INTRODUCTION

Oxidative stress historically is defined as the production of reactive oxygen species (ROS) in excess of cellular capacity to remove them. This overly simplistic definition suggests that oxidative stress is a balance between oxidants and antioxidants and proposes that all prooxidants on one side are equally important, as are all antioxidants on the opposite side. Furthermore, the definition of oxidative stress as a balance implies that cells have the same sensitivities to a given oxidative stimulus or an equivalent ability to respond to it. Experimental evidence suggests that cellular adaptations and damage vary widely with response to different oxidant species and, similarly, cellular antioxidants have a range of capabilities to offset the oxidants produced. Thus, a more useful definition of oxidative stress may be a “disruption of redox signaling and control” (18). Despite the lack of consensus about an appropriate definition, more than 100 human diseases involve an oxidative stress component in the etiology or exacerbation of disease, including cardiovascular diseases (CVD). Oxidative stress is evident in both the etiology and progression of myocardial infarction, congestive heart failure, atherosclerosis, and hypertension (24). The purpose of this review is to outline the role of oxidative stress in CVD and summarize evidence suggesting that activation of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) enhances endogenous antioxidant defenses and counteracts the oxidative stress associated with chronic diseases including CVD. Specifically, we will highlight exercise and phytochemical supplementation as potential Nrf2-activating CVD interventions.

THE FAILURE OF ANTIOXIDANTS AS CVD THERAPY

Antioxidants are defined broadly as substances that decrease the severity of oxidative stress. Antioxidant defenses protect the heart by catalytic quenching of ROS and via direct scavenging of ROS. A large network of endogenous antioxidant enzymes, including superoxide dismutases (SOD), catalases, peroxidases, and reductases catalytically remove ROS. Although often requiring electron donors, antioxidant enzymes scavenge ROS without the need for regeneration. In contrast, vitamins C, E, and beta carotene are dietary antioxidants that serve as redox active nonenzymes with short half-lives. Exogenous antioxidants are consumed in the process of their antioxidant action and, therefore, must be reduced back to their active form to react with another oxidant. In addition, some exogenous antioxidants have the potential to produce prooxidant effects, suggesting that they may be less effective at mitigating oxidative stress biomarkers than endogenous antioxidant defenses (12).

Although supplementation with exogenous antioxidant vitamins for prevention or treatment of human diseases is well studied, vitamins C and E are still only presumed effective for improving CVD outcomes. Early preclinical trials suggested

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that antioxidants may be useful in preventing oxidative damage during cardiovascular insults such as ischemia-reperfusion injury (13). Although proof of principle exists with vitamin C and E supplementation in animal models of CVD, the doses administered in clinical trials are much higher than the doses used in many of these preclinical trials (12). In fact, large-scale clinical trials of vitamins C and E generally have been disappointing, and a highly publicized meta-analysis of 68 randomized clinical trials with placebo or no-intervention controls concluded that supplementation with vitamin E, vitamin A, and beta carotene increased all-cause mortality, whereas vitamin C and selenium resulted in no improvement in overall mortality or CVD outcomes (2).

It still is unclear why there has been a lack of efficacy in dietary antioxidant supplement trials. Hypotheses include incorrect dosage or route of administration, inappropriate time points for assessment of primary outcomes and stage of disease progression, or lack of efficacy of dietary antioxidants in scavenging the oxidants exerting the greatest oxidative stress (13). Despite the lack of success in improving CVD outcomes, exogenous antioxidant vitamins may be beneficial for specific subpopulations of individuals (12,13). Identifying these individuals may aid in administration and efficacy of the vitamins.

Disappointing outcomes of clinical trials with exogenous antioxidants underscore the need for alternative approaches to regulating redox balance in CVD. One promising approach is via upregulation of endogenous networks of antioxidants, providing the potential for more profound cellular protection than antioxidant vitamin supplementation because of the enhanced ability of enzymatic antioxidants to scavenge ROS (14). The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) has emerged recently as the master regulator of cellular antioxidant defenses (15) and a promising therapeutic target for promoting redox balance.

### Nrf2: THE MASTER REGULATOR OF CELLULAR ANTIOXIDANT DEFENSES

#### Regulation of Nrf2 Signaling

Nrf2 is a member of the basic leucine zipper transcription factor family and controls basal and inducible expression of more than 200 genes (21). Nrf2 is remarkably conserved across species, both in structure and in function, suggesting an integral role of Nrf2 in detoxification processes and mitigating oxidative stress. Under normal conditions, Nrf2 is sequestered in the cytoplasm by its involvement in an inactive complex with Kelch-like ECH-associated protein 1 (Keap1). Keap1, an actin-binding protein unique to Nrf2, targets Nrf2 for ubiquitination and degradation by the 26S proteasome system, resulting in basal low-level expression of Nrf2 target genes (Fig. 1A). Under these conditions, the Nrf2 protein has a half-life of approximately 15 to 20 min (16).

The best understood mechanism of Nrf2 activation is its induction by ROS. On exposure to oxidants, cysteine residues...
on the Keap1/Nrf2 complex become oxidized, altering the structure of Keap1. The Keap1/Nrf2 complex then dissociates, allowing Nrf2 to escape ubiquitination and proteasomal degradation (6). As shown in Figure 1B, modification of the Keap1 cysteine residues stabilizes Nrf2, facilitating its translocation to and accumulation within the nucleus. After nuclear translocation, Nrf2 forms heterodimers with Maf and Jun bZip transcription factors, which bind to the 5' upstream cis-acting regulatory sequence known as the antioxidant or electrophile response element (ARE/EpRE) and induce transcription of phase II antioxidant enzymes. The ARE sequence contains a core 5'-GT(AG)AC(G)/C-3' nnnGCA(/C)-3' cis-acting element shared among Nrf2-regulated genes (36).

In addition to ROS and other electrophilic species, phytochemicals such as curcumin, resveratrol, and sulforaphane also can activate Nrf2 (Fig. 1C) (34). Although phytochemicals likely activate Nrf2 by a variety of mechanisms, it seems that some can induce Nrf2 independent of electrophilic reaction with Keap1 cysteine residues. Sulforaphane and resveratrol activate various kinase signaling cascades that phosphorylate Nrf2, resulting in release from Keap1 (34). Although ROS-mediated Nrf2 activation also can coincide with stimulation of redox-sensitive mitogen-activated protein kinases (MAP kinases), activation of MAP kinases is not compulsory for electrophile-induced Nrf2 activation. Kinases implicated in the phosphorylation and subsequent activation of Nrf2 include phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MEK/ERK), p38MAPK, c-Jun N-terminal kinase (JNK), and protein kinase C (34). Although activation of Nrf2 by ROS or other electrophiles necessitates compensation for the initial oxidative insult, activation by nonoxidative methods avoids this need for compensation. Individual phytochemicals induce Nrf2 using these stress-signaling pathways, with curcumin contributing to Nrf2 activation through p38MAPK and epigallocatechin-3-gallate upregulating Nrf2 target genes in endothelial cells through PI3K/Akt-dependent induction. Activation of Nrf2 by combinations of phytochemicals, therefore, can result in a synergistic upregulation of target genes by using various signaling pathways (35). When activated by phytochemical treatment, the Nrf2 protein becomes stabilized, facilitating its nuclear translocation and transcriptional regulation of antioxidant enzymes.

There is accumulating evidence that Nrf2 activators can act by two nonmutually exclusive mechanisms: attenuation of Keap1-mediated ubiquitination and enhanced translation of Nrf2 mRNA (29). Until recently, the mechanisms of enhanced translation of Nrf2 mRNA under periods of oxidative stress were unknown. Electrophilic compounds have been shown to activate cap-independent translation of Nrf2, allowing preferential translation of the transcription factor during periods of cell stress. An internal ribosomal entry site permits redox-sensitive translation of Nrf2 and allows for increased polysomal loading under conditions of cell stress (22). An internal ribosomal entry site in the 5' untranslated region of the Nrf2 mRNA allows preferential translation of the protein under conditions where global protein synthesis is diminished. The tight regulation of Nrf2 signaling, as well as mechanisms that allow translation to occur during periods of environmental or cellular challenge, further highlights the importance of Nrf2 activation in responding to cell stress and the diseases associated with oxidative stress.

**Cytoprotective Functions of Nrf2 Target Genes**

Classical Nrf2-regulated genes support cellular redox homeostasis and phase I detoxification functions (Fig. 1) (21). Transcription of more than 100 genes, including phase II antioxidant enzymes such as heme-oxygenase 1, catalase, glutathione peroxidase (GPx), superoxide dismutase, thioredoxin, NAD(P)H quinone oxidoreductase-1 (NQO1), and glutathione S-transferase, is regulated directly by activated Nrf2 (21). The coordinated induction of Nrf2-mediated gene expression is crucial for cells to maintain redox homeostasis. The expression of detoxification and antioxidant enzymes is blunted significantly in Nrf2-deficient mice, and these animals are more sensitive to carcinogenesis (20). Although transcript levels of 292 genes were elevated in wild-type mice 24 h after treatment with known Nrf2 activator 3H-1,2-dithiole-3-thione (D3T), only 15 of these antioxidant enzymes were induced in Nrf2-deficient mice (20). In addition to inducing transcription of a battery of antioxidant and chemoprotective enzymes, Nrf2 regulates its own expression. Two ARE-like motifs in the 5' flanking region of the Nrf2 promoter are responsible for the induction of Nrf2 on Nrf2 activation (19). Therefore, a feed-forward process ensues, with Nrf2 activation promoting its own expression, thus facilitating a profound cellular response to stress.

In addition to regulating a battery of antioxidant enzymes, Nrf2 also regulates transcription of genes not directly involved in antioxidant activities (Fig. 2). Nrf2 is involved in regulation of mitochondrial biogenesis through an ARE motif in the promoter of nuclear respiratory factor 1 (NRF1) (27). When oxidant production activated Nrf2, subsequent induction of NRF1 resulted in upregulated mitochondrial biogenic signaling and synthesis of mitochondrial DNA. Direct interactions with cell cycle regulators p21 and p53 suggest that Nrf2 may regulate cell proliferation and apoptosis (4), placing Nrf2 in a position to regulate cell survival versus cell death decisions. Furthermore, the identification of an ARE motif in the promoter of selective autophagy cargo receptor p62 (17) as well as various proteasomal subunits (28) suggests that Nrf2 may regulate removal of oxidatively damaged proteins and organelles. These novel functions of Nrf2 that extend beyond classical antioxidant functions further highlight the importance of Nrf2 in maintaining homeostasis in response to cellular oxidative insult.

Nrf2 activators are not one size fits all and can activate differential gene expression based on the mechanism of activation. For example, microarray and proteomic analyses confirm that only 14% of the genes modulated by sulforaphane are similarly modulated by genetic Keap1 knockdown (14). Therefore, it is possible that decisions about the type of Nrf2 activator selected for specific disease states could be based on predicted target gene activation. Nrf2 binds to a variety of other proteins in addition to Keap1, which compete to stabilize or destabilize Nrf2 (4). For example, the cell cycle regulator p21 competes with Keap1 for Nrf2 binding, allowing Nrf2 to escape Keap1-mediated proteasomal degradation and translocate to the nucleus (Fig. 2). The selective autophagy cargo receptor p62 also interacts in the Keap1-Nrf2 complex and...
promotes Nrf2 activation by selective autophagic degradation of the Keap1 protein (17). Therefore, induction of Nrf2 binding partners can activate Nrf2 and may result in differential target gene expression. Understanding how Nrf2 is stabilized and activated by interactions with other proteins is required to optimize the therapeutic potential of Nrf2 activation in CVD and other chronic diseases.

**EXERCISE AND CVD PROTECTION**

Endurance exercise is a well-established intervention to improve the tolerance of the myocardium and vasculature against oxidative injury. Although high concentrations of ROS are detrimental to cellular function, mild oxidative stress, such as the levels produced during moderate exercise, produces a stimulus for physiological antioxidant adaptation (9). This stress without distress has led to the current understanding of exercise as an example of hormesis, whereby a moderate degree of oxidative stress during exercise results in beneficial adaptation (9). Activation of redox-sensitive cell signaling is not only responsible for, but perhaps necessary for, the adaptations that occur after exercise. When redox signaling is blunted through exogenous antioxidant supplementation, many beneficial exercise adaptations are attenuated (9). Although untested experimentally, it is possible that Nrf2 might be well positioned to regulate exercise-induced adaptations to redox-sensitive cell signaling. A member of the phosphoglycerate mutase family, PGAM5, is a Keap1-binding protein (23). Hypothetically, the PGAM5-Keap1-Nrf2 complex could translocate to the mitochondria during periods of increased ROS production (Fig. 2), allowing the Nrf2 complex to sense changes in redox balance and facilitate decisions of cellular survival. A direct relationship between Nrf2 and redox-sensitive cell signaling in response to exercise has yet to be established. Nrf2 regulates cell survival pathways in response to electrophilic and oxidative stresses, therefore, Nrf2 also may play an integral role in mediating beneficial cellular adaptations to exercise.

Human, as well as animal, studies extensively reviewed elsewhere document that chronic aerobic exercise protects the heart and vasculature against maladaptive stress (8,30). The earliest evidence for exercise as a protector against cardiac oxidative stress came from studies of ischemia-reperfusion injury. Ischemic heart disease can be tied directly to other lethal cardiac dysfunctions, including arrhythmias and congestive heart failure. Although initially acknowledged that the interruption in blood flow was responsible for cellular damage, it is now known that the subsequent restoration of myocardial blood flow with tissue reoxygenation also leads to cell injury. Collectively, this interruption and restoration of blood, termed ischemia-reperfusion (IR) injury, results in contractile dysfunction, myocyte injury, and cell death mediated at least in part by oxidative stress (30).

Chronic exercise training (10 d) and acute exercise bouts both afford cardioprotection in rodent models, with protection persistent for up to 9 d after cessation of exercise (30). An ongoing challenge is to determine how much exercise...
(intensity and duration) is necessary for protection against IR injury. Rat treadmill running of 30 to 60 min at speeds of 27 to 33 m·min⁻¹ typically is used as the exercise stimulus (8), an intensity which amounts to approximately 75% VO₂max and consistently confers protection against myocardial infarction–mediated cell injury. Lower intensity treadmill running yields equivoc al results, with some groups suggesting improved functional recovery of the heart after IR injury with exercise and others finding no protection (8). The direct relationship between exercise intensity/duration and cardioprotection is still unclear and should be addressed in future studies. Despite extensive knowledge that exercise is cardioprotective against IR injury, the exact mechanisms responsible for this protection have remained elusive. Proposed cellular adaptations, including improvements in calcium handling, heat shock protein expression, and adenosine triphosphate-sensitive potassium channels (30) may contribute to exercise-induced cardioprotection. However, for the purposes of this review, we will focus on the role of endogenous antioxidants in exercise-induced cardioprotection.

Nonpathological production of ROS during exercise activates a transcriptional program of antioxidant enzymes. Of these antioxidant enzymes, manganese superoxide dismutase (MnSOD), the mitochondrial isoform of SOD, is the most consistently increased antioxidant enzyme after exercise training (30). An early investigation of the role of MnSOD in exercise-induced cardioprotection reported that prevention of exercise-induced increases in MnSOD by oligonucleotide gene silencing abolished protection against myocardial infarction (37). A subsequent investigation confirmed these findings and demonstrated that exercise-induced increases in MnSOD protect against ischemia-reperfusion–induced cardiac arrhythmias (reviewed in Quindry and Hamilton (30)). Moreover, exercised-induced increases in myocardial MnSOD have been found to be partially responsible for the protective effect of exercise against IR-induced cardiac apoptosis (reviewed in Quindry and Hamilton (30)). Alongside increases in MnSOD, chronic exercise training results in attenuation of lipid peroxidation and protein carbonylation, as well as increases in total cardiac glutathione content (8). Intensity of exercise also seems to be critical in determining activity of MnSOD, with low-intensity treadmill running less effective at stimulating antioxidant enzyme changes than high-intensity training. Although it is clear that not all antioxidant enzymes respond similarly to acute or chronic exercise, and intensity seems to be an important determining factor, cellular antioxidant defenses generally increase with endurance exercise training.

**ACTIVATION OF Nrf2 AS CVD THERAPY**

The therapeutic potential of Nrf2 activation in neurodegenerative diseases (3), cancer (32), and hepatic/gastrointestinal diseases (1) has been reviewed. However, identification of the therapeutic potential for Nrf2 activation in cardiovascular diseases is in the early stages. Here, we present data from our group on phytochemical-induced Nrf2 activation, as well as highlight two studies of exercise-induced Nrf2 activation, and the potential for these mechanisms in attenuating oxidative damage associated with CVD.

Nrf2 activators vary in their chemical properties as well as the mechanisms by which they activate the transcription factor. One well-described synergistic combination of phytochemicals, commercially available as Protandim (LifeVantage Corp.), elicits robust increases in Nrf2-regulated gene expression and an improved capacity to maintain redox balance in a variety of cell types (7,31). Protandim is a phytochemical Nrf2 activator composed of Bacopa monniera (45% bacosides), Silibum marianum (70%–80% silymarin), Withania somnifera (0.5% withaferin A), Camellia sinensis (98% polyphenols and 45% epigallocatechin-3-gallate), and Curcuma longa (95% curcumin). Together, treatment with the phytochemical combination in Protandim results in an activation of Nrf2 that far exceeds that achieved by any single phytochemical compound (35). Because of this synergistic effect on Nrf2 activation, the dose of each phytochemical required is very low and use of Protandim in humans is safe, with no reported adverse side effects. The first trial of Protandim supplementation in humans demonstrated that within 5 to 12 d, plasma thiobarbituric acid reactive substances (TBARS), a measure of lipid peroxidation, was significantly attenuated in the treatment group compared with that in controls. Although at baseline subjects displayed an age-related trend of increasing TBARS, subjects in the Protandim-supplemented group demonstrated a 40% decrease in TBARS, resulting in a redox status corresponding to a comparatively younger age than their control counterparts (26). Furthermore, the treatment group demonstrated a significant increase in erythrocyte SOD and catalase activity, with a strong trend toward increased uric acid, an endogenous antioxidant.

Using in vitro models of cardiovascular oxidative stress, we show that Nrf2 activation by Protandim protects against apoptotic cell death in coronary artery endothelial cells (7) and cardiac myocytes (31). Activation of Nrf2 resulted in a robust induction of heme-oxygenase 1, a novel therapeutic target in the management of CVD. In addition, treatment of cultured endothelial cells and cardiomyocytes with the phytochemical Nrf2 activator resulted in significant induction of NQO1, CuZnSOD, and glutathione reductase. Although we have yet to investigate an extensive array of antioxidants, it is expected that other phase II enzymes with ARE motifs will be similarly upregulated, resulting in a battery of antioxidant response mechanisms. In particular, activation of Nrf2 by Protandim should enhance synthesis and reduction of glutathione via regulation of glutathione S-transferase, glutamate-cysteine ligase, and glutathione reductase. Reduced glutathione (GSH) is a critical and abundant cellular antioxidant, and decreased GSH levels correlate with numerous risk factors for CVD, including smoking, aging, and obesity. Impaired Nrf2 activation may be responsible for the diminished capacity to maintain GSH with age (33). By inducing phase II enzymes and regulating cellular GSH homeostasis, activation of Nrf2 enhances antioxidant protection and prevents dysregulation of redox balance with far greater therapeutic potential than exogenous antioxidants such as vitamins C and E.

Activation of Nrf2 is critical in the defense against a variety of cardiovascular stresses, including high glucose–induced oxidative damage and oxidized phospholipids, and Nrf2 activation protects the heart against pathological cardiac hypertrophy (21). Compared with wild-type cells, Nrf2 knockout cardiomyocytes are significantly more susceptible to hydrogen
peroxide—, peroxynitrite—, and 4-hydroxy-2-nonenal—induced cell injury (39). Although treatment of wild-type cardiomyocytes with the synthetic Nrf2 activator D3T upregulated cellular defenses and protected cells against oxidant-induced death, treatment of Nrf2-deficient cardiomyocytes did not. Atheroprone regions of mouse aorta exhibit diminished Nrf2 activation compared with regions that are protected against atheroma development (5), highlighting the role of Nrf2 in protecting against atherogenesis. Furthermore, ischemic preconditioning, which leads to robust cardioprotective effects, also activates Nrf2 and subsequently protects the myocardium against oxidative stress and IR injury (38). Thus, in vitro and in vivo models of cardiovascular disease indicate that activation of Nrf2 by a variety of phytochemical and synthetic compounds protects the heart and vasculature against oxidative stress. Therefore, these models suggest that Nrf2 activators may have a significant therapeutic potential against CVD.

Exercise also has been shown to activate Nrf2, resulting in cytoprotection against subsequent oxidative insult. Exercise-induced Nrf2 activation was first elucidated in rat kidney and human skeletal muscle (25). More recently, in an acute exercise model in mice, exercise activated Nrf2 and Nrf2 binding to the ARE. In this investigation, Nrf2−/− mice, on exposure to the exercise stimulus, exhibited increased cardiac oxidative stress caused by lower basal and exercise-induced expression of antioxidant enzymes (25). The authors concluded that acute exercise stress promotes Nrf2 activation through ROS signaling, but disruption of Nrf2 increases susceptibility of the heart to oxidative damage. This group later reported that aging impairs transcriptional Nrf2 activity and is associated with increases in myocardial oxidative stress, an impairment that can be reversed by moderate exercise training (11). Therefore, endurance exercise training, an intervention leading to cardioprotection via a variety of mechanisms, also results in Nrf2 activation, increased cardiac antioxidant capacity, and reversal of age-related myocardial oxidative stress.

CONCLUSIONS AND FUTURE DIRECTIONS

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

Although much is understood about Nrf2-mediated regulation of cellular antioxidant networks, much remains to be elucidated regarding the cross talk between Nrf2 and signaling pathways regulating apoptosis and proliferation caused by interactions with cell cycle regulator p21 (4). Furthermore, Nrf2 may regulate autophagy and proteasomal removal of oxidatively damaged proteins. Enhanced proteasomal activity and autophagy are partially responsible for beneficial adaptations to exercise (10), suggesting that activation of Nrf2 by exercise could further enhance the removal of damaged cellular components and mitochondria (mitophagy). The identification of an ARE in the promoter of NRF1 has led to the hypothesis that Nrf2 activators may promote mitochondrial biogenesis (27), and that Nrf2 may localize to the mitochondria, via an interaction with a member of the phosphoglycerate mutase family PGAM5 (23), allowing Nrf2 to act in cell survival decisions (Fig. 2). Localization of Nrf2 to the mitochondria via an interaction with Keap1 and PGAM5 would put Nrf2 in a critical place to rapidly and robustly induce the transcriptional program of antioxidant and survival enzymes. However, investigations into how Nrf2 may integrate mitochondrial biogenic and cell survival decision signaling with exercise and cardioprotection are still in early stages. Future research should be aimed at elucidating other mechanisms by which Nrf2 activation likely mediates protection against CVD pathologies.

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References


