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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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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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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)