DISSERTATION

OXIDATIVE AND ENERGETIC STRESS: REGULATION OF Nrf2 AND MITOCHONDRIAL BIOGENESIS FOR SLOWED AGING INTERVENTIONS

Submitted by

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ABSTRACT

OXIDATIVE AND ENERGETIC STRESS: REGULATION OF Nrf2 AND MITOCHONDRIAL BIOGENESIS FOR SLOWED AGING INTERVENTIONS

The following dissertation describes a series of experiments with the overall aim to understand the cellular energetic and oxidative stresses associated with aging, and to investigate treatments which may attenuate these stresses and promote healthspan. The specific aims of the four sets of experiments were 1) to determine if treatment with the phytochemicals in Protandim activates Nrf2 and 2) the mechanisms by which this activation occurs; 3) to assess sexually dimorphic Nrf2 signaling across three rodent models of longevity; and 4) to determine if mitochondrial related proteins are preferentially translated under energetic stress. In Experiments #1 and #2, we found that phytochemicals activate Nrf2 and protect cells against oxidant stress. None of the mechanisms we investigated appear to be responsible for phytochemical-induced Nrf2 activation, and continued investigations must be undertaken to identify how Protandim robustly induces Nrf2 nuclear accumulation. In Experiment #3, we found that Nrf2 signaling was not consistently upregulated in tissues from long-lived models compared to controls, but we did elucidate important sex differences, with female mice generally displaying greater Nrf2 signaling than male mice. We believe this finding, in the context of sexual dimorphism in aging, warrants future investigations into Nrf2, stress resistance, and longevity between males and females. In Experiment #4, we found that mitochondrial proteins were preferentially translated upon pharmaceutical energetic stress, and that this selective translation occurred in the vicinity of the mitochondria. Our results indicate activation of Nrf2 protects cells against oxidant stress, and may be a therapeutic target for cardiovascular diseases and other age-related diseases. Further, we assess selective translation of mitochondrial proteins during energetic stress as a means of understanding how energetic stress mimetics selectively

facilitate the translation of key mitochondrial proteins. Taken together, these studies provide the basis for future work aimed at attenuating diseases with oxidant stress and mitochondrial dysfunction components.

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CHAPTER I- INTRODUCTION/EXPERIMENTAL AIMS

Aging and age-associated diseases such as cardiovascular disease (CVD), sarcopenia, and diabetes are associated with increases in cellular oxidative stress and a diminished capacity to deal with the stress [1]. An increase in cell stress results in damage to cellular macromolecules including lipids, proteins, and DNA. In addition, damage to mitochondria results in dysfunctional mitochondrial respiration and increased reactive oxygen species production, thus further perpetuating cell oxidative damage [2]. Interventions that attenuate the stresses associated with aging, as well as promote the maintenance of mitochondria, could have profound implications on the aging process and age-associated disease.

The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is suggested to be the "master regulator" of cellular antioxidant and cytoprotective responses [3]. Activation of Nrf2 has been proposed for treatment of various diseases including neurodegeneration [4] and hepatic/gastrointestinal diseases [5], yet the role of Nrf2 in CVD is less established. CVD is the leading cause of death and disability within the United States. Oxidant stress is implicated in the etiology and exacerbation of CVD [6]. Therefore, it is important to elucidate the role Nrf2 may play in attenuating cardiac oxidative stress. Understanding mechanisms by which Nrf2 is activated and imparts cytoprotection will open opportunities for targeting Nrf2 for therapeutic purposes.

Since aging is characterized by increased cellular stress, it is plausible that Nrf2 signaling may be involved in the aging process. Long-lived animals display enhanced stress resistance, but the mechanism(s) for the enhanced stress resistance remain unknown [7, 8]. Nrf2 regulates the expression of over 200 cytoprotective genes, and it is proposed that long-lived animals, or interventions that promote longevity, promote enhanced Nrf2 signaling [9]. Females live longer than males across species, and interventions that promote longevity often

have sexual dimorphic responses [10]. Therefore, an assessment of Nrf2 signaling in long-lived versus control mammals with a focus on sexually-dimorphic responses is warranted.

Mitochondria are causally involved in the aging process and age-related diseases, and many slowed-aging interventions promote mitochondrial biogenesis [11]. Many of these slowed-aging interventions are forms of cellular energy restriction, or energy-restriction mimetics, and are thought to act through inhibition of the mechanistic target of rapamycin (mTOR) [12] and simultaneous activation of the cellular energy sensor AMP-activated protein kinase (AMPK) [13]. Inhibition of mTOR results in global inhibition of protein synthesis [14], an energetically expensive process. However, activation of AMPK promotes mitochondrial biogenesis [15], purportedly in an effort to increase oxidative energy production. Therefore, it is paradoxical how in the same cell, under the same stress, global synthesis of proteins can be inhibited alongside selective translation of mitochondrial proteins. We believe mechanisms must exist to facilitate sustained translation of these key proteins under energetic stress. Elucidation of these mechanisms could have an impact on age-related disease, as it may be possible to target the maintenance of the synthesis of key proteins involved in mitochondria and cellular repair processes, thus attenuating age-associated cellular stress.

The overall objective of our projects was to understand the roles of oxidative and energetic stress on age-related diseases, since these represent potential therapeutic targets to attenuate age-related diseases. Specifically, we suggest that activation of Nrf2 may protect cardiac cells against oxidant-induced stress. Further, we characterize Nrf2 signaling across tissues from long-lived models, and propose that future studies determine whether a heightened ability to activate Nrf2 upon stressful stimuli may explain the enhanced stress resistance of long-lived animals. Lastly, we assess selective translation of mitochondrial proteins during treatments that activate cell signaling that is characteristic of energetic stress. We aimed to understand how treatments that are known to promote longevity selectively facilitate the translation of key mitochondrial proteins during inhibited global protein synthesis.

Overall Hypotheses: Activation of Nrf2 by the phytochemicals in Protandim will protect heart cells against oxidant stress and Nrf2 signaling will be greater in tissues from long-lived animals. Further, selective translation of mitochondrial related proteins will occur in the vicinity of the mitochondria under pharmaceutical energetic stress.

Specific Aim of Experiment #1: To determine if treatment of cardiac myocytes with the phytochemicals in Protandim activates Nrf2 and protects cells against oxidant-induced apoptosis.

Specific Aim of Experiment #2: To assess the mechanisms of Protandim-induced Nrf2 activation.

Specific Aim of Experiment #3: To assess sexual dimorphic Nrf2 signaling across three rodent models of longevity.

Specific Aim of Experiment #4: To determine if mitochondrial related proteins are selectively translated during energetic stress, and whether this translation is spatially localized to the mitochondria.

CHAPTER II- MANUSCRIPT I

Upregulation of phase II enzymes through phytochemical activation of Nrf2 protects cardiomyocytes against oxidant stress[1]

Summary

Increased production of reactive oxygen species (ROS) has been implicated in the pathogenesis of cardiovascular disease (CVD), while enhanced endogenous antioxidants has been proposed as a mechanism for regulating redox balance. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcriptional regulator of phase II antioxidant enzymes, and activation of Nrf2 has been suggested to be an important step in attenuating oxidative stress associated with CVD. A well-defined combination of five widely studied medicinal plants derived from botanical sources [*Bacopa monniera*, *Silybum marianum* (milk thistle), *Withania somnifera* (Ashwagandha), *Camellia sinensis* (green tea), and *Curcuma longa* (turmeric)] has been shown to activate Nrf2 and induce phase II enzymes through the antioxidant response element (ARE). The purpose of these experiments was to determine if treatment of cardiomyocytes with this phytochemical composition, marketed as Protandim®, activates Nrf2, induces phase II detoxification enzymes, and protects cardiomyocytes from oxidant-induced apoptosis in a Nrf2-dependent manner. In cultured HL-1 cardiomyocytes, phytochemical treatment was associated

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with nuclear accumulation of Nrf2, significant induction of phase II enzymes, and concomitant protection against hydrogen peroxide-induced apoptosis. The protection against oxidant stress was abolished when Nrf2 was silenced by shRNA, suggesting that our phytochemical treatment worked through the Nrf2 pathway. Interestingly, phytochemical treatment was found to be a more robust activator of Nrf2 than oxidant treatment, supporting the use of the phytochemicals as a potential treatment to increase antioxidant defenses and protect heart cells against an oxidative challenge.

Introduction

Oxidative stress has been implicated in the development or exacerbation of over 100 human diseases [16] including cardiovascular disease (CVD), the leading cause of death and disability within the Western world [17]. Cells contain enzymatic and nonenzymatic antioxidants to prevent damage caused by reactive oxygen species (ROS). Antioxidants may act by directly scavenging ROS, by recycling or reducing other direct antioxidants, or by indirectly upregulating endogenous antioxidant defenses. Direct exogenous antioxidants including vitamin C, beta-carotene, and vitamin E, have been the focus of extensive research but are still only presumed effective in the treatment of CVD [18]. Recent clinical trials [19, 20] fail to show therapeutic benefit of exogenous antioxidant supplementation in CVD and suggest the need for a new approach to regulating cellular redox status.

As a result of the apparent ineffectiveness of antioxidant vitamins in attenuating oxidative stress, recent research has focused on novel ways to induce endogenous antioxidant responses [21, 22]. The upregulation of endogenous antioxidant defenses provides the potential for more profound cellular protection than antioxidant vitamin supplementation due to the enhanced ability of enzymatic antioxidants to scavenge ROS compared to traditional antioxidant vitamins. Some phytochemicals can increase endogenous antioxidant enzyme

activity through the activation of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [21, 23]. Nrf2 is a member of the basic leucine zipper transcription factor family [23, 24] and controls both basal and inducible expression of more than 200 genes [25]. Due to the profound number of genes it transcriptionally regulates, Nrf2 has been termed the "master regulator" of antioxidant defenses [26]. Under normal conditions, Nrf2 is sequestered in the cytoplasm by its involvement in an inactive complex with Kelch-like ECH-associated protein 1 (Keap1) [27, 28]. Keap1, a ubiquitin ligase actin-binding protein [29], targets Nrf2 for ubiquitination and degradation by the 26S proteasome, resulting in basal low-level expression of Nrf2 target genes [30]. Upon exposure to oxidants or chemoprotective compounds, cysteine residues on the Keap1/Nrf2 complex sense cellular redox changes, resulting in alteration of the structure of Keap1. When the cysteine residues on Keap1 are oxidized, dissociation of the Keap1/Nrf2 complex occurs to prevent Nrf2 ubiquitination and degradation [21, 31]. Modification of the Keap1 cysteine residues stabilizes Nrf2, facilitating its translocation into the nucleus. After nuclear import, Nrf2 forms a heterodimer with Maf and Jun bZip transcription factors, which bind to the 5'-upstream cis-acting regulatory sequence known as the antioxidant response element (ARE) [32] and induce transcription of genes with functions that favor survival including mitochondrial biogenesis [33], phase II antioxidant and detoxification [23] , and anti-inflammation [34]. Additionally, Nrf2 gene targets may facilitate cross talk with pathways regulating cell death through interaction with autophagy and apoptosis signaling pathways [35]. The coordinated transcriptional activation of Nrf2-mediated antioxidant and pro-survival enzymes is a potential mechanism to maintain redox homeostasis and avoid the deleterious effects of oxidative stress. However, whether Nrf2 activation can protect against the oxidative stress associated with CVD is still unknown.

The combination of five 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), has

been shown to synergistically induce the AREc32-based bioassay for Nrf2 activation in a concentration-dependent manner [22]. Activation of the ARE by these phytochemicals, marketed as Protandim®, far exceeds the activation elicited by the known Nrf2 activator sulforaphane by nearly 7-fold, highlighting the potency of Protandim [22]. Data from our group shows that this phytochemical combination provides Nrf2-dependent protection of human coronary artery endothelial cells against oxidant induced apoptosis [36], suggesting the potential of Nrf2 activators in protecting against the oxidative stress associated with coronary artery disease [37].

In addition to being causally involved in atherogenesis, oxidative stress has also been implicated in the etiology and progression of ischemic heart disease. Ischemia-reperfusion injury results in accelerated production of reactive oxygen species [38, 39], thereby promoting oxidative injury within the heart. Previous experiments utilizing exogenous antioxidants to attenuate cardiac cell damage have been ineffective [40, 41], and suggest the need for a new approach to maintain redox balance. Recent literature highlights the potential for activation of Nrf2 to protect cardiac myocytes against the oxidative stress associated with CVD [42, 43] however, the ability of phytochemicals to activate Nrf2 and protect the heart against oxidative stress is still unknown. Therefore, we tested the hypothesis that treatment of cardiomyocytes with the phytochemicals in Protandim would result in the activation of Nrf2 and upregulation of phase II enzymes. Further, we hypothesized that treatment with Protandim would protect cultured cardiomyocytes against oxidant-induced apoptosis and that the activation of Nrf2 by the phytochemicals would be superior to that achieved by the cellular response to oxidative stress *per se.*

Materials and Methods

Materials and Reagents

Tert-butyl hydroperoxide (tBH) and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich. Antibodies for western blotting were purchased from Santa Cruz Biotechnology (Nrf2 sc-722, superoxide dismutase-1 (SOD-1) sc-8637, actin sc-1616, and HRP and FITC conjugated secondary antibodies). Antibodies to HO-1 were purchased from Thermo Scientific (PA3-019) and Calbiochem (374087), and antibody to glutathione reductase (GR) from Abcam (ab16801). Protandim was a kind gift from LifeVantage Corp., Salt Lake City, UT. MISSION lentiviral transduction particles (control: SHC001V; Nrf2: TRCN0000054662) were purchased from Sigma-Aldrich. PCR reagents and pre-validated primers and probes (Nrf2: Mm00477784_m1; TATA box binding protein: Mm01229165_m1) were purchased from Applied Biosystems.

Culture of HL-1 Cells

A cardiomyocyte line (HL-1) derived from murine atrium was a generous gift from Dr. William Claycomb. With a similar phenotype to human adult cardiomyocytes, HL-1 cells maintain contractile activity, as well as contain electrophysiological properties and pharmacological responses similar to adult cardiac myocytes through passage 240 [44], providing an appropriate model for cardiomyocyte investigations. Cells (passages 65-92) were maintained in Claycomb supplemented medium with 10% fetal bovine serum, 100 U/mL:100 µg/mL penicillin/streptomycin, 2 mM L-glutamine, and 0.1 mM norepinephrine. Cells were plated on 0.5% fibronectin in 0.02% gelatin coated plates, and were grown to confluence in 37˚C, 5% CO_2 humidified environment.

Cell Treatments and Phytochemical Preparation

The phytochemical combination in commercially available Protandim® was chosen because of its established synergism in activating the ARE [45]. Full microbial and analytical testing of the raw materials and finished product was conducted prior to use. An ethanol extract of the five phytochemicals: *W. somnifera*, *B. monniera*, *S. marianum*, *Ca. sinesis* and curcumin was prepared by shaking 500 mg with 5 mL 100% ethanol overnight at room temperature. The extract was centrifuged at 3,000xg for 15 minutes, and the supernatant was stored at room temperature, protected from direct light. Cardiomyocytes were treated with this phytochemical ethanol extraction (0-100 µg/mL) in supplemented Claycomb medium. Control cells were treated with ethanol vehicle at a concentration that did not exceed 1 µL/mL of medium.

Lentiviral Knockdown of Nrf2 Using shRNA

HL-1 cardiomyocytes were seeded in 60 mm plates at approximately 50% confluence and were transduced with lentiviral particles carrying Nrf2 or control shRNA sequence at a multiplicity of infection (MOI) of 1. Hexadimethrine bromide was added at a final concentration of 8 µg/mL to increase transduction efficiency. Transduced cells were selected in 1 µg/mL puromycin for 3 days before experiments were performed to allow puromycin resistant cells to reach confluence. Subsequent passages were confirmed to be stably transfected by puromycin selection.

Real Time RT-PCR

Real-time RT-PCR was used to verify knockdown of Nrf2 by lentiviral transduction. Total RNA was extracted from 60 mm culture plates using standard TRIzol methods and RNA concentration and protein contamination were determined using spectrophotometry. RNA degradation was determined by agarose gel separation. RNA was reversed transcribed and 20ng of cDNA were amplified using target sequence primer-probe reagents. PCR conditions

were as follows: hot start (15 minutes at 95°C) followed by 40 cycles of denaturing and annealing (15 s at 95°C, 1 minute at 60°C). The relative quantification of the target gene was normalized to an endogenous control (TATA box binding protein) and compared against the control (untreated, untransduced) sample. Fold changes were determined using the cycle threshold ($2^{-\Delta\Delta Ct}$) method [46].

Immunocytochemistry

To determine whether Protandim treatment results in Nrf2 nuclear accumulation, HL-1 cardiomyocytes were grown to confluence on cover slips and treated with phytochemical extract or ethanol vehicle for 15 and 30 minutes, and 1, 2, and 4 hours. The cells were then washed with PBS and fixed in 10% formalin for 30 minutes. Fixed cells were gently washed with PBS and permeabilized with acetone for 15 minutes at 4°C. The samples were gently washed three times and incubated in blocking solution (0.05% goat serum in 5% BSA) for one hour at room temperature. After three gentle washes, the samples were incubated for one hour at room temperature with the primary antibody (1:100), followed by the FITC conjugated secondary antibody (1:200) for 45 minutes. The slides were washed and mounted with DAPI containing mounting medium to allow for nuclear identification. The images were viewed using a fluorescence microscope (Nikon TE2000) and Metamorph software version 7.5 (Universal Imaging Corporation).

Western Blot Analysis

After 12 hours of Protandim treatment, cardiomyocytes were washed two times with ice cold PBS and lysed with buffer containing 200 µL RIPA buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), 0.1 M protease inhibitors, and 0.01 M Na_3VO_4. Samples were sonicated and lysate protein concentration was measured by using the bicinchoninic acid assay. Diluted samples containing equal amounts of protein were prepared

in Laemmli Sample Buffer and 2-mercaptoethanol and heat denatured for 5 minutes at 98˚C. Proteins were resolved on a 10% Tris/glycine SDS-polyacrylamide gel in running buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. Proteins were transferred to a nitrocellulose membrane for 75 minutes at 100 V using a transfer buffer containing 25 mM Tris, 192 mM glycine, 0.02% SDS, and 20% methanol, pH 8.3. Non-specific proteins were blocked by incubation of membrane in 5% nonfat dry milk in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5) at 4˚C overnight. Membranes were incubated overnight in primary antibody (SOD-1 1:500, HO-1 1:500 (Thermo) and 1:1000 (Calbiochem), GR 1:1000) at 4˚C followed by secondary antibody conjugated to HRP for 1 hour at room temperature with 30 minute washes of TBST between primary and secondary incubations. Proteins were detected with chemiluminescence reagents and imaging followed by densitometric analysis using VisionWorks software. Membranes were probed for actin (1:500) to verify equal loading of protein.

Assessment of Apoptosis

To investigate whether Protandim® pretreatment affords protection against apoptosis, nuclear condensation, a hallmark of apoptosis, was assessed. HL-1 cells were cultured to confluence on cover slips, treated with phytochemicals or ethanol vehicle for 12 hours and then exposed to 1.25 mM hydrogen peroxide for 4 hours. The cells were then washed with PBS and fixed in 10% formalin for 45 minutes at room temperature. Fixed cells were gently washed with PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for two minutes. Following washes with PBS, samples were mounted with 25 µL DAPI mounting medium and images viewed using a fluorescence microscope (Nikon TE2000) and Metamorph software version 7.5 (Universal Imaging Corporation).

Statistical Analysis

Significance was set *a priori* at $p<0.05$. Data were analyzed by one-way ANOVA followed by Tukey's test for post hoc multiple comparisons where appropriate (SPSS Version 17). When data did not meet assumptions for equal error variance, log transformations were performed prior to ANOVA. Data are presented as means ± SEM.

Results

Cardiomyocytes cultured in concentrations of Protandim® extract ranging from 0-100 µg/mL grew normally as assessed by the maintenance of a normal morphology and viability (Supplemental Figure 1.1). The expression of the ARE-responsive phase II proteins HO-1, SOD-1, and GR were measured to determine whether phytochemical treatment can upregulate endogenous antioxidant enzymes in cardiomyocytes. Treatment of HL-1 cells with 50, 75, and 100 µg/mL of the phytochemical combination induced phase II enzyme expression ($p<0.05$) with the greatest induction observed at 100 µg/mL (Figure 1.1). In addition to increased expression of phase II enzymes, treatment with 75 µg/mL and 100 µg/mL increased nuclear Nrf2 compared to vehicle treatment (Figure 1.2A). Nrf2 nuclear accumulation was assessed at 15 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours. Nuclear accumulation was observed after 15 minutes of Protandim treatment and was greatest at 1 hour of treatment (data not shown). Western blotting showed significantly ($p<0.05$) greater Nrf2 protein expression with 75 and 100 µg/mL treatment for 48 hours (Figure 1.2B) compared to 0 µg/mL, indicating total cell lysate expression of Nrf2 increases and accumulates with longer duration treatment. No changes were observed in Keap1 protein expression with Protandim treatment (Supplemental Figure 1.3), suggesting Protandim acts independent of Keap1 to activate Nrf2 and induce phase II enzyme expression.

To investigate whether phytochemical-induced Nrf2 activation affords cellular protection against hydrogen peroxide-induced oxidative stress, nuclear condensation, a hallmark of apoptosis, was assessed. Compared to controls, pretreatment with 50, 75, and 100 µg/mL

resulted in attenuated apoptosis following a four hour exposure to 1.25 mM hydrogen peroxide (Figure 1.3 and Supplemental Figure 1.2). No protection was observed with the 10 µg/mL treatment.

To determine if Nrf2 activation is necessary for the phytochemical induced upregulation of phase II enzymes and protection against oxidant stress, shRNA was used to silence Nrf2 expression. While Protandim treatment induced a significant three-fold increase in Nrf2 mRNA compared to vehicle, the induction of Nrf2 mRNA was completely abolished with shRNA compared to untransduced cells (Figure 1.4A). Additionally, upregulation of HO-1 protein was abolished with Nrf2 knockdown, but was not attenuated following transduction with control shRNA (Figure 1.4B). Protection against oxidant-induced apoptosis was significantly attenuated following Nrf2 silencing (Figure 1.5), suggesting Protandim acts through the Nrf2 signaling pathway, and Nrf2 is required for the protection of phytochemical-treated cardiomyocytes against an oxidant stress.

In addition to phytochemicals, direct oxidants are known to activate Nrf2 and induce expression of phase II antioxidant enzymes [24]. To determine whether Protandim treatment or an oxidant challenge results in a more profound activation of Nrf2 and induction of phase II enzymes, HL-1 cardiomyocytes were treated with a range of concentrations of tertbutyl hydroperoxide (tBH), and expression of antioxidant enzymes was determined by immunoblotting. The 12 hour micromolar concentrations and the 2 hour 10mM concentration of tBH were experimentally determined by concentration and duration curves to result in a mild oxidant stress without overt cellular stress. Therefore, under these conditions, no changes in cell morphology or signs of cell stress were observed. Protandim treatment of 100 µg/mL resulted in a more robust activation of Nrf2 than tBH treatment (Figure 1.6A) as well as a significantly greater induction of HO-1 (Figure 1.6B).

Discussion

The purpose of this study was to investigate whether treatment with a combination of phytochemicals, known to activate the ARE, can provide protection against an oxidant challenge in cardiomyocytes, and whether Nrf2 activation is essential for the protection. Treatment of cultured cardiomyocytes with Protandim resulted in nuclear accumulation of Nrf2, upregulation of key endogenous phase II antioxidant enzymes, and Nrf2 dependent protection of cardiomyocytes from apoptosis following an oxidative stress. Further, the upregulation of phase II enzymes and activation of Nrf2 observed with phytochemical treatment was superior to the changes in phase II enzyme expression observed with exposure to oxidants. This study is the first to examine the effects of treatment with the phytochemicals in Protandim in a cardiac myocyte model, and one of few studies to support the use of Nrf2 activating phytochemicals in protection of cardiac myocytes against an oxidative insult. These data support the use of phytochemicals as treatment against the oxidative stress associated with cardiovascular disease.

Nrf2 Activation

Immunofluorescence studies confirmed that Nrf2 content increased in the nucleus within 15 minutes of treatment with Protandim concentrations of 75 and 100 µg/ml. Furthermore, western blot analyses suggested that total Nrf2 protein levels increased with 48 hour treatment, presumably as a result of decreased sequestering and degradation by Keap1 and the 26S proteasome. Nrf2 has been suggested to contain an ARE site in its promoter [47], ensuring that activation of Nrf2 results in nuclear accumulation and upregulation of its own transcription and translation. Nrf2 silencing experiments indicated that Nrf2 is necessary for the induction of phase II enzymes and the observed protection against oxidant stress. In the current experiment, when Nrf2 was silenced with lentiviral shRNA transduction, the phytochemical-induced upregulation of Nrf2 expression was attenuated. Not surprisingly, we were not able to

detect a decrease in Nrf2 expression with the silencing under basal conditions. The lack of decrease of Nrf2 expression may be due to achieving only partial knockdown of the Nrf2 gene with lentiviral transduction, or due to the lack of changes in Nrf2 transcription in the basal state. Other groups have reported that only under conditions of Nrf2 activation are changes in gene expression observed [48], and under basal conditions changes in Nrf2 mRNA expression cannot be detected. It has been speculated that the reason why changes in Nrf2 mRNA expression are so difficult to detect may be due to the long-lived nature of the Nrf2 transcript, despite the reported short half-life of the protein under unstressed conditions [49]. However, we were able to abolish the induction of Nrf2 expression with the lentiviral knockdown. The Nrf2 knockdown was further confirmed by a near complete attenuation of HO-1 induction. Only minimal induction of HO-1 was observed with Protandim treatment in the Nrf2 knockdown cells, which we believe is due to Nrf2 independent mechanisms of HO-1 induction [50].

Phytochemicals have been shown to activate Nrf2 in a variety of cell types by phosphorylation of serine 40, resulting in dissociation of Nrf2 from Keap1 and its translocation to and activation within the nucleus. Kinases implicated in the phosphorylation and subsequent activation of Nrf2 include PI3-kinase [27, 32], MEK/ERK [51], p38MAPK [52], JNK [53], and protein kinase C [54]. Individual phytochemicals have been demonstrated to induce Nrf2 utilizing these stress-signaling pathways, with curcumin contributing to HO-1 induction through p38MAPK [27], and epigallocatechin-3-gallate upregulating HO-1 in endothelial cells through PI3K/Akt-dependent induction [55]. Previous work with the phytochemicals in Protandim suggests the concomitant stimulation of various parallel pathways may be involved in Nrf2 activation, resulting in the observed synergy of ARE activation [45]. When activated by phytochemical treatment, the Nrf2 protein becomes stabilized, therefore allowing it to regulate transcription of antioxidants. Nrf2 has recently been suggested to exist in a complex with various other protein-protein interactions in addition to Keap1, which compete to stabilize/destabilize the protein [35]. Ongoing investigations in our laboratory and others seek to

identify which interactions with Nrf2 assist in its activation and stabilization, and how this can be utilized to optimize Nrf2 activation for treatment of oxidative-stress associated diseases.

Induction of Phase II Enzymes

HO-1, SOD-1, and GR were induced in cardiomyocytes with 75 and 100 µg/mL of Protandim treatment. The doses of the phytochemicals in Protandim ingested by humans [56] induce expression of phase II enzymes comparable to the 100 µg/mL concentration used in this study. Interestingly, the induction of HO-1 with Protandim treatment in this study far exceeded the upregulation of SOD-1 and GR, with maximal induction of HO-1 nearly 400 fold over vehicle. HO-1 is known to have a highly responsive ARE promoter, and therefore is frequently used as an indicator of phase II enzyme induction [57]. Our group [36] and others [58] have previously shown that HO-1 responds the robustly upon Nrf2 activation in comparison to other Nrf2-regulated phase II enzymes. The induction of HO-1 with phytochemical treatment is so large and consistent that HO-1 has been suggested to be a novel therapeutic target in the management of cardiovascular disease [59]. HO-1 has antioxidant, anti-inflammatory, and anti-apoptotic effects [60-62] in a number of tissues; and various chronic diseases including hypertension, atherosclerosis, and myocardial infarction [63], are all associated with HO-1 downregulation. SOD-1 is also suggested to be important in cardioprotection associated with chronic exercise training [64] and pharmacologic interventions [65]. Phytochemical induction of SOD-1 and HO-1 thus represents an effective approach to combat the consequences of oxidative stress associated with cardiovascular disease.

Because phytochemical treatment was found to induce HO-1, SOD-1, and GR via activation of Nrf2, it could be hypothesized that other phase II enzymes containing ARE sites in their promoters including glutathione-S-transferase (GST) and glutamate-cysteine ligase (GCL) [23], could also be induced by supplementation. GR, GST, and GCL are pivotal enzymes in the regulation of intracellular redox status through glutathione (GSH) homeostasis. Genetic

knockouts of the enzymes involved in glutathione biosynthesis indicate the importance of GSH in cellular antioxidant properties [66], as do the observations that lowered GSH levels are correlated with several human diseases. By inducing phase II enzymes and regulating cellular GSH homeostasis, phytochemical treatment allows the opportunity for enhanced antioxidant protection over that available by small molecular weight redox active compounds like vitamins C and E. The upregulation of a battery of ARE-regulated enzymes provides the potential for an effective means of bolstering antioxidant defenses against the diseases associated with redox dysregulation while avoiding the prooxidant actions of direct antioxidants.

Protandim Treatment Affords Protection in Cardiomyocytes

In addition to inducing phase II enzymes and activating Nrf2, cardiomyocytes treated with Protandim demonstrated attenuated oxidant-induced cell death. Nuclear condensation assessment indicated that phytochemical pretreatment resulted in protection against hydrogen peroxide induced apoptosis when compared to hydrogen peroxide without Protandim pretreatment. Interestingly, no significant differences were found between the three concentrations 50, 75, and 100 µg/mL. Presumably due to the synergy of the phytochemicals, the combination of phytochemicals in this investigation can protect cardiomyocytes from hydrogen peroxide induced oxidative insult at lower concentrations, even though maximum induction of phase II enzymes is observed with higher concentrations.

Oxidant stress is known to activate Nrf2 and induce upregulation of phase II enzymes [23], providing a compensatory response. However, often the antioxidant response elicited by ROS cannot overcome the initial damage imparted by the oxidative insult. Our data suggest Protandim treatment can induce a more robust endogenous antioxidant response than mild oxidant stress, and could be utilized preemptively to increase phase II enzyme expression so that the subsequent oxidative challenge is less damaging. The synergistic and cytoprotective properties of the combination of phytochemicals and their superior activation of Nrf2 compared

to oxidants and individual phytochemicals, suggest that phytochemical therapy could be effectively used to treat chronic diseases with oxidative stress components.

Concluding Remarks

The phytochemicals in Protandim were found to be a novel inducer of phase II antioxidant enzymes, to activate Nrf2, the “master regulator” of cellular defense mechanisms and protect cardiomyocytes against hydrogen peroxide induced oxidative stress. The protection afforded by the phytochemicals was dependent on Nrf2 activation, as knockdown of Nrf2 abolished the protective effects. These results support the use of phytochemicals in protection of cardiac myocytes against oxidant stress, and suggest their potential use in treatment of cardiovascular disease. It remains to be determined *in vivo* if phytochemical Nrf2 activators hold promise for prevention and treatment of oxidative stress associated with chronic human diseases.

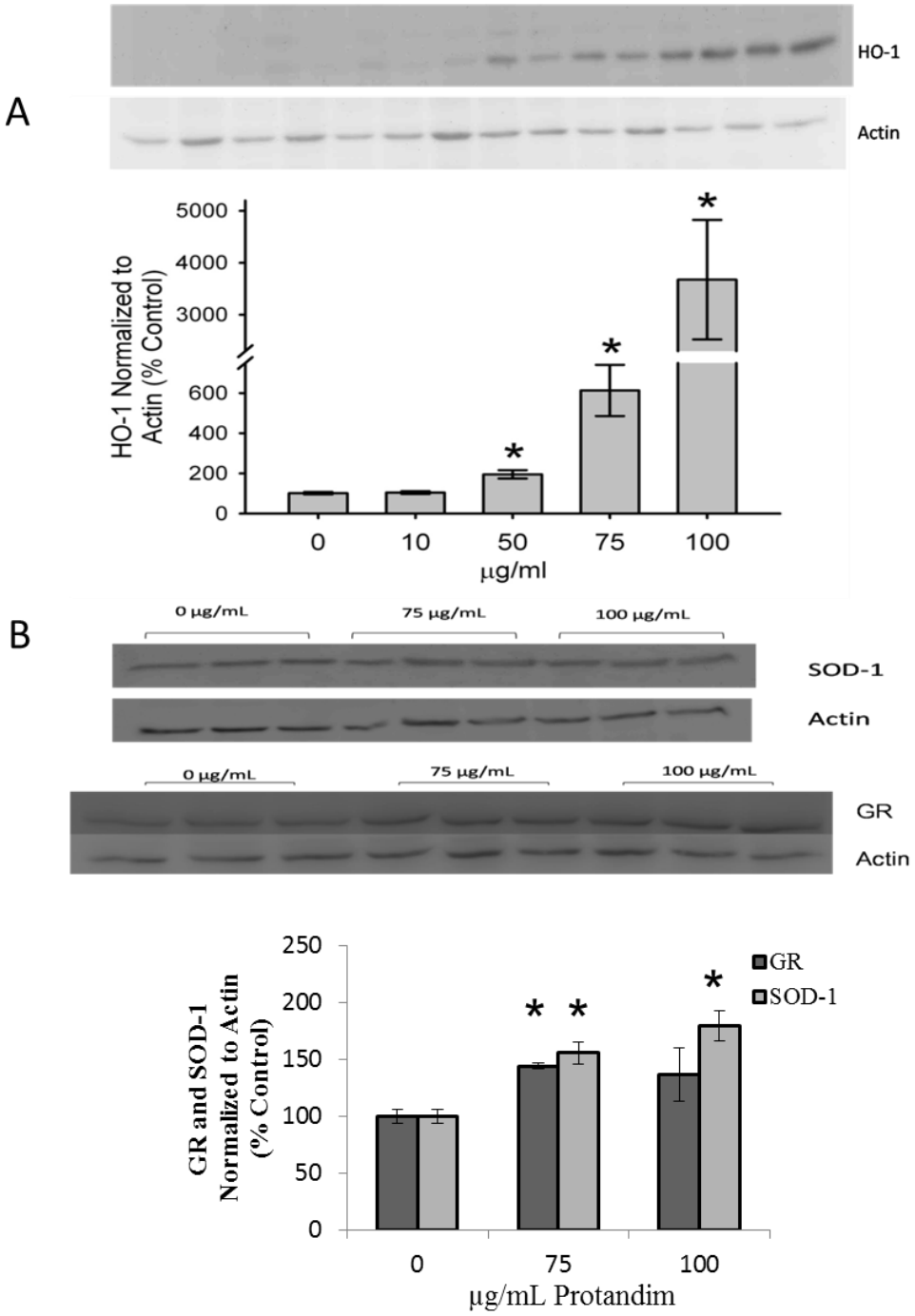


Figure 1.1. Treatment with phytochemicals upregulated the expression of phase II enzymes in a concentration dependent manner. A. 12 hours of Protandim treatment significantly upregulated HO-1 expression at 50, 75, and 100 µg/mL, with greatest induction of 300 fold over control (0 µg/mL) observed with 100 µg/mL. B. 12 hours of phytochemical treatment significantly upregulated expression of SOD-1 and GR. *p<0.05 compared to control (0 µg/mL). n=9 in each condition

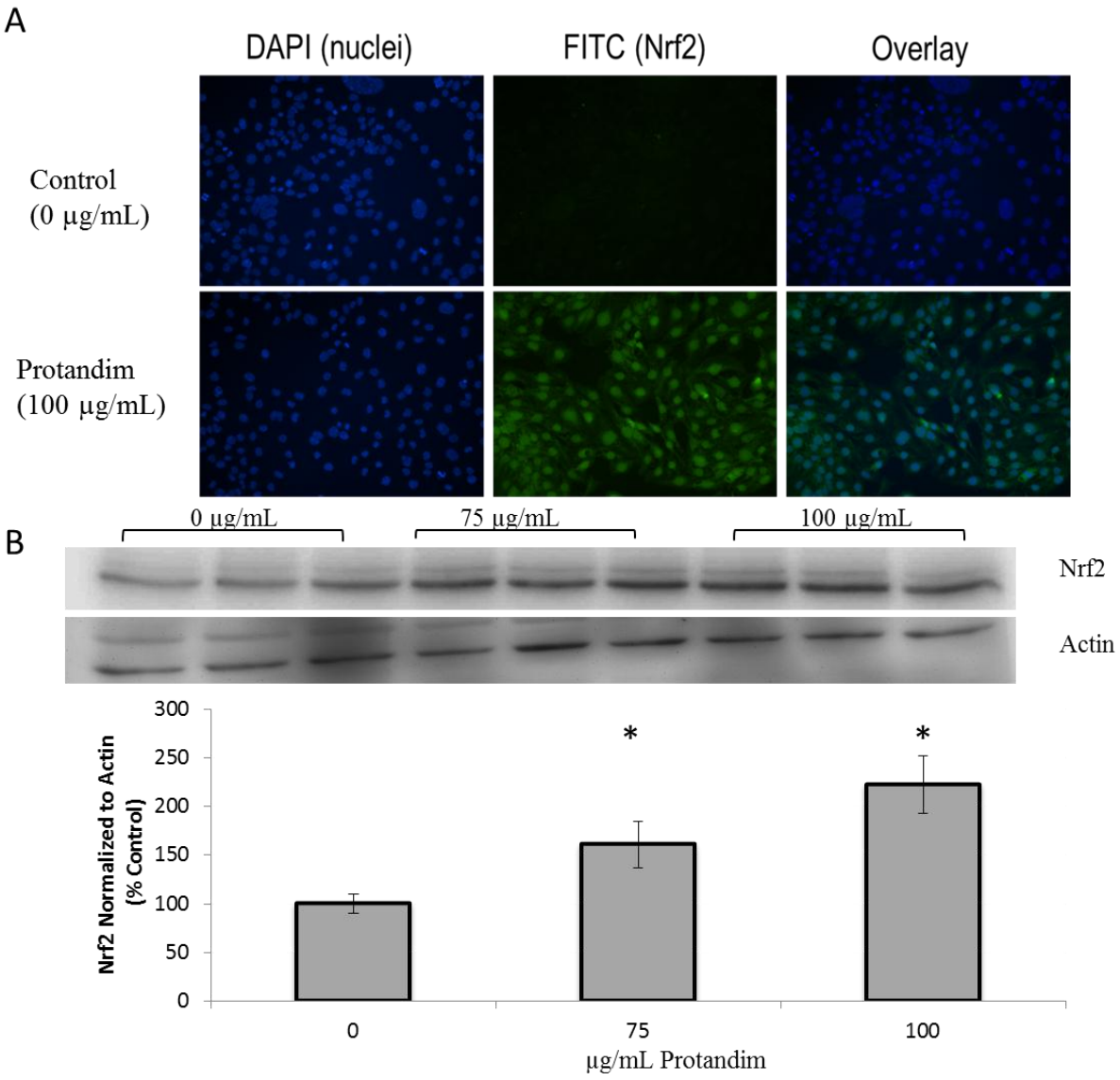


Figure 1.2. Phytochemical treatment activated Nrf2. A. HL-1 cells were treated with Protandim for 1 hour and incubated with anti-Nrf2 antibody to assess Nrf2 nuclear accumulation. DAPI identifies myonuclei, and FITC identifies Nrf2. B. Long term treatment (48 hours) with Protandim upregulated the expression of Nrf2. HL-1 cardiomyocytes were treated with 0, 75, and 100 µg/mL of Protandim, and Nrf2 protein in the whole cell lysate was normalized to actin. *p<0.05 compared to control (0 µg/mL). n=9 in each condition.

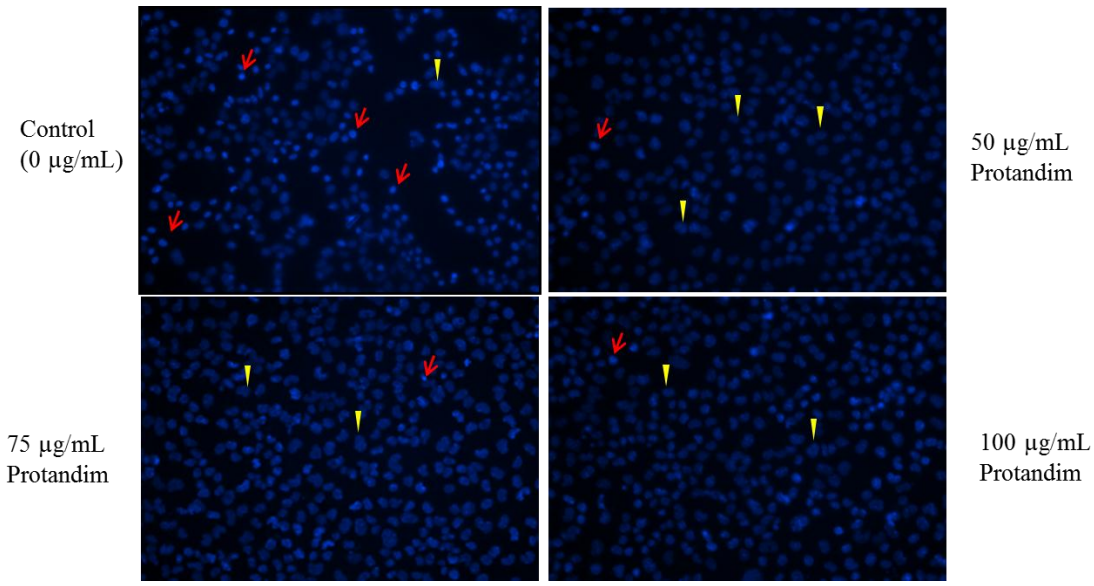


Figure 1.3. Pre-treatment with 50, 75, and 100 µg/mL of Protandim protected cardiomyocytes against oxidant-induced death. Phytochemical treatment was administered for 12 hours prior to H_2O_2 insult, and apoptosis was assessed by nuclear condensation. Red arrows identify condensed nuclei that have undergone apoptosis, and yellow triangles identify healthy nuclei that were resistant to the oxidant stress.

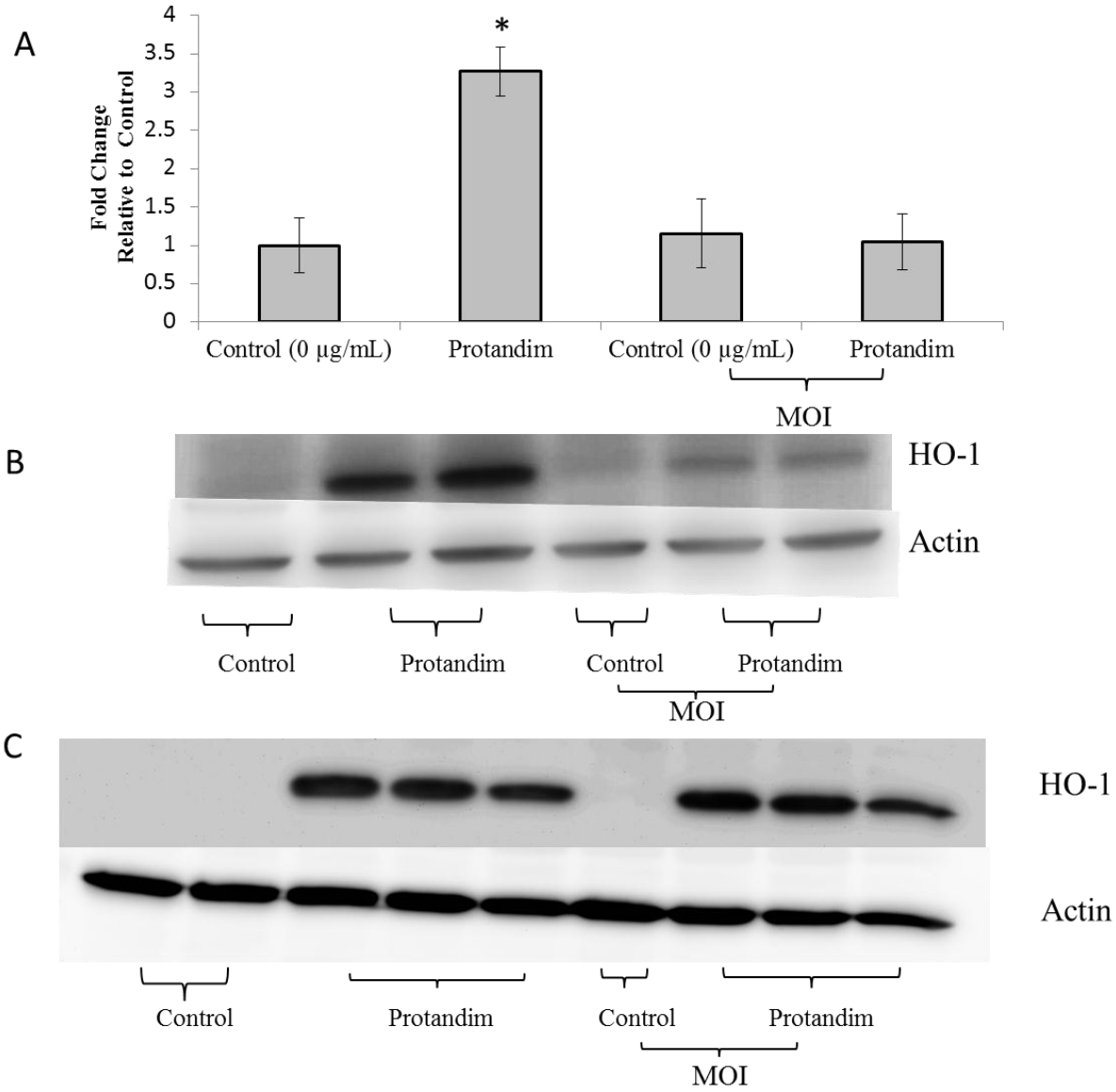


Figure 1.4. Lentiviral shRNA silencing of Nrf2 abolished Protandim-induced upregulation of Nrf2 and phase II enzymes. A. Low-density (~50% confluence) HL-1 cardiomyocytes were transduced with an MOI of 1, followed by an 8 hour 100 µg/mL Protandim treatment. B. Silencing of Nrf2 abolished phytochemical-induced upregulation of HO-1 protein expression. Following transduction of shRNA, HL-1 cells were treated for 12 hours with 100 µg/mL Protandim and HO-1 was verified by western blotting, with actin as a loading control. C. HO-1 induction with phytochemical treatment was maintained with control shRNA sequence. *$p<0.05$ compared to all other conditions.

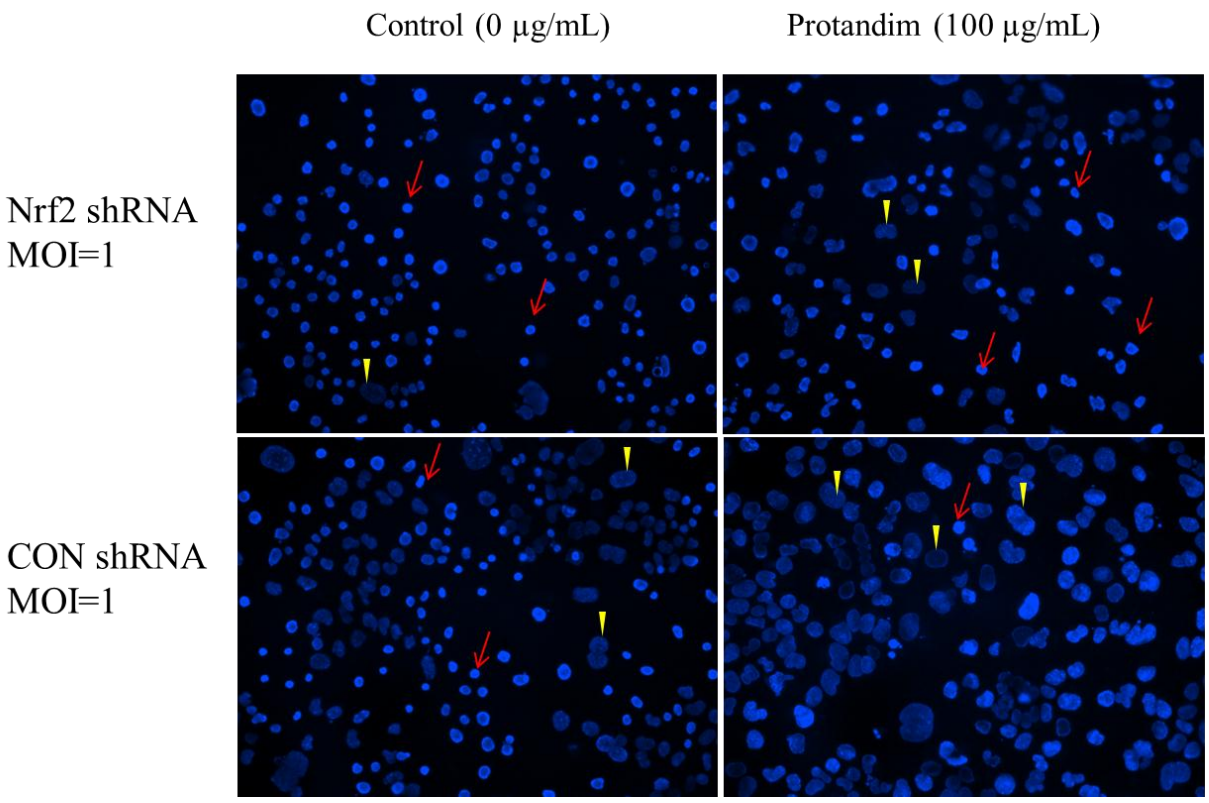


Figure 1.5. Lentiviral knockdown of Nrf2 abolished protection against oxidant stress by Protandim treatment. Transduced cells were treated with 100 µg/mL Protandim or control (0 µg/mL) for 12 hours, followed by a 4 hour 1.25 mM H_2O_2 insult. Apoptotic nuclei were identified by nuclear DAPI condensation and are identified by red arrows, while healthy nuclei are identified with yellow triangles. Protection remained when control shRNA was transduced.

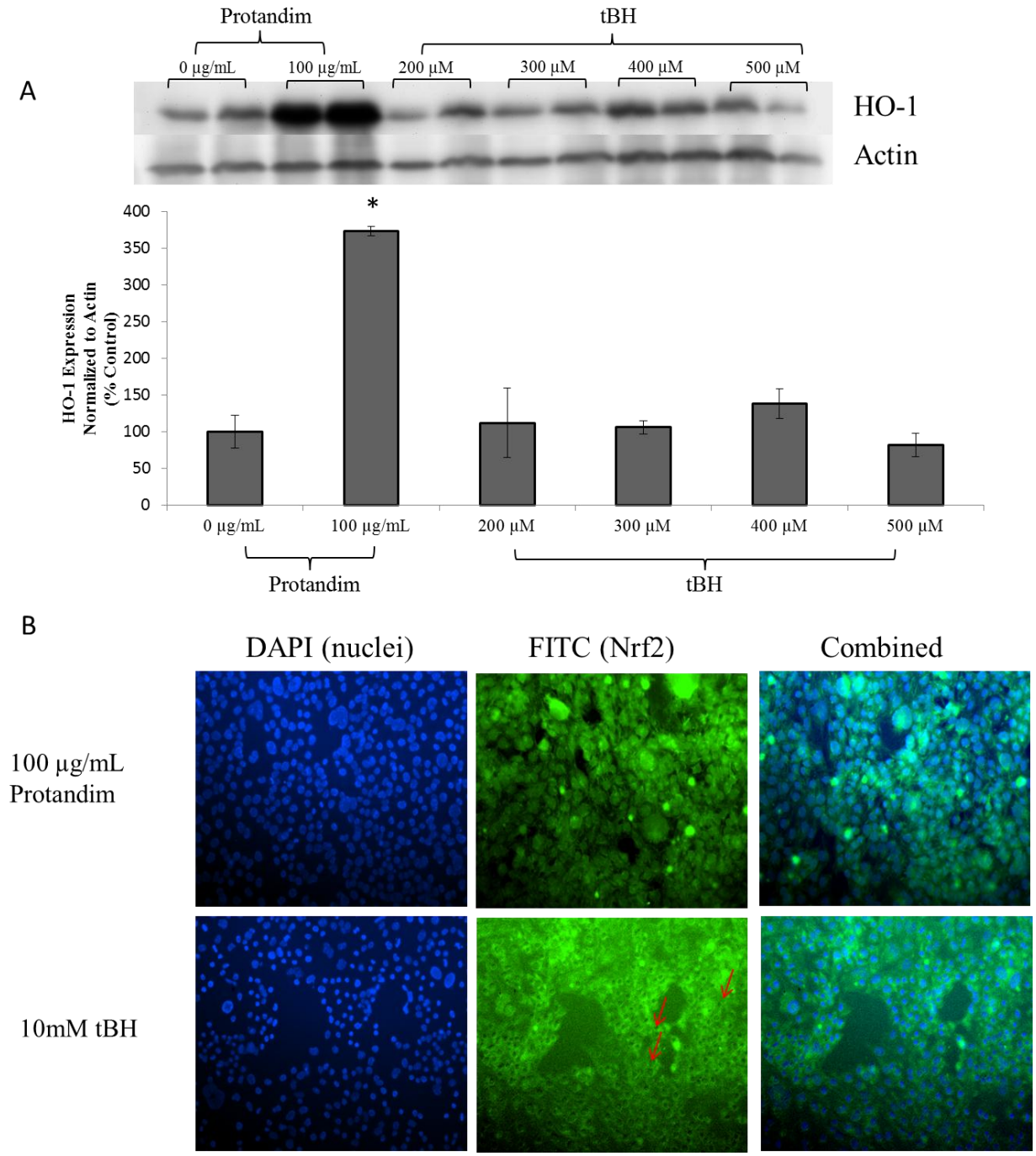
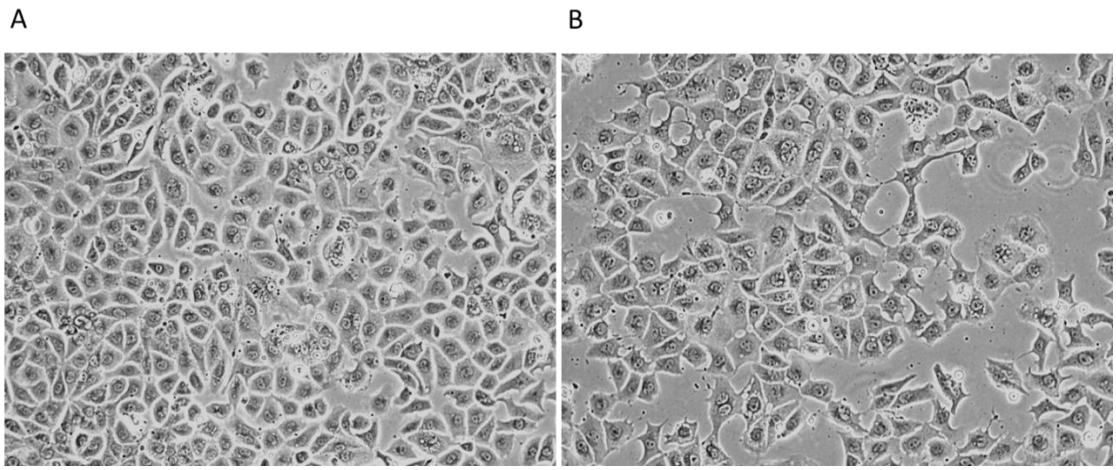
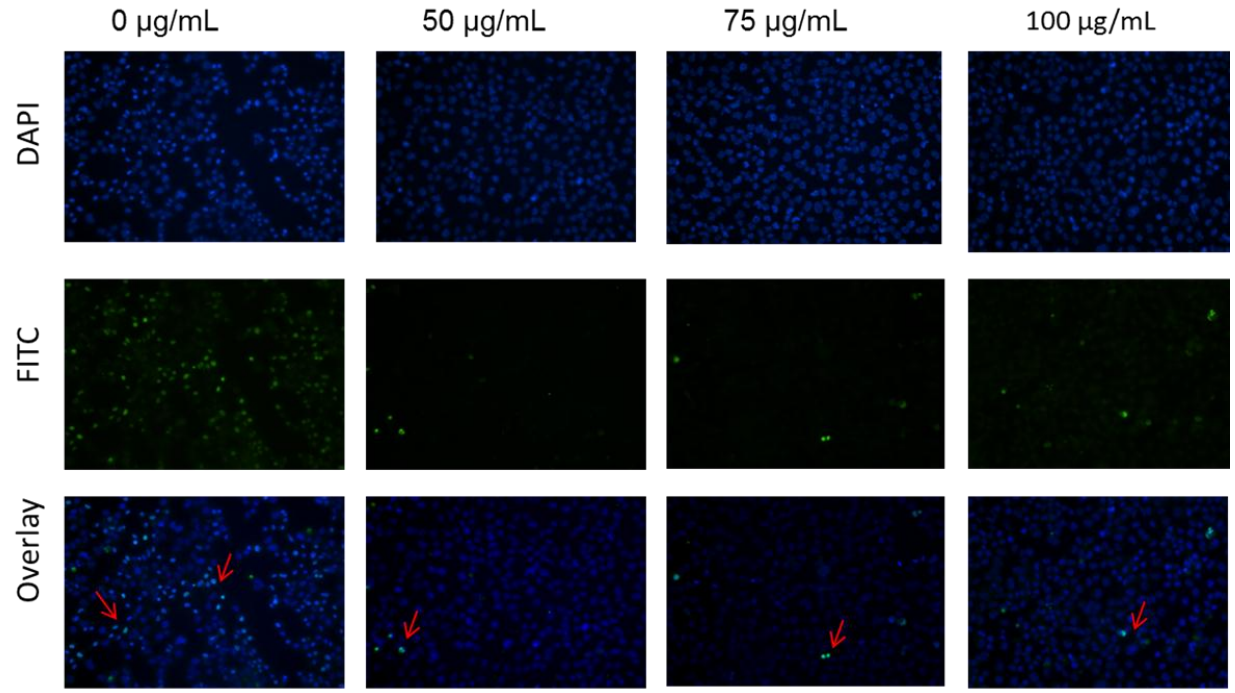


Figure 1.6. Upregulation of phase II enzymes and activation of Nrf2 by Protandim treatment was more robust than by mild oxidant stress. A. HL-1 cardiomyocytes were treated for 12 hours with 100 µg/mL of Protandim, or for 12 hours with varying concentrations of tBH. HO-1 expression was normalized to actin, and expressed as a percent of the 0 µg/mL control. n=2 in each condition. B. Oxidant stress did not activate Nrf2 and induce nuclear accumulation. While 2 hour treatment with Protandim resulted in nuclear accumulation of Nrf2, 2 hours of 10mM tBH insult did not induce nuclear accumulation, but tended to promote nuclear exclusion and cytosolic enrichment. Arrows identify nuclear exclusion.



Supplemental Figure 1.1. HL-1 cardiomyocytes treated with ethanol vehicle (A) and 100 µg/mL phytochemicals (B) maintain normal morphology and grow to confluency.



Supplemental Figure 1.2. To investigate whether phytochemical pretreatment affords protection against apoptosis, tdt dUTP nick end labeling (TUNEL) was carried out. HL-1 cells were cultured to confluence on cover slips, treated with phytochemicals or ethanol vehicle for 12 hours and then exposed to 1.25 mM hydrogen peroxide for 4 hours. The cells were then washed with PBS and fixed in 10% formalin for 45 minutes at room temperature. Fixed cells were gently washed with PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for two minutes. Samples were then incubated with TUNEL reaction mixture, label (FITC-labeled dUTPs) and enzyme (terminal deoxynucleotidyl transferase) solution for 60 minutes at 37˚C. Following washes with PBS, samples were mounted with 25µL DAPI mounting medium and images viewed using a fluorescence microscope (Nikon TE2000) and Metamorph software version 7.5 (Universal Imaging Corporation). DAPI was used to identify nuclear condensation, which was verified with FITC(+) overlay.

CHAPTER III- MANUSCRIPT II

Mechanisms of phytochemical-induced Nrf2 activation

Summary

Cardiovascular disease (CVD) is associated with increased production of reactive oxygen species (ROS). Enhanced endogenous antioxidant defenses have been proposed as a mechanism for regulating redox balance, thus protecting the heart against oxidant stress and CVD. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcriptional regulator of phase II antioxidant enzymes, and activation of Nrf2 has been suggested to be an important step in attenuating the oxidative stress associated with CVD. Activation of Nrf2 is complex, involving many steps of regulation including protein stability, phosphorylation of Nrf2 by upstream kinases, and attenuated proteasomal degradation, amongst others. Our group has previously shown that treatment with a well-defined combination of phytochemicals activates Nrf2 and protects cardiac myocytes against oxidant-induced death in a Nrf2-dependent manner. However, the mechanism of phytochemical-induced Nrf2 activation has remained elusive. The purpose of these experiments was to assess potential mechanisms of Nrf2 activation by phytochemical treatment. Treatment with the phytochemicals significantly increased expression of Nrf2 stabilizer p21. No changes were observed in Keap1 or p62 protein expression, suggesting autophagic degradation of Keap1 is not the mechanism of Nrf2 activation. mRNA half-life was unchanged by phytochemical treatment. Kinase signaling cascades were also not activated with phytochemical treatment. Identification of the mechanism of Nrf2 action by phytochemical treatment remains elusive; however, identification of this mechanism will facilitate development of treatments against oxidant stress and CVD.

Introduction

Cardiovascular disease (CVD), the leading cause of death and disability in industrialized countries, is associated with increased production of reactive oxygen species (ROS) [6]. One promising approach for regulating redox balance in CVD is via upregulation of endogenous networks of antioxidants [22]. The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is the "master regulator" of cellular antioxidant defenses, and thus represents a robust means to combat the redox imbalance associated with CVD.

Nrf2 is a member of the basic leucine zipper transcription factor family and controls basal and inducible expression of more than 200 genes [25]. Nrf2 is remarkably conserved across species, both in structure and in function, suggesting an integral role of Nrf2 in detoxification processes and mitigating oxidative stress. Under normal conditions, Nrf2 is sequestered in the cytoplasm by its involvement in an inactive complex with Kelch-like ECH-associated protein 1 (Keap1). Keap1, an actin-binding protein, binds to Nrf2 and targets it for ubiquitination and degradation by the 26S proteasome system, resulting in basal low-level expression of Nrf2 target genes. Under basal conditions, the Nrf2 protein has a half-life of approximately 15-20 minutes [67]. Upon exposure to oxidants, cysteine residues on the Keap1/Nrf2 complex become oxidized, altering the structure of Keap1. The Keap1/Nrf2 complex then dissociates, allowing Nrf2 to escape ubiquitination and proteasomal degradation [31]. Modification of the Keap1 cysteine residues stabilizes Nrf2, facilitating its translocation to and accumulation within the nucleus. After nuclear translocation, Nrf2 forms heterodimers with Maf and Jun bZip transcription factors, which bind to the 5'-upstream cis-acting regulatory sequence known as the antioxidant or electrophile response element (ARE/EpRE) and induces transcription of phase II antioxidant enzymes. The ARE sequence contains a core 5'-G(/A)TGAC(/G)nnnGCA(/C)-3' *cis*-acting element shared among Nrf2-regulated genes [68].

Accumulating evidence suggest that Nrf2 activators can act by additional mechanisms apart from direct modification of Keap1 residues. Sulfurophane and resveratrol, phytochemicals known to activate Nrf2, activate various kinase signaling cascades that phosphorylate Nrf2 , allowing Nrf2 to release from Keap1 [23]. Kinases implicated in the phosphorylation and subsequent activation of Nrf2 include phosphatidylinositide 3-kinase (PI3K), extracellular signal-regulated kinase mitogen-activated protein kinase (MEK/ERK), p38 mitogen-activated protein kinase (p38MAPK), c-Jun N-terminal kinases (JNK), and protein kinase C [23]. In addition, besides Keap1, Nrf2 binds to a variety of other proteins, which compete to stabilize or destabilize Nrf2 [69]. For example, the cell cycle regulator p21 competes with Keap1 for Nrf2 binding, allowing Nrf2 to escape Keap1-mediated proteasomal degradation and translocate to the nucleus [70]. The selective autophagy cargo receptor p62 also interacts in the Keap1-Nrf2 complex, and promotes Nrf2 activation by selective autophagic degradation of the Keap1 protein [71]. Thus, various Nrf2 activators result in the translocation and accumulation of Nrf2 within the nucleus and stimulate transcriptional activation of ARE-responsive enzymes. However, regulation of Nrf2 activation is complex, with various transcriptional, post-transcriptional, and localization signals mediating Nrf2 antioxidant programs. Therefore, many regulatory steps of Nrf2 activation are possible, are still poorly described, and likely differ between Nrf2 activators.

Our group has previously shown that treatment of cultured cardiac myocytes with a well-defined phytochemical combination marketed as Protandim® robustly activates Nrf2 and protects cardiac myocytes and human coronary artery endothelial cells against oxidant-induced cell death in a Nrf2-dependent manner [36, 72]. Interestingly, we also found phytochemical treatment to be a more robust activator of Nrf2 than oxidant treatment, supporting the use of the phytochemicals as a potential treatment to protect heart cells against an oxidative challenge as well as a model by which to understand Nrf2 signaling [72]. However, the mechanism(s) of

phytochemical-induced Nrf2 activation have remained elusive. Therefore, the purpose of the current study was to characterize the mechanisms of phytochemical activation of Nrf2, and the potential signaling proteins involved in Nrf2 activation. We hypothesized that Nrf2 would be activated by upstream mitogen-activated protein kinase (MAPK) signaling, and that the Nrf2 protein would be stabilized through interaction with Nrf2-Keap1 binding partners. We also assessed transcriptional regulation of Nrf2 through analysis of mRNA stability, and hypothesized this would not be a mechanism of enhanced Nrf2 nuclear accumulation. Understanding how Nrf2 is stabilized and activated by interactions with other proteins could enhance the therapeutic potential of Nrf2 activation in CVD and other chronic diseases.

Methods

Materials

Unless otherwise noted, all reagents and chemicals were purchased from Sigma-Aldrich (St Louis, MO) and ThermoFisher Scientific (Pittsburg, PA). Antibodies were purchased from Santa Cruz (Dallas, TX) (Nrf2 sc722, p21 sc6246, actin sc1616), Abcam (Cambridge, MA) (Keap1 ab66620, p62 ab56416), and Cell Signaling (Danvers, MA) (phospho Erk1/2 (Thr202/Tyr204) #4370, Erk1/2 #4695, p38MAPK #9212, phospho p38MAPK (Thr180/Tyr182) #9211). PCR reagents and pre-validated primers and probes (Nrf2: Mm00477784_m1; 18S ribosomal RNA: Mm03928990_g1) were purchased from Applied Biosystems (Grand Island, NY). Protandim was provided as a generous donation from Life Vantage (Sandy, UT).

Culture and treatment of cardiac myocytes

A cardiomyocyte line (HL-1) derived from murine atrium was a generous gift from Dr. William Claycomb. Cells (passages 85-100) were maintained in Claycomb supplemented medium with 10% fetal bovine serum, 100 U/mL:100 µg/mL penicillin/streptomycin, 2 mM L-

glutamine, and 0.1 mM norepinephrine. Cells were plated on 0.5% fibronectin in 0.02% gelatin coated plates, and were grown in 37˚C, 5% CO_2 humidified environment.

HL-1 cells were grown to confluence and treated with the phytochemical extract (75 and 100 µg/mL), concentrations previously shown to activate Nrf2 in this cell type, or with ethanol vehicle (control) [72]. Actinomycin D was added at a final concentration of 3 µg/mL and cycloheximide (CHX) at 10 µg/mL, concentrations shown in preliminary experiments to inhibit the transcription and translation in this cell type.

Immunoblotting

After phytochemical treatment, cardiomyocytes were washed two times with ice cold PBS and lysed with RIPA buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), and protease and phosphatase inhibitors. Samples were sonicated and lysate protein concentration was measured by using the bicinchoninic acid assay. Diluted samples containing equal amounts of protein were prepared in Laemmli Sample Buffer and 2-mercaptoethanol and heat denatured for 5 minutes at 98˚C. Proteins were resolved on a 10% Tris/glycine SDS-polyacrylamide gel in running buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. Proteins were transferred to a nitrocellulose membrane for 75 minutes at 100 V using a transfer buffer containing 25 mM Tris, 192 mM glycine, 0.02% SDS, and 20% methanol, pH 8.3. Non-specific proteins were blocked by incubation of membrane in Superblock (ThermoScientific; Rockford, IL) for 1 hour at room temperature. Membranes were incubated overnight in primary antibody at 4˚C followed by secondary antibody conjugated to HRP for 1 hour at room temperature with 30 minute washes of TBST between primary and secondary incubations. Proteins were detected with chemiluminescence reagents and imaging followed by densitometric analysis using VisionWorks software. Membranes were probed for actin to verify equal loading of protein.

Real Time RT-PCR

Total RNA was extracted from 60 mm culture plates using standard TRIzol methods. RNA concentration and protein contamination were determined using spectrophotometry. RNA was reversed transcribed and cDNA was amplified using target sequence primer-probe reagents. PCR conditions were as follows: hot start (15 minute at 95°C) followed by 40 cycles of denaturing and annealing (15 s at 95°C, 1 minute at 60°C). The relative quantification of Nrf2 was normalized to an endogenous control (18s rRNA) and calculated as a percent of the initial transcript. The data were log transformed, linear regression was performed, and half-life of the transcript calculated from the equation $t_{1/2}=\ln 2/k_{decay}$ [73].

Immunofluorescence

HL-1 cardiomyocytes were grown to confluence on cover slips and treated with phytochemical extract or ethanol vehicle for 1 hour with or without cycloheximide. The cells were then washed with PBS and fixed in 10% formalin for 30 minutes. Fixed cells were gently washed with PBS and permeabilized with acetone for 15 minutes at 4°C. The samples were gently washed three times and incubated in blocking solution (0.05% goat serum in 5% BSA) for one hour at room temperature. After three gentle washes, the samples were incubated for one hour at room temperature with Nrf2 primary antibody (1:100), followed by the FITC conjugated secondary antibody (1:200) for 45 minutes. The slides were washed and mounted with DAPI containing mounting medium for nuclear identification. The images were viewed using a fluorescence microscope (Nikon TE2000) and Metamorph software version 7.5 (Universal Imaging Corporation; Sunnyvale, CA).

Statistical Analyses

Significance was set *a priori* at $p<0.05$. Data were analyzed by one-way ANOVA followed by LSD post hoc analyses where appropriate (SPSS Version 17). When data did not

meet assumptions for equal error variance, log transformations were performed prior to ANOVA. Data are presented as means ± SEM.

Results

Treatment of cardiac myocytes with Protandim does not influence Keap1 protein expression or result in attenuated expression of Keap1 binding partner and selective autophagy cargo receptor p62.

To determine the roles of Keap1 and p62 in phytochemical Nrf2 activation, p62 and Keap1 protein expression were assessed via immunoblotting. Keap1 protein content did not decrease with 75 or 100 µg/mL phytochemical treatment compared to control (0 µg/mL) at 12 hours (Figure 2.1A). Further, p62 protein content did not decrease with 75 or 100 µg/mL phytochemical treatment compared to control (0 µg/mL) (Figure 2.1B).

p21 protein expression is upregulated by phytochemical treatment.

To determine whether p21 may be involved in Protandim-induced Nrf2 activation, p21 protein expression was examined with phytochemical treatment. Treatment of cultured cardiac myocytes with the phytochemical combination significantly increased p21 expression compared to control at both 75 and 100 µg/mL, with nearly a two-fold increase over control with 100 µg/mL Protandim (Figure 2.2).

Inhibition of translation does not attenuate Protandim-induced nuclear accumulation of Nrf2 protein.

Cardiac myocytes were treated with the phytochemical extract plus the translational inhibitor CHX to assess if the accumulation of Nrf2 protein is dependent on the translation of new Nrf2 protein. Simultaneous treatment of Protandim plus CHX for 1 hour did not attenuate Protandim-induced nuclear accumulation (Figure 2.3).

Treatment with the phytochemicals in Protandim does not influence Nrf2 mRNA stability.

Nrf2 mRNA half-life was assessed to determine whether Protandim treatment stabilizes the Nrf2 transcript. HL-1 cardiac myocytes were treated for 8 hours with Protandim, followed by addition of actinomycin D to inhibit transcription. Decay of Nrf2 expression was calculated using ribosomal 18s RNA as a reference gene. Nrf2 mRNA half-life was calculated to be between 2 and 4 hours following Protandim treatment and 2 and 4 hours for control (Figure 2.4), with no significant difference between the rates of decay between treatments.

Involvement of MAP kinase signaling in Protandim-induced Nrf2 activation

MAPK signaling was assessed to determine if activation of p38MAPK and ERK are involved in Protandim-induced Nrf2 activation. Treatment with 100 µg/mL phytochemical extract for 1 hour did not activate ERK (Figure 2.5A), nor did ERK show increased activation with 4 hours treatment (Supplemental Figure 2.1A). One hour phytochemical treatment suppressed p38MAPK (Figure 2.5B), a suppression which was attenuated by four hours (Supplemental Figure 2.1B).

Discussion

Our group has previously shown that the phytochemicals in Protandim are a robust Nrf2 activator, and that activation of Nrf2 protects heart cells against oxidant stress [72]. It is becoming increasingly apparent that the regulation of Nrf2 activation is complex, with the involvement of upstream signaling cascades and various proteins that compete with Keap1 to stabilize/destabilize Nrf2. Understanding the mechanisms of Nrf2 activation could facilitate therapeutic potential of Nrf2 in CVD. Here, we show that alterations in p62 and Keap1, enhanced Nrf2 mRNA stability, and de novo Nrf2 protein synthesis are not responsible for Protandim-induced Nrf2 activation. Upregulation of p21 protein may be involved in Protandim-induced Nrf2 activation, but future studies are warranted to identify what effect increased p21

expression has on Nrf2 stabilization. We found p38MAPK may be involved in Nrf2 activation, but the involvement of upstream kinases warrants future investigation to identify which signaling cascades activate Nrf2.

Mechanisms of Nrf2 nuclear translocation

The best described mechanism of Nrf2 activation is its activation by oxidants and electrophiles. Under these conditions, electrophiles modify Keap1 cysteine residues, resulting in a conformational change of Keap1 and subsequent release of Nrf2 from Keap1 binding sites. However, the specifics of Nrf2 release from Keap1 are conflicting. Some studies say electrophiles dissociate the Nrf2-Keap1 complex [74, 75], while others cite the dissociation of Keap1 from the ubiquitin ligase Cul3, thus preventing Nrf2 ubiquitination and degradation [76, 77]. Even others indicate that under electrophilic stress, ubiquitination of Keap1 increases, thus freeing Nrf2 from proteasomal degradation and allowing its translocation to the nucleus [78]. Despite a lack of consensus on the mechanisms of Nrf2-Keap1 dissociation, these studies agree that upon cellular oxidant stress, Nrf2 is released from Keap1, accumulates in the nucleus, and activates its transcriptional program.

Phytochemical-induced Nrf2 activation is even less characterized than oxidant Nrf2 activation. Several studies cite activation of upstream kinase signaling cascades as the mechanism for Nrf2 escape from Keap1 [79], as treatment with sulforaphane and resveratrol results in activation of PI3K and the MAPK ERK [23]. It is thought that the phosphorylation of Nrf2 by these upstream kinases results in a conformational change of the Keap1-Nrf2 complex facilitating Nrf2 release and nuclear accumulation. Whether Protandim activates similar upstream signaling cascades is unknown. Here, we show that ERK/MEK is not involved in Protandim-induced Nrf2 activation. Surprisingly, we show that p38MAPK is suppressed with Protandim treatment. While we initially hypothesized that MAP kinases would phosphorylate and activate Nrf2, a previous report suggests that p38MAPK actually phosphorylates and

inhibits Nrf2 via its association with Keap1 [80]. In this study, activation of p38MAPK isoforms in human hepatoma cells phosphorylated Nrf2, increased its association with Keap1, and suppressed Nrf2 nuclear translocation [80]. Additional upstream kinases implicated in inhibition of Nrf2 include tyrosine kinase Fyn and glycogen synthase kinase 3β (GSK3β). GSK3β activates Fyn, stimulating its nuclear accumulation, and resultant nuclear Nrf2 export and degradation [81]. The role of these inhibitory kinases in Protandim-induced Nrf2 activation are unknown, as is the involvement of activating kinases such as JNK, PI3K, and PKC. However, it is apparent that Nrf2 escapes Keap1 and degradation, since the transcription factor accumulates within the nucleus within 15 minutes of phytochemical treatment [82]. Upstream kinases likely mediate both activation and inhibition of Nrf2 nuclear accumulation. Future studies should be undertaken with specific kinase inhibitors in order to elucidate which are necessary for Protandim-induced Nrf2 activation.

Nrf2-Keap1 binding partners

Recently, an increasing number of Keap1-Nrf2 binding partners have been identified. Here, we assessed the contribution of two proteins involved in the Keap1-Nrf2 complex: the selective autophagy cargo receptor p62 and the cyclin dependent kinase p21. While it was well established that p21 contributes to cytoprotection against oxidant stress, the relationship between Nrf2 and p21 was not elucidated until 2009 when Chen and colleagues identified that the two proteins associate *in vitro* [70]. Chen et al. found that Nrf2 and p21 expression both increase in response to the oxidant tert-butyl hydroquinone (tBHQ). Further elegant reciprocal immunoprecipitation studies indicated that p21 competes with Keap1 for Nrf2 binding, thus stabilizing the Nrf2 protein [70]. Chen's group went on to show that this association occurs both during basal conditions, as well as upon administration of tBHQ. Upon oxidative stimuli, association between p21 and Nrf2 increases, likely due to increased expression of both proteins

in response to oxidant treatment. The increased association of p21 and Nrf2 increases the half-life of the Nrf2 protein, facilitating a more robust activation of ARE-responsive enzymes [70].

Here, we assessed p21 expression with phytochemical treatment and found a significant increase in p21 protein expression with Protandim treatment. Various questions still remain regarding this relationship. First, it remains to be determined what effect Protandim treatment has on the association between p21 and Nrf2, and whether an enhanced association between p21 and Nrf2 influences Nrf2 protein half-life. While we show that Nrf2 nuclear accumulation still occurs with treatment of protein synthesis inhibitor cycloheximide, it is unknown whether the Nrf2 protein is stabilized with phytochemical treatment, and whether p21 contributes to this stability. Increased p21 protein may imply more absolute Nrf2 stabilization, perhaps facilitating nuclear accumulation of Nrf2 protein. Second, the cellular localization of the p21-Nrf2 complex is incompletely understood. p21 contains a nuclear localization signal, thus allowing it to regulate cell cycle progression [83]. It is, therefore, feasible that the p21-Nrf2 complex could localize to the nucleus, facilitating stabilization of the Nrf2 protein at a position where it is poised to activate its transcriptional cytoprotective program.

We also assessed the role of p62 in Protandim-induced Nrf2 activation. While initially believed that autophagy, a form of cellular degradation, was non-selective, it has recently become apparent that autophagy can target specific proteins through the selective autophagy cargo receptor p62. Ectopic expression of p62 decreases Keap1 protein expression, thus activating Nrf2. Similarly, siRNA knockdown of p62 increases Keap1, thus decreasing Nrf2 protein content without corresponding changes in Nrf2 mRNA [84]. Thus, upregulation of p62 could facilitate the autophagic degradation of Keap1, thereby activating Nrf2. However, in the current study, with 12 hours of phytochemical treatment, we did not show any changes in p62 or Keap1 protein content. Thus, in the 12 hour context examined here, autophagic degradation of Keap1 cannot explain Nrf2 activation, and points to another mechanism for phytochemical-induced Nrf2 activation.

Additional mechanisms of phytochemical-induced Nrf2 activation

Many phytochemicals known to activate Nrf2 have electrophilic potential. Therefore, Protandim or its individual components may act as a mild oxidant and activate Nrf2 through modification of Keap1 cysteine residues. For example, epigallocatechin gallate (EGCG), one of the five phytochemicals in Protandim, stimulates the production of endogenous hydrogen peroxide, as well as generates oxidative products via its intracellular degradation [85]. These endogenous processes produce mild oxidant stress, resulting in Nrf2 activation [85]. This mild stress, with resultant upregulation of stress defenses, is an example of hormesis whereby a small amount of a stressful stimulus produces beneficial adaptations, as opposed to large amounts of stressful stimuli that damage cells and cellular components. Thus, it is plausible that the phytochemical combination in Protandim act in a hormetic manner, producing a mild stress, and a compensatory antioxidant response that prepares the cell for subsequent stressful stimuli. Additionally, while much research has focused on cysteine residues on Keap1, Nrf2 also has cysteine residues that can be modified, providing another possible mechanism for its regulation [86]. Li et al characterized a nuclear export sequence on Nrf2 which contains a reactive cysteine residue. Mutating this residue attenuated oxidant-induced nuclear translocation of Nrf2 [86]. If Protandim is redox-active, it is possible that it could act through redox-sensitive cysteine residues on Nrf2 or Keap1, thus facilitating its nuclear accumulation via a hormetic adaptation.

Preferential translation of Nrf2 during cell stress

Under unstressed conditions, cytosolic Nrf2 protein expression is low [87]. Therefore, it is difficult for existing cellular Nrf2 pools to account for the large increase in nuclear Nrf2 upon stress or phytochemical treatment. Since we show here that Nrf2 mRNA stability is unchanged by Protandim treatment, enhanced translation of cytosolic mRNA pools doesn't appear to explain the rapid Nrf2 nuclear accumulation either. De novo Nrf2 protein translation is an

important mechanism of rapid elevation of Nrf2 protein by oxidant stress [88]. A surplus of newly synthesized Nrf2 protein may overwhelm the ability of Keap1 to bind and repress it, allowing nuclear accumulation [87]. However, during periods of cell stress, canonical cap-dependent translation is inhibited [89]. Thus, it has been suggested that electrophilic compounds activate cap-independent translation of Nrf2, allowing preferential translation of the transcription factor [87, 88]. An internal ribosomal entry site (IRES) has been identified in the 5' untranslated region of the Nrf2 mRNA. The IRES permits redox-sensitive translation of Nrf2 and allows for increased polysomal loading under conditions of cell stress [90] where global protein synthesis is inhibited. It remains to be assessed whether other mechanisms of Nrf2 activation, such as phytochemical treatment, may also promote selective translation of Nrf2. However, the data shown here suggest that during global protein synthesis inhibition with cycloheximide, Nrf2 nuclear accumulation still persists. Therefore, the increase in Nrf2 nuclear protein must persist via a mechanism that does not require canonical cap-dependent translation, suggesting preferential translation of Nrf2 may occur via regulatory structures in its mRNA or via an unidentified mechanism.

Concluding Remarks

Activation of Nrf2 is a promising target for CVD therapy. Our group has previously shown that treatment with the phytochemicals in Protandim activates Nrf2 and protects heart and vascular endothelial cells against oxidant-induced stress. Here, we show that treatment with the phytochemicals in Protandim significantly increased expression of p21. No changes were observed in Keap1 or p62 protein expression, suggesting autophagic degradation of Keap1 is not the mechanism of Nrf2 activation. mRNA half-life was unchanged by phytochemical treatment. Kinase signaling cascades were also not activated with phytochemical treatment, however phytochemical treatment inhibited p38MAPK, and future directed studies should identify if this attenuates Nrf2 ubiquitination and degradation. Despite

characterization of Nrf2 binding partners, mRNA stability, and upstream signaling cascades, identification of the mechanism of Nrf2 action by phytochemical treatment remains elusive.

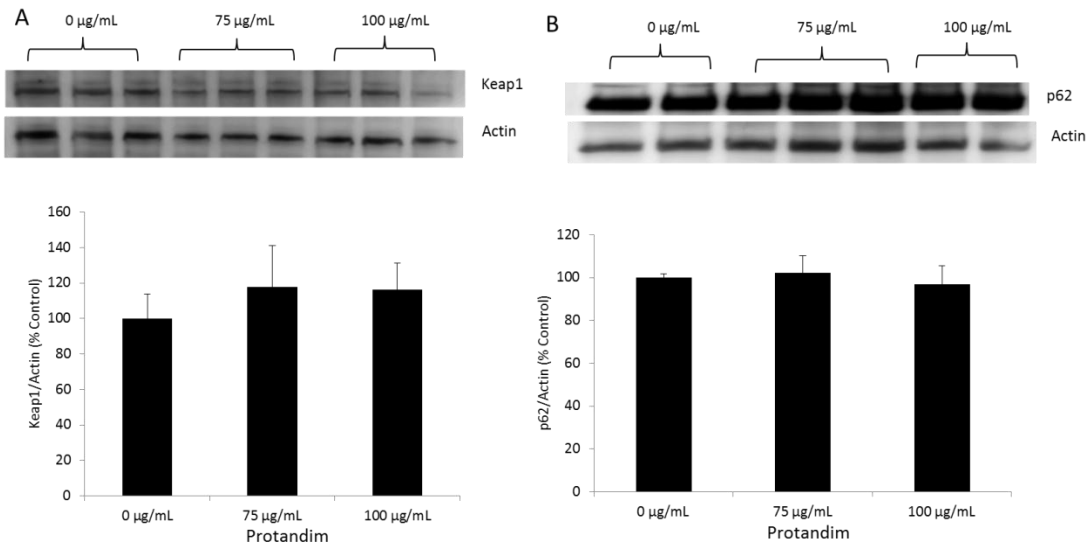


Figure 2.1. To determine if Protandim treatment results in changes in Keap1 protein expression, Keap1 and selective autophagy cargo p62 protein expression were assessed by western blotting. Treatment with 75 and 100 µg/mL Protandim for 12 hours did not significantly change Keap1 or p62 protein expression. Data were normalized to actin and expressed as a percent of control (0 µg/mL). n=9 in each condition for Keap1; n=3 in each condition for p62.

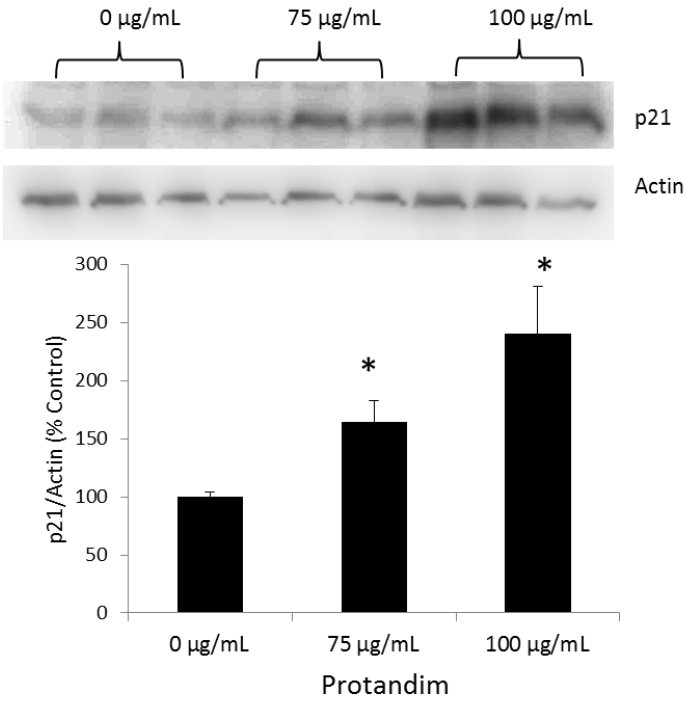


Figure 2.2. Treatment with the phytochemicals in Protandim upregulates the expression of p21 protein. HL-1 cardiac myocytes were treated for 12 hours with the phytochemical extract, and p21 was assessed by western blotting. Data were normalized to actin, and expressed as a percent of the untreated control. *$p<0.05$ compared to 0 µg/mL. n=9 in each condition.

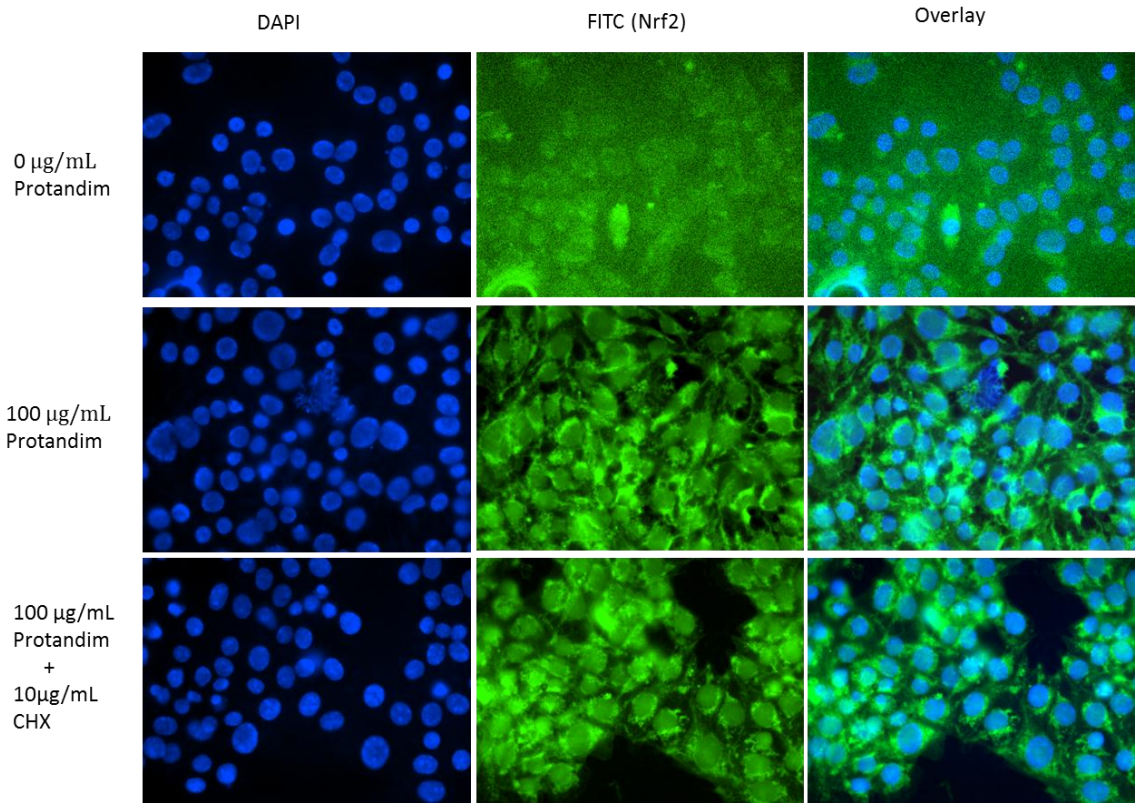


Figure 2.3. Inhibition of protein synthesis by cycloheximide (CHX) treatment does not prevent nuclear accumulation of Nrf2 protein. HL-1 cells were treated for 1 hour with the phytochemical extract (Protandim) with or without CHX. Nuclear accumulation was assessed by immunocytochemistry. DAPI (blue) identifies myonuclei, FITC (green) identifies Nrf2 protein. The overlay of the two fluorophores shows Nrf2 accumulation in the nucleus with Protandim treatment that persists with CHX. The experiment was run three separate times in triplicate, and representative images are presented.

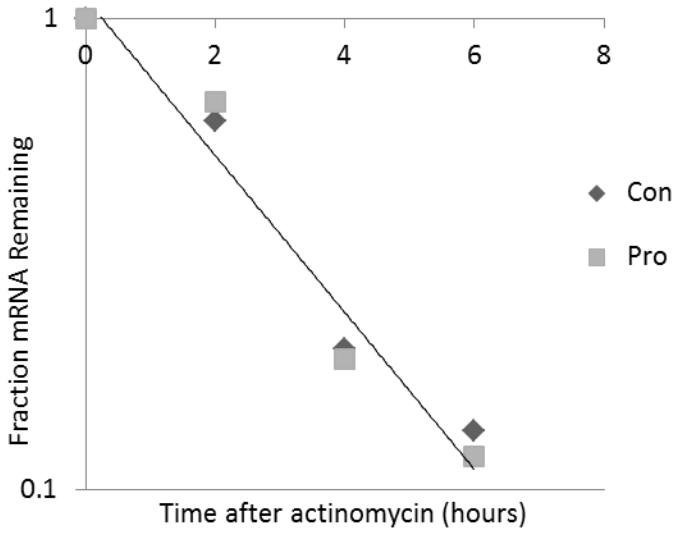


Figure 2.4. Treatment with the phytochemicals in Protandim does not alter Nrf2 mRNA stability. HL-1 cardiomyocytes were treated for 8 hours with the phytochemical extract (Pro) or vehicle (Con). The extract was removed, and actinomycin D was added to culture media. Nrf2 half-life was calculated from the percent remaining following addition of the transcriptional inhibitor. n=3 in each condition. The experiment was run 3 separate times in triplicate, and a representative image is shown. The calculated half-life of Nrf2 mRNA was between 2 and 4 hours for both Protandim and control, with no significant difference between treatment and control conditions.

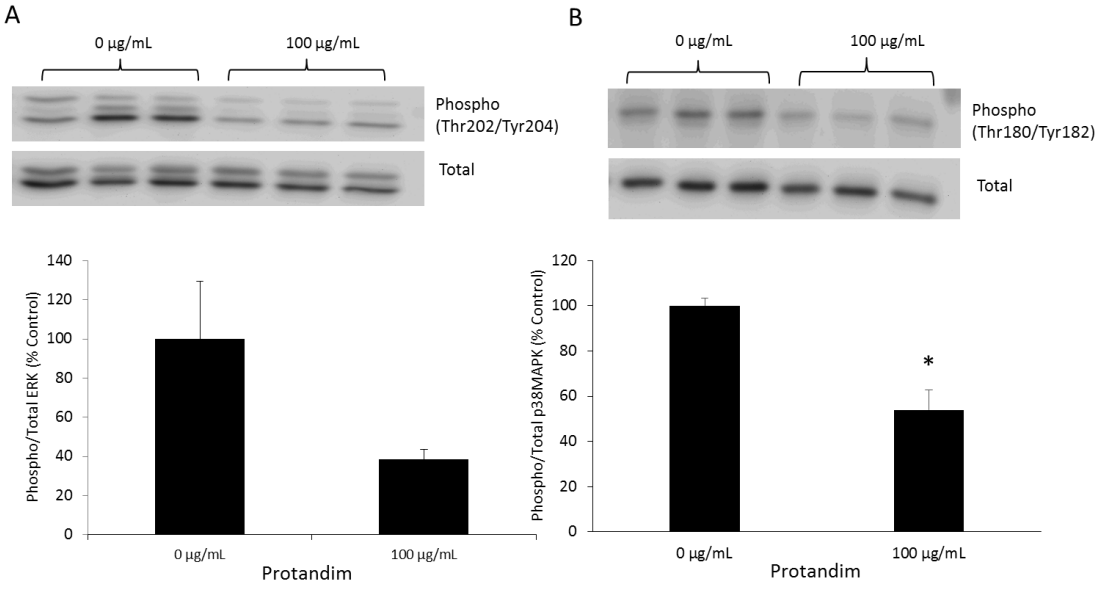
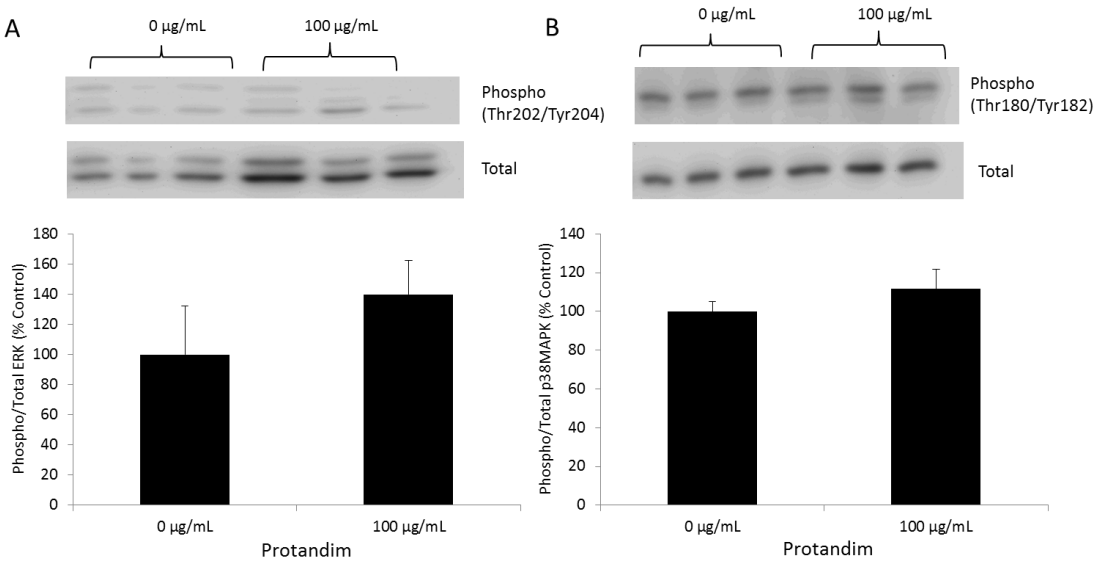


Figure 2.5. Involvement of MAPK in Protandim-induced Nrf2 activation. A. MAP kinase signaling pathway ERK is not activated with one hour Protandim treatment. B. One hour Protandim treatment blunts p38MAPK activity. MAPK were assessed by western blotting. A phospho/total ratio was calculated for each protein, and expressed as a percent of the untreated control. *p<0.05 compared to 0 µg/mL. n=3 in each condition.



Supplemental Figure 2.1. MAPK activation in Protandim-induced Nrf2 activation. A. MAP kinase signaling pathway ERK is not activated with four hours Protandim treatment. B. Four hour Protandim treatment does not activate p38MAPK activity. MAPK were assessed by western blotting. A phospho/total ratio was calculated for each protein, and expressed as a percent of the untreated control. n=3 in each condition.

CHAPTER IV- Manuscript III

Sexual dimorphism in Nrf2-regulated antioxidant defenses across rodent models of longevity

Summary

Lifespan is extended in mice by rapamycin treatment, litter crowding (CL) and in genetic models such as the Snell dwarf mouse. Various mechanisms have been proposed by which lifespan is extended in these rodent models, including improved stress resistance. The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) has been suggested to be the “master regulator” of cellular antioxidant and cytoprotective defenses. However, whether these rodent longevity models show enhanced Nrf2 activation in a sex-specific manner is unknown. The purpose of this study was to determine the tissue specific expression of Nrf2 and Nrf2-regulated antioxidant enzymes in three mouse models of longevity between male and female mice. Endogenous antioxidant enzyme and Nrf2 expression were not consistently increased in long-lived animals, with considerable variability between tissues, models and sex. Nrf2 and some of its targets were greater in females compared to males in liver and heart from mice in the rapamycin cohort, while the males displayed significantly greater Nrf2 and target expression in the muscle. While Nrf2 and some of its targets were increased in tissues from Snell compared to control, no differences between the long-lived and control model were observed with the CL animals. Not all models of longevity displayed similar Nrf2-regulated antioxidant enzymes, and these data suggest that sex, age, and tissue specific differences must be considered with regard to stress resistance and longevity.

Introduction

Aging is a multifactorial process characterized by a decline in genomic integrity, accumulation of oxidative damage, and an increase in disease and mortality [1]. As an organism ages, damage to cellular components occurs, resulting in cell dysfunction and an aging phenotype. The stress resistance theory of longevity suggests that the ability to respond to a stress is directly related to aging and senescence [91]. According to the theory, manipulations that extend lifespan should also improve cellular stress resistance, and mechanisms that improve cellular stress resistance should improve healthspan. This theory is supported by long-lived models which have increased stress resistance [7, 8]. Skin fibroblasts derived from long-lived rodent species and long-lived mutant mice have increased resistance to oxidative, heat, heavy metal, high glucose, and xenobiotic stresses [92, 93], suggesting that it is the resistance to variety of stressors, rather than resistance against a particular stress, that is a good indicator of lifespan extension. Long-lived bird species also have increased resistance to oxidants, the DNA alkylating agent methyl methanesulfonate, and low-glucose medium compared to short-lived bird species, as well as in comparison to rodents of similar body size [94], further supporting the paradigm of longevity and associated stress resistance against cytotoxic agents. Thus, multiple models of longevity lend support to the hypothesis that lifespan extension occurs through the activation of multi-factorial cytoprotective pathways that protect against a wide variety of cellular stresses by activation of a diverse battery of defenses. However, while the relationship between stress tolerance and longevity is well-established, the mechanism for increased stress defenses is not.

The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is integral in the regulation of cellular pro-survival defenses [3]. Recently it was hypothesized that Nrf2 is the “master regulator” of the aging process, and may play a role in determination of species longevity [9]. Under normal conditions, Nrf2 is sequestered in the cytoplasm by its involvement

in an inactive complex with Kelch-like ECH-associated protein 1 (Keap1). Keap1, an actin-binding protein, targets Nrf2 for ubiquitination and degradation by the 26S proteasome system, resulting in basal low-level expression of Nrf2 target genes. However, when Nrf2 is activated by a variety of stresses, pharmaceuticals, or phytochemicals, it transcriptionally upregulates over 200 cytoprotective genes including those involved with xenobiotic metabolism, phase II antioxidant enzymes [36, 95], anti-inflammation [96], autophagy [71], and proteasomal degradation [97]. The Nrf2 pathway is increased in fibroblasts from the extremely long-lived naked mole rat, with higher constitutive nuclear Nrf2 protein expression compared to similar-sized mice [98]. Additionally, skin-derived fibroblasts from long-lived Snell dwarf mutant mice show elevated Nrf2 signaling [99]. Given the cytoprotective properties of Nrf2, it is likely that enhanced Nrf2 activation and Nrf2 target genes are a component of stress resistance in long-lived species.

Lifespan is greater in females than males, and many interventions that improve lifespan have greater lifespan extending effects in females than males including rapamycin feeding [100], insulin-like growth factor 1 (IGF-1) receptor knockouts [101] and the Ames dwarf mouse [102]. Previous work from our laboratory suggests some of the growth signaling that contributes to aging display sexual dimorphism [103]. These previous data from our laboratory suggest that other facets of longevity-related signaling may also respond in a sex-specific manner, and led us to analyze sex specific contributions to Nrf2 signaling.

To address the role of Nrf2 in the association between stress response and the aging process, we retrospectively investigated Nrf2-regulated antioxidant defenses in three rodent models of longevity; crowded litter (CL), chronic rapamycin treatment, and the genetic Snell dwarf model. Recent evidence has shown that very short-term nutrient restriction, limited only to the pre-weaning phase, can extend lifespan [104]. Although the mechanism responsible for lifespan extension remains unknown, it is thought that this model of CL represents a

reprogramming of gene expression during early development, and thus an epigenetic means of improving lifespan. Treatment with rapamycin, a well-defined inhibitor of the mechanistic target of rapamycin (mTOR) not only extends lifespan [105], but also slows multiple components of the aging phenotype [106]. Lastly, the Snell dwarf mouse has a single-gene mutation at the *Pit1* locus resulting in an underdeveloped pituitary, decreases in growth hormone (GH) and IGF-1 signaling, and a 40% increase in mean and maximal longevity [107]. Despite sufficient data supporting the ability of these interventions to increase lifespan, much less is understood about the mechanisms of improved stress resistance that could lead to increased lifespan. Therefore, this study aimed to assess Nrf2 and Nrf2-regulated antioxidant enzyme expression across three rodent models of lifespan extension, with a secondary aim to understand the sexual dimorphism of Nrf2 signaling in longevity. We hypothesized that tissues from long-lived models would display greater Nrf2 and phase II antioxidant enzyme expression compared to their respective controls, with females displaying enhanced Nrf2 signaling compared to males.

Materials and Methods

Animal care

All procedures and conditions at the animal care facilities meet or exceed the standards for animal housing as described in the Animal Welfare Act regulations, the Guide for the Care and Use of Laboratory Animals and the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching and were approved by the University of Michigan Committee on Use and Care of Animals. Snell, CL, and rapamycin treated animals were euthanized following an overnight fast by anesthetization via CO_2 overdose, according to the AVMA Guidelines on Euthanasia. Complete loss of pedal reflexes was confirmed before tissues were collected. The posterior aspect of the distal hind limbs (mixed skeletal muscle:

gastrocnemius, soleus, and plantaris), heart, and liver were dissected and immediately frozen in liquid nitrogen for later analyses.

Snell cohort

Snell dwarf (*dw/dw*) and heterozygote (*dw/+)* control mice were bred as the progeny of (DW/J X C3H/HeJ)F1 *dw/*+ females and (DW/J X C3H/HeJ)F1 *dw/dw* males [107]. Both the Snell and control cohorts were composed of equal numbers of male (n=5) and female (n=5) mice at 7 months of age. All animals were given free access to food and water, and were maintained using standard specific pathogen-free husbandry techniques at the University of Michigan.

Rapamycin cohort

UM-HET3 mice were generated by the offspring of crosses between (BALB/cByJ X C57BL/6J)F1 females and (C3H/HeJ X DBA/2J)F1 males. Mice were fed chow mixed with encapsulated rapamycin (n=6 female, n=6 male) at 14 mg/kg food (equivalent to 2.24 mg of rapamycin/kg body weight/day) or normal chow (n=6 female, n=6 male) in accordance with the original study describing lifespan extension [100]. Diets were administered for 12 weeks beginning at 4 months of age and mice were sacrificed at 7 months of age.

Crowded litter cohort

CL mice of 3-4 months of age (n=8 male, n=8 female) were generated following the previously described procedure [104]. UM-HET3 litters were culled to eight pups (control) or supplemented by transfer of newborn mice to produce litters of 12 mice (CL). Litters were weaned at three weeks of age and housed thereafter at 3 male or 4 female mice per cage. To control for differing levels of nutrition experienced in utero, litters originally containing fewer than 8 or more than 10 pups were not used. After weaning, both groups were fed ad libitum.

Tissue preparation and western blotting

Skeletal muscle, heart and liver tissues were fractionated according to our previously published procedures [103, 108]. Tissue (25-60 mg) was homogenized 1:10 in 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) with phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL, USA) using a bead homogenizer (Next Advance Inc, Averill Park, NY, USA). After homogenization, the samples were centrifuged at 800 x g for 10 minutes at 4 °C (Eppendorf, model 5415R, Hauppauge, NY, USA). The supernatant from the 800 g spin was centrifuged at 9,000 g for 10 minutes and the supernatant saved for western blot analyses.

Protein concentration was determined by bicinchoninic acid assay (Thermo Fisher, Rockford, IL, USA). Samples were boiled with Laemmli buffer, and protein was resolved on a 10% Tris/glycine SDS-polyacrylamide gel in running buffer containing 25 mMTris, 192 mM glycine, pH 8.3. Proteins were transferred at 100 V for 75 minutes in 20 % w/v methanol, 0.02 % w/v SDS, 25 mMTris Base, 192 mM glycine, pH 8.3 to nitrocellulose paper and incubated in Superblock (Thermo Fisher, Rockford, IL, USA) for 1 hour. Antibodies were purchased from Abcam (Cambridge, MA, USA; NAD(P)H dehydrogenase quinone 1 (NQO1) #34173, heme oxygenase-1 (HO-1) #13248), Cell Signaling Technologies (Danvers, MA, USA; peroxiredoxin1 (Prdx1) #8499) or Santa Cruz Biotechnology (Santa Cruz, CA, USA; superoxide dismutase-1 (SOD-1) sc-8637, Nrf2 sc-722, β-tubulin sc-5274, α-tubulin sc-23948, actin sc-1616). Blots were incubated overnight with primary antibodies diluted 1:1000 (NQO1, Prdx1), 1:500 (SOD-1, HO-1, actin, and tubulin), or 1:250 (Nrf2). Blots were washed in TBST and incubated with HRP-conjugated secondary antibody diluted 1:5000 in Superblock with subsequent chemiluminescence detection (West Dura; Pierce, Rockford, IL, USA). Images were captured and densitometry analyzed using a UVP Bioimaging system (Upland, CA, USA). Equal loading of protein was verified using ponceau-s staining as well as with tubulin or actin.

Statistical analyses

Statistical analyses were performed using SPSS (Version 17) with significance set *a priori* at $p<0.05$. Data were assessed by two-way ANOVA (longevity model by sex). Where necessary, data were transformed to equalize variance. Data are presented as means ± standard error of the mean.

Results

Expression of Nrf2 target genes does not differ between background strains of long-lived mice.

To date, no studies have assessed Nrf2 and target protein expression across varying mouse genetic strains. However, differential responses of genetic backgrounds to longevity interventions such as caloric restriction have been reported [109], suggesting important differences may exist between genetic strains with regard to Nrf2 signaling. Therefore, we assessed expression of Nrf2 and target genes NQO1 and SOD-1 in the liver across the two genetic backgrounds. No significant differences existed between strains with regard to Nrf2, NQO1, or SOD-1 expression (Supplemental Figure 3.1). However, NQO1 was significantly greater in female mice from all strains compared to male (Figure 3.1).

Genetic mutation of the Pit1 locus in Snell dwarf mice is associated with increased Nrf2 target protein in some, but not all tissues, with no sexual dimorphism.

The Nrf2 target SOD-1 was significantly greater and HO-1 was significantly less in the liver of Snell mice compared to controls (Figure 3.2). Skeletal muscle SOD-1 was paradoxically significantly lower in Snell (Figure 3.3) and in the heart Nrf2 was significantly greater in Snell compared to control (Figure 3.4). We did observe a significant interaction between sex and

model in some liver proteins (Figure 3.2), suggesting that the Pit1 mutation differentially influences HO-1 and SOD-1 expression in male versus female mice.

Chronic rapamycin treatment does not influence Nrf2 and target protein expression; however expression of Nrf2 and targets was significantly different between male and female mice.

In mice treated with rapamycin for 12 weeks, NQO1 in the liver was significantly lower in rapamycin-fed mice compared to control (Figure 3.5), and Prdx1 expression in the muscle was significantly lower than control (Figure 3.7). No other significant differences were observed between the long-lived and control animals.

We have previously shown sex differences in mTOR signaling and protein synthesis with chronic rapamycin treatment [103]. Therefore, we aimed to determine whether Nrf2 and its target genes would also be differentially regulated with regard to sex. While Nrf2 and NQO1 expression were significantly greater than control in the liver of female mice, HO-1 was significantly greater in males (Figure 3.5). Similarly, SOD-1 and Prdx1 were significantly greater in hearts from female mice compared to males (Figure 3.7). However, in contrast to the heart and liver, Nrf2, NQO1, and SOD-1 were all significantly greater in males (Figure 3.6).

Litter crowding does not change Nrf2 and target protein expression, but does display liver-specific sex differences.

In the liver, NQO1 was significantly greater in female mice compared to male, while HO-1 was significantly higher in male mice than female (Figure 3.8). However, no significant model differences were observed in the liver. Neither sex nor model-specific differences were observed in the skeletal muscle from CL mice (Figure 3.9).

Discussion

It is well established that naturally long-lived animals have greater stress resistance [7, 8], and interventions that extend lifespan also result in improved cellular resistance to some forms of stress [93]. However, the mechanisms for improved stress resistance have remained elusive. We hypothesized that Nrf2, the master regulator of cellular antioxidant enzymes and proposed gatekeeper of longevity [9], would be upregulated in non-proliferative tissues from long-lived models. Contrary to our hypothesis, Nrf2 and its targets were not consistently upregulated in long-lived models, with considerable variation existing among tissues and sex (Supplemental Figure 3.2). However, this study is the first to describe differences in Nrf2 regulated protein expression across varying genetic backgrounds, and we provide novel results showing sex-specific differences in Nrf2-regulated antioxidant expression. These data are important because they provide insight into Nrf2 regulation under non-stressed conditions and establish a foundation for studies further investigating stress resistance in long-lived models.

Sexual dimorphism in mouse models of longevity

Lifespan is greater in females than males, and many interventions that improve lifespan have greater lifespan extending effects in females than males including IGF-1 receptor knockouts [101] and the Ames dwarf mouse [102]. In addition, two of the models examined here, chronic rapamycin feeding and CL, have both shown sexual dimorphic responses to lifespan extension [100, 104]. Rapamycin feeding has been shown to increase mean lifespan by 16% in females, compared to only 8% in males in the UM-HET3 strain, with significant sexual dimorphism across other examined background mouse strains [100]. In lifespan studies in CL mice, median survival increased by 7% in males, and 16% in females, indicating a significant difference between sexes [104]. Previous work from our laboratory suggests some of the mechanisms behind aging display sexual dimorphism. We have shown that female mice show

significantly less mixed and mitochondrial protein synthesis compared to males, as well as differential mTORC1 signaling (both protein synthesis and mTORC1 signaling are associated with age and slowed aging interventions) [103]. These previous data from our laboratory suggest that other facets of longevity-related signaling may also be regulated in a sex-specific manner, and led us to analyze sex specific contributions to Nrf2 signaling.

To our knowledge, few studies have examined sex-specific differences in Nrf2 and its antioxidant response element-regulated targets. Some previous literature suggests liver mitochondria from female rats show enhanced glutathione peroxidase activity [110], and liver NQO1 and HO-1 mRNA were higher in female than male rats [111]. In the current study, liver Nrf2 and NQO1 protein expression from the rapamycin cohort were both significantly greater in female than male mice, and SOD-1 and Prdx1 were greater in the hearts of female mice. However, these patterns contrast with muscle, where SOD-1, Nrf2, and NQO1 were significantly greater in males. In the liver from CL animals, NQO1 protein was significantly greater in females, while HO-1 was significantly higher in males. A significant interaction between sex and treatment indicated that rapamycin differentially affected female mice compared to male mice. While rapamycin feeding decreased Nrf2 expression in males, it increased Nrf2 expression in females (Figure 3.5). How chronic rapamycin treatment may upregulate Nrf2 in females, while downregulating its expression in males remains unknown. To our knowledge, sex differences have not been reported for Nrf2 and its protein targets, particularly between tissue types. Given the disparate lifespan between males and females, it is important to understand sex-specific signaling of the Nrf2 cytoprotective pathway, as these data may provide insight into the mechanisms behind enhanced longevity in females.

Tissue-specific Nrf2 and target protein expression

Liver, heart, and muscle vary with regard to mitotic capability. Liver cells, which are quiescent until stimulated to proliferate, have a greater capacity to replace damaged cells than heart and muscle tissue, which are semi and non-mitotic, respectively (reviewed in [112]). One of the strengths of this investigation was the assessment of Nrf2 and regulated protein expression in three tissues, allowing us to discern tissue-specific differences. Previous work from our laboratory has shown tissue-specific protein synthetic and mTORC1 related signaling in tissues from long-lived models [103, 108], suggesting characteristics of these tissues may be important in determining tissue-specific responses to longevity interventions. An assessment of tissue-specific differences in cellular stress responses is lacking, and may play an important role in determining the response of Nrf2 to the different longevity interventions.

Mechanisms of Nrf2 activation: inducible versus basal activity

Investigations of Nrf2 knockout mice suggest that although these animals are more sensitive to chemical insult, basal differences in phenotype and ROS signaling are not apparent in young animals. For example, Nrf2 knockout has no effect on cardiac structure and function at a young age [43]. However, when these knockout mice undergo an acute exercise stress, the ability to respond to and recover from the stress is attenuated, resulting in diminished glutathione levels, increased ROS production, and dysregulation of cardiac antioxidants. Aging is sufficient to result in diminished Nrf2 activity [113], and aged Nrf2 knockout mice exhibit increased cardiac and skeletal muscle ROS production, glutathione depletion, and decreased expression of Nrf2 target genes [114]. Therefore, it appears that in the absence of stress, the loss of Nrf2 has little effect on skeletal muscle and cardiac redox state in young mice. Upon acute administration of a stress or chronic stress caused by aging, the deficits in Nrf2 signaling become apparent, and result in accelerated tissue and cell dysfunction. The animals in the

current investigation were young (3-4 and 7 months of age), with presumably no impairment of the endogenous antioxidant program and no overt oxidative damage. If older animals were assessed, or younger animals administered a stressful stimulus, perhaps then differences in Nrf2 signaling between long-lived and control animals would be apparent.

Conclusions

Despite previously reported evidence for augmented stress resistance, the longevity models examined in this investigation do not all demonstrate enhanced Nrf2 signaling. This study is the first to describe Nrf2 and target protein expression in young, unstressed mice across two genetic strains, and across three rodent models of lifespan extension. We are the first to examine Nrf2 expression in various tissues from long-lived models, and the first to examine Nrf2 expression in tissues that vary based on mitotic capacity. Further, we assessed sex-specific outcomes, which serve to highlight the need for future sex-specific longevity studies. We provide the basis for future studies which aim to assess whether an enhanced ability to activate Nrf2 contributes to lifespan extension, and whether the age-related decline in Nrf2 activation is attenuated by slowed aging interventions. We hypothesize that pharmaceutical, genetic, epigenetic, and dietary manipulations that extend lifespan will enhance Nrf2 activation at advanced ages and under stressful cellular conditions, contributing to stress resistance and extended healthspan. An analysis of Nrf2 and its antioxidant program in aged long-lived animals is warranted, and may provide insight into the mechanisms of slowed aging in these rodent models of longevity.

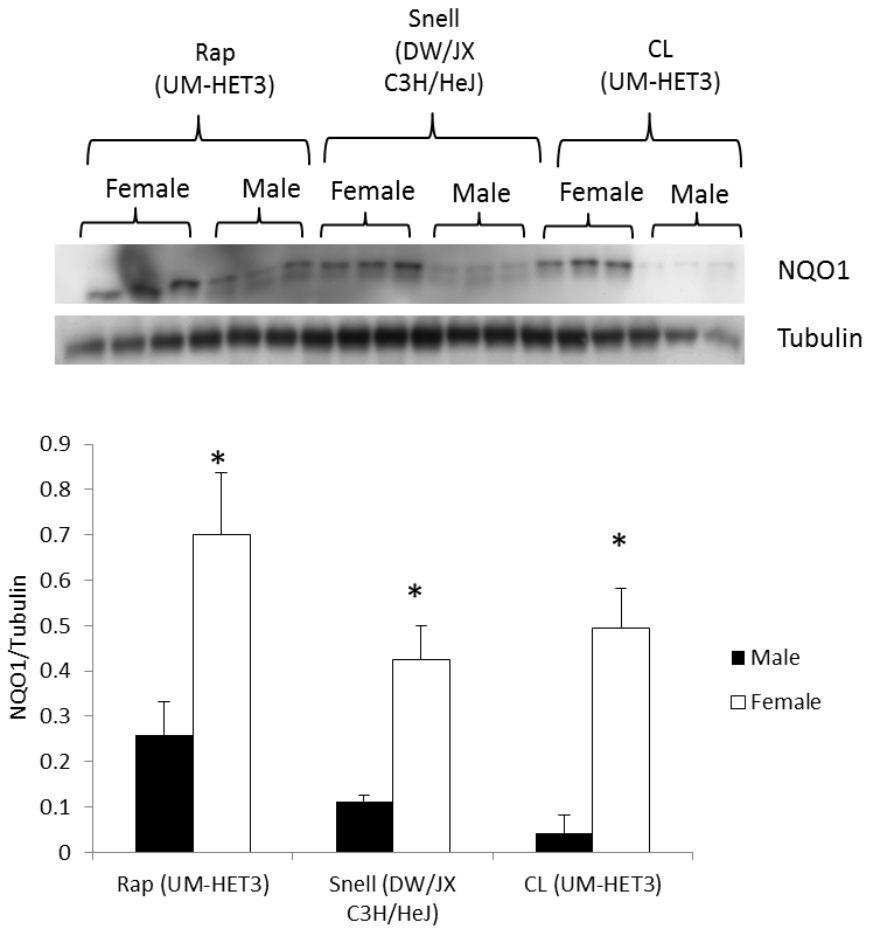


Figure 3.1. Female mice from both genetic backgrounds show significantly greater expression of NQO1 compared to males. NQO1 was assessed by western blotting and data are expressed as a ratio of NQO1 to tubulin. * Sex difference. n=3 in each condition.

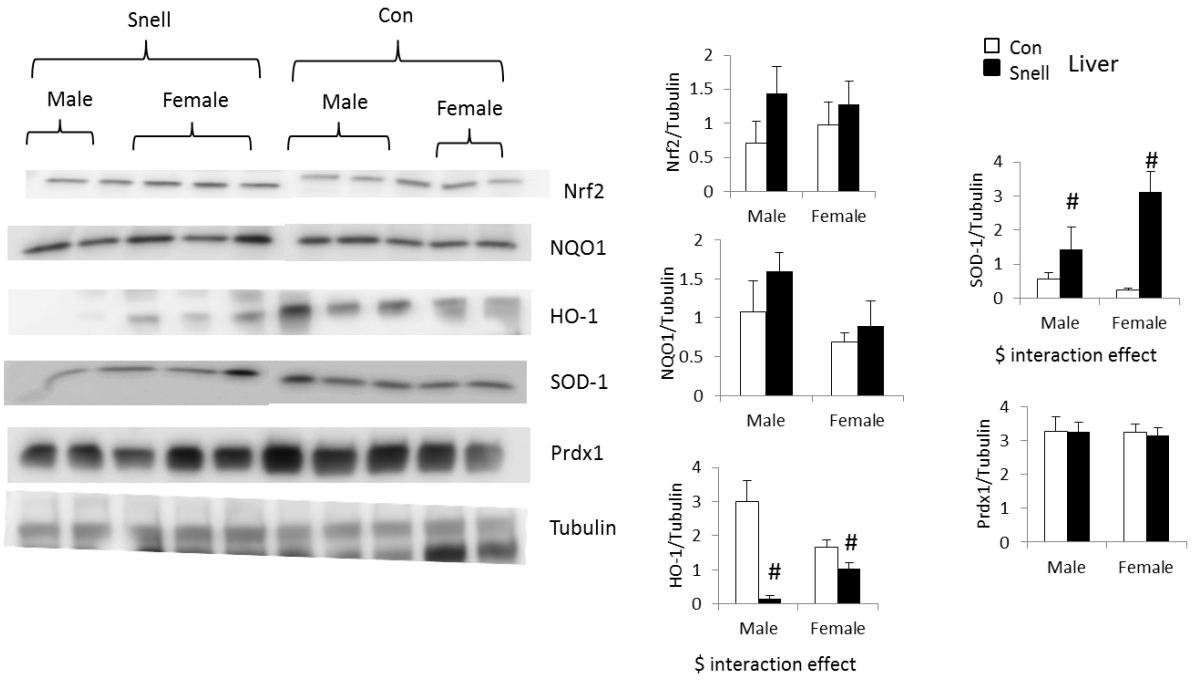


Figure 3.2. The *Pit1* mutation is associated with increased SOD-1 expression and decreased HO-1 expression in the liver compared to controls. Nrf2, NQO1, HO-1, SOD-1, and Prdx1 were analyzed by western blotting, and normalized to tubulin. Data are expressed as a ratio of target protein to tubulin. # Condition difference (Snell vs. Control), $ significant interaction between condition and sex. n=5 in each condition.

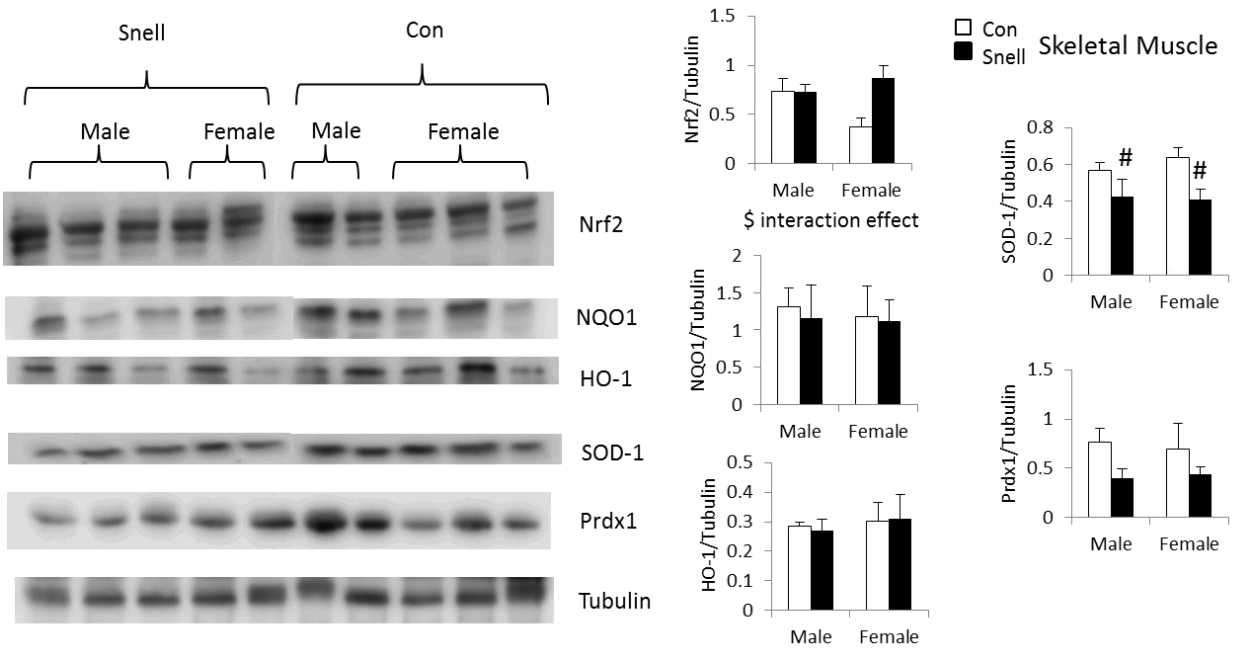


Figure 3.3. Skeletal muscle from Snell mice show decreased SOD-1 expression. Nrf2, NQO1, HO-1, SOD-1, and Prdx1 were analyzed by western blotting, and normalized to tubulin. Data are expressed as a ratio of target protein to tubulin. # Condition difference (Snell vs. Control). n=5 in each condition.

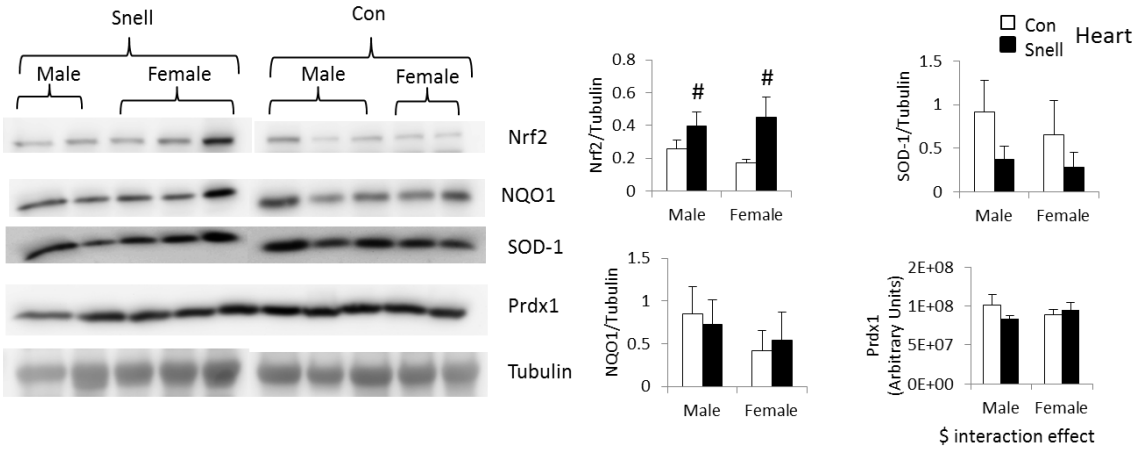


Figure 3.4. The *Pit1* mutation in the Snell mouse increases Nrf2 expression in the heart. Nrf2, NQO1, SOD-1, and Prdx1 were analyzed by western blotting, and normalized to tubulin. HO-1 could not be detected in the hearts from this model. Data are expressed as a ratio of target protein to tubulin. # Condition difference (Snell vs. Control), $ significant interaction between condition and sex. n=5 in each condition.

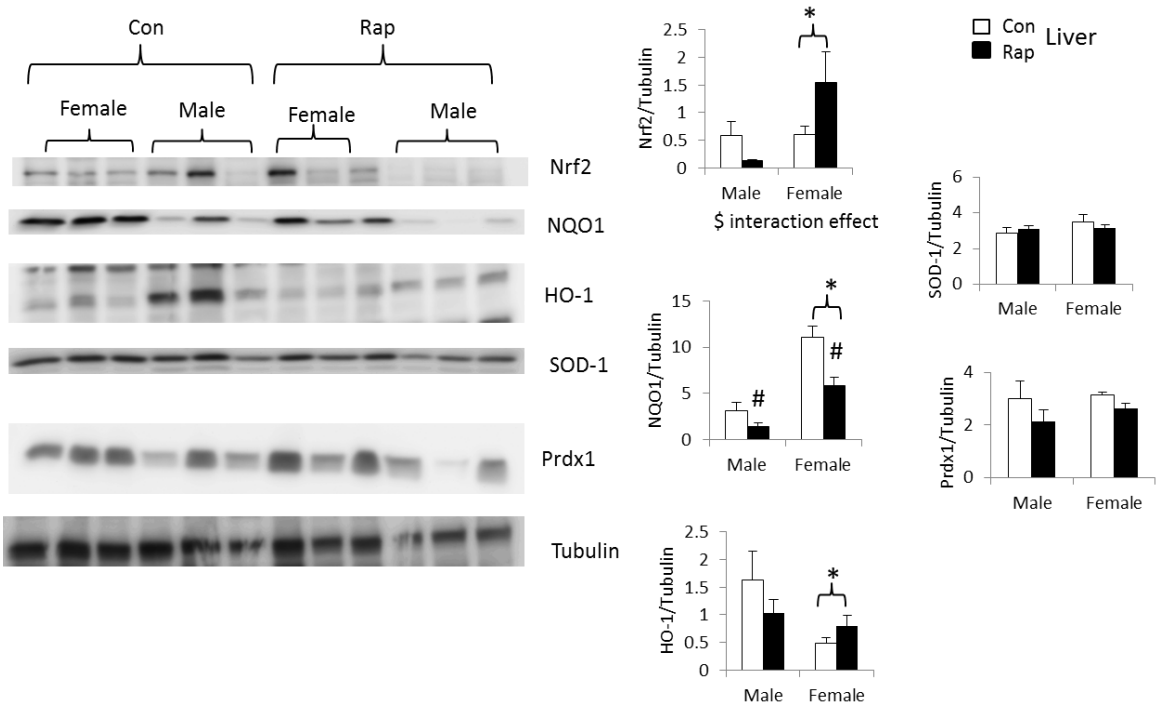


Figure 3.5. Chronic rapamycin (Rap) feeding significantly depresses Nrf2 and NQO1 in livers from female mice compared to males, while significantly upregulating HO-1 in males. Nrf2, NQO1, SOD-1, and Prdx1 were analyzed by western blotting, and normalized to tubulin. # Condition difference (Rap vs. Control), * Sex difference, $ significant interaction between condition and sex. n=6 in each condition.

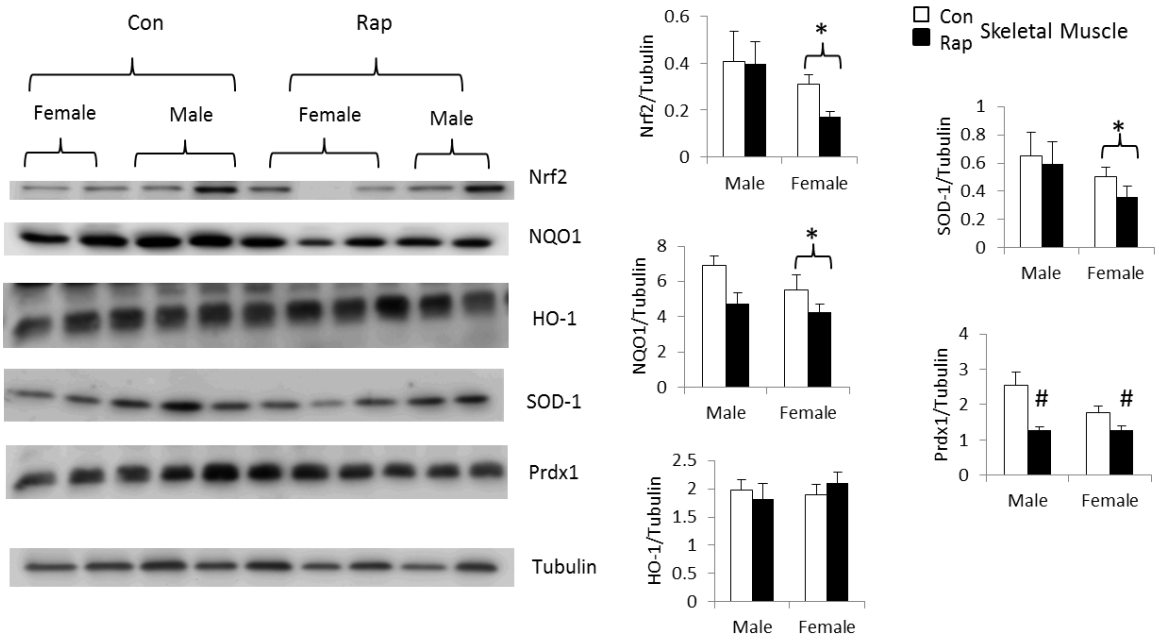


Figure 3.6. Nrf2, NQO1, and SOD-1 were significantly greater in skeletal muscle from male rapamycin (Rap) treated mice. Chronic rapamycin feeding suppressed Prdx1 expression compared to controls. Nrf2, NQO1, SOD-1, and Prdx1 were analyzed by western blotting, and normalized to tubulin. # Condition difference (Rap vs. Control), * sex difference. n=6 in each condition.

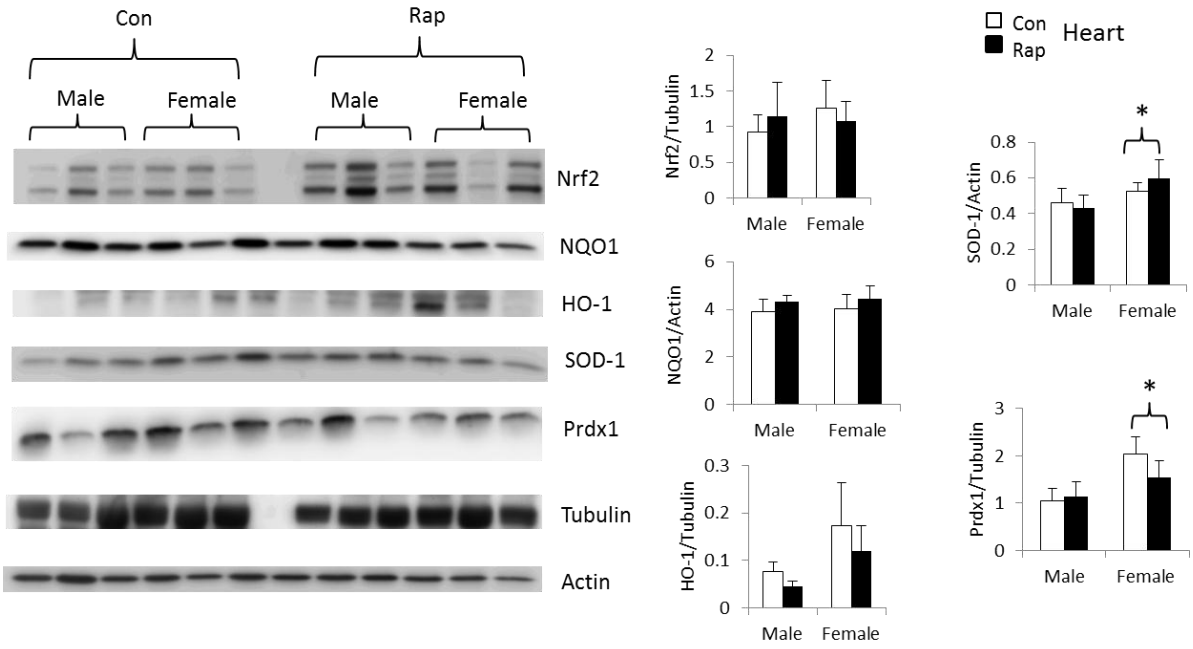


Figure 3.7. SOD-1 and Prdx1 were significantly greater in hearts from female rapamycin (Rap) treated mice compared to males. Nrf2, NQO1, SOD-1, and Prdx1 were analyzed by western blotting, and normalized to tubulin or actin. * Sex difference. n=6 in each condition.

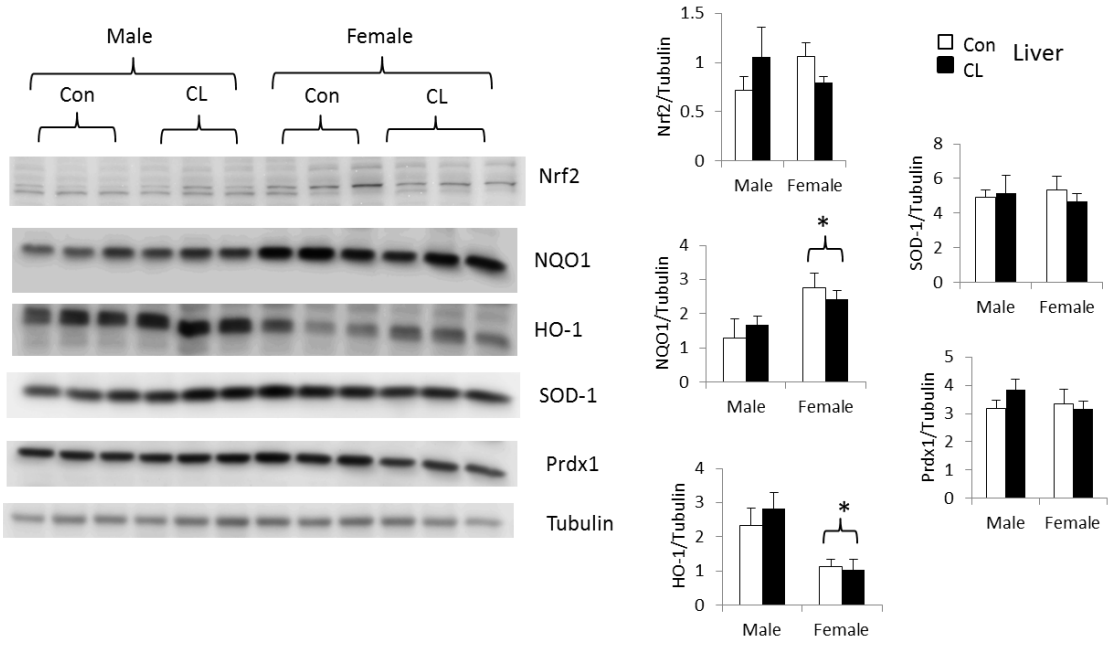


Figure 3.8. Liver NQO1 was significantly greater in crowded litter (CL) female mice, while HO-1 was significantly greater in male mice. No differences were observed between CL and control. Nrf2, NQO1, SOD-1, and Prdx1 were analyzed by western blotting, and normalized to tubulin. *Sex difference. n=8 in each condition.

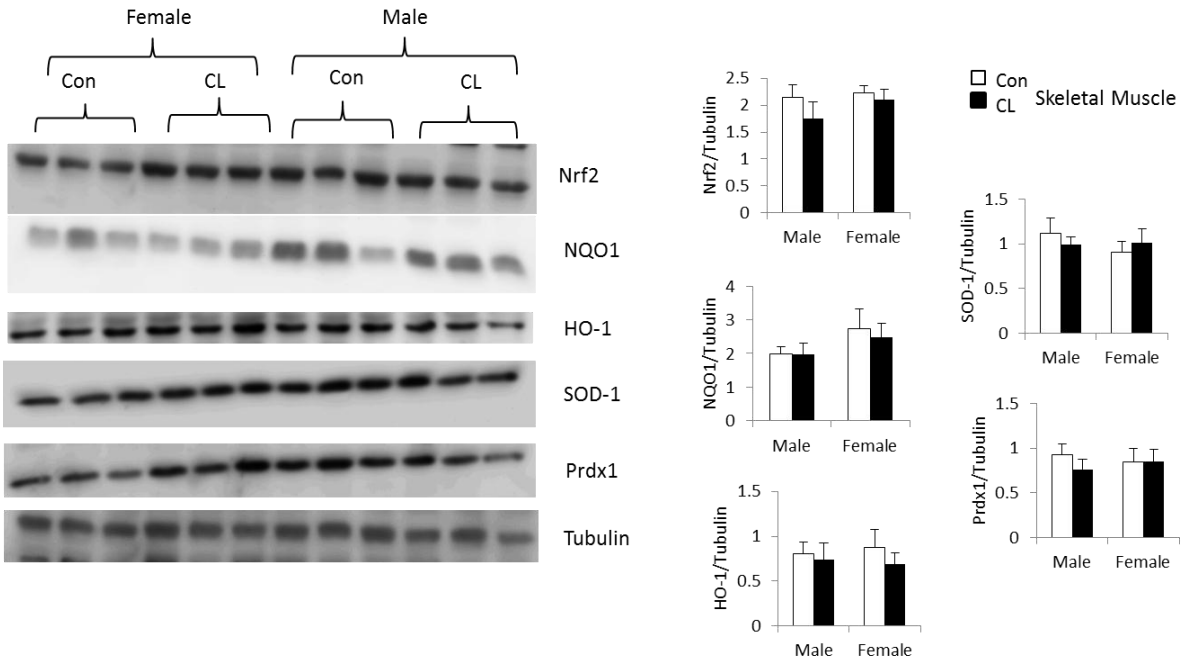
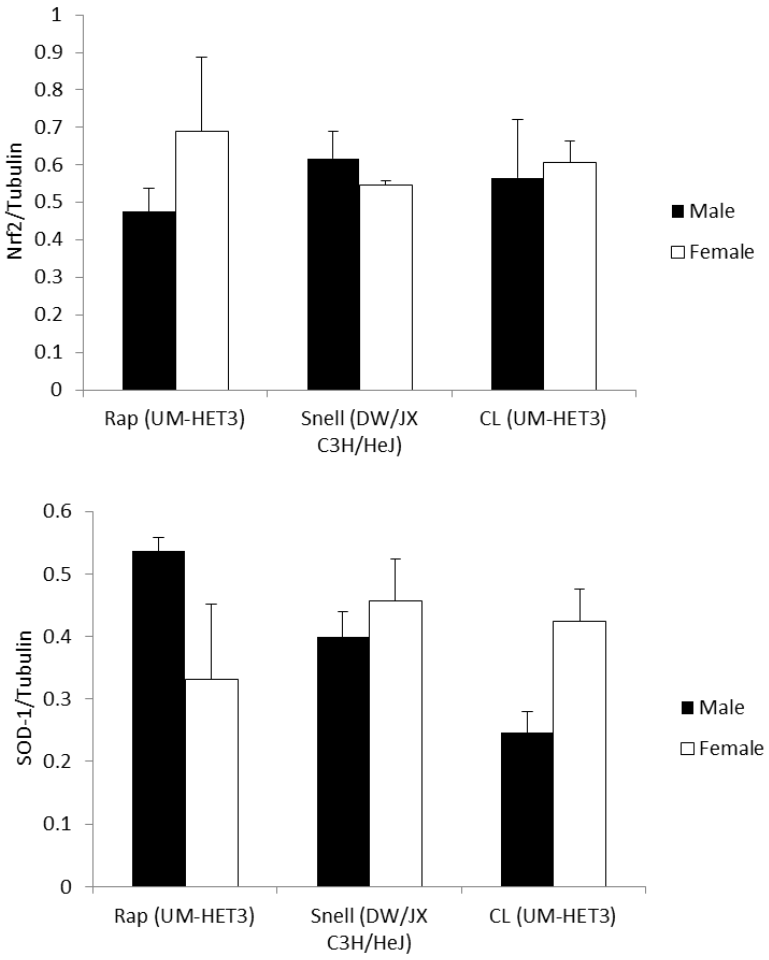


Figure 3.9. No significant sex or model differences were observed in skeletal muscle from crowded litter (CL) animals. Nrf2, NQO1, SOD-1, and Prdx1 were analyzed by western blotting, and normalized to tubulin. n=8 in each condition.



Supplemental Figure 3.1. No significant sex or background differences were observed in SOD-1 or Nrf2 expression in the liver. SOD-1 and Nrf2 were assessed by western blotting, and normalized to tubulin.

Model/Tissue	Nrf2	NQO1	HO-1	SOD-1	Prdx
SNELL					
Liver	⟺	⟺	↓	↑	⟺
Heart	↑	⟺	--	⟺	⟺
Muscle	⟺	⟺	⟺	↓	⟺
RAPAMYCIN					
Liver	F>M ⟺	F>M ↓	M>F ⟺	⟺	⟺
Heart	⟺	⟺	⟺	F>M ⟺	F>M ⟺
Muscle	M>F ⟺	M>F ⟺	⟺	M>F ⟺	↓
CL					
Liver	⟺	F>M ⟺	M>F ⟺	⟺	⟺
Muscle	⟺	⟺	⟺	⟺	⟺

Supplemental Figure 3.2 Summary of Nrf2 and targets in all tissues across all longevity models. Arrows indicate the change in protein expression compared to corresponding control condition. A green arrow (↑) indicates an increase in target in the long-lived model compared to control, an orange arrow (⟺) no difference, and a red arrow (↓) a significant decrease in protein expression in the long-lived model. In addition, significant sex differences are noted.

CHAPTER V- MANUSCRIPT IV

Selective translation of mitochondrial proteins during treatment with rapamycin and metformin

Summary

Age and age-related disease are associated with a decline in mitochondrial function and biogenesis. Conditions that exert energy stress, such as caloric restriction or its mimetics, maintain mitochondrial biogenesis and slow age-related disease. Energetic stress activates AMP-activated protein kinase (AMPK) and inhibits mechanistic target of rapamycin (mTOR). Under these conditions, global protein synthesis is diminished, while translation of key nuclear encoded mitochondrial proteins is maintained. In order for mitochondrial proteins to still be translated under conditions of limited energy availability, there must be a mechanism that facilities their sustained translation. We assessed preferential translation of mitochondrial proteins *in vitro* in response to treatments that activate cell signaling associated with energetic stresses, including metformin, rapamycin, and their combination treatment in a murine myotube model. We show that with pharmaceutical energetic stress, selected mitochondrial proteins are preferentially translated, even when global protein synthesis is suppressed. One of the mechanisms by which this selective translation may occur is via spatial organization of mRNA, as we show that nuclear-encoded mitochondrial mRNAs are associated with the mitochondria. These data provide insight into the mechanisms through which energetic stress promotes mitochondrial biogenesis, despite energy deficits, and may explain how other slowed aging interventions attenuate the mitochondrial dysfunction associated with chronic disease.

Introduction

Mitochondrial dysfunction is involved in the etiology or progression of a variety of chronic diseases including type II diabetes [115], cardiovascular disease [116], and sarcopenia [117]. Over time and through diseases processes, oxidative stress damages mitochondria. Damaged

mitochondria and defective mitochondrial proteins produce greater reactive oxygen species, further perpetuating oxidative damage to mitochondrial DNA and proteins [118].

Caloric restriction (CR), the most consistent and robust means to increase lifespan, increases or maintains mitochondrial biogenesis [11]. Caloric restriction activates AMP-activated kinase (AMPK) [13], the cellular energy sensor [119] and inhibits the mechanistic (formerly mammalian) target of rapamycin (mTOR) [12, 120]. Activation of AMPK increases activation of peroxisome proliferator-activated receptor gamma co-activator (PGC-1α) [15, 121], the transcriptional regulator of mitochondrial biogenesis, presumably in an effort to increase aerobic energy production. Inhibition of mTOR results in the hypophosphorylation of the downstream substrate eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), thereby suppressing translation initiation [14]. Thus, under energetic constraints, activation of AMPK presumably results in global inhibition of protein translation alongside activation of PGC-1α and an increase in the signaling for mitochondrial biogenesis. This apparently antagonistic effect is paradoxical and it is unclear how energetic stress can promote mitochondrial biogenesis while simultaneously inhibiting global protein synthesis.

Studies of mitochondrial protein synthesis in yeast indicate that intracellular mRNA organization is important for the efficiency of the translation process [122]. Since mitochondrial related proteins (MRPs) need to be imported into mitochondria to be functional, it has been suggested that translation may occur spatially proximal to the mitochondria [122, 123]. Translationally active ribosomes with mRNAs encoding mitochondrial proteins accumulate on the surface of mitochondria in yeast [124]. A qRT-PCR array was performed to identify the genes in spatial proximity to the mitochondria, and nearly half of mRNAs from nuclear genes encoding mitochondrial products were found to be translated in the vicinity of mitochondria [124]. In HeLa cells, mRNAs encoding mitochondrial proteins were shown to exhibit the same distribution pattern between free and mitochondrial-bound polysomes as in yeast [125]. These data support the conservation of intracellular mRNA localization from yeast to human cells as a

necessary mechanism by which to tightly couple translation and translocation of mitochondrial proteins.

Lifelong CR animals and mice chronically fed rapamycin maintain mitochondrial protein synthesis with age, in addition to a significant decrease in cellular proliferation [11, 103], suggesting an energy trade-off may contribute to longevity. Therefore, interventions that promote the maintenance of mitochondria during periods of limited energy availability may represent an effective method to combat the cellular stresses of chronic disease. Here, we utilized pharmaceutical means to induce energy stress pathways through activation of AMPK and inhibition of mTOR. We hypothesized that activation of AMPK activates the translation of MRP while suppressing global protein synthesis, resulting in preferential translation of mitochondrial related proteins. Further, we hypothesized that spatial localization of MRP mRNA near the mitochondria allows MRP synthesis to occur when global protein synthesis is suppressed.

Methods

Culture of C2C12 cells

Proliferating myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (ATCC; Manassas, VA). When cells reached confluence, the growth media was replaced with DMEM plus 10% horse serum for 3 days to induce differentiation. Metformin (Merck Millipore; Billerica, MA) or rapamycin (Merck Millipore) was added to culture dishes in growth media at a final concentration of 2 mM and 5 nM, respectively, for 24 hours. The combination of metformin plus rapamycin was also added in growth media for 24 hours, in 2 mM and 5 nM final concentrations. DMSO was added to culture dishes as the vehicle for rapamycin, and metformin was prepared in media (control).

Immunoblotting

Myocytes were washed 2X with ice cold PBS and lysed with buffer containing Halt protease and phosphatase inhibitors (Pierce; Rockford, IL). Samples were sonicated and lysate protein concentration measured by bicinchoninic acid assay (ThermoScientific; Waltham, MA). Diluted samples containing equal amounts of protein were prepared in Laemmli Sample Buffer and 2-mercaptoethanol (BioRad; Hercules, CA) and heat denatured for 5 minutes at 98˚C. Proteins were resolved on a Tris/glycine SDS-polyacrylamide gel in running buffer, and proteins transferred to a nitrocellulose membrane. Following blocking of nonspecific proteins (Superblock; ThermoScientific, Waltham, MA) membranes were incubated overnight in primary antibody (Cell Signaling; Boston, MA, phospho-S6 ribosomal protein (Ser235/236) #4858, total S6 ribosomal protein #2217, phospho-AMPKα (Thr172) #2531, total AMPKα #2532) at 4˚C followed by a secondary antibody conjugated to horseradish peroxidase for 1 hour at room temperature with 30 minute washes of TBST between primary and secondary incubations. Proteins were detected with chemiluminescence reagents (SuperSignal West Dura; Thermo Scientific) and imaged, (BioSpectrum AC Imaging System, UVP; Upland, CA) followed by densitometric analysis using VisionWorks, UVP software. Membranes were also incubated with actin (Santa Cruz 1616; Santa Cruz, CA) followed by chemiluminescent detection and densitometry to verify equal loading of protein.

Isolation of mitochondrial-enriched fractions

Mitochondrial-enriched cellular fractions were isolated by differential centrifugation. Briefly, cells were scraped in mitochondrial isolation buffer 1 (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH 7.5). The cell lysate was spun at 800 x g for 10 minutes to pellet myofibrillar and mixed cellular components. The supernatant of this spin was centrifuged for 30 minutes at 9,000 x g to pellet mitochondria. The mitochondrial pellet was washed in mitochondrial isolation buffer 2 (100 mM KCl, 10 mM Tris HCl, 10 mM Tris Base,

1 mM $MgCl_2$, 0.1 mM EDTA, 1.5% BSA, pH 7.4), centrifuged for 10 minutes at 8,000 x g followed by another wash with mitochondrial isolation buffer 2, and spun 10 minutes at 6,000 x g.

Real-time RT-PCR

RNA was extracted from mitochondrial-enriched fractions by standard TRIzol methods, followed by overnight isopropanol precipitation. RNA concentration and protein contamination were determined using spectrophotometry, and RNA degradation was determined by agarose gel separation. RNA was reversed transcribed and cDNA was amplified using specific oligonucleotide primers outlined in Table 4.1. PCR conditions were as follows: hot start (10 minutes at 95°C) followed by 40 cycles of denaturing and annealing (15 seconds at 95°C, 1 minute at 60°C), followed by a dissociation step. The pBiEx (kindly supplied by Stephen Coleman) was added to each mitochondrial fraction to serve as a reference gene. The relative quantification of the mitochondrial target gene was calculated by subtraction of reference gene (pBiEx), and calculation of ΔΔCt values from corresponding vehicle controls. Fold changes were determined using the cycle threshold ($2^{-\Delta\Delta Ct}$) method.

Deuterium labeling of mitochondrial proteins

Deuterium labeling allows long-term measurement of protein synthesis in different subcellular protein fractions. Here, we adapted our previously published methods [11, 103, 126] to an *in vitro* system to assess cytosolic and mitochondrial protein synthesis rates. Differentiated myotubes were treated for 24 hours with rapamycin, metformin, or the combination, in 10% deuterium oxide (D_2O) enriched media. To precipitate cytosolic proteins, a portion of the supernatant from the 9,000 x g spin from the above isolation procedure was incubated with 14% sulfosalicylic acid for 1 hour, and centrifuged at 16,000 g for 10 minutes. The pellet was washed with ethanol and water, and the mitochondrial pellet was washed with

water, and both pellets were resuspended in 1M NaOH. Proteins from the cytosolic and mitochondrial fractions were then hydrolyzed by incubation in 6M HCl for 24 hours at 120°C. The hydrolysates were cation exchanged, dried down by vacuum, and suspended in 1 mL of 50% acetonitrile, 50mM K_2HPO_4, pH 11. Samples were derivatized by adding 20 µL pentafluorobenzyl bromide. The sealed mixture was incubated at 100°C for 1 hour and derivatives were extracted into ethyl acetate. The organic layer was removed and dried by N_2, followed by vacuum centrifugation. Samples were reconstituted in 1 mL ethyl acetate for analysis.

For calculation of deuterium enrichment of the media, 120 µL of media was placed inverted on a heating block overnight. 2 µL of 10 M NaOH and 20 µL of acetone were added to samples and capped. Samples were vortexed at low speed and kept overnight at room temperature and were extracted with addition of 200 µL hexane. The organic layer was transferred through anhydrous Na_2SO_4 into gas chromatography (GC) vials and analyzed.

GC-mass spectrometry analysis of derivatized amino acids

Derivatized amino acids were analyzed on an Agilent 7890A GC coupled to an Agilent 5975C MS. GC separation used an Agilent DB-5MS GC column (30 m × 0.25 mm × 0.25 µm) while mass spectrometry was performed with negative chemical ionization (NCI). Samples were injected in 1 µL volumes using splitless mode (inlet temperature 220°C). Helium was the carrier and methane the reagent gas. The mass-to-charge ratios of 448, 449, and 450 were monitored for the pentafluorobenzyl-*N*,*N*di(pentafluorobenzyl)alaninate derivative. In all cases, these mass-to-charge ratios represented the primary daughter ions that included all of the original hydrocarbon bonds from the given amino acid. ^2H enrichment was calculated as the M+1 divided by the sum of the abundance of M+0 and M+1. The newly synthesized fraction of proteins was calculated from the enrichment of alanine in the hydrolyzed protein divided by the precursor enrichment estimated from media enrichment as determined from the media

enrichment of water with subsequent adjustment using mass isotopomer distribution analysis (MIDA) [11, 103, 127, 128].

Fluorescent in situ hybridization (FISH)

Myotubes were grown on #1 coverslips and fixed in 10% formalin for 10 minutes. After two washes with PBS, cells were permeabilized with 70% ethanol for 90 minutes at 4°C. Cells were washed for five minutes with wash buffer (10X saline-sodium citrate, 10% formamide), and hybridized (10X saline-sodium citrate, 1% dextran sulfate, 10% formamide) with a custom Stellaris FISH probe set designed against the β subunit of ATP synthase (Biosearch Technologies; Novato, CA) in a dark humidified chamber at 37°C for 5 hours. For co-identification of mitochondria, immunofluorescent staining of total OXPHOS (Total OXPHOS Rodent Antibody Cocktail Abcam 110413) was conducted with FITC conjugation of anti-mouse secondary antibody for visualization. The OXPHOS antibody recognizes one subunit of each of the five electron transport complexes, thus allowing identification of mitochondria. Cells were mounted with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories; Burlingame, CA) for nuclear identification. Images were captured using a Zeiss Axiovert 200M inverted fluorescence microscope equipped with a Hammatsu ORCA-ER-cooled charge-coupled device camera (Hammamatsu Photonics, Hamamatsu City, Japan) and representative images created using Slidebook software (version 5.0; Intelligent Imaging Innovations, Denver, CO).

Statistical analyses

Statistical analyses were performed using SPSS Version 16.0 (SPSS Inc., Chicago, IL). The distribution of each variable was assessed, and if data were unequally variable, appropriate transformations were performed. The main outcome variables were assessed between groups

using one-way ANOVA and LSD post hoc tests where appropriate. Statistical significance was set *a priori* at $p \leq 0.05$.

Results

Cell treatments

Three days of incubation in 10% horse serum media was sufficient to induce differentiation, as evident by the formation of myotubes (Figure 4.1). Treatment with metformin activated AMPK (Figure 4.2A) and inhibited mTOR as assessed by hypophosphorylation of downstream substrate ribosomal protein S6 (rps6) (Figure 4.2B). Treatment with rapamycin also activated pathways indicating energetic stress, as assessed by AMPK activation and mTOR inhibition (Figure 4.2). The combination of the two drugs did not further activate AMPK or inhibit mTOR over the single drug treatments (Figure 4.2).

Protein synthesis

Cytosolic and mitochondrial protein synthesis rates were calculated using deuterium labeling at 12, 18, and 24 hours. For simplicity, only the 24 hour data are reported here. Rapamycin and metformin treated cells displayed significantly blunted cytosolic protein synthesis (Figure 4.3A). In addition, mitochondrial protein synthesis was decreased in the rapamycin and metformin-treated cells compared to control and DMSO at 24 hours of treatment (Figure 4.3B).

Nuclear-encoded mitochondrial transcripts localize to the mitochondria for translation.

FISH was carried out to determine localization of MRP translation. Under control conditions, MRP mRNA were localized in the vicinity of the mitochondria, and this localization persisted during administration of rapamycin, metformin, and the combination (Figure 4.4).

RT-PCR was conducted to verify assessment of localization by FISH. Following isolation of a mitochondrial-enriched fraction, mitochondrial RNA was extracted, and PCR was conducted on Mrps12. Treatment with rapamycin, metformin, and the combination significantly increased expression of Mrps12 in the mitochondrial fraction by five, two, and three-fold, respectively (Figure 4.5).

Discussion

Caloric restriction activates AMPK and inhibits mTOR, resulting in an upregulation of mitochondrial biogenesis and simultaneous inhibition of global protein synthesis. Here, we utilize pharmaceutical mimetics of CR and show that although the fractional synthesis of mitochondrial proteins is suppressed with rapamycin and metformin treatment, mitochondrial related proteins may be preferentially translated despite inhibition of protein synthesis. Further, we show that this preferential translation occurs in the vicinity of the mitochondria, thus proposing a mechanism by which mitochondrial protein synthesis may be maintained during energetic stress. These data are important in the context of aging, as the selective translation of mitochondrial proteins may contribute to longevity.

Selective translation of mitochondrial related proteins

Pharmaceutical treatment of cultured myotubes with compounds that mimic cellular energy stress resulted in AMPK activation and mTOR inhibition. Contrary to our hypothesis, treatment with rapamycin and metformin resulted in inhibition of cytosolic and mitochondrial protein synthesis. A possible explanation for the decrease in mitochondrial protein synthesis rate in this model relates to the content of mitochondrial protein in the fractionation protocol. Mitochondrial protein content in this study was low, and even minimal cytosolic contamination could have an impact on calculated synthetic responses. We therefore assessed the purity of our mitochondrial isolation. While our mitochondrial marker, voltage dependent anion channel 1

(VDAC1) was absent from the cytosolic fraction, the cytosolic marker desmin was still detectable in the mitochondrial fraction (Supplemental Figure 4.1). While in smaller abundance than the cytosolic fraction, the contamination of cytosolic proteins could have an impact on calculated protein synthesis measurements. Rapamycin and metformin are both potent inhibitors of mTOR [129, 130], and thus inhibitors of cytosolic protein synthesis. Therefore, we anticipate that small cytosolic contamination of an already small mitochondrial protein pool may have artificially skewed our results. Our previous publications *in vivo* have shown less cytosolic contamination in the mitochondrial fraction, in addition to a larger quantity of mitochondrial proteins [131], perhaps providing more confidence in a pure mitochondrial protein synthesis measure. Therefore, subsequent studies should aim to optimize the mitochondrial protein isolation protocol for *in vitro* models in order to eliminate cytosolic protein contamination.

An additional explanation has to do with the methodology employed to measure synthesis rates. The D_2O method measures the synthesis of all proteins. Thus, while mitochondrial protein synthesis appears to be suppressed by metformin and rapamycin treatment, it is feasible that specific proteins within the mitochondrial fraction may be stimulated. It is therefore of interest to identify mitochondrial targets which may be similarly preferentially translated during energetic stress to attempt to understand which mitochondrial proteins may be key in mediating the energy-trade off and associated longevity. Current studies are ongoing in our laboratory to measure the synthesis rates of specific mitochondrial proteins, to elucidate which proteins are preferentially translated under conditions of limited energy availability.

Mitochondrial related proteins localize to the mitochondria for translation

mRNA organization is important for the efficiency of translation, and is a widespread mechanism that allows local control of protein synthesis to occur at a specific time and place within the cell [122, 132]. Since MRPs need to be imported into mitochondria to be functional, it has been suggested that translation may occur in the vicinity of the mitochondria, thus allowing

for co-translational import. In yeast, active ribosomes encoding mitochondrial-destined proteins accumulate on the surface of the mitochondria, with more than 50% of nuclear-encoded mitochondrial transcripts spatially located near the organelle [124]. The Puf family of proteins is a family of translational regulatory proteins that repress the translation of their target RNA [133]. One of the best known family members is Puf3, an mRNA binding protein located in mitochondria [134]. The Puf3 protein has been shown to facilitate the localization of nearly half of nuclear genes encoding MRPs close to the mitochondria to subsequently be imported [123]. Puf3 is tethered to the outer mitochondrial membrane via an interaction with translocase of the mitochondrial outer membrane (TOM20) and represses translation until in the vicinity of the mitochondria [123]. Here, we qualitatively show that under basal conditions, the nuclear-encoded MRP β-ATP synthase localizes to the mitochondria, and this localization persists during energetic stress. Isolation of mitochondrial RNA confirmed our FISH results, as the expression of the nuclear-encoded Mrps12 increased in the mitochondrial fraction with treatment of rapamycin and metformin. Thus it appears that translational segregation is important in the translation of mitochondrial proteins in our mammalian cultured cell model, and may allow translational control of MRPs.

Elegant genome-wide characterization studies of mitochondrial localized mRNA have identified the structural element responsible for mRNA localization [124]. The translocation of specific mRNA to the mitochondria requires a *cis*-acting elements present in the RNA molecule. mRNA located close to the organelle were shown to have a 3' untranslated region (3'UTR) conferring mitochondrial targeting properties [124]. These data suggest that mitochondria targeted mRNA are likely to possess a similar 3'UTR that acts as a common zip code, and could aid in the identification of mRNA that should be preferentially translated during global translation inhibition.

Additional mechanisms of preferential translation: mRNA structure

We have shown that MRP can be preferentially translated during energetic stress, when global mTOR regulated protein synthesis is downregulated. In order for MRPs to be selectively translated during these conditions, there must be signals within the MRP mRNA that facilitate their translation. Three possible locations for regulation within MRP mRNA have been identified- the 5'UTR, the 3'UTR, and the region of the open reading frame encoding the mitochondrial sequence.

Caloric restriction studies in *drosophila* have identified a common signal in the 5'UTR of translationally active mRNA [135]. Under CR, when global translation is suppressed, mitochondrial genes were shown to be translationally upregulated [135]. Among the classes of genes with increased ribosome loading were those associated with electron transport chain function. Analysis of the sequence of these genes suggested that messages with a simple 5'UTR had increased ribosome loading compared to complex sequences. The 5'UTR of these proteins were found to be significantly shorter, with weaker secondary structure compared to the whole genome [135]. Although the mechanism for translational upregulation of mitochondrial proteins under energy stress remains unknown, it appears to be independent of an internal ribosomal entry site (IRES). Thus, control of mRNA translation by 5'UTR structure may represent a means of regulating preferential translation of key proteins under energy restriction. Future directions will use a bioinformatics approach to investigate whether mammalian 5'UTR display similar weaker and shorter secondary structure, and whether this facilitates sustained translation under energetic stress.

Perspectives and conclusions

Age and age-related diseases are characterized by mitochondrial dysfunction. CR, the most consistent and robust means to increase lifespan, activates AMPK and upregulates mitochondrial biogenesis. CR also inhibits mTOR and protein synthesis. Therefore, CR

maintains mitochondrial biogenesis in the face of decreased global protein synthesis by incompletely understood mechanisms. Lifelong CR animals maintain mitochondrial protein synthesis with age, in addition to a significant decrease in cellular proliferation [11], suggesting an energy trade-off may contribute to longevity. The hypothesis of an energy trade-off contributing to longevity is also supported by studies of long-lived mice chronically fed rapamycin, which show maintained mitochondrial protein synthesis despite decreased cytosolic protein synthesis [103]. Therefore, interventions that promote the maintenance of mitochondria during periods of limited energy availability may represent an effective method to combat the cellular stresses of chronic disease.

Here, we contribute to the understanding of this mechanism by using pharmaceutical mimetics of energy stress to activate AMPK and inhibit mTOR and show that mitochondrial related proteins can be preferentially translated. Further, we show that this translation occurs in the vicinity of the mitochondria, thus providing a mechanism by which the preferential translation may be permitted. Understanding the mechanisms by which mitochondrial biogenesis is maintained during limited energy availability may provide investigators with future means through which to attenuate the mitochondrial dysfunction associated with aging and chronic diseases.

Table 4.1. Oligonucleotide sequences

	Forward Primer	Reverse Primer
Mrps12	GAGGGCTAGGCCACGACT	CGCTAGGTTGGTGAGGGAC
ATP synthase	AGGCCCTTTGCCAAGCTT	TTCTCCTTAGATGCAGCAGAGTACA
pBiEx	GGGAATTGTGAGCGGATAAC	TGTCGTCGTCATCAATCGTA

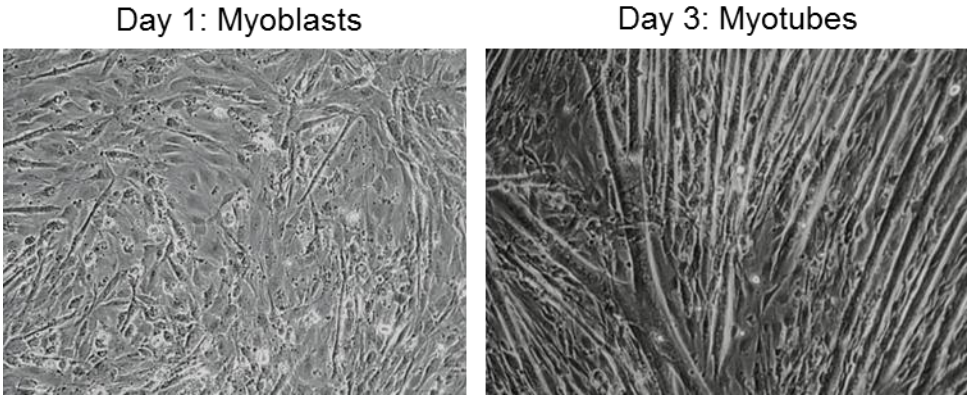


Figure 4.1. 10% horse serum media for 3 days was sufficient to induce differentiation. Proliferating myoblasts were maintained in DMEM supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. When cells reached confluence, the growth media was replaced with DMEM plus 10% horse serum for 3 days to induce differentiation.

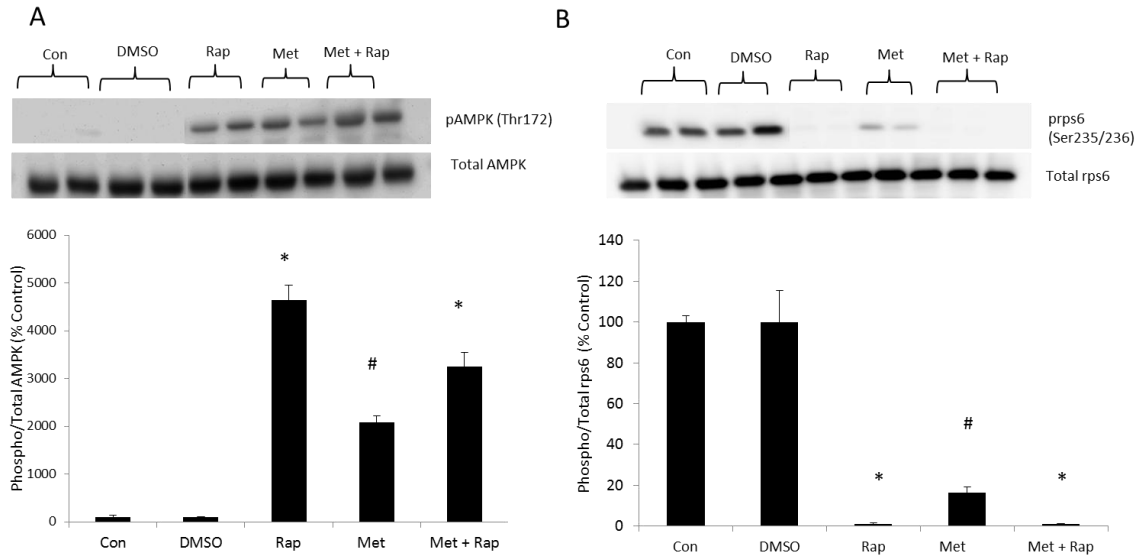


Figure 4.2. A. Treatment of cultured myotubes with rapamycin (Rap) or metformin (Met) activates AMPK. Co-treatment with Met and Rap does not further AMPK activation. B. Treatment of myotubes with Rap, Met, and the combination of Rap + Met inhibits mTOR as assessed by phosphorylation of downstream substrate ribosomal protein S6. AMPK and rps6 were assessed by western blotting and are expressed as a ratio of phospho/total, and percent of control. *p<0.05 compared to DMSO (the vehicle for Rap), #p<0.05 compared to Con (the media-only vehicle of Met). n=4 in each condition.

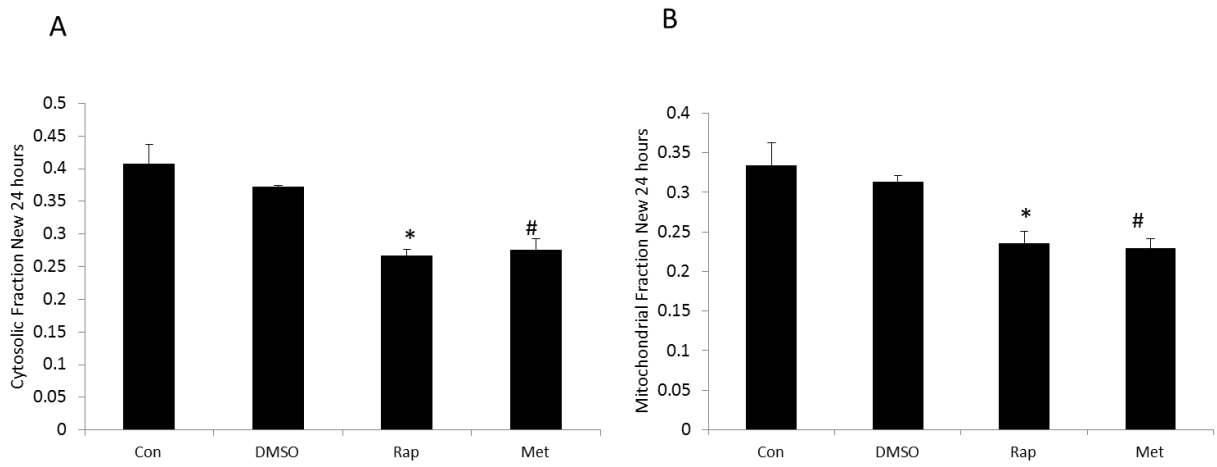


Figure 4.3. Treatment with metformin (Met) and rapamycin (Rap) significantly inhibits cytosolic (A) and mitochondrial (B) protein synthesis over a 24 hour period. Differentiated myotubes were treated for 24 hours with Met or Rap, with D_2O supplemented media to allow for calculation of new protein synthesis. *$p<0.05$ compared to DMSO (the vehicle for Rap), #$p<0.05$ compared to Con (the media-only vehicle of Met). n=3 in each condition.

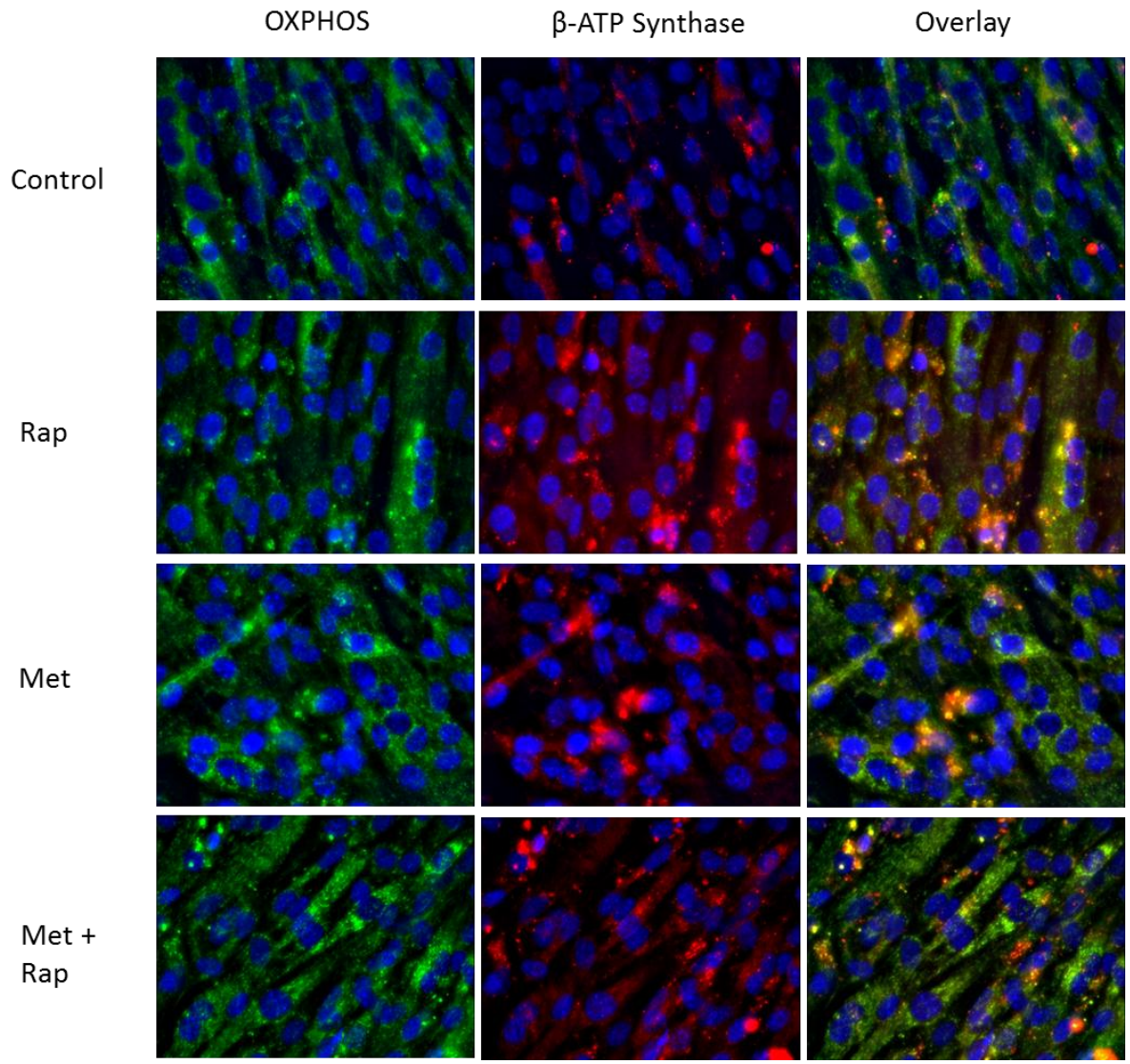


Figure 4.4. The nuclear-encoded mitochondrial transcript β-ATP synthase localizes to the mitochondrial for translation, and this localization persists during energetic stress. Localization of the mRNA was assessed by fluorescent in situ hybridization, alongside identification of mitochondria by total OXPHOS. Green fluorescence identifies mitochondrial proteins, red indicates β-ATP synthase mRNA, and the overlay (yellow/orange) shows overlap of these two fluorophores. The experiment was repeated on three separate occasions, with an n=2 in each condition, and representative images are shown.

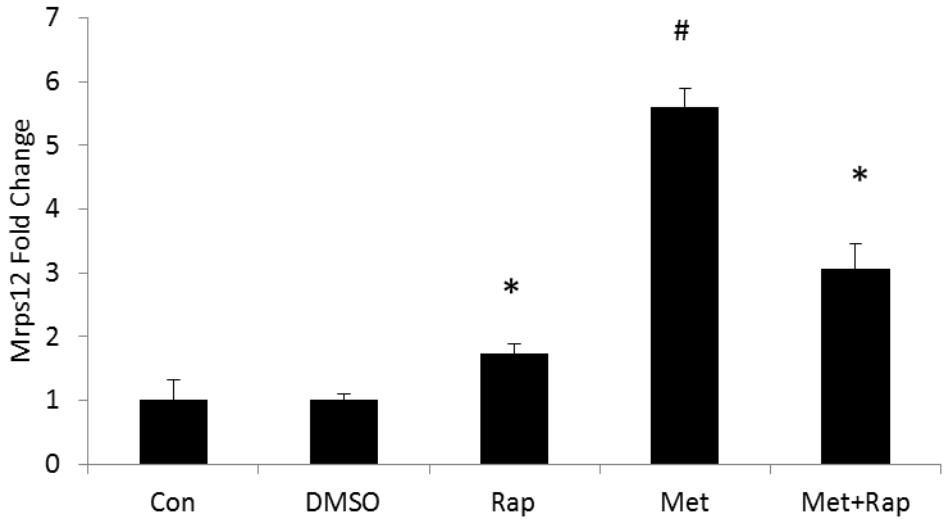
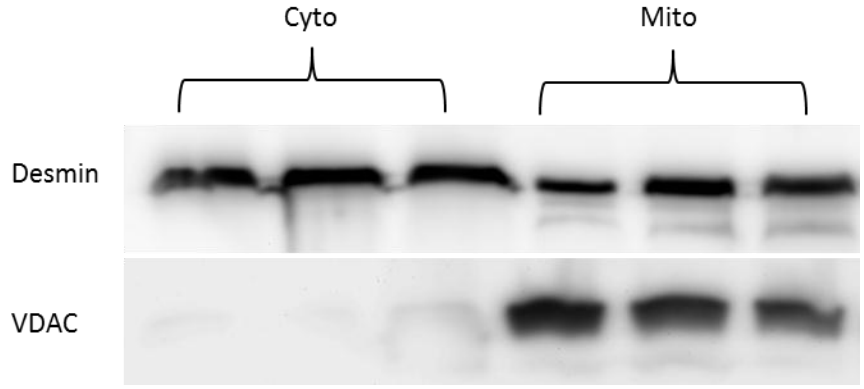


Figure 4.5. Mitochondrial localization of mitochondrial ribosomal protein S12 (Mrps12) increases following energetic stress. Myotubes were treated with metformin (Met), rapamycin (Rap), or the combination for 24 hours, and mitochondrial RNA was isolated and reverse transcribed. Real-time PCR was conducted on the nuclear-encoded mitochondrial transcript Mrps12, and fold changes were calculated relative to an exogenous spike and corresponding control. *$p<0.05$ compared to DMSO (the vehicle for Rap), #$p<0.05$ compared to Con (the media-only vehicle of Met). n=5 (Con) and 6 (all other conditions).



Supplemental Figure 4.1. Purity of mitochondrial isolation. Myotubes were fractioned per previously published methods *in vivo* [103]. Equal quantities of cytosolic (Cyto) and mitochondrial (Mito) proteins were prepared for immunoblotting. Desmin (Santa Cruz 14026) and voltage dependent anion channel 1 (VDAC1) (Santa Cruz 32063) were used to probe for cytosolic and mitochondrial proteins, respectively.

CHAPTER V- OVERALL CONCLUSIONS

Cardiovascular disease (CVD), the leading cause of death and disability within industrialized countries, is associated with increased production of reactive oxygen species. Enhanced endogenous antioxidant defenses are a potential means to counteract the oxidant stress associated with CVD. The transcription factor Nrf2 controls the expression of over 200 cytoprotective genes, making it a therapeutic target for CVD. We show that treatment of cardiac myocytes with a well-defined phytochemical combination activates Nrf2 and induces the expression of cytoprotective antioxidant enzymes. Treatment with the Nrf2 activator significantly protects cells against oxidant-induced apoptosis in an Nrf2-dependent manner. Here, we show that stabilization of Nrf2 mRNA, enhanced degradation of inhibitor Keap1, and stabilization of Nrf2 protein by p21 appear to not be the mechanisms responsible for increased Nrf2 activation by our phytochemical combination. Future research is warranted to identify the means by which these phytochemicals activate Nrf2. However, these results support the use of phytochemicals in protection of cardiac myocytes against oxidant stress and suggest their potential use in treatment of CVD.

We hypothesized that tissues from long-lived animals would display greater Nrf2 signaling than controls, as a potential mechanism for their enhanced stress resistance. However, we found that in the three models examined, Nrf2 and its target antioxidant enzymes were not significantly greater in the long-lived animals with significant variation existing between the longevity models and between tissues. In addition, we examined sexual dimorphism in Nrf2 and its targets since in most models of slowed aging, longevity is known to be greater in females than males. We provide novel results that female mice across two background strains display significantly greater expression of NQO1, a highly responsive Nrf2 target gene, compared to males. We also elucidated model by sex interactions, suggesting some of these

longevity interventions differentially influence male versus female mice, perhaps as a result of the differing hormonal milieu between sexes. These results provide a foundation to further elucidate Nrf2 responses in long-lived models during stress treatments in order to further elucidate how Nrf2 may mediate stress resistance and longevity.

Lifespan can be extended through interventions that inhibit mTOR and promote mitochondrial protein synthesis through activation of AMPK through incompletely understood mechanisms. Mitochondrial protein synthesis is also energetically expensive, and under the same energetic constraints as global protein synthesis. Therefore, there must be a mechanism that permits mitochondrial proteins to be translated when global protein synthesis is inhibited. Here, we show that under pharmaceutical energetic stress, that mitochondrial related proteins may be preferentially translated, even when mTOR and cytosolic protein synthesis are inhibited. We show two nuclear-encoded mitochondrial proteins have increased ribosomal loading under energy stress. Further, we begin to establish one of the mechanisms by which this preferential translation may occur, through mitochondrial localization for translation, which may facilitate their sustained translation during inhibited cytosolic translation.

The present body of work aimed to understand the roles of oxidative and energetic stress on age-related disease, with the goal of optimizing this information for interventions that attenuate these diseases. Specifically, we demonstrated that activation of Nrf2 may protect cardiac cells against oxidant-induced stress and we explore the mechanism by which phytochemicals activate Nrf2. Further, we characterize Nrf2 signaling across tissues from long-lived models, and future studies should assess whether a heightened ability to activate Nrf2 upon stressful stimuli may explain the enhanced stress resistance of long-lived animals. Last, we assess selective translation of mitochondrial proteins during energetic stress as a means of understanding how energetic interventions that promote longevity selectively facilitate the translation of key mitochondrial proteins. Taken together, these studies provide the basis for

future work aimed at attenuating diseases with oxidant stress and mitochondrial dysfunction component.

REFERENCES

1. Kenyon, C.J., *The genetics of ageing.* Nature, 2010. **464**(7288): p. 504-12.
2. Hepple, R.T., *Why eating less keeps mitochondria working in aged skeletal muscle.* Exerc Sport Sci Rev, 2009. **37**(1): p. 23-8.
3. Innamorato, N.G., et al., *Different susceptibility to the Parkinson's toxin MPTP in mice lacking the redox master regulator Nrf2 or its target gene heme oxygenase-1.* PLoS One, 2010. **5**(7): p. e11838.
4. Calkins, M.J., et al., *The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease.* Antioxid Redox Signal, 2009. **11**(3): p. 497-508.
5. Aleksunes, L.M. and J.E. Manautou, *Emerging role of Nrf2 in protecting against hepatic and gastrointestinal disease.* Toxicol Pathol, 2007. **35**(4): p. 459-73.
6. Madamanchi, N.R., A. Vendrov, and M.S. Runge, *Oxidative stress and vascular disease.* Arterioscler Thromb Vasc Biol, 2005. **25**(1): p. 29-38.
7. Kapahi, P., M.E. Boulton, and T.B. Kirkwood, *Positive correlation between mammalian life span and cellular resistance to stress.* Free Radic Biol Med, 1999. **26**(5-6): p. 495-500.
8. Murakami, S., *Stress resistance in long-lived mouse models.* Exp Gerontol, 2006. **41**(10): p. 1014-9.
9. Lewis, K.N., et al., *Nrf2, a guardian of healthspan and gatekeeper of species longevity.* Integr Comp Biol, 2010. **50**(5): p. 829-43.
10. Vina, J. and C. Borras, *Women live longer than men: understanding molecular mechanisms offers opportunities to intervene by using estrogenic compounds.* Antioxid Redox Signal, 2010. **13**(3): p. 269-78.
11. Miller, B.F., et al., *A comprehensive assessment of mitochondrial protein synthesis and cellular proliferation with age and caloric restriction.* Aging Cell, 2011.
12. Miller, B.F., et al., *Calorie restriction does not increase short-term or long-term protein synthesis.* J Gerontol A Biol Sci Med Sci, 2013. **68**(5): p. 530-8.
13. Shinmura, K., et al., *Cardioprotective effects of short-term caloric restriction are mediated by adiponectin via activation of AMP-activated protein kinase.* Circulation, 2007. **116**(24): p. 2809-17.
14. Bolster, D.R., et al., *AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling.* J Biol Chem, 2002. **277**(27): p. 23977-80.
15. Wright, D.C., et al., *Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1alpha expression.* J Biol Chem, 2007. **282**(1): p. 194-9.
16. Aruoma, O.I., H. Kaur, and B. Halliwell, *Oxygen free radicals and human diseases.* J R Soc Health, 1991. **111**(5): p. 172-7.
17. Ergin, A., et al., *Secular trends in cardiovascular disease mortality, incidence, and case fatality rates in adults in the United States.* American Journal of Medicine, 2004. **117**(4): p. 219-27.
18. Willcox, B.J., J.D. Curb, and B.L. Rodriguez, *Antioxidants in cardiovascular health and disease: key lessons from epidemiologic studies.* American Journal of Cardiology, 2008. **101**(10A): p. 75D-86D.
19. Bjelakovic, G., et al., *Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis.* Journal of the American Medical Association, 2007. **297**(8): p. 842-57.
20. Lonn, E., et al., *Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial.* JAMA, 2005. **293**(11): p. 1338-47.

21. Eggler, A.L., K.A. Gay, and A.D. Mesecar, *Molecular mechanisms of natural products in chemoprevention: induction of cytoprotective enzymes by Nrf2.* Mol Nutr Food Res, 2008. **52 Suppl 1**: p. S84-94.
22. Hybertson, B.M., et al., *Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation.* Mol Aspects Med, 2011. **32**(4-6): p. 234-46.
23. Surh, Y.J., J.K. Kundu, and H.K. Na, *Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals.* Planta Med, 2008. **74**(13): p. 1526-39.
24. Itoh, K., K.I. Tong, and M. Yamamoto, *Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles.* Free Radical Biology and Medicine, 2004. **36**(10): p. 1208-13.
25. Li, J., et al., *Targeting the Nrf2 pathway against cardiovascular disease.* Expert Opin Ther Targets, 2009. **13**(7): p. 785-94.
26. Clements, C.M., et al., *DJ-1, a cancer- and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2.* Proc Natl Acad Sci U S A, 2006. **103**(41): p. 15091-6.
27. Pugazhenthi, S., et al., *Regulation of heme oxygenase-1 expression by demethoxy curcuminoids through Nrf2 by a PI3-kinase/Akt-mediated pathway in mouse beta-cells.* Am J Physiol Endocrinol Metab, 2007. **293**(3): p. E645-55.
28. Kang, K.W., S.J. Lee, and S.G. Kim, *Molecular mechanism of nrf2 activation by oxidative stress.* Antioxididant Redox Signaling, 2005. **7**(11-12): p. 1664-73.
29. Jaiswal, A.K., *Nrf2 signaling in coordinated activation of antioxidant gene expression.* Free Radical Biology and Medicine, 2004. **36**(10): p. 1199-207.
30. Zhang, D.D., *Mechanistic studies of the Nrf2-Keap1 signaling pathway.* Drug Metab Rev, 2006. **38**(4): p. 769-89.
31. Dinkova-Kostova, A.T., et al., *Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants.* Proceedings of the National Academy of Science U S A, 2002. **99**(18): p. 11908-13.
32. Martin, D., et al., *Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol.* J Biol Chem, 2004. **279**(10): p. 8919-29.
33. Piantadosi, C.A., et al., *Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via Nrf2-mediated transcriptional control of nuclear respiratory factor-1.* Circ Res, 2008. **103**(11): p. 1232-40.
34. Chen, X.L. and C. Kunsch, *Induction of cytoprotective genes through Nrf2/antioxidant response element pathway: a new therapeutic approach for the treatment of inflammatory diseases.* Current Pharmaceutical Design, 2004. **10**(8): p. 879-91.
35. Stepkowski, T.M. and M.K. Kruszewski, *Molecular cross-talk between the NRF2/KEAP1 signaling pathway, autophagy, and apoptosis.* Free Radic Biol Med, 2011. **50**(9): p. 1186-95.
36. Donovan, E.L., et al., *Phytochemical activation of Nrf2 protects human coronary artery endothelial cells against an oxidative challenge.* Oxid Med Cell Longev, 2012. **2012**: p. 132931.
37. Judkins, C.P., et al., *Direct evidence of a role for Nox2 in superoxide production, reduced nitric oxide bioavailability, and early atherosclerotic plaque formation in ApoE-/- mice.* Am J Physiol Heart Circ Physiol, 2010. **298**(1): p. H24-32.
38. McCord, J.M., *Oxygen-derived free radicals in postischemic tissue injury.* N Engl J Med, 1985. **312**(3): p. 159-63.

39. Raedschelders, K., D.M. Ansley, and D.D. Chen, *The cellular and molecular origin of reactive oxygen species generation during myocardial ischemia and reperfusion.* Pharmacol Ther, 2012. **133**(2): p. 230-55.
40. Jakovljevic, V., D.Z. Djordjevic, and D.M. Djuric, *The effects of vitamin C and nitric oxide synthase inhibition on coronary flow and oxidative stress markers in isolated rat heart.* Gen Physiol Biophys, 2011. **30**(3): p. 293-300.
41. Motl, J., et al., *Vitamin C Compromises Cardiac Resuscitability in a Rat Model of Ventricular Fibrillation.* Am J Ther, 2012.
42. Yu, J., et al., *17beta-Estradiol regulates the expression of antioxidant enzymes in myocardial cells by increasing Nrf2 translocation.* J Biochem Mol Toxicol, 2012.
43. Muthusamy, V.R., et al., *Acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium.* Free Radic Biol Med, 2012. **52**(2): p. 366-76.
44. Claycomb, W.C., et al., *HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte.* Proceedings of the National Academy of Science U S A, 1998. **95**(6): p. 2979-84.
45. Velmurugan, K., et al., *Synergistic induction of heme oxygenase-1 by the components of the antioxidant supplement Protandim.* Free Radical Biology Medicine, 2009. **46**(3): p. 430-40.
46. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
47. Kwak, M.K., et al., *Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter.* Mol Cell Biol, 2002. **22**(9): p. 2883-92.
48. Niture, S.K. and A.K. Jaiswal, *Nrf2 protein up-regulates antiapoptotic protein Bcl-2 and prevents cellular apoptosis.* J Biol Chem, 2012. **287**(13): p. 9873-86.
49. Nguyen, T., et al., *Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome.* J Biol Chem, 2003. **278**(7): p. 4536-41.
50. Schwer, C.I., et al., *Up-regulation of heme oxygenase-1 by sevoflurane is not dependent on Kupffer cells and associates with ERK1/2 and AP-1 activation in the rat liver.* Int J Biochem Cell Biol, 2010. **42**(11): p. 1876-83.
51. Papaiahgari, S., et al., *NADPH oxidase and ERK signaling regulates hyperoxia-induced Nrf2-ARE transcriptional response in pulmonary epithelial cells.* J Biol Chem, 2004. **279**(40): p. 42302-12.
52. Alam, J., et al., *Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells. Role of p38 kinase and Nrf2 transcription factor.* Journal of Biological Chemistry, 2000. **275**(36): p. 27694-702.
53. Wu, C.C., et al., *Upregulation of endothelial heme oxygenase-1 expression through the activation of the JNK pathway by sublethal concentrations of acrolein.* Toxicol Appl Pharmacol, 2006. **214**(3): p. 244-52.
54. Huang, H.C., T. Nguyen, and C.B. Pickett, *Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2.* Proc Natl Acad Sci U S A, 2000. **97**(23): p. 12475-80.
55. Dreger, H., et al., *Characteristics of catechin- and theaflavin-mediated cardioprotection.* Experimental Biology Medicine (Maywood), 2008. **233**(4): p. 427-33.
56. Nelson, S.K., et al., *The induction of human superoxide dismutase and catalase in vivo: a fundamentally new approach to antioxidant therapy.* Free Radic Biol Med, 2006. **40**(2): p. 341-7.

57. Alam, J. and J.L. Cook, *Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway.* Curr Pharm Des, 2003. **9**(30): p. 2499-511.
58. Kanno, T., et al., *A novel small molecule, N-(4-(2-pyridyl)(1,3-thiazol-2-yl))-2-(2,4,6-trimethylphenoxy) acetamide, selectively protects against oxidative stress-induced cell death by activating the Nrf2-ARE pathway: Therapeutic implications for ALS.* Free Radic Biol Med, 2012.
59. Chan, K.H., M.K. Ng, and R. Stocker, *Haem oxygenase-1 and cardiovascular disease: mechanisms and therapeutic potential.* Clin Sci (Lond), 2011. **120**(12): p. 493-504.
60. Pachori, A.S., et al., *Heme-oxygenase-1-induced protection against hypoxia/reoxygenation is dependent on biliverdin reductase and its interaction with PI3K/Akt pathway.* J Mol Cell Cardiol, 2007. **43**(5): p. 580-92.
61. Exner, M., et al., *The role of heme oxygenase-1 promoter polymorphisms in human disease.* Free Radical Biology and Medicine, 2004. **37**(8): p. 1097-104.
62. Bussolati, B. and J.C. Mason, *Dual role of VEGF-induced heme-oxygenase-1 in angiogenesis.* Antioxidant Redox Signaling, 2006. **8**(7-8): p. 1153-63.
63. Deshane, J., M. Wright, and A. Agarwal, *Heme oxygenase-1 expression in disease states.* Acta Biochim Pol, 2005. **52**(2): p. 273-84.
64. Lee, Y., et al., *Exercise protects cardiac mitochondria against ischemia-reperfusion injury.* Med Sci Sports Exerc, 2012. **44**(3): p. 397-405.
65. Umemoto, S., et al., *Calcium antagonist reduces oxidative stress by upregulating Cu/Zn superoxide dismutase in stroke-prone spontaneously hypertensive rats.* Hypertens Res, 2004. **27**(11): p. 877-85.
66. Dalton, T.P., et al., *Genetically altered mice to evaluate glutathione homeostasis in health and disease.* Free Radic Biol Med, 2004. **37**(10): p. 1511-26.
67. Itoh, K., et al., *Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles.* Genes Cells, 2003. **8**(4): p. 379-91.
68. Wasserman, W.W. and W.E. Fahl, *Functional antioxidant responsive elements.* Proc Natl Acad Sci U S A, 1997. **94**(10): p. 5361-6.
69. Chen, W., et al., *Does Nrf2 Contribute to p53-Mediated Control of Cell Survival and Death?* Antioxid Redox Signal, 2012. **17**(12): p. 1670-5.
70. Chen, W., et al., *Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response.* Mol Cell, 2009. **34**(6): p. 663-73.
71. Jain, A., et al., *p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription.* J Biol Chem, 2010. **285**(29): p. 22576-91.
72. Reuland, D.J., et al., *Upregulation of phase II enzymes through phytochemical activation of Nrf2 protects cardiomyocytes against oxidant stress.* Free Radic Biol Med, 2012. **56C**: p. 102-111.
73. Chen, C.Y., N. Ezzeddine, and A.B. Shyu, *Messenger RNA half-life measurements in mammalian cells.* Methods Enzymol, 2008. **448**: p. 335-57.
74. Levonen, A.L., et al., *Cellular mechanisms of redox cell signalling: role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products.* Biochem J, 2004. **378**(Pt 2): p. 373-82.
75. Niture, S.K., A.K. Jain, and A.K. Jaiswal, *Antioxidant-induced modification of INrf2 cysteine 151 and PKC-delta-mediated phosphorylation of Nrf2 serine 40 are both required for stabilization and nuclear translocation of Nrf2 and increased drug resistance.* J Cell Sci, 2009. **122**(Pt 24): p. 4452-64.
76. Gao, L., et al., *Novel n-3 fatty acid oxidation products activate Nrf2 by destabilizing the association between Keap1 and Cullin3.* J Biol Chem, 2007. **282**(4): p. 2529-37.

77. Eggler, A.L., et al., *Cul3-mediated Nrf2 ubiquitination and antioxidant response element (ARE) activation are dependent on the partial molar volume at position 151 of Keap1.* Biochem J, 2009. **422**(1): p. 171-80.
78. Zhang, D.D., et al., *Ubiquitination of Keap1, a BTB-Kelch substrate adaptor protein for Cul3, targets Keap1 for degradation by a proteasome-independent pathway.* J Biol Chem, 2005. **280**(34): p. 30091-9.
79. Balogun, E., et al., *Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element.* Biochemical Journal, 2003. **371**(Pt 3): p. 887-95.
80. Keum, Y.S., et al., *Mechanism of action of sulforaphane: inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element-mediated heme oxygenase-1 in human hepatoma HepG2 cells.* Cancer Res, 2006. **66**(17): p. 8804-13.
81. Jain, A.K. and A.K. Jaiswal, *GSK-3beta acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2.* J Biol Chem, 2007. **282**(22): p. 16502-10.
82. Reuland, D.J., et al., *Upregulation of phase II enzymes through phytochemical activation of Nrf2 protects cardiomyocytes against oxidant stress.* Free Radic Biol Med, 2013. **56**: p. 102-11.
83. Rodriguez-Vilarrupla, A., et al., *Identification of the nuclear localization signal of p21(cip1) and consequences of its mutation on cell proliferation.* FEBS Lett, 2002. **531**(2): p. 319-23.
84. Copple, I.M., et al., *Physical and functional interaction of sequestosome 1 with Keap1 regulates the Keap1-Nrf2 cell defense pathway.* J Biol Chem, 2010. **285**(22): p. 16782-8.
85. Elbling, L., et al., *Hydrogen peroxide mediates EGCG-induced antioxidant protection in human keratinocytes.* Free Radic Biol Med, 2010. **49**(9): p. 1444-52.
86. Li, W., S.W. Yu, and A.N. Kong, *Nrf2 possesses a redox-sensitive nuclear exporting signal in the Neh5 transactivation domain.* J Biol Chem, 2006. **281**(37): p. 27251-63.
87. Shay, K.P., et al., *Cap-independent Nrf2 translation is part of a lipoic acid-stimulated detoxification stress response.* Biochim Biophys Acta, 2012. **1823**(6): p. 1102-9.
88. Purdom-Dickinson, S.E., et al., *Translational control of nrf2 protein in activation of antioxidant response by oxidants.* Mol Pharmacol, 2007. **72**(4): p. 1074-81.
89. Holcik, M. and N. Sonenberg, *Translational control in stress and apoptosis.* Nat Rev Mol Cell Biol, 2005. **6**(4): p. 318-27.
90. Li, W., et al., *An internal ribosomal entry site mediates redox-sensitive translation of Nrf2.* Nucleic Acids Res, 2010. **38**(3): p. 778-88.
91. Johnson, T.E., G.J. Lithgow, and S. Murakami, *Hypothesis: interventions that increase the response to stress offer the potential for effective life prolongation and increased health.* J Gerontol A Biol Sci Med Sci, 1996. **51**(6): p. B392-5.
92. Salmon, A.B., et al., *Fibroblast cell lines from young adult mice of long-lived mutant strains are resistant to multiple forms of stress.* Am J Physiol Endocrinol Metab, 2005. **289**(1): p. E23-9.
93. Harper, J.M., et al., *Skin-derived fibroblasts from long-lived species are resistant to some, but not all, lethal stresses and to the mitochondrial inhibitor rotenone.* Aging Cell, 2007. **6**(1): p. 1-13.
94. Harper, J.M., et al., *Fibroblasts from long-lived bird species are resistant to multiple forms of stress.* J Exp Biol, 2011. **214**(Pt 11): p. 1902-10.
95. Reuland, D.J., et al., *Upregulation of phase II enzymes through phytochemical activation of Nrf2 protects cardiomyocytes against oxidant stress.* Free Radic Biol Med, 2012.
96. Kim, J., Y.N. Cha, and Y.J. Surh, *A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders.* Mutat Res, 2010. **690**(1-2): p. 12-23.

97. Pickering, A.M., et al., *Nrf2-dependent induction of proteasome and Pa28alphabeta regulator are required for adaptation to oxidative stress.* J Biol Chem, 2012. **287**(13): p. 10021-31.
98. Lewis, K.N., et al., *Stress resistance in the naked mole-rat: the bare essentials - a mini-review.* Gerontology, 2012. **58**(5): p. 453-62.
99. Leiser, S.F. and R.A. Miller, *Nrf2 signaling, a mechanism for cellular stress resistance in long-lived mice.* Mol Cell Biol, 2010. **30**(3): p. 871-84.
100. Harrison, D.E., et al., *Rapamycin fed late in life extends lifespan in genetically heterogeneous mice.* Nature, 2009. **460**(7253): p. 392-5.
101. Holzenberger, M., et al., *IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice.* Nature, 2003. **421**(6919): p. 182-7.
102. Brown-Borg, H.M., et al., *Dwarf mice and the ageing process.* Nature, 1996. **384**(6604): p. 33.
103. Drake, J.C., et al., *Assessment of Mitochondrial Biogenesis and mTORC1 Signaling During Chronic Rapamycin Feeding in Male and Female Mice.* J Gerontol A Biol Sci Med Sci, 2013.
104. Sun, L., et al., *Life-span extension in mice by preweaning food restriction and by methionine restriction in middle age.* J Gerontol A Biol Sci Med Sci, 2009. **64**(7): p. 711-22.
105. Miller, R.A., et al., *Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice.* J Gerontol A Biol Sci Med Sci, 2011. **66**(2): p. 191-201.
106. Wilkinson, J.E., et al., *Rapamycin slows aging in mice.* Aging Cell, 2012. **11**(4): p. 675-82.
107. Flurkey, K., et al., *Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6736-41.
108. Miller, B.F., et al., *A comprehensive assessment of mitochondrial protein synthesis and cellular proliferation with age and caloric restriction.* Aging Cell, 2012. **11**(1): p. 150-61.
109. Swindell, W.R., *Dietary restriction in rats and mice: a meta-analysis and review of the evidence for genotype-dependent effects on lifespan.* Ageing Res Rev, 2012. **11**(2): p. 254-70.
110. Valle, A., et al., *Sexual dimorphism in liver mitochondrial oxidative capacity is conserved under caloric restriction conditions.* Am J Physiol Cell Physiol, 2007. **293**(4): p. C1302-8.
111. Kratschmar, D.V., et al., *Suppression of the Nrf2-dependent antioxidant response by glucocorticoids and 11beta-HSD1-mediated glucocorticoid activation in hepatic cells.* PLoS One, 2012. **7**(5): p. e36774.
112. Burkle, A., *Mechanisms of ageing.* Eye (Lond), 2001. **15**(Pt 3): p. 371-5.
113. Gounder, S.S., et al., *Impaired Transcriptional Activity of Nrf2 in Age-Related Myocardial Oxidative Stress Is Reversible by Moderate Exercise Training.* PLoS One, 2012. **7**(9): p. e45697.
114. Miller, C.J., et al., *Disruption of Nrf2/ARE signaling impairs antioxidant mechanisms and promotes cell degradation pathways in aged skeletal muscle.* Biochim Biophys Acta, 2012. **1822**(6): p. 1038-50.
115. Victor, V.M., et al., *Oxidative stress and mitochondrial dysfunction in type 2 diabetes.* Curr Pharm Des, 2011. **17**(36): p. 3947-58.
116. Ballinger, S.W., *Mitochondrial dysfunction in cardiovascular disease.* Free Radic Biol Med, 2005. **38**(10): p. 1278-95.
117. Hiona, A., et al., *Mitochondrial DNA mutations induce mitochondrial dysfunction, apoptosis and sarcopenia in skeletal muscle of mitochondrial DNA mutator mice.* PLoS One, 2010. **5**(7): p. e11468.

118. Druzhyna, N.M., G.L. Wilson, and S.P. LeDoux, *Mitochondrial DNA repair in aging and disease.* Mech Ageing Dev, 2008. **129**(7-8): p. 383-90.
119. Winder, W.W. and D.G. Hardie, *AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes.* Am J Physiol, 1999. **277**(1 Pt 1): p. E1-10.
120. Fok, W.C., et al., *Short-term treatment with rapamycin and dietary restriction have overlapping and distinctive effects in young mice.* J Gerontol A Biol Sci Med Sci, 2013. **68**(2): p. 108-16.
121. Jager, S., et al., *AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha.* Proc Natl Acad Sci U S A, 2007. **104**(29): p. 12017-22.
122. Eliyahu, E., et al., *Tom20 mediates localization of mRNAs to mitochondria in a translation-dependent manner.* Mol Cell Biol, 2010. **30**(1): p. 284-94.
123. Saint-Georges, Y., et al., *Yeast mitochondrial biogenesis: a role for the PUF RNA-binding protein Puf3p in mRNA localization.* PLoS One, 2008. **3**(6): p. e2293.
124. Marc, P., et al., *Genome-wide analysis of mRNAs targeted to yeast mitochondria.* EMBO Rep, 2002. **3**(2): p. 159-64.
125. Sylvestre, J., et al., *The role of the 3' untranslated region in mRNA sorting to the vicinity of mitochondria is conserved from yeast to human cells.* Mol Biol Cell, 2003. **14**(9): p. 3848-56.
126. Robinson, M.M., et al., *Long-term synthesis rates of skeletal muscle DNA and protein are higher during aerobic training in older humans than in sedentary young subjects but are not altered by protein supplementation.* FASEB J, 2011. **25**(9): p. 3240-9.
127. Busch, R., et al., *Measurement of protein turnover rates by heavy water labeling of nonessential amino acids.* Biochim Biophys Acta, 2006. **1760**(5): p. 730-44.
128. Miller, B.F. and K.L. Hamilton, *A perspective on the determination of mitochondrial biogenesis.* Am J Physiol Endocrinol Metab, 2011.
129. Guertin, D.A. and D.M. Sabatini, *The pharmacology of mTOR inhibition.* Sci Signal, 2009. **2**(67): p. pe24.
130. Mihaylova, M.M. and R.J. Shaw, *The AMPK signalling pathway coordinates cell growth, autophagy and metabolism.* Nat Cell Biol, 2011. **13**(9): p. 1016-23.
131. Robinson, M.M., et al., *Acute {beta}-adrenergic stimulation does not alter mitochondrial protein synthesis or markers of mitochondrial biogenesis in adult men.* Am J Physiol Regul Integr Comp Physiol, 2010. **298**(1): p. R25-33.
132. Russo, A., et al., *cis-acting sequences and trans-acting factors in the localization of mRNA for mitochondrial ribosomal proteins.* Biochim Biophys Acta, 2008. **1779**(12): p. 820-9.
133. Wickens, M., et al., *A PUF family portrait: 3'UTR regulation as a way of life.* Trends Genet, 2002. **18**(3): p. 150-7.
134. Garcia-Rodriguez, L.J., A.C. Gay, and L.A. Pon, *Puf3p, a Pumilio family RNA binding protein, localizes to mitochondria and regulates mitochondrial biogenesis and motility in budding yeast.* J Cell Biol, 2007. **176**(2): p. 197-207.
135. Zid, B.M., et al., *4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila.* Cell, 2009. **139**(1): p. 149-60.
136. Lo, S.C. and M. Hannink, *PGAM5 tethers a ternary complex containing Keap1 and Nrf2 to mitochondria.* Exp Cell Res, 2008. **314**(8): p. 1789-803.

APPENDIX I- Manuscript V

The Role of Nrf2 in the Attenuation of Cardiovascular Disease[2]

Summary

Oxidative stress is a component of many human diseases, including cardiovascular diseases (CVD). Exercise and various phytochemicals activate nuclear factor (erythroid-derived 2)-like 2 (Nrf2), the "master regulator" of antioxidant defenses, and attenuate CVD. This review highlights Nrf2 regulation by exercise and phytochemicals, and the role of Nrf2 as a therapeutic target in CVD.

Activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) by exercise and phytochemical supplementation represents a novel potential therapeutic target for cardiovascular diseases.

Key Words: antioxidants, cardioprotection, phytochemicals, oxidative stress, exercise

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INTRODUCTION

Oxidative stress is historically defined as the production of reactive oxygen species (ROS) in excess of cellular capacity to remove them. This overly simplistic definition suggests oxidative stress is a balance between oxidants and antioxidants and proposes that all pro-oxidants on one side are equally important, as are all antioxidants on the opposite side. Further, the definition of oxidative stress as a balance implies cells have the same sensitivities to a given oxidative stimulus, or an equivalent ability to respond to it. Experimental evidence suggests that cellular adaptations and damage vary widely with response to different oxidant species and, similarly, cellular antioxidants have a range of capabilities to offset the oxidants produced. Thus, a more useful definition of oxidative stress may be a “disruption of redox signaling and control” [1]. Despite the lack of consensus about an appropriate definition, over 100 human diseases involve an oxidative stress component in the etiology or exacerbation of disease, including cardiovascular diseases (CVD), the leading cause of death and disability in industrialized countries. Oxidative stress is evident in both the etiology and progression of myocardial infarction, congestive heart failure, atherosclerosis, and hypertension [2]. The purpose of this review is to outline the role of oxidative stress in CVD, and summarize evidence suggesting that activation of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) enhances endogenous antioxidant defenses and counteracts the oxidative stress associated with chronic diseases including CVD. Specifically, we will highlight exercise and phytochemical supplementation as potential Nrf2 activating CVD interventions.

THE FAILURE OF ANTIOXIDANTS AS CVD THERAPY

Antioxidants are broadly defined as substances that decrease the severity of oxidative stress. Antioxidant defenses protect the heart by catalytic quenching of ROS and via direct scavenging of ROS. A large network of endogenous antioxidant enzymes including superoxide dismutases (SOD), catalases, peroxidases, and reductases catalytically remove ROS. While

often requiring electron donors, antioxidant enzymes scavenge ROS without need for regeneration. In contrast, vitamins C, E, and beta carotene are dietary antioxidants that serve as redox active nonenzymes with short half-lives. Exogenous antioxidants are consumed in the process of their antioxidant action and, therefore, must be reduced back to their active form to react with another oxidant. Additionally, some exogenous antioxidants have the potential to produce pro-oxidant effects, suggesting that they may be less effective at mitigating oxidative stress biomarkers than endogenous antioxidant defenses [3].

Although supplementation with exogenous antioxidant vitamins for prevention or treatment of human diseases is well-studied, vitamins C and E are still only presumed effective for improving CVD outcomes. Early pre-clinical trials suggested antioxidants may be useful in preventing oxidative damage during cardiovascular insults such as ischemia reperfusion injury [4]. Although proof of principle exists with vitamins C and E supplementation in animal models of cardiovascular disease, the doses administered in clinical trials are much higher than the doses utilized in many of these pre-clinical trials [3]. In fact, large scale clinical trials of vitamins C and E have been generally disappointing, and a highly-publicized meta-analysis of 68 randomized clinical trials with placebo or no-intervention controls concluded that supplementation with vitamin E, vitamin A, and beta carotene increased all-cause mortality, while vitamin C and selenium resulted in no improvement in overall mortality or CVD outcomes [5].

It is still unclear why there has been a lack of efficacy in dietary antioxidant supplement trials. Hypotheses include incorrect dosage or route of administration, inappropriate time points for assessment of primary outcomes and stage of disease progression, or lack of efficacy of dietary antioxidants in scavenging the oxidants exerting the greatest oxidative stress [4]. Despite the lack of success in improving CVD outcomes, exogenous antioxidant vitamins may be beneficial for specific sub-populations of individuals [3, 4]. Identifying these individuals may aid in administration and efficacy of the vitamins.

Disappointing outcomes of clinical trials with exogenous antioxidants underscore the need for alternative approaches to regulating redox balance in CVD. One promising approach is via upregulation of endogenous networks of antioxidants, providing the potential for more profound cellular protection than antioxidant vitamin supplementation due to the enhanced ability of enzymatic antioxidants to scavenge ROS [6]. The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) has recently emerged as the "master regulator" of cellular antioxidant defenses [7] and a promising therapeutic target for promoting redox balance.

Nrf2: THE "MASTER REGULATOR" OF CELLULAR ANTIOXIDANT DEFENSES

Regulation of Nrf2 Signaling

Nrf2 is a member of the basic leucine zipper transcription factor family and controls basal and inducible expression of more than 200 genes [8]. Nrf2 is remarkably conserved across species, both in structure and in function, suggesting an integral role of Nrf2 in detoxification processes and mitigating oxidative stress. Under normal conditions, Nrf2 is sequestered in the cytoplasm by its involvement in an inactive complex with Kelch-like ECH-associated protein 1 (Keap1). Keap1, an actin-binding protein unique to Nrf2, targets Nrf2 for ubiquitination and degradation by the 26S proteasome system, resulting in basal low-level expression of Nrf2 target genes (Figure A.1A). Under these conditions, the Nrf2 protein has a half-life of approximately 15-20 minutes [9].

The best understood mechanism of Nrf2 activation is its induction by ROS. Upon exposure to oxidants, cysteine residues on the Keap1/Nrf2 complex become oxidized, altering the structure of Keap1. The Keap1/Nrf2 complex then dissociates, allowing Nrf2 to escape ubiquitination and proteasomal degradation [10]. As shown in Figure 1B, modification of the Keap1 cysteine residues stabilizes Nrf2, facilitating its translocation to and accumulation within the nucleus. After nuclear translocation, Nrf2 forms heterodimers with Maf and Jun bZip transcription factors, which bind to the 5'-upstream cis-acting regulatory sequence known as the

antioxidant or electrophile response element (ARE/EpRE) and induce transcription of phase II antioxidant enzymes. The ARE sequence contains a core 5'-G(/A)TGAC(/G)nnnGCA(/C)-3' *cis*-acting element shared among Nrf2-regulated genes [11].

In addition to reactive oxygen and other electrophilic species, phytochemicals such as curcumin, resveratrol, and sulforaphane can also activate Nrf2 [12] (Figure A.1C). Although phytochemicals likely activate Nrf2 by a variety of mechanisms, it appears that some can induce Nrf2 independent of electrophilic reaction with Keap1 cysteine residues. Sulforophane and resveratrol activate various kinase signaling cascades that phosphorylate Nrf2 resulting in release from Keap1 [12]. Although ROS-mediated Nrf2 activation can also coincide with stimulation of redox sensitive mitogen activated protein kinases (MAP Kinases), activation of MAP Kinases is not compulsory for electrophile-induced Nrf2 activation. Kinases implicated in the phosphorylation and subsequent activation of Nrf2 include phosphatidylinositide 3-kinase (PI3K), extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MEK/ERK), p38MAPK, c-Jun N-terminal kinases (JNK), and protein kinase C [12]. While activation of Nrf2 by ROS or other electrophiles necessitates compensation for the initial oxidative insult, activation by non-oxidative methods avoids this need for compensation. Individual phytochemicals induce Nrf2 utilizing these stress-signaling pathways, with curcumin contributing to Nrf2 activation through p38MAPK, and epigallocatechin-3-gallate upregulating Nrf2 target genes in endothelial cells through PI3K/Akt-dependent induction. Activation of Nrf2 by combinations of phytochemicals, therefore, can result in a synergistic upregulation of target genes by utilizing various signaling pathways [13]. When activated by phytochemical treatment, the Nrf2 protein becomes stabilized, facilitating its nuclear translocation and transcriptional regulation of antioxidant enzymes.

There is accumulating evidence that Nrf2 activators can act by two non-mutually exclusive mechanisms; attenuation of Keap1 mediated ubiquitination and enhanced translation of Nrf2 mRNA [14]. Until recently, the mechanisms of enhanced translation of Nrf2 mRNA

under periods of oxidative stress were unknown. Electrophilic compounds have been shown to activate cap-independent translation of Nrf2, allowing preferential translation of the transcription factor during periods of cell stress. An internal ribosomal entry site (IRES) permits redox-sensitive translation of Nrf2 and allows for increased polysomal loading under conditions of cell stress [15]. An IRES in the 5' untranslated region of the Nrf2 mRNA allows preferential translation of the protein under conditions where global protein synthesis is diminished. The tight regulation of Nrf2 signaling, as well as mechanisms that allow translation to occur during periods of environmental or cellular challenge, further highlight the importance of Nrf2 activation in responding to cell stress and the diseases associated with oxidative stress.

Cytoprotective Functions of Nrf2 Target Genes

Classical Nrf2-regulated genes support cellular redox homeostasis and phase I detoxification functions [8] (Figure A.1). Transcription of over 100 genes, including phase II antioxidant enzymes such as heme-oxygenase1 (HO-1), catalase, glutathione peroxidase (GPx), superoxide dismutase, thioredoxin, NAD(P)H quinone oxidoreductase-1 (NQO1), and glutathione S-transferase (GST), is directly regulated by activated Nrf2 [8]. The coordinated induction of Nrf2-mediated gene expression is crucial for cells to maintain redox homeostasis. The expression of detoxification and antioxidant enzymes is significantly blunted in Nrf2 deficient mice, and these animals are more sensitive to carcinogenesis [16]. While transcript levels of 292 genes were elevated in wild type mice 24 hours after treatment with known Nrf2 activator 3H-1,2-dithiole-3-thione (D3T), only 15 of these antioxidant enzymes were induced in Nrf2-deficient mice [16]. In addition to inducing transcription of a battery of antioxidant and chemoprotective enzymes, Nrf2 regulates its own expression. Two ARE-like motifs in the 5' flanking region of the Nrf2 promoter are responsible for the induction of Nrf2 upon Nrf2 activation [17]. Therefore, a feed-forward process ensues, with Nrf2 activation promoting its own expression, thus facilitating a profound cellular response to stress.

In addition to regulating a battery of antioxidant enzymes, Nrf2 also regulates transcription of genes not directly involved in antioxidant activities (Figure A.2). Nrf2 is involved in regulation of mitochondrial biogenesis through an ARE motif in the promoter of nuclear respiratory factor 1 (NRF1) [18]. When oxidant production activated Nrf2, subsequent induction of NRF1 resulted in upregulated mitochondrial biogenic signaling and synthesis of mitochondrial DNA. Direct interactions with cell cycle regulators p21 and p53 suggest Nrf2 may regulate cell proliferation and apoptosis [19], placing Nrf2 in a position to regulate cell survival versus cell death decisions. Further, the identification of an ARE motif in the promoter of selective autophagy cargo receptor p62 [20] as well as various proteasomal subunits [21], suggests Nrf2 may regulate removal of oxidatively damaged proteins and organelles. These novel functions of Nrf2 that extend beyond classical antioxidant functions further highlight the importance of Nrf2 in maintaining homeostasis in response to cellular oxidative insult.

Nrf2 activators are not “one size fits all” and can activate differential gene expression based on the mechanism of activation. For example, microarray and proteomic analyses confirm that only 14% of the genes modulated by sulforaphane are similarly modulated by genetic Keap1 knockdown [6]. Therefore, it is possible that decisions about the type of Nrf2 activator selected for specific disease states could be based on predicted target gene activation. Nrf2 binds to a variety of other proteins in addition to Keap1, which compete to stabilize or destabilize Nrf2 [19]. For example, the cell cycle regulator p21 competes with Keap1 for Nrf2 binding, allowing Nrf2 to escape Keap1-mediated proteasomal degradation and translocate to the nucleus (Figure A.2). The selective autophagy cargo receptor p62 also interacts in the Keap1-Nrf2 complex, and promotes Nrf2 activation by selective autophagic degradation of the Keap1 protein [20]. Therefore, induction of Nrf2 binding partners can activate Nrf2 and may result in differential target gene expression. Understanding how Nrf2 is stabilized and activated by interactions with other proteins is required to optimize the therapeutic potential of Nrf2 activation in CVD and other chronic diseases.

EXERCISE AND CVD PROTECTION

Endurance exercise is a well-established intervention to improve the tolerance of the myocardium and vasculature against oxidative injury. Although high concentrations of ROS are detrimental to cellular function, mild oxidative stress, such as the levels produced during moderate exercise, produces a stimulus for physiological antioxidant adaptation [22]. This "stress without distress" has led to the current understanding of exercise as an example of hormesis, whereby a moderate degree of oxidative stress during exercise results in beneficial adaptation [22]. Activation of redox-sensitive cell signaling is not only responsible for, but perhaps necessary for, the adaptations that occur following exercise. When redox signaling is blunted through exogenous antioxidant supplementation, many beneficial exercise adaptations are attenuated [22]. While untested experimentally, it is possible that Nrf2 might be well-positioned to regulate exercise induced adaptations to redox-sensitive cell signaling. A member of the phosphoglycerate mutase family, PGAM5, is a Keap1-binding protein [23]. Hypothetically the PGAM5-Keap1-Nrf2 complex could translocate to the mitochondria during periods of increased ROS production (Figure 2), allowing the Nrf2 complex to "sense" changes in redox balance and facilitate decisions of cellular survival. A direct relationship between Nrf2 and redox sensitive cell signaling in response to exercise has yet to be established. Nrf2 regulates cell survival pathways in response to electrophilic and oxidative stresses, therefore, Nrf2 may also play an integral role in mediating beneficial cellular adaptations to exercise.

Human as well as animal studies, extensively reviewed elsewhere, document that chronic aerobic exercise protects the heart and vasculature against maladaptive stress [24, 25]. The earliest evidence for exercise as a protector against cardiac oxidative stress came from studies of ischemia-reperfusion injury. Ischemic heart disease can be directly tied to other lethal cardiac dysfunctions including arrhythmias and congestive heart failure. While initially acknowledged that the interruption in blood flow was responsible for cellular damage, it is now known that the subsequent restoration of myocardial blood flow with tissue reoxygenation also

leads to cell injury. Collectively, this interruption and restoration of blood, termed ischemia reperfusion (IR) injury, results in contractile dysfunction, myocyte injury, and cell death mediated at least in part by oxidative stress [25].

Chronic exercise training (10 days) as well as acute exercise bouts both afford cardioprotection in rodent models with protection persistent for up to 9 days following cessation of exercise [25]. An ongoing challenge is to determine how much exercise (intensity and duration) is necessary for protection against IR injury. Rat treadmill running of 30-60 minutes at speeds of 27-33 m/min is typically used as the exercise stimulus [24], an intensity which amounts to approximately 75% VO_2max and consistently confers protection against myocardial infarction-mediated cell injury. Lower-intensity treadmill running yields equivocal results, with some groups suggesting improved functional recovery of the heart following IR injury with exercise, and others finding no protection [24]. The direct relationship between exercise intensity/duration and cardioprotection is still unclear and should be addressed in future studies. Despite extensive knowledge that exercise is cardioprotective against IR injury, the exact mechanisms responsible for this protection have remained elusive. Proposed cellular adaptations including improvements in calcium handling, heat shock protein expression, and ATP-sensitive potassium channels [25] may contribute to exercise induced cardioprotection. However, for the purposes of this review, we will focus on the role of endogenous antioxidants in exercise-induced cardioprotection.

Non-pathological production of ROS during exercise activates a transcriptional program of antioxidant enzymes. Of these antioxidant enzymes, manganese superoxide dismutase (MnSOD), the mitochondrial isoform of SOD, is the most consistently increased antioxidant enzyme following exercise training [25]. An early investigation of the role of MnSOD in exercise-induced cardioprotection reported that prevention of exercise-induced increases in MnSOD by oligonucleotide gene silencing abolished protection against myocardial infarction [26]. A subsequent investigation confirmed these findings and demonstrated that exercise-

induced increases in MnSOD protect against ischemia-reperfusion induced cardiac arrhythmias (Reviewed in [25]). Moreover, exercised-induced increases in myocardial MnSOD have been found to be partially responsible for the protective effect of exercise against IR induced cardiac apoptosis (Reviewed in [25]). Alongside increases in MnSOD, chronic exercise training results in attenuation of lipid peroxidation and protein carbonylation, as well as increases in total cardiac glutathione content [24]. Intensity of exercise also appears to be critical in determining activity of MnSOD, with low-intensity treadmill running less effective at stimulating antioxidant enzyme changes than high intensity training. Although it is clear that not all antioxidant enzymes respond similarly to acute or chronic exercise, and intensity appears to be an important determining factor, cellular antioxidant defenses generally increase with endurance exercise training.

ACTIVATION OF Nrf2 AS CVD THERAPY

The therapeutic potential of Nrf2 activation in neurodegenerative diseases [27], cancer [28], and hepatic/gastrointestinal diseases [29] has been reviewed. However, identification of the therapeutic potential for Nrf2 activation in cardiovascular diseases is in the early stages. Here, we present data from our group on phytochemical induced Nrf2 activation, as well as highlight two studies of exercise induced Nrf2 activation, and the potential for these mechanisms in attenuating oxidative damage associated with CVD.

Nrf2 activators vary in their chemical properties as well as the mechanisms by which they activate the transcription factor. One well described synergistic combination of phytochemicals, commercially available as Protandim (LifeVantage Corp), elicits robust increases in Nrf2 regulated gene expression and an improved capacity to maintain redox balance in a variety of cell types [30, 31]. Protandim is a phytochemical Nrf2 activator composed of *Bacopa monnera* (45% bacosides)*, Silybum marianum* (70-80% silymarin), *Withania somnifera* (0.5% withaferin A), *Camellia sinensis* (98% polyphenols and 45% epigallocatechin-

3-gallate), and *Curcuma longa* (95% curcumin). Together, treatment with the phytochemical combination in Protandim results in an activation of Nrf2 that far exceeds that achieved by any single phytochemical compound [13]. Because of this synergistic effect on Nrf2 activation, the dose of each phytochemical required is very low and use of Protandim in humans is safe, with no reported adverse side effects. The first trial of Protandim supplementation in humans demonstrated that within 5-12 days, plasma TBARS, a measure of lipid peroxidation, was significantly attenuated in the treatment group compared to controls. Although at baseline subjects displayed an age-related trend of increasing TBARS, subjects in the Protandim-supplemented group demonstrated a 40% decrease in TBARS, resulting in a redox status corresponding to a comparatively younger age than their control counterparts [32]. Further, the treatment group demonstrated a significant increase in erythrocyte SOD and catalase activity with a strong trend towards increased uric acid, an endogenous antioxidant.

Using *in vitro* models of cardiovascular oxidative stress, we show that Nrf2 activation by Protandim protects against apoptotic cell death in coronary artery endothelial cells [30] and cardiac myocytes [31]. Activation of Nrf2 resulted in a robust induction of HO-1, a novel therapeutic target in the management of CVD. Additionally, treatment of cultured endothelial cells and cardiomyocytes with the phytochemical Nrf2 activator resulted in significant induction of NQO1, CuZnSOD, and glutathione reductase (GR). Although we have yet to investigate an extensive array of antioxidants, it is expected that other phase II enzymes with ARE motifs will be similarly upregulated, resulting in a battery of antioxidant response mechanisms. In particular, activation of Nrf2 by Protandim should enhance synthesis and reduction of glutathione via regulation of glutathione-S-transferase (GST), glutamate-cysteine ligase (GCL), and GR. Reduced glutathione (GSH) is a critical and abundant cellular antioxidant, and decreased GSH levels correlate with numerous risk factors for CVD including smoking, aging, and obesity. Impaired Nrf2 activation may be responsible for the diminished capacity to maintain GSH with age [33]. By inducing phase II enzymes and regulating cellular GSH

homeostasis, activation of Nrf2 enhances antioxidant protection and prevents dysregulation of redox balance with far greater therapeutic potential than exogenous antioxidants such as vitamins C and E.

Activation of Nrf2 is critical in the defense against a variety of cardiovascular stresses including high glucose-induced oxidative damage and oxidized phospholipids, and Nrf2 activation protects the heart against pathological cardiac hypertrophy [8]. Compared to wild type cells, Nrf2 knockout cardiomyocytes are significantly more susceptible to hydrogen peroxide, peroxynitrite, and 4-hydroxy-2-noneal (4HNE) induced cell injury [34]. While treatment of wild-type cardiomyocytes with the synthetic Nrf2 activator D3T upregulated cellular defenses and protected cells against oxidant-induced death, treatment of Nrf2-/- cardiomyocytes did not. Atheroprone regions of mouse aorta exhibit diminished Nrf2 activation compared with regions that are protected against atheroma development [35], highlighting the role of Nrf2 in protecting against atherogenesis. Further, ischemic preconditioning, which leads to robust cardioprotective effects, also activates Nrf2, and subsequently protects the myocardium against oxidative stress and IR injury [36]. Thus, *in vitro* and *in vivo* models of cardiovascular disease indicate that activation of Nrf2 by a variety of phytochemical and synthetic compounds protects the heart and vasculature against oxidative stress. Therefore, these models suggest Nrf2 activators may have significant therapeutic potential against CVD.

Exercise has also been shown to activate Nrf2, resulting in cytoprotection against subsequent oxidative insult. Exercise-induced Nrf2 activation was first elucidated in rat kidney and human skeletal muscle [37]. More recently, in an acute exercise model in mice, exercise activated Nrf2 and Nrf2 binding to the ARE. In this investigation, Nrf2 -/- mice, upon exposure to the exercise stimulus, exhibited increased cardiac oxidative stress due to lower basal and exercise-induced expression of antioxidant enzymes [37]. The authors concluded that acute exercise stress promotes Nrf2 activation through ROS signaling, but disruption of Nrf2 increases susceptibility of the heart to oxidative damage. This group later reported that aging impairs

transcriptional Nrf2 activity and is associated with increases in myocardial oxidative stress, an impairment that can be reversed by moderate exercise training [38]. Therefore, endurance exercise training, an intervention leading to cardioprotection via a variety of mechanisms, also results in Nrf2 activation, increased cardiac antioxidant capacity, and reversal of age-related myocardial oxidative stress.

CONCLUSIONS AND FUTURE DIRECTIONS

While reactive oxidant species are emerging as important cell signaling molecules, it is well established that unremitting oxidative stress has a negative impact on human health and is part of the pathogenesis of many chronic diseases including CVD. Because exogenous antioxidants have largely failed to improve disease outcomes in clinical trials, new approaches to combat dysregulation of redox status are necessary to attenuate CVD. Activation of Nrf2 regulates transcription of phase II antioxidant defenses to promote maintenance of redox regulation. By activating the “master regulator” of cellular antioxidant defenses, a more robust cellular response can occur that far exceeds the response elicited by single antioxidant enzymes or exogenous antioxidant vitamin supplements. If targeted to the heart and vasculature, organs that are regularly exposed to oxidant stress during disease, it may be possible to protect cells and tissues against oxidative stress and attenuate CVD pathologies.

Although much is understood about Nrf2 mediated regulation of cellular antioxidant networks, much remains to be elucidated regarding the crosstalk between Nrf2 and signaling pathways regulating apoptosis and proliferation due to interactions with cell cycle regulator p21 [19]. Further, Nrf2 may regulate autophagy and proteasomal removal of oxidatively damaged proteins. Enhanced proteasomal activity and autophagy are partially responsible for beneficial adaptations to exercise [39], suggesting that activation of Nrf2 by exercise could further enhance the removal of damaged cellular components and mitochondria (mitophagy). The identification of an ARE in the promoter of NRF1 has led to the hypothesis that Nrf2 activators

may promote mitochondrial biogenesis [18], and that Nrf2 may localize to the mitochondria, via an interaction with a member of the phosphoglycerate mutase family PGAM5 [23], allowing Nrf2 to act in cell survival decisions (Figure A.2). Localization of Nrf2 to the mitochondria via an interaction with Keap1 and PGAM5 would put Nrf2 in a critical place to rapidly and robustly induce the transcriptional program of antioxidant and survival enzymes. However, investigations into how Nrf2 may integrate mitochondrial biogenic and cell survival decision signaling with exercise and cardioprotection are still in early stages. Future research should be aimed at elucidating other mechanisms by which Nrf2 activation likely mediates protection against CVD pathologies.

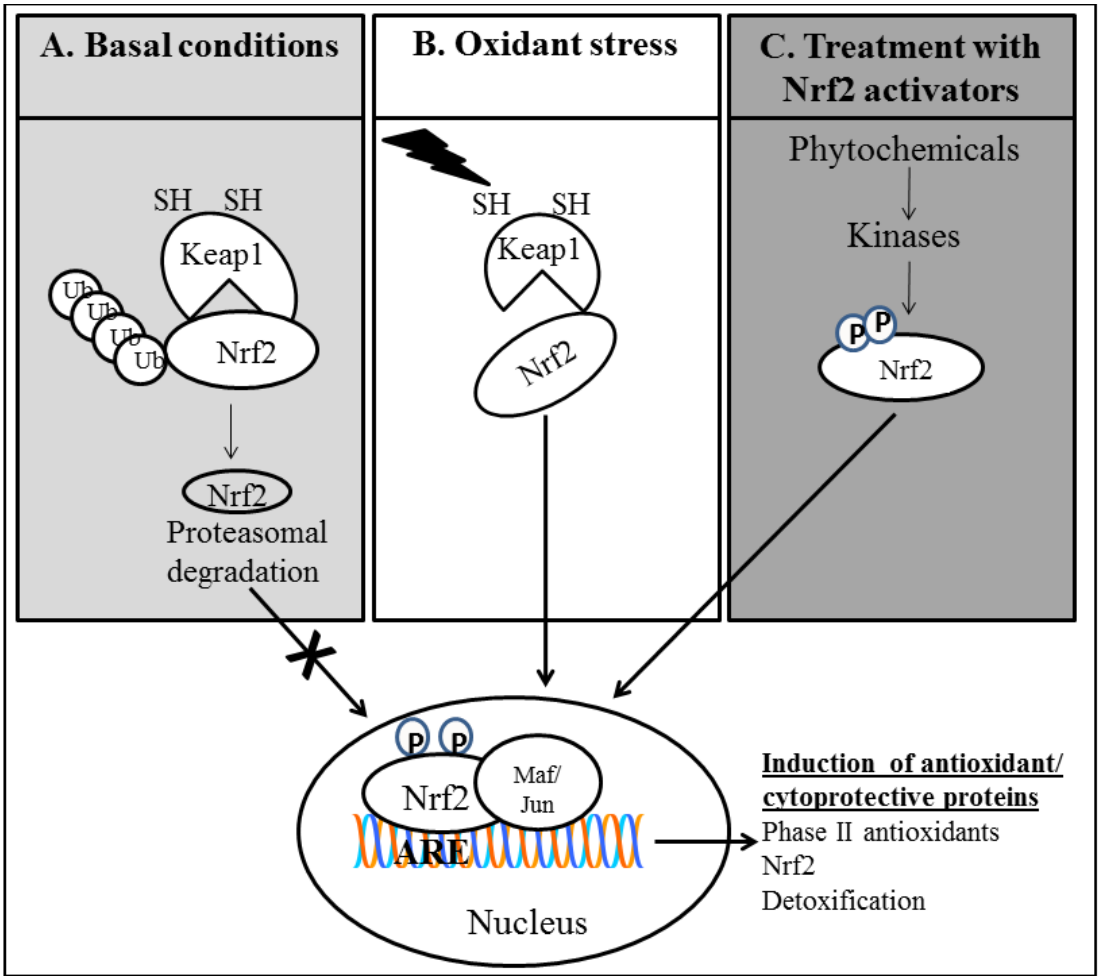

Figure A.1. Regulation of Nrf2 signaling and the endogenous antioxidant network. A. Under basal conditions, Keap1 tethers Nrf2 in the cytosol, resulting in polyubiquitination (Ub) and proteosomal degradation of the Nrf2 protein. B. Under conditions of oxidant stress, cysteine residues on Keap1 are oxidized, resulting in release of Nrf2 from Keap1, and nuclear translocation of Nrf2. C. Alternatively, non-oxidant activators including a variety of phytochemicals, can activate Nrf2 through phosphorylation of Nrf2 by extracellular signal-regulated kinase (ERK), phosphatidylinositide 3-kinase (PI3K), protein kinase C, c-Jun N-terminal kinases (JNK), and MAPKp38. Once in the nucleus, Nrf2 binds to Maf/Jun binding partners to activate the antioxidant response element (ARE) gene program. Following activation of Nrf2 by either oxidant (B) or non-oxidant (C) activators, transcription of cytoprotective genes occurs, including phase II antioxidants, detoxification proteins, and Nrf2 itself.

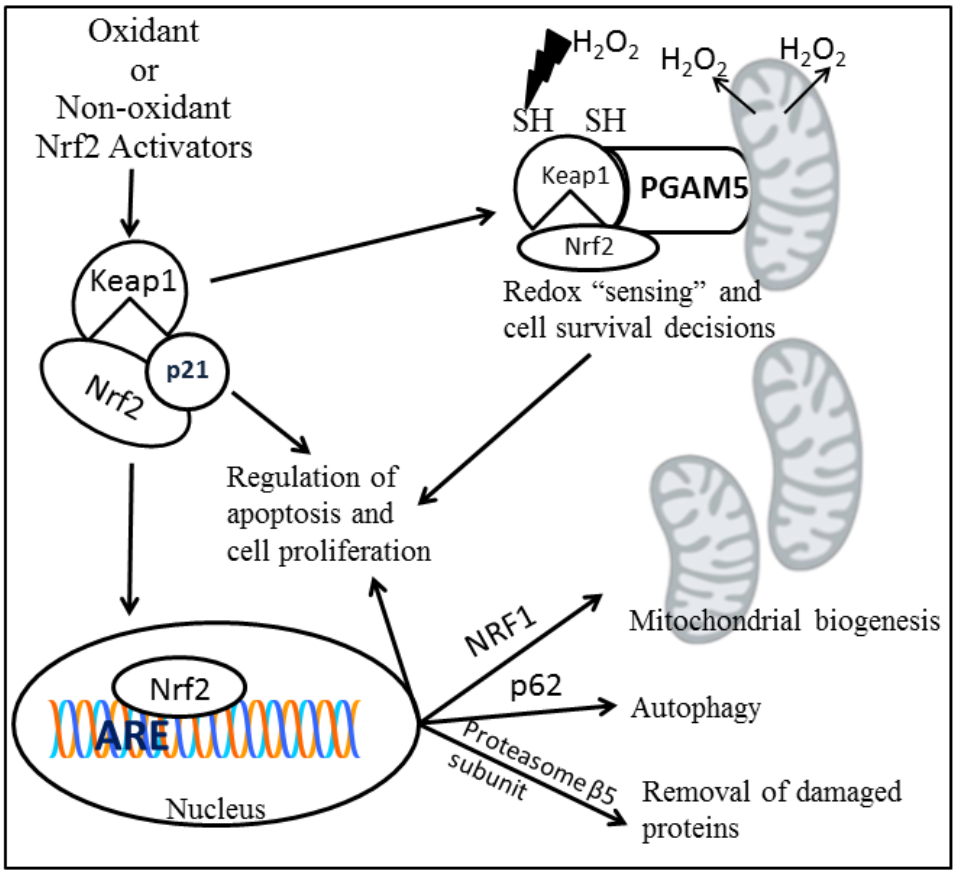

Figure A.2. Cross-talk between Nrf2 and non-antioxidant pathways. Nrf2 signaling overlaps with apoptosis and proliferation pathways due to a direct interaction with cell cycle regulator p21. p21 competes with Keap1 for Nrf2 binding which stabilizes Nrf2, protecting it from ubiquitination and proteosomal degradation [19]. Nrf2 may be involved in the regulation of autophagy and proteasomal removal of oxidatively damaged proteins, based on the identification of an ARE motif in the promoter of the specific autophagy cargo receptor p62 and subunits of the 19S and 20S proteasome [20, 21]. Nrf2 activators may promote mitochondrial biogenesis via an ARE in the promoter of nuclear respiratory factor 1 (NRF1) [18]. Evidence suggests that Keap1-Nrf2 may localize to the mitochondria via an interaction with phosphoglycerate mutase 5 (PGAM5) [23]. This mitochondrial localization suggests that the Keap1-Nrf2 complex might act as a redox sensor, well positioned to sense ROS produced by the mitochondria and facilitate decisions between cell survival and cell death pathways.

REFERENCES

1. Jones, D.P., *Redefining oxidative stress.* Antioxid Redox Signal, 2006. **8**(9-10): p. 1865-79.
2. Madamanchi, N.R., A. Vendrov, and M.S. Runge, *Oxidative stress and vascular disease.* Arterioscler Thromb Vasc Biol, 2005. **25**(1): p. 29-38.
3. Griendling, K.K. and G.A. FitzGerald, *Oxidative stress and cardiovascular injury: Part II: animal and human studies.* Circulation, 2003. **108**(17): p. 2034-40.
4. Hamilton, K.L., *Antioxidants and cardioprotection.* Med Sci Sports Exerc, 2007. **39**(9): p. 1544-53.
5. Bjelakovic, G., et al., *Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis.* JAMA, 2007. **297**(8): p. 842-57.
6. Hybertson, B.M., et al., *Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation.* Mol Aspects Med, 2011. **32**(4-6): p. 234-46.
7. Innamorato, N.G., et al., *Different susceptibility to the Parkinson's toxin MPTP in mice lacking the redox master regulator Nrf2 or its target gene heme oxygenase-1.* PLoS One, 2010. **5**(7): p. e11838.
8. Li, J., et al., *Targeting the Nrf2 pathway against cardiovascular disease.* Expert Opin Ther Targets, 2009. **13**(7): p. 785-94.
9. Itoh, K., et al., *Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles.* Genes Cells, 2003. **8**(4): p. 379-91.
10. Dinkova-Kostova, A.T., et al., *Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants.* Proceedings of the National Academy of Science U S A, 2002. **99**(18): p. 11908-13.
11. Wasserman, W.W. and W.E. Fahl, *Functional antioxidant responsive elements.* Proc Natl Acad Sci U S A, 1997. **94**(10): p. 5361-6.
12. Surh, Y.J., J.K. Kundu, and H.K. Na, *Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals.* Planta Med, 2008. **74**(13): p. 1526-39.
13. Velmurugan, K., et al., *Synergistic induction of heme oxygenase-1 by the components of the antioxidant supplement Protandim.* Free Radic Biol Med, 2009. **46**(3): p. 430-40.
14. Purdom-Dickinson, S.E., et al., *Translational control of nrf2 protein in activation of antioxidant response by oxidants.* Mol Pharmacol, 2007. **72**(4): p. 1074-81.
15. Li, W., et al., *An internal ribosomal entry site mediates redox-sensitive translation of Nrf2.* Nucleic Acids Res, 2010. **38**(3): p. 778-88.
16. Kwak, M.K., et al., *Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway. Identification of novel gene clusters for cell survival.* J Biol Chem, 2003. **278**(10): p. 8135-45.
17. Kwak, M.K., et al., *Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter.* Mol Cell Biol, 2002. **22**(9): p. 2883-92.
18. Piantadosi, C.A., et al., *Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via Nrf2-mediated transcriptional control of nuclear respiratory factor-1.* Circ Res, 2008. **103**(11): p. 1232-40.
19. Chen, W., et al., *Does Nrf2 Contribute to p53-Mediated Control of Cell Survival and Death?* Antioxid Redox Signal, 2012. **17**(12): p. 1670-5.
20. Jain, A., et al., *p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription.* J Biol Chem, 2010. **285**(29): p. 22576-91.

21. Pickering, A.M., et al., *Nrf2-dependent induction of proteasome and Pa28alphabeta regulator are required for adaptation to oxidative stress.* J Biol Chem, 2012. **287**(13): p. 10021-31.
22. Gomez-Cabrera, M.C., E. Domenech, and J. Vina, *Moderate exercise is an antioxidant: upregulation of antioxidant genes by training.* Free Radic Biol Med, 2008. **44**(2): p. 126-31.
23. Lo, S.C. and M. Hannink, *PGAM5 tethers a ternary complex containing Keap1 and Nrf2 to mitochondria.* Exp Cell Res, 2008. **314**(8): p. 1789-803.
24. Frasier, C.R., R.L. Moore, and D.A. Brown, *Exercise-induced cardiac preconditioning: how exercise protects your achy-breaky heart.* J Appl Physiol, 2011. **111**(3): p. 905-15.
25. Quindry, J.C. and K.L. Hamilton, *Exercise Induced Cardioprotection: An Overview of a Unique Form of Preconditioning.* Current Cardiology Reviews, 2007. **3**: p. 255-263.
26. Yamashita, N., et al., *Exercise provides direct biphasic cardioprotection via manganese superoxide dismutase activation.* J Exp Med, 1999. **189**(11): p. 1699-706.
27. Calkins, M.J., et al., *The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease.* Antioxid Redox Signal, 2009. **11**(3): p. 497-508.
28. Saw, C.L. and A.N. Kong, *Nuclear factor-erythroid 2-related factor 2 as a chemopreventive target in colorectal cancer.* Expert Opin Ther Targets, 2011. **15**(3): p. 281-95.
29. Aleksunes, L.M. and J.E. Manautou, *Emerging role of Nrf2 in protecting against hepatic and gastrointestinal disease.* Toxicol Pathol, 2007. **35**(4): p. 459-73.
30. Donovan, E.L., et al., *Phytochemical activation of Nrf2 protects human coronary artery endothelial cells against an oxidative challenge.* Oxid Med Cell Longev, 2012. **2012**: p. 132931.
31. Reuland, D.J., et al., *Upregulation of phase II enzymes through phytochemical activation of Nrf2 protects cardiomyocytes against oxidant stress.* Free Radic Biol Med, 2012. **56C**: p. 102-111.
32. Nelson, S.K., et al., *The induction of human superoxide dismutase and catalase in vivo: a fundamentally new approach to antioxidant therapy.* Free Radic Biol Med, 2006. **40**(2): p. 341-7.
33. Suh, J.H., et al., *Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3381-6.
34. Zhu, H., et al., *Nuclear factor E2-related factor 2-dependent myocardiac cytoprotection against oxidative and electrophilic stress.* Cardiovasc Toxicol, 2008. **8**(2): p. 71-85.
35. Dai, G., et al., *Biomechanical forces in atherosclerosis-resistant vascular regions regulate endothelial redox balance via phosphoinositol 3-kinase/Akt-dependent activation of Nrf2.* Circ Res, 2007. **101**(7): p. 723-33.
36. Zhang, X., et al., *Participation of protein kinase C in the activation of Nrf2 signaling by ischemic preconditioning in the isolated rabbit heart.* Mol Cell Biochem, 2012.
37. Muthusamy, V.R., et al., *Acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium.* Free Radic Biol Med, 2012. **52**(2): p. 366-76.
38. Gounder, S.S., et al., *Impaired Transcriptional Activity of Nrf2 in Age-Related Myocardial Oxidative Stress Is Reversible by Moderate Exercise Training.* PLoS One, 2012. **7**(9): p. e45697.
39. Gottlieb, R.A., K.D. Finley, and R.M. Mentzer, Jr., *Cardioprotection requires taking out the trash.* Basic Res Cardiol, 2009. **104**(2): p. 169-80.