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Nuclear factor erythroid 2 related factor 2 (NrF2), is an essential transcription factor and a master regulator of the antioxidant defense system, which increases antioxidants in response to the production of reactive oxygen and nitrogen species (RONS). Disproportional increases in RONS compared to antioxidant defense capabilities can induce a state of oxidative stress (OS). Aerobic exercise of sufficient intensity and duration is well-known to increase the production of RONS in the blood, skeletal muscle and brain. Chronic aerobic exercise has been shown to mediate OS through increases in antioxidant factors. However, there are only two studies that have reported changes in NrF2 in response to aerobic exercise in a healthy cohort, but were limited to chronic exercise and examined the striatum brain region and whole brain only.

The purpose of this study was to 1) determine the extent to which markers of OS (MDA, GSSG, GSSG/TGSH) change with acute aerobic exercise in the blood, skeletal muscle and brain, 2) determine the extent to which antioxidant defense factors (GSH, TGSH, Mn-SOD) change with chronic aerobic exercise in skeletal muscle and brain, and 3) determine the extent to which NrF2 protein concentration changes with acute and chronic aerobic exercise in skeletal muscle and specific brain regions (cerebral cortex, hippocampus, and cerebellum).

To accomplish these objectives, three groups of rats (n=6-13/group), sedentary (SD), acute exercise (AE) and chronic exercise (ET) (5-7 weeks), ran at an intensity equal to 75% VO_{2max} or served as controls. Gastrocnemius skeletal muscle and cerebral cortex,

hippocampus, and cerebellum brain regions were analyzed using multiple methods to examine markers of OS, antioxidant factors and NrF2 protein concentration.

AE significantly increased MDA concentration in the blood (~69%) and the hippocampus brain region (~36%). However, the OS response did not reflect a significant increase in NrF2 protein concentration with AE in the brain or skeletal muscle. In fact, NrF2 was significantly reduced in all brain regions and muscle with AE compared to SD. ET significantly increased GSH (~27%) and TGSH (~26%) in the hippocampus brain regions, which was concomitant with a significant increase in NrF2 protein concentration in the hippocampus with ET. In contrast, GSH, TGSH, and Mn-SOD (~44%) were significantly reduced in the in gastrocnemius skeletal muscle with ET. There were no significant differences in antioxidant factors in the cortex or cerebellum brain regions, which coincided with the lack of significant elevation in NrF2 with training.

In summary, these data suggest that ET for 5-7 weeks at 75% VO_{2max} was sufficient to increase NrF2 protein concentration and antioxidant factors in the hippocampus, a brain region that is highly susceptible to neurodegeneration. However, the single bout of acute aerobic exercise appears to be inadequate to sufficiently stress the brain and skeletal muscle tissue to increase markers of OS or NrF2 protein concentration.

ACUTE AND CHRONIC EXERCISE EFFECTS ON NrF2 AND ANTIOXIDANTS IN THE MUSCLE AND BRAIN TISSUE OF SPRAGUE DAWLEY RATS

by

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Approved by

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APPROVAL PAGE

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If we knew what it was we were doing, it would not be called research, would it?

~Albert Einstein

After 10.5 years of motivated, focused education, I realize that I am down to my last few weeks as a “student”. This process has been a long and difficult journey, especially the PhD. There were many times that I wanted to quit and maybe I should have quit for my own sanity, but here I am. I have always been the never give up, “quitting” isn’t in my vocabulary, follow your heart, make it to the finish line no matter what, kind of girl. And even with my fiercely independent character, I would not have made it this far without the help and guidance of my mentors and friends.

First, I would like to thank my sister, Sarah. You have been a constant source of support and a willing participant to listen to my tragic tales of life as a PhD lab rat. Thank you for your patience and allowing me to vent, all the time! I would also like to thank my friend and mentor Matt Laurent. I would not have made it this far without all your support and encouragement. I often reflect on your words of advice; “You are capable of doing anything you want, but you have to ask yourself one question, how bad do you want it?”

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CHAPTER I

INTRODUCTION

Aerobic exercise is well recognized as a proactive solution to reduce the incidence of chronic disease such as cancer, stroke, cardiovascular disease, heart disease, and type 2 diabetes (Haskell 2007) as well as to improve mood and endorphin release (Farooqui 2014). Recently, it has been suggested that exercise may also play a role in neuroprotection and prevention of central related disorders such as dementia and Alzheimer's disease (Eldar 2000; Cotman 2000; Smith 2003). Exercise has been demonstrated to increase the production and/or activity of cellular antioxidants and cytoprotective enzymes (Li 2015; Aguiar 2010), thereby attenuating the production of reactive oxygen and nitrogen species (RONS) (Fig.1). RONS formation occurs as part of normal aerobic metabolism and is well known to increase during aerobic exercise, but is

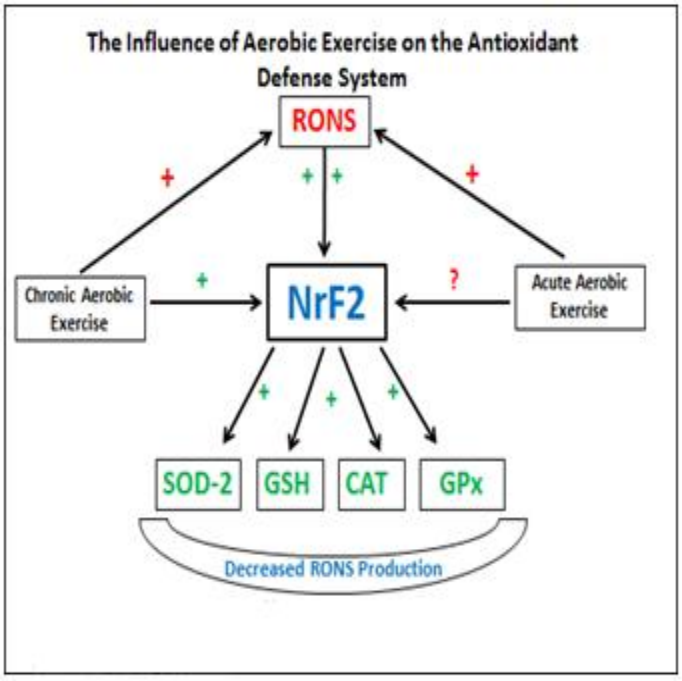

Figure 1. Theoretical Model.

quickly mitigated by cellular antioxidants (Powers 2008). However, if RONS accumulate disproportionally compared to antioxidant defense capabilities, peroxidation of lipids, DNA, and proteins commonly occurs, inducing a state of oxidative stress (OS) (Bloomer 2005). Nuclear factor erythroid 2 related factor 2 (NrF2), promotes the increase of cellular antioxidant production to effectively neutralize RONS.

NrF2 is an essential transcription factor and a master regulator of the antioxidant defense system aiding in cellular defense, protection, survival, and redox balance (Fig. 1) (Pall 2015). Existing in a wide variety of cells, including neural cells, NrF2 is tightly bound in the cytosol by kelch-like ECH associated protein 1 (KEAP1). In eustress conditions, NrF2 concentration is maintained through proteosomal ubiquitination regulated by KEAP1 (Surh2008). Upon activation by proteasome inhibitors, RONS or other stress agents, NrF2 dissociates from KEAP1, stabilizing in the cytosol, followed by translocation to the nucleus (Surh 2008). Nuclear localized NrF2 binds to the antioxidant response element (ARE) and promotes the transcription of numerous antioxidants like glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx) (Sandburg 2014).

The brain is a highly aerobic organ and compared to tissues like skeletal muscle, is extremely sensitive to changes in oxygen content, increasing the susceptibility to OS. RONS induced OS has been implicated in numerous disease states including neurodegeneration and activation of NrF2 is critical to maintain redox balance and brain homeostasis (Tsou 2015; Aguiar 2016). Aerobic exercise has been demonstrated to increase RONS production (Bloomer 2005) and activate NrF2 in skeletal muscle, cardiac

muscle and brain (Donato 2014; Muthusamay 2012; Radak 2001). However, there are few studies that have reported changes in NrF2 in response to exercise and are limited to peripheral regions of the body. NrF2 activation has been reported in the brain with chronic exercise, but with respect to the striatum and whole brain only (Moiron2015; Tsou 2015). Moreover, NrF2 concentration with chronic exercise in brain regions that are susceptible to OS, the cortex, cerebellum, and hippocampus, has not been studied. Further still, it is unclear if changes in the brain are associated with chronic exercise or are occurring as a result of the last bout of exercise. In addition, there are no studies that have compared the extent to which NrF2 changes between brain and active tissues like skeletal muscle and to date there are no studies reporting the acute effects of exercise on NrF2 in the brain.

Therefore, the current objectives of this study are 1) to determine the extent to which NrF2 changes with acute and chronic aerobic exercise in skeletal muscle and brain 2) to determine the extent to which antioxidant defense factors (glutathione, SOD) and markers of OS change with acute and chronic aerobic exercise in skeletal muscle and brain, and 3) to compare the extent to which NrF2, antioxidant factors, and markers of OS change with acute and chronic exercise, between skeletal muscle and individual brain regions. To accomplish these objectives, three groups of rats (n=6-13/group), sedentary (SD), acute exercise (AE) and exercise trained (ET) (5-7 weeks), will be compared to determine changes in NrF2 and antioxidant factors in gastrocnemius skeletal muscle and cerebral cortex, hippocampus, and cerebellum brain regions.

Purpose

The purpose of this study was to examine changes in NrF-2, antioxidant defense factors, and markers of OS within skeletal muscle and specific brain regions with acute and chronic aerobic exercise.

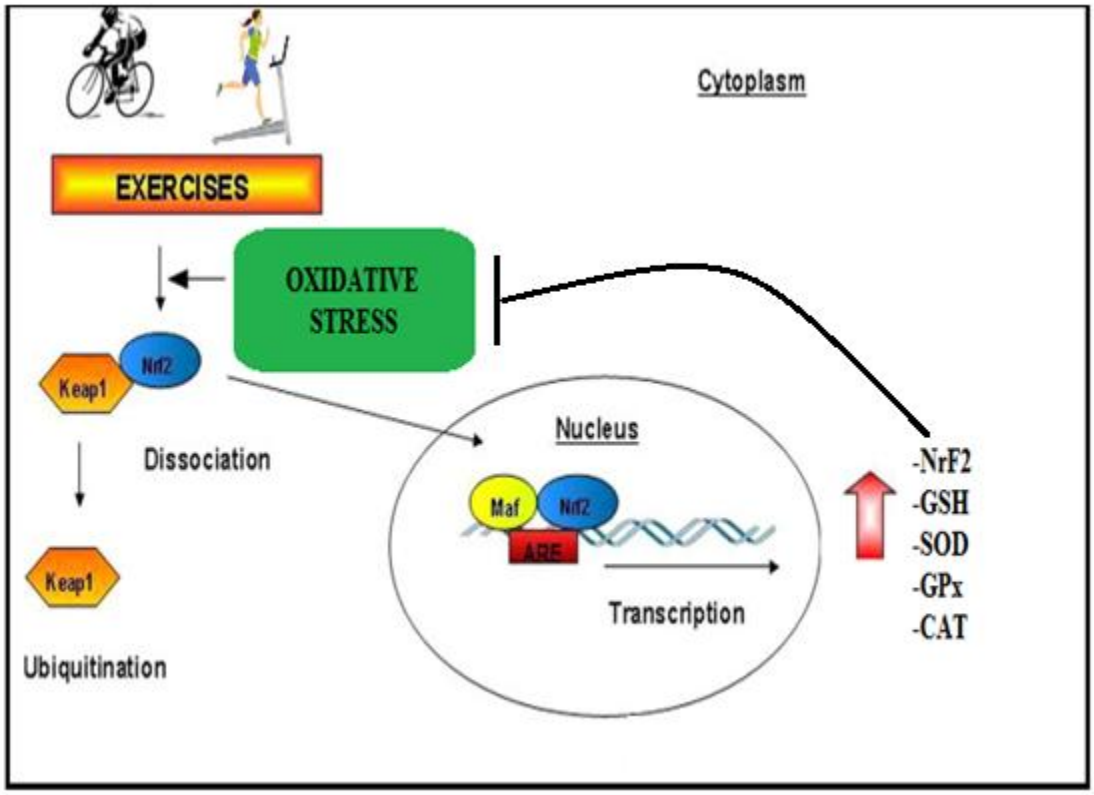

Figure 2. Proposed Model. Exercise Induced NrF2 Activation. Adapted from Abreu et al. 2015.

Specific Aims

Specific Aim #1: To determine the effect of a single bout of acute aerobic exercise on NrF2 and markers of oxidative stress in the brain, skeletal muscle, and blood and to examine the relationship between skeletal muscle and each brain region.

- Hypothesis 1: A single bout of acute aerobic exercise (AE) will be significantly different when compared to sedentary controls (SD).
    - H1A: The cortex brain region:
        - AE NrF2 protein concentration = SD NrF2 protein concentration.
        - AE MDA concentration > SD MDA concentration.
        - AE GSSG concentration > SD GSSG concentration.
        - AE GSSG/TGSH ratio > SD GSSG/TGSH ratio.
    - H1B: The cerebellum brain region:
        - AE NrF2 protein concentration = SD NrF2 protein concentration.
        - AE MDA concentration > SD MDA concentration.
        - AE GSSG concentration > SD GSSG concentration.
        - AE GSSG/TGSH ratio > SD GSSG/TGSH ratio.
    - H1C: The hippocampus brain region:
        - AE NrF2 protein concentration = SD NrF2 protein concentration.
        - AE MDA concentration > SD MDA concentration.
        - AE GSSG concentration > SD GSSG concentration.
        - AE GSSG/TGSH ratio > SD GSSG/TGSH ratio.

- H1D: The blood:
    - AE MDA concentration > SD MDA concentration.
    - AE GSSG concentration > SD GSSG concentration.
    - AE GSSG/TGSH ratio > SD GSSG/TGSH ratio.
- H1E: The gastrocnemius muscle:
    - AE NrF2 protein concentration = SD NrF2 protein concentration.
    - AE MDA concentration > SD MDA concentration.
    - AE GSSG concentration > SD GSSG concentration.
    - AE GSSG/TGSH ratio > SD GSSG/TGSH ratio.

- Hypothesis 2: The relationship between skeletal muscle and each brain region with respect to a single bout of acute aerobic exercise (AE) will be significantly correlated.
    - Muscle NrF2 concentration > Brain NrF2 concentration.
    - Muscle MDA concentration > Brain MDA concentration.
    - Muscle GSSG concentration > Brain GSSG concentration.
    - Muscle GSSG/TGSH > Brain GSSG/TGSH.

Specific Aim #2: To determine the effect of chronic aerobic exercise on NrF2 and antioxidant factors in the brain and skeletal muscle and to examine the relationship between skeletal muscle and each brain region.

- Hypothesis 1: Chronic aerobic exercise (ET) will be significantly different when compared to sedentary controls (SD).
    - H1A: The cortex brain region:

        - ET NrF2 protein concentration > SD NrF2 protein concentration.
        - ET glutathione concentration > SD glutathione concentration.
        - ET MnSOD activity > SD MnSOD activity.
    - H1B: The cerebellum brain region:
        - ET NrF2 protein concentration > SD NrF2 protein concentration.
        - ET glutathione concentration > SD glutathione concentration.
        - ET MnSOD activity > SD MnSOD activity.
    - H1C: The hippocampus brain region:
        - ET NrF2 protein concentration > SD NrF2 protein concentration.
        - ET glutathione concentration > SD glutathione concentration.
        - ET MnSOD activity > SD MnSOD activity.
    - H1D: The gastrocnemius muscle:
        - ET NrF2 protein concentration > SD NrF2 protein concentration.
        - ET glutathione concentration > SD glutathione concentration.
        - ET MnSOD activity > SD MnSOD activity.
- Hypothesis 2: The relationship between skeletal muscle and each brain region with respect to chronic aerobic exercise (ET) will be significantly correlated.
    - Muscle NrF2 concentration > Brain NrF2 concentration.
    - Muscle glutathione concentration > Brain glutathione concentration.
    - Muscle MnSOD activity > Brain MnSOD activity.

Significance

Regular physical activity has been shown to be beneficial not only systemically, but centrally through increases in neuronal plasticity and improvement in mental function (Farooqui 2014).The extent of these exercise benefits is highly dependent on the level of training and the duration and intensity of exercise (Aguiar 2010). Moderate to vigorous exercise maintained at or above 70% VO_{2max} has been demonstrated to increase markers of oxidative stress (OS) in the blood and skeletal muscle (Bloomer 2004). OS is characterized as a disproportional increase and accumulation in reactive oxygen and nitrogen species (RONS) compared to antioxidant defense capabilities. The increase in RONS is a key mediator of the antioxidant defense system (Fig. 1). Nuclear erythroid 2 related factor 2 (NrF2) is the essential transcription factor and a master regulator of the antioxidant defense system aiding in cellular defense and survival against toxins and RONS (Jaramillo 2013). NrF2 activation promotes the up regulation of antioxidant factors such as glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which are also well known to increase in the blood and skeletal muscle with acute and chronic aerobic exercise (Powers 2008).

NrF2 is bound by Kelch-like ECH associated protein 1 (KEAP1) in the cytoplasm in low-stress conditions (Regoli 2014). Under stress, KEAP1 dissociates from NrF2 enabling translocation to the nucleus to activate the antioxidant response element (ARE), inducing transcription and production of antioxidant factors (Regoli 2014). In addition, NrF2 has been shown to increase antioxidant capacity and suggested to increase the expression of numerous protective proteins like BDNF, anti-inflammatory cytokines, and

mitochondrial transcription factors (Reviewed in Sandburg et al. 2014). Therefore, increasing NrF2 in combination with the known benefits of habitual exercise on the brain provides insights to a potential mechanism of neuroprotection.

There is a plethora of researchexaminingNrF2 regulation in a wide variety of tissues in regards to antioxidant defense capabilities to maintain redox balance. NrF2 has been suggested to be a primary factor in neuroprotection due its vast distribution within the CNS. Multiple studies have demonstrated that NrF2 deficiency exacerbates neuronal injury and CNS dysregulation by increasing brain vulnerability to neurotoxins and OS (Reviewed in Sandburg et al. 2014). Aging has also been reported to reduce translocation of NrF2 to the nucleus preventing antioxidant gene expression (Done 2016). With age and genetic anomaly's to credit for deficiencies in NrF2 concentration, it is imperative that a way to maintain this innate defense system is discovered. Acute and chronic aerobic exercise offers a noninvasive solution to maintain or enhance NrF2 and antioxidant factors in skeletal muscle and heart tissue (Wang 2016; Laughlin 1990; Strobel 2011; Muthusamy 2012); however, there is limited research available detailing the effects of aerobic exercise on NrF2 and antioxidants in the brain (Moiron 2015).

Our contribution is to determine the extent to which NrF2 and antioxidants (GSH, SOD) change with acute and chronic aerobic exercise in specific brain regions that are susceptible to OS: cortex, cerebellum, and hippocampus, compared to skeletal muscle. This is significant because NrF2 is activated under stress conditions to increase antioxidant factor production and reduce OS. RONS have been demonstrated as a potent stimulator of the antioxidant defense system and if OS is not properly regulated, long

term, may be a hallmark indicator of neurodegeneration. Therefore, exercise may provide a non-pharmacological stimulus to activate and maintain the NrF2 pathway in the brain leading to enhanced neuroprotection and attenuation of RONS and OS.

Innovation

The role of NrF2 in the antioxidant defense system has been commonly studied in cardiac muscle, skeletal muscle and brain in regards to exercise, health, and chronic disease. However, in brain tissue, studies are limited by specific brain regions of interest and measurement of associated antioxidant factors. Currently, the extent to which NrF2 changes with acute and chronic aerobic exercise in brain regions susceptible to OS induced neurodegeneration: cortex, cerebellum, hippocampus, remains unclear. The proposed study is innovative because it will be the first study to compare changes in NrF2 protein concentration and antioxidant defense factors in brain and skeletal muscle tissue in response to both acute and chronic aerobic exercise. OS is a hallmark indicator of a variety of diseases, including neurodegeneration, and exercise may provide the necessary stimulus to activate and maintain the NrF2 pathway to promote neuroprotection.

In this study, a combination of western blot and high pressure liquid chromatography (HPLC) with electrochemical detection will be used to examine factors within the antioxidant defense system. The latter will provide greater sensitivity and detection above and beyond the current methods used in the recent literature. Together, both methods will serve to identify critical areas of NrF2 and antioxidant activation in the biological pathway that has not been currently shown. This innovative research will set

the stage to 1) expand our understanding of the physiological effects of acute and chronic aerobic exercise on the NrF2 pathway in these brain regions, 2) to determine if aerobic exercise enhances antioxidant defense factors with training, and 3) to evaluate a potential neuroprotective mechanism in the central nervous system that occurs with a non-pharmacological treatment. Effective managerial treatments providing neuroprotection against OS linked diseases, neurodegeneration, and neurological disorders are warranted to protect quality of life.

Limitations

1. A five hour delay post the final bout of chronic exercise was used; however, this time frame may be too long to detect changes in NrF2 and may not be long enough to detect transcription of antioxidant defense factors.
2. Exercise may not affect these specific brain regions studied.
3. Other brain regions may also be susceptible to changes in NrF2.

Delimitations

1. This study implemented a non-pharmacological treatment that has well-known health benefits to identify changes in an essential redox sensitive transcription factor, NrF2.
2. Young male Sprague Dawley Rats of similar age, body weight, and dietary intake were all controlled for to limit variability of scientific measures.
3. Multiple brain regions will be examined post, acute or chronic, exercise.
4. High Pressure Liquid Chromatography (HPLC) will be used, providing a more sensitive measure of glutathione in both the oxidized and reduced forms.

Term Definitions

- Akt – A serine/threonine specific protein kinase, also known as PKB, that regulates multiple cellular processes related to cell growth and development.
- Antioxidants– A substance that inhibits oxidation and removes potentially damaging oxidizing agents.
- Autophagy – A natural regulated process that occurs to disassemble and destroy unnecessary or dysfunctional cellular components.
- ARE – Antioxidant Response Element. A cis-acting enhancer sequence located in regulatory regions of antioxidant genes.
- Autophagosome – Formed during the elongation process of a phagosome when portions of the cytoplasm are isolated.
- Autophagolysosome – A digestive vacuole formed from the fusion of a lysosome with a phagophore during the process of autophagy.
- BHA – Butylated hydroxyanisole. A well-known food preservative, stabilizer and antioxidant.
- BDNF – Brain Derived Neurotrophic Factor. A growth factor, part of the neurotrophin family, found in the brain and periphery, which stimulates nerve growth.
- BTB –Broad complex, Tramtrack and Bric-à-Brac.A primary interaction domain located on the N-terminal of KEAP1 that binds with and E3 ubiquitin ligase through the Rbx-1 and cullin-3 complex.

- bZIP Domain –Basic Leucine Zipper. A domain found in the DNA binding region of eukaryotic proteins.
- CAT – Catalase. A major cellular enzyme that provides cellular protection from hydrogen peroxide by converting it to water and oxygen.
- CNC – Cap 'in' collar. Family of leucine zipper transcription factors that regulate gene expression of various proteins and enzymes.
- Cu^{2+} – Ion Cupric Ion. Copper found in the +2 oxidation state.
- Cytoprotective agents – Chemical compounds that provide cellular protection agents potentially damaging agents.
- CTR – C-Terminal Region. KEAP1 domain that interacts with the DGR tethered to the Neh2 region of NrF2.
- Cullin-3 Complex – A protein subunit associated with E3 ligases with the ubiquitin-proteasome system.
- DGR – Double Glycine Repeat. KEAP1 domain that interacts with the CTR tethered to the Neh2 region of NrF2.
- DLG –A motif within the Neh2 region of NrF2 that tethers to KEAP1 as a low affinity latch site to lock and unlock NrF2 for ubiquitination.
- ETGE – A high affinity hinge motif within the Neh2 region of NrF2 that remains attached to KEAP1 in the event of stress.
- Electrophile – A molecule or ion with a vacant orbital, either positively charged or neutral, that is attracted to electrons to form a nucleophile.

- ERK – Extracellular Signal-Related Kinase. This kinase is part of a chain of proteins in the MAPK pathway that communicates signals from the receptor to the DNA in the cell nucleus.
- ETC. Electron Transport Chain. A series of compounds, located in the mitochondrion, that transport electrons from donors to acceptors.
- E3 Ligases – Enzymes that catalyze the formation of a new chemical bond.
- F-2 Isoprostanes – Prostaglandin like compounds formed from free radical oxidation. Isoprostanes are the gold standard measure of lipid oxidation.
- Fe^{2+} – Ferrous Iron. Iron in the +2 oxidation state.
- GABA – Gamma-aminobutyric Acid. A major inhibitory neurotransmitter in the central nervous system.
- GPx – Glutathione Peroxidase. A selenium dependent enzyme, with multiple isoforms located in both the cytosol and mitochondria that catalyzes the reaction of lipid hydroperoxide and hydrogen peroxide to alcohol and water, respectively.
- GR – Glutathione Reductase. An enzyme that catalyzes the reaction of cellular GSSG to GSH.
- GSH/GSSG/TGSH – Glutathione. A tripeptide (L-ɣ-glutamyl-L-cysteinyl-glycine) containing thiol that serves as the major endogenous antioxidant of the body.
- GST – Glutathione – S – transferase. A major phase II detoxification enzyme found in the cytosol of cells.

- GS3Kβ – Glycogen synthase kinase 3 beta. A serine/threonine protein kinase that mediates the phosphorylation of proteins.
- HO-1 – Heme Oxygenase. A molecular responder to oxidative stress.
- Hormesis – A concept that engages various levels of exposure to stressto improve health or overall stress tolerance, where on either side of the continuum (too much or too little stress) leads to chronic disease or dysfunction.
- HPLC –High Pressure Liquid Chromatography. An analytical chemistry technique that utilizes high pressure to elute a sample through a packed column to separate and quantify each component.
- H_2O_2 – Hydrogen Peroxide. A chemical compound produced from the neutralization of superoxide that is a well-known free radical.
- IKK complex – I Kappa Kappa. An element of the NF_KB cascade that incorporates the IKKα and IKKβ subunits.
- IkB – I kappa beta. A regulatory protein that serves as an inhibitor of NF-kB nuclear localization.
- IKKβ – Inhibitor of nuclear factor kappa-B kinase subunit beta. An enzymatic subunit of IkB kinase, when activated phosphorylates IkB, activating NF-kB for nuclear localization.
- IRV – Intervening Domain. A domain on KEAP1 linked to various cysteine residues that are important for repression of NrF2 and stress sensing.
- JNK – c-Jun N-terminal Kinases. A protein kinase that belongs to the MAPK family that is responsible for binding and phosphorylating c-Jun.

- KEAP1 –Kelch-like ECH Associated Protein 1. A substrate adaptor protein that regulates the cellular transcription factor NrF2.
- LOOH – Lipid Hydroperoxide. A factor produced during lipid peroxidation and a common marker of oxidative stress.
- Maf Proteins – Serve as cellular transcription factors, commonly associated with NrF2 activation of the antioxidant response element.
- MAPK – Mitogen Activated Protein Kinases. A family of serine/threonine protein kinases that serve multiple cellular processes (motility, apoptosis, survival, etc.) that include extracellular signal regulated kinases such as: p38, ERK, JNK, and c-jun.
- MDA – Malondialdehyde. A marker of lipid peroxidation related to oxidative stress.
- MG132 – A cell permeable proteasome inhibitor that reduces the rate of ubiquitination and protein degradation.
- Mn^{+} – Manganese. A chemical element that serves to form agents that protect the cell from stress agents and disease.
- MPP toxicity –1-Methyl-4-phenylpyridinium. A toxic agent that selectively targets dopaminergic neurons and has been used to study Parkinson's disease.
- mTOR – Mechanistic Target of Rapamycin. A serine/threonine protein kinase, part of the PI3K protein family, responsible for cell growth and development.

- NADPH Oxidases – Nicotinamide Adenine Dinucleotide Phosphate. Produced from phagocytic cells and are key contributors to hydrogen peroxide accumulation.
- Neh domain – N terminal regulatory domain located on NrF2 that includes 6 different isoforms (Neh 1-6).
- NFE2L2 – NrF2 gene responsible for transcription of NrF2 protein in the cell.
- NF-kB – Nuclear factor kappa-light-chain-enhancer of activated B cells. A protein complex regulating gene transcription of cytokines and promoting cell survival.
- NrF2 - Nuclear factor erythroid 2 related factor 2. A master transcription factor, regulating gene expression of antioxidants that aid in cellular defense, protection, survival, and redox balance.
- NO – Nitric Oxide. A nitrogen based chemical compound synthesized from arginine that can freely diffuse across cells and is a commonly known free radical.
- OS - Oxidative Stress. A condition where reactive oxygen species accumulate disproportionally compared to antioxidant defense capabilities.
- PC – Protein Carbonyls. A marker of protein oxidation related to oxidative stress.
- Phagophore – An isolating membrane formed during autophagy containing fragments of cellular components.
- PKB – Protein Kinase B. A serine/threonine protein kinase, also known as Akt, that regulates multiple cellular processes related to cell growth and development.

- PKC – Protein Kinase C. A family of protein kinase enzymes that mediate the phosphorylation of proteins.
- Pro-oxidant – Chemicals that induce oxidative stress by generating reactive oxygen species or by inhibiting antioxidants.
- p62 – Also known as Sequestrosome-1, p62 is a protein that engages in the process of autophagy by recognizing cellular waste products.
- Redox Balance – A state of equilibrium achieved through cellular oxidation-reduction reactions.
- RONS – Reactive Oxygen and Nitrogen Species. An array of potentially damaging, chemically reactive molecules, produced in the mitochondria or in the cytosol, containing an unpaired electron.
- RT-qPCR – Real Time – Quantitative Polymerase Chain Reaction. A method used to detect messenger RNA expression.
- SERCA –Sarco-endoplasmic Reticulum Calcium ATPase. A cellular pump that transports calcium ions from the cytoplasm back into the SR.
- SFN – Sulforaphane. A naturally occurring isothiocyanato with known antioxidant properties.
- SOD – Super Oxide Dismutase. An antioxidant responsible for the reduction of super oxide anions to hydrogen peroxide. Two forms are present within the cell, a mitochondrial form (Manganese SOD; MnSOD) and a cytosolic form (Copper-Zinc; Cu-Zn-SOD).

- Src Tyrosin Kinases – A family of kinases located on the C-terminal that include: Fyn, Src, Yes, Fgr.
- Superoxide – A reactive oxygen anion produced in the mitochondria during aerobic cellular metabolism.
- TBARS – Thiobarbituric Acid Reactive Substances. A byproduct formed from lipid peroxidation.
- tBHQ – tert-Butylhydroquinone. A phenolic compound with antioxidant properties that is well-known to prevent ubiquitination of NrF2.
- Transcription – The initial step in the gene expression pathway in which DNA is copied into RNA.
- Translation – Process in which RNA, copied from DNA, is decoded by ribosomes to synthesize a new protein or polypeptide.
- Ubiquitination – An enzymatic process that regulates the degradation of cellular proteins through the ubiquitin-proteasome system.
- XO – Xanthine Oxidase. A cellular enzyme that generates reactive oxygen species.

CHAPTER II

REVIEW OF LITERATURE

The Antioxidant Defense System

On a recurrent basis, the cells of the body are exposed to both exogenous and endogenous agents that are known to cause stress and if chronically exposed can lead to disease and/or dysfunction. In eustress conditions, these agents allow the body to adapt and overcome the stress, while in distress conditions, this can lead to a weakened immune response and futile efforts to combat infection and maintain homeostasis. Aerobic exercise is well known to reduce the risk of chronic diseases and has been documented on numerous occasions to maintain or enhance general health and wellness as well as quality of life (Haskell 2007). In addition, the body is equipped with its own innate defense system to control and counteract stress agents. Nuclear factor erythroid 2 related factor 2 (NrF2) is a basic leucine zipper protein in the cap'in'collar family (CNC), responsible for coordinating the response of multiple factors that provide protection against stress agents. NrF2 is most commonly known for its role as a transcription factor, activating genes for transcription of phase II protective proteins, antioxidants, and cytoprotective enzymes (Pall 2015; Surh 2008).

As an essential transcription factor and a master regulator of the antioxidant defense system, NrF2 aids in cellular defense and survival against toxins, various stress agents, and most notably, reactive oxygen and nitrogen species (RONS) (Jaramillo2013).

RONS are commonly produced as natural byproducts of aerobic metabolism, which are thought to be insignificant in eustress conditions as they are effectively neutralized by various antioxidants. However, in situations of disease or distress, RONS accumulate above and beyond antioxidant capacity, challenging redox balance and inducing a state of oxidative stress (OS). As a redox sensitive factor, NrF2 confronts the stress through initiation of antioxidant gene expression to correct for the imbalance between RONS production and antioxidant capacity (Singh 2014). RONS will be discussed in extensive detail later on, but due to their role in the NrF2 pathway merited notation.

Under eustress conditions, NrF2 resides in the cytosol bound to Kelch-like ECH associated protein 1 (KEAP1) (Surh 2008). Under stress, KEAP1 unlocks and dissociates from NrF2. Once dissociated, NrF2 stabilizes in the cytosol and prepares for translocation to the nucleus to activate the antioxidant response element (ARE), inducing gene transcription of protective proteins and cytoprotective enzymes (Fig. 2) (Surh 2008). Within the nucleus, other proteins such as Maf and JNK have been suggested to assist in NrF2 activation to the ARE and/or repression of other proteins (Katsuoka 2005; Sandburg 2014). Once redox balance has been achieved, NrF2 activity is repressed and exported back to the cytoplasm for ubiquitination. The premise behind the NrF2 system is to keep NrF2 concentration low under basal conditions with the potential for rapid activation in states of OS (Sandburg 2014).

NrF2 and KEAP1

The covalent interaction between NrF2 and KEAP1 is highly complex and dependent on the conditions within and surrounding the cell. NrF2 is comprised of a three

domain structure where each domain regulates the level of interaction with other proteins. The N-terminal is the regulatory domain termed Neh2 that bindsKEAP1 (Itoh 1999); the C-terminal contains a bZIP domain for heterodimerization with various Maf proteins in the nucleus, and a central domain allows for transactivation of antioxidant genes (Lo 2006). As an adaptor protein KEAP1 functions to maintain constant levels of NrF2 to regulate redox balance, but can dissociate in conditions of OS. KEAP1 forms a homodimer that retains four binding domains termed BTB (Broad complex, Tramtrack and Bric-à-Brac) (N-terminal domain), IVR (intervening domain), DGR (double glycine repeat domain), and CTR (C-terminal region) (Ogura 2010).The BTB domain is responsible for KEAP1 homodimerization and suppression of NrF2 (Zipper 2002). The DGR and CTR interact with the Neh2 domain on NrF2 tethering it in the cytosol bound to KEAP1 (Ogura 2010). Additionally, the BTB is bound to an E3 ubiquitin ligase through the cullin-3 complex (Sandburg 2014) and together with the IVR are linked to various cysteine residues that are important for repression of NrF2 and redox sensing (Ogura 2010).

KEAP1 forms a dimer to recognize two DGR, CTR binding motifs located on the Neh2 domain of NrF2, the ETGE (AFFAQLQLDE<u>ETGE</u>FL) and the DLG (ILWRQDI<u>DLG</u>VSREV) (Fig. 3) (Canning 2015; Tong 2006). Each motif binds with different affinities known as the “hinge” ETGE (high affinity) and the “latch” DLG (low affinity) (Bryan 2013). Just as the physics of these structures suggest, the ETGE hinge allows subtle movement in NrF2, while the DLG latch is restrictive to movement impeding NrF2 release and positioning it for ubiquitination. However, the DLG latch is

rather susceptible to modification via stress. Once unlatched, NrF2 is released and the ubiquitination complex is disrupted, stabilizing NrF2 in the cytosol (Bryan 2013).

Under eustress conditions, the DLG latch and the ETGE hinge serve as structural mediators between NrF2 and KEAP1 for ubiquitination and degradation of NrF2. Furthermore, unbound NrF2 may translocate to the nucleus and activate the NrF2 gene (NFE2L2) producing more NrF2 protein (Bryan 2013). However, KEAP1 imposes negative feedback to maintain cellular NrF2 protein levels and the current redox state through ubiquitination. Under stress, feedback by KEAP1 is inhibited allowing NrF2 to accumulate in the cell. Elevated protein concentration of NrF2 through release by KEAP1 and by NFE2L2 enhances the post translational production of cytoprotective agents, which may improve antioxidant capacity.

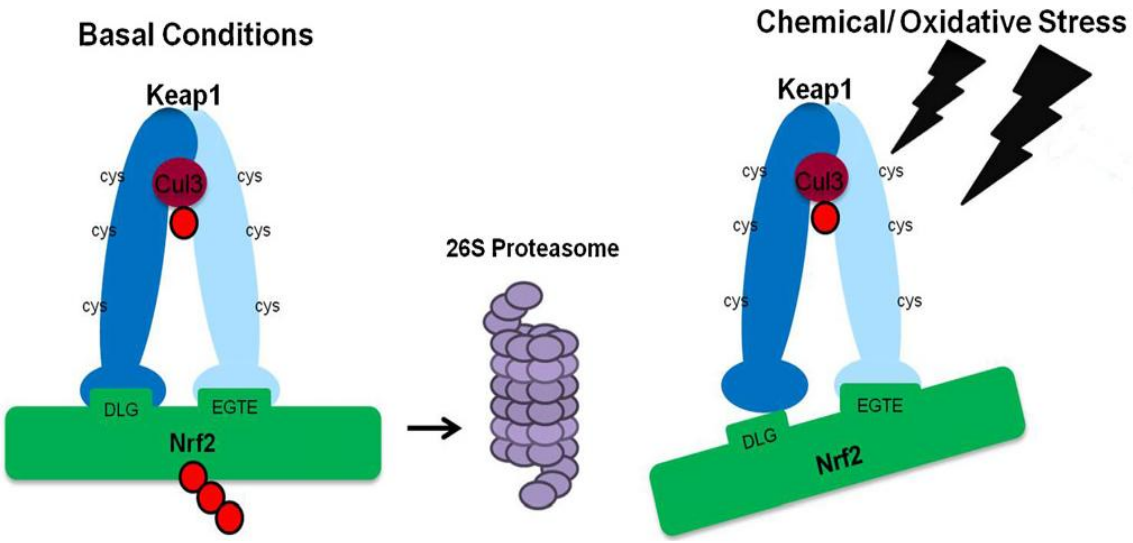

Figure 3. NrF2 and KEAP1 Structural Interaction. Adapted from Bryan et al. 2013.

NrF2 Activation

Multiple theories have been developed to explain NrF2 activation with exposure to RONS being a critical component. However, numerous factors have been identified in the process of NrF2 activation including: exercise, direct oxidant or electrophile interaction, diet, biological crosstalk, transcriptional co-effectors, post translational phosphorylation, proteasome inhibition, and autophagy. NrF2 activation fosters the potential to induce either transcription of NFE2L2 or post translational effects including gene expression of antioxidants and various cytoprotective agents. The activation pathways have been suggested to promote or enhance NrF2 dissociation from KEAP1 and foster binding of NrF2 at the ARE in the nucleus. NrF2-ARE binding stimulates NFE2L2 gene transcription and/or post translational transcription of antioxidant factors (Bryan 2013). However, NrF2 predominantly exerts its effects post translationally through protein stabilization and increasing nuclear concentration to expand the production of antioxidants and cytoprotective agents, enhancing antioxidant capacity and cellular protection (Sandburg 2014).

Oxidant and Electrophilic Interaction

The extent to which NrF2 responds to stress is highly dependent upon KEAP1's ability to "sense" potential stress agents such as pro-oxidant or electrophilic molecules and most commonly, RONS. KEAP1 is a 69.7kda protein (Tkachev 2011) with a cysteine rich moiety surrounded by basic amino acids, which increase the reactivity of the cysteine residues allowing KEAP1 to "sense" oxidative stresses (Bryan 2013). Cysteine residues on the DRG binding motif, near the DLG latch, have been reported to react with various

electrophilic molecules forming covalent adducts and subsequently RONS, allowing NrF2 dissociation within the cytosol (Lau A 2010; Bryan 2013; Tong 2007). Irrespective of electrophiles, RONS alone can activate NrF2. This process aims to extend the half-life of NrF2, normally 13-20 minutes beyond basal conditions (Itoh 2003; Bryan 2013). The ETGE hinge, however, is relatively unaffected and allows NrF2 to navigate freely within the cell. In this premise, NrF2 concentration is controlled at normal aerobic production of RONS and increased concentrations are a direct result of OS.

Additionally, the cysteine residues on KEAP1 are susceptible to reactivity with oxidized proteins and peptides like glutathione. Holland et al. reported glutathionylation of cysteine residues on KEAP1 by oxidized glutathione (GSSG) (Holland 2008). These modifications on KEAP1 contribute to the release and stabilization of NrF2 within the cell. When RONS accumulate in the cell the balance between reduced glutathione (GSH) and GSSG is affected, resulting in an increased GSSG/GSH ratio (Bryan 2013). Increased GSSG indicates redox imbalance and may serve as an additional regulator of NrF2 during times of stress. However, others suggest a cysteine independent activation of NrF2 through modified protein folding and structure of KEAP1 (Bryan 2013).

Upon immediate dissociation from KEAP1, NrF2 lacks subsequent covalent adducts and has a molecular weight of approximately 66kda (605 amino acid chain-Human; 604 amino acid chain-Rat) (The UniProt Consortium 2017; Abed 2015).With a typical half-life of 13-20 minutes, NrF2 concentrations accumulate and stabilize within the cytosol for up to 20min (Abed 2015; Itoh 2003; Bryan 2013). To maintain cellular redox balance and restore oxidative balance, NrF2 must translocate to the nucleus to

promote transcription of antioxidant responsive genes. These signaling events require the formation of heterodimeric bonds and phosphoprotein interactions. A number of proteins have been identified to interact with NrF2 and affect NrF2 localization within the cell (Itoh 2004).Of which only the most noted in the literature will be discussed in detail in the following sections.

Aerobic Exercise

Exercise is well known to increase the production of cellular RONS and has been demonstrated with both aerobic and anaerobic exercise, with aerobic exercise contributing the greatest effect (Bloomer 2004; Wang 2016; Li 2015; Morion 2015). Aerobic exercise, independent of RONS production, has also been reported to indirectly increase NrF2 expression, encouraging a state of proactive cellular protection. Both high and low intensity aerobic exercise has been demonstrated to increase p38 mitogen activated protein kinase (MAPK) and Akt-mTOR during the post exercise recovery period (Donato 2014). Furthermore, Akt and MAPK have been reported to inhibit glycogen synthase kinase 3 beta (GSK3β) through multisite phosphorylation (Bryan 2013). GSK3β is associated with NrF2 phosphorylation at the Neh6 domain, which stabilizes the connection with an E3 ligase and promotes NrF2 degradation (Rada 2012). Therefore, inhibition of GSK3β by Akt or MAPK serves to increase NrF2 activation. Specifically, selective GSK3β inhibition results in greater NrF2 protein concentration as well as increased expression of NrF2 target genes in the hippocampus (Rada 2012).

Phosphorylation

Protein phosphorylation is a post translational modification of amino acid residues located on one of the multiple NrF2 domains by a protein kinase, forming a covalent adduct with NrF2. These covalent associations are necessary to stabilize NrF2 in the cytosol and to aid in the translocation to the nucleus or binding to the ARE. Each covalent adduct increases the molecular weight of NrF2 (depending on the size of the protein adduct), which has been a source of confusion regarding the size and appearance of NrF2 in the recent literature (Lau 2013). Numerous proteins such as MAPK and Src family kinases have been demonstrated to phosphorylate NrF2 affecting KEAP1-NrF2 interaction causing NrF2 localization and ARE binding, while preventing protein degradation (Bryan 2013; Niture 2011). Increased NrF2 phosphorylation has been shown to occur through activation of the PI3K/Akt pathway and MAPKs such as c-jun N-terminal kinase 1/2 (JNK 1/2) and extracellular signal-regulated kinase (ERK), respectively (Nguyen 2003) (Yuan 2006). Typically these pathways increase muscle hypertrophy, mitochondrial biogenesis, and subsequently NrF2 protein concentration.

Similar to KEAP1, NrF2 is a residue rich motif containing multiple serine, threonine, and tyrosine residues that act as targets for protein phosphorylation (Rojo 2012). Various protein kinases have been reported to phosphorylate NrF2, liberating it from KEAP1, and promoting stabilization within the cytosol and translocation to the nucleus. Protein kinase C (PKC) has been demonstrated as a key factor in the subsequent activation of NrF2. PKC phosphorylates NrF2 at NrF2-Ser-40 forming a covalent adduct, which has been shown to be a critical signaling event in the NrF2 pathway (Huang 2002).

Furthermore, NrF2 activation and ARE binding is diminished by PKC inhibitors (Huang 2002). Additionally, protein kinase B (PKB) has also been shown to be an important activator of the NrF2-ARE connection in neuroblastoma cell lines, which suggests an important link to neuroprotection (Lee 2001).

Transcriptional Co-effectors

In addition to NrF2 dissociation and translocation under stress conditions, Maf proteins have been suggested as an important component in the cellular stress response. By acting as site specific ARE sensors in the promoter region, they contribute to transcriptional activation and/or repression based on their interaction with various heterodimeric partners (Katsuoka 2005). Maf proteins have been most recently noted for their heterodimeric formation with transcriptional proteins of the CNC family, including NrF2 (Katsuoka 2005). To date, three Maf proteins have been identified; MafG, MafK, and MafF, but no functional differences have been detected (Motohashi 2002). Maf proteins have been reported as "co-effectors" which are essential for the regulation of gene transcription and to induce the expression of NrF2 and other ARE dependent genes (Katsuoka 2005). However, other proteins like c-Jun (Jeyapaul 2000) and ATF4 (He 2001) have been reported to heterodimerize and co-activate NrF2. Although Newman et al. demonstrated a greater binding affinity between Maf and CNC family proteins suggesting a stronger association among Maf proteins and NrF2 in this pathway (Newman 2003).

Proteasome Inhibition

Antioxidants and known proteasome inhibitor compounds like tert-Butylhydroquinone (tBHQ), Butylated Hydroxyanisole (BHA), Sulforafane (SFN), and MG132 have been reported to increase NrF2 concentration in the cytosol (Niture 2011; Lee 2011; Li 2005; Cui 2013). Induction of NrF2 leads to an accumulation of NrF2 protein, which may increase subsequent transcription and translation of phase II antioxidants or activation of the NFE2L2 gene. NrF2 induction also increases the activation of other signaling pathways such as PKC and ERK, which have been demonstrated to sever NrF2 from KEAP1 allowing nuclear translocation (Huang 2002; Lee 2011). However, some have reported that exposure to antioxidants like tBHQ promote phosphorylation of NrF2 by Src kinases leading to nuclear export and degradation (Niture 2011). Others suggest that compounds like MG132, in certain cell lines, may cause cell apoptosis and cell damage (Reviewed in Cui 2013).

The mechanism of NrF2 induction depends on the type of synthetic compound used, the duration and concentration of the treatment, and potentially cell type as conflicting results have been reported (Cui 2013). MG132 acts through inhibition of the 26S proteasome. Ubiquitination is prevented through inhibiting activation of β subunits on the proteasome allowing NrF2 release from KEAP1 and accumulation in the cytosol where it can be further phosphorylated by kinases such as MAPK or PKC (Cui 2013; Lee 2011). Induction through antioxidants like SFN and tBHQ is not entirely understood. Some have suggested that tBHQ works in a similar manner to MG132 via inhibition of KEAP-1 mediated ubiquitination (Li 2005). However, Li et al. demonstrated that the

protective affects exerted by tBHQ was not through inhibition of ubiquitination, but rather through selective autophagy (Li 2014). This concept will be discussed in more detail next.

Selective Autophagy

Acute NrF2 activation has been demonstrated through selective-autophagy in connection with the phosphorylation of the substrate adaptor Sequestosome1 (p62) (Ichimura2013). Autophagy is a self-destructive operation similar to that of apoptosis where dysfunctional or mutated cells and even excessive cellular components are degraded through a self-eating process mediated by lysosomal actions. Autophagic conditions are stimulated with various types of injury, infection, dysregulation, and metabolic stress. Initially a phagophore isolates portions of the cytoplasm to form an autophagosome. Later, autophagosomes fuse with lysosomes to form autophagolysosomes complete with hydrolytic enzymes to recycle various components back to the original building blocks (Wu 2015). Controlled autophagic activities are essential for maintaining brain homeostasis; however, unregulated situations have been linked with neurological disease and cancer (Wu 2015; Ichimura 2013). Under stress-like conditions p62 is phosphorylated and localizes to the autophagosome and the associated ubiquitinated proteins (Ichimura2013). Once NrF2 dissociates from KEAP1 at the DLG latch, p62, with a high affinity for KEAP1, binds to the empty domain on KEAP1 (Bryan 2013). Together, KEAP1-p62 is degraded by autophagy, thus stabilizing NrF2 in the cytosol (Ichimura 2013; Bryan 2013).

Biological Cross-talk

Other cellular transcription factors have been reported to regulate the NrF2 pathway, one of which operates in a similar manner to that of NrF2. Nuclear factor k-light-chain-enhancer of activated B cells (NF-kB) is a transcription factor that is well known to mediate cellular apoptosis, inflammation, and plays a critical role in aging (Bryan 2013). Like NrF2, NF-kB is bound in the cytosol by I_KB, until phosphorylation and subsequent dissociation of I_KB allows nuclear translocation. Interestingly, an NF-kB binding site has been located in the promoter region of NrF2, suggesting inter-regulation with antioxidant gene expression (Nair 2008). However, when freed, NF-kB has been demonstrated to suppress ARE-dependent genes by inhibiting Maf-NrF2 binding (Liu 2008). Although, KEAP1 has been associated with ubiquitinating IKKβ, a member of the IKK complex that is responsible for phosphorylating I_KB, negatively regulating NF-kB and preventing activation (Kim 2010). Therefore, KEAP1 assumes dual roles in the regulation of NrF2 antioxidant gene expression by preventing NF-kB mediated suppression and maintaining cellular concentrations of NrF2 to commensurate with the redox state of the cell. In conclusion the regulation and interaction between NrF2 and NF-kB is highly complex and warrants further inquiry to establish the exact nature of the biological cross talk.

Dietary Factors

In main stream society today dietary factors have been advocated to enhance the innate defense systems of the body and promote systemic health and protection. Consumption of flavonoids, allyl sulfides, isothiocynates, polyphenols have been

demonstrated to increase NrF2 stabilization and expression. These compounds have been the target of a vast body of research for many years given their suggested physiological functions and the presence of phytochemicals. Plant based compounds such as sulforaphane, curcumin, and resveratrol have been demonstrated to induce NrF2 activation and attenuate RONS production (Kode 2008; Chen 2005). High protein, low carbohydrate diets have also been shown to increase NrF2 concentration compared to normal protein/carbohydrate diets with no significant differences in body weight after 12 weeks (Morion 2015). In addition, chronic aerobic exercise in combination with a high protein diet further elevated NrF2 concentration above sedentary subjects (Morion 2015), promoting current recommendations for physical activity and weight management. Furthermore, high fat diets as commonly observed in the 'standard western diet' have been implicated in NrF2 impairment and increased oxidative stress, potentiating morbidity and mortality (Morrison 2010).

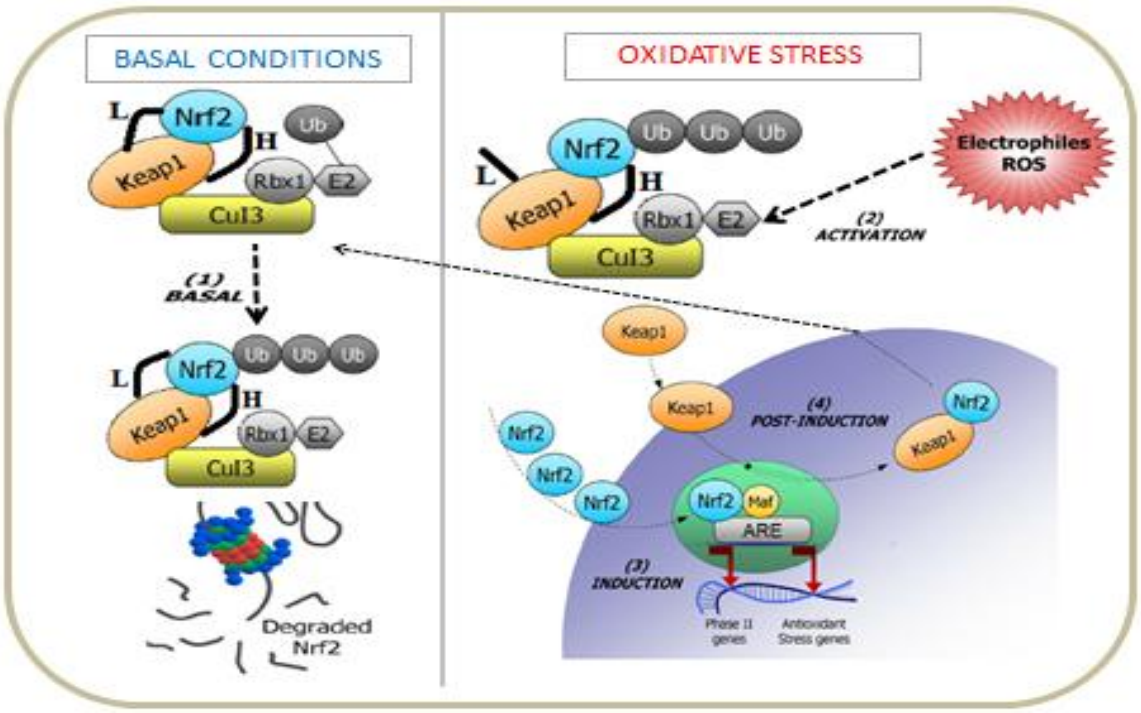

Figure 4. NrF2 Activation Pathway. L= latch; H= hinge; E2/E3= ligase; Ub= ubiquitin ligase; Cul3/Rbx1= anchoring proteins; Adapted from Regoli et al. 2014.

NrF2 Activation Timeline

The process of gene transcription and subsequent protein translation is highly dependent on the target protein of interest. In addition, a stimulus of sufficient intensity and duration is necessary to activate the target protein to a level that is detectable by current biochemical and analytical techniques. Some transcription factors have been shown to significantly increase product protein concentration via translation in as little as 1-hr. These factors are denoted as "immediately early genes" and have been suggested to increase in response to RONS (Nose 1991). Nuclear NrF2 protein has been demonstrated to significantly increase within 30min of diethylmaleate (DEM) treatment (Itoh 2003). DEM is an electrophile classified as a Michael reaction acceptor (Itoh 2003). Chronic

aerobic exercise (4 weeks) was also demonstrated to significantly increase NrF2 DNA binding activity1-hr post exercise with peak activity occurring 8-hrs post exercise (Tsou 2015). In addition, NrF2 binding activity remained elevated 24-hrs post exercise and was similar to the activity observed 1-hr post exercise (Tsou 2015).

Basal levels of NrF2 in whole cell lysate are minutely detectable compared to cells treated with DEM as demonstrated by Itoh et al. 2003. This indicates that only a small concentration of cytosolic NrF2 exists in non-stressed cells. Nuclear accumulation of NrF2 occurs in response to a rapid, stress induced, activation of cytosolic NrF2 as demonstrated by an elevated NrF2 nuclear protein concentration and detectable cytosolic concentration (Itoh 2003). Furthermore, the extent to which NrF2 protein concentration increases is dependent on the duration and intensity of the stress stimulus as demonstrated through exercise induced activation. Exhaustive exercise significantly increased NrF2 protein concentration compared to moderate-high intensity exercise (Li 2015), while a minimum duration of 45min was required to detect a noticeable difference in NrF2 whole cell lysate concentration (Wang 2016).

NrF2 Degradation

In a quiescent state NrF2 is slated for ubiquitination and proteasomal degradation through the cullin-3, E3 ligase complex on the N-terminal region of KEAP1 (Sandburg 2014). Acting as an anchoring protein, KEAP1 secures NrF2 to the cytoskeleton at the DLG latch, which impedes translocation to the nucleus and interaction with other proteins (Singh 2014). Furthermore, the latch positions NrF2 in line with the E3 ligase on the cullin-3 complex and initiates ubiquitination through the 26S proteasome,

diminishing the half-life of NrF2 (Fig. 4) (Sandburg 2014; Tong 2007; Itoh 2003). This is the typical KEAP1-NrF2 pathway regulating cellular NrF2 concentration; however, other biological mechanisms can influence this pathway.

Protein kinases also serve a role in the degradation process by preserving the connection between NrF2 and KEAP1 or through nuclear export of NrF2, all leading to ubiquitination. In this situation NrF2 and/or bound NrF2-KEAP1 are phosphorylated by upstream targets. MAPKs, like p38, commonly phosphorylate serine/threonine residues on NrF2 fixing the association between NrF2 and KEAP1 (Keum 2006). Src subfamily tyrosine kinases such as Fyn, Src, Yes, and Fgr, and GSK-3β, have been reported to regulate NrF2 phosphorylation on multiple levels (Sandburg 2014; Ingley 2008). In a state of OS, the Src tyrosine kinases: Fyn, Src, Yes, and Fgr, have been demonstrated to accumulate in the nucleus and phosphorylate NrF2 (Niture 2011). GSK-3βhas been documented as an upstream activator of Src tyrosine kinases (Jain 2007). Stress activated GSK-3β has been reported to phosphorylate Src kinases resulting in NrF2 phosphorylation and subsequent degradation (Niture 2011; Rada 2012). Independent of the particular mechanism of phosphorylation once NrF2 is phosphorylated it appears to be then programmed for subsequent degradation.

NrF2 Detection

Unbound NrF2, independent of KEAP1, has a predicated molecular weight of approximately 66kda (605 amino acid chain-Human; 604 amino acid chain-Rat) (The UniProt Consortium 2017; Abed 2015); however, this has been a major source of controversy in the literature in regards to western blot protein detection (Lau 2013;

Kremmerer 2015). Despite the relatively moderate size of the protein, its migration pattern by sodium dodecyl sulfate– polyacrylamide gel electrophoresis (SDS-PAGE) differs from its predicted molecular weight causing misinterpretation of data and confusion in the literature surrounding NrF2. Lau et al. recently published a report demonstrating evidence through a series of experiments including: chemical activation, vector expression, and recombinant expression, that despite its predicted molecular weight, biological NrF2 migrates between 95-110kda under reduced or denatured conditions on SDS-PAGE gels and commercially available gradient gels (Lau 2013). The authors cite "the abundance of acidic residues" as the explanation for the unpredicted migration of NrF2.

Furthermore Kremmerer et al. substantiated findings presented by Lau and colleagues regarding the migration of NrF2. Additionally, they reported specific differences in the reduced protein mobility phenomenon between Tris-glycine gels and Bis-Tris gels. Per the report, Tris-glycine gels demonstrate NrF2 protein migration with a band resulting near 100kda vs. migration for Bis-Tris gels with a band resulting near 80kda (Kremmerer 2015). Therefore, it is important to note that despite the undisputed molecular weight of NrF2 (66kda), evidence has demonstrated that the migration pattern detected in the procedure of western blot displays significantly different results in terms of protein band location is highly dependent on the type of gel used during the process of gel electrophoresis.

NrF2 and Antioxidants

Upon activation, NrF2 promotes the up regulation of genes that code for enzymatic and non-enzymatic antioxidant production such as glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (Powers 2008) as well as numerous protective proteins like BDNF, anti-inflammatory cytokines, and mitochondrial transcription factors (Reviewed in Sandburg et al. 2014).These genes are known as "NrF2 regulon" (Singh 2010) and to date over 500 genes have been documented to be influenced by NrF2 (Pall 2015; Itoh 2003). NrF2 and its "regulon" are ubiquitously expressed in various cells throughout the body, compartmentalized in the cytoplasm and cellular organelles, acting as multi-organ protectors. By way of the "regulon", antioxidants are produced and together serve as a protective mechanism against cellular RONS.

Cellular antioxidants are ubiquitous molecules that scavenge and neutralize free radicals through stable electron donation (Lobo 2010). Electron pairing reduces the capacity to oxidize cellular components or damage DNA. The antioxidant system is not 100% effective; however, it can delay and inhibit free radical capacity and subsequent oxidative stress. Antioxidants are produced through endogenous systems in the body or are taken in through the diet. Typically, these are divided into two major classifications, enzymatic and non-enzymatic. Enzymatic antioxidants include superoxide dismutase, catalase, and glutathione peroxidase. Non-enzymatic antioxidants include glutathione, ascorbic acid (Vitamin C), melatonin, uric acid, tocopherols and tocotrienols (Vitamin E). These antioxidants possess variability in strength and line of defense in prevention of

oxidative stress, repair and de novo, radical scavenging, and cell signaling adaptation (Lobo 2010). Cells interact with a network of antioxidants to maintain hormesis and prevent free radical production.

Superoxide Dismutase

Superoxide Dismutase (SOD) is a cellular bound enzyme responsible for catalyzing the dismutation of super oxide anions to hydrogen peroxide and oxygen (Zelko 2002).

$$(O_2^{-}\cdot + O_2^{-}\cdot + 2\ H^{+} \xrightarrow{SOD} O_2 + H_2O_2)$$

SOD exists in two forms (copper-zinc, manganese), with three distinct isoforms (SOD1, SOD2, SOD3) which are found in two different locations within the cell. SOD1, also known as copper-zinc superoxide dismutase (Cn-Zn-SOD) is located in the cytosol or interstitial cellular fluid, bound to the cell membrane (Zelko 2002). SOD3 is the other copper-zinc form usually found in the extracellular fluid, but is the least distinguished of the SOD's (Zelko 2002). SOD2 is the most common form, also known as manganese superoxide dismutase (Mn-SOD), which is typically found in the mitochondria (Zelko 2002). The existence of SOD1 and SOD2 is typically abundant in cells throughout the body; however, SOD3 thrives in tissues where its activity can easily surpass that of the other two isoforms (Zelko 2002).

SOD, specifically SOD2, functions as one of the major antioxidants against RONS, most importantly from superoxide anion. Although, SOD is stimulated by an array of biological phenomenon (heat shock, heavy metals, hydrogen peroxide, nitric

oxide, arachidonic acid, t-butyl, inflammatory cytokines, lipopolysaccharides, histamine, serotonin, etc.) (Reviewed in Zelko et al. 2002). SOD1 and SOD2 mRNA expression and activity increase in a positive linear fashion from birth to adulthood, but SOD3 demonstrates a negative linear decrease in mRNA expression with age (Zelko 2002). Despite increases in mRNA expression, the activity of SOD does necessarily reflect changes in gene expression and protein concentration, which may be related to the specific assay used or the type of cell analyzed.

Cellular regulation of SOD activity and mRNA expression is highly dependent on the tissue of origin and the biological stimulator. Clearly, SOD responds to the presence of RONS as well as other noxious agents, but it may do so utilizing different signaling pathways. Mn-SOD has been demonstrated to increase in the presence of tumor necrosis factor alpha (TNFα) and hydrogen peroxide (Warner 1996). However, the temporal pattern of induction was significantly different between these two stressors suggesting distinct signaling pathways. Furthermore, SOD acts as a primary scavenger of superoxide anions produced in the electron transport chain and peroxisomes (Zelko 2002). Therefore, increases in aerobic metabolism and beta oxidation stimulate an increase in SOD2 and SOD3 activity, respectively.

Furthermore, the necessity for cellular SOD2 is crucial to survival in aerobic environments. MnSOD gene knockout models have been reported lethal in animal studies, although heterozygous animals with decreased SOD activity reported greater susceptibility to OS and RONS linked injury (Candas 2014). In response to stressful stimulators, i.e. RONS, SOD2 activity is transiently affected with significant increases in

the brain occurring immediately post an acute bout of aerobic exercise (Asku 2009). However, bodily tissues demonstrate variation in the upregulation of SOD with exercise. Skeletal muscle has shown significant increases in SOD 1-hr to 1-day post an acute bout of exercise (Radak 1995). In addition, MnSOD mRNA is increased in as little as 1-hr post acute exercise, with protein concentration significantly altered at 10 and 24-hrs post exercise (Hollander 2001). Repeated exercise training has also been reported to increase SOD chronically in skeletal muscle and various regions of the brain (Devi 2004, Higuchi 1989). Radiation is also a potent stimulator of RONS production and activation of SOD with peak activity occurring 6-hr post treatment; however, no change in protein concentration was detected (Jin 2015).

Glutathione

Serving as the major endogenous antioxidant in the body, glutathione is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) formed from cysteine residues (Lushchak 2012). Existing in two forms, an oxidized form (GSSG) and a reduced form (GSH), the reduced form, GSH, is the most predominant within the cell. Together these forms (2 GSSG + GSH) make up total glutathione (TGSH) and act to maintain cellular redox balance.GSH is formed in the cytosol of the cell through a two-step, energy requiring process:

1. L-glutamate + L-cysteine + ATP → γ-glutamyl-L-cysteine + ADP + Pi
2. γ-glutamyl-L-cysteine + L-glycine + ATP → GSH + ADP + Pi

However, this is a rate limited process regulated by the availability of cysteine (Wu 2003). After formation, GSH has a half-life lasting 2-3 hours (Lu 2009). GSH contains an active thiol group and retains the ability to be directly consumed through oxidation by RONS or conjugation by electrophiles to GSSG (Lushchak 2012; Zhang 2009).GSH is also commonly used as a co-substrate by glutathione peroxidase to neutralize RONS (Lushchak 2012).In the oxidized form, GSSG will either be excreted from the cell or undergo a salvage cycle to reform GSH by glutathione reductase, expending NADPH (Lushchak 2012; Lu 2009). The former is the most common outcome of glutathione consumption, whereas the latter is usually blocked by methionine (Lushchak 2012). GSH can also be hydrolyzed by peptidases and the resulting amino acids (glycine, cysteine or glutamate) will be taken up the by cells to be used in metabolism or reform glutathione.

$$(ROOH + 2GSH \longrightarrow ROH + GSSG + H2O)$$

$$(GSSG + NADPH + H^{+} \longrightarrow 2GSH + NADP)$$

Outside of the cellular protection function, GSH also plays a role in metal ion homeostasis and metabolism, specifically of copper and iron. GSH is well documented for its involvement in the mobilization, transport, reduction, and incorporation of iron and copper from various sources into target locations (Lushchak2012). The brain contains a localized concentration of copper and iron regionally located in the telencephalon (hippocampus, cerebral cortex, cerebral lobes). Iron is most commonly found in the body bound to hemoglobin, myoglobin, and cytochromes or in stored form as ferritin and hemosiderin (Reddy 2004). Similarly, copper is most commonly found in stored form

within the brain, liver, and kidney (Manto 2014). With a high capacity to store divalent metals, iron and copper are rarely found in free form; however, when these metals are free, iron and copper have a natural proclivity to generate RONS. GSH rapidly sequesters copper and iron reducing their availability for Fenton based reactions and the production of stress agents (Manto 2014).

GSH and GSSG concentrations are tightly regulated and remain relatively constant through balanced consumption, production, and transportation of glutathione within and surrounding the cell. Over 90% of GSH is contained within the cytosol with the other 10% scattered between the mitochondria and the endoplasmic reticulum (Meredith 1982; Hwang 1992) with negligible GSSG. In times of OS the cellular ratio of GSSG/TGSH increases, allowing greater consumption of GSH to protect the cell. The alteration in GSH and GSSG concentration with acute aerobic exercise is typically transient, dependent on exercise duration and intensity, with significant increases in the transition of GSH to GSSG with longer duration or more intense activity (Wang 2016; Li 2015). Additionally, it has been reported that cellular GSH levels in can increase with supplementation of cysteine precursors (Lushchak 2012; Sen 1999; Goldfarb-Ribcys Unpublished data), exercise training in skeletal muscle, blood, and brain (Sen 1999; Elokda 2007; Aguiar 2010; Tsou 2015) and through activation of the NrF2 pathway, enhancing cellular protection. Knockout models have identified NrF2 as the critical component in the maintenance of GSH, which demonstrated significantly lower GSH levels and a greater susceptibility to RONS induced injury (Chan 2001). NrF2 has also been demonstrated to regulate GSH levels by affecting de novo synthesis and redox

balance through activation of glutathione reductase (Harvey 2009) and GSH utilizing enzymes like glutathione peroxidase. Furthermore, NrF2 mediates the cysteine/glutamate exchange transporter, regulating cellular cysteine influx (Sasaki 2002).

Glutathione Peroxidase

Glutathione peroxidase (GPx) is a family of selenium dependent and independent enzymes found in a variety of cells that utilize GSH to reduce hydrogen peroxides to water, oxygen, and GSSG (Espinoza 2008).

$$(2H_2O_2 \xrightarrow[GSH]{GPx} 2H_2O + O_2)$$

GPx exists in multiple categories including a selenium dependent form, two sub-forms of GPx-like proteins, and a family of selenium independent enzymes. Irrespective of the class, GPx is found within the cytosol or mitochondria of cells throughout the body (Arthur 2000). The table below provides an inclusive list of GPx sub-forms and locations (Table 1).

GPx functions to protect the cell from OS in a specific manner related to its existence within a cell type and cellular compartment. In the presence of hydrogen peroxides, particularly lipid hydroperoxide (LOOH), GPx expression increases, as seen with chronic OS resulting in increased GPx activity (Espinoza 2008). However, acute OS tends to employ a transient over production of RONS leading to increased GPx activity and subsequent degradation or reduction of GSH (Comhair 2005). GPx expression may also be duration dependent in terms of physical activity induced OS. A 6-hr exercise increased the mRNA expression of GPx, while a 1-hr exercise bout was insufficient to

induce change (Li 2015).Glutathione S-transferases (GST) are responsible for transforming electrophilic compounds into less reactive substances for cell survival. Formally serving as mediators, GST's regulate GSH conjugation with electrophiles and reduction/neutralization of LOOH (Lushchak 2012). Despite biological differences between GPx and GST, both are regulated through the activation of NrF2 to enhance activity of phase II detoxifying enzymes and to increase synthesis of GSH (Lushchak 2012).

Table 1. Forms of Glutathione Peroxidase.

Name	Symbol	Type	Location
Glutathione Peroxidase 1	GPx1	Selenium dependent	RBC's; cytosolic
Glutathione Peroxidase 2	GPx2	Selenium dependent	Gastrointestinal Tract; cytosolic
Glutathione Peroxidase 3	GPx3	Selenium dependent	Kidneys, Lungs, Placenta, Liver, Gastrointestinal Tract, Plasma
Glutathione Peroxidase 4	GPx4	Selenium dependent	A variety of cells; mitochondrial
Glutathione Peroxidase 5	GPx5	GPx like protein	Epididymis
Glutathione Peroxidase 6	GPx6	GPx like protein	Bowman's gland
Glutathione-S-transferases	GST	Selenium dependent	A variety of cells; cytosolic, mitochondrial, and microsomal

Adapted from Arthur 2000.

Catalase

Functioning in a similar manner to GPx, catalase (CAT) is an enzymatic tetramer with a 3D structure that catalyzes the dismutation of hydrogen peroxide to water and oxygen (Sharma 2012).

(Birben ($2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$)

CAT contains 4 identical monomers in the form of heme that play a role in removal of hydrogen peroxide. Occurring in a two-step process whereby two molecules of hydrogen peroxide form an oxyferryl species and regeneration of the resting enzyme, followed by production of water and molecular oxygen (Sharma 2012). SOD is a primary producer of hydrogen peroxide for which CAT scavenges.

CAT activity depends on the availability of hydrogen donors including: phenols, methanol, ethanol, and etc. (Goyal 2012). Its role in most cells is tolerance and adaptivity to RONS induced OS. Specifically in the brain, CAT serves a crucial role in the protection and development of neurons (Mann 1997). Expressed primarily in the cytosol, CAT is also located in mitochondria and peroxisomes, scavenging hydrogen peroxide (Sharma 2012). CAT demonstrates a high sensitivity for hydrogen peroxide, but a low affinity compared to ascorbate peroxidase, another heme based antioxidant (Sharma 2012).

In response to RONS, the activity of CAT clearly differs between acute and chronic stress and has also exhibited tissue dependence (Laughlin 1990, Higuchi 1989, Devi 2004). Acute exercise of varying intensities has not been demonstrated to increase CAT activity. In addition, duration does not affect the extent of CAT activity, with 1-hr and 6-hr exercise durations eliciting similar effects (Li 2015). However, it has been proposed that CAT is responsible for the body's adaptive nature to RONS production

(Goyal 2012). Therefore, the change in CAT activity may only be necessary during chronic stress, while other antioxidants forge protection against more transient stress.

Brain Derived Neurotrophic Factor

In addition to the numerous antioxidants that are expressed through NrF2 activation, other protective proteins such as brain derived neurotrophic factor (BDNF) have also been demonstrated to increase in response to NrF2 activation. BDNF is a key protein that has been reported to influence learning, memory, neuronal development and survival, regulation of body weight, energy homeostasis, and signaling of other cellular factors (Reviewed in Rasmussen et al. 2009). The central nervous system is the primary site of BDNF production; however, skeletal muscle, vascular endothelium, and platelets have also been reported to secret BDNF (Rasmussen 2009; Matthews 2009; Yamamoto 1990; Hohn 1990). The hippocampus and cerebral cortex brain regions have been attributed to the most substantial increases in BDNF reported in the central nervous system (Wetmore 1990). Low levels of circulating BDNF have been associated with depressive disorders and Alzheimer's disease (Tsai 2003). Therefore, BDNF is a commonly targeted factor for treatment of neurological disorder and dysfunction. Use of antidepressant medications are a common therapeutic technique to increase BDNF expression (Russo-Neustadt 2000), although exercise has been noted as an effective treatment to upregulate BDNF. The effects of exercise on BDNF will be discussed in more detail in a later section.

Oxidative Stress

It is well understood that under stressed conditions and even as part of normal aerobic metabolism that cells release noxious agents in the form of reactive oxygen and nitrogen species (RONS). RONS are disruptive to homeostasis and can potentially cause damage. Normally cellular damage is uncommon due to the ability of endogenous antioxidants to quickly neutralize toxic agents. However, in situations of elevated stress, pro-oxidants accumulate beyond antioxidant defense capabilities, exceeding physiological capacity. This condition is termed oxidative stress (OS). When antioxidant defenses capabilities are unmatched to RONS production, damage commonly occurs to cellular proteins, lipids and nucleic acids.

Reactive Oxygen and Nitrogen Species

All cells in the body have the potential to produce RONS, which can lead to OS and affect the redox balance of the cell. RONS have been implicated with exercise, aging, and disease, which can impact the life span of the cell. RONS are produced both aerobically and anaerobically, with aerobic production contributing the greatest propensity for OS. Molecules with an unpaired electron have the ability to exist independently, but are highly unstable and were commonly referred to as “free radicals” (Powers 2008). Free radicals are typically generated as products of redox centered reactions (donation), as well as homolytic and heterolytic reactions (Powers 2008). These processes exist to allow free radicals to fill their outer orbital with an additional electron for stabilization. Electron reactions usually involve lipids, proteins, and nucleic acids

forming reactive species. However, available antioxidants scavenge free radicals and neutralize them preventing the formation of reactive species (Powers 2008).

A multitude of free radicals and reactive species are produced within the cell under stress and as part of normal metabolism; an inclusive list of RONS are listed below (Table 2). The primary radicals generated within the cell are superoxide ($O2^{\bullet -}$), singlet oxygen (O^{-}), and nitric oxide (NO), which readily react to form RONS (Powers 2008).

Table 2. Common Reactive Oxygen and Nitrogen Species.

Name	Symbol	Reactive Species
Superoxide	$O_2^{\bullet -}$	ROS
Hydroxyl radical	$OH^{\bullet}$	ROS
Hydroperoxyl	$HO_2^{\bullet}$	ROS
Peroxyl radical	$RO_2^{\bullet}$	ROS
Alkoxyl radical	$RO^{\bullet}$	ROS
Carbon dioxide radical	$CO_2^{\bullet -}$	ROS
Singlet oxygen	O^{-}	ROS
Nitric Oxide	NO	RNS
Hydrogen peroxide	H_2O_2	ROS
Organic peroxides	ROOH	ROS
Peroxynitrite	$ONOO^{-}$	RNS

Adapted from Halliwell 2006. ROS are oxygen based radicals and RNS are nitrogen based radicals.

RONS Production

RONS are produced from multiple sources within the cell, through both aerobic and anaerobic metabolism, and from external sources such as chemicals, air pollutants including smoke, and radiation (Lobo 2010). Radical forming reactions are well known to occur in the mitochondria, peroxisomes, the endoplasmic reticulum, and plasma membranes. Formation also occurs through phagocytosis, inflammation, interaction with metals, catecholamine metabolism, calcium dysregulation, xanthine oxidase and NADPH generation (Sharma 2012). The primary targets of pro-oxidants include nucleic acids, the building blocks of DNA, lipids, and proteins. In situations of OS, there is an increased risk of genetic mutations, diminished membrane quality, metabolic dysregulation, unregulated cellular exchange, etc. The figure below outlines the results of RONS induced oxidative damage to the lipids, proteins and DNA and a short list of subsequent markers of oxidative stress (Fig. 5).

The extent to which RONS accumulate within the cell is highly dependent on multiple interacting factors. As mentioned earlier, cellular antioxidant capacity is a key regulator of RONS formation and degradation during times of stress. Antioxidant activity increases with RONS production in the short term, while long duration or chronic OS induces antioxidant gene expression to increase the protein concentration of available antioxidants and subsequent activity. The duration of RONS production in a given period of time defines the potential for OS of that specific cell. Additionally, the frequency that cells are stimulated to produce RONS can also potentiate OS. Finally, RONS vary in

location of their production as well as their ability to oxidize macromolecules within the cell.

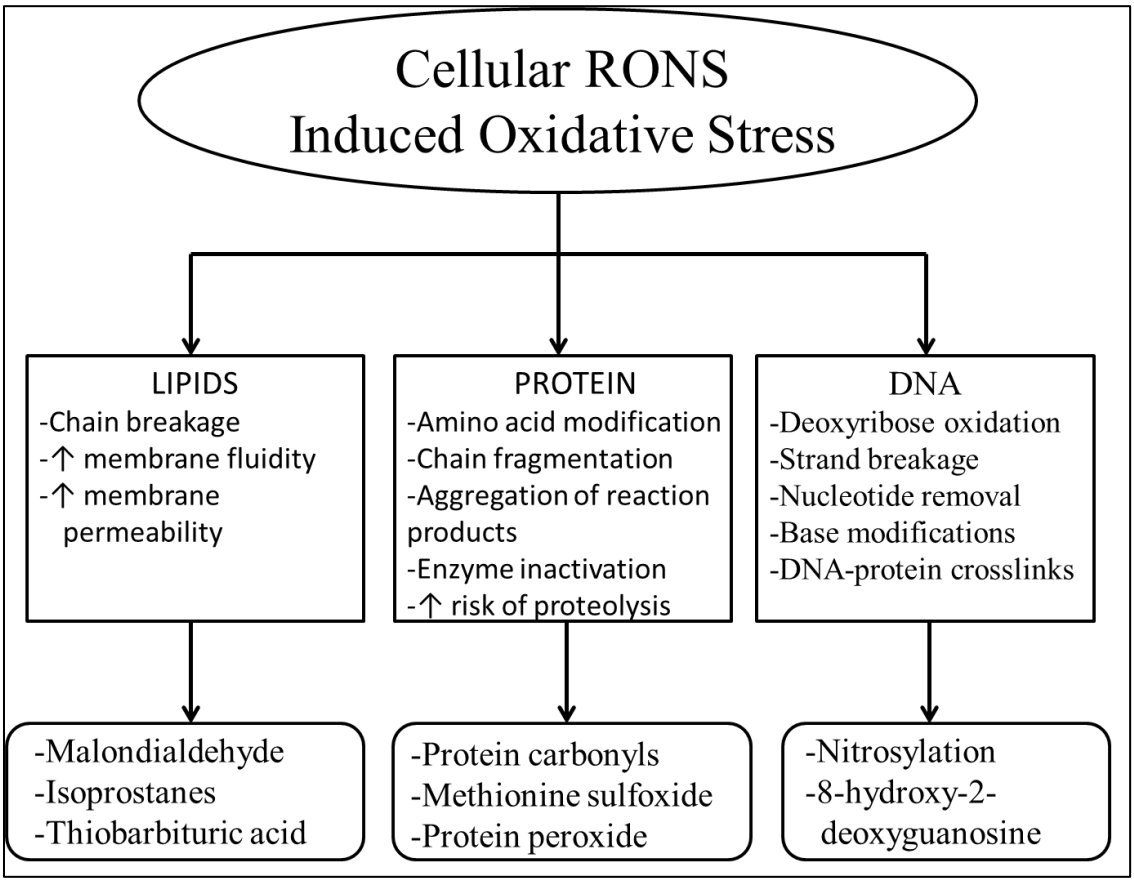

Figure 5. Markers of Oxidative Damage. Adapted from Sharma et al. 2012.

Protein Carbonyls

One of the most common reactive species formed under stress conditions is the protein carbonyl (PC), which results from direct oxidation of side chain amino acids and aggregation of reactive products (Sharma 2012; Suzuki 2010). Protein modifications leading to PC production have been identified in various disease states (Suzuki 2010), although, they are also produced during exercise. The extent to which carbonyls are

generated in response to exercise is related to the intensity of the exercise bout. Exercise intensities at or above 70-75% VO_{2max} have been suggested to be critical to observe changes in markers of oxidative stress (McKenzie 2013). Detection of PC is also duration dependent. Rapid generation of PC occurs through direct oxidation of proteins, cleavage of peptide bonds, or through secondary reactions (Dalle-Donne 2006). And removal is comparative by means of the ubiquitin-proteasome pathway or the kidney from both tissue and blood (Shang 2001). Peak PC concentration has been reported immediately post exercise in both tissue and blood (Lui 2000; McKenzie 2013), but returns to baseline within 2-hrs post exercise (Vezzoli 2014) or less depending on the extent of increase. By 2-hrs^{+} post exercise, PC concentration is unchanged from baseline or has been attenuated (Lui 2000; Radak 2001). PC generation also differs among bodily tissues. Skeletal muscle production of RONS is highly dependent on the muscle fiber type, with type 1 fibers imparting the greatest contribution to protein oxidation (Lui 2000). PC levels are typically unchanged in brain tissue immediately post an acute exhaustive bout of exercise (Lui 2000; Radak 2001); however, blood PC concentration has been demonstrated to increase immediately post exercise (McKenzie 2013). This is most likely a collective homogenate from multiple active tissues and does not accurately reflect concentration in any individual tissue.

Malondialdehyde

When RONS handling exceeds antioxidant capabilities, lipid peroxidation of unsaturated fatty acids and phospholipids accelerates. Produced primarily from lipid hydroperoxides and secondarily from both enzymatic and non-enzymatic reactions,

malondialdehyde (MDA) is a common marker of omega-6 and omega-3 fatty acid peroxidation and is largely responsible for cell membrane and organelle damage (Sharma 2012; Ayala 2014). Lipid peroxidation occurs in a 3-step process where hydroxyl radicals form carbon centered radicals, which react with singlet oxygen and then are eventually neutralized by antioxidants; termed "initiation", "propagation" and "termination" (Ayala 2014). MDA is metabolized through interaction with mitochondrial aldehyde dehydrogenase, phosphoglucose isomerase, or excreted through the kidney (Ayala 2014).

Intense aerobic exercise has been shown to increase MDA concentration in blood and a myriad of tissues. MDA has been reported to increase in blood immediately post exercise (30min at 70% VO_{2max}) in both Caucasians and African Americans with ethnic disparity (McKenzie 2013). Skeletal muscle, liver, and brain have also been demonstrated to produce MDA in response to acute and chronic exercise in a time dependent manner. MDA appears to be unchanged immediately post exercise up to 1-hr post exercise in most tissues (Moirón 2015); however, peak expression has been reported as late as 6-hrs post exercise (Paula 2005) and remains elevated 48-62-hrs post exercise (Paula 2005; Strobel 2011). Interestingly, unlike other markers of OS, MDA does not appear to be influenced by antioxidant treatment (Strobel 2011) and is elevated to a greater extent with chronic vs. acute exercise (Lui 2000). One study reported that high levels of MDA in the brain leads to cerebral suppression and depression of many physiological functions (Li 2010).

RONS Production in the Brain

The brain is a highly complex organ with a high rate of metabolism, vast storage of copper and ion, and a primarily aerobic means of energy production, making it the

most susceptible to OS (Radak 2013). Super oxide anions and hydrogen peroxide are generated by numerous processes within the brain that interact with phospholipid membranes, DNA, and various proteins, increasing the production of RONS (Radak 2013). Multiple regions within the brain, including astrocytes, neurons, glial cells, various organelles, and others, have been reported to produce RONS (Gershon 1990). The blood brain barrier acts as an important protective sheath against toxic agents outside of the central nervous system. However, it cannot protect the brain against RONS as well as inflammatory agents produced within the brain itself (Farkas 2006).

The mitochondrion is a primary region of pro-oxidant production. In the respiratory chain, molecular oxygen (singlet oxygen) interacts with free electrons forming hydrogen peroxide (H_2O_2) (Aguiar2007). Hydrogen peroxide easily reacts with reduced metals like iron (Fe^{2+}), copper (Cu^{2+}) and manganese (Mn^{+}) by means of the Fenton reaction (Haber-Weiss 1930).The production of hydrogen peroxide combines with available metals, like iron, inducing the formation of potent hydroxyl radicals that can inflict oxidation to cellular proteins and lipids.

$$(Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^{-})$$

$$(Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^{+})$$

Nicotinamide adenine dinucleotide (NADPH) oxidases are key contributors to hydrogen peroxide accumulation within the cell due to their potent generation of super oxide anions. Produced from phagocytic cells, NADPH oxidases are well known to interact with lipids generating RONS (Lambeth 2007), although their role in the brain is

still in question. Neuronal membranes are highly sensitive to RONS produced through these processes, posing a potential risk to neuronal membrane integrity and their communicative properties.

Furthermore, the brain is constantly receiving sensory information and sending efferent responses in the form of excitatory and inhibitory impulses by way of neurotransmitters to regulate autonomic functions and energy production. Glutamate is the central nervous systems most prominent excitatory neurotransmitter that serves as a precursor for GABA, the brains inhibitory neurotransmitter. To release neurotransmitters and mediate cognitive function, both intracellular and extracellular calcium must be released (Bading 2013). The endoplasmic reticulum serves as the primary reservoir for calcium intracellularly, but at rest may contain only small concentrations of calcium, providing minor contributions to nuclear calcium influx (Garaschuk 1997). When activated by glutamate, hormones, and inflammatory molecules, a calcium wave is propagated along a neuron allowing subsequent activation of neighboring receptors on neurons (Bading 2013; Berridge 2000). Nuclear calcium signaling has been demonstrated as an important regulator in gene transcription, memory formation and neuroprotection. However, due to calcium's central role in the brain, calcium dysregulation has been reported to be involved in neurodegeneration and various neurological disorders (Reviewed in Bading 2013).

In addition, increased neural communication in the brain promotes extensive activation and inhibition between neurons. Continuous afferent and efferent transmission perpetuates the release of calcium and glutamate (Radak 2013). Heightened synaptic

transmission increases cellular glutamate concentration and sustained exposure has been reported to destroy neurons (Choi 1988). Concomitantly, increases in cellular calcium are prevalent with synaptic transmission and general neuronal function. Excitotoxicity has been shown to stimulate calcium dysregulation through activation of calcium dependent proteases and caspases resulting in receptor degradation and OS (Rodriguez 2001).

Auto-oxidation of catecholamines is well known to increase the production of RONS and increase the risk of neurological disorders due to the formation of neuromelanin (Smythies 1998; De Iuliis 2008). Furthermore, chronic auto-oxidation has been implicated in the destruction of dopaminergic neurons (Miller 1996). Additionally, changes occurring outside of the brain, as in blood and skeletal muscle, can indirectly affect brain metabolism and RONS production. It has been demonstrated that gradual exercise increases both serum and brain calcium levels and to a greater extent during forced exercise (Sutoo 1996). What's more is in addition to the calcium stored centrally, serum calcium is transported into the brain stimulating dopamine synthesis (Sutoo 1985). Increased dopamine production, especially in a low antioxidant state and in the presence of transition metals, may result in accelerated auto-oxidation with various quinones forming toxic radical species (Smythies 1998; De Iuliis 2008).

Being a highly aerobic organ, the brain is extremely sensitive to changes in oxygen content and redox balance. Aerobic exercise has been demonstrated to increase RONS production throughout the body, including the brain (Devi 2004; Radak 2001; Somani 1995; Radak 1995; Brickson 2001; Laughlin 1990). RONS generation can occur in all brain regions, but tends to be elevated in more active areas with exercise (i.e. motor

cortex, cerebellum), specifically in the neuronal and glial mitochondrial membranes (Radak 2013). Moreover, brain oxidative capacity has been shown to increase in some brain regions in response to chronic exercise (Dishman 2006), but may not occur as readily in other regions, like the hippocampus (McCloskey 2001). The extent of RONS accumulation in the brain with exercise is minimal and may be due the adaptability to its highly oxidative environment or the time of sacrifice following exercise, this being highly variable among studies. The following tables outline markers of RONS in the brain with exercise (Table 3, 4). It appears there is minimal change in markers of oxidative stress in whole brain in response to acute exercise. This might be related to no change in these markers with acute exercise or related to the dilution of these changes with different regions of the brain. In general, the extent of the increase in changes of oxidative stress markers with exercise training is either an attenuated response or no difference in these markers.

Table 3. Markers of Oxidative Stress in the Brain with Acute Exercise.

Acute Exercise Training	Marker of Oxidative Stress	Time of Sacrifice	Authors
Exercised for 1hr at 10, 15, 20m/min or to exhaustion	Nitrate-nitrate ↔ TBARS ↔	Immediately post exercise	Asku et al. 2009
Swimming exercise for 2hr at 32°C	TBARS ↔ PC ↔ 8-OHdG ↔	Immediately post exercise	Radak et al. 2001
Exercised until exhaustion at 1.6km/min	MDA ↔ PC ↔ GSSG ↔ GSH/GSSG ↔	Immediately post exercise	Liu et al. 2000
Electrical stimulation in the tibialis anterior muscle with 10s of 5V for 10min and a frequency of 100Hz at a 1:1	MDA ↑ Nitrite ↑	Immediately post exercise	Paula et al. 2005

The above results are from animal studies and unless otherwise noted are from whole brain.

Table 4. Markers of Oxidative Stress in the Brain with Chronic Exercise.

Chronic Exercise Training	Marker of Oxidative Stress	Time of Sacrifice	Authors
Swimming exercise 5-30min/d for 12 weeks with 3%BW tied to tail	MDA ↔ LF-like AFS ↔	Same day	Devi et al. 2004
Exercised 3-4d/wk, at 35cm/s for 12wks.	TBARS ↑ Protein carbonyls ↔	Same day	Moiron et al. 2015
Swimming exercise for 1hr/d, 5d/wk for 9wks at 32°C	PC ↓ TBARS ↔ 8-OHdG ↔	2hrs post exercise	Radak et al. 2001
Exercised for 30min/d at 8-54m/min at 6-10° for 7 weeks.	Cortex GSSG ↓ Brainstem GSSG ↓ Striatal GSSG ↑	18hrs post exercise	Somani et al. 1995
Chronic exercise for 2hrs 5d/wk for 8wks at 1.6kg/hr. Sacrificed 48hrs post exercise.	MDA ↓ PC ↔ GSSG ↔ GSH/GSSG ↔	12hrs post exercise	Lui et al. 2000

The above results are from animal studies and unless otherwise noted are whole brain.

RONS Production in the Skeletal Muscle

Skeletal muscles are continuous producers of RONS, which is accentuated during aerobic exercise and exacerbated during eccentric exercises (Hellsten 1997; Brickson 2001). Superoxide anions are the primary molecules generated through mechanisms including the handling of aerobic energy production process. Superoxide anions spontaneously interact with free electrons resulting in the formation of hydrogen peroxide and other hydroxyl radicals. Peroxide and radical generation increases with exercise intensity and may be a potential contributor to muscle fatigue and muscle damage with

strenuous, long duration activity (Reid 2001). Numerous intracellular sources contribute to superoxide anion production as either by-products of metabolism, through enzymatic reactions, as a result of calcium dysregulation, and auto-oxidation of catecholamine's (Reid 2001).

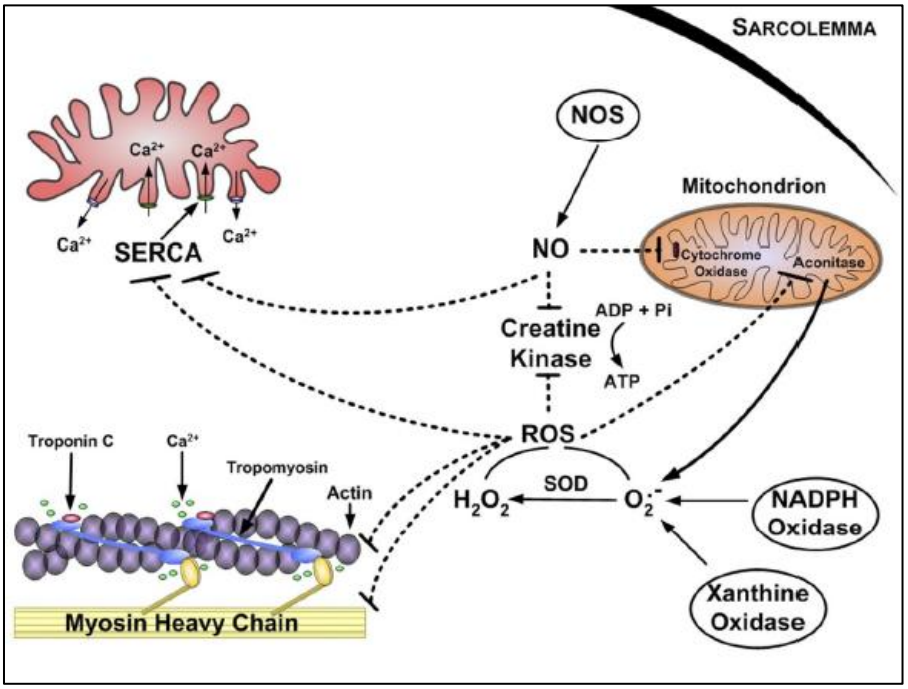

Figure 6. Factors of RONS Production in Skeletal Muscle. Adapted from Powers et al. 2008.

The mitochondrial electron transport chain (ETC) has been implicated as the primary source of RONS production, specifically superoxide anions. Superoxide is most commonly produced in complexes one and three of the ETC with leakage of free electrons in complex three and Q_{10} semiquinone step (Barja 1999; Muller 2004). It has been reported that under eustress conditions 2-5% of total oxygen consumption results in superoxide generation (Boveris 1973; Loschen 1974). During exercise mitochondrial

activity and total oxygen consumption increase triggering a 50-100 fold increase in superoxide production with aerobic exercise (Kanter 1994; Urso 2003). Given a normal cellular antioxidant state, superoxide anions are quickly neutralized to hydrogen peroxide, which has the potential to create toxic hydroxyl radicals. In addition, the rise in RONS may be partially related to excess calcium accumulation during continuous muscle contractions.

The sarcoplasmic reticulum ATPase pump (SERCA) regulates calcium uptake from the cytosol during skeletal muscle contraction. SERCA is a redox sensitive pump, whose function is influenced by RONS induced OS (Grover 1997). Production of RONS from other cellular locations, target the SR and can cause calcium dysregulation and accumulation within the cytosol of skeletal muscle (Reid 2001). Xanthine oxidase (XO), found in skeletal muscle, is an additional source of superoxide anions. Clearly produced during exhaustive exercise (Radak 1995), XO may contribute to the disruption in calcium uptake and the further accumulation in the cytosol (Okabe 1985). In addition, cytosolic calcium accumulation acts as a cell signaling agent in a feed forward mechanism triggering greater XO formation (Radak 1995). This tends to be accentuated with eccentric exercise contractions with enhanced cytosolic calcium (Hellsten 1997).

Nitric oxide (NO) is continuously produced within skeletal muscle and is most commonly associated with muscle mitochondria. NO production increase with skeletal muscle contractions and has been suggested to have a negative impact of muscle force generation and may contribute to cellular calcium dysregulation (Powers 2008). Additionally, NADPH oxidase produced from the sarcoplasmic reticulum and plasma

membranes in skeletal muscle (Xia 2003) and phagocytic cells is well known to generate superoxide anions contributing to OS in muscle (Powers 2008). Finally, catecholamines are commonly released during exercise and increase with the intensity and duration of exercise. Typically, catecholamines are responsible for increasing skeletal muscle oxidative metabolism to enhance energy production (Romijn 1993) However; auto-oxidation of epinephrine has also been reported to produce superoxide anions, often observed in the heart, but may also occur in skeletal muscle (Simpson 1987; Ji 1999).

In summary, due to the contractive nature of skeletal muscle, RONS production is common. Both aerobic and anaerobic mechanisms have been identified in the production of RONS in multiple locations within the muscle cell. The aforementioned factors of RONS production within skeletal muscle are outlined in Figure 6 with specific detail as to where each factor induces free radical production. Acute and chronic exercise accelerates the production of RONS and is exacerbated with longer duration exercise or more intense exercise. The following tables outline the production of RONS with acute (Table 5) and chronic (Table 6) exercise in various skeletal muscles.

Table 5. Markers of Oxidative Stress in the Muscle with Acute Exercise.

Acute Exercise Training	**Muscle**	**Marker of Oxidative Stress**	**Time of Sacrifice**	**Authors**
Exercised for 45, 90, 120, 150min. Loads = 0° at 8.2 m/min, 5° at 15m/min, 10° at 19.3m/min for 15min each until time was reached	Gastrocnemius	>45min H_2O_2 ↑	Immediately post exercise	Wang et al. 2016
Exercised 1hr or 6hrs at 20m/min, 5%	Hind limb	1hr exercise GSSG ↔ 6hr exercise GSSG ↑	Immediately post exercise	Li et al. 2015
Exercised to exhaustion at 24m/min and 15% grade	Soleus and tibialis anterior	TBARS ↑	Immediately post exercise and 1 day post exercise	Radak et al. 1995
Exercised to exhaustion at 1.6km/hr	Vastus lateralis	PC ↔ MDA ↔ GSSG ↔ GSH/GSSG ↔	12hrs post exercise	Lui et al. 2000
Electrical stimulation: 10s of 5V for 10min and a frequency of 100Hz at 1:1	Tibialis anterior	MDA ↑ Nitrite ↑	Immediately post exercise	Paula et al. 2005

The above results are from animal studies.

Table 6. Markers of Oxidative Stress in the Muscle with Chronic Exercise.

Chronic Exercise Training	Muscle	Marker of Oxidative Stress	Time of Sacrifice	Authors
Exercised for 90min/d, 4d/wk, 14wks, at 70-75% VO_{2max}	Gastrocnemius	MDA ↑	62±2hrs post exercise	Strobel et al. 2011
Exercised for 2hrs 5d/wk for 8wks at 1.6kg/hr	Vastus lateralis	MDA ↑ PC ↑ GSSG ↔ GSH/GSSG ↔	12hrs post exercise	Lui et al. 2000

The above results are from animal studies.

Aerobic Exercise and the Antioxidant Defense System

Aerobic exercise is well known to increase the production of cellular RONS (Bloomer 2004; Wang 2016; Li 2015; Morion 2015), although the extent of production is highly dependent on the type of exercise (aerobic vs. anaerobic), current training status, and the duration and/or intensity of exercise performed (Farooqui 2014). Extensive research has been conducted on NrF2 activation in cell culture and various tissues in the presence of OS where RONS and electrophilic molecules have been demonstrated as the key elements in the activation of NrF2 (Bryan 2013; Sandburg 2014; Surh 2008; Jaramillo 2013). When cellular mechanisms enhance NrF2 nuclear concentration, the body responds through evasion of the normal KEAP1-NrF2 pathway. Activity can be classified as either acute or chronic. Acute exercise consists of a single bout of exercise that may result in the production of RONS and antioxidant defense factors (Bloomer

2005). Chronic exercise or exercise training incorporates repeated bouts of exercise by which the body's response to exercise adapts over time.

Exercise induced NrF2 activation via acute and chronic exercise demonstrates key differences in cellular protection against OS through antioxidant expression or changes in antioxidant activity. Furthermore, NrF2 over expression has been demonstrated to increase antioxidant expression with and without pro-oxidant stimulation (Itoh 1999; Kobayashi 2002). This suggests that multiple biological phenomena are responsible for the increase in NrF2 activation. However, NrF2 activation capabilities may be tissue dependent related to the extent of RONS production and the overall stress response.

Skeletal Muscle

Hind limb skeletal muscles, including the gastrocnemius, soleus and plantaris, are normally recruited during locomotion. Aerobic exercise typically engages the gastrocnemius (red fibers) and soleus muscles to a greater extent compared to the plantaris, which is primarily a fast twitch muscle (Armstrong 1985). The amount of stress placed on the muscle during an exercise bout is related to the intensity and duration of the exercise session. In addition, the production of RONS and measurement of markers associated with OS can also indicate the level of stress and the amount of antioxidant capacity of the muscle. It has been well established that aerobic exercise increases RONS production, not only in skeletal muscle, but a variety of tissues (Reviewed in Radak et al. 2001). Both acute and chronic exercise precipitate the generation of RONS, but the extent of production is highly dependent on the amount of stress placed on the exercising muscle and the current training status of the individual.

Radak et al. demonstrated that an acute bout of exercise to exhaustion increased the production of TBARS in soleus muscle and the tibialis anterior muscle (over 200%) and XO in plasma (900%) (Radak 1995). In response to exercise induced RONS production, both the activity and protein concentration of Cu-ZnSOD and MnSOD increased in soleus and tibialis muscles. Furthermore, a second group of animals were dosed with a superoxide dismutase derivative that attenuated the production of RONS relative to control in both the plasma and skeletal muscle with exercise. In summary, increased RONS production with acute exercise increased endogenous antioxidant activity and furthermore was remediated with SOD supplementation. However, long term supplementing with antioxidants has been shown to have a negative impact on performance (Nikolaidis 2012) and may foster reduced activation of NrF2.

Li et al. examined the differences between an acute bout of long duration exercise for 1-hr and an acute exhaustive bout of exercise for 6-hrs (Li 2015). In response to an acute bout of long duration aerobic exercise, skeletal muscle antioxidant gene expression increased up to 12 fold with the exhaustive exercise, but was relatively unchanged with the l-hr exercise. Concomitantly, only the exhaustive exercise demonstrated an increase in GSSG formation and a reduced GSH/GSSG ratio. The associated increase in antioxidant expression with exhaustive exercise was most likely mediated by an increase in NrF2 activation and DNA binding to reduce RONS production. However, there were no changes in markers of RONS concentration, NrF2 concentration or NrF2 DNA binding with the 1hr exercise. This suggests a possible necessity for RONS induced NrF2 activation.

Similarly, Wang et al. detected changes in NrF2, antioxidants, and RONS in the gastrocnemius with individual acute bouts of exceedingly longer duration exercise (45, 90, 120, 150min) (Wang 2016). Exercise beyond 45min increased the production of hydrogen peroxide, and a progressive increase in antioxidant activity, and NrF2 gene expression. These results clearly illustrate the previously mentioned facet that the greater the duration of stress placed on the muscle can result in greater RONS production and response of the antioxidant defense system. Table 7 below summarizes the change in antioxidant expression, activity, and protein concentration with acute aerobic exercise.

Table 7. Antioxidant Activity in the Muscle with Acute Exercise.

Acute Exercise Training	Muscle	Antioxidants	Authors
Acute exercise for either 45, 90, 120, 150min. Loads = 0° at 8.2 m/min, 5° at 15m/min, 10° at 19.3m/min for 15min each until time was reached	Gastroc.	90, 120, 150m/min MnSODactivity ↑ GSH concentration ↑ Cu-ZnSOD activity ↔	Wang et al. 2016
Acute exercise either 1hr or 6hrs at 20m/min and 5% grade	Hind limb	1hr exercise expression SOD1 ↔ SOD2 ↔ GR ↔ GPx ↔ CAT ↑ Ho-1 ↔ GSH ↔ 6hr exercise expression SOD1 ↑ SOD2 ↑ CAT ↑ Ho-1 ↑ GSH ↑	Li et al. 2015
Acute exercise to exhaustion at 24m/min and 15% grade	Soleus and Tibialis Anterior	Muscle Activity Cu-Zn SOD ↑ Mn SOD ↑ GPx↔ CAT ↔ Muscle Concentration Cu-Zn SOD ↑ Mn SOD ↑	Radak et al. 1995

Changes in antioxidant activity listed by brain region. ↔ no change, ↑ increase, ↓ decrease. Animals were sacrificed immediately post acute exercise.

Chronic aerobic exercise is associated with multiple health related benefits; however, it also has the ability to increase RONS production to a greater extent than acute exercise, but in some cases shows greater protection (Bloomer 2005). The excitable

difference with chronic exercise is the associated increase in antioxidant capacity providing extensive cellular protection against RONS induced OS. Chronic aerobic exercise (6-12wks) has been reported to increase antioxidant activity and protein concentration with respect to: GPx, CAT, MnSOD, and total SOD within skeletal muscle (Laughlin 1990; Higuchi 1985).

Strobel et al. reported that 14 weeks of exercise training increased MnSOD protein concentration over 30%, but mRNA expression was unchanged and activity decreased (Strobel 2011). Furthermore, NrF2 protein concentration increased, most likely precipitating the increase in MnSOD protein concentration, providing greater cellular protection. Additionally, mitochondrial biogenesis increased as a result of exercise training, a well know positive outcome of exercise. However, this adaptation was reduced with antioxidant supplementation, suggesting a negative impact on cellular hormesis. Table 8 below summarizes the change in antioxidant expression, activity, and protein concentration with chronic aerobic exercise.

In summary, both acute and chronic aerobic exercise increase NrF2 protein concentration, gene expression, and DNA binding activity. Greater NrF2 activation most likely contributed to the increase in antioxidant activity, gene expression, and protein concentration, most notably observed with longer duration exercise. Therefore, acute exhaustive exercise and chronic exercise training may be demonstrating similar changes in NrF2 and antioxidants through different stress induced adjustments; either as a product of RONS production or as a result of exercise, independent of RONS.

Table 8. Antioxidant Activity in the Muscle with Chronic Exercise.

Chronic Exercise Training	**Muscle**	**Antioxidants**	**Authors**
Chronic exercise for 2hr/d, 5d/wk for 6wks, at 32min/m and 8% grade	Soleus	CAT activity ↑ GPx activity ↑ SOD activity ↔	Laughlin et al. 1990
Chronic exercise for 90min/d, 4d/wk, 14wks, at 70-75% VO_{2max} *	Gastrocnemius	GPx activity ↔ SOD2 expression ↔ SOD2 protein ↑ SOD2 activity ↓	Strobel et al. 2011
Chronic exercise for 2hr/d, 5d/wk, 6wks, at 32min/m and 8% grade	Gastrocnemius	CAT activity ↑ GPx activity ↑ SOD activity ↔	Laughlin et al. 1990
Chronic exercise for 2hr/d, 5d/wk for 12wks at 31m/min and 15% grade	Soleus	Total SOD activity ↑ Mn SOD activity ↑ Cu-Zn SOD activity↔ CAT activity ↔	Higuchi et al. 1989

*Changes in antioxidant activity listed by brain region. ↔ no change, ↑ increase, ↓ decrease. *Animals were sacrificed 62±2hrs post exercise.*

Brain

The brain is a highly aerobic organ and extremely sensitive to changes in oxygen content. During exercise, energy usage increases in the cortex, cerebellum, striatum, hypothalamus, hippocampus and other cortices (Dishman 2006). Aerobic exercise has been shown to increase oxygen extraction in the brain despite little change in cerebral blood flow (Dalsgaard 2003). Furthermore, brain cells, specifically in the hippocampus, have been shown to discharge at a faster rate in conjunction with increases in running velocity (Czurko 1999). It is well known that regular physical activity has many physiological benefits, especially in the brain such as neurogenesis, synaptogenesis, the

production of neurotrophic factors, and gene activation of cytoprotective products (Reviewed in Farooqui 2014). However, there is limited information characterizing the effects of acute exercise on the brain as well as the comparative physiological outcomes between acute and chronic exercise.

Early reports of RONS and antioxidant changes in the brain with exercise are inconclusive. There are multiple reports of increased markers of RONS concentration with exercise, while other studies refute these findings will similar bouts of exercise. Dichotomous findings may be associated with the exercise intensity and or duration utilized as well as the brain region analyzed. Additionally, the effects of acute aerobic exercise in the brain, to date, have only reported changes in markers of RONS concentration and antioxidant activity. To our knowledge, there are no published studies that report changes in NrF2 protein, binding, or expression, as well as antioxidant protein concentration or gene expression with acute exercise. Tables 9 and 10 summarize literature reports of subsequent changes in antioxidants with acute (Table 9) and chronic (Table 10) exercise. The following paragraphs will characterize each study individually, followed by a summary section.

Radak et al. analyzed the hippocampus brain region to compare changes in markers of RONS and antioxidant activity with immobilization and an acute bout of swim exercise for 2-hrs (Radak 2001). TBARS, protein carbonyls, and 8-OHdG were significantly greater in the immobilized group, but were not different after the acute exercise. Additionally, glutamine synthetase decreased in the immobilized group, but was not different with acute exercise. The study reports that acute exercise does not increase

the concentration of RONS and it demonstrates key benefits of physical exercise on the brain as well as inhibiting any cognitive impairment that may be associated with a sedentary lifestyle.

Aguiar et al. demonstrated tissue dependent changes in antioxidants with an acute bout of aerobic exercise (Aguiar 2010). Animals exercised for 2, 20min segments that were separated by 10min. The cortex, hippocampus and striatum were analyzed for GPx and GSH. Antioxidant activity of GPx increased over 30% in the cortex and hippocampus, but not the striatum after the exercise. However, GSH content increased over 40% in the striatum and cortex, but decreased in the hippocampus. Changes in markers of RONS concentration were not measured. This study suggests that particular regions of the brain may be activated to a greater extent than others during exercise. In addition, some brain regions may contain greater amounts of one antioxidant than other.

Interestingly, Aksu et al. reported no changes in GPx activity in response to a 1-hr acute exercise to various intensities (10, 15, 20m/min or exhaustive) in either the hippocampus, striatum, or cortex brain regions (Asku 2009). However, SOD activity increased by 70% in the striatum only during the 20m/min exercise intensity compared to the non-exercising group. In relation to the previous study, this suggests that not only do antioxidants differ by brain region, but the intensity of exercise may be a key factor influencing cellular antioxidant activity. Despite minimal change with exercise, these results comparatively reflect the absence of RONS accumulation during the exercise bout. Therefore, the stress imposed may not have been challenging enough in the brain or due to the already high concentration of antioxidants, RONS accumulation was

suppressed. Aksu and colleagues also compared acute exercise to chronic exercise training and found similar results.

Table 9. Antioxidant Activity in the Brain with Acute Exercise.

Acute Exercise Training	**Antioxidants**	**Authors**
Acute exercise for 1hr at 10, 15, 20m/min or to exhaustion	Hippocampal Activity SOD ↔ GPx ↔ Striatal Activity SOD ↑ GPx ↔ Cortex Activity SOD ↔ GPx ↔	Asku et al. 2009
Acute swimming exercise for 2hr at 32°C	Hippocampal Activity GS ↔	Radak et al. 2001
Acute exercise with 2, 20min bouts separated by 10min recovery	Cortex Activity GPx ↑ NPSH ↑ Hippocampal Activity GPx ↑ NPSH ↓ Striatal Activity GPx ↔ NPSH ↑	Aguiar et al. 2010

Changes in antioxidant activity listed by brain region. ↔ no change, ↑ increase, ↓ decrease. All animals were sacrificed immediately post acute exercise.

Chronic exercise has been reported to show similar responses to that of acute exercise in relation to antioxidant activity and RONS concentration. Asku et al. reported that 1-hr exercise sessions for 8wks at varying exercise intensities demonstrated no change in RONS concentration and little change in antioxidant activity, similar to that of acute exercise (Asku 2009). No changes in antioxidant activity were observed in the

hippocampus, but SOD activity increased in the striatum only, and GPx activity increased in the cortex only. This study suggests that repeated bouts of exercise may have favorable effects on some brain regions, like hippocampus, by decreasing OS, negating the need for increased antioxidant activity.

Somani et al. reported similar findings in the hippocampus, cortex, and striatum. In addition, they measured the brainstem after chronic exercise training and found increased GSH concentration and SOD activity (Somani 1995). Furthermore, RONS production in the form of GSSG was increased in the striatum only. Therefore, chronic exercise was demonstrated to promote favorable effects within specific brain regions. However, it should also be noted that detection of RONS and cellular antioxidants is highly dependent on assay procedures and equipment. This may be a fundamental reason for the discrepancy between experimental findings.

Similar to the production of RONS and antioxidants observed with exercise in tissues like skeletal muscle, brain production of these may also be a reflection of the intensity of exercise and subsequent amount of stress placed on the body during an exercise session. Devi et al. noted increased antioxidant activity in GPx, SOD, and CAT in the hippocampus and cortex brain regions with swimming exercise (Devi 2004). Swimming has been shown to have a greater effect on stress compared to forced treadmill exercise (Aguiar 2007). Furthermore, most swimming studies have animals swim with 3% of their body weight tied to their tail, imposing an even greater intensity of exercise and possible perceived stress.

To date, only 2 studies have reported changes in NrF2 activation in the brain with exercise. Moiron et al. demonstrated increased whole brain NrF2 protein concentration with 12wks of chronic exercise (Moiron 2015). However, only CAT activity increased and there were no changes in RONS concentration. Tsou et al. assessed striatal NrF2 with only 4wks of exercise training in an MPP toxicity model. NrF2 binding activity increased by 200%along with total GSH concentration and HO-1 expression in the control group and loss of total GSH was attenuated in the MPP toxicity group (Tsou 2015). With only 2 studies available and only 1 individual brain region observed, conclusions regarding NrF2 adaptations with chronic exercise are negligible.

Table 10. Antioxidant Activity in the Brain with Chronic Exercise.

Chronic Exercise Training	**Antioxidants**	**Authors**
Chronic exercise 3-4d/wk, at 35cm/s for 12wks Sacrificed: same day	Whole Brain Activity Mn-SOD ↔ Cu-Zn SOD ↔ CAT ↑	Moiron et al. 2015
Chronic swimming exercise 30min/d for 12 wks with 3% of BW tied to tail Sacrificed: same day	Hippocampal Activity GPx ↑ SOD ↑ CAT ↑ Cortex Activity GPx ↑ SOD ↑ CAT ↑	Devi et al. 2004
Chronic exercise for 30min/d at 8-54m/min and 6-10° grade for 7wks Sacrificed: 18hrs post	Cortex Activity SOD ↔ GPx ↔ GSH ↔ Brainstem Activity SOD ↑ GPx ↔ GSH ↑ Striatal Activity SOD ↑ GPx ↓ GSH ↔ Hippocampal Activity SOD ↔ GPx ↔	Somani et al. 1995
Chronic exercise for 1hr/d, 5d/wk, for 8wks at 10, 15, or 20m/min Sacrificed: 3d post	Hippocampal/Striatal Activity SOD ↓ GPx ↔ Cortex Activity SOD ↓ GPx ↑	Asku et al. 2009

Changes in antioxidant activity listed by region. ↔ no change, ↑ increase, ↓ decrease.

Given, NrF2's suggested protective properties and abundant expression within the brain, it has been indicated as primary means for neuroprotection. In a Parkinson's induced rat model, 4wks of exercise training was demonstrated to have neuroprotective effects in the striatum by not only maintaining antioxidant levels, but preventing a disease associated decline in NrF2 and GSH concentration compared to controls (Tsou 2015). Aguiar et al. demonstrated increased NrF2 mRNA expression (150 fold) and antioxidant HO-1 protein concentration with 6wks of exhaustive exercise training in the striatum (Aguiar 2016). Therefore, NrF2 mediated changes in antioxidant gene expression and subsequent increases in antioxidant concentration are the primary factors necessary for neuroprotection. Greater concentration of antioxidants in the brain necessitates improved antioxidant activity in times of RONS induced OS.

In summary, there is both limited and conflicting evidence on the effects of acute and chronic exercise on NrF2 and antioxidant changes in the brain. Furthermore, there is limited verification of markers of RONS concentration and or the extent of RONS accumulation with exercise in the brain. It is the intent of this study proposal to tease out the effects of acute and chronic exercise on NrF2 gene expression and protein concentration as well as changes in antioxidant gene expression within multiple brain regions that have been associated with OS induced neurodegeneration.

Aerobic Exercise and BDNF

Exercise has been demonstrated as an effective treatment to increase BDNF concentration. Specifically, low intensity exercise significantly increases BDNF concentration to a greater extent than moderate intensity exercise in the hippocampus

(Table 11) (Soya 2007). Hippocampal BDNF expression has also been reported to increase as a result of acute "severe" exercise stress, but not in response to moderate intensity exercise (Huang 2005). Furthermore, 3 days of moderate intensity exercise demonstrated similar increases in BDNF concentration compared to a single bout of "severe" exercise (Table 11) (Huang 2005), suggesting intensity and training dependent effects on BDNF production. Elevated serum concentrations of BDNF immediately following a graded exercise test also reflect the importance of intensity (Ferris 2007). There also appears to be a duration dependent effect of exercise with longer exercise bouts contributing to a greater elevation in BDNF concentration (Rasmussen 2009).

The chronology of BDNF upregulation within tissue and blood in response to acute and chronic exercise also appears to be time dependent. Given sufficient intensity and exercise duration, BDNF mRNA expression and protein concentration have been report to peak in the cortex and hippocampus 2hr post-acute exercise and remain elevated as long as 5 to 6-hrs post-acute exercise (Rasmussen 2009; Huang 2011; Soya 2007). Chronic exercise has also been indicated to increase BDNF expression as late as 36-hrs post exercise in both cerebral cortex and hippocampus brain regions (Seifert 2009). In serum, BDNF concentration has been reported to increase as early as 60min post exercise and remain elevated 90min post exercise (table 7) (Griffin 2011).

Table 11. BDNF Concentration in the Blood and Brain with Exercise.

Treatment	Concentration/ Expression	Time of Sacrifice/Sampling	Authors
Acute 30min run; sub-LT 15m/min and supra-LT 25m/min	All Hippocampus Sub-LT [BDNF] ↑ vs. pre	Immediately post exercise	Soya et al. 2007
<u>Human Study</u> Exercise trained (3mo). Pre and post training incremental cycling for 4min at 60%, 70%, 80%, 90%, and 100% of $VO_{2\ max}$ with 6min recovery between <u>Mouse Study</u> Exercise trained 1hr/d, 5d/wk for 5wks at 10% grade, up to 16.7m/min	<u>Human Study</u> Jugular Vein Blood [BDNF] ↑ with training Arterial Blood [BDNF] ↑ vs. rest <u>Mouse Study</u> Hippocampus [BDNF] ↑ Cerebral Cortex [BDNF]↔	<u>Human Study</u> 48-hrs post last training session <u>Mouse Study</u> 36-hrs post last training session	Seifert et al. 2009
<u>Human Study</u> Acute continuous 4hr ergometer row at ≤10-15% LT <u>Mouse Study</u> Acute continuous 2hr run at 18m/min, 10% grade	<u>Human Study</u> Jugular Vein Blood [BDNF] ↑ at 4hrs Arterial Blood [BDNF] ↑ at 4hrs <u>Mouse Study</u> Cerebellum BDNF mRNA ↔ Hippocampus BDNF mRNA↑2,6hrs Cerebral Cortex BDNF mRNA↑2,6hrs	<u>Human Study</u> 0hr, 2, and 4hrs, and 1hr post <u>Mouse Study</u> 0hr, 2, 6, or 24hrs post exercise	Rasmussen et al. 2009
Exercise trained 5d/wk, 60min/d,	All Hippocampus	<u>Exercise Trained</u> 2hr or 2d post	Huang et al. 2005

at 70% VO_{2max} for 4wks. Final 2d Morris water maze Acute moderate exercise at 15m/min for 30min for 1 or 3d. Acute intense exercise up to 24m/min to exhaustion	<u>Exercise Trained</u> BDNF mRNA↑2hrs post [BDNF]↑2hrs post <u>Acute Moderate Exercise</u> [BDNF]↑ <u>Acute Intense Exercise</u> BDNF mRNA↑ [BDNF]↑ <u>Acute Intense Exercise w/no familiarization</u> BDNF mRNA↓ vs. CON [BDNF]↓ vs. CON	<u>Acute Exercise</u> 2hrs post	
Acute graded exercise test to exhaustion on a cycle ergometer Exercise trained 3d/wk for 60min at 60% VO_{2max} for 3wk or 5wk	All Serum <u>Acute Exercise</u> [BDNF]↑ 60min post <u>Exercise Trained 5wks</u> [BDNF]↑ 90min post <u>CON post 3wks & 5wks</u> [BDNF]↑ 60, 90min post	0hr and 30min pre, then 60 and 90min post	Griffin et al. 2001
Acute graded exercise test to exhaustion on a cycle ergometer Acute exercise for 30min on a cycle ergometer at 20% below VT or 10% above VT	All Serum <u>Acute Graded Exercise</u> [BDNF]↑ post <u>20% Below VT</u> [BDNF] ↔ <u>10% Above VT</u> [BDNF]↑ post	Immediately pre and post	Ferris et al. 2007

Despite neuronal activation and redistribution of blood flow to multiple brain regions during exercise, the cerebral cortex and hippocampus are the only two regions that have been reported to increase BDNF concentration. The cerebellum is typically activated during exercise as it plays a role in motor response and coordination; however, it has not been shown to increase BDNF concentration in response to stress (Rasmussen 2009). Therefore, BDNF concentration is not only specific to the intensity and duration of exercise, but may be dependent on the brain region of activated. It has been suggested that more complex motor learning tasks impart a greater stress compared to moderate intensity exercise or familiar tasks in regards to BDNF secretion (Klintsova 2004). Finally, the protective role of BDNF typically occurs during the recovery period in response to stress with transient increases leading to a cumulative effect of elevated protein concentration.

Overall Conclusions

In conclusion, the role of NrF2 in the body is essential to maintaining antioxidant defense capacity and reducing OS. Aerobic exercise has been demonstrated to increase the concentration of NrF2 as well as antioxidant factors and cytoprotective agents in the brain and skeletal muscle. In alignment with this information, we and others conclude that behavioral modifications like exercise, which have been shown to increase NrF2 activation, may be important avenues to reduce OS, increase cognitive factors, and prevent neurodegenerative diseases (Sandburg 2014). In addition, it is also pertinent that we distinguish between acute and chronic exercise responses and their impact on neuroprotection (Dishman 2006). Therefore, the purpose of this study is 1) to determine

the effect of a single bout of acute aerobic exercise on NrF2 protein concentration and markers of oxidative stress in the brain and skeletal muscle, 2) to determine the effect of chronic aerobic exercise on NrF2 protein concentration and antioxidant factors in the brain and skeletal muscle, and 3) to compare changes in NrF2, antioxidant factors, and markers of OS between the skeletal muscle and individual brain regions.

CHAPTER III

METHODS

Animals

Male, Sprague Dawley rats (N=32), 4 weeks of age, were randomly selected into one of three groups, sedentary (SD) (n=13), exercise trained (ET) (n=13) or acute exercise (AE) (n=5). Sample sizes were determined using Gpower3 (P=0.8). Animals were housed individually in standard caging with a 12:12 light/dark cycle with *ad libitum* access to water and food. All animals were fed a standard chow diet. Upon arrival and prior to testing, the animals were acclimated to the animal research facility for a minimum of 4 days. Following initial acclimation, the animals were habituated to the motorized treadmill using brief exposure training for 1 week. After habituation, ET animals were aerobically trained for 5-7 weeks on a motorized treadmill. AE animals performed a single bout of exercise on the treadmill, similar to a single ET session and SD animals were placed on a non-operational treadmill and were allowed to engage in regular daily activities. The University of North Carolina Greensboro Institutional Animal Care and Use Committee (IACUC) approved all procedures and experiments prior to animal testing.

Exercise Training Protocol

ET (n=13) animals were trained for 5-7 weeks with a 1 week habituation period (week 0) on a motorized treadmill. A ramp up protocol was implemented for weeks 1-4

to gradually increase the speed and duration of the exercise to 30m/min for 60min (Table 12). All animals consistently exercised at a 6° grade (equal to 10.5% grade) and an intensity equal to 70-75% of VO_{2max} (Cartee 1987). AE animals (n=5) were acclimated to the animal facility and habituated to the treadmill in a similar manner as the ET group prior to performing a single bout of exercise for 60 minutes at 20m/min up a 6° grade (equal to 10.5% grade). The single bout of acute exercise was equal to an intensity of 70-75% VO_{2max} (Cartee 1987) and the duration mimicked that of the ET animals. SD animals were placed on a non-operational treadmill to simulate an environment similar to the exercised animals.

Table 12. Ramp Protocol. Ramp treadmill protocol for 5-7 weeks of training with 1 week habituation performed at a constant 6° grade (equal to 10.5% grade).

Week:Day	Monday	Tuesday	Wednesday	Thursday	Friday
Week 0	**10m/min, 5min**	**15m/min, 5min**	**15-20m/min, 10min**	**20m/min, 10min**	**20m/min, 15min**
Week 1	**20m/min, 15min**	**20m/min, 25min**	**20-25m/min, 30min**	**20-25m/min, 30min**	**20-25m/min, 30min**
Week 2	**25m/min, 30min**	**25m/min, 30min**	**25m/min, 35min**	**25m/min, 40min**	**25m/min, 45min**
Week 3	**25m/min, 50min**	**25m/min, 55min**	**25m/min, 60min**	**26m/min, 60min**	**27m/min, 60min**
Week 4	**28m/min, 60min**	**29m/min, 60min**	**30m/min, 60 min**	**30m/min, 60 min**	**30m/min, 60 min**
Week 5-7	**30m/min, 60 min**	**30m/min, 60 min**	**30m/min, 60 min**	**30m/min, 60 min**	**30m/min, 60 min**

Tissue Collection

After completion of the treatment, immediately after exercise (AE) or approximately 5hrs from the last exercise session (ET, SD) the animals were anesthetized using Pentobarbital and then once sedated (approximately 3-5min) were euthanized by guillotine. Pentobarbital was chosen to reduce stress associated with euthanasia and has been shown to suppress free radical production and synergistically increase antioxidant abilities (Ureda 2007). Immediately following euthanasia, tissue samples were collected and placed into a container under dry ice. Brain regions including the cortex, cerebellum, and hippocampus were dissected, separated and sealed into collection tubes. Skeletal muscle tissue from the gastrocnemius was collected and placed into collection tubes. Whole blood was collected and a small amount was aliquoted into 5% 5-sulfosalicylic acid and vortexed and centrifuged and the remaining blood was centrifuged for plasma. Plasma and treated blood were placed into collection tubes. All collection tubes were immediately stored at -80°C until further analysis.

Sample Preparation

Total protein fractions were obtained for each brain region (cortex, cerebellum, hippocampus) and skeletal muscle (gastrocnemius) tissue through suspension in RIPA buffer (50mM Tris, 1mM EDTA, 150mM NaCl, 1% NP-40, 1% Sodiumdeoxycholate, 1% SDS, pH 7.4) with protease inhibitors (Aprotinin, Leupeptin, E-64, Pepstatin A, Phenanthroline, PMSF) and phosphatase inhibitors (Sodium Orthovanadate, Sodium Fluoride, β-glyderophosphate). Samples were homogenized using an electric mixer and then sonicated (Sonic Dismembrator-50) for 20s, followed by centrifugation (Beckman

Allegra X-15R) at 5250rcf (4756rpm) for 30min at 4°C. Tissue homogenates were aliquoted into separate collection tubes for NrF2, MnSOD, BDNF, and MDA and stored at -80°C until further analysis. Glutathione preparation will be explained in detail in the chromatography section.

Protein Determination

Prior to analysis, all cell lysates were analyzed for protein concentration using the bicinchoninic acid assay technique (BCA). An aliquot of homogenate (3ul) was placed in an Eppendorf tube and mixed with deionized water (22ul) for a total volume of 25ul. Standards were prepared from serum bovine albumin in a serial dilution. BCA working solution (500ul) was added to each tube and left to incubate for 30min at 37°C. Each sample was read on a microplate reader (Gen5) at a wavelength of 562nm and compared against know standards. All samples were performed in duplicate.

Western Blot

Tissue homogenates containing 60ug protein from brain and skeletal muscle were electrophoresed on a 10% Bis-TrisSDS-PAGE gel and then transferred to a polyvinylidene difluoride (PVDF) membrane for 6 hours at 4°C. The membrane was blocked with 5% non-fat powdered dry milk dissolved in Tris buffered saline Tween-20 (TBST) for 1 hour at room temperature. Subsequently, the membrane was probed with a polyclonal rabbit anti-NrF2 antibody in 5% BSA (1:250) (Abcam; ab31163) overnight at 4°C. After the overnight incubation, the membrane was washed 3X (10min) in 5% TBST milk at room temperature. The membrane was further incubated with a goat anti-rabbit immunoglobulin G secondary (1:2000) (Abcam; ab97080) in 5% milk for 1 hour at room

temperature. The membrane was wash 4X (10min) in TBST at room temperature. The bands were detected using chemiluminescence (Bio-Rad) and quantified by densitometry using Bio-Rad software. The bands were normalized relative to anti beta-actin expression (arbitrary units) (Abcam; ab8229). Differences were determined by comparing the band intensity to the control group.

Cytochrome c Oxidase Activity

The activity of cytochrome *c* oxidase was determined at 37°C using a Clark-type oxygen electrode following the methods described previously (Mitchell 2002). Homogenates (30 μl) were treated with Triton X-100, then added to 1.47 ml of assay medium (50mM K_2HPO_4, 0.1mM EDTA, 0.62mM tetramethylpentadecane, 12.5mM sodium ascorbate, and 0.04mM cytochrome *c*, pH 7.4) and centrifuged at 600*g* for 10min. Supernatants, prepared in duplicate, were used to determine the activity of cytochrome c oxidase spectrophotometrically. The reduced (2 mM cyanide)-oxidized spectrum at 605–630 nm with an extinction coefficient of 10.8 mM/cm was used to calculate the concentration.

Superoxide Dismutase Activity

The activity of SOD was measured at 25°C using xanthine oxidase as a substrate. Tissue homogenate (10-40ul) was added to a mixture containing 1.3ml of a cytochrome c solution (50mM KH2PO4, 0.1mM EDTA, 0.05mM xanthine, 0.01mM cytochrome c pH 7.8) and 10ul of potassium cyanide to inhibit Cu-Zn-SOD. To begin the reaction, 10ul of xanthine oxidase was added to the mixture and absorbance of Mn-SOD was read at 30sec and 150sec at a wavelength of 550nm on a spectrophotometer (Varian Cary 50 Bio UV-

spectrophotometer). One unit of activity was equal to the amount of SOD required to result in a 50% inhibition of baseline activity of cytochrome c. Each sample was performed in duplicate.

Chromatography

High pressure liquid chromatography (HPLC) was used to measure glutathione in both the oxidized (GSSG) and reduced (GSH) forms to obtain a total glutathione measurement (TGSH) in brain tissue and whole blood samples. Brain tissue homogenates (50ul) were added to 450ul of a 5% 5-sulfosalicylic acid solution, vortex mixed, and centrifuged at 11,000rpm for 10min at room temperature (Benani 2007). Whole blood samples were vortex mixed in a 1:1 ratio with 10% 5-sulfosalicylic acid and centrifuged at 10,000rpm for 10min at 4°C for supernatant collection. Each sample (20ul), performed in duplicate, was injected into the HPLC (Shimadzu) and compared to known standards. All samples were analyzed on a SD-30DS column (3.0X100mm) with a 0.1M Phosphate buffer (pH 2.5) mobile phase run at a flow rate of 0.4 ml/min at 25°C. Peaks for GSH and GSSG were identified using the LC software (LC Solutions) and compared to their respective standards and total glutathione concentration was calculated automatically.

Lipid Peroxidation

Malondialdehyde (MDA) was analyzed in blood plasma and brain tissue samples using a colorimetric assay kit from Oxis (BIOXYTECH MDA-586) per manufacturer's instructions. MDA standards and unknown samples were prepared in duplicate following the tissue sample preparation procedures explained previously. Each tube contained 10ul of probucol, 200ul of prepared standard or unknown sample, 640ul of reagent 1 (R1) and

150ul of reagent 2 (R2). Samples were incubated for 60min at 45°C then centrifuged for 10min at 10,000 X g for supernatant collection. The clear supernatant was transferred to a 96-well plate with absorbance detected at 586nm (Gen5). MDA concentration was determined using the following equation:

$$(\text{MDA}= \frac{Absorbance-Intercept}{Slope} x\ Dilution\ factor).$$

Statistical Analysis

All data was analyzed using SPSS version 24.0 (Chicago, IL). Gpower3 was used to determine the minimum sample size required (N=32) to analyze the effects of treatments in the population (P=0.8, f=0.4). Independent T-test's were used to determine differences between AE vs. SD or ET vs. SD with respect to NrF2, antioxidant factors, and markers of oxidative stress in the skeletal muscle, blood (AE only) and each brain region. A Pearson's Correlation was implemented to compare the relationship between skeletal muscle and each brain region with respect to NrF2, antioxidant factors, and markers of oxidative stress. All data is reported as the mean ± SEM. The significance level was set a-priori at the 0.05 level.

CHAPTER IV

RESULTS

The results section is organized as follows: Animals, Markers of Oxidative Stress in the blood, skeletal muscle and brain, Antioxidant Factors in the skeletal muscle and brain, and Nuclear Factor Erythroid 2 Related Factor 2 (NrF2) concentration with acute and chronic exercise. All the values presented in this section are presented as the mean ± the standard error of the mean (SEM).

Animals

Male, Sprague Dawley rats were ordered in two groups from Charles River Labs. Animals designated for chronic exercise, originally weighing 150-200g, were randomly assigned into either exercise trained (ET) (n=13) or sedentary control (SD) (n=13). Animals designated for acute exercise (AE) (N=10), originally weighing 250-275g, 10-12 weeks of age, were randomly assigned into either acute exercise (AE) (n=5) or sedentary control (SD) (n=5). Acute animals were ordered heavier to match the chronic exercise group weight at the time of sacrifice. ET animals were aerobically trained for 5-7 weeks for 60min/day, five days/week at 30m/min at 6° grade. AE animals performed a single bout of exercise similar to a single ET session for 60min at 20m/min at 6° grade. At the end of the experimental period, prior to sacrifice, all animals were weighed. There were no significant differences in body weight in either the chronic exercise (ET = 370.60 ± 8.9, SD = 392.19 ± 14g) or acute exercise groups (AE = 339.08 ± 5.6, SD = 349.02 ±

7.4g). At five hours after the final exercise training bout (ET) or immediately after the acute bout of exercise (AE), the animals were sacrificed by injection of Pentobarbital and the brain regions: cortex, hippocampus, and cerebellum, and skeletal muscle gastrocnemius, were rapidly removed.

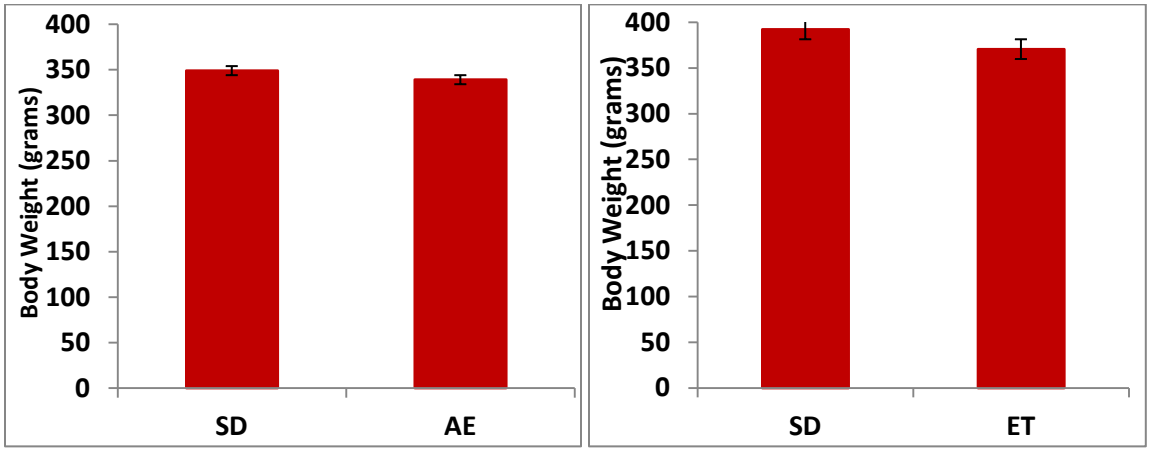

Figure 7. Final Body Weight. All values are displayed as the mean ± SEM. Non-significant (NS) using an independent T-test to compare ET to AE or SD.

Cytochrome c Oxidase

To determine if 5-7 weeks of chronic aerobic exercise elicited a training adaptation in the animals, cytochrome c oxidase activity was measured in the plantaris, soleus, white gastrocnemius, red gastrocnemius and whole gastrocnemius skeletal muscles. Cytochdrome c oxidase activity was significantly increased with 5-7 weeks of chronic aerobic exercise in all the skeletal muscles compared to sedentary controls (the data is not shown).

Blood Markers of Oxidative Stress

A primary aim of this study was to determine if acute aerobic exercise significantly increased markers of oxidative stress including malondialdehyde (MDA), oxidized glutathione (GSSG) and the ratio of GSSG to total glutathione (TGSH), the GSSG/TGSH ratio. In order to validate that acute exercise stimulates oxidative stress, blood plasma was examined for MDA, GSSG, and the GSSG/TGSH ratio. A single bout of acute aerobic exercise significantly increased plasma MDA concentration (AE = 3.31 ± 0.38, SD = 1.95 ± 0.22 uM) compared to sedentary controls. However, there were no significant differences in GSSG concentration (AE = 0.272 ± 0.029, SD = 0.292 ± 0.008 mM) and the GSSG/TGSH ratio (AE = 12.52 ± 0.56, SD = 11.38 ± 1.48 %) with acute exercise. The results are shown below.

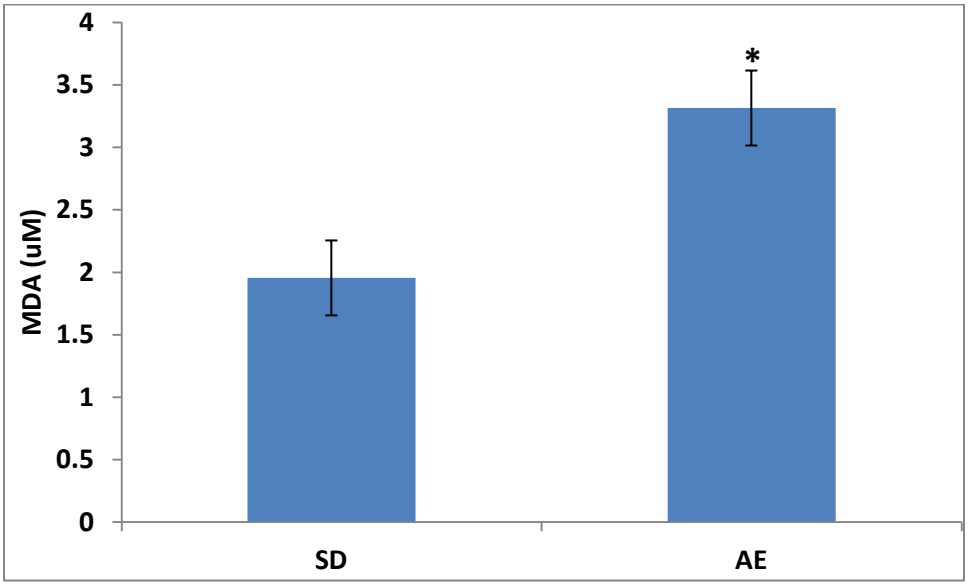

Figure 8. Plasma Concentration of MDA. All values are displayed as the mean ± SEM. The * denotes significance at $p \leq 0.05$ using an independent T-test to compare AE to SD.

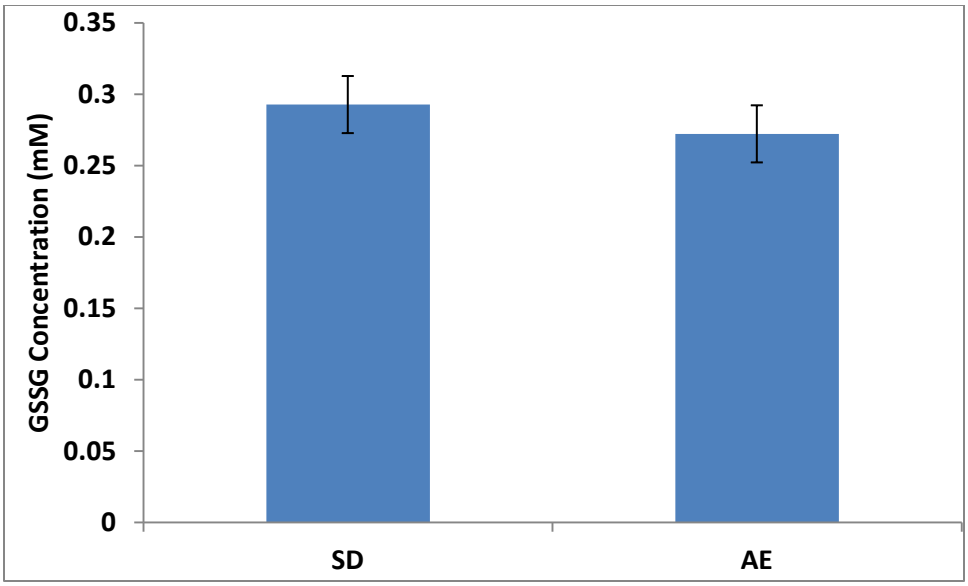

Figure 9. Plasma Concentration of GSSG. All values are displayed as the mean ± SEM. Non-significant (NS) using an independent T-test to compare AE to SD.

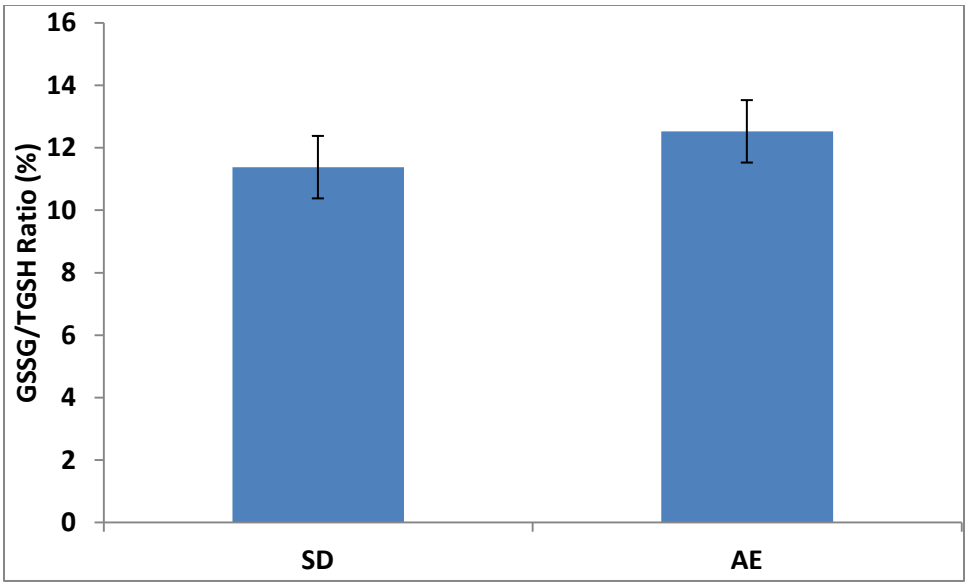

Figure 10. Plasma Concentration of GSSG/TGSH ratio. All values are displayed as the mean ± SEM. Non-significant (NS) using an independent T-test to compare AE to SD.

Tissue Markers of Oxidative Stress

Additionally, this study sought to understand the effect of acute aerobic exercise on markers of oxidative stress in specific tissues. Markers of oxidative stress, MDA, GSSG, and the GSSG/TGSH ratio were examined in the brain regions: hippocampus, cortex, and cerebellum. The results are listed in Table 13. Gastrocnemius skeletal muscle was also examined for markers of oxidative stress. There were no significant differences in muscle MDA concentration (AE = 0.20 ± 0.03, SD = 0.28 ± 0.06 umol/mg protein), GSSG concentration (AE = 0.55 ± 0.16, SD = 1.28 ± 0.34 umol/mg protein) or the GSSG/TGSH ratio (AE = 0.31 ± 0.09, SD = 0.27 ± 0.05 %) with acute exercise.

Table 13. Acute Exercise and Markers of Oxidative Stress in the Brain.

	Cerebral Cortex	**Hippocampus**	**Cerebellum**
	Sedentary	**Sedentary**	**Sedentary**
	Exercise	**Exercise**	**Exercise**
GSSG (umol/mg protein)	10.81±0.22 4.10±0.27*	4.35±0.24 4.60±0.49	8.38±0.41 3.55±0.44*
GSSG/TGSH (%)	3.21±0.03 2.85±0.21	3.07±0.21 2.60±0.29	2.64±0.32 2.63±0.10
MDA (umol/mg protein)	1.89±0.08, 0.78±0.07*	0.42±0.03 0.58±0.06*	1.16±0.12 0.76±0.05*

Values are presented as the mean ± SEM; sample sizes for each variable n = 5 for all.
* $P < 0.05$ main effect of exercise.

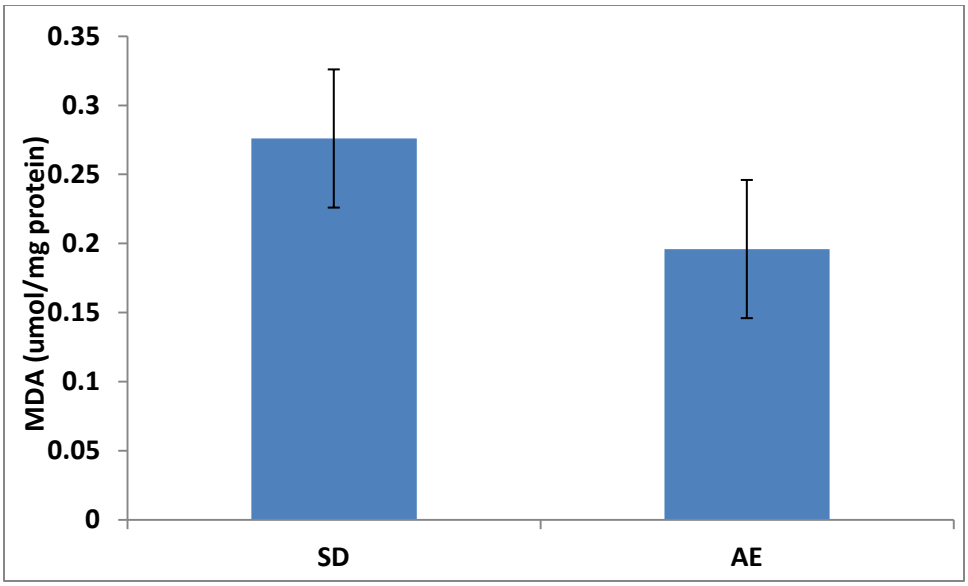

Figure 11. Muscle Concentration of MDA. All values are displayed as the mean ± SEM. Non-significant (NS) using an independent T-test to compare AE to SD.

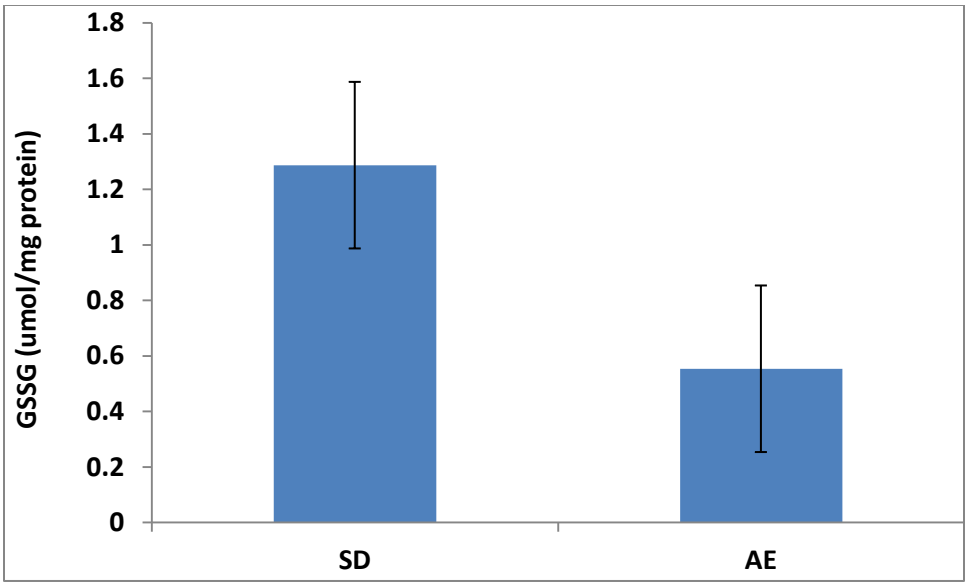

Figure 12. Muscle Concentration of GSSG. All values are displayed as the mean ± SEM. Non-significant (NS) using an independent T-test to compare AE to SD.

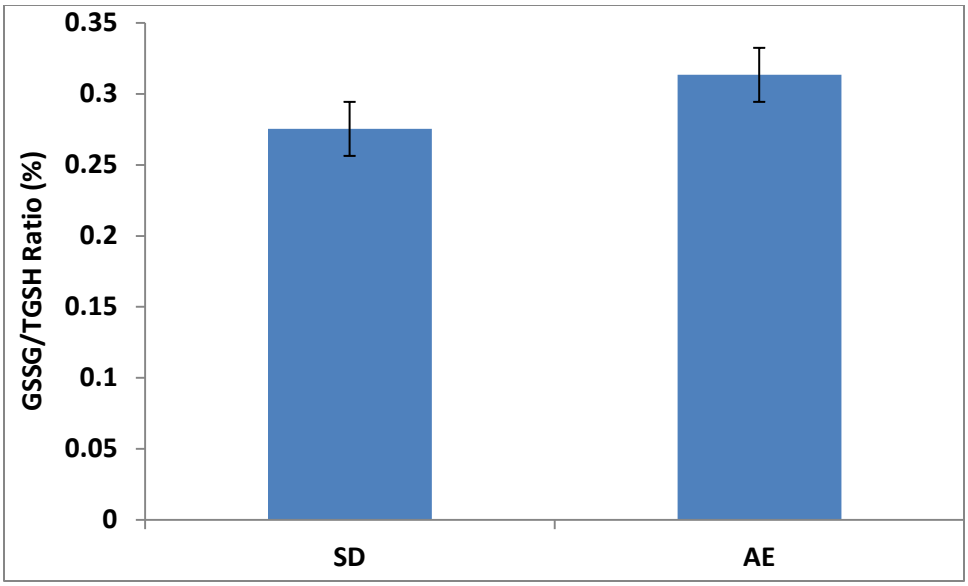

Figure 13. Muscle Concentration of GSSG/TGSH ratio. All values are displayed as the mean ± SEM. Non-significant (NS) using an independent T-test to compare AE to SD.

Oxidative Stress Correlations between Muscle and Brain

A key area of interest in this study was to determine the relationship between skeletal muscle and brain tissue with respect to markers of oxidative stress, MDA, GSSG, the GSSG/TGSH ratio. A Pearson's Correlations was used to examine the relationship between skeletal muscle gastrocnemius and each brain region. There were no significant correlations between skeletal muscle gastrocnemius and individual brain regions in regards to MDA concentration (CT vs. M r = 0.118; HP vs. M r = -0.569; CB vs. M r = 0.121), GSSG concentration (CT vs. M r = 0.329; HP vs. M r = 0.627; CB vs. M r = 0.702), and the GSSG/TGSH ratio (CT vs. M r = 0.029; HP vs. M r = 0.296; CB vs. M r = 0.572). The results are shown below.

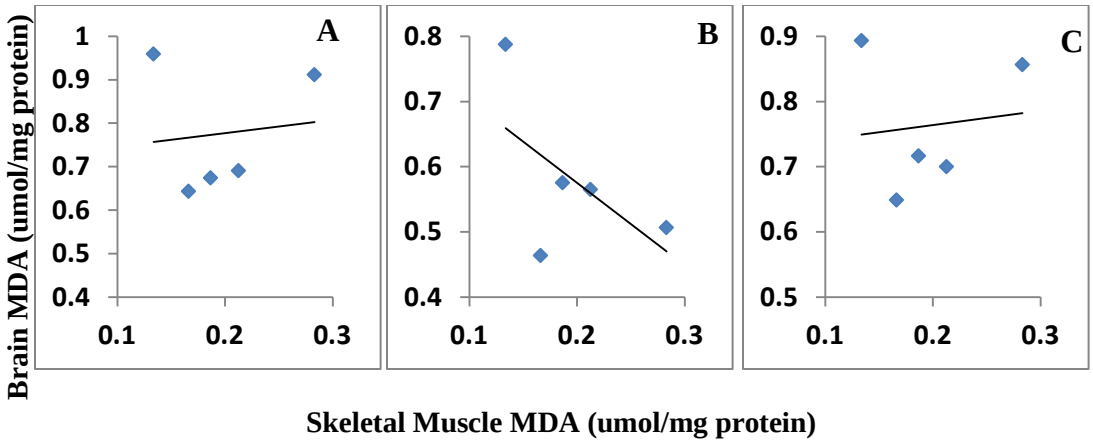

Figure 14. Correlations between Muscle and Brain [MDA].
A) Cortex, B) Hippocampus, C) Cerebellum.

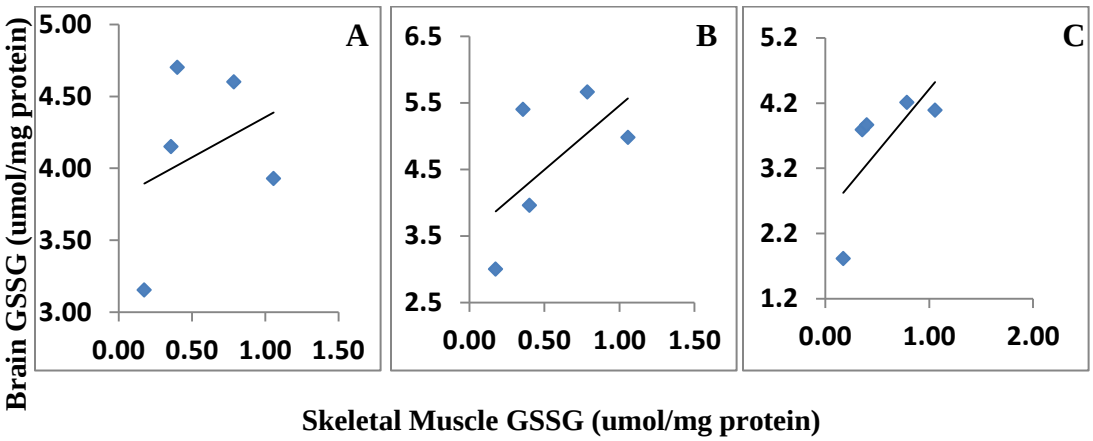

Figure 15. Correlations between Muscle and Brain [GSSG].
A) Cortex, B) Hippocampus, C) Cerebellum.

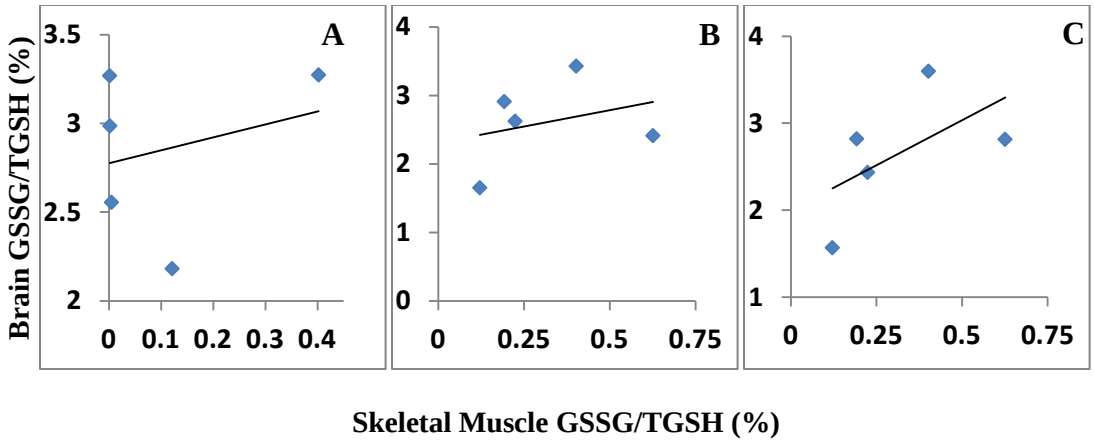

Figure 16. Correlations between Muscle and Brain GSSG/TGSH. A) Cortex, B) Hippocampus, C) Cerebellum.

Antioxidant Factors

A second aim of this study was to understand the effect of chronic aerobic exercise on antioxidant factors. Multiple antioxidant factors were examined including manganese superoxide dismutase (Mn-SOD), reduced glutathione (GSH), and total glutathione (TGSH) in the brain regions: cortex, hippocampus, and cerebellum. The results are listed below in Table 14. Skeletal muscle gastrocnemius was also examined for the Mn-SOD, GSH, and TGSH with chronic aerobic exercise. Chronic aerobic exercise demonstrated a significant decrease in Mn-SOD (ET = 30.85 ± 1.33, SD = 55.85 ± 2.04 units/mg protein) activity, and GSH (ET = 355.38 ± 16.44, SD = 475.29 ± 21.02 umol/mg protein), and TGSH (ET = 357.29 ± 16.68, SD = 477.30 ± 21.12 umol/mg protein) concentration in skeletal muscle gastrocnemius. The results are shown below in Figures 17, 18, and 19. In regards to Mn-SOD activity, all brain regions including the cortex, hippocampus, and cerebellum (Table 14) exhibited greater overall activity in the

sedentary animals and with chronic aerobic exercise compared to skeletal muscle gastrocnemius.

Table 14. Chronic Exercise and Antioxidant Factors in the Brain.

	Cerebral Cortex	Hippocampus	Cerebellum
	Sedentary	**Sedentary**	**Sedentary**
	Exercise	**Exercise**	**Exercise**
TGSH (umol/mg protein)	229.57±28.60	135.54±4.89	331.65±15.66
	243.02±26.39	182.76±4.64*	300.46±6.70
GSH (umol/mg protein)	221.83±27.78	131.36±4.83	323.12±15.45
	234.56±25.57	178.94±4.59*	291.54±6.55
Mn-SOD (units/mg protein)	64.21±3.40	61.06±2.51	98.39±3.67
	65.16±2.45	56.46±0.99	97.14±3.32

Values are presented as the mean ± SEM; sample sizes for each variable ranged from n = 13 - 14 for all.

* $P < 0.05$ main effect of exercise.

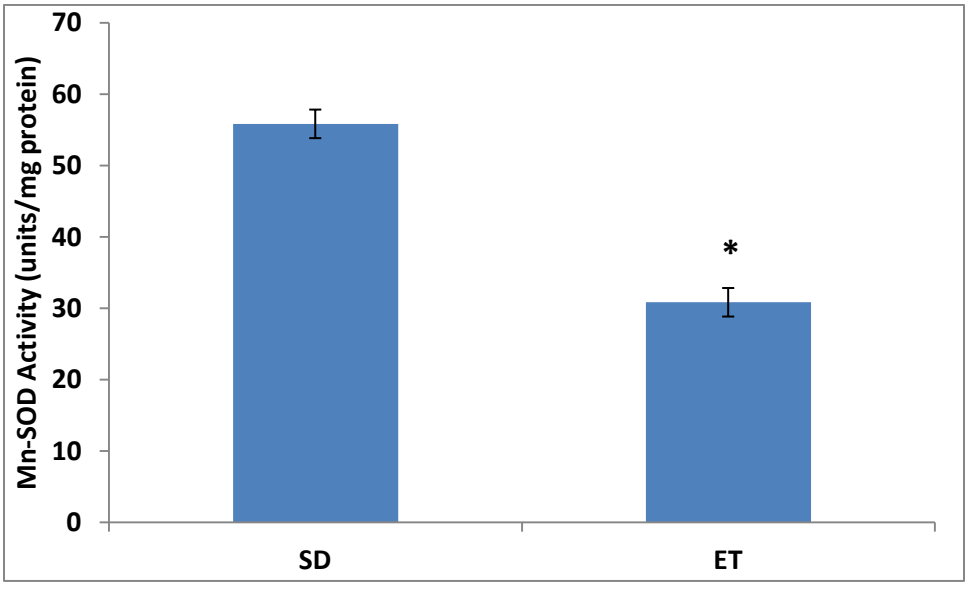

Figure 17. Muscle Activity of Mn-SOD. All values are displayed as the mean ± SEM. The * denotes significance at $p \leq 0.05$ using an independent T-test to compare ET to SD.

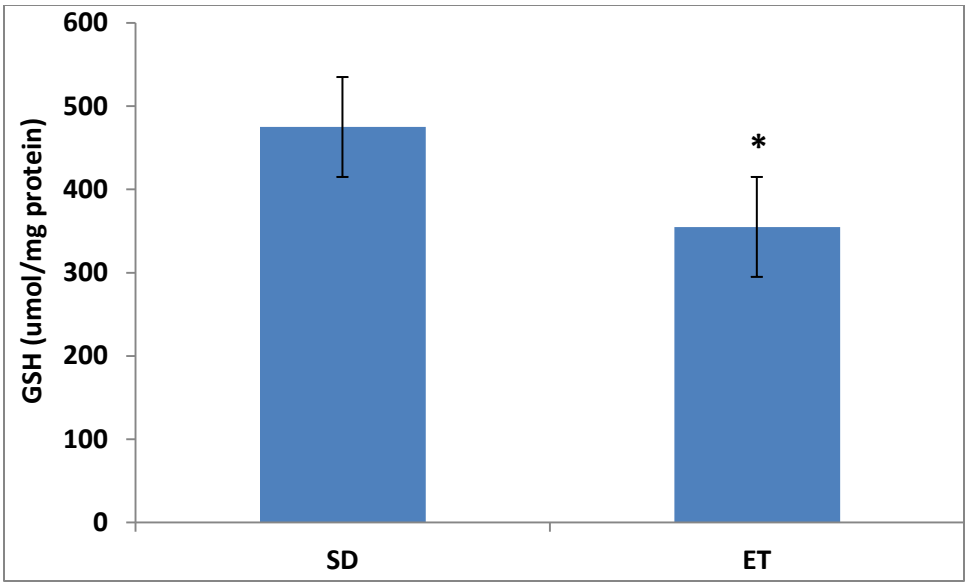

Figure 18. Muscle Concentration of GSH. All values are displayed as the mean ± SEM. Non-significant (NS) using an independent T-test to compare ET to SD.

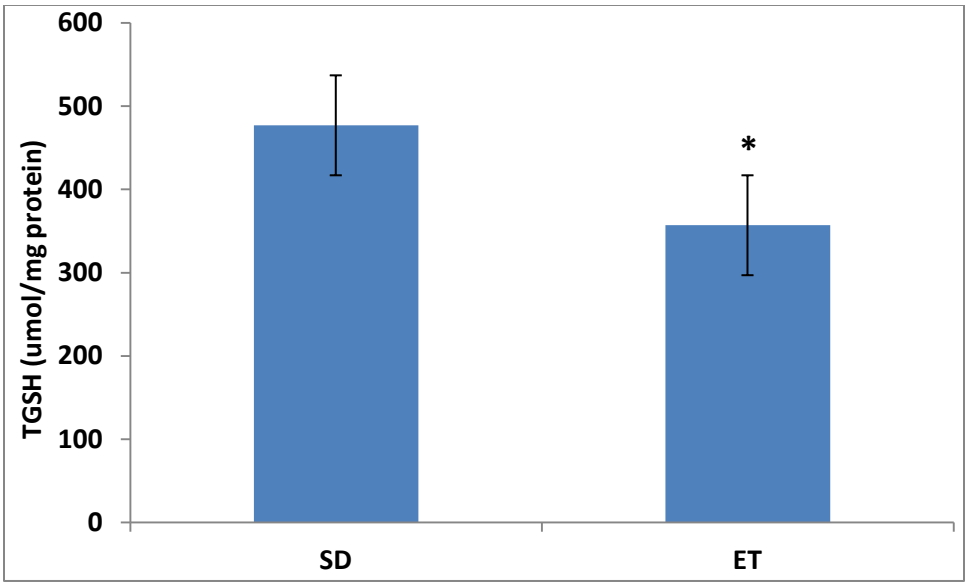

Figure 19. Muscle Concentration of TGSH. All values are displayed as the mean ± SEM. Non-significant (NS) using an independent T-test to compare ET to SD.

Antioxidant Factor Correlations between Muscle and Brain

Additionally, this study sought to determine the relationship between skeletal muscle and brain with respect to antioxidant factors, Mn-SOD, GSH, and TGSH. A Pearson's Correlations was used to examine the relationship between skeletal muscle and each brain region. There were no significant correlations between skeletal muscle gastrocnemius and individual brain regions with regards to Mn-SOD activity (CT vs. M r = -0.226; HP vs. M r = -0.003; CB vs. M r = 0.090), GSH (CT vs. M r = 0.495; HP vs. M r = 0.284; CB vs. M r = -0.097) and TGSH (CT vs. M r = 0.495; HP vs. M r = 0.287; CB vs. M r = -0.101) concentration. The results are shown below.

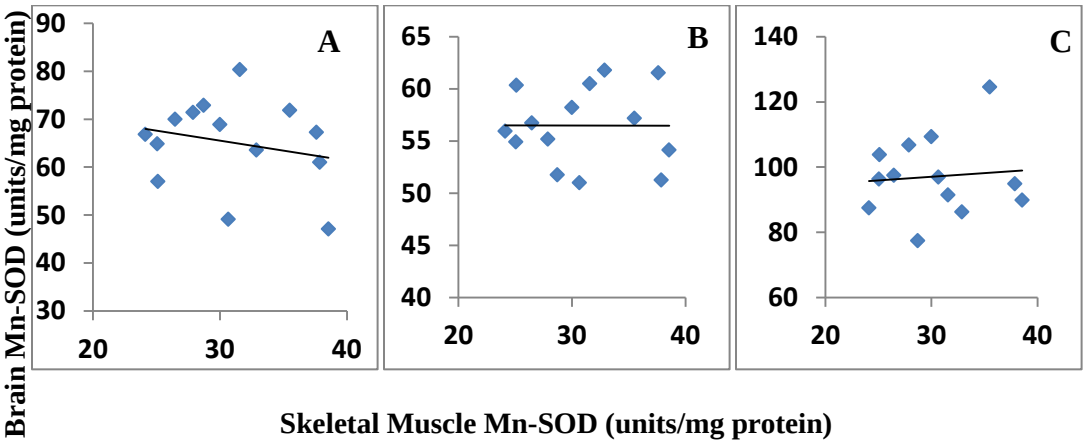

Figure 20. Correlations between Muscle and Brain Mn-SOD Activity. A) Cortex, B) Hippocampus, C) Cerebellum.

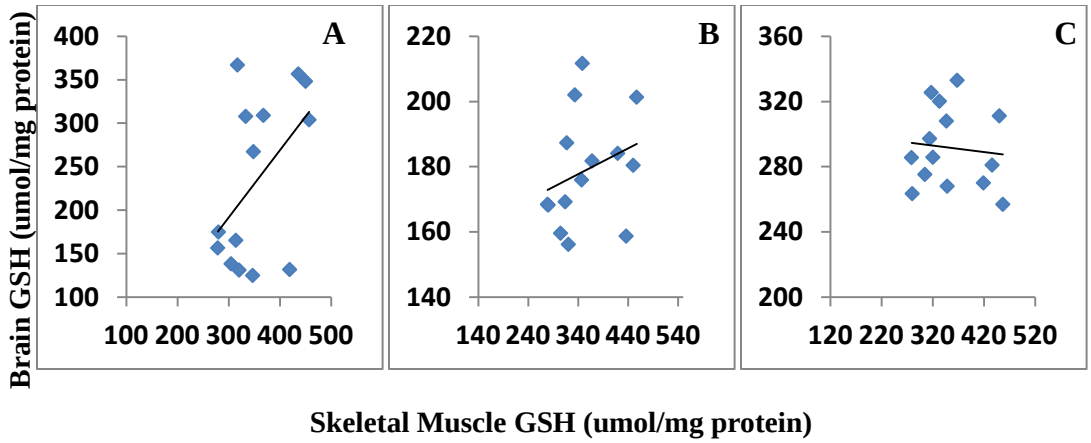

Figure 21. Correlations between Muscle and Brain [GSH]. A) Cortex, B) Hippocampus, C) Cerebellum.

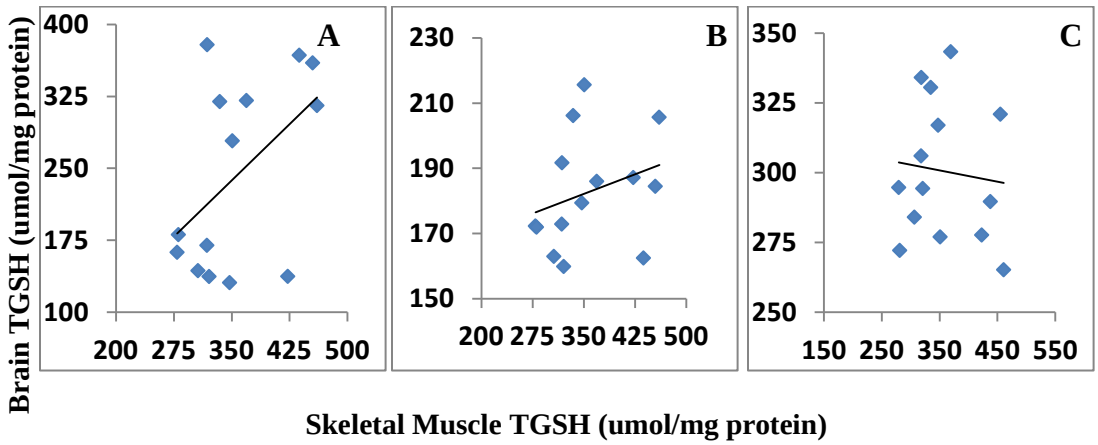

Figure 22. Correlations between Muscle and Brain [TGSH]. A) Cortex, B) Hippocampus, C) Cerebellum.

NrF2

The central focus of this study was to determine the effect of acute aerobic exercise and chronic aerobic exercise on Nuclear Factor Erythroid 2 Related Factor 2 (NrF2) protein levels in the brain and skeletal muscle by means of western blot analysis. The acute aerobic exercise group was sacrificed immediately post exercise to understand

the effects of a single bout of strenuous exercise on the brain and muscle. Overall, NrF2 protein concentration was significantly reduced in all regions of the brain and skeletal muscle with acute exercise (Fig. 23). The chronic aerobic exercise group was sacrificed 5-hrs post exercise to prevent any acute exercise effects and to understand if training influences brain and muscle NrF2 differently than acute exercise. Chronic aerobic exercise showed a significant decrease in NrF2 protein concentration in the cortex, cerebellum, and skeletal muscle regions. However, the hippocampus was the only brain region to demonstrate a significant increase in NrF2 protein concentration with exercise compared to sedentary controls. Refer to Figures 23 and 24 for the results.

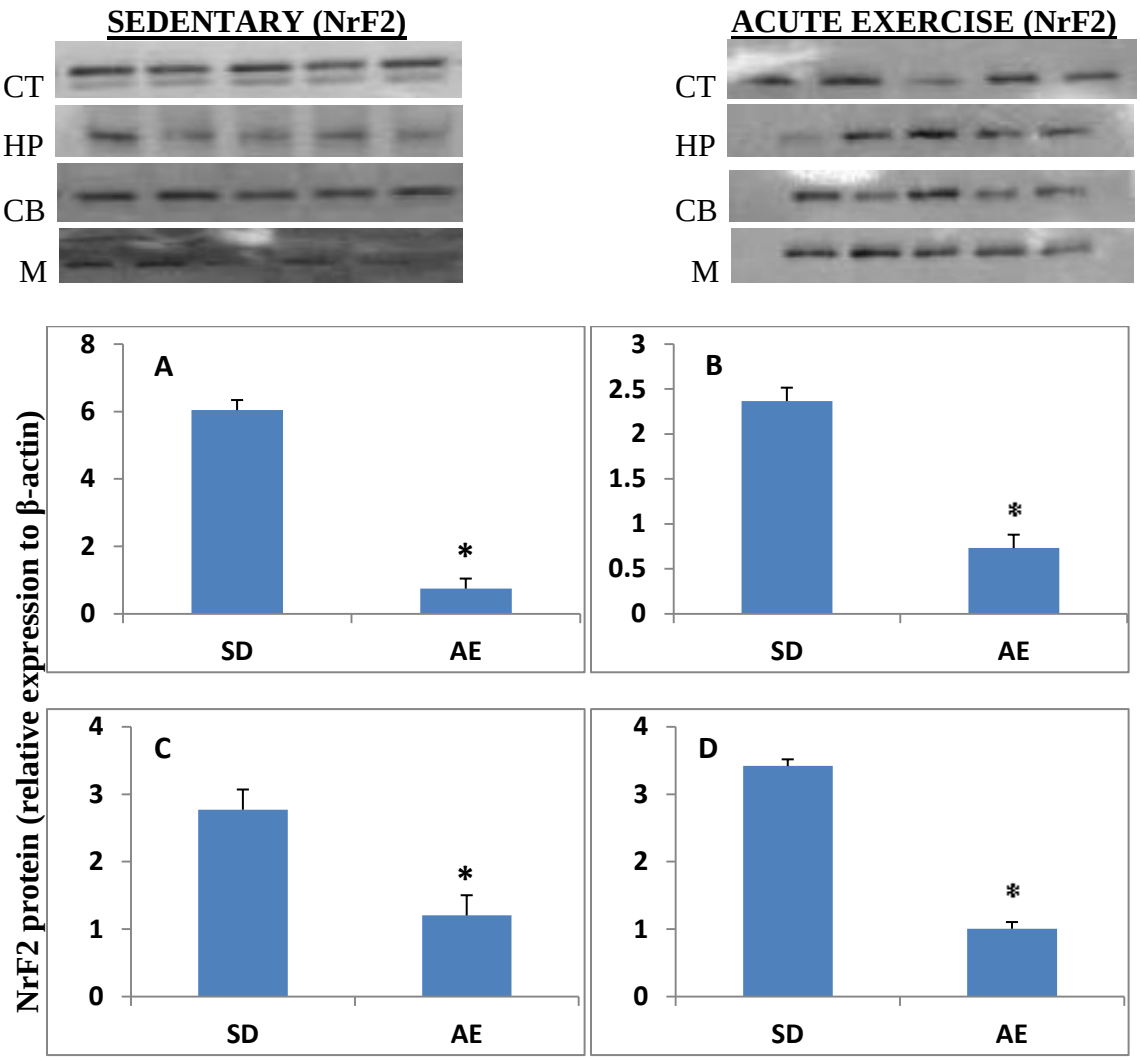

Figure 23. Acute Aerobic Exercise NrF2 Protein. (n=5/group). A) Cortex, B) Hippocampus, C) Cerebellum, D) Muscle. All values are displayed as the mean ± SEM. The * denotes significance at $p \leq 0.05$ using an independent T-test to compare AE to SD. CT=Cortex; HP=Hippocampus; CB=Cerebellum; M=Skeletal Muscle.

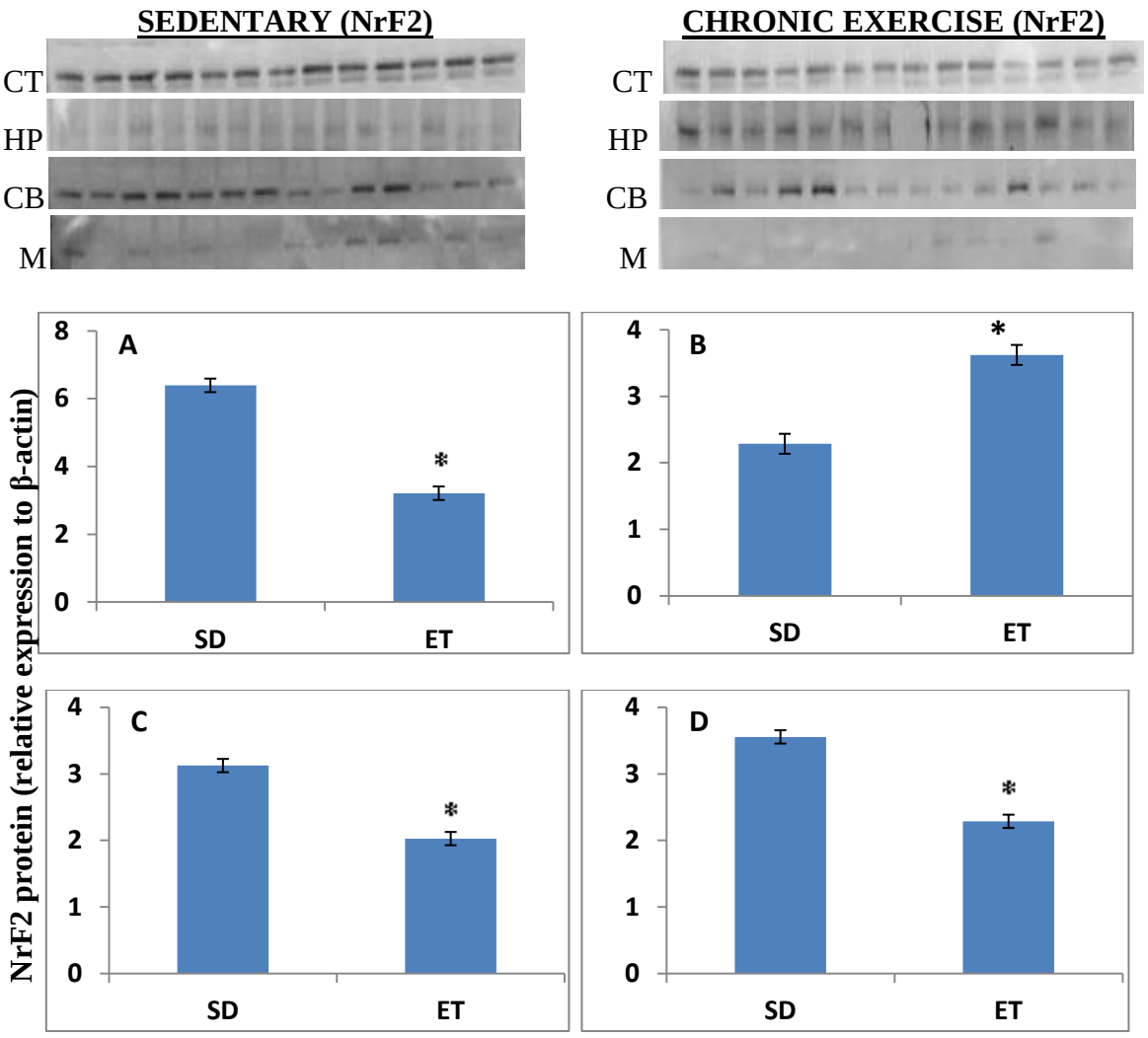

Figure 24. Chronic Aerobic Exercise NrF2 Protein. (n=13-14/group). A) Cortex, B) Hippocampus, C) Cerebellum, D) Muscle. All values are displayed as the mean ± SEM. The * denotes significance at $p \leq 0.05$ using an independent T-test to compare ET to SD. CT=Cortex; HP=Hippocampus; CB=Cerebellum; M=Skeletal Muscle.

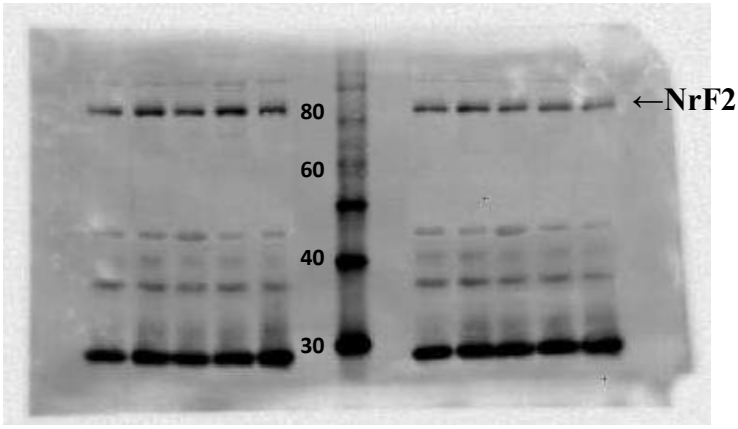

Figure 25. Example Blot. Skeletal Muscle NrF2 Protein with Acute Exercise.

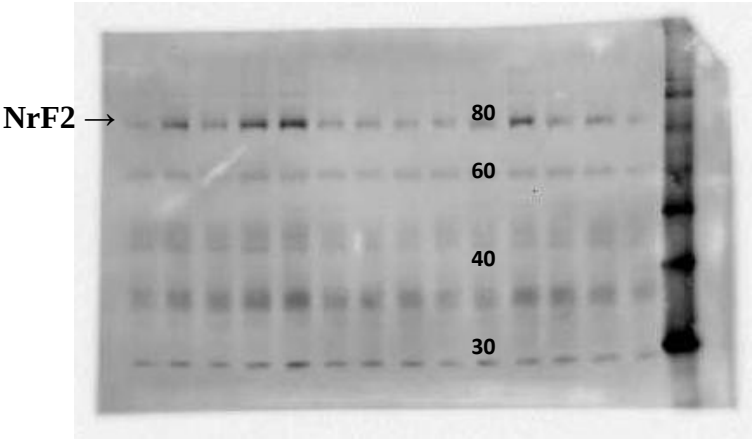

Figure 26. Example Blot. Cerebellum NrF2 Protein with Chronic Exercise.

NrF2 Correlations between Muscle and Brain

Furthermore, this study sought to determine the relationship between skeletal muscle and brain with respect to NrF2 protein concentration. A Pearson's Correlations was used to examine the relationship between skeletal muscle gastrocnemius and each brain region. Acute exercise showed a significant negative correlation in NrF2 protein concentration between skeletal muscle and cerebellum ($r = -0.857$, $p<0.05$). No

significant correlations in NrF2 were noted between skeletal muscle and hippocampus or cortex (r = 0.725 and r = 0.386, respectively) with acute exercise. Chronic exercise showed a significant positive correlation in NrF2 protein concentration between skeletal muscle and cortex (r = 0.716, $p<0.01$) and cerebellum (r = 0.737, $p<0.01$). No significant correlation in NrF2 was observed between skeletal muscle and hippocampus (r = -0.307).

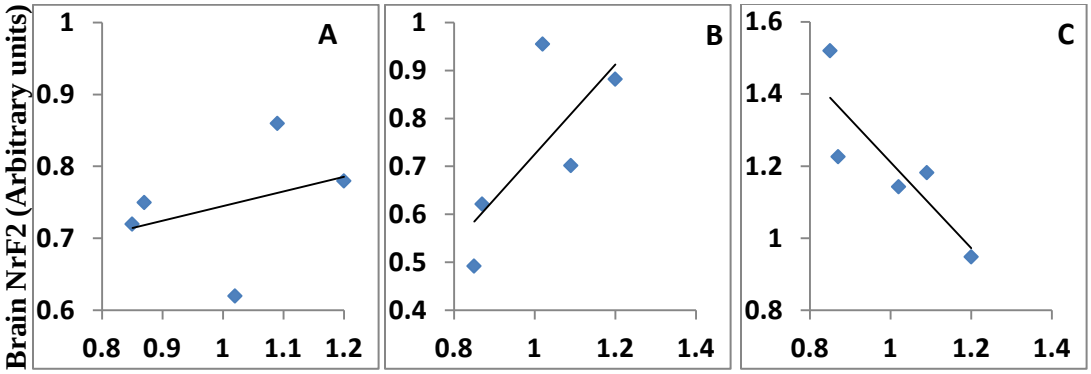

Figure 27. Correlations between Muscle and Brain [NrF2].
A) Cortex, B) Hippocampus, C) Cerebellum (n=5/group).

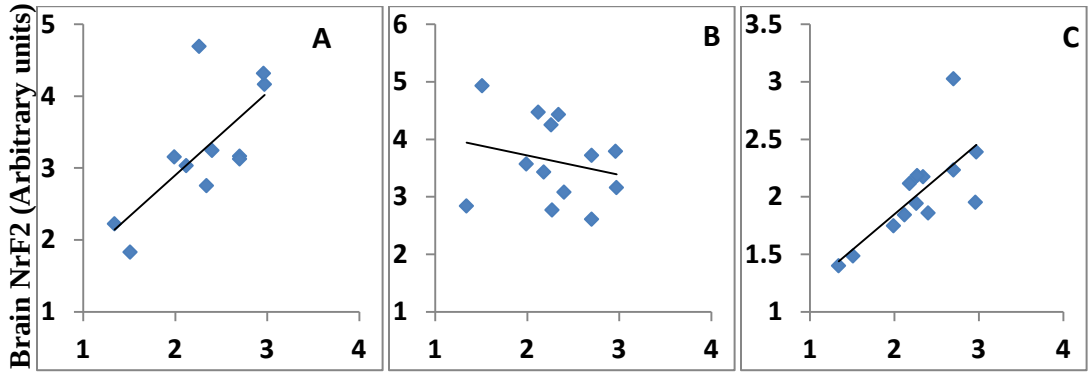

Figure 28. Correlations between Muscle and Brain [NrF2].
A) Cortex, B) Hippocampus, C) Cerebellum (n=12-13/group).

CHAPTER V

DISCUSSION AND CONCLUSIONS

This dissertation was focused on two primary aims. 1) To determine the effect of a single bout of acute aerobic exercise on NrF2 protein concentration and markers of oxidative stress in the brain and skeletal muscle. 2) To determine the effect of chronic aerobic exercise on NrF2 protein concentration and antioxidant factors in the brain and skeletal muscle. Firstly, to our knowledge, this is the first study to examine changes in NrF2 protein concentration with acute aerobic exercise in the brain. Secondly, this is the first study to our knowledge to examine changes in NrF2 with chronic aerobic exercise in the cortex, hippocampus, and cerebellum brain regions and if those changes are occurring as a result of the last bout of exercise. In addition, this is the first study to compared NrF2 protein concentration changes between these brain regions and an active tissue like skeletal muscle.

In regards to NrF2 protein concentration it was hypothesized that there would be no significant difference between acute exercise and sedentary controls. NrF2 protein concentration was significantly reduced in all brain regions and skeletal muscle gastrocnemius; therefore, these hypotheses were rejected. It was also hypothesized that there would be a significant increase in NrF2 protein concentration with chronic exercise compared to sedentary controls. This hypothesis was accepted in relation to the

hippocampus brain region only. In all other brain regions and skeletal muscle NrF2 was significantly reduced, therefore the hypotheses were rejected and the null hypotheses were accepted in those regions.

In regards to markers of oxidative stress with acute exercise it was hypothesized that there would be a significant increase in malondialdehyde (MDA), oxidized glutathione (GSSG) and the ratio of GSSG to total glutathione (TGSH), (GSSG/TGSH), in the brain and skeletal muscle. In regards to the GSSG/TGSH ratio, there were no significant differences in all brain regions and skeletal muscle; therefore the hypotheses were rejected. GSSG was significantly reduced in the cortex and cerebellum regions with acute exercise, but there were no differences detected in the hippocampus or skeletal muscle; therefore the hypotheses were rejected. With respect to MDA, there was a significant increase in the hippocampus region; therefore, the hypothesis was accepted. However, there was a significant decrease in MDA in the cortex and cerebellum regions and no difference in skeletal muscle; therefore, the hypotheses were rejected. With minimal evidence in brain and muscle tissue regarding an exercise oxidative stress response, this study sought to determine if the acute exercise had a cumulative stress response amongst all tissues. Therefore, blood markers of oxidative stress were measured. It was hypothesized that there would be a significant increase in blood MDA, GSSG, and the GSSG/TGSH ratio with acute exercise compared to sedentary controls. The hypothesis was accepted in regards to MDA concentration in the blood, but was rejected in regards to GSSG concentration and the GSSG/TGSH ratio.

In regards to antioxidant factors with chronic exercise it was hypothesized that there would be a significant increase in manganese superoxide dismutase (Mn-SOD) activity, reduced glutathione (GSH) and total glutathione (TGSH) concentration in the brain and skeletal muscle. These hypotheses were accepted in relation to GSH and TGSH concentration in the hippocampus region; however, these hypotheses were rejected in the cortex, cerebellum, and skeletal muscle. GSH and TGSH concentration were significantly reduced in skeletal muscle. Furthermore, Mn-SOD activity was significantly reduced with chronic exercise compared to sedentary controls in skeletal muscle, but there were no significant differences in any brain region. Therefore, these hypotheses were rejected.

Another objective in this dissertation was to compare the concentration and/or activity of markers of oxidative stress, antioxidant factors, and NrF2 protein between each of the brain regions (hippocampus, cortex, cerebellum) and skeletal muscle gastrocnemius. It was hypothesized that there would be a significant correlation between skeletal muscle and brain in regards to markers of oxidative stress, MDA, GSSG, and the GSSG/TGSH ratio. No relationships were detected between skeletal muscle and brain for any marker of oxidative stress; therefore, these hypotheses were rejected. It was also hypothesized that there would be a significant correlation between skeletal muscle and brain in regards to antioxidant factors, GSH, TGSH, and Mn-SOD. No relationships were detected between skeletal muscle and brain for any antioxidant factor; therefore, these hypotheses were rejected.

Finally, it was hypothesized that there would be a significant correlation between skeletal muscle and brain in regards to NrF2 protein concentration with acute and chronic

aerobic exercise. There was a significant negative correlation between skeletal muscle and cerebellum with acute exercise, supporting the original hypothesis, but no significant relationships were detected in cortex or hippocampus; therefore, these hypotheses were rejected in these regions. With chronic exercise there was a significant positive correlation between skeletal muscle and cortex and skeletal muscle and cerebellum, which is in support of the original hypotheses; however, there was no relationship detected between skeletal muscle and hippocampus; therefore the hypothesis for this brain region was rejected.

Thus, the primary findings of this dissertation demonstrate that 1) acute aerobic exercise induced a systemic and central oxidative stress response through MDA, but was unable to sufficiently stress tissue specific regions for all markers of oxidative stress. 2) Chronic aerobic exercise (5-7 weeks) increased the concentration of GSH and TGSH in the hippocampus only, but was unable to sufficiently increase antioxidant factors in all other tissue regions. 3) Acute aerobic exercise significantly reduced NrF2 protein concentration in the brain and skeletal muscle; however, chronic aerobic exercise significantly increased NrF2 protein concentration in the hippocampus only, a primary brain region linked to neurodegeneration. All other brain regions and skeletal muscle showed a significant decrease in NrF2 protein concentration

These data suggest that an acute bout of exercise for 60min at 20m/min up a 6° grade in rats was insufficient to induce a substantial oxidative stress response in the blood, brain and gastrocnemius skeletal muscle tissue. Chronic aerobic exercise for 60min/day, 5days/week for 5-7 weeks minimally increased antioxidant factors in the

brain, but not in the skeletal muscle. Furthermore, these data suggest that regional differences in markers of OS, antioxidant factors and NrF2 exist and that changes in NrF2 protein concentration may not be significantly related to this particular exercise treatment, but regulated by other mediating factors. Additionally, these data suggest that NrF2 protein concentration with exercise training is correlated between skeletal muscle and the cortex and cerebellum brain regions. This chapter will discuss in detail the results of the present study in comparison to previous peer reviewed research in regards to NrF2 protein concentration, antioxidant factors, and markers of oxidative stress in the brain and skeletal muscle.

Acute and Chronic Exercise Animals

The rats utilized in this study were subjected to one of three conditions: acute exercise (AE), chronic exercise (ET), or sedentary controls (SD). All animals were fed *ad libitum* and had access to food prior to and after an exercise session, except for the acute exercise animals who were sacrificed immediately post a single bout of acute aerobic exercise. Body weight measures were not significantly different between AE and SD or between ET and SD at the time of sacrifice.

Blood Markers of Oxidative Stress

Aerobic exercise of sufficient intensity and duration is well known to increase markers of oxidative stress (OS) in the blood and tissue (Bloomer 2004). OS is characterized as a disproportional increase and accumulation in reactive oxygen and nitrogen species (RONS) compared to antioxidant defense capabilities. Because OS, and particularly RONS, have been identified as key mediators in the NrF2 activation pathway

it was of significant importance to establish the presence of markers of OS with acute aerobic exercise. Therefore, as a means of systemic assessment of OS, blood was collected from acute exercised animals immediately after treatment.

As previously reported, of the three markers of oxidative stress, only MDA concentration was significantly elevated with acute exercise. The GSSG/TGSH ratio trended to increase with acute exercise, but failed to reach significance. Collectively, this suggests that OS was present in the blood after the single bout of acute exercise, but it is inconclusive from this measure as to where it was originally produced (i.e. tissue of origin). In order to confirm whether or not these animals were stressed by a single bout of acute exercise, tissue analysis would need to be performed for markers of OS. This will be discussed in detail in the next section.

In regards to plasma MDA, the results from this study are consistent with findings by McKenzie et al. 2014. In this study, untrained human subjects completed a graded exercise test until exhaustion (8-10min). In agreement with the present study, MDA concentration was significantly increased (~55%) with acute exercise. In addition, a primary product of lipid peroxidation, LOOH, was significantly increased (~68%) with exercise. Despite differences in species (human vs. rat), the concentration of MDA, pre to post exercise, was similar to the current study.

Laaksonen et al. 1999 examined another marker of lipid peroxidation, thiobarbituric acid reactive substances (TBARS). Because TBARS are a derivative of MDA, they are commonly used to quantify MDA concentration. In this particular study, untrained subjects cycled for 40min at 60% of VO_{2max}. The concentration of TBARS was

significantly increased (~67%) with acute exercise. Radak et al. 1995 also demonstrated an increase in TBARS with acute treadmill exercise at 24m/min at 15% grade until exhaustion. Another study by Spirlandeli et al. 2013 examined both TBARS and MDA concentration with acute exercise. In this study, human subjects completed the "Running based Anaerobic Sprint Test" twice, with 2min of recovery in between each test. In agreement with the current study, Spirlandeli et al. 2013 reported a significant increase in plasma MDA concentration (~18%) immediately post exercise and a significant increase in plasma TBARS concentration (~56%) immediately post exercise with both returning to baseline within 1-hr post exercise.

However, not all data reported on lipid peroxidation with acute exercise are in agreement. Bloomer et al. 2005 reported in a similar study to Laaksonen and colleagues that MDA concentration was unchanged with acute cycling exercise at 70% VO_{2max} for 30min (Bloomer 2005). Furthermore, Quindry et al. 2003 utilized an acute graded exercise test and reported similar findings to Bloomer and colleagues; no significant differences in plasma MDA concentration. Elikov 2016 reported that rats subjected to acute exercise stress by swimming for 20min with a load equal to 10% of body weight demonstrated no change in plasma MDA concentration (Elikov 2016). Another study by You et al. 2005 reported no changes in plasma MDA in rats subjected to an acute downhill run.

Given the current inconsistencies in findings of lipid peroxide markers, researchers recommend using MDA as the primary measure of OS as TBARS have been suggested to overestimate MDA quantity (Meagher 2000) and are a non-specific marker.

Additionally, there may be discrepancies between human and animal models and the production of RONS with exercise. Based on the reports listed above, lipid peroxidation seems to more readily occur in response to acute exercise in animals compared to humans. Furthermore, mode of exercise may also be a contributing factor. General running, sprinting, and graded exercise have been suggested in the previous studies to increase lipid peroxidation; however, downhill running, swimming, and cycling tend to demonstrate the opposite effect.

In the current study we demonstrated a trend for an increase in the GSSG/TGSH ratio ($p=0.182$), which failed to meet significance, but inclined in the direction of the original hypothesis to support the notion. Several previous studies have demonstrated significant findings in the GSSG/TGSH ratio with a single bout of acute aerobic exercise. Laaksonen et al. 2016 demonstrated a ~60% increase in the GSSG/TGSH ratio with an acute cycling bout. This finding was substantiated in previous studies with a similar exercise treatment (McKenzie 2014; Bloomer 2005). However, those findings are in humans. Reports from animal studies, specifically rats, are consistent with the current study's findings and inconsistent with human observations. You et al. 2005 reported no changes in the ratio of GSSG/TGSH with an acute bout of downhill running.

In addition, this study demonstrated a significant decrease in GSSG concentration with exercise, which was not expected. Theoretically and empirically, with an acute exercise stress, oxidized glutathione concentration should increase, while reduced glutathione concentration should decrease. Multiple studies have demonstrated this effect in glutathione. Laaksonen et al. 2016 reported an increase in GSSG concentration (~52%)

immediately post acute exercise. Bloomer et al. 2005 noted a ~22% increase in plasma GSSG concentration immediately post exercise. And McKenzie et al. 2014 reported an increase in GSSG concentration (>100%) with an acute exercise bout. However, in the present study, glutathione concentrations, oxidized and reduced, were unchanged with acute exercise. In agreement with the current study, You et al. 2005 indicated no significant differences with an acute strenuous exercise. Multiple factors may be responsible for the lack of significant findings.

It is imperative to note for the purpose of this section and the next that markers of oxidative stress are highly dependent on the intensity and duration of the exercise (Alessio 1993) and the mode of exercise (Elikov 2016). In addition, the presence or absence of these markers, reported in the literature, differ between not only human and animal studies, but also sex differences (male vs. female) and training status (trained vs. untrained subjects) (Bloomer 2008; Bloomer 2006). Timing of the measurement of various markers of OS has also been shown to be a critical factor for proper detection of changes with exercise. According to Michailidis et al. 2007 timing of the measurement of markers of OS is specific to the marker. This particular study demonstrated that optimal timing for blood measurement of TBARS is 1-hr post acute exercise and glutathione is 2-hr post acute exercise in humans. Also, the rather small sample size (n=5) may have been a major contributing factor to the lack of a significant finding.

Therefore, it is important to consider that in this study, animals were subjected to an acute bout of treadmill exercise for 60min at a speed of 20m/min up a 6° grade (Cartee 1987). Blood was collected from these animals immediately post exercise for

examination of MDA and glutathione. Findings from this study may only be representative of animals, in particular rats, and running as a form of exercise as compared to cycling or swimming. In addition, the timing of blood sampling may not have captured the peak in the blood to analyze markers of OS in these animals. In the next section, the measurement of markers of oxidative stress will be expanded to tissues.

Markers of Oxidative Stress in Skeletal Muscle Tissue

To further demonstrate the presence of OS with a single bout of acute aerobic exercise and to confirm the results of the blood analysis, a skeletal muscle tissue assessment, using mixed gastrocnemius muscle, was completed on MDA, GSSG, and the GSSG/TGSH ratio. Markers of OS were not significantly different with acute exercise compared to sedentary controls in skeletal muscle. In regards to MDA and GSSG concentration, there was a non-significant decrease with acute exercise. In addition, the GSSG/TGSH ratio, a highly sensitive marker of OS, demonstrated a non-significant increase with acute exercise, similar to the blood analysis. The findings from this study are inconsistent with older reports (1970-1997) on OS in the muscle with acute aerobic exercise (Reviewed in Radak et al. 2001). These reports suggested that acute exhaustive aerobic exercise significantly increased lipid peroxidation and reactive carbonyl derivatives in various skeletal muscles (hindlimb, gastrocnemius, soleus). However, more current research (1999-2017) both refutes and supports prior reports.

Original research by Radak et al. 1995 suggested acute exercise increased markers of OS. Exercise was performed at a similar intensity to that of the current study, rats were subjected to running at 24m/min up a 15% grade until exhaustion and soleus and tibialis

anterior muscles were measured for TBARS. Radak and colleagues reported significant increases in TBARS (>200%) with acute exercise compared to controls. Additionally, Bejma et al. 1999 reported significant increases in MDA (~26%) and GSSG (~37%) concentration in vastus lateralis muscle, but no changes in PC with acute exhaustive exercise. Paula et al. 2005 reported a significant increase in TBARS (~967%) 6-hrs and 1-hr post exercise and nitrite (~90.5%) 1-hr post exercise, in the tibialis anterior muscle following exhaustive exercise. This study in particular demonstrates that there may be an optimal "window" for measurement of MDA. In the current study, we measured MDA immediately after exercise and showed no difference, whereas Paula and colleagues demonstrated that waiting 1-hr post exercise may be necessary to detect a difference. It should also be noted that the above studies used different skeletal muscles compared to the current study to measure markers of OS, suggesting that similar tissues respond differently to the same exercise stress.

Furthermore, a recent study by Li et al. 2015 subjected rats to a nearly identical protocol to the current study and reported a significant increase in GSSG concentration by ~25%. However, Liu et al. 2000 examined markers of OS in rats following an acute bout of exercise until exhaustion. Vastus lateralis muscle, fast and slow fibers, were measured for MDA, GSSG, and protein carbonyls (PC) concentration. In agreement with the current study, MDA and PC were not significantly different with acute exercise in both fast and slow muscle. Furthermore, GSSG concentration was not significantly different with exercise in fast muscle and was not detectable in slow muscle.

Interestingly, Liu and colleagues also examined the effects of chronic aerobic exercise (8 weeks) on the same markers of OS. They reported significant increases in MDA in fast and slow muscle, significant increases in PC in slow muscle, but no change in GSSG concentration. These reports indicate that not only are there tissue specific differences in the production of markers of OS, but regional tissue assessment of these markers may require repeated stress leading to the accumulation of RONS to a detectable concentration. Blood represents the accumulation of RONS from all active tissues, allowing greater overall detection in markers of OS.

These findings are not consistent with the original hypotheses nor do they conform to previous findings. This may be due to a number of reasons. First, the small sample size (n=5) reduced the overall power of the study and sensitivity to measure subtle changes in markers of OS. Second, this study used mixed gastrocnemius muscle tissue to measure makers of OS. Mixed muscle contains both slow-twitch and fast-twitch muscle fibers and given that the exercise was aerobic in nature suggests that these fibers were not activated equally during the 60min run. Therefore, if more fast-twitch fibers were contained in the sample used for analysis, these fibers may not have been activated to the extent of slow-twitch fibers and show a reduced level of OS. Third, the leg muscle analyzed differs between studies (soleus, white or red gastrocnemius, tibialis anterior) making it difficult to conclude if aerobic exercise induces OS equally in all muscles of the hindlimb (Alessio 1993).

Additionally, the intensity and duration of exercise may have been insufficient to increase OS in skeletal muscle as most studies implemented a running speed of

>20m/min with a duration >2hrs or to exhaustion. In this study, a 60min bout of acute exercise was implemented. Furthermore, as mentioned previously, optimal timing for the measurement of markers of OS differs significantly between various markers. These animals were sacrificed immediately post exercise; however, slow tissue extraction at the time of euthanasia may have influenced tissue clearance rates or reduction reaction rates in markers of OS.

Blood flow through active skeletal muscles post exercise may have contributed to the tissue clearance rates, which may be why we detected a significant increase in plasma MDA and a trended increase in the plasma GSSG/TGSH ratio, but no differences in the muscle tissue. Blood flow in skeletal muscle increases rapidly in the first few seconds of acute exercise and can increase up to 20-fold during high intensity exercise. The rapid redistribution of blood flow is highly controlled by local mediating factors as well as various integrative mechanisms. Increased production of local factors such as: hydrogen ions, ADP, pCO_2, and even RONS have been demonstrated to increases muscle blood flow. However, workload demands may not be evenly distributed throughout all skeletal muscles leading to differences in the activation of these signaling pathways and the subsequent redistribution of blood flow (Reviewed in Sarelius et al. 2010)

Markers of Oxidative Stress in Brain Tissue

As previously indicated, changes in markers of OS varied with acute exercise compared to sedentary controls all brain region (cortex, hippocampus, cerebellum). As shown in the results, there was a significant increase in MDA in the hippocampus, but a significant decrease in MDA and GSSG in the cortex and cerebellum and no change in

the GSSG/TGSH ratio in any brain region in with acute exercise compared to controls. The findings from this study are consistent with recently published literature by Liu et al. 2000. In this study, rats were exercised until exhaustion and whole brain was assessed for multiple markers of OS. Liu and colleagues reported no significant differences with exercise in MDA, PC, and GSSG concentration. These findings were also supported by Radak et al. 2001. In this particular study, rats were exposed to swimming for 2-hrs with a water temperature of 32°C. In the hippocampus, TBARS and PC were not significantly different with exercise.

In another study by Asku et al. 2009, exercised rats ran until exhaustion using a speed protocol similar to the current study. Using whole brain, markers of OS, TBARS and nitrite were measured, but no significant differences were observed with exercise. However, Paula et al. 2005 demonstrated an increase in whole brain TBARS (~109%) and nitrite (~60%) with electrical stimulation of the tibialis anterior muscle. Electrical stimulation is a direct means of activation of the skeletal muscle and a more intense treatment compared to motivated exercise as it circumvents the potential for central fatigue and overrides the typical exertion patterns exhibited during normal exercise. Therefore, these results may not be correlative under normal physiological conditions.

Unfortunately, most of the current literature focuses on whole brain rather than regional assessment of markers of OS. It may be that changes in markers of OS in the individualized regions are insignificant, but when collectively analyzed in the whole brain, the results show significant increases with exercise, potentially leading to the exacerbation of cognitive function. For example, Li et al. 2010 demonstrated that whole

brain increases in MDA elicited cerebral suppression and depression of physiological functions due to decreased neuronal firing rates, which are not easily detected in individual brain regions. As mentioned previously, the intensity and duration of exercise may have been insufficient to increase OS in individual tissue regions as most studies implemented a running speed of >20m/min with a duration >2hrs or to exhaustion. Or, the intensity and duration may have been sufficient to increase OS in the whole brain, as demonstrated in previous studies, which could be indicative of an accumulated response from multiple individual brain regions. Future studies are warranted to corroborate findings of OS in specific brain regions as well as to expand our understanding as to how the individualized regions respond to exercise stress compared to the whole brain. It is also a possibility that by the time the brain regions were harvested post exercise, the OS that had transiently accumulated in individual tissues reverted back to normal or even below normal values.

Cerebral blood flow and neuronal protection via the blood brain barrier may also be contributing factors to the minimal increase in markers of OS with acute exercise. During exercise, blood flow increases in the brain are functionally dependent on the intensity of exercise and regions specific to locomotion (Delp 2001). Additionally, these same active regions require greater oxygen consumption and neuronal ATP demand, which puts them at a greater risk of free radical production (Freeman 2012). However, in the current study, the brain regions measured have not been demonstrated to show significant alterations in blood flow with exercise (Delp 2001), reducing the risk of OS.

Based on these reports, our findings related to OS in brain regions not associated with locomotion are congruent.

Furthermore, in this particular study, the correlation between each brain region and gastrocnemius skeletal muscle was examined in regards to markers of OS to identify if a relationship exists between changes in OS in the brain compared to skeletal muscle with acute exercise. As previously mentioned, there were no significant correlations between gastrocnemius muscle and each brain region in respect to any marker of OS. These correlative findings coincide with the lack of a significant increase in most markers of OS in each tissue region with acute exercise.

Acute Exercise and NrF2

In the current study, NrF2 protein concentration exhibited a significant reduction with acute aerobic exercise in all brain regions and skeletal muscle. To our knowledge, this study is the first report on the changes in NrF2 protein concentration in the brain with an acute bout of aerobic exercise. Other research has examined changes in NrF2 in the brain with acute "stress", but this did not include exercise. One study by Sethy et al. 2011 exposed rats to hypobaric hypoxia at an altitude of 25,000ft. Peak NrF2 concentration was significant in whole brain after 3-hrs of exposure to hypobaric hypoxia. However, the findings from this study and this type of stress may not be relevant to the current study's findings, given that the exercise was aerobic in nature. Although, this study may provide an indication of how whole brain and various brain regions such as the hippocampus, cortex, and cerebellum respond to acute stress.

Previous research in skeletal muscle indicates an increase in NrF2 with acute aerobic exercise. In a study by Li et al. 2015, NrF2 was examined in whole hindlimb muscle in response to a similar acute exercise protocol as the current study, 20m/min on a 5% grade for either 1-hr or 6-hrs. NrF2 binding affinity and nucleoprotein expression was significantly increased with 6-hrs of exercise compared to controls. No changes were reported with the 1-hr exercise. These findings are in disagreement with the current study, suggesting that it may require a greater stress in exercise duration to detect changes in NrF2 concentration. In the current study, the animals were exercised for 60min in duration. It may be that these animals did not spend long enough on the treadmill to elicit detectable increases in NrF2 concentration.

In another study by Wang et al. 2016, NrF2 was examined with an exercise intensity identical to the one utilized in the current study. Rats ran for predetermined durations of 45, 90, 120, or 150 minutes and gastrocnemius and quadriceps femoris skeletal muscles were analyzed. NrF2 mRNA was significantly elevated in the 90, 120, and 150min exercise groups, with the peak occurring at 120 minutes. NrF2 protein concentration was significantly elevated (~60%) in a similar manner across all exercise groups, 90, 120, and 150min, compared to controls. This study suggested that an exercise duration of at least 90mins is necessary to illicit changes in NrF2 protein concentration given that the exercise intensity, speed and grade, were identical to the current study.

Overall, there are minimal published findings on the effects of acute exercise on NrF2 in brain and muscle tissues and the extent to what has been reported is concentrated in skeletal muscles such as hindlimb, gastrocnemius, and the quadriceps femoris.

However, blood mononuclear cells have been reported to significantly increase NrF2 protein concentration and markers of OS in whole cell and nuclear cell lysates with 30 minutes of cycling at 70% VO_2max in young men (Done 2016). These findings may be indicative of a significant systemic response to OS rather than a regional assessment of individualized tissues. Based on the theoretical model of the current study, because markers of OS were minutely increased or unchanged in the brain and gastrocnemius muscle tissues with a single bout of acute aerobic exercise, NrF2 remained bound to KEAP1 in the cytosol. This may be reflective as to why there were no observed changes in NrF2 protein concentration in the brain and muscle with acute exercise compared to controls.

Brain is a highly protected tissue via the blood brain barrier which is characterized as a selective filter by means of tight junction proteins. These proteins are adhesive to one another and provide an anchor point to the basement membrane. In addition to multiple protective layers of the barrier, the brain is equipped with an augmented supply of glutathione, catalase, and Mn-SOD (Reviewed in Freeman et al. 2012). The formation of RONS is necessary to activate NrF2 in the brain, but if RONS are immediately neutralized by antioxidants, NrF2 will remain bound to KEAP1 and is rapidly degraded in the cytosol by the ubiquitin-proteasome system (Itoh 2003). However, if RONS successfully activate NrF2 causing dissociation from KEAP1, NrF2 will stabilized in the cytosol for up to 20min (Bryan 2013). To translocate to the nucleus, NrF2 must be phosphorylated by one of many phosphoproteins, such as Maf. If phosphorylation does not occur, NrF2 cannot translocate to the nucleus and degradation

of NrF2 occurs through autophagy, as discussed earlier in the literature review. Despite the lack of a protective barrier in skeletal muscle, phosphorylation is still a necessary component to activate NrF2. This may be the major contributing factor for the significant reduction in NrF2 protein concentration not only in the brain, but skeletal muscle with acute aerobic exercise.

Furthermore, in this particular study, the correlation between each brain region and skeletal muscle was examined in regards to NrF2 protein concentration to identify if a relationship exists between NrF2 activation in the brain compared to skeletal muscle under conditions of acute stress. To our knowledge, this is the first study to examine the relationship between skeletal muscle and brain tissue NrF2 concentration. As previously stated, there was no significant relationship in NrF2 detected between skeletal muscle and any brain region. This may primarily be due to the fact that there were significant reductions in NrF2 concentration in all tissues with acute exercise. Additionally, it may also be due to tissue specific differences or that peripheral tissue changes are not reflective of central tissue changes.

Chronic Exercise and NrF2

The current study also examined changes in NrF2 protein concentration with chronic aerobic exercise, which unlike acute exercise, has been researched in regards to skeletal muscle and more importantly, the brain. As indicated in the results, NrF2 protein concentration was significantly increased in the hippocampus brain region in response to exercise training. However, NrF2 concentration was significantly reduced in the cortex, cerebellum, and gastrocnemius muscle with 5-7 weeks of training. Previous research is in

agreement with the current study findings in the hippocampus brain region; however, it should be noted that these reports of NrF2 are confined to the whole brain or striatum only. To our knowledge, this is the first study to examine changes in NrF2 in multiple individual brain regions. However, in regards to skeletal muscle, the current research is in opposition to the findings in this study.

Tsou et al. 2015 examined NrF2 DNA binding activity in the striatum in response to 4 weeks of exercise training at 70% VO_{2max}. NrF2 binding activity significantly increased with training. Furthermore, there was a time dependent increase with binding activity significantly increasing as early as 1-hr post exercise and reaching a peak activity at 8-hrs post the final bout of exercise. In the current study, animals were sacrificed 5-hrs post the final bout of exercise and NrF2 protein concentration was significantly increased in the hippocampus only. Despite differences in the measure of NrF2 activation, these findings suggest that changes in NrF2 are time dependent and thus require a longer duration post exercise to identify changes in the pattern of NrF2 activation. Therefore, the current study may need to be repeated with a greater time lapse between exercise and euthanasia to detect more robust changes in NrF2 in other brain regions and muscle.

Additionally, Moiron et al. 2015 subjected rats to running at 35cm/s for 12 weeks and measured NrF2 protein concentration in the whole brain. Moiron and colleagues reported a significant increase in NrF2 protein concentration with exercise training in both normal fed (~75%) and high protein fed (~45%) animals. This was an interesting study as it demonstrated that greater amounts of dietary protein increased basal concentrations of NrF2 which ultimately led to a blunted increase in NrF2 protein

concentration with exercise training compared to normal fed animals. This reiterates a point that will be discussed in detail in an upcoming section regarding limitations to changes in protein concentration based on basal levels of NrF2 protein.

Another study by Aguiar et al. 2016 aerobically trained rats for 6 weeks and examined the change in NrF2 mRNA in the striatum in response to a bout of exhaustive exercise. Aguiar and colleagues demonstrated a ~50% increase in NrF2 mRNA. Based on the previous study by Moiron and colleagues, that lasted 12 weeks, in this study changes in NrF2 after only 6 weeks are similar to the normal fed animal group who displayed lower basal levels of NrF2 protein concentration. Because Aguiar and colleagues used an acute exhaustive bout of exercise to measure changes in NrF2 after training, this suggests that NrF2 may respond similarly to both acute and chronic exercise and that mediating factors, like phosphoproteins, may play a greater role in regulating basal NrF2.

As previously mentioned, the brain is a highly protected region via the blood brain barrier, especially in a healthy population. Under normal circumstances the brain produces an abundant amount of endogenous antioxidant defense factors through the NrF2 system. These factors are readily activated in response to RONS production. This suggests that basal NrF2 protein concentrations are already elevated in the brain in a healthy cohort and exercise training may only provide modest increases or no change at all (Reviewed in Freeman et al. 2012). Additionally, not all areas of the brain are activated homogenously during exercise, meaning some regions of the brain exhibit greater neuronal excitation compared to others.

The diencephalon and mesencephalon brains regions have been demonstrated to show the greatest excitation during treadmill running in rats (Iwamoto 1996). These regions do not contain the brain tissues that were examined in this study, cortex, hippocampus, and cerebellum. However, the hippocampus is located on the exterior portion of the dienchephalon and contains a vast neural network with multiple regions within the dienchephalon, as they are part of the limbic system. Cross activation in adjoining neural networks may be responsible for the increase in hippocampal NrF2 despite low neuronal excitation. Furthermore, the hippocampus has also been reported to be a highly plastic brain region, readily adaptable to the benefits of exercise training (Devi 2004) which was corroborated in the current study as it was the only region to respond positively to exercise. This finding in the hippocampus merits distinction as it is primary region linked to neurodegenerative disorders such as Alzheimer's and dementia and based on these findings, may be a potential target for adaptive exercise treatment.

Reports of increased NrF2 activation with training are also demonstrated in skeletal muscle. A study by Strobel et al. 2011 aerobically trained rats for 14 weeks at 70% VO_{2max} and measured NrF2 protein concentration in red gastrocnemius skeletal muscle. With training, NrF2 protein concentration increased by ~73% in red gastrocnemius muscle. In addition, there was a significant reduction in Mn-SOD activity, but no changes in GPx activity. Furthermore, MDA concentration was significantly increased, but not xanthine oxidase, a primary source of OS in skeletal muscle with training. In relation to the current study, gastrocnemius Mn-SOD activity was also

reduced with training; however, in contention, NrF2 concentration was significantly reduced, suggesting that the muscle tissue was already highly protected.

The evidence from Strobel and colleagues suggests that increases in OS via MDA induced NrF2 activation, but failed to alter antioxidant factor production. In this study, the reduction in NrF2 may be related to the use of mixed gastrocnemius muscle and the unequal activation of fast and slow fibers with aerobic exercise. Because slow fibers require greater blood flow to accommodate oxygen consumption (Armstrong 1983), this increases the risk of RONS production. We hypothesized that if OS was present with exercise, it would lead to an increase in NrF2 concentration. Interestingly, MDA concentration was elevated in the gastrocnemius muscle with exercise training (data not shown), but may not have been severe enough as antioxidant activity and concentration were significantly reduced. However, if the sample contained a greater amount of type 2 fibers, which are activated to a lesser extent during aerobic exercise, this would explain the reduction in NrF2.

Additionally, despite regional differences between brain and skeletal muscle, the report from Strobel et al. shares congruency with the hippocampal observations in the current study. A significant increase in MDA concentration in the hippocampus was present with training (data not shown) along with an increase in NrF2 concentration, but the activity of antioxidants, Mn-SOD, was unchanged, while GSH and TGSH concentration were increased. This suggests that 5-7 weeks of exercise training may have potentiated multiple adaptations including: greater oxidative capacity via an increase in cytochrome c oxidase, better handling of RONS, or an enhanced capacity to protect

against RONS. It should also be considered that the overall OS response in MDA may not have been severe enough to prompt an increase in antioxidant activity.

Lastly, the correlation between each brain region and gastrocnemius skeletal muscle was examined in regards to NrF2 protein concentration to identify if a relationship exists between the brain and skeletal muscle with prolonged exercise training. As previously indicated, a significant positive correlation in NrF2 protein concentration was observed between gastrocnemius muscle and both the cortex and cerebellum brain regions with exercise training. These findings are the first comparisons between brain and skeletal muscle in regards to NrF2 concentration in the literature. This study indicates that changes in mixed gastrocnemius muscle may be reflective of central adaptations in NrF2 with 5-7 weeks of chronic aerobic exercise. Furthermore, these findings may potentially allow for the expansion of research and cross comparison between animals and humans to understand how NrF2 concentrations may be affected with chronic aerobic exercise.

Antioxidant Factors

To understand the physiological change in antioxidant production within tissues in response to exercise, both sides of the paradigm were examined; activity and concentration. Antioxidant activity is a measure of the current biological condition and represents the ability to inhibit RONS formation and subsequent OS. Antioxidant concentration is the relative amount of a chemical represented per unit volume. This does not necessarily reflect the biological activity of a chemical, but rather the capacity to which that tissue can respond during times of stress. Therefore, given that in the current

study, the chronic exercise group was sacrificed 5-hrs post the final bout of exercise, to eliminate the effects of acute exercise, we sought to determine how antioxidant factors were affected long term in the brain and skeletal muscle tissues.

Antioxidant Factors in Skeletal Muscle Tissue

In the present study, antioxidant activity and concentration was assessed in the skeletal muscle with chronic aerobic exercise. Mn-SOD, TGSH, and GSH were measured in mixed gastrocnemius muscle. As previously indicated in the results, Mn-SOD activity was significantly reduced (~44.7%) with chronic aerobic exercise compared to controls. Furthermore, both TGSH and GSH concentration were significantly reduced by ~33% with chronic exercise. These findings are meaningful in relation to changes in NrF2 concentration with chronic aerobic exercise. As a major regulator of the antioxidant defense system, increases in NrF2 activation should coincide with increases in antioxidant factor production. However, in this particular study, NrF2 protein concentration was significantly reduced with exercise training in gastrocnemius skeletal muscle; therefore, no changes in antioxidant activity or concentration should be expected in gastrocnemius muscle. These expectations align with the findings in antioxidant factors with chronic aerobic exercise.

In agreement with the current study, Strobel et al. 2011 reported a significant reduction in Mn-SOD activity (~75%) after 14 weeks of chronic aerobic exercise in red gastrocnemius skeletal muscle. However, total SOD2 protein was significantly increased by 20% with exercise training, as determined by western blot analysis. This suggests that although biological activity was reduced, the capacity to protect against OS may have

increased with aerobic training in gastrocnemius skeletal muscle. According to the current study results, the adaptive response to chronic aerobic exercise may have reduced the production of RONS and the subsequent need for a change in activity of antioxidant factors. However, the current study did not show an increase in the concentration of antioxidant factors in skeletal muscle, most likely due to the reduction in cellular NrF2 concentration limiting transcription of antioxidant defense factors.

In agreement with the current study, Laughlin et al. 1990 aerobically trained rats for 12 weeks on a ramp protocol with a conditioned speed of 32m/min on an 8% incline. They reported no significant differences in SOD activity in either the red or white gastrocnemius muscle or the triceps brachii muscle. In addition, catalase activity was significantly reduced with chronic exercise in the red and white gastrocnemius, the soleus, and in the triceps brachii muscle tissues. In the current study, mix gastrocnemius was used to measure antioxidant factors. However, based on Laughlin's findings in separated gastrocnemius muscle, increases in antioxidant factors were not apparent in either portion of the muscle with training and therefore, should not be expected to be significantly increased in mixed gastrocnemius muscle.

Another study by Leeuwenburgh et al. 1994 trained rats for 10 weeks at 27m/min on a 15% incline. They demonstrated no significant differences in SOD activity and catalase activity with training in soleus muscle and vastus lateralis muscle. In addition, TGSH concentration was not significantly different in the vastus lateralis, but was significantly reduced in the soleus muscle. Futhermore, GSH concentration was significantly reduced in the soleus muscle with training, but was unchanged in the vastus

lateralis muscle. Although not significant, these findings align with the current study's observations in antioxidant factor concentration and activity, as well as the pattern of change. In a healthy subject, the skeletal muscle is highly responsive to RONS production via antioxidant defense factors to keep cellular stress low. Therefore, enhancing antioxidant production is unnecessary given the current state of stress and no changes should be expected to occur.

However, an earlier study by Higuchi et al. 1985 used a similar protocol to Laughlin where rats were subjected to 12 weeks of training at 31m/min on a 15% incline. They reported a significant increase in SOD activity in soleus muscle (~29%) and red vastus lateralis muscle (~21%), but not white vastus lateralis muscle. In addition, Mn-SOD activity was significantly increased in the soleus (~36%) and red (~37%) and white (~14%) vastus lateralis muscles with exercise. However, Cu-Zn-SOD activity and catalase activity was unchanged in all muscles with exercise training. Although Higuchi and colleagues reported significant changes in antioxidant activity with training, they also reported a significant decrease in Mn-SOD activity to cytochrome c activity and to citrate synthase activity. The electron transport chain is the primary site of radical formation and with training they demonstrated robust increases in the respiratory chain enzymes, but rather small changes in antioxidant activity. This suggests that despite the changes in activity, there is little to no overall effect on tissue protection from free radicals with training.

There may be multiple factors that contributed to the decrease in skeletal muscle antioxidant activity and concentration with chronic aerobic exercise. First, the duration of

training in the current study was significantly shorter compared to other studies (10-14 weeks). Secondly, in the current study peak intensity of the ramp protocol was reached in the middle of week 4 and plateaued. The duration of time spent at the peak intensity (30m/min for 60min) was only between 1.5-2.5 weeks long. It may be that the animals adapted to the exercise or that the peak intensity was not great enough to elicit a change in the concentration of antioxidant factors.

Additionally, with an adaptive response to training and the plateau in the intensity of exercise in the middle of week 4, RONS production may have been reduced or minimal, negating a need for an increase the activity or concentration of antioxidant factors. Tissue specific responses and activation of specific skeletal muscles during the exercise may also be a concern when measuring antioxidant factors. Gastrocnemius muscle is a mixed muscle in terms of fiber type. Given that the exercise was aerobic in nature, type 1 (slow twitch) fibers were mostly likely activated to a greater extent compared to type 2 (fast twitch fibers) in gastrocnemius muscle. Furthermore, during a running exercise, rats engage multiple hindlimb muscles with the red gastrocnemius, plantaris, and soleus being the most highly activated muscles (Armstrong 1983). In the current study, if more white gastrocnemius muscle was present in the sample, this could be a contributing factor as to why no significant differences in antioxidant factor concentration were noted.

Antioxidant Factors in Brain Tissue

The current study demonstrated regional brain tissue differences in antioxidant factors with chronic aerobic exercise. As previously indicated, the cortex and cerebellum

regions demonstrated no significant differences in Mn-SOD activity, TGSH, or GSH concentration with exercise training. However, the hippocampus region demonstrated a significant increase in both TGSH and GSH concentration, but not in Mn-SOD activity.

These findings are in agreement with the theoretical model, which demonstrated that increased NrF2 protein concentration in the brain would elicit an increase in antioxidant factor production via dissociation from KEAP1 and translocation to the nucleus. NrF2 concentration was significantly elevated in the hippocampus brain region only, which coincides with the significant increase in glutathione concentration reported in the hippocampus. This demonstrates the paradigm of NrF2-ARE binding promoting transcription of antioxidant defense factors.

Furthermore, significant decreases were observed in NrF2 concentration in both the cortex and cerebellum regions, which is reflective of the lack of change in antioxidant factors with training. With an already augmented basal concentration of antioxidant factors in a healthy brain, exercise training may not have been a severe enough insult to trigger NrF2 activation and subsequent transcription of additional antioxidant factors. Moreover, with an already elevated concentration of antioxidants, RONS would be immediately neutralized, suppressing NrF2 stabilization and translocation to the nucleus.

A study by Somani et al. 1995 examined the effects of exercise training on the antioxidant system in multiple brain regions. Rats were trained for 7.5 weeks on a ramp protocol with a conditioned speed of 30m/min on a 10° incline. Antioxidant factors were measured in response to training in the cortex, hippocampus, striatum, and brainstem regions. Somani and colleagues demonstrated a ~30% increase in SOD activity in both

the brainstem and cortex regions. GSH concentration significantly increased in the brainstem (~9%) region only and was undetectable in the hippocampus. However, GPx activity was not significantly different in any of the brain regions with exercise. These reports are disparate to the current study findings, where hippocampus was noted to exhibit not only detectable, but greater changes in TGSH and GSH concentration with exercise training.

Somani and colleagues also observed regional brain tissue differences in antioxidant factors with exercise training. SOD was noted to exhibit the greatest activity in the cortex (40 units/mg protein) and the lowest activity in the hippocampus brain region (24 units/mg protein) (Cortex>Brainstem>Striatum>Hippocampus). Furthermore, GSH was reported to exhibit the greatest concentration in the cortex and the lowest concentration in the brainstem region (Cortex>Striatum>Brainstem). These observations substantiate the current study findings that antioxidant factors are variable by individual brain region. This suggests that exercise activation of specific brain regions is varied (Iwamoto 1996) or that changes in blood flow during exercise may be altered in particular regions (Dishman 2006; Delp 2001), increasing the susceptibility to OS (Freeman 2012) and eliciting an increase in antioxidant activity or concentration.

In the current study, similar to the findings by Somani et al 1995, it is interesting to note that the individual brain regions demonstrated regional variations in Mn-SOD activity, despite no significant differences with training. The cortex and hippocampus brain regions demonstrated similar Mn-SOD activity (60 units/mg protein). Although, the cerebellum demonstrated a greater Mn-SOD activity (>1.5-fold) compared to the cortex

and hippocampus regions. However, Somani and colleagues reported the hippocampus as having the lowest activity/concentration of antioxidants, whereas in the current study the hippocampus was noted to have an overall greater TGSH and GSH concentration compared to areas like cortex, which was reported as the most concentrated region by Somani et al. 1995.

In another study by Moiron et al. 2015, reports of antioxidant changes with exercise training are in agreement with the current study. Moiron and colleagues trained rats for 12 weeks with a conditioned speed of 35cm/s. Superoxide dismutase activity was measured in three forms, total, Mn-SOD, and Cu-Zn-SOD. In all three forms, there were no significant differences between exercise trained and controls on a normal diet. However, the one noted difference was in catalase activity, which significantly increased with exercise by ~50% compared to controls. In addition, Asku et al. 2009 utilized an identical exercise protocol to the current study and reported no significant differences in SOD and GPx activity with training. These studies suggest that exercise at intensities well-known to increase markers of OS in the skeletal muscle and blood (Donato 2014; Bloomer 2005) minimally affect brain antioxidant factor responses to adaptive exercise training.

In a study by Devi et al. 2004, rats were trained to swim for 12 weeks with 3% of their body weight tied to their tail (water temperature unknown). Devi and colleagues reported significant increases in GPx (~17-26%), SOD (~22-32%), and catalase (~32%) activity in the hippocampus and cortex brain regions with exercise training. This study was in complete contention from the current study. Swimming imposes a greater stress

response than running via the hypothalamic-adrenal-pituitary-axis (HPA-axis) (Concarteze 2008) and may also induce a hypoxic stress as well. This suggests that the mode of exercise and the extent of activation of the HPA-axis may be contributing factors to the change in antioxidant factors with exercise training. In fact, whole body stress may cause a more robust change in antioxidant activity and/or concentration compared to activation of concentrated muscle groups.

As suggested by Leeuwenburg et al. 1994, exercise training may increase antioxidant factors, but these adaptations are limited by basal antioxidant levels. Tissues with greater antioxidant activity prior to training demonstrate little change in antioxidant activity after training compared to lower initial basal levels. Therefore, increases in antioxidant factors with training may be transient and peak at a critical point during the training regime and then return to baseline as protection is mediated. Some studies, including the current study, have demonstrated significant decreases in antioxidant factors with training (Strobel 2011). This may be indicative of a time dependent adaptation in basal antioxidant factors that could have been missed with such a short training period (5-7 weeks) compared to previous literature which implemented longer training periods (10-14 weeks).

Finally, in this particular study, the correlation between each brain region and gastrocnemius skeletal muscle was examined in regards to antioxidant factors to identify if a relationship exists between changes in antioxidant concentration and/or activity in the brain compared to skeletal muscle with chronic exercise. As previously mentioned, there were no significant relationships between skeletal muscle and any brain region in regards

to antioxidant factors. Reports of skeletal muscle and brain antioxidant responses to exercise tend to vary depending on the antioxidant factor measured and the muscle tissues examined. This may explain the lack of any significant correlations. Additionally, peripheral tissue responses may not be reflective of central tissue responses with exercise.

Summary

The effects of acute and chronic aerobic exercise on NrF2 protein concentration is highly complex in nature and will require additional research to fully elucidate this paradigm. In this particular study, compared to others, it seems that intensity of exercise and degradation processes are the primary factors responsible for the change in NrF2. Furthermore, the increase in antioxidant factors is directly related to an increase in NrF2 protein concentration in the brain. Finally, individual tissues, including blood, demonstrated variable responses to aerobic exercise. Based on these findings, it appears that in a healthy population, skeletal muscle and brain tissue are highly protected by endogenous defense systems and demonstrate little variation in markers of OS and antioxidant factors with acute and chronic aerobic exercise in these rats.

Recommendations

A greater body of research is warranted to fully elucidate the response of NrF2 in both skeletal muscle and brain tissue. In addition, more evidence is required to validate the changes in both markers of OS and antioxidant factors in tissues. A more clear understanding of tissue OS responses with exercise will allow for optimal design of exercise programs to increase NrF2 concentration and subsequent antioxidant factors.

Finally, the knowledge gained from the antioxidant defense system could lead to future roles in neurodegenerative diseases and overall neuroprotection.

1. The present experiment should be repeated with a greater sample size for acute aerobic exercise and measurement of markers of OS in the blood, skeletal muscle, and brain tissue. The OS markers demonstrated a non-significant pattern of increase with exercise that may be more robust with greater power.
2. Based on previous evidence suggesting an optimal measurement window for markers of OS and antioxidant factors (Michailidis 2007), it may be of interest to complete a time course experiment or to sacrifice a small number of animals over a period of time for a more accurate detection of markers of OS and antioxidant factors.
3. It would also be of interest to examine the effects of nutrition on NrF2 and antioxidant factors. It is well known that antioxidant supplements can increase activity and/or concentration of various antioxidants. Repeating this experiment with a supplement group could aid in the mechanistic understanding of NrF2 and antioxidants with exercise.
4. These experiments, acute and chronic, utilized mix gastrocnemius muscle, which contains both type 1 (slow twitch) and type 2 (fast twitch) muscle fibers. It may be more compelling to separate this muscle into type 1 and type 2. Furthermore, other muscles such as soleus and hindlimb, which are more commonly measured in the literature, should be examined.

5. To more efficiently detect changes in NrF2, antioxidant factors, and markers of oxidative stress, it may be of interest to exercise train animals for multiple weeks and examine the adaptive response of training to a single bout of acute aerobic exercise.
6. Based on the conflicting evidence in the literature surrounding NrF2, it may be advantageous to examine changes in KEAP1 in addition to NrF2 as well as the basal regulating factors of autophagy and proteasome degradation on this system with acute and chronic aerobic exercise.
7. Finally, it may be of interest to examine the difference in NrF2 changes with exercise compared to a greater insult like ischemia-reperfusion injury to understand the degree to which stress enhances or suppresses the antioxidant defense system.

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APPENDIX A

CHEMICALS

Western Blot Chemicals

RIPA Buffer (pH 7.4)–
50mM Tris Base
1mM EDTA
150mM NaCl
1% NP-40
1% Na Deoxycholate
1% SDS

Resolving Gel Tris 1.5M (pH 8.8)-
90.85g Tris Base
17ml HCl
In 500ml deionized water pH 8.8

Stacking Gel Tris 1.0M (pH 6.8)-
30.3g Tris Base
20ml HCl
In 250ml deionized water pH 6.8

APS (10%)-
4g Ammonium Persulfate
In 40 ml of deionized water

2x Loading Buffer-
25ml Tris 6.8
40ml SDS (4%)
10ml glycerol (10%)
8mg bromophenol blue (0.008%)
Makes 100ml

Electrophoresis Buffer (10x)-
90.8g Tris Base
432.4g glycine
30g SDS
In 3 liters deionized water

Transfer Buffer (10x)-
30.26g Tris Base
144.13g Glycine
20% methanol
In 1000ml deionized water

DTT-
154mg Dithiothreitol
In 10ml of deionized water

10x TBST –
24.23g Tris Base
87.66g NaCl
10ml Tween 20x
In 1000ml deionized water pH 7.6

Protease Inhibitor Part A –
1mg Aprotinin
3 x 1mg E-64
5mg Leupeptin
Makes 5ml

Protease Inhibitor Part B –
15mg 1,10-Phenanthroline
132mg PMSF
15ml Methanol (100%)
1mg Pepstatin A

Tyrosine Phosphatase Inhibitor–
1.1g Sodium Orthovanadate
In 25ml deionized water pH 10

Phosphatase Inhibitor –
4.3mg NaF
43.2mg β-Glycerophosphate
In 1ml RIPA buffer

HPLC Chemicals

Mobile Phase 0.1M –
13.80g Sodium Phosphate Monobasic
In 1000ml deionized water pH 2.5

5% 5-sulfosalicylic acid –
5g 5-sulfosalicylic acid
In 100ml deionized water

20% Methanol Solution –
200ml of 100% Methanol
800ml of deionized water
In 1000ml HPLC grade container

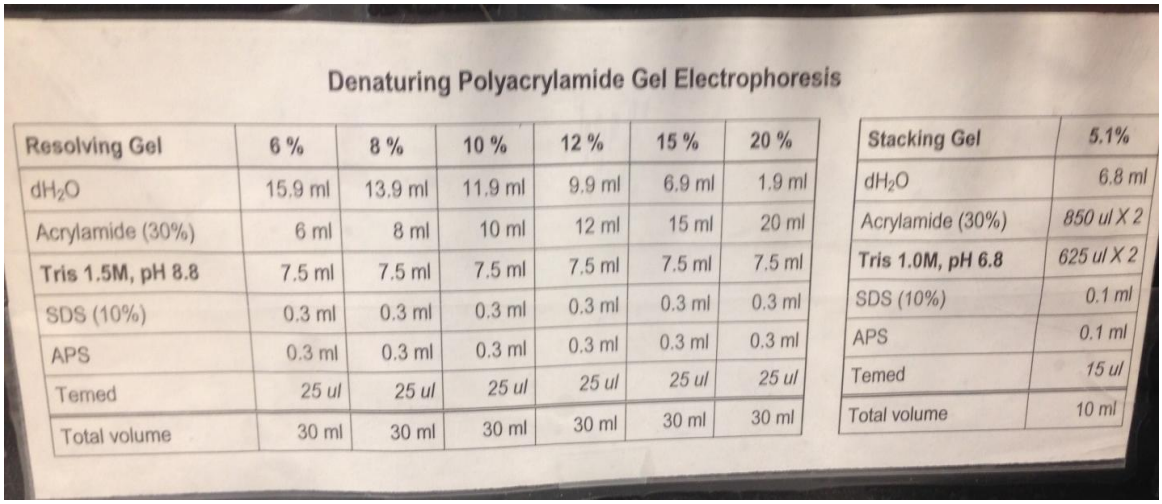
APPENDIX B

WESTERN BLOTTING

Gel Electrophoresis

To determine the NrF2 protein content of samples, a western blotting analysis was performed. First a 10% acrylamide resolving gel was made for the gel electrophoresis (Fig. B1). This solution was poured into a glass plate cartridge to polymerize and capped with deionized water for approximately 30min (Fig. B2). Electrophoresis buffer was made using 100ml of 10x electrophoresis buffer and 900ml of deionized water. When the resolving gel polymerized, the water cap was poured off and the cartridge containing two glass plates was gently dried with filter paper. The cartridge was placed in the tank of the Hoeffer SE400 vertical electrophoresis unit. Electrophoresis buffer was placed in the bottom tank once the resolving gel polymerized.

A stacking gel was made and poured into the cartridge above the 10% resolving gel for the loading of the samples (Fig. B1). The stacking gel was poured into the glass plate cartridge above the resolving gel once the deionized water was removed (Fig. B2). A 15 tooth comb was cleaned with 70% ethanol and dried before insertion into the cartridge to form the loading lanes. The stacking gel was then allowed to polymerize for approximately 30min.



Denaturing Polyacrylamide Gel Electrophoresis

Resolving Gel	6 %	8 %	10 %	12 %	15 %	20 %
dH_2O	15.9 ml	13.9 ml	11.9 ml	9.9 ml	6.9 ml	1.9 ml
Acrylamide (30%)	6 ml	8 ml	10 ml	12 ml	15 ml	20 ml
Tris 1.5M, pH 8.8	7.5 ml	7.5 ml	7.5 ml	7.5 ml	7.5 ml	7.5 ml
SDS (10%)	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml
APS	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml
Temed	25 ul	25 ul	25 ul	25 ul	25 ul	25 ul
Total volume	30 ml	30 ml	30 ml	30 ml	30 ml	30 ml

Stacking Gel	5.1%
dH_2O	6.8 ml
Acrylamide (30%)	850 ul X 2
Tris 1.0M, pH 6.8	625 ul X 2
SDS (10%)	0.1 ml
APS	0.1 ml
Temed	15 ul
Total volume	10 ml

Figure B1. Polyacrylamide Gel Protocol for Western Blotting.

Sample Preparation

Once the stacking gel had polymerized, the comb was gently removed, and lanes were straitened using a straight edge dull needle (only if deemed necessary). Electrophoresis buffer was used to fill the lanes as well as to remove any bubbles. A sample protein concentration of 60ug/well was prepared for each sample. Appropriate sample volumes were determined by the total protein concentration (ug/ul) and combined with an equal amount of 2x loading buffer (500ul) and DTT (25ul). After combining the protein with the loading buffer, the samples were boiled at 80°C and briefly centrifuged to ensure all sample was at the bottom of the Eppendorf tube. The samples were kept on ice throughout the preparation process until they were ready to boil and be loaded into the gel for electrophoresis. The samples were then pipetted into the appropriate lanes. The Hoeffer SE400 electrophoresis top tank was then secured to the top of the cartridge (Fig. B2). The remaining electrophoresis buffer was added to the top and bottom tank for the

vertical electrophoresis chamber (SE400 Air-Cooled Vertical Electrophoresis Unit, Hoefer, Inc. Holliston, MA) and the gel electrophoresis was run over night (approximately 12-15 hours) at 55-65V.

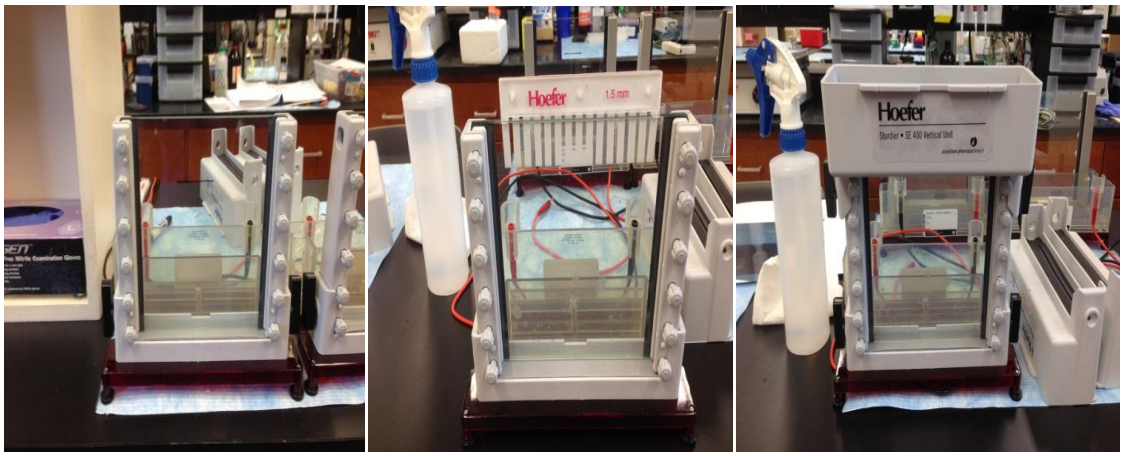


Figure B2. Hoeffer SE400 Electrophoresis Unit.

Transfer Electrophoresis

Once the electrophoresis process was complete, the gels were removed from the glass plate cartridge. The stacking gel was cut away and the gel was then placed in transfer buffer. A polyvinylidene fluoride (PVDF) membrane that was briefly soaked in 100% methanol was placed flush against the gel outside of the transfer buffer. The membrane was smoothed against the gel, careful to remove any and all bubbles, and then sandwiched between 2 sponges and 2 pieces of filter paper. Together this was secured into a plastic cartridge for transfer electrophoresis (Fig. B3). The cartridge was placed into a transfer electrophoresis box and filled with transfer buffer (Fig. B3). The transfer electrophoresis was then run, on ice, for 6 hours at 200mA. Ice was added throughout the 6 hour transfer as necessary to maintain a cool temperature for the transfer buffer.

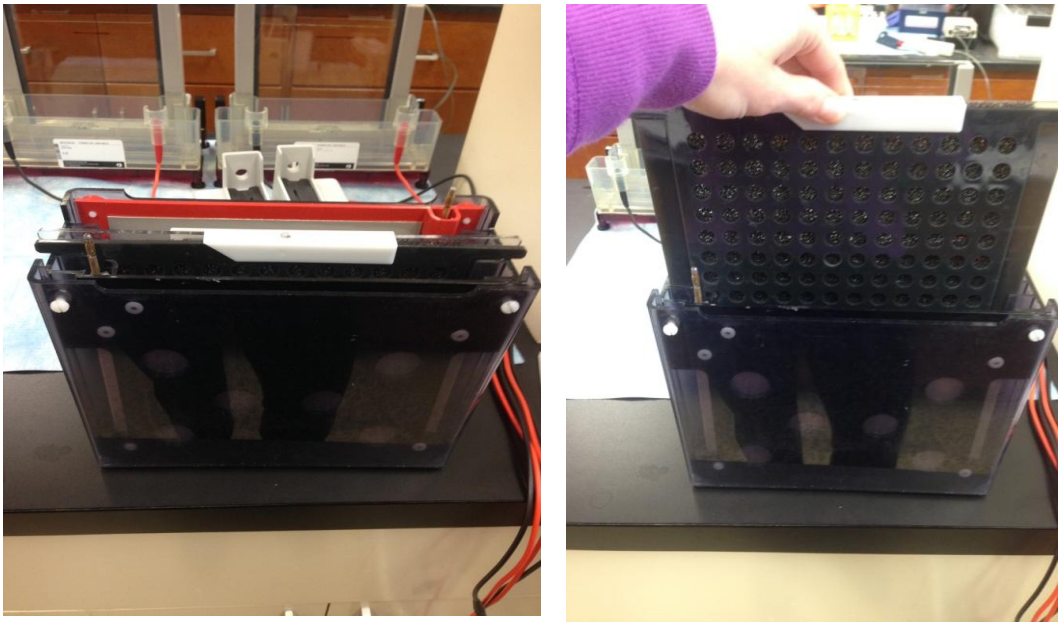


Figure B3. Transfer Electrophoresis Apparatus.

Western Blotting

After the transfer electrophoresis was complete, the gels were discarded and the PVDF membrane was washed in 1X TBST for 5min on an orbital shaker set at approximately 100rpm. The TBST was discarded and the PVDF membrane was blocked in a 5% TBST milk solution for 1 hour on an orbital shaker set at approximately 100rpm.After the blocking step, the 5% TBST milk was discarded. The membrane was washed again 3X for 5min each in TBST, on an orbital shaker. The TBST was discarded after each wash and new TBST was added. Meanwhile, plastic boxes for administration of primary antibodies were blocked for approximately 10min in 5% TBST milk to reduce non-specific binding of the antibody to the plastic surface of the box (Fig. B4). The 5% TBST milk was then discarded. After washing, the membrane was placed in a box on a rocker and 15ml of primary antibody in bovine serum albumin (BSA) was added. The

membrane was then probed overnight with primary antibody on the rocker at 4°C (≥12hrs). The primary antibody for NrF2 was purchased from Abcam (ab31163).

When probing with the primary antibody was done, the antibody was saved for reuse. The membrane was washed 3X for 10min in a 5% TBST milk solution. The solution was discarded after each wash. Meanwhile, the plastic box for the administration of secondary antibody was blocked for ≥10min in5% TBST milk. The 5% TBST milk was then discarded (Fig. B4). After washing, the membrane was placed in a box on a rocker and then 15 ml of 5% milk and antibody was added to the membrane. The secondary antibody in 5% milk was put on the rocker at room temperature for 1 hour.

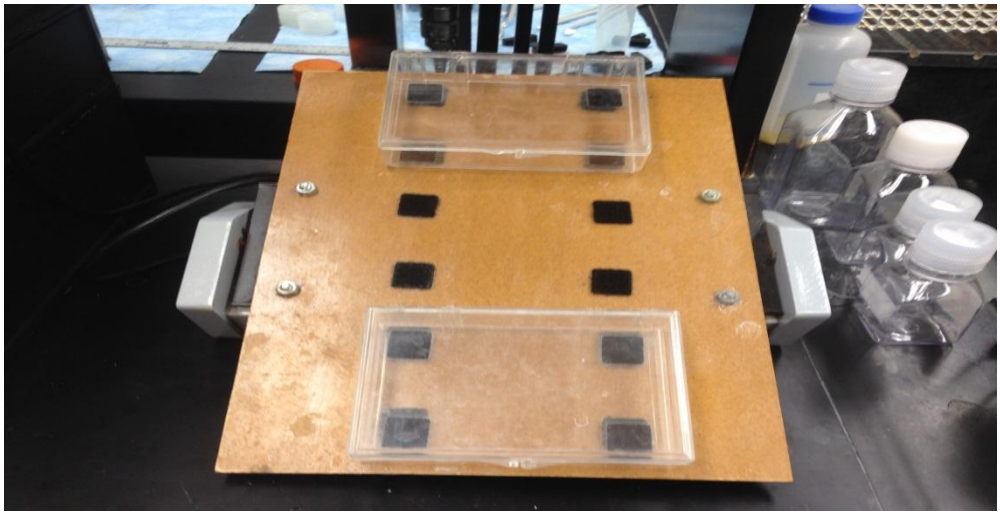


Figure B4. Plastic Box and Rocker. This apparatus was used to administer primary and secondary antibodies.

Western Blot Imaging

Once the secondary antibody was done incubating, the antibody was discarded. The membrane was washed 4X for 10min each in TBST, discarding used TBST with

each wash. The membrane was then immersed in ECL plus (1:1 ratio) (PerkinElmer Inc. Waltham, Massachusetts) for 2 minutes and imaged on BioRad Chemidoc Station. An example of these images for the NrF2 can be seen in Figure 5B. The band density was assessed using Quantity One software on the same computer connected to the Chemidoc. NrF2 was normalized to the loading control beta-actin. An average density was collected for each sample (NrF2 or beta-actin) and subtracted from the average background density to obtain a "clean density". Finally, the clean density for NrF2 was divided by the clean density for beta actin to obtain a normalized protein concentration value (arbitrary units).

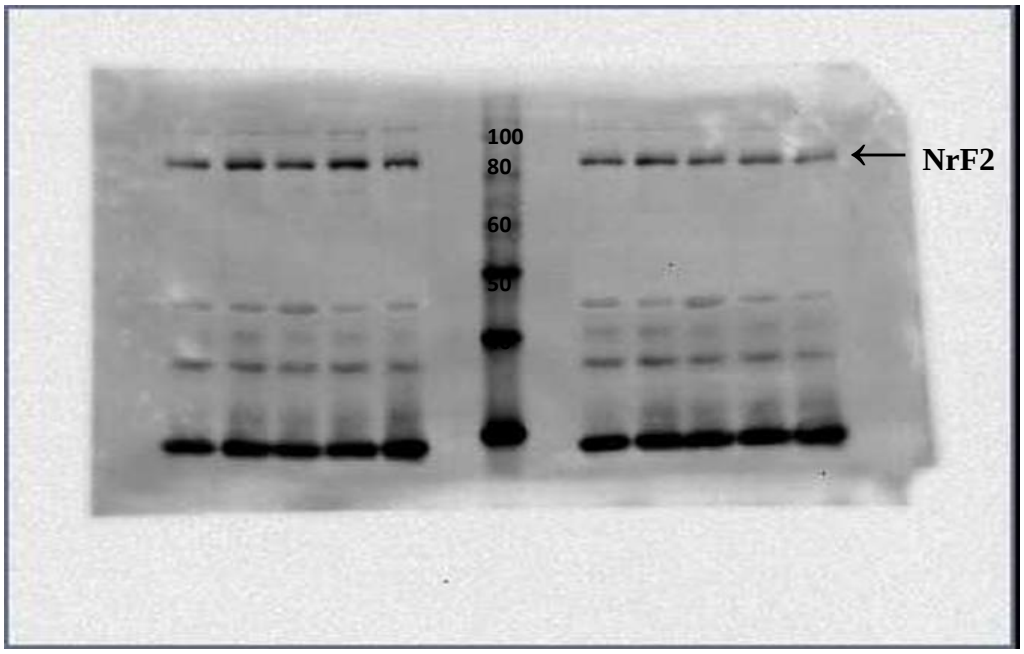


Figure B5. Example Image. NrF2 Protein Banding in the Gastrocnemius Muscle Using the BioRadChemidoc Station.

APPENDIX C

CELL CULTURE

NrF2 protein identification through western blot analysis has been called into question in recent years. Beginning as early as 2013, Lau and colleagues wrote a letter to the editor suggesting that the current literature has been misidentifying NrF2 protein bands on western blot analyses and reporting these inaccurate findings. This has led to a critical debate as to whether or not the data surrounding NrF2 changes in any tissue are valid. Therefore, a positive control is warranted when detecting NrF2 to ensure proper identification of the correct protein band. Induction of NrF2 through treatment with known proteasome inhibitors like tert-Butylhydroquinone (tBHQ) or MG132 prevent ubiquitination and allow NrF2 to accumulate in the cytosol (Li 2014). Hydrogen peroxide (H_2O_2) is a pro-oxidant capable of inducing a state of oxidative stress and has been shown to increase cellular NrF2 concentration (Pickering 2012). Additionally, cardiac muscle is the most oxidative tissue in the body with a high antioxidant capacity (Venditti 1997) and ability to increase NrF2 protein concentration under stress conditions (Muthusamy 2012; He 2009; Valcarcel-Ares 2012). Furthermore, gastrocnemius muscle is composed of both fast and slow twitch fibers making it a moderately oxidative tissue with a relatively high antioxidant capacity (Venditti 1997) due to its propensity to induce oxidative stress. Finally, as outlined in the "Literature Review" chapter, chronic exercise has also been demonstrated to increase NrF2 concentration in the whole brain and striatum.

To contribute additional clarity to the literature surrounding the migration pattern and mobility of NrF2 protein using a Bis-Tris SDS-PAGE gel, a cell culture experiment was conducted using adipocytes. Cells were treated for 4-hrs with either tBHQ (25uM), H_2O_2 (300uM), MG132 (10uM), or were given no treatment. After the treatment cells were collected and labeled in Eppendorf tubes and stored at -80°C until further analysis. Prior to western blot analysis, a protein determination was conducted using the standard BCA procedures, which are outline in the "Methods" chapter. Western blot procedures were conducted in the same manner as those listed above (Appendix B). A second cell culture experiment was repeated in a similar manner to the first, but with additional treatment groups. Cells were treated for 6-hrs with either tBHQ (25uM), H_2O_2 (300uM), MG132 (10uM), H_2O_2 + MG132, tBHQ + MG132, or were given no treatment.

The purpose of the cell culture experiment was to provide a positive control to aid in the correct identification of NrF2 protein bands on a western blot. However, the outcome of the cell culture experiment was inconclusive. The accepted and undisputed molecular weight of NrF2 is 66kda; however, as discussed previously in the "Literature Review" chapter, based on the type of gel, Bis-Tris vs. Tris-glycine, and whether an SDS-PAGE or a gradient gel were used, effects the mobility and migration pattern of NrF2. Based on the treated cells, no protein bands were detected between 95-110kda as Lau et al. 2013 suggested (Fig. 1C). However, protein bands were detected at approximately 66kda and 80kda. The adipocytes demonstrated bands at the known molecular weight of 66kda, but heart and skeletal muscle tissue did not display any bands in this region (Fig. 1C). Furthermore, heart and skeletal muscle displayed bands at both

100kda and 80kda (Fig. 1C) as Lau et al. 2013 and Kremmerer et al. 2015 suggested, respectively.

Given the current literature suggesting differences in the mobility and migration pattern of NrF2 in western blot and the fact that our western blot procedure implements a Bis-Tris SDS-PAGE gel, the results confer with Kremmerer and colleagues (Kremmerer 2015). The only protein band that is expressed consistently across cell types (adipocytes, cardiac muscle, skeletal muscle) and various treatments (tBHQ, H_2O_2, MG132) is located at approximately 80kda. This phenomenon was also demonstrated in brain tissue including hippocampus, cerebral cortex, and cerebellum along with heart and skeletal muscle (Fig. C2).

In the current experiment, there were no significant changes in NrF2 protein concentration with any of the treatments. This is contradictory to previous reports of NrF2 activation in a variety of tissues, including skeletal muscle, cardiac muscle, and adipocytes. However, despite the appearance of consistent banding at 80kda, this still does not account for the lack of NrF2 induction with known activators such as tBHQ, H_2O_2, sulforaphane, and MG132. In previous literature, others have reported significant increases in Nrf2 protein concentration in various tissues with similar treatments (Li 2014; Lee 2011; Kremmerer 2015; Cui 2013; Li 2005; Pickering 2012).

Although, a major limitation to these studies is the fact that they either do not specify the molecular weight at which they intended to detect NrF2 and/or they do not provide an entire image of their western blot to indicate the band they suspect to be NrF2. Given the recent information regarding migration and mobility differences in western

blot procedures measuring Nrf2, it is unclear if these reports are correctly identifying NrF2 or are misinterpreting their data as Lau and Colleagues suggest. Clearly, future research is warranted to clarify NrF2 detection via western blot and changes associated with known NrF2 activators.

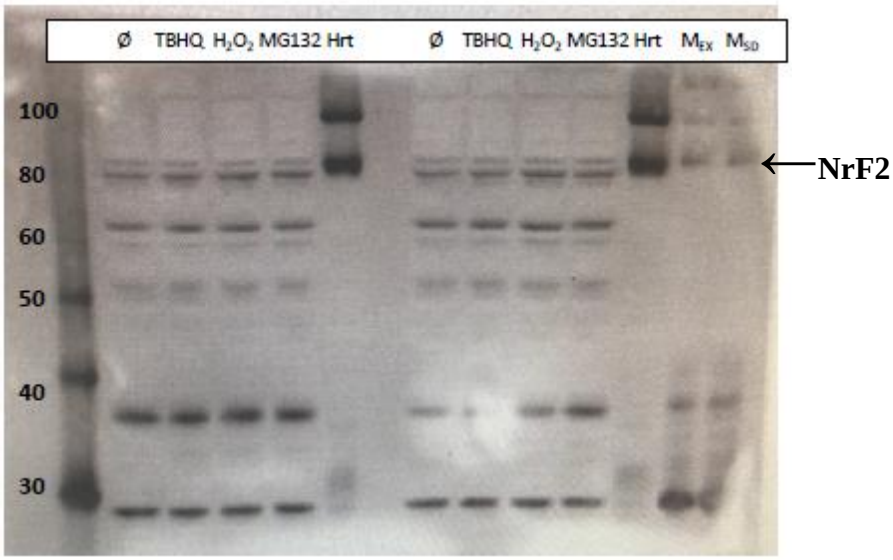


Figure C1. Cell Culture Experiment. Identification of NrF2 protein expression at 80kda. Adipocytes were treated for 4hrs with tBHQ (25uM), H_2O_2 (300uM), MG132 (10uM) or received no treatment.Ø=no treatment, tBHQ=tert-Butylhydroquinone, H_2O_2=Hydrogen Peroxide, MG132=MG132, Hrt=Heart, M_{EX}=Exercise Trained Gastrocnemius Muscle, M_{SD}=Sedentary Gastrocnemius Muscle.

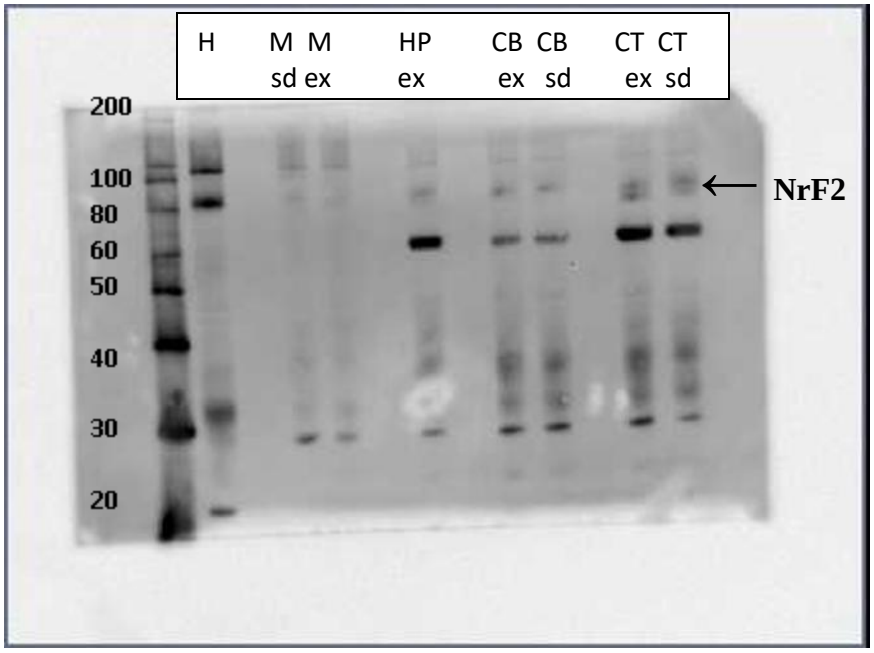


Figure C2. Identification of NrF2 at 80kda Across Multiple Cell Types. H=Heart, M=Muscle, HP=Hippocampus, CB=Cerebellum, CT=Cerebral Cortex, sd=Sedentary, ex=Exercise Trained.