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ARTICLE *in* FREE RADICAL BIOLOGY AND MEDICINE · NOVEMBER 2012

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Original Contribution

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

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ARTICLE INFO

Article history:
Received 9 November 2012
Accepted 11 November 2012
Available online 30 November 2012

Keywords:
Nrf2
Phytochemicals
Oxidative stress
Antioxidants
Free radicals

ABSTRACT

Increased production of reactive oxygen species has been implicated in the pathogenesis of cardiovascular disease (CVD), and enhanced endogenous antioxidants have 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. 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 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.

Oxidative stress has been implicated in the development or exacerbation of over 100 human diseases [1], including cardiovascular disease (CVD), the leading cause of death and disability within the Western world [2]. Cells contain enzymatic and non-enzymatic 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, β-carotene, and vitamin E, have been the focus of extensive research but are still only presumed effective in the treatment of CVD [3]. Recent clinical trials [4,5] 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 [6,7]. The upregulation of endogenous antioxidant defenses provides the potential for more profound cellular protection than antioxidant vitamin supplementation because of 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) [6,8]. Nrf2 is a member of the basic leucine zipper transcription factor family [8,9] and controls both basal and inducible expression of more than 200 genes [10]. Because of the profound number of genes it transcriptionally regulates, Nrf2 has been termed the "master regulator" of antioxidant defenses [11]. Under normal conditions, Nrf2 is sequestered in the

Abbreviations: ARE, antioxidant response element; CVD, cardiovascular disease; GR, glutathione reductase; HO-1, heme oxygenase-1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; ROS, reactive oxygen species; tBH, *tert*-butylhydroperoxide

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http://dx.doi.org/10.1016/j.freeradbiomed.2012.11.016

cytoplasm by its involvement in an inactive complex with Kelch-like ECH-associated protein 1 (Keap1) [12,13]. Keap1, a ubiquitin ligase actin-binding protein [14], targets Nrf2 for ubiquitination and degradation by the 26 S proteasome, resulting in basal low-level expression of Nrf2 target genes [15]. 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 [6,16]. 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) [17] and induce transcription of genes with functions that favor survival, including mitochondrial biogenesis [18], phase II antioxidant and detoxification [8], and anti-inflammation [19]. Additionally, Nrf2 gene targets may facilitate cross talk with pathways regulating cell death through interaction with autophagy and apoptosis signaling pathways [20]. The coordinated transcriptional activation of Nrf2-mediated antioxidant and prosurvival 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 [7]. Activation of the ARE by these phytochemicals, marketed as Protandim, far exceeds the activation elicited by the known Nrf2 activator sulforaphane by nearly sevenfold, highlighting the potency of Protandim [7]. Data from our group shows that this phytochemical combination provides Nrf2-dependent protection of human coronary artery endothelial cells against oxidant-induced apoptosis [21], suggesting the potential of Nrf2 activators in protecting against the oxidative stress associated with coronary artery disease [22].

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 [23,24], thereby promoting oxidative injury within the heart. Previous experiments utilizing exogenous antioxidants to attenuate cardiac cell damage have been ineffective [25,26] 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 [27,28]; 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-butylhydroperoxide (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 horseradish peroxidase (HRP)- and fluorescein isothiocyanate (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, USA). MISSION lentiviral transduction particles (control, SHC001V; Nrf2, TRCN0000054662) were purchased from Sigma–Aldrich. PCR reagents and prevalidated 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 phenotype similar to that of human adult cardiomyocytes, HL-1 cells maintain contractile activity, as well as containing electrophysiological properties and pharmacological responses similar to those of adult cardiac myocytes through passage 240 [29], 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 a 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 [30]. Full microbial and analytical testing of the raw materials and finished product was conducted before use. An ethanol extract of the five phytochemicals, *W. somnifera*, *B. monniera*, *S. marianum*, *Ca. sinensis*, and curcumin, was prepared by shaking 500 mg with 5 ml 100% ethanol overnight at room temperature. The extract was centrifuged at 3000 g for 15 min, 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 20 ng of cDNA was amplified using target sequence primer-probe reagents. PCR conditions were as follows: hot start (15 min at 95 °C)

followed by 40 cycles of denaturing and annealing (15 s at 95 °C, 1 min 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 [31].

Immunocytochemistry

To determine whether Protandim treatment results in Nrf2 nuclear accumulation, HL-1 cardiomyocytes were grown to confluence on coverslips and treated with phytochemical extract or ethanol vehicle for 15 and 30 min and 1, 2, and 4 h. The cells were then washed with phosphate-buffered saline (PBS) and fixed in 10% formalin for 30 min. Fixed cells were gently washed with PBS and permeabilized with acetone for 15 min at 4 °C. The samples were gently washed three times and incubated in blocking solution (0.05% goat serum in 5% bovine serum albumin) for 1 h at room temperature. After three gentle washes, the samples were incubated for 1 h at room temperature with the primary antibody (1:100), followed by the FITC-conjugated secondary antibody (1:200) for 45 min. The slides were washed and mounted with DAPI-containing mounting medium to allow for nuclear identification. The images were viewed using a

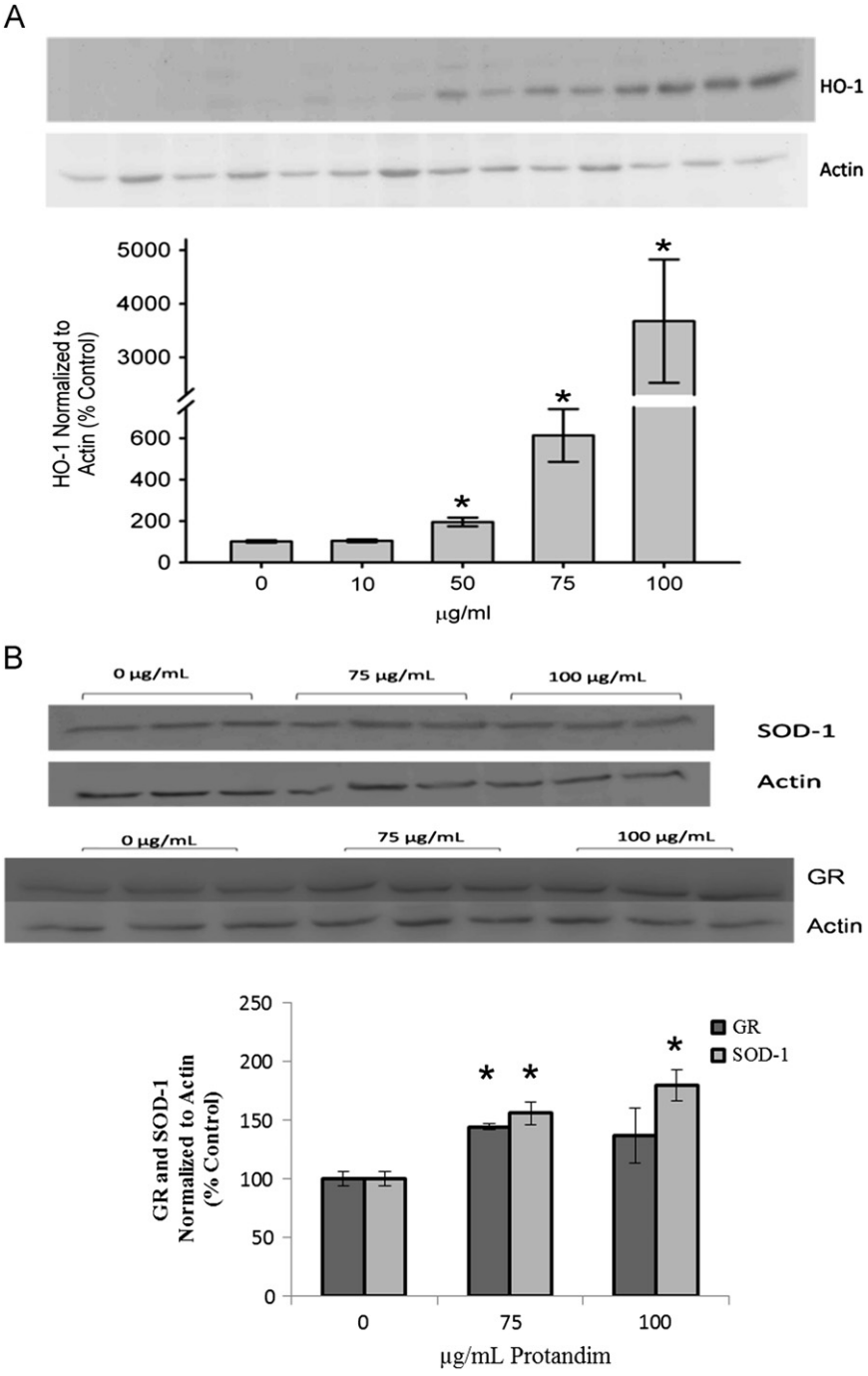

Fig. 1. Treatment with phytochemicals upregulated the expression of phase II enzymes in a concentration-dependent manner. (A) 12 h of Protandim treatment significantly upregulated HO-1 expression at 50, 75, and 100 µg/ml, with greatest induction of 300-fold over control (0 µg/ml) observed with 100 µg/ml.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

fluorescence microscope (Nikon TE2000) and Metamorph software version 7.5 (Universal Imaging Corp.).

Western blot analysis

After 12 h 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 min 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 min at 100 V using a transfer buffer containing 25 mM Tris, 192 mM glycine, 0.02% SDS, and 20% methanol, pH 8.3. Nonspecific proteins were blocked by incubating the 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 h at room temperature with 30-min 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 coverslips, treated with phytochemicals or ethanol vehicle for 12 h, and then exposed to 1.25 mM hydrogen peroxide for 4 h. The cells were then washed with PBS and fixed in 10% formalin for 45 min 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 min. After being washed with PBS, samples were mounted with 25 µl DAPI mounting medium and images

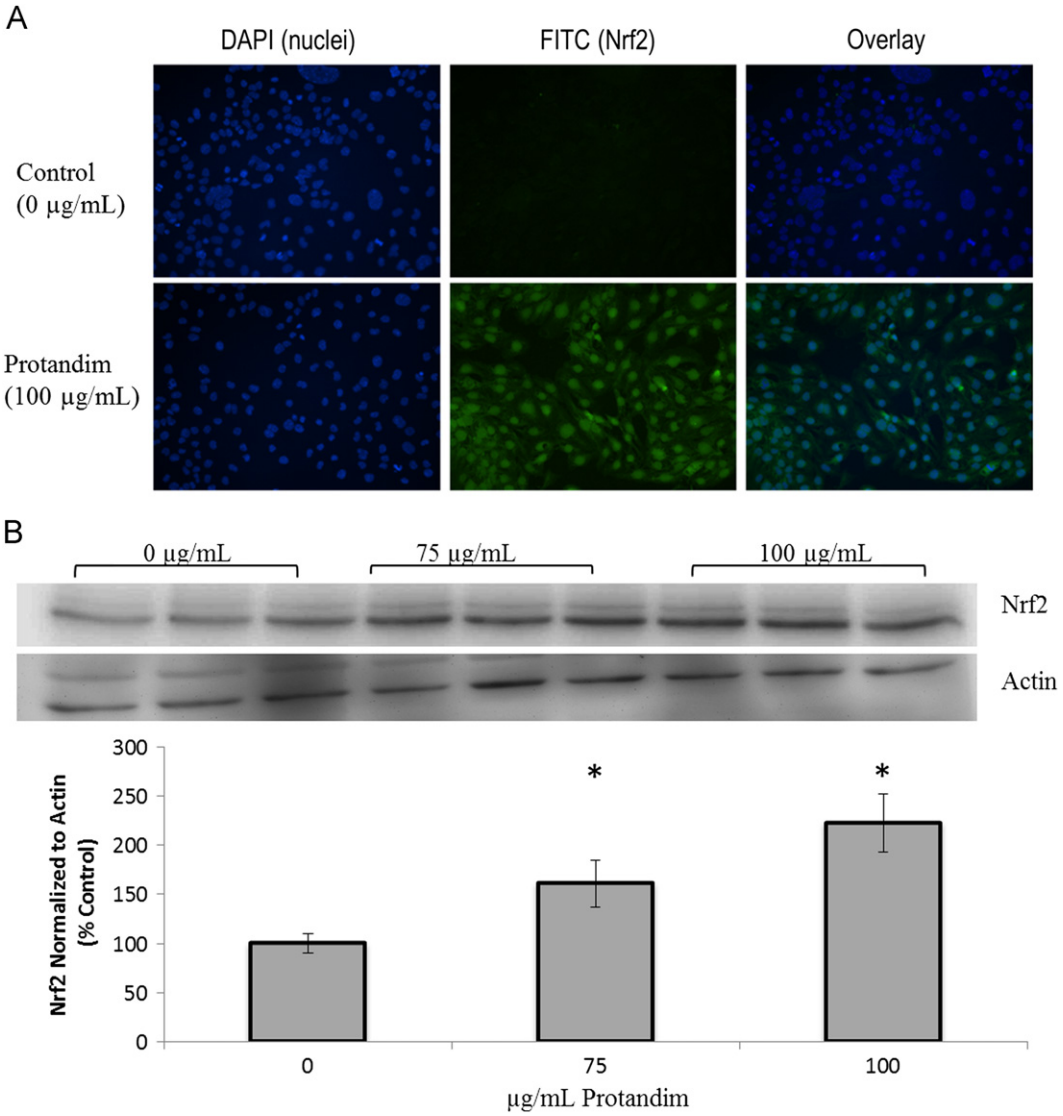

Fig. 2. Phytochemical treatment activated Nrf2. (A) HL-1 cells were treated with Protandim for 1 h and incubated with anti-Nrf2 antibody to assess Nrf2 nuclear accumulation. DAPI identifies myonuclei, and FITC identifies Nrf2. (B) Long-term treatment (48 h) with Protandim upregulated the expression of Nrf2. HL-1 cardiomyocytes were treated with 0, 75, and 100 µg/ml Protandim, and Nrf2 protein in the whole-cell lysate was normalized to actin. $^*p < 0.05$ compared to control (0 µg/ml). $n=9$ for each condition.

viewed using a fluorescence microscope (Nikon TE2000) and Metamorph software version 7.5 (Universal Imaging Corp.).

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 before ANOVA. Data are presented as means ± SEM.

Results

Cardiomyocytes cultured in concentrations of Protandim extract ranging from 0 to 100 μg/ml grew normally as assessed by the maintenance of a normal morphology and viability (Supplementary Fig. 1). The expression of the ARE-responsive phase II proteins HO-1, SOD-1, and GR was 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 (Fig. 1). In addition to increased expression of phase II enzymes, treatment with 75 and 100 μg/ml increased nuclear Nrf2 compared to vehicle treatment (Fig. 2A). Nrf2 nuclear accumulation was assessed at 15 min, 30 min, 1 h, 2 h, and 4 h. Nuclear accumulation was observed after 15 min of Protandim treatment and was greatest at 1 h 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 h (Fig. 2B) compared to 0 μg/ml, indicating that total cell lysate expression of Nrf2 increases and accumulates with longer duration treatment. No changes were observed in Keap1 protein expression with Protandim treatment (Supplementary Fig. 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 after a 4 h exposure to 1.25 mM hydrogen peroxide (Fig. 3 and Supplementary Fig. 2). No protection was observed with the 10 μg/ml treatment.

To determine if Nrf2 activation is necessary for the phytochemically induced upregulation of phase II enzymes and protection against oxidant stress, short hairpin RNA (shRNA) was used to silence Nrf2 expression. Whereas Protandim treatment induced a significant threefold increase in Nrf2 mRNA compared to vehicle, the induction of Nrf2 mRNA was completely abolished with shRNA compared to untransduced cells (Fig. 4A). Additionally, upregulation of HO-1 protein was abolished with Nrf2 knockdown, but was not attenuated after transduction with control shRNA (Fig. 4B). Protection against oxidant-induced apoptosis was significantly attenuated after Nrf2 silencing (Fig. 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 [9]. 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 tBH, and expression of antioxidant enzymes was determined by immunoblotting. The 12 h micromolar concentrations and the 2 h 10 mM 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 (Fig. 6A) as well as a significantly greater induction of HO-1 (Fig. 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

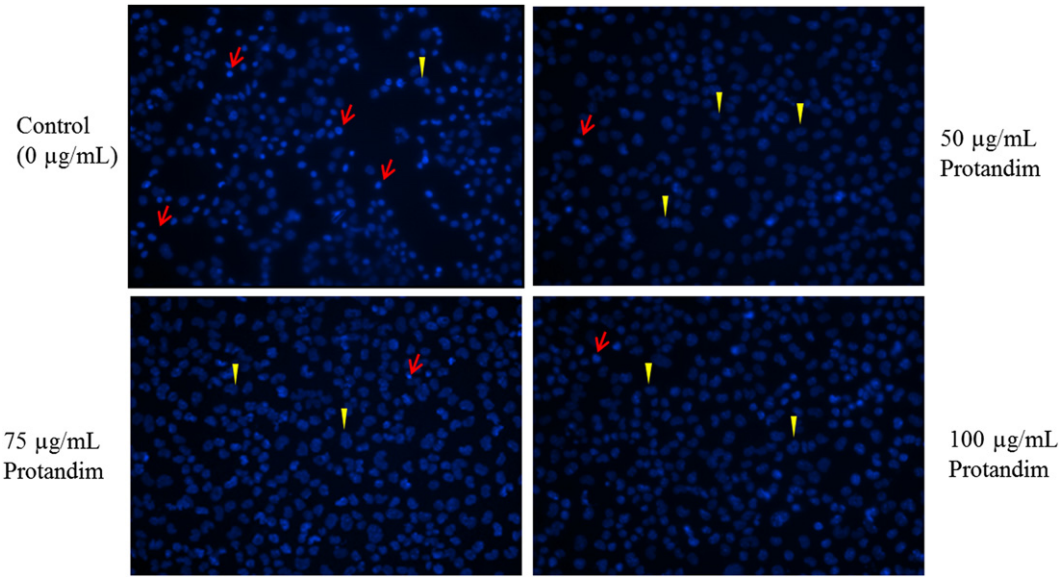

Fig. 3. Pretreatment with 50, 75, and 100 μg/ml Protandim protected cardiomyocytes against oxidant-induced death. Phytochemical treatment was administered for 12 h before H_2O_2 insult, and apoptosis was assessed by nuclear condensation. Red arrows identify condensed nuclei that have undergone apoptosis, and yellow triangles identify healthy nuclei that were resistant to the oxidant stress.

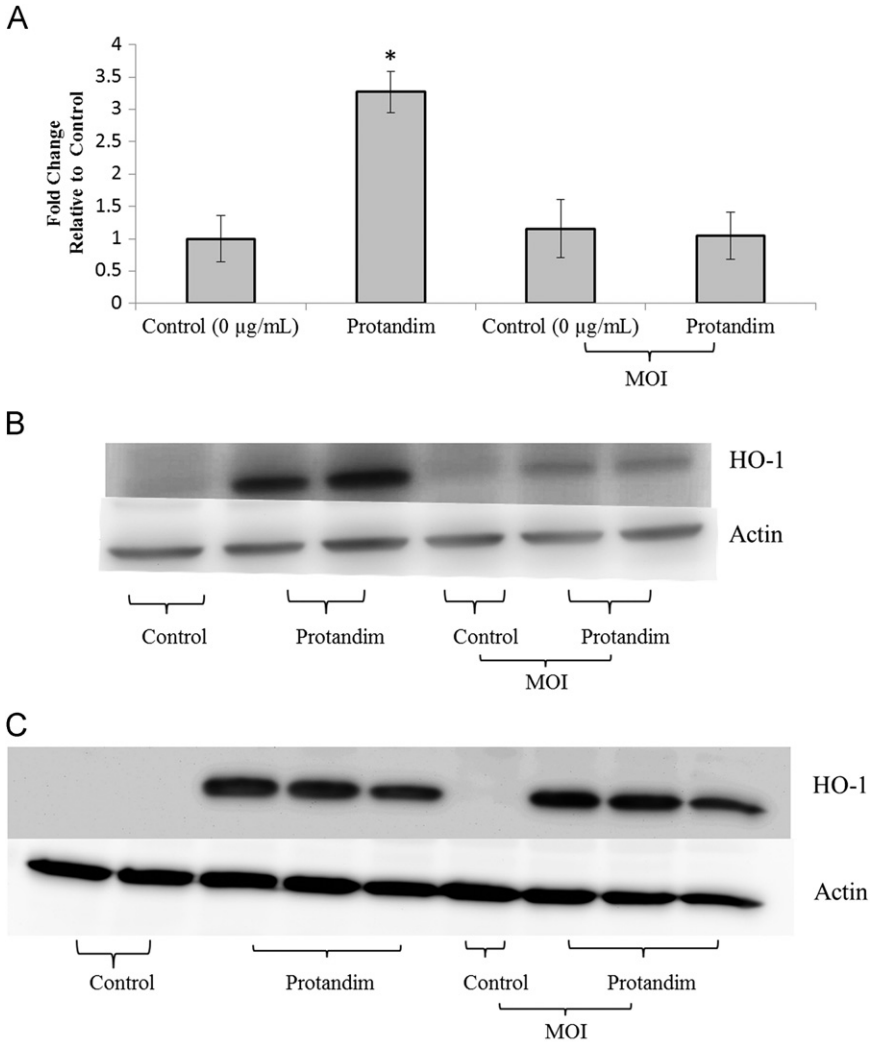

Fig. 4. Lentiviral shRNA silencing of Nrf2 abolished Protandim-induced upregulation of Nrf2 and phase II enzymes. (A) Low-density (~50% confluence) HL-1 cardiomyocytes were transduced with an MOI of 1, followed by an 8 h 100 µg/ml Protandim treatment. (B) Silencing of Nrf2 abolished phytochemically induced upregulation of HO-1 protein expression. After transduction of shRNA, HL-1 cells were treated for 12 h 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.

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 after 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 min of treatment with Protandim concentrations of 75 and 100 µg/ml. Furthermore, Western blot analyses suggested that total Nrf2 protein levels increased with 48 h 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 [32], 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 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 [33], and under basal conditions changes in Nrf2 mRNA expression cannot be detected. It has been speculated that the reason changes in Nrf2 mRNA expression are so difficult to detect may be due to the long-lived

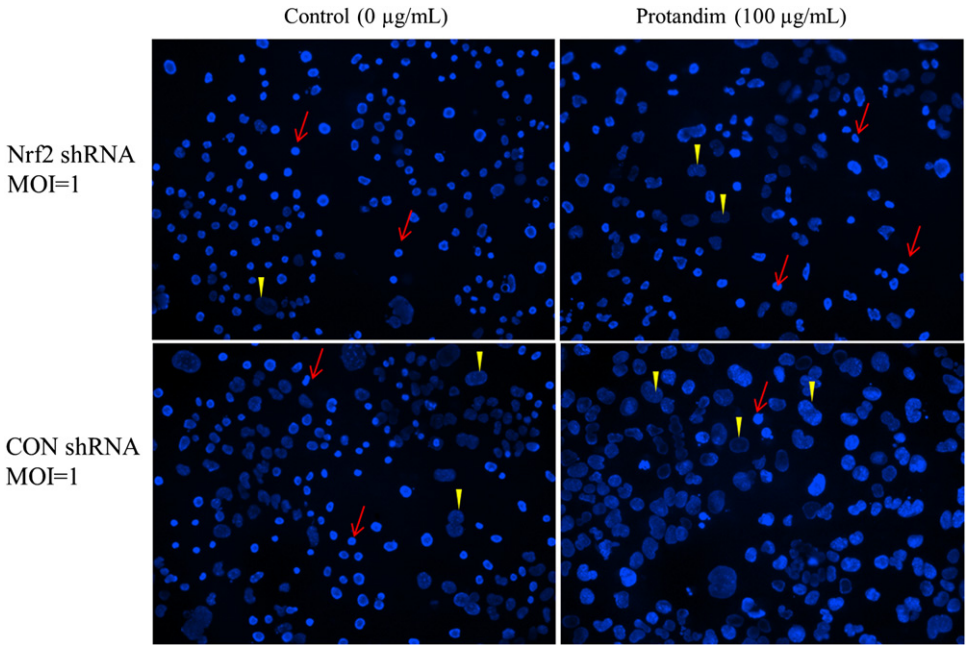

Fig. 5. Lentiviral knockdown of Nrf2 abolished protection against oxidant stress by Protandim treatment. Transduced cells were treated with 100 μg/ml Protandim or control (0 μg/ml) for 12 h, followed by a 4 h 1.25 mM H_2O_2 insult. Apoptotic nuclei were identified by nuclear DAPI condensation and are identified by red arrows, and healthy nuclei are identified with yellow triangles. Protection remained when control shRNA was transduced.

nature of the Nrf2 transcript, despite the reported short half-life of the protein under unstressed conditions [34]. 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 [35]. 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 [12,17], MEK/ERK [36], p38MAPK [37], JNK [38], and protein kinase C [39]. Individual phytochemicals have been demonstrated to induce Nrf2 utilizing these stress-signaling pathways, with curcumin contributing to HO-1 induction through p38MAPK [12] and epigallocatechin-3-gallate upregulating HO-1 in endothelial cells through PI3K/Akt-dependent induction [40]. Previous work with the phytochemicals in Protandim suggests that the concomitant stimulation of various parallel pathways may be involved in Nrf2 activation, resulting in the observed synergy of ARE activation [30]. When activated by phytochemical treatment, the Nrf2 protein becomes stabilized, thereby 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 [20]. 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 [41] 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 [42]. Our group [21] and others [43] have previously shown that HO-1 responds 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 [44]. HO-1 has antioxidant, anti-inflammatory, and antiapoptotic effects [45–47] in a number of tissues; and various chronic diseases including hypertension, atherosclerosis, and myocardial infarction [48] are all associated with HO-1 downregulation. SOD-1 is also suggested to be important in cardioprotection associated with chronic exercise training [49] and pharmacologic interventions [50]. 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) [8], 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 [51], 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 from small-molecular-weight redox-active compounds such as 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 pro-oxidant actions of direct antioxidants.

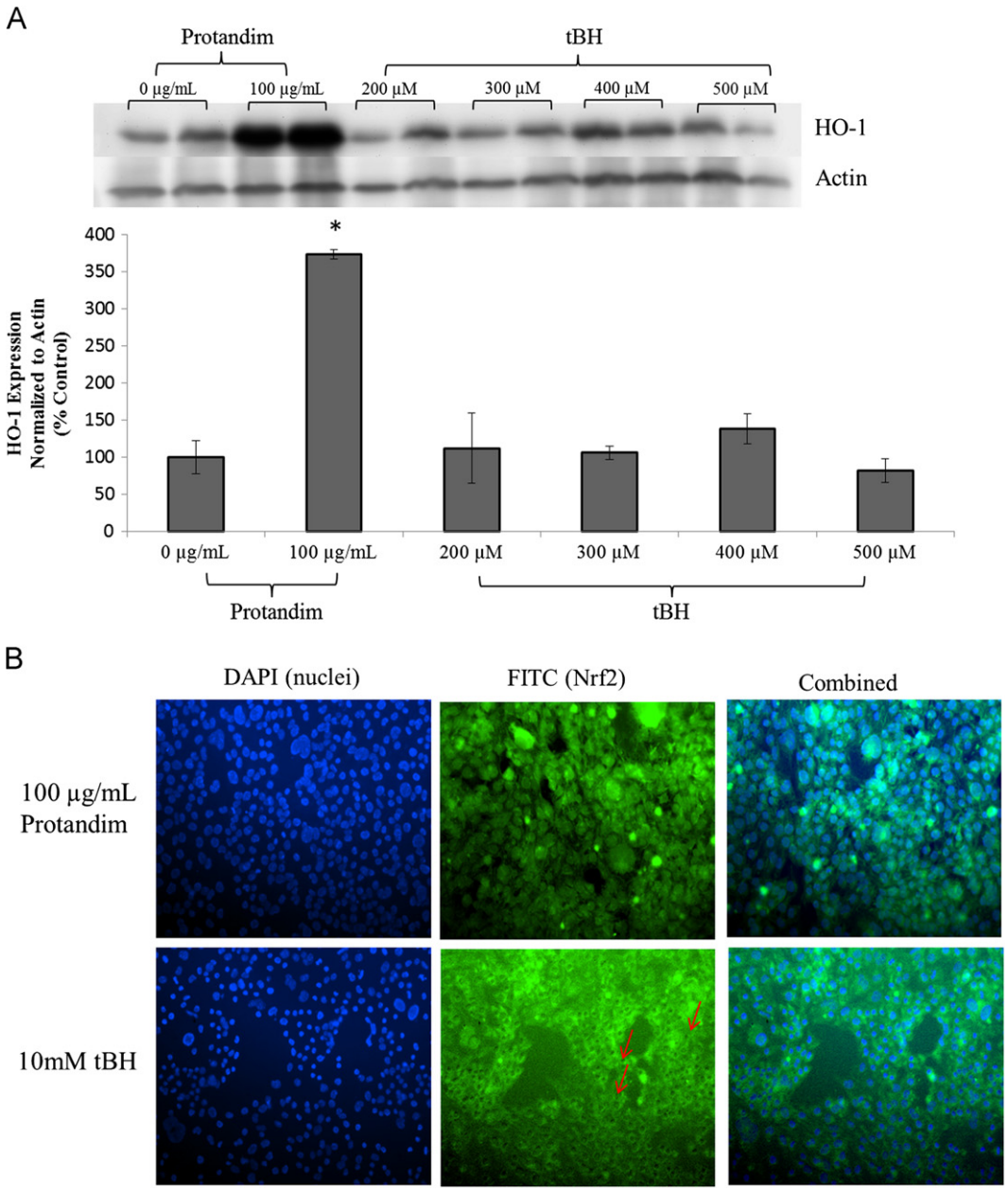

Fig. 6. Upregulation of phase II enzymes and activation of Nrf2 by Protandim treatment was more robust than by mild oxidant stress. (A) HL-1 cardiomyocytes were treated for 12 h with 100 µg/ml Protandim or for 12 h with varying concentrations of tBH. HO-1 expression was normalized to actin and expressed as a percentage of the 0 µg/ml control; $n=2$ in each condition. (B) Oxidant stress did not activate Nrf2 and induce nuclear accumulation. Whereas 2 h treatment with Protandim resulted in nuclear accumulation of Nrf2, 2 h of 10 mM tBH insult did not induce nuclear accumulation, but tended to promote nuclear exclusion and cytosolic enrichment. Arrows identify nuclear exclusion.

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 compared to hydrogen peroxide without Protandim pretreatment. Interestingly, no significant differences were found between the three concentrations 50, 75, and 100 µg/ml. Presumably because of 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 [8], 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.

Acknowledgments

The authors thank Elise Donovan for her assistance with these experiments. This project is supported by a grant from the U.S. Department of Agriculture, Colorado State Agricultural Experiment Station, and by Defense Advanced Research Projects Agency N66001-10-c-2134.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2012.11.016.

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