THESIS

EVALUATION OF A NOVEL PHYTOCHEMICAL NRF2 ACTIVATOR ON CYTOPROTECTIVE GENE EXPRESSION AND PROTEOSTASIS IN VIVO

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ABSTRACT

EVALUATION OF A NOVEL PHYTOCHEMICAL NRF2 ACTIVATOR ON CYTOPROTECTIVE GENE EXPRESSION AND PROTEOSTASIS IN VIVO

Aging is associated with increases in oxidative stress. Redox imbalance occurs when chronic production of reactive oxygen species (ROS) exceeds the capacity of antioxidant enzymes to eliminate ROS. Chronic levels of intracellular ROS can compromise proteostasis by causing irreversible damage to proteins. The transcription factor nuclear factor erythroid-derived 2-like 2 (Nrf2) mediates the cellular endogenous antioxidant defense system by regulating antioxidant enzymes that are cytoprotective against ROS. The phytochemical dietary supplement Protandim activates Nrf2. Previous work from our lab has demonstrated that Protandim treatment can improve proteostasis in skeletal muscle in vivo. Recently, we have begun to characterize a second-generation Nrf2 activator dietary supplement (PB125) that can inhibit components of the Nrf2 shutdown pathway, potentially allowing Nrf2 to stay transcriptionally active for longer. Therefore, we speculated that PB125 might have additional benefits on proteostatic processes. The purpose of the present study was to examine in vivo the effects of three doses (low, medium, high) of PB125 supplementation on Nrf2 activation and proteostasis. We assigned sixty male CB6F1 mice aged 10-11 months to diets containing low, medium, or high doses of PB125 in a 5 week feeding study. Mice were isotopically labeled with 8% deuterium oxide (D_2O) administered in the drinking water to simultaneously measure protein and DNA

fractional synthesis rates in liver, heart, and skeletal muscle. We assessed Nrf2 activation through analysis of gene expression profiles via Affymetrix GeneChip microarray. Proteostatic mechanisms increased in the liver mitochondrial fraction in the low treatment group. There were no differences in proteostatic mechanisms in heart. In the skeletal muscle mixed fraction, there was a reduction in proteostatic mechanisms in the medium treatment group. In the medium treatment group, there was also upregulation of Nrf2-dependent cytoprotective genes (Akr1c19, Akr1d1, Gpx2, Gclm, Fthl17b) as detected by microarray analysis. From our data we were able to conclude that all three doses were safe, and that 100 ppm was effective at activating Nrf2. In addition, there was an indication of increased proteostatic processes in the liver, but not heart or skeletal muscle, perhaps due to the healthy status of the mice.

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CHAPTER I: INTRODUCTION

In the United States, 6 out of 10 of the leading causes of death are age-related chronic diseases (CDC, 2015). By the year 2050 an estimated 20% of the U.S. demographic will be over age 65, and chronic disease prevalence is expected to rise in tandem with this shift (Ortman et al., 2014;Seals et al., 2015). Given that aging is a risk factor shared among many chronic diseases, directing strategies to slow the aging process may delay the onset of multiple chronic diseases simultaneously. Therefore, targeting slowed aging rather than treating individual diseases may extend human healthspan and alleviate the social and economic impact of an aging population (Seals et al., 2015).

There are several key processes underlying the mechanistic relationship between aging and disease pathology, including the loss of protein homeostasis (proteostasis) and oxidative stress. Proteostasis involves a number of cellular processes which maintain the stability of the proteome (Morimoto & Cuervo, 2014). Loss of proteostasis is characteristic of several age-related diseases, and has been implicated as a hallmark of aging (Kennedy et al., 2014). Proteostatic processes may be impaired by oxidative stress (Balch, Morimoto, Dillin, & Kelly, 2008). Oxidative stress is defined as the imbalance between reactive oxygen species production and oxidant scavenging enzymes (Kelly, 2003). When ROS production *chronically* outweighs the capacity of endogenous antioxidant systems to neutralize ROS, irreversible oxidative damage to macromolecules such as proteins can occur (Kelly, 2003).

Oxidative damage can compromise the structural integrity of proteins, leading to irreparable conformational changes and loss of function (Niforou et al., 2014).

Restoring redox balance may help to combat the negative effects of chronic oxidative stress on proteostasis. Cells are equipped with endogenous antioxidant systems that are sensitive to the redox state of the cell, and become activated in response to oxidative stress. The endogenous antioxidant systems are partially regulated by a transcription factor called Nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Upon activation, Nrf2 translocates from the cytosol to the nucleus where it regulates the transcription of target genes that contain an antioxidant response element (ARE) in their promoters (Hybertson, Gao, Bose, & McCord, 2011). Many ARE-dependent genes encode for enzymes that are cytoprotective against ROS, such as superoxide dismutase, catalase, and glutathione peroxidase (Hybertson, Gao, Bose, & McCord, 2011).

In addition to oxidants, plant-based phytochemicals can also activate Nrf2 (Stefanson & Bacovic, 2014). The supplement Protandim contains five phytochemicals that synergistically up-regulate Nrf2 (Nelson, 2006; Velmurugan et al., 2009). In vitro studies have demonstrated that Protandim treated coronary arterial endothelial cells and cardiomyocytes were protected against a hydrogen peroxide stressor in a Nrf2-dependent manner through the up-regulation of ARE-dependent genes (Reuland et al., 2012; Donovan et al., 2012). Unpublished work from our lab has also demonstrated that Protandim treatment in C2C12 myoblasts improves proteostasis when co-treated with a

hydrogen peroxide challenge. In vivo, Protandim was shown to increase the median lifespan of male mice in the NIA funded Interventions Testing Program (ITP) (Strong et al., 2016). Although this evidence supports the therapeutic potential for phytochemical-based Nrf2 activation as an intervention for slowed aging, the sexual dimorphic response that was observed in the ITP warrants further investigation into novel phytochemical-based therapies for lifespan extension.

Statement of the problem

The purpose of this study is to examine the effects of the dietary supplement PB125, a novel phytochemical Nrf2 activator, on proteostasis and Nrf2 activation in vivo.

Hypotheses

Testing PB125 at a range of doses (low (30 ppm), medium (100 ppm), high (300 ppm)), we hypothesize that the high dose of PB125 will be most effective at activating Nrf2 and improving proteostasis in heart, liver, and skeletal muscle tissue.

CHAPTER II: Literature Review

Redox Biology

Reactive Oxygen Species

Oxygen is an indispensable element that supports basic life functions in all aerobic organisms. However, oxygen can also be toxic in nature, as its chemical properties can cause it to behave as a free radical depending on its state. A free radical may be defined as an atom or molecule with an unpaired electron in its valence shell. Oxygen exists as a free radical in its atomic state. When two oxygen atoms combine to form molecular oxygen (O_2), the valence shell electrons remain as two unpaired electrons, making molecular oxygen a bi-radical. Molecular oxygen can then undergo successive reduction reactions, which give rise to reactive oxygen species (ROS) (Davies, 1995).

The term reactive oxygen species (ROS) encompasses a range of radical and non-radical molecules that are derived from molecular oxygen. The partial reduction of molecular oxygen produces highly reactive intermediates such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH) (Turrens, 2003). Reactive oxygen species are highly unstable due to the single unpaired electron contained in their outermost orbital. As such, ROS will readily react with other molecules to achieve a more stable electron configuration (Krumova & Gonzalo, 2016).

Biological Sources of ROS

Organisms are constantly exposed to ROS derived from both exogenous and endogenous sources. Exogenous sources of ROS include UV radiation, air pollutants, and xenobiotics. However, the majority of the endogenous reactive oxygen species are generated by the mitochondria, which represent the primary oxygen consuming organelles of the cell (Bractic & Trifunovic, 2010). The mitochondria generate ROS during an oxygen-dependent process termed aerobic respiration, which involves a series of oxidation-reduction reactions that take place within the mitochondria (Mitchell & Moyle, 1967). The redox reactions that occur in the mitochondria are carried out during a process termed oxidative phosphorylation. Oxidative phosphorylation involves the transfer of electrons from reduced donor substrates (FADH+, NADH+) across a series of five protein complexes embedded in the inner mitochondrial membrane. As electrons are transferred across the complexes, the free energy release is used to pump protons (H^+) into the intermembrane space. Hydrogen ions that are pumped into the intermembrane space establish an electrochemical gradient between the inter- and inner mitochondrial membranes, creating a chemiosmotic potential termed “proton motive force” (Mitchell, 1961). The proton motive force is essential to drive the phosphorylation of ADP to ATP as hydrogen ions are shuttled back to the inner mitochondrial matrix by the molecular motor ATP synthase (Mitchell, 1961; Bratic & Larsson 2013).

Although the process of oxidative phosphorylation is highly coordinated, it is not always perfectly coupled to ATP synthesis—a scenario that favors ROS formation. A majority

of mitochondrial ROS are generated at complexes I-and III during electron transfer (Bratic & Larsson, 2013). Uncoupling proteins in the inner mitochondrial membrane can also act as transporters and return hydrogen ions to the inner matrix uncoupled from ATP synthase activity. The consequences of uncoupled H+ transport result in both dissipation of proton motive force and superoxide (O_2^-) formation as H+ reacts with oxygen (Bratic & Larsson, 2013). It has been estimated that approximately 1% of oxygen consumed by the mitochondria during respiration is converted to O_2^- (Kudin, Malinska & Kunz, 2008)

To a lesser extent, ROS can also be generated by other biological sources, including the NOX family of NADPH oxidases. The NADPH oxidases are membrane-bound enzymes that use NADPH as a donor to catalyze the reaction of oxygen (O_2) to O_2^-. NADPH oxidases are found in various subcellular locations, but are particularly abundant in phagocytes (Bedard & Krause, 2007; Holstrom & Finkel, 2014). NADPH oxidases serve a biologically relevant function in immunity and cell defense. When stimulated, phagocytes utilize the NADPH-oxidase mechanism to generate a large ROS-response termed ‘respiratory burst’, which effectively kills invading bacteria and microorganisms (Holmstrom & Finkel, 2014).

Biological Targets of ROS

The primary biological targets of ROS include macromolecules such as lipids, proteins, and nucleic acids (Kelly, 2003). Paradoxically, the stability achieved when ROS react with macromolecules comes at the cost of instability incurred by their prospective

targets. Poly-unsaturated fatty acids in phospholipid membranes are a prime target for ROS due to their structural abundance of reactive hydrogen atoms. As ROS abstract hydrogen atoms from the methylene-carbon groups of lipid side chains, an unpaired electron on the carbon atom is left behind. Naturally, the carbon radical will react with O_2, resulting in the production of peroxyl radical (Gutteridge & Halliwell, 1990). The peroxidation of lipids is an insidious process, as peroxyl radicals can react with adjacent fatty acid side chains, thus initiating a cascade of oxidation events that disrupts membrane structure, decreases membrane fluidity, and increases permeability (Gutteridge & Halliwell, 1990).

Both nuclear and mitochondrial DNA are also susceptible to oxidative damage by ROS. Oxidative damage to DNA occurs when ROS (most notably the hydroxyl •OH radical) react with nucleobases or the deoxyribose backbone of the DNA strand. Although all of the nucleobases can be affected, the most common target of ROS is guanine. The initial •OH lesion with guanine forms a radical adduct, which is further reduced and eventually oxidized to 8-hydroxy-2'deoxyguanosine (8-OH-dG), which is considered a universal marker of DNA damage (Valavanidis et al., 2009). The consequences of oxidative damage to DNA include impaired replication, translation, and alterations to base pairs that are often mutagenic (Jackson & Bartek, 2010).

The number of types of oxidative modifications that proteins can undergo is extensive. Oxidative modification to proteins typically occurs at the site of amino acid side-chains or at the polypeptide backbone (Davies, 2016). The specific modification of the protein

is dependent on the particular ROS exposure, and can also vary based on select amino acid residues involved (Halliwell & Gutteridge, 1999). The most common amino acid residues involved in oxidative modification are cysteine and methionine, due to the accessibility of the reactive sulfur anion in the –SH thiol group (Zhang et al., 2013). Oxidation of cysteine and methionine produces disulfides and methionine sulfoxide respectively, a process that can be reversed by cysteine and methionine reductase enzymes, as well as the antioxidant glutathione (Berlett & Stadtman, 1997).

Oxidation of other amino acids such as lysine, arginine, proline and threonine are often irreversible (Berlett & Stadtman, 1997). Direct oxidation of these amino acids produces glycated and carbonylated end-products, and the chronic exposure can manifest as the formation of protein-protein cross linkages and insoluble protein aggregates (Dalle-Donne, 2003). Evidence of protein aggregation has been documented in several disease states associated with chronic levels of oxidative stress, such as diabetes, Alzheimer's, and sarcopenia (Dai et al., 2014).

ROS in Disease and Health

Due to their damaging effects, ROS have rightfully earned a reputation as the underlying cause of many chronic diseases. ROS have also been implicated in the pathogenesis of human aging. Denham Harman first introduced the "Free Radical Theory of Aging" in 1955. Harman's original theory posited that the accumulation of ROS-induced cellular damage explains the tissue degeneration that is observed in organismal aging (Harman, 1955). Although Harman's theory is accepted, it has also

been criticized due to the oversimplified notion that ROS are only biologically relevant as toxic byproducts of mitochondrial metabolism. In agreement with Harman's logic, *chronic exposure* to ROS can be deleterious and cause cumulative damage at the tissue level. However, it is now appreciated that *at moderate or low levels,* ROS are responsible for regulating a diverse range of cell functions as signaling molecules, including growth, differentiation, and apoptosis (Veal et al., 2007).

ROS as Signaling Molecules

One of the primary molecular mechanisms for ROS-mediated signaling is through modification of thiol groups, particularly those containing the amino acid cysteine. Compared to other forms of ROS, the non-radical H_2O_2 has a relatively long half-life, and this characteristic makes it a viable signaling molecule (Powers et al., 2010). As previously mentioned, the initial oxidation of thiol groups by H_2O_2 is a reversible event (Zhang et al., 2013). Therefore, the cyclic oxidation and reduction of protein thiol groups serves as a redox sensing mechanism that can transiently alter protein conformation and function (Vazquez-Torres, 2012).

The initial product of cysteine oxidation results in sulfenic acid. Sulfenic acid can form disulfide bonds with other protein thiol groups, or with the coenzyme glutathione (GSH). Consequently, the biological activity of the protein is temporarily altered, and downstream signal transduction is modified. These types of modifications represent "reversible" transformations, and can be reversed by the small thiol proteins thioredoxin, glutaredoxin, or glutathione (Poole & Nelson, 2008; Kiley & Storz, 2004). Alternatively,

when reduced thiols are subjected to sustained ROS exposure, sulfinic and sulfonic acids can form. These modifications represent irreversible forms of ROS modifications, and can permanently alter protein function (Poole & Nelson, 2008).

To summarize, oxidative modifications exist on a continuum, and the physiologic effects are dependent on both the magnitude and length of ROS exposure. The observation that exposure to low, pulsatile doses of ROS can elicit beneficial signaling responses, whereas exposure to higher, sustained doses of ROS can produce deleterious outcomes defines a concept known as hormesis (Alleman et al., 2014). The hormetic effects of ROS have been exemplified in several experimental models where sub-lethal exposures to oxidants can induce redox-signaling pathways that result in beneficial stress adaptations. For example, in the invertebrate model *C.Elegans,* pre-treatment with sub-lethal doses of a variety of oxidative stressors afforded protective effects when animals were subjected to subsequent exposures at a higher dose (Cypser & Johnson, 2002).

Hormetic effects of ROS have also been observed in models of ischemic preconditioning. Ischemic preconditioning involves the restriction of blood flow to a tissue for brief periods of time, inducing a localized hypoxic environment. Upon tissue re-oxygenation, a transient “burst” of ROS is produced (Bell et al., 2011). In a study by Bell et al., 2011, focal cerebral ischemia was induced in mice through occlusion of the middle cerebral artery. During reperfusion, researchers found increased gene expression of endogenous antioxidant enzymes in pre-conditioned animals as

compared controls. Thus, preconditioned animals were protected against oxidative insult through ROS-mediated induction of antioxidant defense pathways. In summary, when produced in excess, ROS can exert maladaptive responses such as tissue damage and physiologic decline. However, acute exposures to ROS can produce adaptive responses that provide an organism with a stress resistant phenotype.

Regulation of the Redox Environment

Antioxidant Defenses

To prevent the physiologic "tipping point" from adaptive versus maladaptive responses to ROS, the redox environment must be under tight regulation (Foyer & Noctor, 2005). The cellular redox environment is often characterized by levels of ROS relative to the activity of ROS-scavenging antioxidants. Under basal conditions, the redox environment is slightly pro-oxidant to permit necessary redox signaling events (Poljsak, Suput, & Milisav, 2013). In contrast, oxidative stress is defined as an imbalance between ROS production and removal by antioxidant defense systems. When ROS production *chronically* outweighs the capacity of endogenous antioxidant systems to neutralize ROS, irreversible oxidative damage to macromolecules such as proteins, lipids, and DNA can occur (Kelly, 2003).

To maintain redox homeostasis, cells are equipped with defense systems that eliminate ROS through reactions with non-enzymatic and enzymatic antioxidants. Non-enzymatic antioxidants include ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), and glutathione (GSH) and contribute to redox homeostasis through direct ROS-scavenging

mechanisms (Trachootham et al., 2008, Valko et al., 2007). Examples of enzymatic antioxidants include superoxide distmutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), which eliminate ROS catalytically.

Non-Enzymatic Antioxidants

Vitamin C

Vitamin C (ascorbic acid) represents an antioxidant that is derived from food sources. As humans lack the appropriate enzyme to synthesize vitamin C, it must be obtained from the diet. High sources of vitamin C include citrus fruits, peppers, and cruciferous vegetables (Halliwell & Gutteridge, 1990). Vitamin C is water-soluble, and readily dissociates into ascorbate anion (AH^-) at physiological pH (Beyer, 1994). Ascorbate serves as a reducing agent, donating electrons to stabilize free radicals in a process termed “free radical quenching”. During the reaction, ascorbate is oxidized to form the ascorbyl radical. The ascorbyl radical is relatively weak and unreactive, and can be further oxidized to produce dehydroascorbic acid, or reduced back to ascorbate by antioxidants such as glutathione. (Padayatty et al., 2003).

Vitamin E

Vitamin E (alpha-tocopherol, alpha-tocotrienol) is a fat-soluble compound that is obtained from dietary sources such as vegetable oils, nuts, and grains (Halliwell & Gutteridge, 1990). Vitamin E functions as an antioxidant by scavenging peroxyl radical, which is a product of lipid peroxidation chain reactions. As such, vitamin E is often referred to as a “chain-breaking” antioxidant due to its inhibition of peroxidation events

propagated in lipid membranes (Burton & Traber, 1990). During its interaction with peroxyl radical, vitamin E donates a hydrogen atom from its phenol group to form tocopheroxyl radical (Rock et al., 1996). In vivo, it has been suggested that vitamin E and vitamin C operate synergistically, as ascorbate can reduce tocopheroxyl radical for vitamin E re-synthesis (Van den Berg et al., 1990).

Glutathione

Glutathione is a versatile antioxidant and major regulator of redox homeostasis. Glutathione in itself is a thiol, composed of the amino acids glutamate, cysteine, and glycine. Glutathione is primarily responsible for maintenance of the reduced state of protein thiol groups, reducing oxidized disulfides to cysteine. However, glutathione can also interact directly with number of ROS as a scavenging antioxidant (Pavarino et al., 2013; Halliwell & Gutteridge, 1990). Glutathione also serves a critical co-factor for many glutathione-dependent enzymes such as glutathione peroxidase (GPX) (Halliwell & Gutteridge, 1990). In its reduced form (GSH), glutathione is oxidized to GSSG by the GPX enzyme, and converted back to GSH by glutathione reductase (GR). Due to its ubiquitous nature, the ratio of reduced to oxidized glutathione (GSH/GSSG) is often used as a measure of cellular redox status (Zitka et al., 2012).

Enzymatic Antioxidants

Superoxide Dismutase

Superoxide dismutase is an important antioxidant enzyme that exists in three isoforms in humans (SOD1, SOD2, SOD3) localized to the cytosolic, mitochondrial and

extracellular compartments respectively (Matés et al., 1999). SOD was first discovered by McCord & Fridovich in 1968, who demonstrated that SOD catalyzes the dismutation of superoxide anion O_2^- to O_2 and H_2O_2 (McCord & Fridovich, 1968; Matés et al., 1999). Transgenic mice deficient in SOD have been shown to accrue substantial oxidative damage in a number of tissues, accompanied by an accelerated pathogenesis of several chronic diseases such as cancer, myocardial injury, and diabetes (Elchuri et al., 2005; Lebovits et al., 1996; DeRubertis et al., 2007). These studies confirm the critical role of SOD in protection against oxidative stress.

Catalase

The dismutation of O_2^- by SOD produces H_2O_2 that can be broken down further to O_2 and H_2O by the heme-containing enzyme catalase. Catalase (CAT) is found in high concentrations in the liver, although it is also detected in erythrocytes, brain, heart, and skeletal muscle. Within tissues, catalase is contained in peroxisomes (Halliwell & Gutteridge, 1990). The catalase enzyme is highly efficient, and it has been estimated that one molecule of catalase can drive the dismutation of 6 million H_2O_2 molecules each minute (Kurutas, 2016). The degradation of H_2O_2 is critical due to its potential to form the highly reactive •OH through interaction with Fe^{2+} during Fenton's Reaction (Halliwell & Gutteridge, 1990).

Glutathione Peroxidase

The H_2O_2 that is produced from the dismutation of O_2^- can also be degraded by the GPX enzyme. The GPX enzyme superfamily consists of four main isozymes (GPX1-4).

GPX1 is often referred to as the “classic” GPX, as it is the most abundant of the GPX family members. GPX1 is found in nearly all tissues with particularly high expression in the liver, and exists in the cytosolic, mitochondrial, and nuclear cell compartments (Lubos, Loscalzo, & Handy, 2011).

GPX enzymes use reduced glutathione (GSH) as an electron donor to catalyze the reaction of H_2O_2 to H_2O and GSSG (Li et al., 2000). Although CAT is also capable of degrading H_2O_2, its activities are limited to peroxisomes. Further, GPX enzymes are thought to be more effective than catalases at dismantling H_2O_2 (Antunes, Han, & Cadenas, 2002; Lubos, Loscalzo, & Handy, 2011).

Nrf2: Master Regulator of the Endogenous Defense System

Together, SOD, CAT, and GPX represent the major enzymatic antioxidants of the endogenous antioxidant defense system. The biosynthesis and activity of the endogenous antioxidant systems are in part regulated by a transcription factor called Nuclear factor E2-related factor 2 (Nrf2). Nrf2 is a cap’n’collar (CNC) basic leucine zipper (bZIP) family member that controls both basal and inducible gene expression of phase-II drug detoxifying and antioxidant enzymes through the Nrf2-ARE signaling pathway (Hayes & Dinkova-Kostova, 2014).

The activity of Nrf2 is subject to regulation by redox-sensitive proteins. Under homeostatic conditions, Nrf2 is sequestered in the cytosol through Kelch-like ECH-associated protein 1 (Keap1) binding. Keap1 is an actin-bound cytoplasmic protein, and

interacts directly with the Neh2 (Nrf2-ECH homology domain 2) domain of Nrf2. The actin-Keap1-Nrf2 scaffolding mechanism prevents the nuclear translocation of Nrf2 (Kang et al., 2003). Keap1 also functions as an adaptor for the Cul3-based E3 ligase (Kobayashi et al., 2004), which targets Nrf2 for poly-ubiquitination and degradation by the 26S proteasome. When Nrf2 is bound to Keap1, it is constitutively degraded with an approximate half-life of ~15 minutes (Nguyen, Nioi, & Pickett, 2009).

The Keap1 protein has an abundance of cysteine thiol groups, which are target sites for modification by oxidants (Dinkova-Kostova et al., 2002). Upon exposure to oxidants or electrophiles, cysteine residues on Keap1 are oxidized, inducing conformational changes to Keap1 and subsequent release of Nrf2. Once released from Keap1, free Nrf2 translocates to the nucleus and binds to target genes that contain an ARE regulatory sequence in their promoter region. The ARE core consensus sequence *5'-GTGACxxxGC-3'* has been identified in a wide range of genes with antioxidant and cytoprotective function including SOD, GPX, CAT, heme-oxygenase 1 (HMOX1), NAD(P)H quinone dehydrogenase 1 (NQO1) (Lewis et al., 2010).

The transcriptional activity of Nrf2 is controlled by a number of factors that are responsive to the oxidative environment of the cell. Acute oxidative stress induces the nuclear import of Nrf2, whereas persistent oxidative stress activates components of the Nrf2 shutdown pathway (Kaspar & Jaiswal, 2011). The nuclear export of Nrf2 is initiated by glycogen synthase kinase-3beta (GSK3B). In response to oxidative stress, GSK3B is activated by phosphatidylinositol-3 kinase (PI3K) (Salazar et al., 2006). Once

activated, GSK3B phosphorylates its downstream effector Fyn kinase. Phosphorylation of Fyn kinase induces its nuclear translocation, where Fyn phosphorylates Nrf2 thus causing its nuclear export (Kaspar & Jaiswal, 2011).

Pleiotropic Effects of Nrf2

In addition to antioxidant defense, Nrf2 can elicit pleiotropic responses in the cell through crosstalk with NF-κB signaling pathways (Khor et al., 2006; Tornatore et al., 2012). Nuclear factor kappa beta (NF-κB) is a transcription factor that is activated in response to oxidative stress and inflammation. Inflammatory cytokines can initiate NF-κB activation by stimulating inhibitor of nuclear factor kappa beta kinase (IKK), an enzymatic complex that degrades inhibitor of κB (IκB) proteins on NF-κB (Tornatore et al., 2012). Degradation of IκB proteins results in NF-κB activation and nuclear translocation, where the transcription factor regulates gene expression of pro-inflammatory cytokines such as interleukin 1-beta (IL-1β) and tumor necrosis factor alpha (TNF-α) (Tornatore et al., 2012).

Studies in Nrf2 knockout models have indicated that Nrf2 activation may have anti-inflammatory properties by functioning as a NF-κB antagonist. In a mouse model of inflammatory colitis, Nrf2 deficient (Nrf2 -/-) mice had an exaggerated disease severity that was associated with decreased expression of antioxidant enzymes, as well as increased expression of inflammatory cytokines such as cyclooxygenase-2 (COX-2), IL-1β, and TNF-α (Khor et al., 2006). Although a causative link between Nrf2 and NF-κB was not established, the inflammatory cytokines that were measured are known

transcriptional targets of NF-κB (Khor et al., 2006). Up-regulation of inflammatory cytokines in Nrf2 deficient mice suggests that suppression of Nrf2 may promote NF-κB activity (Wardyn et al., 2015).

Studies performed in Nrf2 deficient mice have also defined a role Nrf2 in mitochondrial biogenesis (Zhang, Wu, & Klaassen, 2013). Mitochondrial biogenesis can be induced by oxidants, which is also the main stimulus for Nrf2 activation. As such, Zhang et al. attempted to examine the role of Nrf2 in mitochondrial biogenesis using a mouse model of fasting-induced oxidative stress. Results from the study showed that Nrf2 deficient mice had lower mitochondrial content as compared to controls, suggesting Nrf2-dependence in the regulation of mitochondrial biogenesis (Zhang, Wu, & Klaassen, 2013).

Nrf2 can promote mitochondrial biogenesis though interaction with peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and nuclear respiratory factor 1 (NRF1) (Dinkova-Kostova & Abramov, 2015; Piantadosi et al, 2008, Brose et al. 2012). PGC-1α is a prominent transcriptional co-activator for DNA-binding transcription factors NRF1 and Nrf2, and mitochondrial transcription factor A (TFAM). While NRF1 and Nrf2 transcribe nuclear-encoded mitochondrial proteins, TFAM is involved with replication of the mitochondrial genome (Koltai et al., 2012).

Interestingly, although PGC-1α can serve as a co-activator for Nrf2, Brose et al. 2012, identified that Nrf2 may reciprocally regulate the activity of PGC-1α. In human

fibroblasts treated with a small-molecule Nrf2 activator sulforaphane, gene expression of PGC-1α was increased, suggesting that Nrf2 can promote the transcription of PGC-1α. Nrf2 can also promote mitochondrial biogenesis through transcriptional regulation of NRF1 (Piantadosi et al., 2008). Piantadosi et al. showed that NRF1 has several ARE motifs in its promoter region, and oxidative stress increases the occupancy of Nrf2 at each of the identified ARE sites. Further, the transcriptional regulation of NRF1 by Nrf2 was associated with increased mitochondrial biogenesis (Piantadosi et al., 2008).

Nrf2 and Longevity

Nrf2 serves multifaceted roles as a regulator of redox homeostasis, inflammation, and mitochondrial biogenesis. As such, Nrf2 has been recognized for its cytoprotective effects in several chronic diseases such as cardiovascular disease, cancer, and neurodegenerative disorders (Gao, Doan, & Hybertson, 2014). More recently, Nrf2 has also been proposed to beneficially influence organismal healthspan and longevity (Lewis et al., 2010).

The study of long-lived models has provided insight into the unifying cellular mechanisms that impart longevity. Several long-lived models, including genetic mutants and naturally long-lived species display increased resistance to oxidative stress (Salmon et al., 2005; Lewis et al., 2015). The Snell dwarf mouse is a genetic model of extended longevity, harboring a mutation in the Pit-1 gene which results in deficient production of growth hormone, insulin-like growth-factor 1, and thyroid stimulating hormone. The abnormal hormonal profile observed in the Snell dwarf is thought to give

rise to its slowed-aging phenotype, where mutants live 40-45% longer than their wild-type counterparts (Salmon et al., 2005). Primary dermal fibroblasts derived from Snell dwarf mice are resistant to a number of oxidant stressors in culture (Murakami, Salmon, & Miller, 2003). Further, Snell dwarf mice also exhibit elevated levels of Nrf2 and Nrf2-inducible genes such as glutamine cysteine ligase modifier subunit (GCLM), heme-oxygenase 1 (HMOX1), and NAD(P)H quinone dehydrogenase 1 (NQO1) (Leiser & Miller, 2010). This observation indicates that enhanced Nrf2 signaling may impart stress resistance in the Snell dwarf.

The naked mole rat is a naturally long-lived species with remarkable stress resistance. The naked mole rat lives nine times longer than rodents of comparable body size, with an impressive maximal lifespan of ~28.3 years. The naked mole rat is also an intriguing model of “successful aging”, due to the fact that despite high levels of oxidative stress, the rodent experiences relatively low rates of disease or declines in physiological function (Buffenstein, 2008). Compared with wild-type mice, the naked mole rat has 50% higher Nrf2:ARE binding, elevated antioxidant gene expression, and ~5 fold higher Nrf2 protein levels. Further, maximal lifespan has been positively correlated with Nrf2 signaling in the naked mole rat (Lewis et al., 2015).

Nrf2 and Proteostasis

Nrf2 signaling may also may also influence longevity by promoting protein homeostasis (proteostasis). Maintenance of proteostasis has been identified as a hallmark of slowed aging (Morimoto & Cuervo, 2014), and involves the coordinate processes of protein

synthesis, folding, localization, and breakdown to ensure proteome stability (Miller et al., 2014; Morimoto & Cuervo, 2014). Because enzymatic capacity to repair proteins is limited, damaged proteins must be broken down to recycle amino acids for new protein synthesis (Ryazanov & Nefsky, 2012). The balance of protein synthesis and breakdown defines the process of protein turnover. Maintaining adequate rates of protein turnover ensures the degradation of damaged proteins, and renewal and replacement of damaged structures through new protein synthesis. Therefore, new protein synthesis is essential to maintain adequate concentrations of protein in the cell, and is a key feature of proteostatic maintenance (Miller et al., 2014).

In order to accurately assess the contribution of protein synthesis to proteostasis, it is essential to measure both protein and DNA synthesis. To illustrate, new protein synthesis is observed solitarily as an adaptive response to environmental stressors. However, new protein synthesis also accompanies DNA synthesis during the process of cellular replication. In one scenario, protein synthesis has increased in preexisting cells, whereas in the other, protein synthesis increases to provision daughter cells with an equal complement of proteins. Therefore, measuring the ratio of protein to DNA synthesis (PRO:DNA) indicates whether increases in protein synthesis are allocated toward somatic maintenance (enhanced proteostasis) or cellular proliferation (growth) (Miller et al., 2014).

Previous work from our lab has shown that the long-lived Snell dwarf mouse has enhanced proteostatic mechanisms as indicated by an increased PRO:DNA synthesis

ratio (Drake et al., 2015). Although a Nrf2 influence was not measured in the study, others have shown that the Snell dwarf has elevated Nrf2 signaling, and better maintains the reduced state of protein thiol groups in response to an oxidative stress (Leiser & Miller, 2010). However, it is possible that elevated Nrf2 signaling is tissue-specific in Snells, as we were not been able to detect differences in Nrf2 signaling in skeletal muscle (Bruns et al., 2015). In older individuals, there is evidence of impaired proteostatic maintenance in skeletal muscle as determined by increased protein carbonyl content, an irreparable product of protein oxidation. Dysregulated Nrf2-ARE signaling measured in the study suggests that older individuals may be more susceptible to protein damage due to age-related decrements in stress resistance (Safdar, deBeer, & Tarnopolsky, 2010). Accumulation of protein damage could also reflect overwhelmed proteosomal systems, which are responsible for protein degradation. Interestingly, Nrf2 also partially mediates the activity of the 20S and 26S proteasomes by transcribing catalytic subunits of the complexes (Kapeta, Chondrogianni, & Gonos, 2010; Kwak et al., 2003).

To summarize, Nrf2 signaling enhances stress resistance and preserves proteostatic maintenance in the face of oxidative stress. These characteristics have been identified in long-lived models such as the Snell dwarf mouse, suggesting that elevated Nrf2 signaling may confer a slowed-aging phenotype. Age-related chronic diseases are associated with increased oxidative stress, as well as declines in proteostasis. Given that several chronic diseases share aging as a common risk factor, directing strategies to the slow the aging process may address the treatment of several chronic diseases

simultaneously (Seals et al., 2015). The insight that Nrf2 appears to be at the nexus of cellular cytoprotective and longevity pathways has led to the emerging prospect that Nrf2 may represent a novel therapeutic target for slowed aging and increased healthspan (Bruns et al., 2015; Lewis, Mele, Hayes, & Buffenstein, 2010).

Therapeutic Potential of Nrf2

Attempts to alleviate oxidative stress in chronic disease has typically been done through oral administration of exogenous antioxidants such as vitamin C + E. In clinical trials, increased consumption of diets rich with vitamin C + E was associated with a reduction in markers of oxidative damage to DNA (Thompson et al., 1999). However, in a study by Darko et al., 3-weeks of supplementation with vitamin C in diabetic subjects failed to improve serum measures of 8-epi-prostaglandin F2α, a marker of lipid oxidation. In healthy individuals, Rehman et al. found that six weeks of co-supplementation of vitamin C and iron *increased* biomarkers of oxidative DNA damage in plasma. This finding suggested that vitamin C supplementation could potentially have pro-oxidant effects for individuals who already meet dietary recommendations. Ristow et al. further established the negative implications of antioxidant supplementation by showing that co-supplementation of vitamin C and E may interfere with beneficial ROS signaling events that mediate exercise-induced mitochondrial adaptations (Ristow et al., 2009). In a randomized controlled trial, Lee et al. demonstrated that vitamin E supplementation does not reduce risk of chronic conditions such as cancer or cardiovascular disease in healthy women (Lee et al., 2006). The failure of exogenous antioxidants to prevent oxidative damage or chronic disease incidence supports the notion that up-regulation of

the endogenous antioxidant systems via Nrf2 could represent a promising alternative strategy to attenuate oxidative stress in aging and disease (Reuland, 2012; Nelson et al., 2006).

Phytochemical Activation of Nrf2

Phytochemicals are bioactive compounds found in plants that include phenolic acids, flavonoids, carotenoids, and isothiocyanates (Dinkova-Kostova & Talalay, 2008). The consumption of phytochemicals from fruit and vegetable sources has been associated with reduced chronic disease risk, which is thought to be partially due to their antioxidant function (Prior & Cao, 2000). The observation that plant-derived phytochemicals can induce the expression of antioxidant enzymes suggests that phytochemicals may exert their antioxidant effects indirectly through Nrf2 activation (Dinkova-Kostova & Talalay, 2008). Indeed, numerous phytochemical compounds have been documented to covalently modify cysteine residues on Keap1 as electron acceptors during Michael addition reactions to upregulate Nrf2 activity (Stefanson & Bacovic, 2014). Additionally, phytochemicals can also activate Nrf2 indirectly by up-regulating kinases in the PI3K/Akt signaling pathway of which Nrf2 is a downstream target (Martin et al., 2004).

Although individual compounds are capable of activating Nrf2 in isolation, the antioxidant effects of phytochemicals are thought to be more potent when combined as they are naturally found in whole-foods (Liu, 2004). Furthermore, Nrf2 activation by isolated compounds can be difficult to achieve in vivo due to lack of bioavailability

(Hybertson et al., 2011). Therefore, phytochemical-based supplements that have been formulated for Nrf2 activation include several phytochemical compounds for their additive effects (Velmurugan et al., 2009). Protandim is a commercially available supplement that contains five plant-derived phytochemicals (*Bacopa monniera (*Water hyssop*)*, *Silybum marianum* (milk thistle), *Withania somnifera* (Ashwagandha), *Camellia sinensis* (green tea), and *Curcuma longa* (turmeric)) that synergistically up-regulate Nrf2 activity (Nelson, 2006; Velmurugan et al., 2009). In vitro, Protandim treatment protected arterial endothelial cells and cardiomyocytes from a hydrogen peroxide stressor in a Nrf2-dependent manner (Reuland et al., 2012; Donovan et al. 2012).
Unpublished work from our lab has also shown that co-treatment of C_2C_{12} myoblasts with Protandim and H_2O_2 improves proteostatic maintenance. In the NIA-funded Interventions Testing Program, Protandim supplementation extended the median lifespan of male, but not female, HET3 mice by 7% (Strong et al., 2016). Together, this evidence highlights the potential for phytochemical Nrf2 activation as an intervention for slowed aging.

PB125: The Next Generation

Success of phytochemical Nrf2 activation by Protandim has led to the recent development of a novel Nrf2 activator called PB125. PB125 was formulated by Pathways Bioscience, and contains a combination of three plant-derived phytochemicals (*Carnosol* (Rosemary extract), *Withaferin A* (Ashwaganda), Luteolin*)* selected for their individual documented effects on Nrf2 activation (Tong et al., 2016;Heynick et al., 2016;Zhang et al., 2013). In vitro studies performed by Pathways

Bioscience have demonstrated that PB125 may be a more robust Nrf2 activator than Protandim due to its inhibitory effects on components of the Nrf2 shutdown pathway, which would allow Nrf2 to stay transcriptionally active for a longer period of time (unpublished data). However, whether or not PB125 can activate Nrf2 in vivo has yet to be determined. Further, whether or not up-regulation of the antioxidant system by PB125 is associated with improvements in proteostasis remains unknown. In the present study, we examined if five weeks of dietary supplementation with PB125 at a range of doses (low, medium, and high) could activate Nrf2 and improve proteostasis in 10-11 month old male CB6F1 mice. Because the efficacy of plant-derived phytochemicals can be impacted by low bioavailability (Holst & Williamson, 2008; Hybertson et al., 2011), we hypothesized that the most effective Nrf2 activation would be achieved with the high dose of PB125, and this would be reflected as an increase in proteostatic mechanisms in skeletal muscle, heart, and liver tissues.

CHAPTER III: METHODS

Animal Care

10-11 month old male CB6F1 (BALB/cBY x C57BL/6) mice were purchased from the National Institute of Aging (NIA) colony. All animals were housed at the CSU Laboratory Animal Resource Center at 18-26 C° (dry bulb), 30-70% humidity, and a 12-hr light/dark cycle. All procedures at the facility meet or exceed the standards for facilities housing animals as described in the Animal Welfare Act regulations, the Guide for the Care and Use of Laboratory Animals, and the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching and was approved by the CSU Animal Care and Use Committee.

Following a one-week acclimatization period, animals were randomized to receive one of four doses of PB125 (AIN-93 diet with 0, 30, 100, or 300 ppm of PB125 added). Mouse body weights and food intake were recorded at baseline and every three days until the end of the five-week feeding trial, and the data was averaged to obtain average body weight (gm) and average food intake (gm). Two separate cohorts of mice were used in the study. The isotopically labeled cohort (n=40 mice) received deuterium oxide (D_2O) in the drinking water to measure protein and DNA synthesis. Mice received a bolus intraperitoneal (i.p.) injection of D_2O (Sigma-Aldrich, St. Louis, MO, USA) relative to 60% of body weight as previously described, and D_2O was added to the drinking water of TRACD mice for the last two weeks of the feeding trial (Miller et al., 2013). D_2O incorporates into non-labile sites on alanine and deoxyribose to determine

fractional synthesis rates (FSR) of protein and DNA synthesis. The microarray cohort (n=20) was not labeled with D_2O. 12 hours prior to sacrifice, all food was removed from the cage of TRACD mice. The mice in the microarray cohort were not fasted overnight to improve sensitivity of Nrf2 activation measurements. All animals were anesthetized with 0.5-1.0 ml i.p. injection of ketamine (80mg/kg)/xylazine (20mg/kg) mixture. Blood was obtained by cardiac venipuncture (approx. 1.0ml), followed by excision of heart, liver, gastrocnemius, soleus, and marrow from the tibia. All tissues were flash frozen in liquid nitrogen and stored at -80 C° until later analysis.

Diet Composition

The PB125 supplemented diets contained a combination of three plant-derived phytochemicals (*Carnosol* (Rosemary extract), *Withaferin A* (Ashwaganda), Luteolin*)* at varying doses in pellet form (Dyets Inc, Bethlehem, PA). The dietary composition for each dose is listed as follows: Low dose (30 ppm) - Rosemary extract (2.05×10^{-5} mg/g diet), Ashwaganda extract (1% withaferin A) (6.82×10^{-6} mg/g diet), Luteolin (2.73×10^{-6} mg/g diet). Medium dose (100 ppm) – Rosemary extract (6.82×10^{-6} mg/g diet), Ashwaganda extract (1% withaferin A) (2.27×10^{-5} mg/g diet), Luteolin (9.09×10^{-6} mg/g diet). High dose (300 ppm) – Rosemary extract (2.05×10^{-4} mg/g diet), Ashwaganda extract (1% withaferin A)(6.82×10^{-5} mg/g diet), Luteolin (2.73×10^{-5} mg/g diet).

Tissue and Analyte Preparation

Differential Centrifugation and Mitochondrial Isolation

Whole skeletal muscle, heart, and liver tissue samples were pulverized under liquid nitrogen. Approximately 40-50 mg of tissue was used for differential centrifugation to isolate mitochondrial (Mito), cytosolic (Cyto), and mixed (Mix) subcellular protein fractions according to our previously published procedures (Miller et al., 2013). Pulverized tissues were bead homogenized in 1:10 isolation buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH 7.5) in addition to phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford IL). The initial tissue homogenate was centrifuged at 800g for 10 min at 4°C. The resulting supernatant was removed and added to a new tube, and the pellet was saved as Mix. The supernatant from the previous spin was centrifuged at 10,000g for 30 min at 4°C, and the resulting pellet was saved as Mito. From the supernatant, 400 uL was removed and an equal volume (400 uL) of 14% SSA was added. The tube incubated on ice for 1hr and labeled as Cyto. The remaining volume of supernatant from the Mito spin was saved for protein quantification. The Mito pellet was washed with 200 uL buffer #2 (100 mM KCl, 10 mM Tris-HCl, 10 mM Tris Base, 1 mM $MgSO_4$, 0.1 mM EDTA, 0.02 mM ATP, and 1.5% BSA, pH 7.4) and centrifuged at 8000g for 10 min at 4°C. The supernatant was removed and the pellet was washed a second time with 100uL buffer #2 and centrifuged at 6000g for 10 min at 4°C.

Following the 6000g spin a final wash step with 1ml ddH_2O was performed. After the 1 hr incubation, the Cyto tube was centrifuged at 16000g for 10 min at 4°C to yield the

Cyto protein pellet. The Cyto and Mix pellet were washed with 500 uL 100% ethanol, centrifuged at 1000g for 4 min at 4°C, and washed with 500 uL ddH_2O and centrifuged again at 1000g for 4min at 4°C. Cyto and Mix wash steps were repeated once. Mito, cyto, and mix pellets were solubilized in 250 uL 1M NaOH for 15 min at 50°C and hydrolyzed in 6M HCl for 24 hours at 120°C.

Body Water Derivation and Analysis

Body water enrichment was determined from plasma as previously described by our lab (Scalzo et al., 2014). 125 uL of plasma was pipetted into the inner well of an o-ring screw cap, and tubes were inverted and incubated on a heating block overnight at 80°C. The following day, samples were cooled to room temperature and 2 uL of 10M NaOH and 20mL of acetone were added to each sample and 0%-20% D_2O standards. Samples were gently vortexed and allowed to sit overnight at room temperature. The following morning, samples were prepared for acetone extraction by simultaneously adding 200 uL of hexanes to all samples and standards. The organic layer was transferred to GC vials via 200 uL tips filled with Na_2SO_4. The mass-to-charge ratios of 58 and 60 were monitored for the acetone derivative, and quantified using ChemStation software (Agilent Technologies, Santa Clara, CA, USA). Samples were analyzed via GC/MS on EI Mode with a DB-17MS column.

Alanine Derivation and Analysis

Protein was hydrolyzed overnight in 6M HCl at 120°C. Protein hydrolysates were ion exchanged and dried under vacuum according to our previous published procedures

(Miller et al., 2012). Dried hydrolysates were resuspended in 1 mL of molecular biology grade water, and approximately 500 uL of sample was used for derivation. 500 uL acetonitrile, 50 uL1M K2HPO4, and 20 uL of pentafluorobenzyl bromide were added to all samples and standards. Tubes were vortexed, and incubated on a heating block at 100°C for 1 hour. Next, samples were removed from the heating block and cooled to room temperature. Once cooled, 600 uL of ethyl acetate was added to each sample and vortexed vigorously to allow for phase separation. Using a Pasteur pipette, the top organic layer was transferred to GC vials and dried down under N_2. After drying, samples were reconstituted in 700 uL ethyl acetate, vortexed, and tightly capped for analysis on GC/MS. Samples were analyzed by negative chemical ionization in selective ion monitoring mode. A DB225 gas chromatograph column was used to separate amino acid derivatives. Starting temperature was 100°C and increased to 220°C at a rate of 10°C per minute with helium as the carrier gas and methane as the reagent. The mass-to-charge ratios of 448, 449, and 450 were monitored for the pentafluorobenzyl-*N*,*N*-di(pentafluorobenzyl)alaninate derivative, and quantified using ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The mass-to-charge ratios represented the primary daughter ions that included all of the original hydrocarbon bonds from the given amino acid. ^{2}H enrichment was calculated as the M+1 mass isotopomer divided by the sum of the M+1 and M+0 mass isotopomers (Hellerstein & Neese, 1999). The newly synthesized fraction of proteins was calculated by dividing the ^{2}H enrichment of alanine from protein by the precursor enrichment from body water (plasma). The precursor enrichment was determined from the enrichment of deuterium in body water, and adjusted using mass isotopomer distribution analysis

(MIDA) to determine alanine enrichment (Hellerstein & Neese, 1999). Protein synthetic rates were calculated by dividing fraction new by time, and expressed as fractional synthesis rates (%FSR/day).

DNA Isolation and Derivation

DNA was isolated from whole tissue and bone marrow (QIAamp DNA mini kit, Qiagen, Valencia, CA, USA) and hydrolyzed overnight at 37°C with nuclease S1 and potato acid phosphatase. Next, 80uL of glacial acetic acid and 100 uL pentafluorobenzyl hydroxylamine solution were added to sample hydrolysates and standards (1-30 ug/ml range deoxyribose) and incubated on a heating block for 30 min at 100°C. Following incubation, samples were cooled to room temperature and then reacted with 1ml acetic anhydride and 100 uL n-methylimidazole for 15-20 minutes. Once reaction was complete, 2ml molecular biology grade water and 750uL methylene chloride was added to each tube and vortexed to induce phase separation. The bottom organic layer was extracted and expelled into a new tube containing granular anhydrous Na_2SO_4. An additional 750uL methylene chloride was added to samples, and extraction steps were performed a second time. Extraction was then transferred from tubes containing Na_2SO_4 to GC vials. GC vials were dried under vacuum for one hour and reconstituted in 70uL ethyl acetate. Ethyl acetate was transferred into a tapered GC vial insert and placed back into GC vials. Vials were tightly capped for GC/MS analysis. Samples were analyzed by GC/MS with a DB-17MS column using negative chemical ionization with helium as the carrier gas and methane as the reagent. The pentafluorobenzyl triacetyl derivative of purine dR was monitored for the fractional molar isotope

abundances at m/z 435 and 436, and quantified using ChemStation software (Agilent Technologies, Santa Clara, CA, USA). DNA fraction new was calculated in comparison to bone marrow, which represents a fully turned over population of cells (Miller et al., 2012). DNA synthesis rates were calculated by dividing DNA fraction new by time and expressed as fractional synthesis rates (FSR%/day). The protein FSR was then expressed over the DNA FSR to obtain the PRO:DNA synthesis ratio.

Microarray Analysis

Two Affymetrix Clariom S Chips were used for Nrf2-inducible gene expression in skeletal muscle. In skeletal muscle (soleus), five samples from both control and medium dose (100 ppm) PB125-treated mice were pooled for analysis. Total RNA (150ng) was extracted from mouse skeletal muscle by RNeasy Total RNA isolation kit (Qiagen Inc, Valencia, CA, USA). The concentration of each sample was determined based on the absorbance at 260 nm (A260), and purity was determined based on the ratio of A260 to A280. A range of 1.9-2.1 is considered adequately pure. The integrity of total RNA samples was examined by Agilent 4200 Tape Station. RNA was converted to double-stranded cDNA (ds-cDNA) using cDNA synthesis kit (Affymetrix). An oligo-dT primer containing a T7 cDNA was utilized. The ds-cDNA was then purified and recovered using purification beads (Affymetrix).

Next, in vitro transcription was performed to generate biotin-labeled cRNA using a RNA Transcript Labeling Kit (Affymetrix). Biotin-labeled cRNA was purified using purification beads (Affymetrix). cRNA was fragmented to ensure optimal hybridization to the

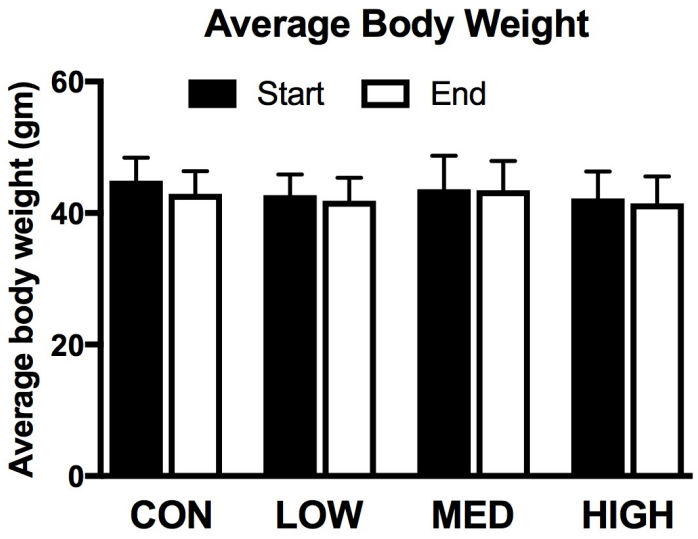
oligonucleotide array. Fragmentation was performed such that the cRNA fragments were between 40-70 bases in length by incubating the cRNA at 94°C for 35 minutes in a fragmentation buffer. The sample was then added to a hybridization solution containing Control Oligo B2 (2nM), 20X Hybridization Controls (bioB, bioC, bioD, cre), 2x Hybridization Mix, DMSO, and Nuclease-free water. The final concentration of the fragmented cRNA was 0.023 μg/μL. Hybridization was performed by incubating 80 uL of the sample to the Affymetrix GeneChip® Clariom™ S mouse gene expression assay (Affymetrix Inc., Santa Clara, California, USA) at 45 °C for 16 hours using a GeneChip® Hybridization Oven 640 (Affymetrix). After hybridization, the hybridization solutions were removed and the arrays were washed and stained with Streptavidin-phycoerythrin using a GeneChip® Fluidics Station 450 (Affymetrix). Arrays were read at a resolution of 2.5 to 3 microns using the GeneChip Scanner 3000 (Affymetrix). Each gene was represented by the use of ~11 probes per transcript and many control probes. The Command Console GeneChip software program was used to determine the intensity of expression for all genes on the array.

Statistics

Statistical analyses were performed using Prism Version 7 (GraphPad Software, La Jolla, CA). A one-way ANOVA was used to determine differences between the treatment group means. Tukey's Test was performed for post hoc analyses when appropriate. Statistical significance was set a priori at $p<0.05$.

CHAPTER IV: RESULTS

A.



B.

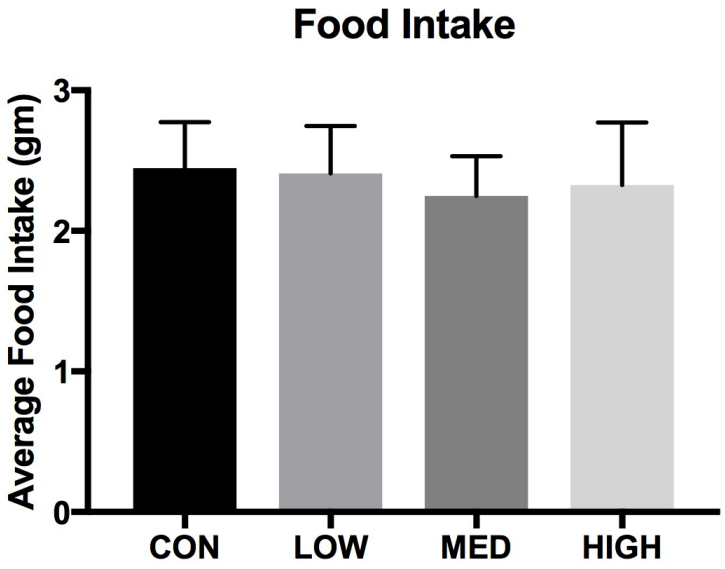


Figure 1. Average body weight (gm) (A) and average weekly food intake (gm) (B) for mice supplemented with low, medium, and high doses of PB125.

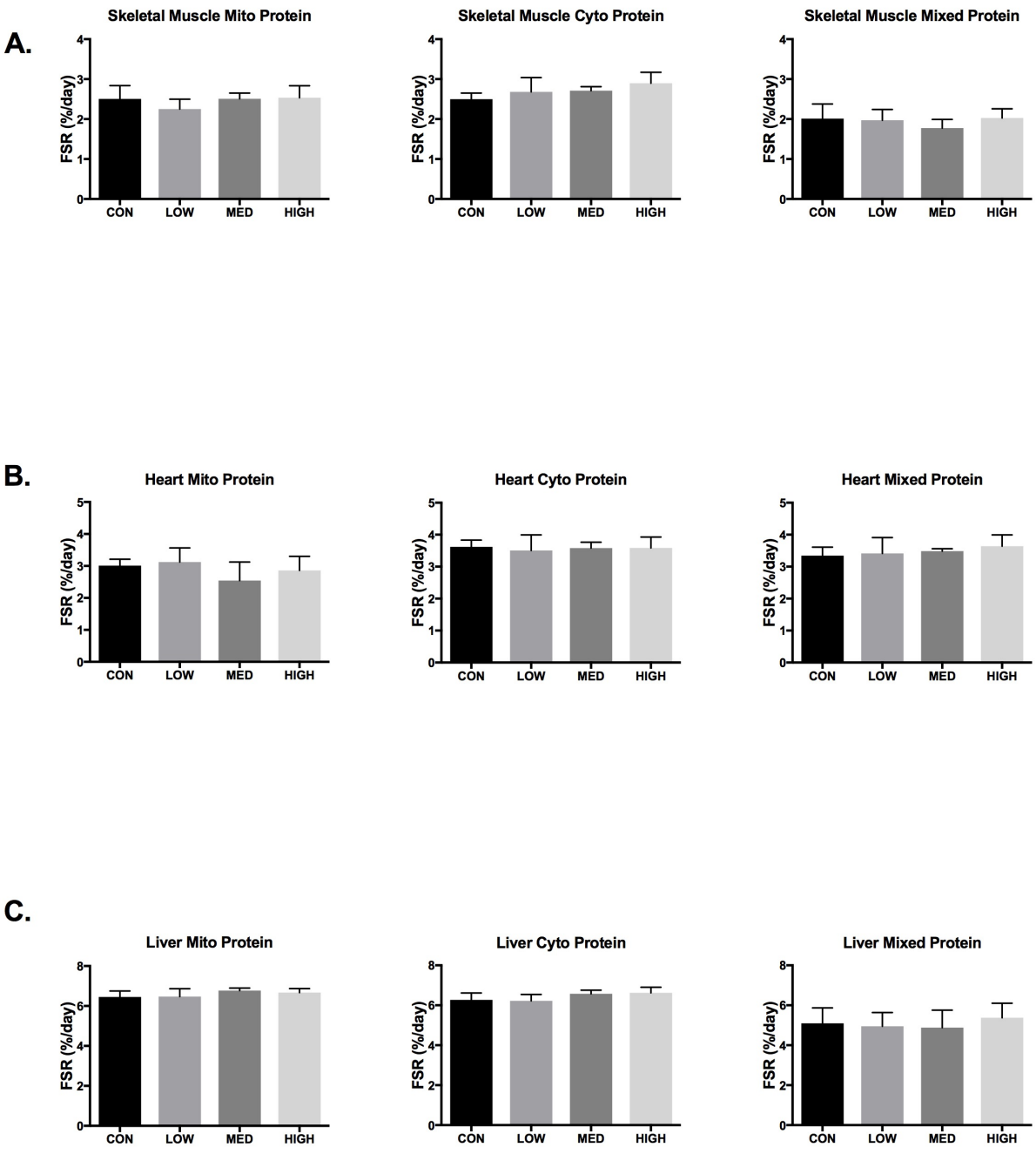


Figure 2. Protein fractional synthesis rates (FSR %/day) at different doses of PB125 across subcellular compartments (Mito, Cyto, Mixed) for skeletal muscle (A), heart (B), and liver (C) tissue. Figures represent protein FSR over a 2-week D_2O labeling period.

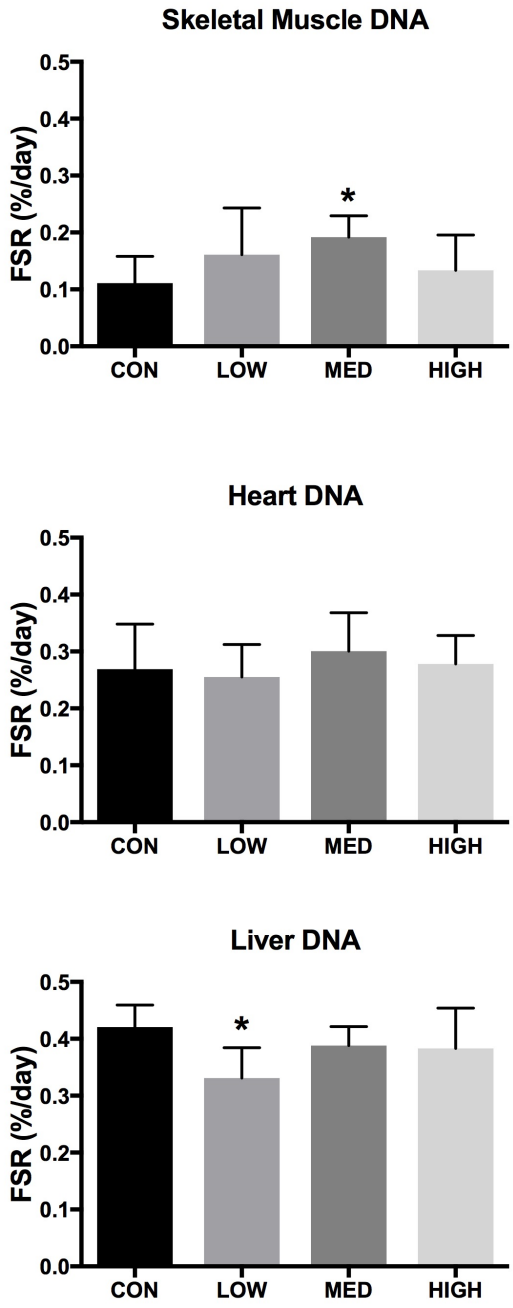


Figure 3. DNA fractional synthesis rates (FSR %/day) for skeletal muscle, heart, and liver tissue at different doses of PB125. *Significantly different from control (P<0.05).

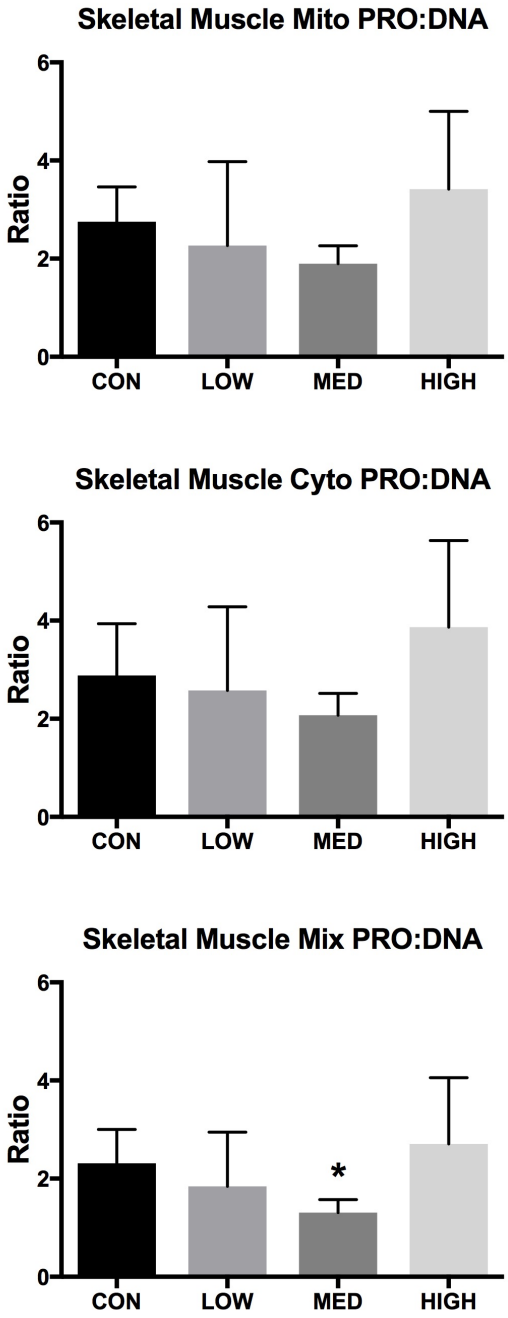


Figure 4. Ratio of protein:DNA by PB125 dose for Mito, Cyto, and Mixed subcellular compartments in skeletal muscle tissue (FSR %/day). *Significantly different from control (P<0.05).

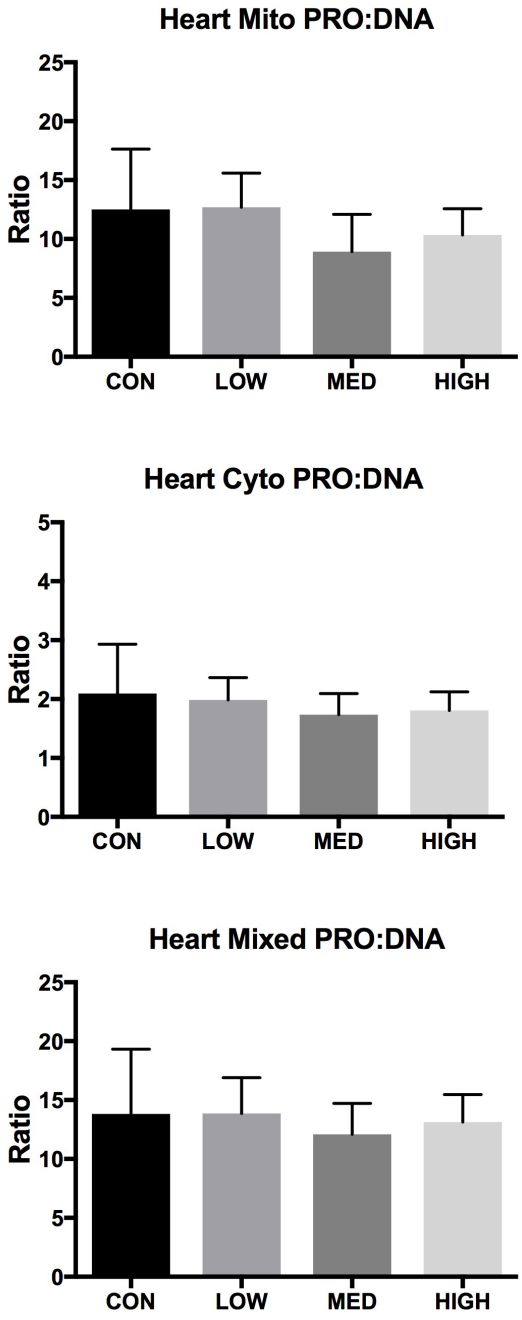


Figure 5. Ratio of protein:DNA by PB125 dose for Mito, Cyto, and Mixed subcellular compartments in heart tissue (FSR %/day).

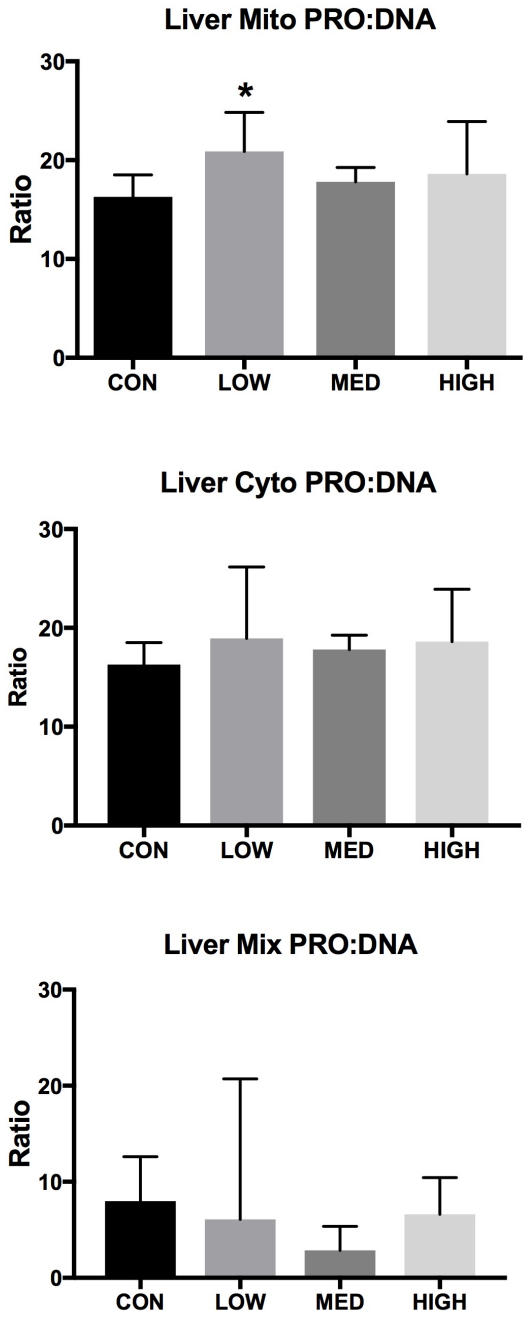


Figure 6. Ratio of protein:DNA by dose for Mito, Cyto, and Mixed subcellular compartments in liver tissue (FSR %/day). *Significantly different from control (P<0.05).

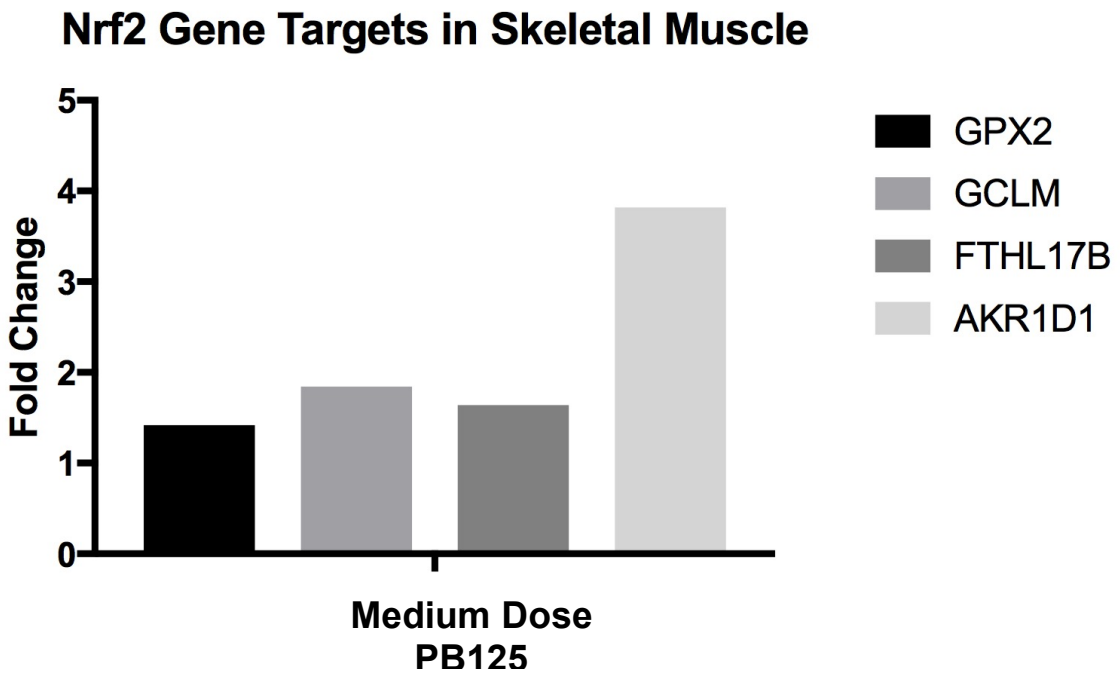


Figure 7. Nrf2 gene targets in skeletal muscle of mice consuming diets supplemented with the medium dose of PB125. Data are presented as fold change from control. Statistical significance could not be determined from the data due to sample pooling.

Body Weight and Food Intake

No significant differences in body weight were detected between the treatment groups before and after the 5-week feeding trial (Figure 1A). There were also no significant differences in food intake between the treatment groups (Figure 1B). Subjectively, the mice did not display any signs of gastrointestinal distress or abnormal behavior while consuming the PB125-supplemented diets.

Proteostasis

Figure 2 shows the FSR (%/day) at different doses of (%/day) in skeletal muscle, heart and liver tissue. There were no significant differences in the protein FSR (%/day) of the mitochondrial, cytosolic, and mixed subcellular compartments for each tissue. Figure 3. shows the dose-dependent effects of PB125 on DNA FSR (%/day) for skeletal muscle, heart, and liver tissue. The group supplemented with a medium dose of PB125 had a significantly greater DNA FSR in skeletal muscle as compared to control ($P<0.05$). DNA FSR was significantly lower in the liver of mice treated with the low dose of PB125 as compared to control ($P<0.05$). No other significant differences were found.

In order to determine if PB125 could affect proteostatic mechanisms, the protein:DNA ratio (FSR %/day) was calculated for skeletal muscle, heart, and liver tissue. In the skeletal muscle mixed fraction, the PRO:DNA ratio was greater in the group supplemented with a medium dose of PB125 as compared to control ($P<0.05$) (Figure 4). In the heart, proteostatic mechanisms were unchanged (Figure 5).

In the liver mitochondrial fraction, there was a greater PRO:DNA ratio in the group supplemented with a low dose of PB125 as compared to control ($P<0.05$) (Figure 6).

Nrf2 Activation

Due to sample pooling, statistical significance could not be determined from the array data. As such, gene expression data was evaluated as the fold change difference from control, with a fold change of 1.5 or higher (up-regulation of gene expression), and .5 or lower (down-regulation of gene expression) considered to be a meaningful response to treatment. From our skeletal muscle microarray analysis, we measured over 20,000 gene transcripts, of which 182 increased by two-fold in response to the medium dose of PB125 supplement. Of the 182 transcripts that increased by 2-fold, we confirmed that 63 contained an ARE regulatory sequence. In skeletal muscle, the Nrf2-regulated cytoprotective gene targets GPX2, GCLM, Akr1d1, and Fthl17b were elevated in response to PB125 supplementation as indicated by a fold change of 1.84, 1.42, 3.82, and 1.64 respectively (Figure 7).

CHAPTER V: DISCUSSION

Principle Outcomes

In the present study, rates of protein and DNA synthesis were measured to assess a mechanism of proteostatic maintenance in the skeletal muscle, heart, and liver of mice consuming diets supplemented with diets containing low, medium, or high doses of the phytochemical Nrf2 activator supplement PB125. Nrf2-regulated gene expression was also measured in skeletal muscle to assess Nrf2 activity. We hypothesized that five weeks of dietary supplementation with PB125 would effectively up-regulate Nrf2-regulated gene expression and improve proteostatic processes, which would be reflected as an increase in the PRO:DNA synthesis ratio. We expected to see enhanced proteostasis and Nrf2 activation in mice consuming the high dose of PB125 supplemented diet. Our main findings were that mice consuming the low dose of PB125 supplemented diet displayed enhanced proteostatic maintenance in the mitochondrial fraction of the liver, while the medium dose of PB125 was effective at inducing the expression of Nrf2-regulated genes in skeletal muscle.

Proteostasis

A primary outcome of the study was to determine if the novel phytochemical based Nrf2 activator PB125 could enhance proteostatic maintenance in vivo. New protein synthesis is a key mechanism of proteostatic maintenance, and the contribution of protein synthesis to proteostasis can be assessed by measuring the ratio of newly synthesized protein to DNA (PRO:DNA) (Miller et al., 2014). An increased PRO:DNA

ratio indicates that protein synthesis is allocated toward somatic (proteostatic) maintenance versus cell growth (Miller et al., 2014). In vitro, our lab has previously shown that the commercially available phytochemical-based Nrf2 activator Protandim enhances proteostasis when co-treated with a H_2O_2 challenge, as evidenced by an elevated PRO:DNA synthesis ratio (Unpublished data). From our current findings, we detected modest evidence of enhanced proteostatic mechanisms in the mitochondrial enriched fraction of the liver in mice consuming the low dose of the second-generation Nrf2 activator PB125 (Fig. 7). In contrast, we saw a reduction in the PRO:DNA ratio in the mixed fraction of the skeletal muscle in mice treated with the medium dose of PB125 (Fig. 4). Although this finding may initially be perceived as negative, the reduction in our ratio appears to be driven by an increase in skeletal muscle DNA synthesis in response to the medium dose PB125 treatment (Fig. 3), while no differences in protein synthetic rates were observed (Fig. 2A). The elevation in DNA synthesis that we observed in our study is consistent with reports that Nrf2 activation can stimulate cellular proliferation and differentiation in various tissues (Murakami & Motohashi, 2015).

We did not detect changes in the PRO:DNA ratio in other subcellular fractions of the skeletal muscle or liver, or in the heart. However, it is important to note that the mice used in our study were relatively young (10-11 months). Studies performed in young $Nrf2^{-/-}$ mice have shown that under basal conditions, mice do not display increased production of ROS or alteration of the redox environment unless exposed to an acute stressor (Muthusamy et al., 2012). The mice in our study were maintained under

conditions that did not impose an oxidative challenge to proteostatic processes. Therefore, due to the young, healthy status of the mice, it may have been difficult to detect differences in proteostatic mechanisms in response to the PB125 treatment. Further, our ability to detect differences in proteostatic mechanism may have been impacted by the late initiation of our two-week labeling period. Were D_2O administered at the onset of the feeding trial, we may have been able to detect early changes in proteostatic mechanisms that we were not able to capture in the last two-weeks of the feeding trial.

Nrf2 Activation

Nrf2 activation was assessed by the expression of Nrf2-inducible antioxidant genes (Fig 8). Phytochemicals can modify cysteine residues on Keap1, thus releasing Nrf2 from Keap1 binding (Hybertson, Gao, Bose, & McCord, 2011). Free Nrf2 then translocates to the nucleus and transcriptionally regulates genes that contain an ARE in their promoters, many of which encode for antioxidant enzymes that are cytoprotective against ROS (Hybertson, Gao, Bose, & McCord, 2011). Overall, we found that the medium dose of PB125 supplement was effective at up-regulating the ARE-driven genes GPX2 (glutathione peroxidase 2), GCLM (glutamate-cysteine ligase modifier), Akr1d1 (aldo-keto reductase family member), and Ferritin (Fthl17b) in skeletal muscle (Fig 8). GPX2 and GCLM represent “traditional” Nrf2 regulated antioxidant genes associated with the GSH-based antioxidant system (Hayes & Dinkova-Kotsova, 2014). Akr1d1 is a Phase 1 drug detoxification enzyme, and Fthl17b is involved with iron and heme metabolism. Both Akr1d1 and Fthl17b have been characterized as positively regulated by Nrf2 (Hayes &

Dinkova-Kostova, 2014). Additionally, we found that 62 other ARE-driven genes were up-regulated by 2-fold in response to the PB125 treatment, although their direct association with Nrf2 has been less characterized.

Although the changes in the antioxidant gene expression profile that we observed were more modest than we had originally anticipated, measurements of Nrf2-related gene expression can be highly time sensitive due to the transient nature of Nrf2 activity (Reuland et al., 2013). Under basal conditions, Nrf2 has a relatively short half-life of ~15 minutes, which increases to approximately 100 minutes once activated (Itoh et al., 2003; Baird & Dinkova-Kostova, 2011). In order to augment the chance of capturing Nrf2-related gene expression in response to the PB125 treatment, the mice in our study were allowed full access to food up until the morning of tissue collection. However, it is possible that the timing of PB125 supplemented food consumption and Nrf2 activation could have impacted the magnitude of our gene expression measurements.

Taken together, our proteostasis and Nrf2 activation measurements reveal a promising role for the phytochemical compound PB125 as a novel slowed aging intervention. Compelling evidence for phytochemical Nrf2 activation and slowed aging comes from the NIA-funded Interventions Testing Program, where the phytochemical-based supplement Protandim extended the median lifespan of male, but not female, HET3 mice (Strong et al., 2015). Consistent with ITP results, we were able to detect evidence of Nrf2 activation in the male mice that were used in our study. The modest yet significant increase in median lifespan that was observed in the ITP supports the potential for phytochemical-

based compounds to increase organismal longevity and healthspan. However, results from the ITP also suggest that further investigation with phytochemical-based compounds is needed in order to address the sexual dimorphic response and lack of effect on maximal lifespan extension observed during the Protandim trial (Strong et al., 2015). In comparison to Protandim, the second-generation phytochemical supplement PB125 has been shown to have more robust effects on Nrf2 activation when each compound was tested at the same doses in vitro (unpublished data). Therefore, PB125 may have potential to elicit more potent lifespan extension effects than previous phytochemical Nrf2 activators. Taking a translational approach, our study represents the first to examine the dose-dependent effects of PB125 on Nrf2 activation *in vivo*, with indication that the medium dose of PB125 is capable of elevating the expression of several Nrf2-related genes.

Our study is among the few that have attempted to examine the effects of phytochemical Nrf2 activators in younger animals. Despite the young, healthy status of the mice used in our study, we were able to detect subtle differences in Nrf2-related gene expression as determined from our microarray analysis. Therefore, it is possible that PB125 could be effective when administered early in life. Qureshi et al., 2010 has shown that phytochemical supplements can be used as a successful preventative therapy in young mice with muscle dystrophy, a disorder where cumulative tissue damage accelerates the disease process beginning in the early stages of life. Given that cumulative oxidative damage to proteins can contribute to the aging process (Harman, 1955), our insights from young mice suggest that PB125 could be also be

used in a preventative manner to maintain redox homeostasis and avoid age-related proteostatic impairments before substantial physiological declines have occurred.

Conclusions and Future Directions

In summary, results from the present study are the first to characterize the cytoprotective potential of the second-generation phytochemical Nrf2 activator PB125. Our data provides modest evidence that the medium dose of PB125 can effectively activate Nrf2 as indicated by up-regulation of Nrf2-inducible gene expression. However, further investigation is needed to determine whether phytochemical activation of Nrf2 by PB125 can improve proteostasis in vivo. As previously stated, model selection may have masked the full potential of PB125 to improve proteostatic outcomes, as the mice used in our study were relatively young and unstressed. Therefore, future studies should consider examining the effects of PB125 in aged mice or in a model of oxidative stress to determine the efficacy of PB125 under conditions where proteostatic mechanisms are likely to be impaired. In vitro studies performed in our lab have shown that phytochemicals can improve proteostasis during conditions of oxidative stress. Therefore, if phytochemical activation of Nrf2 is capable of improving proteostatic maintenance in aged or stressed mice, this may elucidate a mechanism by which phytochemicals confer slowed aging benefits. Overall, we believe that future investigation into the cytoprotective effects of PB125 will support the application of phytochemical Nrf2 activation as a therapeutic intervention for slowed aging and lifespan extension.

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