THESIS

NRF2 ACTIVATION BUT NOT VITAMIN C TREATMENT PROMOTES PROTEOSTATIC MAINTENANCE DURING AN OXIDATIVE CHALLENGE

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ABSTRACT

NRF2 ACTIVATION BUT NOT VITAMIN C TREATMENT PROMOTES PROTEOSTATIC MAINTENANCE DURING AN OXIDATIVE CHALLENGE

Improved proteostasis may be a mechanism of stress resistance, and it is likely that the increased protein turnover with exercise training contributes to adaptation to stress. Exogenous antioxidant treatments such as vitamin C (VitC) target the detrimental effects of reactive oxygen species (ROS), but may simultaneously prevent the beneficial redox signaling associated with exercise. A possible alternative strategy to prevent oxidative damage while permitting redox-sensitive signaling is to increase endogenous antioxidants. The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) increases the transcription of endogenous antioxidants by binding to the antioxidant response element in the promoter region of target genes. Protandim (Pro, LifeVantage), a combination of five phytochemicals, activates Nrf2 by increasing its translocation to the nucleus. We hypothesized that, compared to VitC, treatment with the Nrf2 activator Pro would not blunt ROS induced proteostatic maintenance. To mimic ROS signaling, C2C12 myoblasts were treated with H2O2. Treatment occurred alone or in combination with either VitC or Pro. Deuterium oxide labeling was used to measure protein synthesis in the mitochondrial and cytosolic cell fractions after 2, 4, 8, and 12 hours of treatment. Simultaneously cell proliferation was measured by deuterium incorporation into DNA. Compared to the untreated control, H2O2 alone increased DNA synthesis but did not
increase mitochondrial protein synthesis, resulting in decreased proteostasis. Compared to 
H$_2$O$_2$ alone, Pro decreased protein synthesis in both cytosolic and mitochondrial fractions. 
However, Pro also decreased DNA synthesis. This resulted in a greater protein to DNA ratio 
suggesting maintenance of proteostasis. VitC with H$_2$O$_2$ increased DNA synthesis and decreased 
proteostasis, similar to H$_2$O$_2$ treatment alone. From these data, it appears that although 
treatment with exogenous antioxidants increases proliferation, activation of Nrf2 maintains 
mitochondrial protein synthesis despite a reduction in proliferation. Further study into the role 
of Nrf2 in improving mitochondrial proteostasis to promote stress resistance is warranted.
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# TABLE OF CONTENTS

ABSTRACT...............................................................................................................................................ii

ACKNOWLEDGEMENTS.......................................................................................................................iv

TABLE OF CONTENTS........................................................................................................................v

CHAPTER I: INTRODUCTION ................................................................................................................. 1

Statement of Problem ................................................................................................................... 3

Hypotheses ......................................................................................................................................... 3

CHAPTER II: LITERATURE REVIEW ..................................................................................................... 4

Redox Balance and Signaling ............................................................................................................ 4

ROS Production and Elimination ..................................................................................................... 4

Redox Signaling ................................................................................................................................. 7

Oxidative Stress is Hormetic ............................................................................................................. 9

Proteostasis and Mitochondria ......................................................................................................... 13

Mitochondrial Proteostasis .............................................................................................................. 14

Mitochondrial Redox Signaling ......................................................................................................... 15

Disruption of Exercise-Induced ROS signaling with Antioxidant Supplementation ............ 17

Upregulation of the Endogenous Antioxidant System ................................................................. 19

D₂O Labeling to Measure Proteostasis ............................................................................................. 20

CHAPTER III: METHODS AND PROCEDURES ............................................................................... 22

Method Overview .............................................................................................................................. 22

Cell Model ........................................................................................................................................ 22
Preparation of Samples and GC/MS Analysis ................................................................. 24

Cell Fractionation and Mitochondrial Isolation ......................................................... 24

Media Derivation and Analysis ............................................................................... 25

Alanine Derivation and Analysis ........................................................................... 25

DNA Isolation and Analysis ................................................................................... 26

Statistical Analysis .................................................................................................. 27

CHAPTER IV: RESULTS .............................................................................................. 28

CHAPTER V: DISCUSSION .......................................................................................... 35

Principle Outcomes ................................................................................................. 35

Measuring Proteostasis ............................................................................................ 35

Hydrogen Peroxide ................................................................................................. 37

Vitamin C .................................................................................................................. 38

Nrf2 Activation ......................................................................................................... 39

Conclusions and Future Directions ........................................................................ 41

REFERENCES ............................................................................................................ 43
CHAPTER I: INTRODUCTION

Under normal physiological conditions, reactive oxygen species (ROS) are produced in various cellular processes and are eliminated by the antioxidant defense system [1]. ROS are highly reactive and cause oxidative damage to proteins, lipids and DNA [2]. Consequently, if the redox environment becomes chronically unbalanced in favor of ROS production, an oxidative stress occurs and results in irreparable damage to proteins and can cause cell death [3]. A well-functioning proteostatic network is required for degrading oxidatively damaged proteins and replacing them with newly synthesized proteins in order to maintain quality of the proteome and cellular function [4].

ROS can also act as signaling molecules through reversible post-translational modifications in order to alter protein function [5]. Hydrogen peroxide is thought to be the ROS with the greatest signaling capabilities since it can either directly oxidize protein thiols or react with highly reactive thiol oxidoreductase enzymes, such as peroxiredoxins, which then react with a signaling protein [6]. Hydrogen peroxide has been shown to increase the activity of central redox sensitive transcription factors such as heat shock factor 1 (HSF1), nuclear factor κ-B (NF-kB) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [7]. As a result of activation by hydrogen peroxide, these transcription factors induce the expression of genes including those that code for heat shock proteins and antioxidant enzymes. In this manner, hydrogen peroxide signaling is an adaptive response to improve proteostatic maintenance and stress resistance. Therefore, hydrogen peroxide has a hormetic affect, where an acute increase in concentrations induces adaptive changes, while a chronic exposure could be lethal to the cell.
The ROS-induced stress response also includes the activation of signaling to upregulate mitochondrial biogenesis [8, 9]. Mitochondrial biogenesis is the synthesis of new mitochondrial proteins and their incorporation into the mitochondrial protein network. The combination of mitochondrial biogenesis and degradation of damaged mitochondrial proteins contributes to the maintenance of mitochondrial proteostasis and prevention of mitochondrial degeneration [10]. Notably, mitochondrial proteostasis impacts overall cellular function due to the mitochondria’s central role in energy production, ROS production and apoptotic signaling [11].

Increased skeletal muscle contractility, such as during a bout of exercise, results in an acute increase in ROS production [12]. Supplementation with exogenous antioxidants during exercise has been studied as a means to prevent oxidative damage from a ROS challenge. There are conflicting data in the literature on whether or not antioxidants interfere with the adaptive response to increased ROS levels, specifically regarding mitochondrial function. Some evidence suggests supplementation with vitamin C and/or vitamin E does not alter the response to exercise training [13-15]. However, others have found evidence showing supplementation with vitamin C and/or vitamin E does result in a blunted response to exercise training, as measured by markers of mitochondrial biogenesis [16, 17].

An alternative way to prevent oxidative damage from ROS overproduction but still allow mitochondrial adaptations could be to upregulate the endogenous antioxidant system. The transcription factor Nrf2 is a regulator of the antioxidant defense system. Upon activation, Nrf2 translocates to the nucleus where it induces expression of genes coding for antioxidant enzymes to protect against an oxidative challenge [18, 19]. It has also been determined that Nrf2 has a role in nuclear respiratory factor 1 (NRF-1) expression [20] and peroxisome
proliferator-activated receptor-γ coactivator 1α (PGC1α) activation [21] to regulate mitochondrial biogenesis. Therefore, Nrf2 activation could be a potential mechanism for allowing redox-sensitive adaptations to occur while promoting redox balance.

**Statement of Problem**

The purpose of this study is to determine if either treatment with vitamin C or Nrf2 activation will interfere with hydrogen peroxide signaling as reflected by rates of mitochondrial protein and DNA synthesis in myoblasts.

**Hypotheses**

It is hypothesized that treatment with hydrogen peroxide will increase mitochondrial protein synthesis and proteostasis and vitamin C treatment, but not Nrf2 activation, will blunt this increase.
CHAPTER II: LITERATURE REVIEW

Redox Balance and Signaling

Pro-oxidant species and antioxidant molecules determine the redox balance of the cell. Reactive oxygen species (ROS) includes both free radical and non-radical oxygen containing molecules that undergo electron exchange reactions. A free radical contains an unpaired electron and reacts with other molecules in order to fill its molecular orbital. Non-radicals contain complete sets of paired electrons but are reactive compounds in that they readily generate free radicals. Reactive nitrogen species (RNS) also affect redox balance and can react with oxygen species to form other reactive molecules. ROS are highly reactive and cause oxidative damage to proteins, lipids and DNA [2]. However, ROS can act as signaling molecules through reversible post-translational modifications in order to control protein function (Kramer 2015). The cell has an antioxidant defense system in place to scavenge reactive species before they cause oxidative damage as well as to repair or replace damaged biological molecules. When ROS production is matched by ROS elimination oxidative damage is kept to a minimum. However, when a chronic imbalance occurs in the redox environment, either through over-production of ROS or impaired antioxidant function, oxidative damage can accumulate and cause oxidative stress and disruption of cellular homeostasis.

ROS Production and Elimination

The mitochondria are one of the main sites of ROS production. Specifically, the electron transfer system (ETS) generates superoxide during the process of cellular respiration and ATP synthesis [22]. The ETS is comprised of five distinct protein complexes. These complexes
transfer electrons in order to generate a proton gradient to drive ATP synthesis. Electrons leak from the ETS, mainly at complexes I and III, and transfer to molecular oxygen to generate superoxide anions. Within the mitochondrial matrix, superoxide dismutates into hydrogen peroxide and water. Hydrogen peroxide is a non-radical reactive oxygen species that is unable to react directly with lipids or DNA, making proteins its primary substrate [23]. Hydrogen peroxide homolytically cleaves into hydroxyl radicals and hydroxyl ions. Hydroxyl radicals are highly reactive and have a broad reactivity, meaning they can oxidize a variety of biological molecules [6]. As a consequence, hydroxyl radicals are considered the most damaging ROS and exert their effect close to their site of production.

Non-mitochondrial sources of ROS include the endoplasmic reticulum and NADPH oxidase. The enzymes in the family of NADPH oxidases (NOX) are found in cellular membranes and produce superoxide. The primary substrate of superoxide in the cytosol is [Fe-S] containing proteins. Cytosolic superoxide is also converted into hydrogen peroxide, causing hydrogen peroxide to exist in the cytosol. Nitric oxide is produced by the enzyme nitric oxide synthase from L-arginine and is generated during the inflammatory response. Nitric oxide reacts with superoxide to form peroxynitrite more rapidly than superoxide can dismutate into hydrogen peroxide [23]. Peroxynitrite is a stronger oxidizing agent than nitric oxide and reacts with DNA and proteins.

Non-enzymatic antioxidant molecules that are synthesized endogenously or are introduced exogenously act as electron donors in order to directly scavenge and stabilize ROS. Ascorbic acid (AA), or vitamin C, is a water-soluble vitamin and is a main contributor to the antioxidant defense system in the mitochondria [24]. Skeletal muscle cannot synthesize AA and
therefore imports it from the extracellular fluid and stores it at concentrations of 3-4mg/100g wet tissue weight [25]. AA is a cofactor for the key enzyme of carnitine synthesis as well as an antioxidant reactant with hydrogen peroxide [26]. When oxidized, AA forms ascorbyl free radical (AFR) which rapidly dissociates into AA and dehydroascorbic acid (DHA). The reduced form of AA can be regenerated from both AFR and DHA by cytosolic and mitochondrial reductases [25]. Glutathione (GSH) is a tripeptide non-protein thiol that is synthesized in the liver and transported to tissues through circulation. In addition to being a ROS scavenger, GSH provides electrons to reform other non-enzymatic antioxidants and to serve as a substrate for enzymatic antioxidants in redox reactions. For example, AA can be regenerated through reduction by GSH [24]. The reduced form of GSH can be regenerated through the redox reaction catalyzed by the enzyme glutathione reductase using NADPH as the hydrogen and electron source.

Antioxidant enzymes are selective and catalyze reactions to convert ROS into other reactive molecules or more stable molecules. Superoxide dismutase (SOD) is a primary antioxidant enzyme that catalyzes the dismutation of superoxide radicals into hydrogen peroxide and oxygen while preventing the formation of peroxynitrate. Three isoforms of SOD are found in mammalian cells with SOD1 localized in the cytosol and mitochondrial intermembrane space and SOD2 localized in the mitochondrial matrix. In skeletal muscle, mitochondrial SOD accounts for 15-35% of the total SOD activity while the remaining 65-85% occurs in the cytosol [27]. The enzyme catalase (CAT) degrades hydrogen peroxide into water and oxygen. CAT has a high Km meaning it has a low affinity for hydrogen peroxide at low concentrations. This makes CAT an important part of the antioxidant defense system when
there are relatively high concentrations of hydrogen peroxide. The peroxiredoxin (PRX) system includes 6 isoforms located in cytosolic and mitochondrial fractions of the cell. PRX reduces hydrogen peroxide and peroxynitrate to water by transferring electrons from its catalytic cysteine residue and is then reduced back to its active form using electrons from a thiol, such as GSH [28].

Overall, the balance between ROS production and ROS elimination determines the redox status of the cell. ROS are produced in the cytosol as well as in specific cellular organelles, including the mitochondria. Non-enzymatic antioxidants donate an electron in order to directly scavenge ROS while enzymatic antioxidants catalyze reactions for eliminating ROS.

Redox Signaling

Redox signaling is the process of changing the activity of signaling proteins by oxidative modification [7]. In low concentrations, ROS can cause reversible oxidation of proteins in order to regulate cell signaling pathways. Superoxide and hydroxyl radicals have short half-lives of $10^{-6}$ and $10^{-9}$ sec, respectively, making them poor signaling molecules. On the other hand, hydrogen peroxide has a longer half-life, $10^{-3}$s, and can diffuse through cellular membranes, making it a good signaling molecule. Further, the concentration and localization of hydrogen peroxide can be controlled for example, by altering the membrane permeability with hydrogen peroxide channels [6]. Under normal conditions, human plasma contains 1-8 µM hydrogen peroxide, with an average of 3µM [29]. Intracellular concentrations of hydrogen peroxide are more difficult to measure and can fluctuate rapidly. In the interstitial space of skeletal muscle under basal conditions, hydrogen peroxide concentrations are 10 -15 µM [23]. So far, there has been no discovery of a hydrogen peroxide receptor. However, it is suggested that the cell
contains sensors which detect changes in hydrogen peroxide concentrations and acts as the
signal mediator [7].

Hydrogen peroxide is able to relay signals through the oxidation of cysteine thiols [30].
The amino acid cysteine (Cys) contains a terminal sulfhydryl (thiol) group that is highly reactive
with hydrogen peroxide. Thiol oxidation results in the creation of a sulfenic acid derivative that
can further lead to formation of stable inter- or intra-molecular disulfide bonds with GSH or
other protein thiols [30]. These disulfide intermediates can also be reduced back to the thiol
form making thiol oxidation a reversible modification. However, subsequent oxidations of
sulfenic acid will form sulfinic acid and sulfonic acid which are irreversible modifications and
may render the protein inactive [29]. Reversible oxidation of cysteine residues by hydrogen
peroxide is a post-translational covalent modification that causes conformational changes and
alterations in protein activity (Kramer 2015). Reactivity of specific Cys residues varies
considerably and can change based on the protein environment and pH in order to provide
specificity and control of redox signal transduction [6]. Therefore, it is not likely that all protein
thiols react with hydrogen peroxide to the same degree. Some low reactivity thiol proteins
would require there to be a relatively high concentration of hydrogen peroxide in order to
undergo oxidation [30].

Thiol oxidoreductase enzymes transfer oxidizing equivalents from hydrogen peroxide to
target proteins in order to regulate redox signaling [6]. In addition to its antioxidant role in the
cell, it is suggested that PRX reacts with hydrogen peroxide then forms disulfide bonds with
proteins in order to alter their activity [6]. PRX has an incredibly high affinity for hydrogen
peroxide and therefore reacts very quickly and more often with hydrogen peroxide compared
to other protein thiols [28]. In mammalian cells, PRX was shown to have a direct role in transferring oxidizing equivalents from hydrogen peroxide to the transcription factor, STAT3 [31]. PRX may also be a negative regulator of hydrogen peroxide signaling. The flood-gate hypothesis implicates PRX as a ‘gate’ that controls hydrogen peroxide concentrations, in order to prevent other proteins from becoming oxidized [29]. PRX have a low Km for hydrogen peroxide, thus the decomposition of hydrogen peroxide occurs fairly quickly. Higher hydrogen peroxide concentrations cause hyperoxidation of the catalytic site of PRX causing it to become inactive, leading to accumulation of hydrogen peroxide. Consequently, more proteins come into contact and are oxidized by hydrogen peroxide than when PRX are active [29]. In both scenarios, PRX activity is altered in response to changes in hydrogen peroxide concentrations, suggesting it is involved in redox signaling.

As discussed above, redox signaling involves oxidative modification of the thiol group on protein cysteine residues. Hydrogen peroxide is thought to be the ROS with the greatest signaling capabilities because it is highly selective and its concentrations and localization within the cell can be controlled. The mechanism of hydrogen peroxide signaling includes direct oxidation of protein thiols or reaction with PRX which then forms disulfide bonds with a redox sensitive signaling protein.

Oxidative Stress is Hormetic

The classic definition of oxidative stress is “a disturbance in the oxidant-antioxidant balance in favor of the former” [32]. When an imbalance in the redox environment occurs, intracellular ROS levels increase resulting in lipid peroxidation, protein oxidation and damage to nuclear and mitochondrial DNA [33]. However, fluctuations in the redox environment are
normal under physiological conditions and have a role in cell signaling and adaptation. Thus, Jones proposed a new definition of oxidative stress to be “a disruption of redox signaling and control” [34]. The antioxidant defense and repair systems are in place to respond to increases in ROS and restore damaged biological molecules. When the redox state is uncontrolled or damage is irreparable, cellular function is disrupted. For example, oxidized DNA left unreppaired leads to mutations and deletions in the genome which result in flawed gene transcription and cellular replication [35]. Therefore, it is thought that oxidative stress has a biphasic response where a low dose elicits a favorable response while a high dose elicits a deleterious response. This phenomenon is referred to as hormesis and is commonly seen in pharmacology and toxicology where a low dose of a drug or toxin elicits benefits, but a very high dose would be lethal [36].

An important distinction when applying the concept of hormesis to a biological or physiological system is the difference between adaptation and maladaptation. Adaptation is becoming better suited to an environment and improving fitness. A maladaptation occurs when the system adapts to a new environment, where it may benefit acutely, but experiences negative repercussions over time. Again, this bipartite distinction applies to oxidative stress. ROS are produced under normal physiological conditions and are well controlled by the antioxidant defense system. Mild and acute increases in ROS, such as in contracting skeletal muscle during exercise, leads to signaling events necessary for adaptation and improved stress resistance [37]. For example, upregulation of the antioxidant defense system occurs in response to an oxidative challenge [38]. However, a chronic elevation in ROS, such as that seen in cardio-metabolic disease, is cumulative to the point of maladaptive signaling. At first, the body will
adapt in an attempt to control the oxidative stress but over time the adaptive responses becomes overwhelmed and the stress has deleterious effects [39].

The cellular responses affected by ROS are numerous but incompletely understood. The initial responses to an increase in ROS production are the heat shock response and the oxidative stress response [23]. The heat shock response is a stress response that involves the upregulation of heat shock proteins and chaperones in an attempt to rescue misfolded proteins. The genes that code for these proteins contain a heat shock element (HSE) in the promoter region that is inducible by transcription factors [7]. One such transcription factor is heat shock factor 1 (HSF1), which is the main regulator of the heat shock response. Hydrogen peroxide induces structural changes that expose the nuclear localization signal on HSF1, thus promoting its translocation and accumulation in the nucleus [7]. If the heat shock proteins are unable to repair the protein, the protein is tagged for degradation.

The oxidative stress response involves upregulation of antioxidant enzymes. The main transcription factors that are activated in this response are nuclear factor κ-B (NFκB) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2), both of which are redox-sensitive [37]. NFκB is bound to inhibitory IκB proteins in the cytosol under basal conditions. Through incompletely understood mechanisms, hydrogen peroxide promotes degradation of IκB, thus freeing NFκB to translocate into the nucleus [37]. Nrf2 is sequestered in the cytosol through binding with Keap 1 under basal conditions, and upon activation translocates to the nucleus. There are several mechanisms by which Nrf2 is activated by hydrogen peroxide including upregulation of mRNA translation, activated protein translocation to the nucleus and greater stabilization through phosphorylation [7]. Once in the nucleus, Nrf2 binds to the antioxidant response element (ARE)
found in the promoter region of several dozen genes [40]. Binding of Nrf2 to the ARE increases the expression of genes, including those that code for the antioxidant proteins, CAT, SOD and glutathione peroxidase [41].

The activation of NFκB has many more downstream effects in addition to the oxidative stress response. Together, members from the NFκB and the mitogen-activated protein kinase (MAPK) pathways are involved in a majority of the effects induced by ROS related to gene expression, proliferation, protein breakdown and apoptosis [23]. Activation of the MAPK signaling cascade leads to further activation or inhibition of other pathways via phosphorylation of regulatory proteins. Specifically, the MAPK pathways ERK, JNK and p38 have been shown to be activated by ROS and have effects on protein breakdown and apoptosis [37].

An additional family of redox sensitive proteins is the NAD⁺-dependent deacetylase sirtuin proteins. Sirtuins detect changes in the ratio of NAD⁺/NADH and modulate protein activity through removal of acetyl groups from lysine residues to elicit cell-protective mechanisms such as oxidative stress defense, DNA repair and protein folding [42]. There are seven sirtuin proteins which are localized in different compartments of the cell with SIRT1 being the main isoform in the cytosol. SIRT1 has been shown to be regulated by altered redox status in the cell and activate an antioxidant response via deacetylation of the transcription factor forkhead box O3a (FOXO3a) [43]. When FOXO3a is deacetylated it is a transcriptional activator of the genes that encode for catalase and the mitochondrial localized SOD2 protein [43].

To summarize, mild oxidative stress induces adaptive changes in the expression and activity of proteins in order to improve cellular function and stress resistance [44]. Research into the mechanisms of the hormetic nature of oxidative stress has led to the discovery of
beneficial signaling roles of ROS. The heat shock and oxidative stress responses are activated by ROS signaling mechanisms via central redox sensitive transcription factors including HSF1, NFκB, Nrf2 and FOXO3a in order to elicit adaptive changes within the cell.

**Proteostasis and Mitochondria**

Protein homeostasis, or proteostasis, refers to maintaining adequate amounts of proteins as well as the proper folding, binding and localization of proteins within the cell [45]. Proteins are susceptible to irreversible oxidative damage caused by the formation of hydroxyl and carbonyl groups. Oxidation of the amino acid side chains and peptide backbones of proteins cause function impairment and may result in a complete loss of function [4]. If not repaired or degraded, damaged proteins can aggregate and accumulate causing protein toxicity and disruption of proteostasis [45]. Preserving proteostasis requires the integration of signals and the coordination of many processes to ensure proper quantity and quality of proteins.

Protein synthesis and accurate folding of nascent polypeptides are part of proteome quality control. The synthesis of new proteins requires gene transcription, mRNA translation and protein folding. Translation of mRNA into nascent polypeptides is a multi-step and energy costly process [46]. Excess translation would be a waste of cellular resources and an abundance of nascent polypeptides could overwhelm the molecular chaperones and contribute to protein aggregation [47]. Therefore, translation is under tight regulatory control in order to match cellular requirements. Molecular chaperones are constitutively expressed by the cell to modulate folding and unfolding of proteins [48].

An additional proteome quality control mechanism is the degradation of misfolded or damaged proteins. The ubiquitin/proteasome system (UPS) and chaperone-mediated
autophagy (CMA) are two central processes of protein degradation. The UPS is a two component pathway. First, a protein substrate is targeted for degradation through the covalent linkage of ubiquitin molecules, forming a polyubiquitin chain. Second, the polyubiquitin chain is recognized by chaperones that transport the protein to the proteasome, a multicatalytic complex with a proteolytic core, where degradation takes place [48]. CMA is the selective degradation of cytosolic proteins by lysosomes. Oxidized proteins have a higher susceptibility to being taken up by lysosomes than non-oxidized proteins, suggesting that CMA is activated during oxidative stress [49].

Overall, regulation of protein synthesis ensures proper allocation of cellular resources as well as adequate protein concentrations. The aggregation of inoperative protein fragments is prevented in part by molecular chaperone assisted re-folding and protein degradation by the proteolytic systems. Whole cell proteostasis is also dependent on the dynamics of cellular organelles, including the mitochondria.

*Mitochondrial Proteostasis*

Mitochondria utilize a dynamic network of proteins to participate in many essential cellular processes. A small fraction of mitochondrial proteins are coded by a distinct genome of mitochondrial DNA while a majority of proteins are nuclear encoded [50]. The process of mitochondrial biogenesis is the synthesis of new mitochondrial proteins and replication of mitochondrial DNA. These new mitochondria do not form a separate organelle within the cell but instead become incorporated to expand the existing network [51]. Further, constant remodeling of the mitochondrial reticulum through fusion and fission events establishes the dynamic nature of the mitochondrial network [11]. Fusion is the joining of two regions of the
mitochondrial reticulum in order to interconnect membranes and distribute content among the
network. Fission is the fragmentation of the mitochondrial reticulum in order to remove
damaged mitochondrial proteins that had been sequestered within the network [51]. Both
fusion and fission events are regulated by energy availability and together maintain

In addition to the regulation of mitochondrial biogenesis and restructuring, degradation
of damaged proteins helps to maintain proteostasis and mitochondrial function. Mitochondrial
proteins and DNA are at a higher susceptibility to oxidative damage than nuclear DNA and
cytosolic proteins due to the high ROS production in the mitochondria (Balaban 2005). In order
to maintain protein quality control, DNA repair enzymes, chaperones and proteases localized in
the mitochondria repair or remove damaged proteins and DNA [10].

Ultimately, maintaining mitochondrial proteostasis and function through finely tuned
control of protein synthesis and degradation is important for maintaining overall cellular
function. Increased ROS levels in the mitochondria can cause accumulation of oxidative damage
and disrupt mitochondrial function, but also activate redox-sensitive signaling pathways.

**Mitochondrial Redox Signaling**

Mitochondria house major energy production and ROS production pathways within the
cell and thus are key regulators of energy and redox signaling [36]. Similar to hormesis in the
context of the whole cell, mild stress from decreased levels of metabolic molecules and
increased levels of mitochondrial ROS triggers a hormetic response to improve mitochondrial
function [36]. AMP-activated protein kinase (AMPK) acts as a metabolic sensor and is activated
by changes in the ratio of AMP/ATP, such as in response to oxidative stress [52]. Slight changes
in this ratio will elicit rapid signaling to regulatory proteins to elicit changes in energy production. AMPK promotes the activity of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), the master regulator of mitochondrial biogenesis [53]. PGC-1α is the transcriptional co-activator of nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (TFAM). NRF-1 is the transcription factor that increases the expression of nuclear encoded mitochondrial proteins while TFAM is the transcription factor that increases the expression and replication of mitochondrial DNA [54].

Changes in the redox status are also thought to be an activator of mitochondrial biogenesis as well as increased antioxidant defenses. A modest increase in mitochondrial ROS accumulation activates AMPK [8] and increases PGC-1α expression [9]. Additionally, the expression of SIRT3, the mitochondrial localized sirtuin isoform, is increased with ROS levels [55]. SIRT3 is required for activation of the antioxidant enzyme SOD2 in the mitochondria [56]. Further, up-regulation of SIRT3 enhances PGC-1α activation [57]. Together, these oxidative stress induced signals stimulate mitochondrial biogenesis and antioxidant defenses to support the concept of mitohormesis [42].

Overall, the mitohormetic response involves sensors that detect changes in AMP and NAD⁺ levels in order to alter energy and ROS producing pathways. Activation of mitochondrial signaling proteins results in adaptive changes to improve antioxidant defenses and mitochondrial proteostasis. Evidence suggests a significant role of oxidative stress in mitochondrial adaptive signaling and mitohormesis.
Disruption of Exercise-Induced ROS signaling with Antioxidant Supplementation

During exercise, increased ROS production in skeletal muscle triggers beneficial signaling and adaptations in a mitohormetic fashion. Muscle contraction during a bout of exercise requires increased energy production which contributes to an increased production of ROS. It is well established that one of the adaptations to exercise training is increased mitochondrial content and improved function [58]. Exercise training also increases the expression of antioxidant enzymes [59]. The adaptation of skeletal muscle to exercise training could be in response to oxidative stress in the form of acute increases in ROS production in order to improve mitochondrial function and stress resistance [60].

Although mild oxidative stress is beneficial, too much can be detrimental. One of the first studies to measure increased ROS production from contracting skeletal muscle during a single bout of exercise was published in the 1980s [12]. This study also included evidence of cellular damage from ROS produced during a single bout of exercise. Then, it was determined that an optimal cellular redox state for muscle force production exists where too much oxidative stress impedes force production [61]. Because it was thought that ROS production during exercise led to increased oxidative damage, some have supported the use of exogenous antioxidant supplements to prevent damage and optimize skeletal muscle performance [62]. However, based on the current research investigating beneficial ROS signaling presented earlier, recommendations for supplementation with antioxidants have become a controversial topic. It is unknown whether or not exogenous antioxidant supplementation interferes with the beneficial ROS signaling occurring in cases such as exercise training.
There is conflicting data on whether or not supplementation with exogenous antioxidants interferes with the beneficial signaling activated by an increase in ROS production from exercise. Markers of mitochondrial function were measured before and after a 12 week exercise training study in young moderately trained men [15]. Skeletal muscle citrate synthase activity, a marker of mitochondrial function, increased with the exercise training in both controls and those supplemented with vitamin C and E [15]. Further, markers of cell signaling for increased mitochondrial biogenesis were measured in rats after an acute exercise bout [14]. mRNA levels of PGC1α, TFAM and NRF-1 increased after the bout of exercise in the gastrocnemius muscle of rats fed a control diet and those fed a diet supplemented with vitamin C [14]. In addition, the effect of long-term antioxidant supplementation on exercise-induced increases in mitochondrial protein content and endogenous antioxidant expression was studied [13]. Three weeks of exercise training increased the expression of SOD2 and mitochondrial protein content in the triceps muscles of rats fed a control diet and rats given vitamin C and E supplemented food for eight weeks prior to the exercise training [13]. These data would suggest that antioxidant supplementation does not interfere with the training adaptations to exercise.

Findings from other lab groups show that supplementation with exogenous antioxidants can blunt the positive mitochondrial responses to exercise. For example, a four week exercise training program was conducted in young previously trained and untrained men in order to measure changes in PGC1α RNA levels as a marker of mitochondrial biogenesis [16]. PGC1α RNA levels in skeletal muscle increased with the exercise training in the subjects given a placebo, however this increase was blunted in the subjects supplemented with vitamin C and E
Similar markers of mitochondrial biogenesis were measured in rats before and after three weeks of exercise training [17]. PGC1α protein content and NRF-1 and TFAM RNA levels in skeletal muscle increased with the exercise training in the rats given standard chow, however this increase was blunted in the rats supplemented with vitamin C and E [17]. Additionally, the long-term effects of antioxidant supplementation on exercise-induced increases in mitochondrial protein content were studied [63]. Twelve weeks of exercise training increased mitochondrial protein content in the soleus and gastrocnemius muscles of rats fed a control diet, however this increase was blunted in rats given vitamin C and E supplemented food for fourteen weeks prior to the exercise training [63]. These data would suggest that antioxidant supplementation does interfere with the training adaptations to exercise.

**Upregulation of the Endogenous Antioxidant System**

An alternative approach to supplementing with exogenous antioxidants would be to increase the endogenous antioxidant defense system. As previously described, the Nrf2 pathway is redox sensitive and the antioxidant system within a cell is at least partially controlled by Nrf2 [64]. Activation of Nrf2 has been implicated as an important part of the adaptive response to cellular stress and has been shown to protect against oxidative stress [18, 19]. Further, activation of Nrf2 may stimulate mitochondrial biogenesis since the gene that codes for NRF-1 also contains an ARE in its promoter region and is inducible by Nrf2 binding [20]. It is also suggested that Nrf2 activation induces PGC1α, though only under stress conditions [21]. Therefore, Nrf2 activation by ROS leads to increased NRF-1 and PGC1α activation in order to promote mitochondrial biogenesis. Treatment with an Nrf2 activator
could be an alternative approach to exogenous antioxidants to prevent oxidative damage while still allowing beneficial ROS signaling to occur.

\textit{D}_2\textit{O Labeling to Measure Proteostasis}

In order to test the hypothesis that activating Nrf2 would not interfere with ROS induced mitochondrial biogenesis in skeletal muscle, the appropriate outcome measurements should be considered. Protein turnover is the combination of protein synthesis and degradation, which together determines total protein concentration. Protein concentrations and RNA levels are static measurements and do not accurately depict the dynamic nature of cellular processes. Protein turnover is a dynamic variable, meaning changes cannot always be captured by static measurements. For example, an increase in the synthesis of a particular protein that is matched by a similar increase in the degradation of that same protein would reflect no change in the concentration measurement. However, protein turnover would have increased suggesting an increase in proteostatic maintenance. Therefore, rate measurements are more insightful for assessing protein turnover as well as mitochondrial biogenesis and ultimately, proteostasis [65].

Accurate assessment of proteostasis using protein synthesis rates also depends on rates of cell proliferation. Protein synthesis increases in order to replace proteins that were damaged and degraded. In this case, the newly synthesized proteins are contributing to maintenance of the protein network within the cell. In a proliferating cell type, the entire protein network must be doubled in order to populate the newly replicated cell after division. This would be reflected as a substantial increase in protein synthesis as well as DNA synthesis. Therefore, measuring cell proliferation via rates of DNA synthesis is important to understand how much of the
measured protein synthesis is attributed to the making of new cells versus the maintenance of the existing proteome [66].

Previously, our lab has adapted the tracer method utilizing the stable isotope deuterium oxide (D\textsubscript{2}O) to measure the synthesis of cellular proteins and DNA [67]. The principle of the method relies on the deuterium of D\textsubscript{2}O replacing non-labile hydrogen in alanine and deoxyribose. Free alanine contains four C-H bonds that exchange hydrogen for deuterium to reach equilibrium with the enriched media [68] while deoxyribose contains seven [69]. In the current project, the D\textsubscript{2}O labeling method was adapted into an in vitro model to measure protein and DNA synthesis.
CHAPTER III: METHODS AND PROCEDURES

Method Overview

To measure synthesis, deuterium oxide (D$_2$O) enriched media was added to cultured cells for differing amounts of time. The rates of protein and DNA synthesis were then determined by the rates of incorporation of deuterium-labeled alanine into protein and DNA. The ratio of protein synthesis to DNA synthesis (protein:DNA) is calculated as an indicator of proteostasis. The first experiment conducted was a hydrogen peroxide time course and concentration response for synthesis of proteins to determine the appropriate concentration and duration of treatment. A comparison of cell culture plate size was conducted next to determine if 60mm versus 100mm plates yield sufficient cells for measuring deuterium incorporation into both protein and DNA. The following synthesis experiment compared protein synthesis and DNA synthesis when cells were treated with hydrogen peroxide concurrently with either vitamin C or the Nrf2 activator, Protandim. The final experiment compared protein and DNA synthesis when cells were pre-treated with Protandim before adding hydrogen peroxide to cells treated with Protandim concurrently with hydrogen peroxide to determine if pre-treatment improves proteostasis.

Cell Model

All experiments involved culturing C2C12 myoblasts (passages 1-6) on 60 mm or 100 mm culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 5% CO$_2$ humidified atmosphere at 37°C. The enriched media was made by adding sterilized 99% D$_2$O to supplemented DMEM to yield
final percentages of 4% D₂O enriched media. Cells were harvested in 1 ml or 1.2 ml isolation buffer #1 (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM MgCl₂, 1mM EDTA, 1mM ATP, pH 7.5) with phosphatase and protease inhibitors. Immediately before cell harvesting, 1 ml samples of media were taken from every plate to measure media enrichment over time.

Cells were cultured in non-D₂O enriched media and seeded onto culture plates. When cells reached 70-90% confluence, non-enriched media was removed, cells were washed with sterile PBS, and 4% D₂O enriched media with treatments was added. Pre-treatment with Protandim occurred in non-D₂O enriched media for 15 hours starting when cells had reached 70% confluence. Then, 4% D₂O enriched media plus H₂O₂ and Protandim were added at time 0. Myoblasts were harvested at time points ranging from 1-12 hours (n=3/time point).

Oxidant treatment included H₂O₂ (30% W/W) diluted in DMEM to concentrations 10, 50, and 100 µM. Exogenous antioxidant treatment consisted of vitamin C (Sigma-Aldrich; L-Ascorbic Acid) diluted in DMEM to a final concentration of 50 µM. Nrf2 activation was achieved through treatment with Protandim (LifeVantage Corp., Littleton, CO, USA), a combination of five plant-derived phytochemicals: *Bacopa monniera* (45% bacosides), *Silybum marianum* (70-80% silymarin), *Withania somnifera* (1.5% withanolides), *Camellia sinensis* (98% polyphenols and 45% epigallocatechin-3-gallate), and *Curcuma longa* (95% curcumin). Protandim was extracted using 100% alcohol. The extract was centrifuged at 3,000g for 15 minutes and the supernatant was removed and stored at room temperature protected from direct light. The alcohol extract was diluted in DMEM to a concentration of 100µg/ml. Control cells were treated with ethanol vehicle at a concentration of 1µl/ml diluted in DMEM. Previous work by our group has shown
activation of Nrf2 and subsequent upregulation of endogenous antioxidants in response to Protandim treatment [18, 19].

**Preparation of Samples and GC/MS Analysis**

**Cell Fractionation and Mitochondrial Isolation**

Cells were harvested in buffer #1 (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM MgCl₂, 1mM EDTA, 1mM ATP, pH 7.5). The amount of buffer used was 1 mL when only protein synthesis was being measured and 1.2 mL when DNA synthesis was also being measured. When measuring protein synthesis, we prepared a mitochondrial enriched (Mito) and a cytoplasmic (Cyto) fraction from 1 mL of the cell extract. The initial cell extract was centrifuged at 800g for 10 min at 4°C. The supernatant was removed to another tube and the resulting pellet was saved in 1 ml buffer #1 as a mixed protein fraction. The supernatant was centrifuged at 10,000g for 30 min at 4°C to pellet the Mito fraction. From the supernatant, 400µL was removed to yield the Cyto fraction. The remaining volume of supernatant was saved for protein quantification. Equal volume (400µL) of 14% SSA was added to the 400µL of supernatant now labeled Cyto. The tube was vortexed and incubated on ice for 1 hr. In the meantime, the Mito pellet was washed with 200 µL buffer #2 (100 mM KCl, 10 mM Tris-HCl, 10 mM Tris base, 1 mM MgSO₄, 0.1 mM EDTA, 0.02 mM ATP, and 1.5% BSA, pH 7.4) and then centrifuged at 8000g for 10 min at 4°C. The supernatant was removed and the pellet was washed a second time with 100µL buffer #2 and then centrifuged at 6000g for 10 min at 4°C. The supernatant was removed and the Mito pellet was carefully washed with 1 ml ddH₂O. After the 1 hr incubation, the Cyto tube was centrifuged at 16000g for 10 min at 4°C yielding a protein pellet. The Cyto pellet were washed with 500 µL 100% ethanol, centrifuged at 1000g for 4 min at 4°C, washed with 500 µL ddH₂O
and centrifuged at 1000g for 4 min at 4°C. This washing process with ethanol and ddH₂O was repeated once. Mito and Cyto pellets were then solubilized in 250 µL 1 N NaOH for 15 min at 50°C and hydrolyzed in 1.5 mL 6 N HCl for 24 hrs at 120°C.

Media Derivation and Analysis

To prepare cell culture media for analysis, 125µL of sample were pipetted into the inner well of an o-ring screw cap and the tubes were placed on the heat block overnight at 80°C. Samples were allowed to cool to room temperature the following day then 2µL of 10M NaOH and 20µL of acetone were added to each sample. The tubes were capped immediately after addition of acetone, vortexed at a low speed, and then left overnight at room temperature. Samples were extracted by addition of 200µL hexane and anhydrous Na₂SO₄ and the organic layer was transferred to GC vials and analyzed via EI mode with a DB-17MS column.

Alanine Derivation and Analysis

The protein hydrolysates were ion-exchanged, dried under vacuum, and then resuspended in 1 mL molecular biology grade H₂O. Half of the total volume (500µL) of sample was derivatized by addition of 500µL acetonitrile, 50 mM K₂HPO₄, pH 11, and 20µL of pentafluorobenzyl bromide. Derivatives were sealed and incubated at 100°C for 1 hr. Ethyl acetate was added and the organic layer was removed to a GC vial and dried under N₂. Samples were reconstituted in ethyl acetate then analyzed by negative chemical ionization.

A DB225 gas chromatograph column was used to separate the amino acid derivatives. The starting temperature was 100° C and increased to 220°C at a rate of 10°C per min. Samples then entered the mass spectrometer with helium as the carrier gas and methane as the reagent gas. The mass-to-charge ratios of 448 and 449, which were representative of the M+0 and M+1,
mass isotopomers of alanine, were monitored to measure isotopic abundance. Alanine enrichment was calculated by dividing abundance of M+1 by the sum of the abundance of M+0 and M+1. The alanine enrichment was then divided by the precursor enrichment, which was predicted from media enrichment using mass isotopomer distribution analysis (MIDA), to calculate the newly synthesized fraction of cellular proteins (fraction new) [68]. Protein synthesis rates were calculated by dividing the fraction new by time and were expressed as fractional synthesis rates (FSR %/h) when measuring incorporation of label.

*DNA Isolation and Analysis*

To extract DNA, 200 µL of the initial cell extract was processed using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA). DNA extraction elutions were hydrolyzed overnight at 37°C with nuclease S1 and potato acid phosphatase. Hydrolysates were reacted with pentafluorobenzyl hydroxylamine and acetic acid and incubated at 100°C for 30 min. Then, the samples were acetylated with acetic anhydride and 1-methylimidazole. Methylene chloride was added and the organic layer was removed to a GC vial and dried under vacuum. Samples were reconstituted in ethyl acetate then analyzed.

DNA samples were analyzed by GC-MS on a DB-17 column with negative chemical ionization, using He as carrier and methane as the reagent gas. The fractional molar isotope abundances at m/z 435 (M+0 mass isotopomer) and 436 (M+1) of the pentafluorobenzyl triacetyl derivative of purine dR were quantified using ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The DNA enrichment was then divided by the precursor enrichment, which was predicted from media enrichment using mass isotopomer distribution analysis (MIDA), to calculate the newly synthesized DNA [69]. DNA synthesis rates were
calculated by dividing the fraction new by time and were expressed as fractional synthesis rates (FSR %/h) when measuring incorporation of label.

**Statistical Analysis**

Significance was set *a priori* at p<0.05. Data were analyzed by one-way ANOVA or two-way ANOVA followed by Tukey’s test for post hoc multiple comparisons where appropriate (Prism Version 6). Data are expressed as means ± SEM.
CHAPTER IV: RESULTS

To determine an appropriate concentration of hydrogen peroxide ($H_2O_2$) to induce a mild and acute oxidative stress, fractional synthesis rates (FSR) of the mitochondrial (Mito) fraction were measured (Figure 1). At 4 hours, 10 and 50µM $H_2O_2$ increased Mito FSR compared to control cells ($p<0.0001$) and 100µM $H_2O_2$ treated cells ($p<0.05$). After 8 hours, 10 and 50µM $H_2O_2$ did not change mitochondrial FSR compared to control. However, 10µM $H_2O_2$ increased Mito FSR compared to both 50 and 100µM $H_2O_2$ ($p<0.05$). After 21 hours, there were no significant differences in Mito FSR between the treatments. Based on these results, it was determined that 50µM of $H_2O_2$ was the appropriate concentration to induce an acute oxidative stress.

The next method development experiment was conducted to determine if two 60mm or a single 100mm plate of cells was better to measure protein and DNA fraction new simultaneously. The effect of 1µl/ml ethanol (EtOH), the vehicle for experimental treatment with Protandim, on Mito protein and DNA synthesis was also evaluated. Myoblasts in the 100mm plates were harvested in 1.2ml Mito 1 buffer where 1ml of cell suspension was used to isolate the Mito fraction and the remaining 200µl was used for DNA extraction. Myoblasts from two 60mm plates were harvested in 1ml Mito 1 buffer each where the entire volume from one plate was used to isolate the mitochondrial protein fraction and the other volume was used for DNA extraction. It was determined that a single 100mm plate contains sufficient cell yield to measure both Mito protein and DNA fraction new from the same plate of cells (Figure 2A and Figure 2B). EtOH had no effect on fraction new however 100mm plates had a greater fraction...
new of Mito protein (Figure 2A) and a greater ratio of Mito protein:DNA fraction new than the 60mm plates (p<0.05) (Figure 2C).

Concurrent treatments of 50µM H$_2$O$_2$ and either vitamin C (VitC) or the Nrf2 activator, Protandim (Pro), were used to investigate if either treatment would interfere with the effects of H$_2$O$_2$ on rates of protein synthesis or DNA synthesis. Myoblasts were grown on 100mm cell plates and treated with 4% D$_2$O enriched media. Additional treatments included, 50µM H$_2$O$_2$, 50µM H$_2$O$_2$ plus 1µl/ml EtOH, 50µM H$_2$O$_2$ plus 50µM VitC, or 50µM H$_2$O$_2$ plus 100µg/ml Pro. Myoblasts were harvested in 1.2ml Mito 1 buffer to determine both protein and DNA synthesis at 2, 4, 6 and 8 hours. Mito protein, cytosolic (Cyto) protein and DNA FSR were calculated. At 2 hours, there was an interaction effect between treatment and protein fraction (p<0.05). There were no differences in Mito FSR between treatments, but H$_2$O$_2$ alone, H$_2$O$_2$ plus VitC, and H$_2$O$_2$ plus Pro decreased Cyto FSR compared to control (p<0.05) (Figure 3A). At 6 hours, H$_2$O$_2$ plus Pro decreased Mito FSR compared to H$_2$O$_2$ plus VitC treatment (p<0.05) (Figure 3B), though the difference was not statistically significant at 8 hours (data not shown). Also at 6 hours, H$_2$O$_2$ plus Pro decreased Cyto FSR compared to control and H$_2$O$_2$ plus EtOH, the vehicle control for Pro (p<0.05) (Figure 3B).

At 2 hours, DNA FSR increased in cells treated with H$_2$O$_2$ alone, H$_2$O$_2$ plus VitC, and H$_2$O$_2$ plus Pro compared to control (p<0.05) (Figure 4A). DNA FSR was decreased in cells treated with H$_2$O$_2$ plus EtOH compared to H$_2$O$_2$ alone (p<0.05). Additionally, DNA FSR was decreased in cells treated with H$_2$O$_2$ plus Pro compared to H$_2$O$_2$ alone (p<0.05) and H$_2$O$_2$ plus VitC (p<0.01). At 6 hours, H$_2$O$_2$ plus VitC was the only treatment that increased DNA FSR compared to control.
(p<0.05) (Figure 4B). Also at 6 hours, treatment with H₂O₂ plus Pro decreased DNA FSR compared to all other treatments (p<0.05) (Figure, 4B).

As an indication of proteostasis, the ratio of protein:DNA synthesis rates was calculated from the previously mentioned protein and DNA FSR. At 2 hours, there was an interaction effect between treatment and protein fraction (p<0.05) (Figure 5A). The control condition was the only condition where the Cyto:DNA ratio was significantly different from the Mito:DNA ratio. Treatment with H₂O₂ and H₂O₂ plus VitC decreased Mito:DNA and Cyto:DNA ratio compared to the respective controls (p<0.05). Interestingly, while treatment with H₂O₂ plus Pro decreased Cyto:DNA ratio compared to control (p<0.05), Mito:DNA ratio was maintained compared to control (p<0.05). Treatment with H₂O₂ plus Pro also increased both Mito:DNA and Cyto:DNA ratios compared to H₂O₂ plus VitC (p<0.05) (Figure 5A). At 6 hours, both Mito:DNA and Cyto:DNA ratios were increased in H₂O₂ plus Pro treated cells compared to all other treatments (p<0.001) (Figure 5B).

The final experiment was conducted to determine if pre-treating with Pro for 15 hours before addition of 50µM H₂O₂ would improve proteostasis compared to concurrent treatment with Pro and 50µM H₂O₂. Myoblasts were grown on 100mm cell plates and harvested in 1.2ml Mito 1 buffer to determine both protein and DNA FSR at 2, 4, 6 and 8 hours. At 4 hours, Pro pre-treatment increased both Mito FSR (P<0.05) and Cyto FSR (P<0.01) compared to concurrent treatment of Pro with H₂O₂ (Figure 6A). This effect of pre-treating with Pro was gone after 8 hours where there was no significant difference between Mito FSR and Cyto FSR compared to concurrent H₂O₂ and Pro treatment (Figure 6B). At 8 hours, Cyto FSR in both conditions was greater than Mito FSR (P<0.05). DNA FSR was not significantly different between treatments at
any time points. Additionally, the ratio of protein:DNA was not significantly different between treatments at any time points suggesting proteostasis was not improved with Pro pre-treatment (data not shown).

Figure 1: Fractional synthesis rates (%/hr) of the Mito fraction from a H$_2$O$_2$ concentration response (10μM, 50μM and 100μM) and time course (4, 8 and 21 hours). Control is 4% D$_2$O enriched media. 50μM of H$_2$O$_2$ causes a transient increase in Mito synthesis rate.* Significantly different from control (p<0.0001). #Significantly different from 10 μM (p<0.05). ^Significantly different from 50 μM (p<0.01).
Figure 2: Myoblasts on 60mm or 100mm plates treated with 4% D$_2$O enriched media or 4% D$_2$O enriched media plus 1μl/ml ethanol (EtOH). Protein and DNA were extracted from the same 100mm plate of cells (n=3) while protein and DNA were extracted from different 60mm plates (n=3 protein and n=3 DNA). Mito protein fraction new (A) and DNA fraction new (B) were measured after 4 hours. The ratio of Mito protein fraction new and DNA fraction new was calculated (C). There were no differences between EtOH treated and control cells. The Mito fraction new and the protein:DNA ratio of the 100mm plates was greater than the Mito fraction new and the protein:DNA ratio of the 60mm plates (p<0.05).

Figure 3: Mito and Cyto protein FSR (%/hr) at 2 hours (A) and 6 hours (B). Myoblasts on 100mm plates were treated with 4% D$_2$O enriched media. Treatments included 50μM H$_2$O$_2$, 50μM H$_2$O$_2$ + 1μl/ml EtOH, 50μM H$_2$O$_2$ + 50μM VitC, or 50μM H$_2$O$_2$ + 100μg/ml Pro. **Interaction effect (p<0.05) Significantly different from Mito control. #Significantly different from Cyto control (p<0.05). *Significantly different from Cyto H$_2$O$_2$ + EtOH (p<0.05). +Significantly different from Mito H$_2$O$_2$ + VitC (p<0.05).
Figure 4: DNA FSR (%/hr) at 2 hours (A) and 6 hours (B). Myoblasts on 100mm plates were treated with 4% D₂O enriched media. Treatments included 50µM H₂O₂, 50µM H₂O₂ + 1µl/ml EtOH, 50µM H₂O₂ + 50µM VitC, or 50µM H₂O₂ + 100µg/ml Pro. #Significantly different from control (p<0.05). ^Significantly different from H₂O₂ (p<0.05). *Significantly different from H₂O₂ + EtOH (p<0.01). +Significantly different from H₂O₂ + VitC (p<0.01).

Figure 5: Ratio of protein:DNA FSR at 2 hours (A) and 6 hours (B). Myoblasts on 100mm plates were treated with 4% D₂O enriched media. Treatments included 50µM H₂O₂, 50µM H₂O₂ + 1µl/ml EtOH, 50µM H₂O₂ + 50µM VitC, or 50µM H₂O₂ + 100µg/ml Pro. **Interaction effect (p<0.05) Significantly different from Mito control. #Significantly different from control (p<0.05). ^Significantly different from H₂O₂ (p<0.05). *Significantly different from H₂O₂ + EtOH (p<0.0001). +Significantly different from H₂O₂ + VitC (p<0.05).
Figure 6: Mito and Cyto protein FSR and DNA FSR at 4 hours (A) and 8 hours (B). Myoblasts on 100mm plates were treated with 4% D$_2$O enriched media. Treatments included 50μM H$_2$O$_2$ + 100μg/ml Pro or a 15 hour pre-treatment with 100μg/ml Pro before concurrent treatment with 50μM H$_2$O$_2$. #Significantly different from Mito H$_2$O$_2$ + Pro (p<0.05). *Significantly different Cyto H$_2$O$_2$ + Pro (p<0.01).
CHAPTER V: DISCUSSION

Principle Outcomes

In the current study, rates of mitochondrial protein, cytosolic protein and DNA synthesis were used to measure proteostasis in response to treatment with a low concentration of H$_2$O$_2$ alone or in combination with VitC or the Nrf2 activator, Pro. It was hypothesized that treatment with H$_2$O$_2$ would increase mitochondrial protein synthesis and proteostasis and that VitC treatment, but not Pro would blunt this increase. Further, it was hypothesized that the treatments would have no effect on cytosolic protein synthesis. The main findings were that treatment with H$_2$O$_2$ or VitC concurrently with H$_2$O$_2$ did not stimulate mitochondrial protein synthesis or improve proteostasis. However, concurrent treatment with Pro and H$_2$O$_2$ maintained mitochondrial protein synthesis and decreased DNA synthesis to improve proteostasis. While a low concentration of H$_2$O$_2$ (50µM) stimulated mitochondrial protein synthesis in one experiment, this finding was not reproduced in the second experiment. Further, it was shown that H$_2$O$_2$ or VitC concurrently with H$_2$O$_2$ acutely decreased cytosolic protein synthesis and increased DNA synthesis to decrease proteostasis. Lastly, pre-treating with Pro before concurrent treatment with H$_2$O$_2$ acutely increased rates of protein synthesis, but did not improve proteostasis, compared to no pretreatment with Pro.

Measuring Proteostasis

One aim of the project was to ensure that both deuterium labeled DNA and proteins could be quantified from the same plate of cells. This study showed that there is sufficient yield from a 100mm plate of myoblasts to measure both DNA and Mito protein fraction new from
the same plate of cells (Figure 2). This finding is critical for assessing proteostasis in vitro considering the C2C12 myoblasts used in this study are highly proliferative. During cell division, an entirely new genome and proteome are synthesized in order to populate the new myoblast cell. Therefore, DNA synthesis is a measurement of cell proliferation [69]. In addition to contributing to cell division, synthesis of new proteins is also contributing to protein turnover and proteostatic maintenance of the existing cell population [45]. Measuring the rates of both protein and DNA synthesis provides an indication of how much newly synthesized protein is populating new cells and how much is maintaining the existing cell population. The ratio of newly synthesized protein to newly synthesized DNA (protein:DNA) is an indicator of proteostasis where a higher protein:DNA ratio is indicative of greater proteostatic maintenance [67].

Skeletal muscle is a post-mitotic tissue that recruits new DNA from resident satellite cells during hypertrophy [70]. The skeletal muscle of mice from a crowded litter, an intervention shown to extend lifespan, has increased mitochondrial protein synthesis and maintained DNA synthesis [67]. These changes improve proteostasis through increasing mitochondrial protein synthesis in the existing cell population. In contrast, skeletal muscle of mice treated with Rapamycin, another intervention shown to extend lifespan, has decreased DNA synthesis and maintained mitochondrial protein synthesis [67]. These changes also improve proteostasis though as a result of maintained mitochondrial proteostasis despite a decrease in proliferation. The different responses to the two interventions of lifespan extension illustrate the different mechanisms of improving proteostasis.
The C2C12 myoblasts used in this study are highly proliferative and require measurements of DNA synthesis and protein synthesis from the same sample of cells in order to accurately measure proteostasis. Utilizing this novel method of measuring proteostasis in vitro, it is possible to assess changes in proteostatic maintenance, with mitochondrial proteostasis being of main concern, in response to an acute oxidative challenge.

**Hydrogen Peroxide**

An exogenous ROS challenge in the form of low concentration H$_2$O$_2$ treatment was administered to the cells via incorporation into the cell culture media. We hypothesized that treatment with H$_2$O$_2$ would increase rates of mitochondrial protein synthesis while maintaining DNA synthesis, thus promoting proteostatic maintenance.

In the first experiment, it was shown that mitochondrial protein synthesis increased with low concentrations of H$_2$O$_2$ (10 and 50 µM) (Figure 1). This increase in protein synthesis was seen at 4 hours, but after 21 hours there was no significant difference between H$_2$O$_2$ treatment and control. Previous studies have shown that a low concentration of H$_2$O$_2$ activates signaling molecules associated with mitochondrial biogenesis, such as AMPK and PGC1α [9, 71]. Unfortunately, since we did not measure DNA synthesis or protein breakdown we could not determine if proteostasis was improved.

In our next experiment, 50 µM H$_2$O$_2$ increased DNA synthesis and decreased cytosolic protein synthesis acutely but did not change mitochondrial protein synthesis compared to control (Figures 3 and 4). These date indicate an increased rate of proliferation without an improvement in proteostasis, which is contrary to our hypothesis but supportive of the role of ROS signaling in cell growth.
H$_2$O$_2$ is a signaling molecule in the cellular stress response as well as in cell cycle regulation [7]. The progression of myoblasts through the cell cycle is accompanied by increased levels of H$_2$O$_2$ in the mitochondrial network [72]. Further, treatment with low concentrations of H$_2$O$_2$ for 30 minutes stimulates cell cycle progression *in vitro* through activation of the MAP kinases ERK1/2 [73]. The importance of H$_2$O$_2$ concentrations in cell cycle regulation is also supported by the observation that the pro-survival signaling protein Bcl-2 impedes cell cycle transitioning by depleting intracellular ROS [73]. However, the interacting roles of Bcl-2 and H$_2$O$_2$ in cell signaling are incompletely understood. Low concentrations of H$_2$O$_2$ activate a hormesis response through Bcl-2 activation to increase oxidative stress resistance [74]. Additionally, Bcl-2 expression is highest after 9 hours with low concentration H$_2$O$_2$ treatment which coincides with the highest nuclear localization of Nrf2 [74]. Together, these data suggest H$_2$O$_2$ may have concentration and time-dependent effects on the stress response and cell proliferation. Our results suggest a low concentration of H$_2$O$_2$ acutely increases myoblast proliferation. However, after 6 hours and 8 hours (data not shown) the proliferation rate of H$_2$O$_2$ treated cells was not different from control. Proliferation rates at later time points past 8 hours with H$_2$O$_2$ treatment may yield different outcomes than what was observed at the 2 hour time point.

**Vitamin C**

Concurrent treatment with VitC and H$_2$O$_2$ had no effect on Mito protein synthesis compared to H$_2$O$_2$ alone or control. Further, VitC treatment with H$_2$O$_2$ acutely decreased Cyto protein synthesis and increased DNA synthesis compared to control but was not different compared to H$_2$O$_2$ alone. Unlike treatment with H$_2$O$_2$ alone, treatment with VitC and H$_2$O$_2$
increased DNA synthesis rate at 6 hours compared to control. These data suggest that treatment with VitC did not interfere with the H\(_2\)O\(_2\) signaling to increase cell proliferation compared to control.

VitC is a ROS scavenger and part of the antioxidant defense system to protect the cell from oxidative stress, but VitC has additional functions beyond its antioxidant properties. VitC activates growth signaling by activating ERK1/2 and proliferation in endothelial cells [75]. Further, VitC activation of ERK1/2 is independent of H\(_2\)O\(_2\) since treatment with catalase to degrade H\(_2\)O\(_2\) did not alter the proliferation in response to VitC [75]. VitC supplementation may interfere with beneficial H\(_2\)O\(_2\) signaling in skeletal muscle stress adaptation [16]. In the current study, however, VitC did not interfere with H\(_2\)O\(_2\) signaling since treatment with VitC and H\(_2\)O\(_2\) increased proliferation of myoblasts compared to control. However, since the proliferation rate was not different from H\(_2\)O\(_2\) alone, we cannot conclude whether the effect was specifically from VitC or not. Utilizing a less proliferative cell model, such as myotubes instead of myoblasts to study VitC and H\(_2\)O\(_2\) signaling may yield changes in protein synthesis rates rather than changes in proliferation. Additionally, myotubes may be a better model to assess an interaction effect between VitC and H\(_2\)O\(_2\).

**Nrf2 Activation**

We hypothesized that Nrf2 activation concurrent with H\(_2\)O\(_2\) treatment would result in similar rates of protein synthesis as H\(_2\)O\(_2\) treatment alone and greater synthesis rates compared to control. On the contrary, Nrf2 activation resulted in no change in Mito protein synthesis and decreased Cyto protein synthesis compared to control. However, activation of Nrf2 decreased DNA synthesis at both 2 and 6 hours compared to control and H\(_2\)O\(_2\) treatment alone. Together,
these data demonstrate maintenance of Mito protein synthesis despite a decrease in proliferation, which results in improved proteostasis. These data would suggest that since there are fewer new cells being made, a greater proportion of newly synthesized Mito proteins are going towards maintaining the existing reticulum rather than populating the mitochondrial reticulum of new cells.

The signaling roles and gene targets of Nrf2 related to cellular homeostasis and growth are numerous. Nrf2 is a central transcription factor in cellular adaptation to stress by increasing endogenous antioxidants [64]. Nrf2 activation also contributes to the regulation of mitochondrial biogenesis and protein quality control [21]. During skeletal muscle growth in vivo, newly recruited satellite cells differentiate into myoblasts that proliferate and then fuse into a multi-nucleated myotube [70]. Nrf2 activation promotes the process of skeletal muscle growth, specifically the steps of myoblast proliferation and differentiation into myotubes [76]. However, the current study is the first to measure rates of proliferation and protein synthesis in vitro in response to Nrf2 activation during an oxidative challenge. Further, Nrf2 activation improves proteostatic maintenance as opposed to increasing proliferation.

Protein translation and cell growth are partially under the control of the mammalian target of rapamycin (mTOR). Chronic mTOR inhibition reduces cell proliferation and global protein synthesis in skeletal muscle, but mitochondrial protein synthesis is maintained [77]. In addition, mitochondrial biogenesis signaling and clearance of damaged mitochondrial components are enhanced by mTOR inhibition in vitro resulting in reduced mitochondrial stress [78]. This enhanced mitochondrial signaling is due, in part, to Nrf2 accumulation in the nucleus [78]. When in the nucleus, Nrf2 induces expression of genes that code for NRF1 and TFAM. This
Nrf2 activated signaling promotes mitochondrial protein turnover. Thus, Nrf2 activation via Pro treatment during H$_2$O$_2$ treatment in this study could be contributing to the preservation of mitochondrial protein synthesis rates despite decreased proliferation.

Pre-treating with Protandim before treatment with H$_2$O$_2$ may have enhanced effects on proteostasis since the mechanism of action is via transcriptional regulation of mitochondrial biogenesis. Upon activation with Protandim treatment, Nrf2 translocation into the nucleus occurs within 15 minutes [19]. However, increased protein expression of Nrf2 target genes occurred after 12 hours of Pro treatment [19]. The results from the current study show acute differences in Mito and Cyto FSR between pre-treating with Pro compared to concurrent treatment with Pro and H$_2$O$_2$, which are gone after 8 hours (Figure 6). However, there were no significant differences between protein:DNA ratios between the two treatments suggesting that proteostasis was not different (data not shown). Together, these data suggest a time-dependent effect of Pro treatment and Nrf2 activation on functional outcomes such as protein synthesis, but proteostatic maintenance is not significantly affected.

**Conclusions and Future Directions**

This study supports the role of Nrf2 in improved proteostatic maintenance in addition to its well-documented role in stress resistance. Treatment with a low concentration of H$_2$O$_2$ promoted proliferation but not proteostatic maintenance in myoblasts, an effect that was not altered with concurrent vitamin C treatment. However, activation of Nrf2, an important transcriptional regulator of the antioxidant defense system, improved proteostasis. The significant effects of H$_2$O$_2$ and H$_2$O$_2$ plus VitC treatment on proliferation occurred after 2 hours with results similar to control conditions occurring after 8 hours. Longer duration treatments
may yield different results than what was observed acutely in this study. Proteostasis, and specifically mitochondrial proteostasis, is important for maintaining proper cellular function. Due to the multi-faceted role of Nrf2, improved mitochondrial proteostasis could be a mechanism of stress resistance as more cellular resources are being allocated towards maintaining the current proteome rather than making new cells.

Maintaining proteostasis requires synthesis of new proteins as well as degradation of dysfunctional proteins. Measuring rates of protein breakdown is an important next step in assessing proteostasis in response to a mild oxidative stress and activation of Nrf2. Further, longer duration experiments and a differentiated myotube cell model should be considered to more fully understand the physiologically significant effects of a mild oxidative stress on cellular growth and proteostasis.
REFERENCES


