THESIS

UPREGULATION OF HEME OXYGENASE-1 AND ACTIVATION OF NRF2 BY THE PHYTOCHEMICALS IN PROTANDIM

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERIVSION BY DANIELLE JUDITH REULAND ENTITLED UPREGULATION OF HEME OXYGENASE-1 AND ACTIVATION OF NRF2 BY THE CHEMICALS IN PROTANDIM BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

UPREGULATION OF HEME OXYGENASE-1 AND ACTIVATION OF NRF2 BY THE PHYTOCHEMICALS IN PROTANDIM

Increased production of reactive oxygen species has been implicated in the pathogenesis of cardiovascular disease (CVD), with enhanced endogenous antioxidants proposed as a potential mechanism for promoting redox balance. Protandim is 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)]. The purpose of this study was to determine if treatment of cardiomyocytes with Protandim induces phase II detoxification enzymes, including the endogenous antioxidant heme oxygenase-1 (HO-1), with activation of nuclear factor E2 p45-related factor 2 (Nrf2), and protection from oxidative stress induced apoptosis. In cultured cardiomyocytes, treatment with Protandim was associated with activation of Nrf2 and a significant increase in HO-1. Protandim supplemented cells were protected against hydrogen peroxide-induced apoptosis as assessed by TUNEL (35% apoptotic in untreated vs. 5% apoptotic in Protandim treated). These findings support the use of Protandim as a potential method for

upregulation of antioxidant defenses and protection of heart cells against an oxidative challenge. Future studies will focus on optimizing phytochemical induction of Nrf2-mediated antioxidant defenses in relevant *in vivo* models of CVD.

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CHAPTER 1

INTRODUCTION

Oxidative stress, the result of an imbalance between antioxidants and prooxidants [2], is associated with the aging process as well as over 100 human diseases [3]. Under physiological conditions, cells maintain redox balance through generation and elimination of reactive oxygen species (ROS) by scavenging free radicals and upregulating antioxidant enzymes. At low levels, ROS act as signaling molecules to promote cell survival, while accelerated production of ROS without concomitant increases in antioxidant enzyme capacity can induce damage and cause cell death [4-5]. Cancer [6], diabetes [7], cardiovascular disease [8], pulmonary disease [9], and neurodegenerative diseases including Alzheimer's and Parkinson's disease [10] have all been associated with increased ROS, demonstrating the role of oxidative stress in a wide array of disease processes.

In an effort to counteract the detrimental effects of oxidative stress, investigators have studied supplementation of antioxidants including vitamins C, E, and beta carotene. Recent clinical trials have been equivocal, with antioxidant vitamins failing to improve markers of oxidative disease [11], and in some cases even increase prooxidant concentrations [12-14]. Current research efforts have subsequently turned to novel compounds that increase endogenous antioxidant enzyme activity, providing the potential for more profound antioxidant protection than is achieved with supplemental antioxidant vitamins.

Phytochemicals have recently been suggested to be compounds capable of increasing endogenous antioxidants [15-17]. Protandim, a combination of five plant-derived compounds (bacopa, epigallocatechin-3-gallate, curcumin, silymarin, and withaferin A), has been proposed to upregulate phase II enzymes [18], resulting in an induction of cytoprotective enzymes with long half lives, and without the possibility of evoking prooxidant effects [16]. Protandim has been shown to transcriptionally activate Nrf2 [18], resulting in a coordinated effect of the Antioxidant Response Element (ARE) and subsequent upregulation of an army of antioxidant enzymes. Specifically, Protandim has been shown to upregulate heme-oxygenase 1 (HO-1), the rate-limiting enzyme in heme degradation [18]. HO-1 has been shown to exert cytoprotective properties in various cells including neurons [19], pancreatic beta cells [18], and cardiomyocytes [20]. This induction of phase II enzymes by Protandim may represent a novel approach to combating oxidative stress and the diseases associated with oxidative dysregulation.

Statement of Problem

The purpose of this study is to determine if Protandim can activate Nrf2 and increase expression phase II enzymes as reflected by HO-1 in cardiomyocytes. Additionally, this study will seek to determine if Protandim-supplemented cardiomyocytes are afforded protection from oxidative stress.

Hypotheses

It is hypothesized that treatment of cardiomyocytes with Protandim will result in activation of the transcription factor Nrf2 and increased expression of HO-1. It is proposed that cells treated with Protandim will be protected from hydrogen peroxide-induced apoptosis.

CHAPTER II

LITERATURE REVIEW

Introduction: Reactive Oxygen Species and Redox Balance

Oxidative stress is defined as the imbalance between pro-oxidants and anti-oxidants [2], and is a result of the formation of reactive oxygen species (ROS) in excess of the capacity of antioxidants to remove them [6]. ROS have been defined as substances generated by a one electron reduction of molecular oxygen [21], including oxygen radicals as well as nonradical species, and are often thought to include reactive nitrogen species (RNS) as well. Common radical species include superoxide and the hydroxyl radical, both of which contain an unpaired electron and as such are extremely reactive, allowing them to react immediately with any biological molecule to produce cellular damage. The most common physiologically encountered nonradical species is hydrogen peroxide (H_2O_2). Although it is less reactive than atoms containing unpaired electrons, H_2O_2 still has the capacity to react with transition metals and can easily react with other cellular components and cause damage. RNS include nitric oxide and peroxynitrite. As a result of unpaired electrons, RNS behave similarly to oxygen free radicals.

Reduction of molecular oxygen is the principal mechanism for ROS formation, with the majority of intracellular ROS produced within the mitochondria [22], resulting in the production of the superoxide radical. ROS can be generated from both endogenous and exogenous

sources. Endogenous producers of ROS include the microsomal electron transport chain, mitochondrial electron transport chain, primarily complexes I and III, the oxidant enzymes lipooxygenase, NAD(P)H oxidase, and cytochrome c oxidase, phagocytic cells, autooxidation reactions, and peroxisomes. Under certain conditions, superoxide can also be produced by nitric oxide synthase (NOS) [23]. Exogenous sources of ROS production include sunlight, drug oxidation, cigarette smoke, heat shock, ionizing radiation, and atmospheric pollutants [3, 5].

Under physiological conditions, cells maintain redox balance through the production and subsequent elimination of ROS. Cells are able to protect themselves against oxidative stress by the finely tuned regulation of redox status through endogenous enzymes, antioxidants, and other cellular mechanisms. At low levels, ROS act as signaling molecules that modulate cell proliferation and survival, immunity, enzyme activation, drug detoxification, muscle contraction [24], prostaglandin biosynthesis, and second messenger processes [25].

In excess of physiological concentrations, ROS become detrimental and result in extensive cellular damage. To protect against oxidative damage, cells contain enzymatic and nonenzymatic mechanisms to eliminate or attenuate damage caused by ROS. One of the major classes of enzymes involved in this maintenance of redox homeostasis is the superoxide dismutase (SOD) family. Multiple isoforms of this enzyme exist within different cellular compartments, and serve to dismutate two moles of superoxide to hydrogen peroxide and molecular oxygen. Further maintenance of redox homeostasis occurs as catalase, an enzyme primarily localized in the peroxisomes, converts hydrogen peroxide to water and oxygen. Additional antioxidant enzymes include glutathione peroxidases, present in high concentrations in an array of cell types. These enzymes catalyze the conversion of reduced glutathione (GSH), which can react with oxidants to form oxidized glutathione (GSSG) [5, 25]. Glutathione

peroxidases are the most important enzymes for removing H_2O_2 located within the cell. They require selenium at the active site, and remove hydrogen peroxide by using it to oxidize GSH into GSSG. Following this reduction, glutathione reductase regenerates GSH from GSSG, with NADPH as a source of reducing power [25]. Glutathione can also scavenge hydroxyl radicals and singlet oxygen. These various enzyme and homeostatic processes function together and are upregulated during conditions of increased oxidative stress. The delicate balance between ROS production and elimination is essential in the maintenance of redox balance. When increased production of ROS or diminished scavenging capacity occurs, redox imbalance and associated cellular damage may occur.

Consequences of Oxidative Stress

When the balance between ROS generation and elimination is disrupted, an increase in intracellular ROS levels occurs. Under these conditions, oxidative stress can produce a spectrum of genetic, metabolic, and cellular responses [26]. Ultimately, this may result in irreversible cell damage including lipid peroxidation, protein oxidation, and damage to nuclear and mitochondrial DNA [5, 25, 27-28].

Mild oxidative stress can render cells more resistant to subsequent oxidant insults if it results in the upregulation of antioxidant defenses; however, severe and/or persistent oxidative stress can lead to cell death. In some cell types, oxidative stress activates the apoptotic pathway, the intrinsic suicide pathway of cellular defense mechanisms. This compensatory measure involves loss of mitochondrial transmembrane potential, release of cytochrome c into the cytoplasm, down-regulation of mitochondrial mRNA, rRNA, and DNA, and resultant diminished transcription of the mitochondrial genome [6]. In extreme oxidative conditions, cells

bypass the apoptotic pathway and become necrotic, activating immune and inflammatory responses, as they release cellular contents through lysis near neighboring cells. This lytic outcome of necrotic cell death results in the potential for further damage to surrounding cells and tissues. Oxidative stress-induced necrosis has been implicated in ischemia-reperfusion injuries such as heart attack, stroke, ischemic bowel disease and macular degeneration [26].

Various cellular components are vulnerable to oxidative modification, including lipids, proteins, and DNA. Due to their chemical structure, lipids are highly susceptible to oxidative modification. Lipid peroxidation generates lipid radicals, which through radical chain reaction mechanisms can attack adjacent lipid molecules, resulting in continued propagation. If phospholipids are subject to oxidative modification, membrane damage can result in cell death due to loss of membrane potential, and generation of cytotoxic lipid peroxidation products such as malondialdehyde (MDA) [29]. Severe oxidative stress can also damage proteins by inducing disulfide bond-mediated protein cross-linkage or modifications between oxidized proteins and lipid peroxides. Oxidized proteins can generate large groups of protein complexes, resulting in inactivation of 26S and 20S proteasomes, leading to accumulation of damaged proteins and eventually cell death [5]. DNA is also damaged as a result of oxidative stress. Mitochondrial DNA is highly susceptible to ROS attack compared to nuclear DNA, due to proximity to the electron transport chain, a lack of mitochondrial histones, and inefficient DNA repair mechanisms. During mitochondrial oxidative phosphorylation under pathological conditions, the electron transport chain may become uncoupled, leading to increased superoxide production, further increasing mitochondrial DNA damage, and resulting in loss of mitochondrial function [8]. Despite its protective double helix structure, oxidative modification of DNA may occur and can result in extensive cellular damage. Oxidative damage to the sugar backbone has been

shown to cause single and double strand breaks, and excessive damage or failure of DNA repair mechanisms can disrupt transcription, translation, and DNA replication, give rise to mutations [26], and ultimately induce apoptosis [5].

Diseases Associated with Oxidative Stress

Changes in redox balance can occur as a result of physiological or pathological conditions including aging, exercise, hypertrophy, and various disease processes [21]. More than 100 human diseases have been identified to involve an oxidative stress component with ROS implicated in the development or exacerbation of the pathology [3]. ROS have been identified as potential carcinogens due to their ability to promote mutagenesis, tumor development, and progression of the disease [30]. Prooxidative shifts in plasma thiol/disulfide redox states have been observed in patients with various types of cancers [31]. Additionally, elevated ROS have been implicated in diabetes mellitus [7], neurodegenerative diseases including Alzheimer's and Parkinson's diseases [10], rheumatoid arthritis [32], and pulmonary disease [9].

Cardiovascular Disease and Oxidative Stress

Cardiovascular disease (CVD) affects more than 80 million people in the United States and is the leading cause of death and disability in the Western World [33]. Recent studies have implicated increased production of ROS in the initiation and progression of CVD [34-35], specifically in the etiology of hypertension [36], congestive heart failure (CHF) [37], and stroke [38]. *In vivo*, animal studies have shown increases in oxidative stress during experimental hypoxia and cardiac ischemia, with oxidative stress demonstrated during coronary by-pass grafting, post myocardial infarction, and CHF in humans [29]. These studies suggest an

important role for ROS in the development of CVD, and highlight the need for therapeutic methods to counteract the changes in redox status observed in patients with developing heart disease.

The majority of CVD results from complications of atherosclerosis [8]. In atherogenesis, oxidized lipids contribute to endothelial damage, initiating a chronic inflammatory response. Lipids in the blood are oxidatively modified by hydrogen peroxide, superoxide, and hydroxyl radicals, resulting in the transport of oxidized low-density lipoprotein (ox-LDL) across the endothelium into the artery wall. Upon infiltration of ox-LDL into the artery wall, endothelial cells, leukocytes, and smooth muscle cells secrete growth factors and chemoattractants which augment the migration of monocytes and leukocytes into the arterial lumen. As monocytes ingest the oxidized lipoproteins, they mature into macrophages, which themselves generate ROS, converting oxidized LDL into highly oxidized LDL. This highly oxidized LDL is taken up by macrophages to generate foam cells, which with leukocytes, begin to form the fatty streak that is characteristic of atherosclerosis. As this fatty streak is converted into a lesion, and eventually a plaque, it begins to calcify, resulting in a fibrous cap surrounding a lipid core, wedged within the walls of the artery. This fibrous plaque can rupture, and the release of thrombi can occlude vessels, causing myocardial infarction and stroke [8]. The recent discovery of the progression of LDL through increasing levels of oxidation has resulted in the development of the "oxidative modification hypothesis" of atherosclerosis [8], and serves to describe the important role played by redox dysregulation in the initiation and progression of arterial disease.

The importance of redox balance in CVD can be demonstrated by studies of antioxidant system depletion. In coronary artery disease, glutathione peroxidase 1 (GPx) activity, which is ubiquitous in the endothelium, is significantly attenuated, resulting in a diminished capacity to

regulate redox balance within the endothelium [8]. Additionally, patients with CVD have been observed to have diminished *in vivo* activity of endothelial SOD (eSOD), further contributing to the severity of endothelial dysfunction. Decreases in eSOD activity have also been noted in post myocardial infarction patients, and patients with diminished levels of the enzyme have been observed to suffer from larger infarct sizes [39] than age-matched controls. While antioxidant defenses rise initially to counteract atherogenesis, as atherosclerosis progresses and multiple ROS and inflammatory responses are established, antioxidant enzyme activity begins to decline. As antioxidant defenses can no longer compensate for accelerated ROS production, cytoprotection is diminished and cardiovascular disease progresses [40].

An additional role played by ROS in the development and initiation of CVD is the contributory role it plays in the decline of nitric oxide (NO) bioavailability. NO, a potent vasodilator, is produced within vascular endothelium by endothelial cell NO synthase (eNOS), and serves to dilate the vasculature as a result of shear stress and other stimulants. NO bioavailability has been shown to decrease as a result of decreased expression of eNOS, a lack of cofactors and/or substrate for the enzyme, alterations of cellular signaling resulting in dysfunctional eNOS activation, and accelerated degradation of NO by ROS [41]. Superoxide anions in particular have been shown to react with vascular NO to generate high levels of peroxynitrite, which diminish NO bioavailability and hamper its important physiological role. Additionally, when eNOS becomes uncoupled such as during exposure to LDL [42] or substrate deficiency as a result of increased oxidative stress [43], eNOS increases production of superoxide, favoring the production of ROS over the generation of NO. The alteration of NOS activity in favor of superoxide formation advances proatherogenic conditions [23, 43] as a result of both diminished NO availability and increased superoxide production.

It has been shown that treatment of cholesterol-fed rabbits with glycolated-SOD can augment endothelium-dependent vascular relaxation, with no effect observed in normocholesterolemic animal. This supports the hypothesis that in hypercholesterolemia, nitric oxide bioavailability is diminished by superoxide. Similarly, human patients with hypercholesterolemia have been observed to have increased urinary markers of lipid peroxidation and circulating oxLDL antibodies [44] when compared to age and gender-matched normocholesterolemic controls. Ultimately, although increased oxidative stress is not the only causal factor in the development of CVD, increased ROS production without adequate compensatory responses has been well documented in the progression of CVD [40, 45], suggesting a role for antioxidants in disease treatment or prevention.

Direct Antioxidants

As it has become accepted in the literature that oxidative stress plays a contributory role in the development of CVD, the proposition that antioxidants could offer prevention or treatment options developed. Antioxidants, defined as any substance that decreases the severity of oxidative stress by forming less active radicals or by quenching damage created by free radical chain reactions [24], broadly include any substance that delay or prevent the oxidation of a substrate [46]. Antioxidant effects of a compound may act by two mechanisms: the compound itself may exhibit direct antioxidant effects through scavenging ROS or inhibiting their formation, or the compound may indirectly upregulate endogenous antioxidant defenses. In some cases, antioxidants can exhibit both direct and indirect properties. Direct exogenous antioxidants include vitamin C which reacts stoichiometrically with ROS to scavenge aqueous-state free radicals, beta-carotene, and vitamin E, a membrane-bound antioxidant scavenger. These antioxidants are redox active nonenzymes, with short half lives, and are sacrificed in the

process of their antioxidant action, causing need for regeneration. These compounds are described as acting through an intrinsic short-term response, illustrated by kinetic studies of vitamin E. These studies suggest the rate constant for scavenging superoxide radicals is five orders of magnitude slower than endogenous antioxidant enzyme scavenging through SOD, limiting the efficacy of vitamin E in modulating on biological oxidative outcomes [45]. Additionally, direct antioxidants have the potential to evoke pro-oxidant effects if inappropriately utilized, such as in excess levels of supplementation, due to the oxidation of cellular components by chain reaction mechanisms [16] and potential deleterious effects [45].

Although supplementation of direct antioxidants is a highly researched topic, the compounds are still only presumed effective [13]. Studies of supplementation with a single antioxidant vitamin have shown that this intervention either has no effect or results in increased levels of all-cause mortality [11]. For example, a meta-analysis of 15 clinical trials on cardiovascular outcome failed to show benefits from vitamin E supplementation, regardless of antioxidant dosage or type and severity of cardiovascular disease. Additionally, supplementation of beta carotene was associated with a small but significant increase in all-cause mortality and CVD [14] presumed to be attributed to its actions as a prooxidant. While the oxidative hypothesis of various disease processes is well-supported in the literature, recent clinical trials fail to show benefits gained from exogenous antioxidant supplementation and suggest the need for a new approach to regulating cellular redox status.

Phytochemicals: Indirect Antioxidants

As a result of the apparent ineffectiveness of supplemental antioxidant vitamins in decreasing oxidative stress, recent research has focused on novel ways to induce an

endogenous antioxidant response. Current research efforts have turned to compounds that can be used to increase endogenous antioxidant enzyme activity, providing the potential for more profound antioxidant protection than the traditional approach of antioxidant vitamin supplementation. Phytochemicals, chemical compounds derived from plants, have been examined as a class of these novel inducers of antioxidant enzymes. Also described as indirect antioxidants due to their role in activating phase II cytoprotective enzymes, phytochemicals stimulate a battery of antioxidant responses in addition to directly scavenging ROS. Indirect antioxidant compounds act catalytically and are therefore not consumed in the reaction. Unlike direct antioxidants, they have long half lives, and are unlikely to evoke pro-oxidant effects [16], suggesting the ability for both a more efficient and longer lasting response to oxidative stress.

Bioactive polyphenol compounds exhibit Michael acceptor functions due to the ability to accept free electron pairs. Additionally, polyphenols, diterpenes, isothiocyanates and other bioactive phytochemicals have been demonstrated to activate xenobiotic responses in target cells, resulting in the upregulation of phase II antioxidant enzymes. Therefore, not only do these compounds exert intrinsic, short-term effects through direct ROS scavenging, but they also maintain the ability to work through a long-term response resulting in the increased transcription of enzymes [47]. Polyphenols, a biologically prevalent class of phytochemicals, have been demonstrated to regulate cell growth and differentiation, activate NF-κB and glutathione synthesis, improve endothelial dysfunction and the lipid profile [48], and scavenge ROS directly or via induction of endogenous antioxidant activity [15]. Additionally, studies of phytochemicals support the ability of these compounds to activate the transcription factor Nrf2 [49-51], a critical step in the induction of antioxidant response mechanisms. Cytoprotective effects of polyphenols have been observed with consumption of olive oil [52], chocolate [53],

and tea-derived phytochemicals [54], with reported cytoprotective and cardioprotective properties including vasodilatory, anti hypertensive, and antioxidant effects [54]. Due to their ability to transcriptionally activate cytoprotective proteins, endogenous antioxidants have the capacity to be regenerated, without the risk of being converted into radicals and further proliferating oxidative stress. By coordinating the expression of cytoprotective proteins, indirect antioxidants provide the potential for greater and more profound upregulation of antioxidant properties and cell protection.

The Antioxidant Response Element

The first experimental evidence for the existence of the antioxidant response element (ARE) was found in the late 1980's during studies of xenobiotic metabolism. During these studies, a group of similar compounds was found to induce phase I and II xenobiotic metabolizing enzymes. Initially named the xenobioitic and electrophilic response element due to the common feature of all inducers to attract electrons, it was later renamed the ARE due to the ability of compounds belonging to a group of phenolic antioxidants to induce phase II enzymes [55]. Many natural and synthetic phenols and thiol-containing compounds can increase transcription of the genes regulated by the ARE, as well as heavy metal atoms, thiol-containing compounds, hydroperoxides, and heme complexes. Although all activators differ structurally, they all share the property of electrophilicity [55].

Located in the 5' flanking regulatory region of phase II target antioxidant genes, the cis-acting ARE is a DNA site containing the nucleotide sequence 5'-AGTGACTnnnGCAG-3' [56]. This site binds nuclear transcription factor Nrf2, resulting in transcription in a number of xenobiotic and antioxidant enzymes. Oligonucleotide microarray analyses suggest *tert*-butyl hydroquinone,

an ARE activator, influences the induction of 137 ARE-responsive genes [57]. Under hyperoxic conditions, transcription of 175 genes have increased [58], and the ARE activator sulfurophane has upregulated the transcription of 77 Nrf2-regulated genes [59]. This evidence highlights the impressive number of antioxidant genes regulated by the ARE and its role in mitigating the consequences of oxidative stress.

Nrf2: The "Master Regulator" of the Antioxidant Cellular Defense System

Nuclear factor E2 p45-related factor 2 (Nrf2) is a member of the basic leucine zipper (bZip) transcription factor family [51, 60-61]. A member of the cap'n'collar transcription factor family [47], Nrf2 controls both basal and inducible expression of more than 200 genes [62], suggesting a role as both a constitutively and functionally active transcription factor [63]. Ubiquitously expressed, Nrf2 is essential for normal development due to its regulation of phase II enzymes.

Under normal conditions, Nrf2 is sequestered in the cytoplasm by its involvement in an inactive complex with Kelch-like ECH-associated protein 1 (Keap1) [49, 64]. Keap1, a 624 amino acid long actin-binding protein unique to Nrf2 [65], also targets Nrf2 for ubiquitination and degradation by the 26S proteasome system, resulting in basal low-level expression of Nrf2 target genes [1]. Keap1 contains three major domains; an N-terminal BTB domain, implicated in the homodimerization of Keap1, a linker region, rich in cysteine residues that regulates activity of Keap1, and a C-terminal Kelch domain which binds to Nrf2. Initially thought to passively sequester Nrf2 in the cytoplasm, it is now known that Keap1 plays an active role in targeting Nrf2 for ubiquitination and proteasomal degradation by functioning as a component of the Cul3 E3 ubiquitin ligase complex [1, 66].

Regulation of Nrf2

Nrf2 can be induced "injuriously" by ROS [67] or "noninjuriously" by phytochemicals such as curcumin and sulfurofane [59, 65, 68-69]. Upon exposure to oxidants or chemoprotective compounds, cysteine residues on the Keap1/Nrf2 complex sense cellular redox changes, resulting in an alteration in the structure of Keap1. Keap1 has 27 cysteine residues, 25 of which are highly conserved, suggesting an important role in sensing changes in cellular redox status [1]. The most reactive residues of Keap 1, C257, C273, C288, and C297 are located in the intervening region between BTB and Kelch repeat domains of Keap1, and therefore serve as the direct sensors and activators of the phase II system. Inducers interact with the highly prevalent thiol groups, allowing dissociation of the Keap1/Nrf2 complex to prevent Nrf2 ubiquitination and subsequent degradation [50, 70]. Additionally, molecular studies of Nrf2 induction indicate

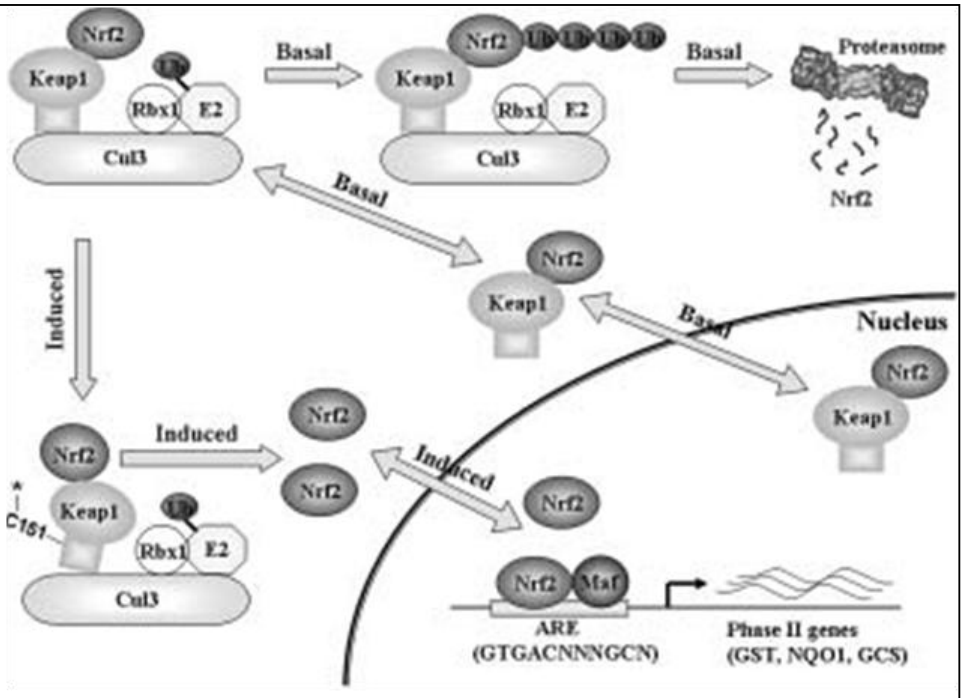


Figure 1: Regulation of Nrf2 [1]. Schematic of Nrf2 regulation. Keap1, an actin-binding protein, retains Nrf2 in the cytosol, where it is ubiquitinated and targeted for proteasomal degradation. Upon induction by Keap1 cysteine residue modification, Nrf2 is released, allowing translocation and accumulation within the nucleus. Once accumulating in the nucleus, Nrf2 binds to the ARE and induces the transcription of phase II antioxidant enzymes.

Keap1 intermolecular disulfide bridges form via Cys151 as a result of oxidation of Keap1 [71], altering the structure of Keap1 to allow release of Nrf2. Inducers of Nrf2-dependent transcription have also been suggested to inhibit Keap-1 dependent degradation mediated by the proteasome pathway [72], resulting in stabilization of Nrf2. As shown in Figure 1, modification of the Keap1 cysteine residues stabilizes Nrf2, facilitating its translocation into and accumulation in the nucleus. After translocation, Nrf2 forms a heterodimer with Maf and Jun bZip transcription factors, which bind to the 5'-upstream cis-acting regulatory sequence known as the ARE [47] and induce transcription of phase II antioxidant enzymes.

Chronic Nrf2 activation within the nucleus has been shown to be harmful and in mouse models results in postnatal death from malnutrition and hyperkeratosis, and increased risk for tumorgenesis [73]. Because of this potentially pathogenic role, mechanisms exist within the cell to degrade the transcription factor shortly following its activation. After translocation and accumulation of Nrf2, nuclear degradation occurs to prevent constitutive activation. A DGR region of Keap1 and prothymosin-α (PTMα), a chromatin remodeling nuclear protein, imports Keap1 into the nucleus in a complex with CuI3 and Rbx1. Upon entering the nucleus, Keap1 releases PTMα, binds to Nrf2, and triggers degradation to shut off downstream gene expression. This autoregulatory loop between Nrf2 and Keap1 controls the abundance of each protein within the cytosol and nucleus, thus allowing for induction of phase II enzymes while preventing chronic constitutive expression of these genes [74].

The role of Nrf2 in cytoprotection

Nrf2 is a major regulator of cellular defense mechanisms in various tissue types including the liver, lung, GI tract, bladder, kidney, brain, skin, ovary, macrophages, and

erythrocytes [62]. Additionally, Nrf2 has been implicated in a number of disease states including a role in the progression of hepatic and gastrointestinal disease [75], drug-induced cellular toxicity [76], pulmonary fibrosis [77],and provides a potential therapeutic mechanism in the treatment of neurodegenerative diseases [78]. The role of Nrf2 in the progression of CVD has also been well documented, as Nrf2 is ubiquitous in the cardiovascular system. In bovine aortic endothelial cells the administration of an NO donor has been shown to increase Nrf2 protein levels, and in human aortic endothelial cells, laminar flow has been shown to drive Nrf2 signaling, highlighting an important role in redox and stress response in the heart [45]. Additionally, several Nrf2 target genes are critical for protecting the heart against maladaptive remodeling and cardiac dysfunction. Through redox regulation, Nrf2 plays a critical role in the control of cardiomyocyte hypertrophy and cardiac fibroblast proliferation, as a loss of Nrf2 has been shown to sensitize cardiomyocytes to oxidative stress-mediated cell death. By suppressing oxidative stress, Nrf2 protects against maladaptive cardiac remodeling and the development of heart failure associated with sustained hemodynamic stress [79], demonstrating its role in maintaining structural and functional integrity of the heart.

Downstream genes of Nrf2 support cellular redox homeostasis, cell growth and apoptosis, mitochondrial biogenesis [80], inflammatory functions [1] and upregulate phase II enzymes. A few of these phase II enzymes include heme-oxygenase1 (HO-1), catalase, glutathione peroxidase (GPx), superoxide dismutase, thioredoxin [5], NADPH:quinine oxidoreductase-1 (NQO1), and glutathione S-transferase (GST) [51]. The coordinated induction of Nrf2-mediated enzymes is crucial for cells to maintain redox homeostasis and avoid the adverse effects of oxidative stress. These cytoprotective proteins serve to directly or indirectly scavenge free radicals and as a result, decrease ROS toxicity. In a study the ability of Nrf2's to

regulate the transcription of antioxidant genes, the expression of detoxification enzymes was significantly blunted in Nrf2 deficient mice, and these animals were more sensitive to carcinogenesis. While transcript levels of 292 genes were elevated in wild type mice 24 hours after treatment with 3H-1,2-dithiole-3-thione, (a chemoprotective agent undergoing clinical investigations), only 15 of these antioxidant enzymes were induced in Nrf2-deficient mice [57].

The role of Nrf2 in cytoprotection has been suggested to be one of the most important pathways for the cell to respond to oxidative stress [74], and studies of Nrf2 knockout and overexpressed animals support this claim. Compared to wild type cells, Nrf2 knockout cardiomyocytes are significantly more susceptible to H_2O_2 induced cell injury [81]. Nrf2 knockout mice are more susceptible to liver injury and die as a result of acetaminophen-induced oxidative stress sooner than wild-type animals [82], and Nrf2 deficient mice were found to develop more gastric tumors, display lower basal expression of phase II enzymes, as well as demonstrate up to a 50% reduction in induction of phase II enzyme activities [83]. Similarly, animals with overexpressed Nrf2 show enhanced cytoprotection, as demonstrated with adenoviral Nrf2 transfer and subsequent protection of endothelial cells from oxidant injury [84]. In a central nervous system model, Nrf2 has been shown to be protective against oxidative stress and has been demonstrated to reduce ischemic brain injury , resulting from an attenuation of neuronal cell death [51], further supporting the role of Nrf2 in cytoprotection against oxidative stress.

Heme Oxygenase-1

Originally identified in 1968, heme oxygenase-1 (HO-1) is a 32kDa protein that catalyzes the degradation of heme to biliverdin, which is rapidly metabolized to bilirubin. An inducible,

rate-limiting phase II enzyme with an ARE-promoter in its regulatory region, HO-1 is expressed in the liver, spleen, pancreas, intestine, kidney, heart, retina, lung, skin, prostate, brain, spinal cord, vascular smooth muscle, and endothelial cells [85]. *In vitro* and animal studies have established antioxidant, antiapoptotic and antiinflammatory effects [86-89] with cytoprotective properties traditionally attributed to the byproducts of heme degradation, namely bilirubin and carbon monoxide (CO). Each of these byproducts exerts antioxidant, anti-inflammatory and antiapoptotic effects. HO-1 deficiency has been implicated in various disease states including hypertension, atherosclerosis, hypoxia-induced lung injury, cancer, transplant rejection, myocardial infarction, Alzheimer's disease, and diabetic kidney disease [15]. Although its main inducer is its substrate heme, HO-1 can also be stimulated by a variety of non-heme factors all of which possess the ability to generate oxidant species including ultraviolent radiation, endotoxins, heavy metals, shear stress, and oxidants such as hydrogen peroxide [90-91].

Recent studies indicate phytochemicals such as curcumin, epigallocatechin-3-gallate (EGCG), and carnosol may induce HO-1 in time and concentration-dependent manners. Carnosol, a diterpine derived from rosemary leaves, has been shown to induce HO-1 via Nrf2 activation in rat pheochromocytoma PC12 cells, a rat adrenal medulla line. Carnosol supplementation allows Nrf2 to accumulate in the nucleus and activate the ARE [47]. Another study conducted by Wu and colleagues [92] suggests HO-1 can be induced by EGCG, the main catechin in green tea, through PI3K and ERK pathways and propose cellular protection against H_2O_2 induced oxidative stress in endothelial cells. Curcumin, a plant polyphenolic compound derived from turmeric, has also been suggested to activate HO-1 via Nrf2 and the ARE, with significant increases in both HO-1 activity and expression observed in renal cells [68].

As noted, most of the cytoprotective properties of HO-1 have been attributed to its products bilirubin and CO, which contain antioxidant and anti-inflammatory effects, respectively. Bilirubin, a product of HO-1 action, is the most abundant endogenous antioxidant in mammalian tissue, and at plasma concentrations, unconjugated bilirubin (UCB) acts as a potent antioxidant. UCB has been shown to restore myocardial function, reduce infarct size, inhibit oxidation of low-density lipoprotein, [93] and reduce mitochondrial damage in a rat model of ischemia/reperfusion injury. Exposure of rat cardiomyocytes to UCB during a hypoxic challenge diminished cell injury caused by subsequent reoxygenation by reducing the generation of ROS, highlighting the protective role of UCB in cardiovascular oxidative stress [94]. CO, the other principle product of heme degradation, has been demonstrated to promote vasorelaxation [90] and serve as a neural messenger. Together, CO and UCB provide HO-1 its protective properties through their function as endogenous antioxidants. The functional role of HO-1 in cardioprotection has been well established, with HO-1 knockout mice experiencing significantly increased myocardial infarct size [95], and greater susceptibility to myocardial ischemia-reperfusion injury [96]. Elevated oxidative stress and enhanced thrombogenesis have been observed in HO-1 null mice, with more profound damage observed in diabetic knockout mice. Suggested to be due to excessive oxidative insult to the left myocardium, animals lacking this critical antioxidant enzyme are subject to decreased survival and diminished recovery of cardiac function.

HO-1 polymorphisms

HO-1 deficiency in an animal model and in one human case study demonstrates the important role of the enzyme in cytoprotection. HO-1 deficient mice show serious impairment of iron metabolism, leading to liver and kidney oxidative damage [47], as well as accumulation

of heme, resulting in reduced antioxidant scavenging [97]. A 6-year old boy with HO-1 deficiency suffered marked growth retardation, intravascular hemolysis, abnormality of coagulation, and erythrocyte fragmentation, suggesting an *in vivo* role for HO-1 [89] in protection against oxidative insult. Overexpression of HO-1 results in increased antioxidant capacity and resistance against oxidative-stress mediated cell death [19].

The ability of patients with diverse genotypes to upregulate HO-1 has been identified as an important factor in the protection against oxidative stress. Two polymorphisms in the promoter region of human HO-1 have been reported to mediate the level of HO-1 activity in response to a given stimulus, and humans differ markedly in their ability to mount an HO-1 response [85]. It has been suggested that HO-1 polymorphisms and activity could play a role in protection against oxidative stress, with individuals with high HO-1 activity responding much quicker, stronger or more effectively than those with reduced HO-1 expression. Therefore, from a therapeutic standpoint, upregulation of HO-1 and identification of enzyme inducers could have large clinical implications in the treatment of oxidative stress-associated diseases.

Protandim: A Novel Inducer of Phase II Enzymes

Protandim is a commercially available supplement composed of 5 different 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) (Figure 2). Each of these phytochemicals has an extensive history of use in Indian and Chinese medicine [2], with extensively documented chemoprotective properties within various cell and tissue types.

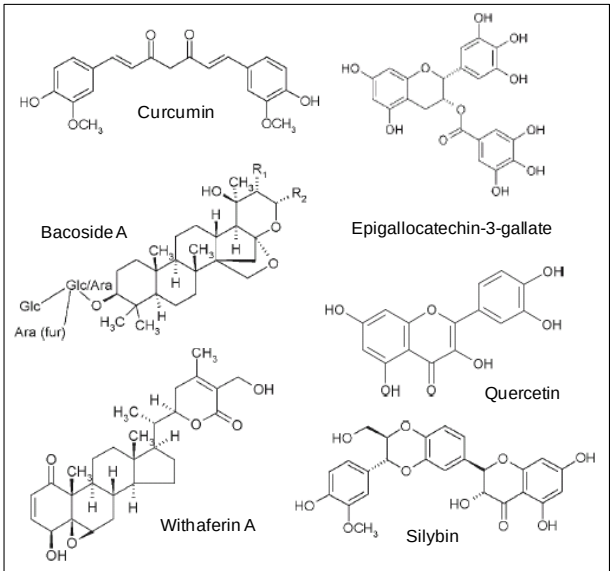


Figure 2: Structure of the phytochemicals in Protandim.

Three published studies on Protandim support its use in treatment and prevention of oxidative stress and its associated disorders. The earliest study of Protandim, a 3 month supplementation study in humans, supports the use of Protandim as a safe supplement, as none of the 29 subjects reported nausea, vomiting, headache, drowsiness, gastrointestinal discomfort, diarrhea, constipation or itching. Furthermore, subjects in the treatment group demonstrated a significant increase in erythrocyte SOD and catalase activity with a strong trend towards increased uric acid, an endogenous antioxidant. Additionally, at baseline, an age-induced trend of increasing TBARS, a marker of lipid peroxidation product MDA, was observed. However, following the month-long trial, subjects in the Protandim-supplemented group demonstrated a 40% drop in TBARS from baseline, suggesting a reversal of

this trend, and a redox status corresponding to a comparatively lower age than their control counterparts [98].

When supplemented in a mouse-derived β-cell line, and a human neuroblastoma cell line, Protandim was found to synergistically induce the HO-1 promoter three to nine-fold in a dose-dependent manner. Similar findings were obtained for HO-1 mRNA and protein expression, and Protandim mediated ARE activation through multiple signaling pathways and the activation of Nrf2 [18]. Because Protandim activates the ARE via Nrf2, it could be expected that the supplement would also activate other phase II enzymes, resulting in a battery of antioxidant response mechanisms. In addition to its induction of phase II antioxidant enzymes, cellular protection has also been observed as a result of Protandim supplementation. When supplemented to a mice diet, Protandim has been shown to significantly increase the expression of SOD and catalase, as well as reduce skin tumor incidence and multiplicity compared to controls. Together, these three studies indicate an anti-tumor, antioxidant, chemoprotective role of Protandim, as a result of its ability to upregulate phase II enzymes through activation of Nrf2. Additionally, the seeming absence of toxic side effects may support its use in human trials in an effort to attenuate damage from oxidative stress and correlated diseases.

Silymarin

A flavanoid extracted from the milk thistle *silybum marianum,* silymarin exhibits free radical scavenging properties. Various studies indicate its function as a hepatoprotective, anti-cancer substance, and imply its cytoprotective properties on prostate, lung, central nervous system, kidney, and pancreas [99-101]. In a 2003 study of oxidative-stress induced diabetes through damage of the pancreatic β-cells via necrosis, silymarin was found to protect against pancreatic damage. This protection was suggested to be due to an increase in activity of SOD,

catalase, and glutathione peroxidase, resulting in an augmented defense system against free radical damage [100]. Silymarin has also been suggested to protect against lipid peroxidation, resulting in decreased rates of oxidized proteins in aged brains, specifically observed in the hippocampus and cortex tissues in mouse models of aging [99]. Both of these studies, along with extensive use of the compound in herbal medicine, support its role in cytoprotection.

Bacopa monnieri

Used in India as a memory-enhancing, antiinflammatory analgesic, *bacopa monnieri* is used as a traditional Ayurvedic medicinal plant [102]. A study of the effects of *bacopa monnieri* in primary cerebral cortex cells showed that cells treated with the phytochemical expressed lower levels of ROS than controls, suggesting attenuation of intracellular oxidative stress, resulting in increased cell viability. *Bacopa* also shows the ability to decrease lipid peroxidation, resulting in prolonged lifespan of the culture neurons [103]. Given these neuroprotective properties, the use of *bacopa* has been suggested for treatment of neurodegenerative disorders with oxidative stress components, such as Alzheimer's disease.

Withania Somnifera

Commonly known as Ashwagandha, *withania somnifera* is a subtropical under-shrub with established sedative, hypotensive, anti-aging, anti-inflammatory, antiperoxidative, anti-tumor, and thyroregulatory effects [104]. In a study of lead-induced oxidative damage, 20 days of Ashwagandha supplementation was found to upregulate activities of SOD and catalase, resulting in significantly decreased lipid peroxidation in a mouse model [105]. This upregulation of antioxidant activity rendered mice kidney and liver tissue more resistant to

lipid peroxidative damage, suggesting a role for the root Ashwagandha in therapeutic treatment of oxidative stress.

Epigallocatechin-3-gallate

Tea is one of the most widely consumed beverages in the world, with green tea constituting the most common of all hot beverages. Epidemiological studies have established a correlation between tea consumption and reduced tumorgenesis, therefore suggesting potential health benefits [106]. Of the components of green tea, epigallocatechin-3-gallate (EGCG) is the most abundant and most active catechin derivative. *In vitro* and *in vivo* studies indicate powerful anti-inflammatory and antiatherogenic properties [107], with consumption associated with a reduced risk of coronary artery disease [108] and other cardiovascular disease processes.

The antiatherogenic properties of EGCG have been attributed to its function as an antioxidant, and indicate its role as both a short-term and long-term regulator of antioxidant defenses. Because of their catechol structure, most tea polyphenols are metal ion chelators that can bind and decrease levels of free iron, therefore reducing the generation of oxygen free radicals. Additionally, tea polyphenols such as EGCG are strong scavengers of superoxide and hydroxyl radicals and have the ability to react with peroxyl radicals to terminate lipid peroxidation chain reactions [106]. Pretreatment with ECGC prior to hydrogen peroxide exposure reduced cell death and protected cardiac myocytes from oxidative stress, reportedly due to the ability of EGCG to directly scavenge ROS [109]. Perhaps even more important than the role of EGCG as a direct antioxidant, EGCG has been found to upregulate HO-1 in a time and concentration-dependent manner, as well as upregulate Nrf2 and to increase ARE-

luciferase activity [92]. By acting to induce phase II antioxidant enzymes, EGCG can induce a more sustained cytoprotective response than is obtained with supplementation of antioxidant vitamins.

Curcumin

Curcumin is a natural extract of turmeric, produced from the plant *Curcuma longa*, part of the ginger family [110], and is recognized in Indian cooking for the flavor and yellow color it gives to curry. In recent years, curcumin has been shown to have cytoprotective properties including anti-carcinogenic, anti-inflammatory, anti-apoptotic, antiviral, and metabolic functions [111]. Due to its structure very rich in phenolics, curcumin is known to possess antioxidant properties as a result of its ability to scavenge superoxide, hydrogen peroxide, and nitric oxide radicals. The phenolic and methoxy groups located on the benzene rings of curcumin, as well as its 1,3-diketone system are the two important structural feature that contribute to this scavenging capability [111], and as a result, allow curcumin to protect against oxidative stress.

Similarly to EGCG, curcumin also has long-lasting endogenous antioxidant activation capabilities. Studies of curcumin have demonstrated its ability to activate Nrf2 as well as enhance enzymes such as SOD, catalase, HO-1, and glutathione peroxidase [68, 112]. By activating Nrf2 and its binding to the ARE, curcumin stimulates the transcription of phase II enzymes, upregulating a battery of antioxidant defenses.

Protandim: A unique synergy of phytochemicals

With the exception of curcumin, none of the five compounds of Protandim has been shown to individually significantly induce antioxidant enzyme activity. Instead, the upregulation

of SOD, catalase, and HO-1 have only been observed with orally-administered pharmacological dosages, which potentially cause toxic side effects. Therefore, the unique combination of Protandim was developed to explore a synergy amongst the phytochemicals to induce the desired endogenous antioxidant response without unwanted toxicity of pharmacological doses. When any single ingredient of Protandim is omitted, the induction of phase II enzymes is significantly attenuated. However, the induction of antioxidant enzymes as a result of Protandim supplementation is larger than the sum of each individual component, suggesting a bioactive synergistic action of Protandim [2]. The benefit of this synergistic response is that lower concentrations of each ingredient are required, thus reducing potential side effects while augmenting protection against oxidative stress.

Conclusion

It is well established that oxidative stress has a negative impact on human health and is highly correlated with many disease processes, including CVD, the leading cause of death and disability in the United States. Because direct antioxidants have fallen short in clinical and epidemiological trials, new mechanisms to combat dysregulation of redox status have been explored, including the use of phytochemicals as inducers of phase II antioxidant enzymes. Protandim, a unique combination of five phytochemicals with demonstrated synergism, is one of these novel inducers of phase II enzymes, and has the potential to upregulate an arsenal of antioxidant responses via activation of the transcription factor Nrf2 and its subsequent regulation of genes through the ARE. HO-1, a phase II enzyme with an ARE sequence in its promoter, has established antioxidant properties, and shows promise in attenuating the damage caused by ROS. Therefore, to elucidate the potential use of Protandim in treatment or

prevention of CVD, we propose to study the upregulation of HO-1, and activation of Nrf2, and the cytoprotection afforded as a result of Protandim supplementation in a cardiac cell line.

CHAPTER III

METHODS AND PROCEDURES

Culture of HL-1 Cells

A cardiomyocyte line (HL-1) derived from murine atrium was a generous gift of Dr. William Claycomb. With a similar phenotype to human adult cardiomyocytes, HL-1 cells maintain contractile activity, as well as contain electrophysiological and pharmacological features similar to adult cardiac myocytes [113], providing an appropriate model for cardiomyocyte investigations. Cells were maintained in Claycomb supplemented medium with 10% fetal bovine serum, 100U/mL:100µg/mL penicillin/streptomycin, 2mM L-glutamine, and 0.1mM norepinephrine. Cells were plated in a 0.5% fibronectin in 0.02% gelatin coated plates, and were grown to confluence in 37˚C, 5% CO_2 humidified environment. Passages 65-92 were used for these experiments, as HL-1 cells have been shown to retain an adult cardiomyocyte phenotype and contractility through passage 240 [113].

Cell Treatments and Protandim Preparation

Protandim (LifeVantage Corp., Littleton, CO, USA) is a commercially available supplement composed of five ingredients: *W. somnifera* (Ashwagandha), *B. monniera* (45% bacosides), *S. marianum* (70-80% silymarin), *Ca. sinesis* (green tea, 98% polyphenols and 45% (-)-epigallocatechin-3-gallate), and curcumin (95%) from turmeric (*Cu. Longa).* An alcohol extract of Protandim was prepared by shaking 500mg Protandim with 5mL 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 ethanol extraction (0-100μg/mL) in supplemented Claycomb medium 8 to 12 hours prior to analyses. Control cells were treated with ethanol vehicle only, in a concentration of ethanol that did not exceed 100 μg/mL of medium.

Preparation of Nuclear Extract

Following an 8 hour treatment with Protandim, myonuclei were extracted as per manufacturer instructions (Active Motif Nuclear Extract Kit). Briefly, cells were washed in phosphate buffer saline (PBS) and then scraped in 3mL PBS with phosphatase inhibitors. The suspension was centrifuged at 500rpm at 4°C for 5 minutes and the pellet was resuspended in 500μL hypotonic buffer and incubated for 15 minutes on ice. To each sample, 25μL of detergent was added, followed by centrifugation for 30 seconds at 14,000xg and 4°C. The supernatant (cytosolic fraction) was stored at -80°C for additional analyses and the pellet was resuspended in 50μL Complete Lysis Buffer and vortexed at highest setting for 10 seconds. The cells were allowed to swell on ice for 30 minutes on a rocking platform, then vortexed and centrifuged for 10 minutes at 14,000xg at 4°C. The supernatant (nuclear fraction) was transferred into a pre-chilled microcentrifuge tube and stored at -80°C.

DNA Binding

NF-E2-related factor (Nrf2) DNA binding activity was assessed using cardiomyocyte nuclear extracts. The oligonucleotide sequence containing the ARE consensus binding site (5'-GTCACAGTGACTAG-CAGAATCTG-3') was purchased from Active Motif and used to detect and quantify Nrf2 activation and DNA binding. Briefly, following protein concentration

determination using the bicinchoninic acid, 40µL of complete binding buffer was added to each well of the 96-well microplate containing the oligonucleotide sequence as per manufacturer suggestion. Samples, diluted in complete lysis buffer, were added in triplicate to each well and incubated 1 hour at room temperature with mild agitation. Wells were washed with 200µL wash buffer and 100µL diluted Nrf2 antibody was added to each well, incubated for 1 hour at room temperature without agitation and washed. Diluted HRP-conjugated secondary antibody was added to each well, incubated without agitation for 1 hour at room temperature and washed. Developing solution was added to all wells and incubated at room temperature protected from direct light until color developed. Stop solution was added to each well and absorbance was read at 450nm with a reference wavelength of 655nm (SoftmaxPro Software; MDS Analytical Technologies).

Western Blot Analysis

After 12 hours of treatment with Protandim extract with 0 (ethanol vehicle) to 100µg/mL, cardiomyocyte were washed 2X with ice cold PBS and lysed with buffer containing 200µL RIPA buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), 0.1M protease inhibitors and .01M Na_3VO_4. Samples were sonicated and lysate protein concentration was measured by using the bicinchoninic acid assay (ThermoScientific). Diluted samples containing equal amounts of protein were prepared in Laemmli Sample Buffer and 2-mercaptoethanol (BioRad) and heat denatured for 5 minutes at 98˚C. Proteins were resolved on a 10% Tris/glycine SDS-polyacrylamide gel in running buffer containing 25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3. Proteins were transferred to a nitrocellulose membrane for 75 minutes at 100V using a transfer buffer containing 25mM Tris, 192mM 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 (20mM Tris, 150mM NaCl, 0.1% Tween 20, pH 7.5) at 4˚C overnight. Membranes were incubated overnight in primary antibody (Stressgen anti-HO-1 1:1,000; Affinity Bioreagents anti-Nrf2 1:500;) at 4˚C followed by secondary antibody conjugated to HRP (Pierce Biotechnology anti-rabbit IgG 1:10,000) 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 imaging (BioSpectrum AC Imaging System, UVP) followed by densitometric analysis using VisionWorks software. Membranes were incubated with actin conjugated to HRP (1:1,000) for 75 minutes at room temperature, followed by chemiluminescent detection and densitometry to verify equal loading of protein.

TUNEL

To investigate whether pretreatment with the Nrf2 activators in Protandim 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 Protandim or ethanol vehicle for 12 hours and then exposed to 1.25mM hydrogen peroxide for 4 hours in Protandim supplemented Claycomb medium. 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 2 minutes. Samples were then incubated with TUNEL reaction mixture, Label (FITC-labeled dUTPs) and enzyme (terminal deoxynucleotidyl transferase) solution (Roche Applied Science) for 60 minutes at 37˚C. Following washes with PBS, samples were mounted with 25µL DAPI mounting medium (Vectashield; Vector Laboratories) and images viewed using a fluorescence microscope (Nikon TE2000) and Metamorph software version 7.5 (Universal Imaging Corporation).

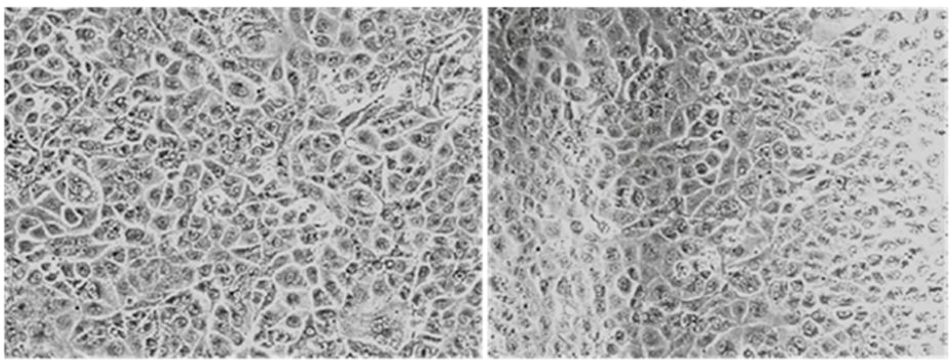
Statistical Analysis

Significance was set *a priori* at $p<0.05$. All 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 expressed as means ± SEM.

CHAPTER IV

RESULTS

Cardiomyocytes cultured in concentrations of Protandim extract ranging from 0-100 µg/mL grew normally maintaining a normal morphology and viability (Figure 3).



0 µg/mL Protandim 100 µg/mL Protandim

Figure 3: HL-1 cells with normal morphology, cultured in Protandim-supplemented medium. HL-1 cells were treated with Protandim (0, 10, 50, 75, and 100 µg/mL) for 8 and 12 hours, and demonstrated normal morphology throughout treatment.

To determine whether Protandim, a unique synergistic combination of phytochemicals, can induce an endogenous antioxidant response in cardiomyocytes, the expression of HO-1, an ARE-responsive, inducible rate-limiting phase II enzyme, was measured. The ethanol extract of Protandim induced HO-1 following treatment with 50, 75, and 100 µg/mL ($p<0.05$), with a 36 fold increase observed over control at 100 µg/mL (Figure 4).

In addition to an induction in HO-1, Protandim-treated cardiomyocytes responded to supplementation with an increase in Nrf2 activation (Figure 5). Nrf2 was assessed by its binding to a known ARE oligonucleotide sequence. Treatment with 75 µg/mL and 100 µg/mL were both found to significantly activate Nrf2 when compared to vehicle treatment ($p<0.05$). Western

blotting for Nrf2 found significant ($p<0.05$) induction of protein expression with 75 and 100 µg/mL supplementation (Figure 6), indicating total cell lysate expression of Nrf2 increases and accumulates with Protandim supplementation.

To investigate whether treatment with Protandim affords cellular protection against hydrogen peroxide-induced oxidative stress, TUNEL was conducted to assess nuclear DNA fragmentation, a hallmark of apoptosis. Compared to controls, protection against oxidative insult and increased survival ($p<0.001$) were demonstrated in Protandim supplemented cells. Pretreatment with Protandim resulted in 4% of nuclei apoptotic with 50 µg/mL, 2% apoptosis with 75 µg/mL, and 2% with 100 µg/mL, compared to 35% apoptotic death in the hydrogen peroxide only treatment (Figure 7).

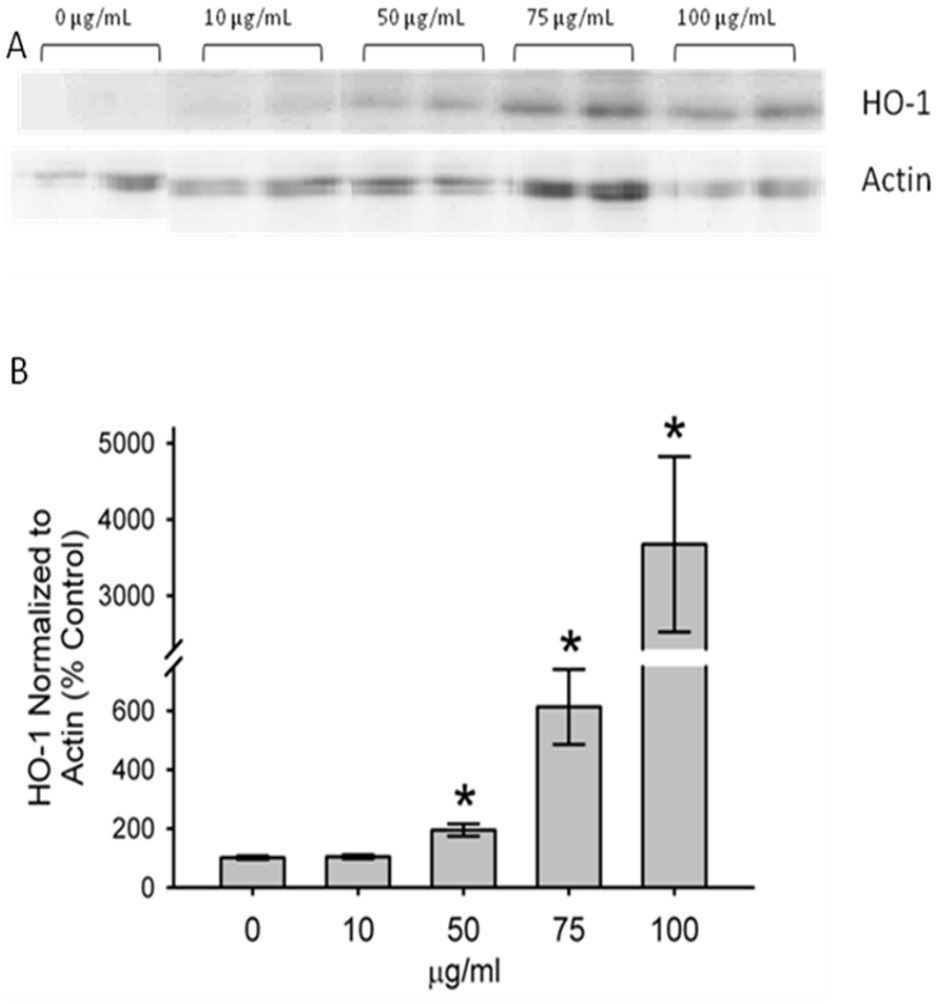


Figure 4: Western blot analysis of the total cell lysate expression of HO-1. A. Representative result of HO-1 induction by 12 hour Protandim treatment. B. Densitometric analyses were conducted, normalized to actin, and expressed as a percent of vehicle treated controls. Data are means ± SEM. *$p<0.05$ compared to 0 µg/mL vehicle controls.

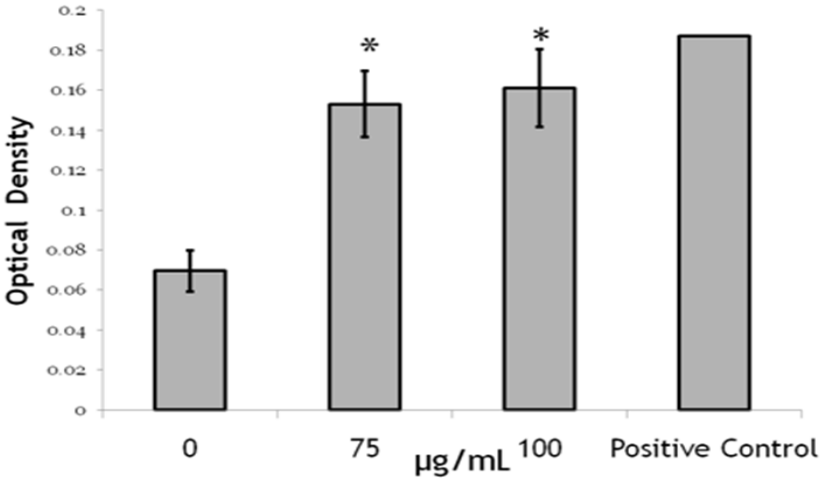


Figure 5: Activation of Nrf2 by Protandim. Nrf2 activation was assessed by DNA binding assay. HL-1 cells were treated for 8 hours with Protandim, and then myonuclei were isolated. A positive transfected control was utilized to confirm presence of Nrf2. *$p<0.05$ compared to 0 µg/mL control.

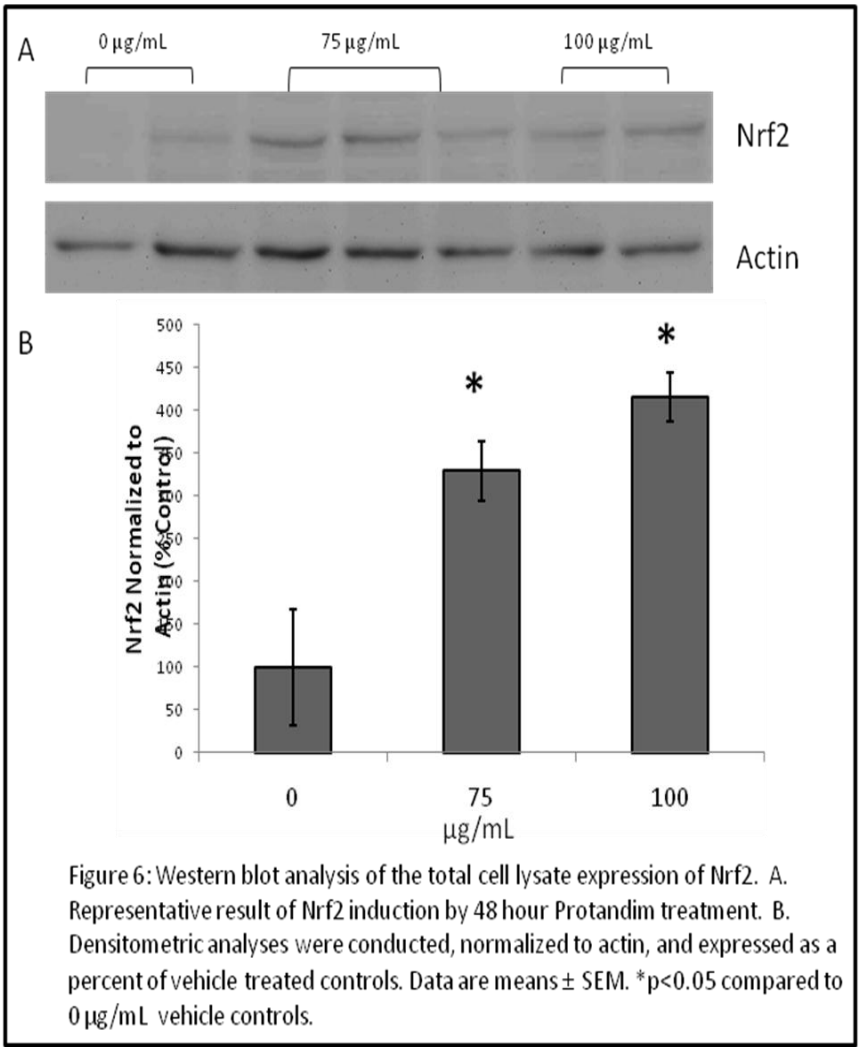


Figure 6: Western blot analysis of the total cell lysate expression of Nrf2. A. Representative result of Nrf2 induction by 48 hour Protandim treatment. B. Densitometric analyses were conducted, normalized to actin, and expressed as a percent of vehicle treated controls. Data are means ± SEM. *p<0.05 compared to 0 µg/mL vehicle controls.

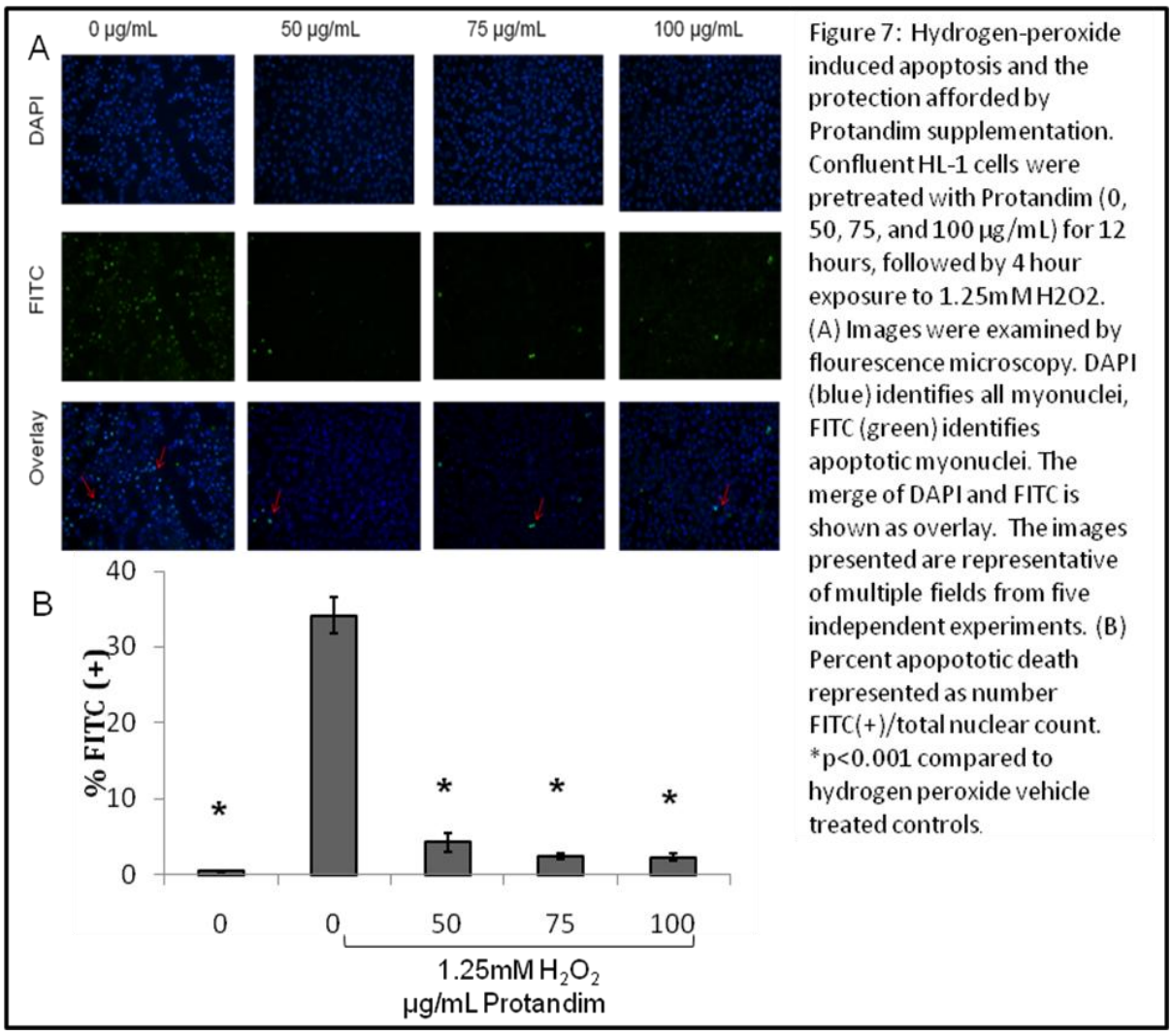


Figure 7: Hydrogen-peroxide induced apoptosis and the protection afforded by Protandim supplementation. Confluent HL-1 cells were pretreated with Protandim (0, 50, 75, and 100 µg/mL) for 12 hours, followed by 4 hour exposure to 1.25mM H2O2. (A) Images were examined by flourescence microscopy. DAPI (blue) identifies all myonuclei, FITC (green) identifies apoptotic myonuclei. The merge of DAPI and FITC is shown as overlay. The images presented are representative of multiple fields from five independent experiments. (B) Percent apopototic death represented as number FITC(+)/total nuclear count. *p<0.001 compared to hydrogen peroxide vehicle treated controls.

CHAPTER V

DISCUSSION

Principle Findings

Oxidative stress is implicated in the progression of many chronic diseases including cardiovascular disease [8]. The cardiovascular outcomes of clinical investigations utilizing antioxidant vitamin supplementation have generally been disappointing [12-14]. The purpose of this study was to investigate whether treatment with a combination of phytochemicals, known to activate the endogenous ARE, can provide protection against an oxidant challenge in cardiomyocytes. Treatment of cultured cardiomyocytes with the phytochemicals in Protandim resulted in activation of Nrf2, upregulation of a key endogenous phase II antioxidant enzyme HO-1, and protection of cardiomyocytes from apoptosis following an oxidative stress. This study is the first to examine the effect of Protandim in a cardiac cell model, and the first to support the use of the supplement in protection of cardiac myocytes against an oxidative insult.

Induction of HO-1

Heme oxygenase-1 was shown to be induced in cardiomyocytes with increasing concentrations of Protandim. This was the first study to investigate the possible concentration-response induction of HO-1 as a result of Protandim supplementation in cardiomyocytes, and supports the use of relevant concentrations of Protandim as capable of inducing a phase II antioxidant response. HO-1 is an important cytoprotective enzyme, with demonstrated antioxidant, antiinflammatory, and anti-apoptotic effects [20, 85-86] in a number of tissue types. HO-1 is known to have a highly responsive ARE promoter, and therefore can be used as an indicator of phase II enzyme induction. Numerous disease states including hypertension,

atherosclerosis, and myocardial infarction [114], have all been linked to HO-1 downregulation, and HO-1 null mice all experienced diminished responsiveness to oxidative stress and CVD functional markers, supporting the key role of the protein in regulating cellular defense against oxidative stress. Current knowledge highlights the importance of HO-1 specifically within the heart, due to the action of the products of heme degradation. Unconjugated bilirubin, a product of HO-1 action and the most abundant endogenous antioxidant in mammalian tissue, has been shown to restore myocardial function, reduce infarct size, and inhibit the oxidation of low-density lipoprotein [93]. Coupled with the ability of CO to promote vasodilation [90], UCB and HO-1 play an important role in maintaining redox status within the heart, and protecting the vital organ against oxidative insult.

Nrf2 Activation

HO-1 is known to be induced by a number of transcription factors, including Nrf2 [115]. This study demonstrated the upregulation of HO-1 and the concomitant activation of Nrf2, suggesting Protandim induces phase II enzymes through the activation of Nrf2 due to its binding to the ARE [116] . Our data from DNA binding experiments suggest Nrf2 increases within the nucleus with response to 75 and 100 μg/mL Protandim supplementation. Furthermore, Western blot analyses suggest total Nrf2 protein levels increase with supplementation, presumably as a result of decreased sequestering and degradation by its cytosolic tethering protein Keap1 and the 26S proteasome. Because Protandim has been found to induce HO-1 via activation of Nrf2 [18], it could be hypothesized that other phase II enzymes including glutathione peroxidase (GPx), thioredoxin, NADPH:quinine oxidoreductase-1 (NQO1), and glutathione S-transferase (GST) [51], could also be induced by supplementation. Although the degree of induction will depend on the number of ARE sites in the promoter region of each of

these cytoprotective proteins, if Protandim can upregulate a coordinated effect of phase II enzymes, the opportunity for antioxidant protection will be enhanced over that allowed by small molecular weight redox active compounds like vitamins C and E. Since GPx, NQO1, and GST, among others, all contain an ARE sequence in their promoter, it would be expected that Protandim, in addition to modulating the upregulation of HO-1 observed in this study, would also induce the expression of these other enzymes. While avoiding the potential deleterious effects of prooxidant actions of direct antioxidants, the upregulation of a battery of ARE-regulated antioxidant enzymes provides the potential for more effective means of bolstering antioxidant defenses against the diseases associated with redox dysregulation.

Phytochemicals have been shown, in a variety of cell types, to activate Nrf2 by phosphorylation of serine 40 resulting in dissociation of Nrf2 from Keap1 and its translocation and activation within the nucleus. Kinases implicated in the phosphorylation and subsequent activation of Nrf2 include PI3-kinase[47, 49], MEK/ERK [117], p38MAPK [118], JNK [119], and protein kinase C [120]. Components of Protandim have been demonstrated to induce Nrf2 utilizing these signaling pathways, with curcumin contributing to HO-1 induction through p38MAPK [49], and EGCG upregulating HO-1 in endothelial cells through PI3K/Akt-dependent induction [109]. Previous studies with Protandim indicate the concomitant stimulation of various parallel pathways may be involved in Nrf2 activation, resulting in the observed synergy [18].

Protandim Affords Cytoprotection in Cardiomyocytes

In addition to inducing HO-1 and activating Nrf2, as hypothesized, Protandim supplemented cells demonstrated attenuated cell death as a result of oxidative stress. TUNEL results indicate protection against hydrogen-peroxide induced stress at Protandim concentrations of 50, 75, and 100 μg/mL when compared to hydrogen peroxide treatment alone. Interestingly, no significant differences were found between the three doses, indicating Protandim supplementation is equally cytoprotective at 50 μg/mL as 100 μg/mL. Therefore, presumably due to the synergy of the supplement, Protandim can protect cardiomyocytes from hydrogen-peroxide induced oxidative insult at lower relevant concentrations.

Each of the compounds of Protandim has a history of extensive use in Eastern medicine and established antioxidative, antiinflammatory, and cytoprotective properties [18, 98]. However, when supplemented together, the benefits of the combination include a synergistic effect [18] that serves to lower the necessary concentration of each ingredient, thus avoiding potential negative side effects. Previous studies of Protandim support its use as a safe supplement, and demonstrate its ability to induce various cytoprotective enzymes including HO-1, SOD, and catalase, through the activation of Nrf2 and the ARE [2, 18, 98]. Together, the synergistic and cytoprotective properties of Protandim suggest therapeutic significance of the compound. As the induction of antioxidant enzymes over the use of direct stoichiometric antioxidants becomes a better established approach to promoting redox balanced compared to antioxidant vitamin supplementation, the use of Protandim will continue to be investigated as a therapy to chronic disease with oxidative stress components.

Conclusions

Protandim, a novel inducer of phase II antioxidant enzymes, was found to upregulate HO-1 in a concentration-dependent manner, as well as activate the "master regulator" of cellular defense mechanisms, Nrf2. Cardiomyocytes supplemented with Protandim exhibited enhanced cytoprotection against hydrogen-peroxide induced oxidative stress, indicating an anti-apoptotic effect of the compound. Taken together, these results support the use of Protandim in protection of cardiac myocytes, and suggest the potential use of Protandim in treatment of the oxidative stress associated with cardiovascular disease.

Future investigations with Protandim supplementation should seek to identify the most synergistic combination of phytochemicals in the induction of phase II antioxidant enzymes and the activation of Nrf2 and the antioxidant response element. While the compounds in Protandim were chosen based on their synergistic effect on inducing the endogenous antioxidant response, many other phytochemicals exist with established antioxidant, anti inflammatory, and cytoprotective properties and have been shown to activate Nrf2, suggesting other potentially superior synergistic combinations could be identified. Additionally, although current studies [18, 121] and this study support the activation of Nrf2 as a critical step in inducing HO-1 and subsequent cellular protection, various other pathways have been cited to be involved in HO-1 upregulation [115]. To elucidate the mechanism through which HO-1 may be upregulated and cardiomyocytes afforded protection, future studies will seek to knockdown Nrf2 through siRNA silencing to determine if HO-1 induction and subsequent cellular protection are still present without the activation of Nrf2. Lastly, future studies will seek to determine whether Protandim's protection extends to relevant *in vivo* models of cardiovascular and

oxidative stresses and ideally, whether phytochemical Nrf2 activators hold promise for prevention and treatment of oxidative stress associated with other chronic human diseases.

CHAPTER VI

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