

Review Article

DIETARY PHYTOCHEMICALS ACTIVATE THE REDOX-SENSITIVE TRANSCRIPTION FACTOR NRF2

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ABSTRACT

Nuclear transcription factor erythroid 2p45-related factor 2 (Nrf2) plays a crucial role in regulating phase-2 detoxifying/antioxidant gene induction. Under physiological conditions, NRF2 is mainly located in the cytoplasm. However, in response to oxidative stress, NRF2 translocates to the nucleus and binds to specific DNA sites, termed "antioxidant response elements" or "electrophile response elements," to initiate the transcription of numerous cytoprotective genes. Many structurally diverse antioxidants derived from various sources of dietary phytochemicals have been found to activate this particular redox-sensitive transcription factor, thereby potentiating the cellular detoxification action of Nrf2. This review focuses on known phytochemical inducers and the mechanism by which they regulate antioxidant responsive element (ARE)/Nrf2-dependent detoxification genes.

Keywords: Nrf2, Keap1, Phase 2 enzymes, Dietary phytochemicals, Sulforaphane.

INTRODUCTION

Naturally occurring plant chemicals, known as phytochemicals, are found in various foods and food products and have been shown to play a protective role in the etiology of various diseases [1]. Upon entering a cell, these phytochemicals directly scavenge free radicals, leading to the generation of "chemical or electrophilic stress signals" that regulate various downstream cellular signaling pathways [2, 3]. For example, these phytochemical-induced stress signals activate the nuclear factor erythroid 2-related factor 2 (Nrf2).

Nrf2 is an oxidative stress-dependent transcription factor that regulates numerous downstream targets, many of which are involved in cytoprotection [4]. Moreover, Nrf2 has emerged as a key player in the induction of a variety of detoxification enzymes, biotransformation enzymes, and xenobiotic efflux transporters [5]. Oxidative or electrophilic stress signals activate Nrf2, which subsequently aids in the detoxification and elimination of potentially harmful exogenous chemicals and their metabolites [6]. Nrf2 is ubiquitously expressed in many different human tissues, with high levels of expression found in key detoxification organs, such as the liver [7, 8]. In these tissues, Nrf2 has been shown to be the most prominent nuclear erythroid 2-related factor, compared to Nrf1 and Nrf3, functioning to activate and induce antioxidant responsive element (ARE)-mediated genes [9, 10]. Further, Nrf2 regulates more than 200 genes, which act to synergistically increase the efficiency of a cell's defense system [11]. Structurally, Nrf2 is composed of six regions, called Neh (Nrf2-ECH homology) 1–6 domains, which are highly conserved across different species. The Neh1 domain contains the cap'n'collar (CNC) homology region and basic-leucine zipper domain [12], which is also found in the CNC family of transcription factors [12, 13]. Importantly, small masculoaponeurotic fibrosarcoma (Maf) proteins form a heterodimeric complex with the Neh1 domain and bind to the AREs within gene promoter regions [14]. Several lysine residues in the Neh1 domain are acetylated by a co-transactivator, histone acetyltransferase p300/cAMP response element binding (CREB)-binding protein (CBP), which also regulates DNA binding [15]. Furthermore, overall protein stability is partially regulated through the binding of the ubiquitin-conjugating enzyme UbcM2 to cysteine (Cys) 136 in the Neh1 domain. UbcM2 has a cysteine residue that functions as a redox sensor; by binding to and stabilizing Nrf2, it enhances the transcriptional activity of the protein during cellular stress [16].

Neh2 contains a degron that interacts with redox-sensitive Kelch-like ECH associated protein 1 (Keap1), an adaptor protein for the

Cullin 3-based E3 ubiquitin ligase complex that also functions as a repressor of Nrf2. The interaction of these two proteins under normal conditions results in rapid ubiquitylation and subsequent degradation by the proteasome with a half-life of approximately 10 minutes [17]. Recently, it has been shown that two sites within the Neh2 domain of Nrf2, termed the DLG and ETGE motifs, mediate binding to the Keap1 double glycine repeats (DGRs) or Kelch repeats region [18, 19]. Neh3, in turn, consists of the carboxyl-terminal region of the protein and is involved in the transcriptional activation of ARE-dependent genes [20, 21]. The Neh4 and Neh5 domains act cooperatively to bind another transcriptional coactivator, CBP [22]. Furthermore, a mutational study performed for the actin-related motif, termed DME, in the Neh5 domain showed a selective decrease in hemoxygenase 1 (HO-1) expression; the expression of NAD(P)H dehydrogenase quinone 1 (NQO-1) or the glutamate-cysteine ligase modifier subunit (GCLM) was not affected [23]. While the Neh4 and Neh5 domains are critical in coordinating the transcriptional machinery necessary for ARE activation, other coactivators can also selectively affect gene expression.

The Neh6 domain (amino acids 329–379) is essential in the Keap1-independent degradation of Nrf2 that occurs in the nucleus of oxidatively stressed cells [21]. However, to date, the most studied regulatory mechanism of Nrf2 activation is its interaction with Keap1, a repressor protein essential for the rapid turnover of Nrf2. Under basal conditions, Nrf2 is sequestered in the cytoplasm by Keap1, which functions as an adaptor protein between Nrf2 and the N-terminal region of Cullin 3 (Cul3). This binding promotes the constant proteasomal degradation of Nrf2 [24]. Under conditions of cellular stress or in the presence of Nrf2 activating compounds, this degradation is hindered and Nrf2 translocates to the nucleus. Here, Nrf2 heterodimerises with small Maf proteins, which in turn facilitate the binding of Nrf2 to the ARE, a cis-acting enhancer sequence (TCAG/CXXXGC) in the promoter region of Nrf2-regulated genes [25, 26]. Keap1 binds to the Neh2 domain of Nrf2 and was initially identified by a yeast two-hybrid assay [20]. Keap1 protein is a cytosolic protein and is composed of five different domains: an amino-terminal region, a Broad complex, a Tram track and bric-a-brac (BTB) domain, an intervening region (IVR), six Kelch/DGRs, and a carboxy-terminal region (CTR) [27]. While each domain has a specific purpose related to Keap1 function, the BTB domain plays two important roles. It is thought to serve as a dimerization domain, maintaining the dimer structure of Keap1 because mutation of Ser140 in this domain leads to the dedimerization of Keap1 and

subsequent release of Nrf2 [28]. Furthermore, the BTB domain also contains the binding site for Cul3 [29].

The DGR/Kelch domain, composed of six Kelch motif repeats, is required for the interaction of Keap1 with the actin cytoskeleton, which anchors Keap1 in the cytoplasm [30, 31]. The Keap1 DGR/Kelch domain is also essential for Nrf2 binding as it interacts with the amino-terminal Neh2 domain [32]. Two proteins, p62 [33] and p21 [34], have been identified as inducers, which disrupt Keap1 repressor function by interrupting DRG/Kelch domain-Nrf2 binding. p62 does this by binding to and/or interacting with eight amino acids in the DGR motif of Keap1, including Y334, S363, N382, and S602, leading to the separation of Nrf2 and Keap1 [33]. p21 competes with Keap1 to directly bind the DLG and ETGE motifs of Nrf2 [34]. Additionally, some kinases can also be considered Nrf2 inducers. For example, MAPK, PI3K, and PKC can all phosphorylate Nrf2 and change its conformation, preventing the association with Keap1 [35]. It is not surprising that the proteins in the PI3K and MAP kinase families lie upstream of Nrf2 activation as these proteins are highly sensitive to cellular stress and often determine the balance of pro-survival or apoptotic signaling cascades. By collaring

Nrf2 and phase II enzymes into the cellular response to stress, these important signaling cascade proteins can help determine cellular fate under conditions of injury. Importantly, many of these inducers also function to block or modify active thiols. Keap1 contains many cysteine residues, 25 in mouse Keap1 and 27 in the human form, of which approximately one-third are cysteines with low predicted pK_a [10]. These reactive thiols are excellent targets for electrophiles and can be modified in vitro by numerous different oxidants [36]. Indeed, several electrophilic reagents have also been shown to modify the thiols directly [37-40]. Covalent modification of the cysteine residues present in the Keap1 protein is believed to constitute a stress-sensing mechanism, and the covalent binding of several electrophiles, including sulforaphane, to the thiol group(s) has been observed in vitro [41].

Based on electrophile-mediated modification, location, and mutational analyses, it appears that three cysteine residues, Cys151, Cys273, and Cys288, are crucial for Keap1 activity [42]. Further, forced expression of recombinant KEAP1 in various cell lines has shown that mutants lacking these three cysteine residues are unable to negatively regulate NRF2 [42-45]. As a nucleophile, Cys151 is sensitive to many inducers and is therefore considered to be a stress sensor [46] and is a critical residue for a subset of Nrf2 activators [39, 47, 48]. Modification of Cys151 likely inhibits the Keap1-Cul3 interaction and prevents the ubiquitination of Nrf2, resulting in the stabilization of Nrf2 [29]. Cys273 and Cys288, found in the IVR domain along with numerous other cysteines, are also indispensable for Keap1 activity [42, 46, 47]. Oxidation of these cysteines changes the structure of Keap and reduces its affinity for Nrf2 [43]. Additionally, Keap1 structural changes caused by oxidation of these two cysteines may also dissociate Cul3 from Keap1, as the N-terminus of the IVR domain is also a Cul3 binding site [44]. A relationship has been proposed whereby the conformational change of Keap1 induced by Cys151 alkylation might expose Cys273 and Cys288 for further alkylation, leading to total inactivation of Keap1 [42, 49], but additional investigation into this phenomenon is necessary. Further, Keap1 Cys151 is the only cysteine consistently and highly modified by all of the phytochemicals tested thus far, including isoliquiritigenin, 10-shogaol, xanthohumol [50], and sulforaphane [51].

Dietary phytochemicals activate Nrf2

Plants are an incredibly rich source of compounds that activate cytoprotective genes. The development of a simple microtiter-plate-based assay [52] to assess the induction of the cytoprotective enzyme NAD(P)H: quinone oxidoreductase 1 (QR1) in mouse Hepa1c1c7 cells has greatly facilitated the ability to screen for and identify cytoprotective phytochemicals. Furthermore, several plant families important for human dietary nutrition are also particularly rich in ARE inducers. These include curcumin, sulforaphane, quercetin, and tert-butylhydroquinone.

Curcumin

Curcumin, a bioactive polyphenol, is present in the rhizome of the plant *Curcuma longa* [53] and has been shown to be an effective anticarcinogenic, antiviral, antioxidant [54-57], and anti-inflammatory substance in human and animal models [58, 59]. Further, the effects of curcumin have been extensively investigated in a number of cell culture models using liver cells [60], human lymphocytes [61], endothelial cells [62], renal epithelial cells [63], astrocytes [64], and murine splenocytes [65]. In the cell, curcumin acts as a direct and an indirect antioxidant as it scavenges both reactive oxygen and nitrogen species [66, 67] and has been shown to attenuate oxidative stress, inflammation, and insulin resistance by activating cytoprotective enzymes, such as glutathione-S-transferase [68] [68], γ -glutamyl cysteine ligase (γ -GCL), and HO-1, among others [69, 70]. Furthermore, the endogenous antioxidant defense mechanisms and activation of detoxification enzymes observed for curcumin have been shown to be modulated by transcription factors, such as Nrf2 [56, 71-73].

Nrf2-regulated genes can be classified into phase II xenobiotic-metabolizing antioxidant enzymes, molecular chaperones, DNA repair enzymes, and anti-inflammatory response proteins [74]. These proteins reduce electrophiles and free radicals to less toxic intermediates whilst also increasing the ability of the cell to repair any subsequent damage [69, 74-76]. In this regard, curcumin is able to induce protection and activate Nrf2-dependent protective responses in cell lines or animal models exposed to oxidative conditions [77].

Further, curcumin has been shown to induce GSTP1 expression with the involvement of transcription factor Nrf2 in human hepatic cells [78]. Dietary administration of curcumin elevated hepatic GST and NQO1, resulting in increased detoxification of benzo(a)pyrene-treated mice [79]. Pretreatment with curcumin can also protect against H₂O₂-induced cell death in retinal cell lines 661W and ARPE-19 by upregulating HO-1 and thioredoxin via Nrf2 [80]. Moreover, mice injected intraperitoneally with curcumin showed a twofold increase in total brain glutathione levels after treatment with buthioninesulfoximine [81]. Oral curcumin administration also resulted in enhanced nuclear translocation, ARE-binding of Nrf2, and a subsequent increase in the liver expression of HO-1, suggesting that curcumin has hepatoprotection potential in dimethylnitrosamine (DMN)-induced hepatotoxicity through Nrf2 activation [72]. Curcumin also activates ARE-mediated expression of antioxidant defense genes in human monocytes via PKC- ζ , p38MAPK, and Nrf2 [82]. The ability of curcumin to reverse functional and structural alterations in rats with 5/6 nephrectomy was clearly associated with enhanced translocation of Nrf2, attenuation of oxidant stress, and preservation of the activity of several antioxidant enzymes [57]. Moreover, Khor et al. [83] demonstrated that curcumin, at least in part, had a chemopreventive effect on prostate cancer through Nrf2, while Carmona-Ramírez et al. [84] found that curcumin had a neuroprotective effect through Nrf2 activation and increased superoxide dismutase and glutathione peroxidase activity in rats with neurodegeneration.

Sulforaphane

Sulforaphane (SFN), 1-isothiocyanate-(4R)-(methylsulfinyl)butane, is a dietary isothiocyanate produced by myrosinase activity on glucopharanin, a 4-methylsulfinylbutyl glucosinolate present in cruciferous vegetables of the genus *Brassica*, which includes broccoli, Brussels sprouts, and cabbage [85]. SFN has garnered particular interest as an indirect antioxidant due to its extraordinary ability to induce expression of several enzymes via the KEAP1/Nrf2 pathway [86, 87]. In diabetic rats, the antioxidant and anti-inflammatory effects of SFN appear to be mediated through increased expression of Nrf2 and downstream targets HO-1 and NQO-1 and the reduction of NF- κ B expression [88]. Treatment with SFN reduced the renal dysfunction and injury caused by ischemia-reperfusion of the rat kidney. These effects were mediated by the induction of phase II enzymes by decreasing the Keap1 protein levels and increasing Nrf2 nuclear translocation [89]. A recent study has also shown that after long-term treatment with SFN, diabetic

mice exhibited significant renal prevention from nephropathy via induction of NRF2-mediated antioxidant pathway [90].

Furthermore, treatment with SFN prevented oxidative stress-induced cytotoxicity in rat striatal cultures by increasing the intracellular GSH content via an increase in γ -GCS expression induced by the activation of the ARE/Nrf2 pathway [91]. SFN also functions in the endothelium, where it induces protective genes during injury and inhibits inflammatory genes via Nrf2 in vivo [92]. In the a rodent brain and microvasculature injury model, SFN has been demonstrated to induce Nrf2-driven genes and reduce brain damage following a traumatic brain injury [93]. Moreover, in a model of neonatal hypoxia-ischemia, pretreatment with SFN increased the expression of Nrf2 and HO-1 in the mouse brain and reduced infarct ratio [94].

Bai et al. [95] investigated whether this compound can prevent diabetic cardiomyopathy. To do this, type-1 diabetes was induced in rats by intraperitoneal injections with low dose streptozotocin. Following SFN treatment, these diabetic rats showed beneficial results triggered by an upregulation in Nrf2 expression and transcription activity that was reflected by increased accumulation of nuclear Nrf2 and phosphorylation as well as increased mRNA and protein expression of downstream Nrf2 antioxidants. In vitro, SFN has also been shown to reduce the risk of tumorigenesis in breast epithelial cells through Nrf2 activation [96].

Notably, SFN increased both the mRNA and protein expressions of Nrf2 as well as the expression of the downstream target gene NQO-1 in TRAMP C1 cells [97]. Baek et al. 2008 [98] further demonstrated the protective effects of SFN and an extract of young radish (*Raphanussativus L.*) cultivated with sulfur (sulfur-radish extract) for carbon tetrachloride-induced liver injury in mice. They showed that both SFN and the sulfur-radish extract ameliorated the carbon tetrachloride induced increase in the serum level of alanine aminotransferase, lipid peroxidation, and necrosis. This hepatoprotective effect was associated with liver phase II enzyme induction. Lii et al. 2010 [99] have shown that SFN also upregulates the expression of GST through the Nrf2 pathway in rat Clone 9 liver cells, while Ernst et al., 2011 have shown that SFN increases Nrf2-dependent gene expression in murine cultured fibroblasts [100].

Quercetin

Quercetin is a dietary polyphenol, predominantly present in citrus fruits and buckwheat. It is a multi-potent bioflavonoid with immense potential for the prevention and treatment of cancer [101]. Quercetin is capable of activating Nrf2 and upregulating phase II enzymes, such as NQO1, in HepG2 cells with an EC50 of 15 μ M as measured using an ARE-luciferase reporter gene assay [102]. Interestingly, in hepatocytes, quercetin increased cellular Nrf2 level not only by inhibiting ubiquitination of Nrf2 but also by increasing the level of Nrf2 mRNA. Low levels (50 μ M) of quercetin also provided marked protection of RAW264.7 macrophages from H₂O₂-induced apoptosis through the upregulation of Nrf2-directed enzymes, including HO-1 [103]. Recently, Yao et al. [104] observed that HO-1 upregulation by quercetin also protects human hepatocytes from oxidative stress. In this study, p38 and ERK, two players in the MAPK signaling pathway, mediate the quercetin-derived Nrf2 translocation into nuclei and subsequent induction of HO-1 activity.

tert-Butylhydroquinone

tert-Butylhydroquinone (tBHQ) is a synthetic phenolic antioxidant that is widely used as a preservative to extend the shelf life of various foods. It has been well-established that tBHQ exerts its antioxidant function by increasing Nrf2 protein stability [105, 106]. Interestingly, tBHQ, like SFN, activates Nrf2 via inhibition of Keap1-mediated ubiquitination through the modification of Cys151 [42, 107]. Several studies have also used tBHQ as a positive control treatment while investigating Nrf2 activation and cellular signaling [108, 109]. Downstream genes, such as NQO1 and GST, are also affected by tBHQ, which was found to induce the synthesis of these factors in mouse liver and intestinal mucosa [110]. More recently, it was found that tBHQ prevents the deposition of amyloid β -protein after oxidative stress in NT2N neurons, a cell line model for

Alzheimer disease, through the activation of Nrf2 [111], and provides effective prophylaxis against cerebral ischemia in vivo [112].

CONCLUSION

It is apparent that phytochemicals play an important role in combating oxidative stress. In this review, we have given a number of examples wherein phytochemical stress signals initiate a cellular response through Nrf2. Although our understanding of the mechanism of action for dietary phytochemical is continually expanding, there is much left to learn. Future work will ideally focus on additional phytochemical isolation, characterization, and clinical application.

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