwood, Peroxiredoxin 1 functions as a signal peroxidase to receive, transduce, and transmit peroxide signals in mammalian cells, Free Radical Biology and Medicine 53(7) (2012) 1522-1530.

[70] L. Parker, C.S. Shaw, N.K. Stepto, I. Levinger, Exercise and Glycemic Control: Focus on Redox Homeostasis and Redox-Sensitive Protein Signaling, Frontiers in Endocrinology 8(87) (2017).

[71] D.A. Moghaddam, A. Heber, D. Capin, T. Kreutz, D. Opitz, E. Lenzen, W. Bloch, K. Brixius, C. Brinkmann, Training increases peroxiredoxin 2 contents in the erythrocytes of overweight/obese men suffering from type 2 diabetes, Wien Med Wochenschr 161(21-22) (2011) 511-8.

[72] A.J. Wadley, S. Aldred, S.J. Coles, An unexplored role for Peroxiredoxin in exercise-induced redox signalling?, Redox Biol 8 (2016) 51-8.

[73] P.A. Kramer, J. Duan, M.J. Gaffrey, A.K. Shukla, L. Wang, T.K. Bammler, W.J. Qian, D.J. Marcinek, Fatiguing contractions increase protein S-glutathionylation occupancy in mouse skeletal muscle, Redox Biol 17 (2018) 367-376.

[74] R.J. Mailloux, Protein S-glutathionylation reactions as a global inhibitor of cell metabolism for the desensitization of hydrogen peroxide signals, Redox Biol 32 (2020) 101472.

[75] R.M. Gill, M. O'Brien, A. Young, D. Gardiner, R.J. Mailloux, Protein S-glutathionylation lowers superoxide/hydrogen peroxide release from skeletal muscle mitochondria through modification of complex I and inhibition of pyruvate uptake, PLOS ONE 13(2) (2018) e0192801.

[76] A.N. Carvalho, C. Marques, R.C. Guedes, M. Castro-Caldas, E. Rodrigues, J. van Horssen, M.J. Gama, S-Glutathionylation of Keap1: a new role for glutathione S-transferase pi in neuronal protection, FEBS Letters 590(10) (2016) 1455-1466.

[77] J.N. Cobley, A. Noble, E. Jimenez-Fernandez, M.T. Valdivia Moya, M. Guille, H. Husi, Catalyst-free Click PEGylation reveals substantial mitochondrial ATP synthase sub-unit alpha oxidation before and after fertilisation, Redox Biol 26 (2019) 101258.

[78] J.N. Cobley, G.K. Sakellariou, H. Husi, B. McDonagh, Proteomic strategies to unravel age-related redox signalling defects in skeletal muscle, Free Radical Biology and Medicine 132 (2019) 24-32.

[79] J.N. Cobley, H. Husi, Immunological Techniques to Assess Protein Thiol Redox State: Opportunities, Challenges and Solutions, Antioxidants (Basel, Switzerland) 9(4) (2020) 315.