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Review

Molecular mechanisms of natural products in chemoprevention: Induction of cytoprotective enzymes by Nrf2

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Cancer chemoprevention involves the use of natural or synthetic compounds to reduce the risk of developing cancer. One of the potential strategies for preventing cancer in the human population is to use food-based natural products to induce cytoprotective enzymes, such as NAD(P)H:quinone oxidoreductase 1, glutathione *S*-transferase, superoxide dismutase, and heme oxygenase-1. The regulatory regions of these inducible genes contain the antioxidant response element (ARE), which is activated upon binding of the nuclear factor E2-related protein 2 (Nrf2) transcription factor protein. Nrf2 has been shown to be essential in the upregulation of these genes in response to oxidative stress and treatment with certain dietary phytochemicals. This review presents the current body of knowledge regarding the molecular mechanisms of Nrf2 regulation, and highlights the need for future investigations into how these mechanisms apply to natural product inducers of cytoprotective enzymes.

Keywords: Antioxidant response element / Cancer prevention / Cul3 / Keap1 / Nrf2

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1 Introduction

Cytoprotective enzyme induction is believed to be an important means of protecting against carcinogenesis. These inducible, cytoprotective enzymes include superoxide dismutase, NAD(P)H:quinone oxidoreductase 1 (QR1), and glutathione *S*-transferase, which lead to detoxification and elimination of carcinogens, antioxidant enzymes such as heme oxygenase-1, and enzymes that regulate the reducing environment of the cell such as the NADPH-regenerating enzyme glucose 6-phosphate dehydrogenase. A promising means for upregulating levels of cytoprotective enzymes in the human population is the consumption of plant-based foods and other natural products [1, 2]. The increased levels of cytoprotective enzymes induced by these phytochemicals over the course of a human lifetime could lead to reduced rates of carcinogenesis. Clinical studies of cytoprotective enzyme induction by natural products in the human population are underway [3]. The success of using natural products to upregulate cytoprotective enzymes in the human population requires knowledge of the molecular mechanisms that govern this upregulation, and this knowledge will facilitate the evaluation of the chemopreventive potential of natural products. In addition, an increased understanding of the molecular basis of cytoprotective enzyme induction may aid in the design of new, targeted therapies for cancer prevention, as well as the development of mechanism-based biomarkers, a key hurdle in bringing chemoprevention to the clinical setting [4].

The antioxidant response element (ARE) has been identified in the regulatory regions of numerous genes that encode cytoprotective enzymes and are upregulated in response to oxidants, electrophiles, and natural products. Compounds that induce cytoprotective enzyme expression in an ARE-dependent manner will be collectively referred to herein as ARE inducers. Induced expression of genes by ARE inducers is highly dependent on the basic region-leucine zipper (bZIP)-type transcription factor nuclear factor E2-related protein 2 (Nrf2) (Fig. 1). A schematic for the pri-

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Abbreviations: ARE, antioxidant response element; **co-IP,** co-immunoprecipitation; **EPA_{ox},** eicosapentaenoic acid oxidation products; **GSK-3 β**, glycogen synthase kinase-3β; **4-HNE,** 4-hydroxynonenal; **Keap1,** Kelch-like ECH-associated protein 1; **MAPK,** mitogen-activated protein kinase; **NES,** nuclear export signal; **Nrf2,** nuclear factor E2-related protein 2; **PI3K,** phosphatidylinositol 3-kinase; **PKC,** protein kinase C; **QR1,** NAD(P)H:quinone oxidoreductase 1; **tBHQ,** *tert*-butylhydroquinone

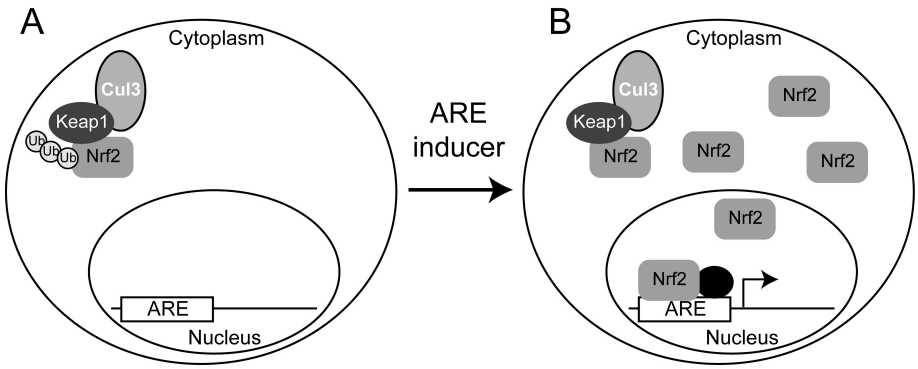


Figure 1. Schematic of Nrf2 nuclear accumulation and ARE upregulation of cytoprotective enzymes in response to ARE inducers. (A) In the absence of ARE inducers, Nrf2 is constitutively ubiquitinated and degraded. Keap1 serves as a bridge between Nrf2 and Cul3. (B) Treatment of cells with an ARE inducer inhibits ubiquitination of Nrf2, leading to its nuclear accumulation and activation of cytoprotective enzymes.

mary sequence of Nrf2 is shown in Fig. 2A. Gene expression profiling studies in the Nrf2 null mouse have shown that Nrf2 is essentially required for upregulation of cytoprotective genes by natural product ARE inducers, including phenethyl isothiocyanate [5] and sulforaphane [6, 7] from cruciferous plants such as broccoli and curcumin [8] from the spice turmeric (Table 1). These natural products have been shown to be cancer preventive in animal models against a wide spectrum of cancer types [9 – 14].

Due to the importance of Nrf2 in mediating the upregulation of cytoprotective enzyme expression by natural product cancer preventive agents, this review will focus on the current body of knowledge regarding the molecular mechanisms of regulation of the Nrf2-signaling pathway by ARE inducers, with particular attention to its regulation by natural products. Because of the large number of publications in this area, the focus of the review will be placed on findings that are mechanistic in nature, excluding several studies which implicate phosphorylation pathways but currently lack a mechanistic component. This review will also present the current studies regarding the potential for cancer prevention in humans through upregulation of the Nrf2 pathway.

2 Regulation of Nrf2 in the absence of ARE inducers

In the absence of ARE inducers, Nrf2 protein is found primarily in the cytoplasm of cells where its concentration is maintained at a low level (Fig. 1A). Upon introduction of ARE inducers to the organism or cell, Nrf2 protein levels increase, and Nrf2 accumulates in the nucleus where it forms heterodimers with other bZIP-domain containing transcription factors (Fig. 1B). These heterodimers bind to the ARE and induce expression of ARE-regulated genes through recruitment of the transcriptional machinery. Details of the events leading to Nrf2 nuclear accumulation in response to ARE inducers will be reviewed in Sections 3 and 4.

In the absence of ARE inducers, Nrf2 is constitutively expressed at high levels, but it is also constitutively ubiquitinated and degraded such that its overall concentration is maintained at a low level [15] (Fig. 1A). The cytoplasmic sequestration of Nrf2 and its ubiquitination are both mediated by the Kelch-like ECH-associated protein 1 (Keap1) protein. A schematic of the primary sequence of Keap1 is shown in Fig. 2B. A Crm1-dependent nuclear export signal (NES) sequence in Keap1, numbered L^{301}VKIFEELTL^{310} in the human Keap1 sequence, helps to maintain the Keap1 – Nrf2 protein complex in the cytoplasm [16 – 18]. The BTB (broad complex, tramtrack, bric-a-brac) domain of Keap1 mediates formation of homodimers and the Kelch repeat domain mediates binding to the Neh2 domain of Nrf2 [19] (Fig. 2). Recent studies have found two sites within the Neh2 domain of Nrf2, termed the DLG and ETGE motifs (Fig. 2A) that mediate binding to the Keap1 Kelch repeats [20, 21].

Keap1 serves as a bridge between Nrf2 and the Cullin3-based E3 – ligase ubiquitination complex, leading to ubiquitination of lysines in the Neh2 domain thereby targeting Nrf2 for degradation by the 26S proteasome [22]. These lysines are located between the two Kelch-binding sites on Neh2 (Fig. 2A), and a model has been proposed whereby binding of a Keap1 homodimer to these two sites allows for ubiquitination to occur [20, 23].

The cellular localization of Nrf2 in the cytoplasm under basal conditions is also mediated by two Crm1-dependent NESs within the protein (Fig. 2A). A canonical redox-insensitive NES of Nrf2 has been found, and there has been some confusion in the literature as to whether two redox-insensitive NESs have been found. In fact, the NES reported as ^{537}LKKQLSTLYL^{546} [24] and the NES reported as ^{545}LKRRLSTLYL^{554} [25] both refer to the same region of

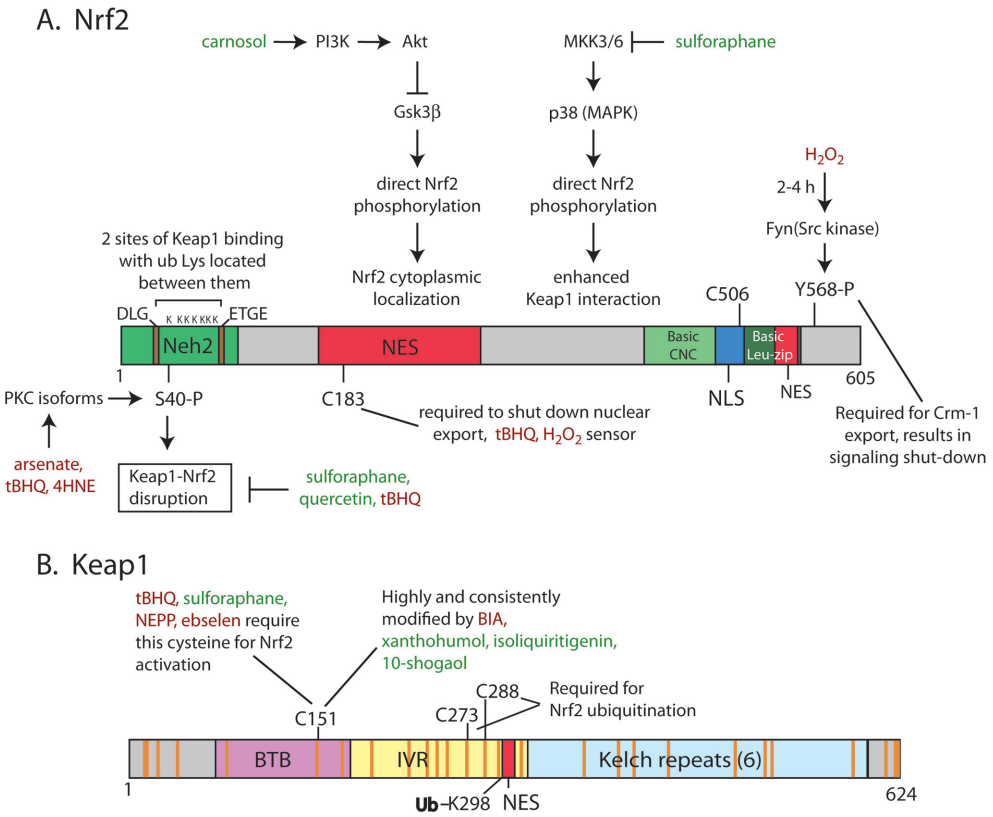


Figure 2. Primary sequences of (A) Nrf2 and (B) Keap1 with proposed regulatory mechanisms illustrated. ARE inducers from natural sources are indicated in green text, and those from non-natural sources are shown in magenta text. Keap1 cysteines are represented as orange lines. Details for the elements and mechanisms as well as their references are given in the text.

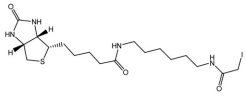
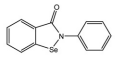
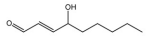
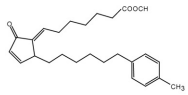
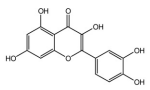
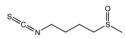
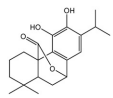
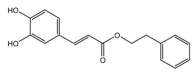
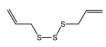
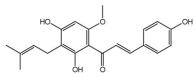
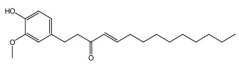
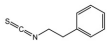
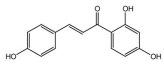
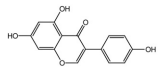
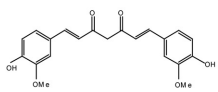
homology in Nrf2 from two different species, human and mouse. An additional NES has been identified, reported as ^{175}LLSIPELQCLNI^{186} [26], which has been shown to be redox sensitive as described in Section 4. In addition to the two NESs, Nrf2 contains a bipartite nuclear localization signal (NLS), numbered R^{494}RRGKQKVAANQCRKRK^{511} in the mouse Keap1 [25] (Fig. 2A), which likely facilitates Nrf2 nuclear localization upon addition of ARE inducers.

In summary, in the absence of ARE inducers, association of Nrf2 with Keap1 in the Cul3 ubiquitin ligase complex ensures that Nrf2 is kept sequestered in the cytoplasm at low, steady-state levels. Therefore under basal conditions there is low expression of the ARE battery of gene products. However, in the presence of ARE inducers, or under conditions of oxidative stress, the system is perturbed, and Nrf2 accumulates in the nucleus leading to increased transcription of genes under control of the ARE (Fig. 1). Since 2002, a growing number of groups have contributed to the understanding of the molecular basis for Nrf2 activation by these inducers. Two general mechanisms for Nrf2 nuclear accumulation in response to inducers have emerged. The first is downregulation of Nrf2 ubiquitination, proposed to occur through disruption of the Keap1–Cul3 and Keap1–Nrf2 complexes, and the other is alteration of the nuclear import/export of Nrf2. The following sections outline the current understanding of these mechanisms, with a focus on mechanistic studies of ARE induction by natural products. However, as many of these studies have been carried out with non-natural inducers, these will be discussed as well, in order to present a more complete picture of our current understanding of induction of this pathway.

3 Effects of ARE inducers on the Cul3–Keap1–Nrf2 complex and ubiquitination of Nrf2 and Keap1

It is widely accepted that Nrf2 protein accumulates and localizes to the nucleus in response to ARE inducers (Fig. 1). Nrf2 ubiquitination is downregulated in response to ARE inducers including sulforaphane [16], *tert*-butylhydroquinone (tBHQ) [16] and quercetin [27] (Table 1), resulting in the accumulation of both cytoplasmic and nuclear levels of Nrf2 protein. Increasing attention is now focusing on the molecular mechanisms of the effects of ARE inducers on the activity of the Cul3–Keap1–Nrf2 complex including downregulation of Cul3-mediated Nrf2 ubiquitination, and a possible increase in Keap1 ubiquitina-

Table 1. ARE inducers

Compound	Structure	Natural product	Plant source	Nrf2 mechanistic investigations	Mechanism references
tBHQ		No	N/A	Yes	[16], [22], [26], [36], [38 – 40], [50], [55], [57]
BIA		No	N/A	Yes	[37]
Phorone		No	N/A	Yes	[41]
Ebselen		No	N/A	Yes	[30]
4-HNE		No	N/A	Yes	[40]
NEPP11		No	N/A	Yes	[29]
Quercetin		Yes	Onions, apples, green and black tea	Yes	[27]
Sulforaphane		Yes	Cruciferous vegetables	Yes	[16], [22], [26], [36], [46]
Carnosol		Yes	Rosemary	Yes	[52]
Caffeic acid phenethyl ester		Yes	Honey	No	N/A
Diallyl trisulfide		Yes	Garlic	No	N/A
Xanthohumol		Yes	Hops	No	N/A
(10)-shogaol		Yes	Ginger	No	N/A
PEITC		Yes	Cuciferous vegetables	No	N/A
Isoliquiritigenin		Yes	Licorice, shallots, and tonka bean seeds	No	N/A
Genistein		Yes	Soy products	No	N/A
Curcumin		Yes	Turmeric	No	N/A

tion. Modification of Keap1 cysteines and phosphorylation of Nrf2 have both been proposed to alter the protein – protein interactions within this complex. The importance of Keap1 cysteine modification, in regards to Nrf2 ubiquitination downregulation, effects on Keap1 interactions with Nrf2 and Cul3, and possible effects on Keap1 ubiquitination, will be discussed first, followed by the effects of Nrf2 phosphorylation on the Keap1 – Nrf2 complex interaction.

Although the chemical nature of ARE inducers varies widely, the vast majority of ARE inducers contain electrophilic moieties capable of reacting with sulfhydryls such as reactive cysteine residues (Table 1). Human Keap1 contains

27 cysteines (Fig. 2B), 25 of which are highly conserved across species. Due to its high cysteine content, Keap1 was proposed to be the sought-after sensor of ARE inducers soon after its discovery as a suppressor of Nrf2 transcriptional activity [28]. Convincing evidence has since accumulated that Keap1 cysteines play an important physiological role in sensing the presence of ARE inducers and oxidative stress. Thus far, C151, located in the BTB domain (Fig. 2B), is the only cysteine identified to be required for stabilization and activation of Nrf2 in response to the ARE inducers sulforaphane and tBHQ [16]. The importance of C151 in sensing ARE inducers was subsequently illustrated by work from several other groups [29 – 32]. Neurite outgrowth-promoting prostaglandin 11 (NEPP11), an endogenous NEPP [29], and ebselen (Table 1), a seleno-organic drug [30], are both highly dependent on Keap1 C151 for their upregulation of the ARE response in cells. Importantly, our group has found C151 to be one of the most reactive cysteines in the human Keap1 protein *in vitro* [31, 33], and the only cysteine consistently and highly modified *in vitro* by the natural product ARE inducers xanthohumol, isoliquiritigenin, and 10-shogaol (Table 1) [32]. Mutation of two additional cysteines, C273 and C288, located in the intervening region (IVR) domain of Keap1 (Fig. 2B), show these cysteines are required for efficient ubiquitination and basal inhibition of Nrf2 in cell culture [16], and these cysteines may play a role as well in sensing of ARE inducers. Therefore, modification of Keap1 cysteines, and in particular C151, by inducers likely impairs the ability of Keap1 to efficiently ubiquitinate Nrf2 and target it for degradation.

There is still little known about the mechanism by which Keap1 cysteine modification leads to the downregulation of Nrf2 ubiquitination. An early model based on *in vitro* experiments was proposed in which modification of Keap1 cysteines by ARE inducers directly disrupts the interaction between Keap1 and the Neh2 domain of Nrf2 [34]. This attractive model had a large impact on the field, and numerous groups have interpreted Nrf2 nuclear accumulation as resulting from the disruption of the Keap1 – Nrf2 interaction and have reported it as such. However, subsequent *in vitro* experiments by our group demonstrated that in fact disruption of the Keap1 – Neh2 complex does not occur upon modification of Keap1 cysteines [31].

While modification of Keap1 cysteines does not alter the affinity of Keap1 for Nrf2, recent data indicate that the Keap1 – Cul3 interaction is altered by Keap1 cysteine modification. The Hannink lab demonstrated by coimmunoprecipitation (co-IP) that less Cul3 precipitated with Keap1 upon treatment with sulforaphane or tBHQ [22]. Interestingly, a C151S mutation largely abrogated this effect, again implying a key role for C151 in ARE induction. Gao *et al*. [35] also observed by co-IP that the oxidative products of n-3 fatty acids such as eicosapentaenoic acid (EPA_{ox}), a major component of fish oil, resulted in a reduced association of Cul3 with Keap1. They also noted that EPA_{ox} reacted with Keap1 cysteines. Collectively, these results suggest that reaction of ARE inducers with Keap1 cysteines leads to a reduced association between Keap1 and Cul3, thereby downregulating Nrf2 ubiquitination. This would in turn lead to Nrf2 accumulation and increased expression of ARE-controlled gene products. However, more precise *in vitro* experiments are required to quantitate any alteration in the dissociation constants of Keap1 from Cul3 when modified by electrophiles, as well as the effect on Nrf2 ubiquitination.

Although the primary focus of the field has been on the regulation of Nrf2 ubiquitination due to its important role, the increased ubiquitination of Keap1 and degradation by a proteasome-independent pathway is beginning to receive attention as it may also play a role in ARE induction. Different ARE inducers appear to have very different effects on Keap1 ubiquitination and stability. Zhang *et al*. [36] first proposed that tBHQ-induced ubiquitination of Keap1 results in increased degradation of Keap1 by a proteasome-independent mechanism. Interestingly, they observed that unlike tBHQ, sulforaphane treatment did not result in Keap1 ubiquitination and degradation. They also noted that the increase in Keap1 degradation occurred by a C151-independent pathway, distinct from the decrease in Nrf2 ubiquitination. Other groups have also observed effects of ARE inducers on Keap1 ubiquitination and stability. For example, Hong *et al*. [37] noted that electrophilic adduction to Keap1 cysteines coincided with increased Keap1 ubiquitination and Nrf2 stabilization in cells exposed to *N*-iodoacetyl-*N*-biotinylhexylenediamine (BIA). They identified the site of ubiquitination as K298 (Fig. 2B). Keap1 ubiquitination increased in the presence of heavy metals such as arsenic and chromium in mouse Hepa1c1c7 cells, at least upon initial exposure to the metal [38, 39]. However, no change was observed in endogenous Keap1 levels in the study. Treatment of HepG2 cells with quercetin was observed to result in decreased endogenous Keap1 levels, although in this study no change in Keap1 ubiquitination was detected [27]. Collectively these results suggest the possibility that a subset of ARE inducers increase ubiquitin transfer to the Keap1 protein, resulting in decreased Keap1 protein levels, which would then lead to increased Nrf2 activation. Further studies are required to elucidate whether the different findings result from the use of different cell lines or different ARE inducers, as well as to determine whether Keap1 cysteine modification or other mechanisms are required for the increase in Keap1 ubiquitination. Further studies are also required to determine how ubiquitination of Keap1 would be increased if the interaction of Cul3 and Keap1 were disrupted upon treatment with ARE inducers, as reported for sulforaphane and tBHQ [22] as well as for EPA_{ox} [35].

Phosphorylation of Nrf2 S40 by a protein kinase C (PKC) isoform appears to play an important role in mediating signaling by ARE inducers (Fig. 2B). The activity of

the PKC enzyme was stimulated by all three of the ARE inducers tested including tBHQ [40], phorone, and 4-hydroxynonenal (4-HNE) [41]. PKC inhibitors downregulated the ARE induction response in these studies. The sole site of phosphorylation by PKC was identified *in vitro* as S40 [41 – 43]. The importance of S40 phosphorylation in mediating ARE induction was illustrated by experiments in which the nuclear translocation of a Nrf2 S40A mutant protein in response to 4-HNE was greatly decreased compared to wild-type protein [41]. This study also found that atypical PKC isoforms are involved [41], while other studies have implicated a novel isoform, PKCδ, in mediating ARE signaling by arsenate [44, 45]. Data from co-IP experiments suggest that *in vitro* phosphorylation of Nrf2 by PKC promotes its dissociation from Keap1, and that this effect is largely abrogated by the S40A mutation [42]. Neither the importance of PKC phosphorylation of S40 in signaling by natural product ARE inducers nor any effect on Nrf2 ubiquitination or Keap1 ubiquitination have been investigated.

A second kinase pathway involving p38 mitogen-activated protein kinase (MAPK) isoforms has also been implicated in ARE signaling, and alteration of the Keap1 – Nrf2 affinity has been proposed as the mechanistic explanation (Fig. 2A). The Kong group found that p38 MAPK isoforms, immunoprecipitated from cell lysates, were able to phosphorylate purified Nrf2 protein [46]. This in turn increased the amount of endogenous Keap1 that coimmunoprecipitated with Nrf2, suggesting that Nrf2 binds more tightly to Keap1 after phosphorylation by p38 MAPK. They further showed that sulforaphane inhibited the upstream kinase MKK3/6. Therefore, sulforaphane is proposed to inhibit the phosphorylation of Nrf2 by p38 MAPK isoforms, resulting in a reduced interaction between Keap1 and Nrf2 and subsequent Nrf2 activation. However, other groups have found that p38 MAPK activation, rather than p38 MAPK inhibition, leads to ARE induction [47 – 49]. One possible explanation for the different findings is that while certain ARE inducers may activate p38 MAPK, others including sulforaphane may inhibit p38 MAPK.

The Keap1 – Nrf2 interaction has been proposed by various groups to be disrupted by ARE inducers, either through direct Keap1 cysteine modification, stimulating phosphorylation of Nrf2 S40 by PKC isoforms, or by inhibition of p38 MAPK isoforms phosphorylation of Nrf2. However, monitoring of the Keap1 – Nrf2 complex in the cellular environment by co-IP assays by several groups has shown that Keap1 and Nrf2 remain associated after treatment of cells with either tBHQ [22, 50], sulforaphane [22], or quercetin [27] (Fig. 2A). Nuclear disruption of the Keap1 – Nrf2 complex has been observed by co-IP in response to the heavy metals arsenic and chromium VI [38, 39]; however conflicting results for tBHQ-induced nuclear disruption of Nrf2 – Keap1 were observed in these two studies [38, 39]. Co-IP is a useful but imprecise means of determining comparative affinities of protein – protein interactions, making it difficult to ascertain whether partial disruption of the Keap1 – Nrf2 complex occurs in response to inducers in the cellular context. It is worthwhile to note that downregulation of Nrf2 ubiquitination is sufficient to promote Nrf2 nuclear accumulation and ARE activation, as shown by treatment of cells with the proteasome inhibitor MG132 [16].

In summary, downregulation of Nrf2 ubiquitination is a key factor in ARE inducer signaling, and changes in Keap1 ubiquitination may play a role as well. Further studies are required to determine how Keap1 cysteine modification might lead to changes in ubiquitination of Nrf2 and possibly Keap1. Alteration of the Keap1 – Cul3 interaction seems to be a likely mechanism, while disruption of the Keap1 – Nrf2 interaction by Keap1 cysteine modification has been ruled out. Alteration of the Keap1 – Nrf2 complex by Nrf2 phosphorylation, leading to decreased Nrf2 ubiquitination, is also an attractive mechanism requiring further investigation.

4 Effects of ARE inducers on the nuclear import and export of Nrf2

Nrf2 nuclear accumulation in response to ARE inducers is clearly a result of an alteration of the Keap1 – Nrf2 – Cul3 ubiquitination complex in some manner, leading to downregulation of Nrf2 ubiquitination. However, recent studies indicate that nuclear import and export of Nrf2 also play a role in the mechanism of Nrf2 nuclear accumulation in response to ARE inducers. Both Nrf2 phosphorylation events and Nrf2 cysteine modification have been proposed to affect Nrf2 nuclear localization. These studies are discussed below, along with initial studies that indicate Keap1 may accumulate in the nucleus in response to inducers as well.

Two Nrf2 phosphorylation events have been shown to regulate Nrf2 nuclear import/export in response to ARE inducers. First, the Cuadrado group has proposed a mechanism involving direct phosphorylation of Nrf2 by glycogen synthase kinase-3β (GSK-3β), downstream of phosphatidylinositol 3-kinase (PI3K) (Fig. 2A). They observed that the ARE induction and long-term antioxidant activities of carnosol (Table 1), a chalcone diterpene from rosemary that stimulates glutathione-*S*-transferase and QR1 activities in the rat liver [51], were dependent on PI3K [52]. Other groups have observed a dependence on PI3K for Nrf2 activation of non-natural product ARE inducers [53 – 55]. Johnson and coworkers [55] noted that a PI3K inhibitor reduced ARE activation of endogenous Nrf2 in response to tBHQ in IMR-32 cells. The PI3K inhibitor treatment did not appear to affect Nrf2 degradation, but it did reduce nuclear localization of Nrf2, suggesting that PI3K activation by ARE inducers results in alteration of the nuclear import/export of Nrf2 rather than the ubiquitination status of Nrf2. The Cuadrado lab then found that the Akt1 kinase, downstream from PI3K, is required for ARE induction by carnosol [52]. This led them to investigate whether GSK-3β, which is inacti-

vated by Akt1, was a negative regulator of Nrf2 activity [56]. They found that Nrf2 is indeed a substrate for GSK-3β, and it promotes cytoplasmic localization of Nrf2. Therefore, it appears that ARE inducers can activate the PI3K pathway, leading ultimately to Nrf2 nuclear accumulation by downregulating Nrf2 phosphorylation by GSK-3β (Fig. 2A). It is not yet known whether phosphorylation of Nrf2 by GSK-3β under basal conditions (in the absence of ARE inducers) promotes nuclear export or inhibits nuclear import, or the identity of the site of phosphorylation.

A second mechanism whereby Nrf2 phosphorylation modulates Nrf2 localization in response to ARE inducers was found by Jain and Jaiswal [57] (Fig. 2A). A temporal Crm1 mediated export of Nrf2 is stimulated several hours postinduction, leading to eventual removal of Nrf2 from the nucleus after ARE induction has occurred. The proposed mechanism of export is stimulation of phosphorylation of Nrf2 Y568, leading to enhanced interaction with Crm1. Using transfected Hepa1 cells, they showed that the Nrf2 mutant protein Y568A predominately localized in the nucleus, and they demonstrated by a co-IP assay that the interaction of Nrf2 with Crm1 was dependent on tBHQ-induced Y568 phosphorylation. Using the inducer H_2O_2 and PP2, an inhibitor of the Src family of tyrosine kinases, they showed in Hep-G2 cells that 2 h after induced nuclear accumulation, endogenous Nrf2 nuclear export was highly dependent on the activity of this family of kinases. Experiments utilizing siRNA against Fyn kinase, an Src kinase, in Hepa1 cells transfected with Nrf2 proteins showed that Fyn is likely responsible for the phosphorylation. Based on this work it appears that ARE inducers may stimulate an Src kinase, likely Fyn kinase, leading to the export of Nrf2 from the nucleus several hours after induction thereby shutting down the signaling response. Further investigations are required to determine whether natural product – ARE inducers utilize this mechanism established for H_2O_2.

Interestingly, the aforementioned study by Jain and Jaiswal [57] was initiated in part by the observation that a Tyr kinase inhibitor, genistein (Table 1), induced-Nrf2 nuclear accumulation, and ARE activation. Genistein is a isoflavanoid found predominantly in soy that has been shown to be an inducer of QR1 in several cell lines [58, 59]. This raises several interesting questions that remain to be answered. For instance, is the observed ARE activation by genistein [57] due mainly to its ability to inhibit phosphorylation of Nrf2 Y568 by Src kinases, or is it also acting at the level of downregulating Nrf2 ubiquitination? Furthermore, might other natural product – ARE inducers similar in structure to genistein act primarily by inhibiting phosphorylation of Nrf2 Y568 or by downregulating Nrf2 ubiquitination?

In addition to Nrf2 phosphorylation, modification of Nrf2 cysteines may also regulate Nrf2 localization, though its importance compared to Keap1 cysteine modification in sensing inducers and its physiological relevance have been disputed [23]. A cysteine is located at residue 183 in the NES identified by Li *et al*. [26] (Fig. 2A). They showed that tBHQ and H_2O_2 were unable to stimulate ARE induction in HeLa cells transfected with the Nrf2 C183A mutant, as compared to wild-type Nrf2 [26]. Similar results were obtained with a construct containing the NES of Nrf2 rather than the full-length Nrf2 protein. These results indicate that modification or oxidation of Nrf2 C183 may shut down the nuclear export of wild-type Nrf2, allowing Nrf2 nuclear accumulation. However, when ARE activation by sulforaphane was tested, using the construct containing only the NES of Nrf2, the dependence on Nrf2 C183 was considerably weaker than that observed for tBHQ and H_2O_2. The experiment with sulforaphane was not carried out using the full-length Nrf2. An interesting possibility is that sulforaphane and other promising cancer preventive agents may rely on modification of C183 to a far lesser extent than oxidizing agents such as tBHQ and H_2O_2. Jaiswal and coworkers [60] found that C506 of Nrf2 was required for nuclear accumulation of overexpressed Nrf2 under basal conditions. Further work is required to determine whether modification of Nrf2 C506 plays a role in sensing ARE inducers.

With regards to the relative importance of Nrf2 cysteine modification in the overall signaling mechanism, Tong *et al*. [23] point out that an Nrf2 self-redox induction model is not well supported by other data in the literature. In the experiments by Li *et al*. [26] examining Nrf2 C183 dependence, only Nrf2 protein, and not Keap1 protein, is overexpressed. However, Keap1 protein exerts a strong inhibitory effect on Nrf2 nuclear accumulation [61], making it unlikely that activation of the ARE would be significantly dependent upon Nrf2 C183 if overexpressed Keap1 were present in those experiments. In support of this idea, an Nrf2 mutant lacking the ability to bind Keap1 was shown to no longer be responsive to ARE inducers in the presence of overexpressed Keap1, despite having an intact NES including C183 [62]. In contrast, the cysteines of Keap1 protein, and C151 in particular, have been demonstrated by multiple groups to play an important role in signaling, including in the presence of overexpressed Nrf2, as described in Section 3.

5 Relevance of the Nrf2 pathway to cancer prevention in humans

While the effects of chemopreventive agents are likely pleotropic in nature [63, 64], there is a large body of evidence accumulating that induction of cytoprotective enzymes by chemopreventive agents through the Nrf2 pathway will be an important and viable part of preventing cancer in the human population. There is clear evidence that this is the case in animals, and the strongest of which is as follows. First, many compounds shown to be chemopreventive in animal models were originally isolated from plants based solely on their ability to induce cytoprotective enzymes [65]. For example, sulforaphane was first isolated

from broccoli as a highly potent inducer of QR1 in murine hepatoma cells [66]. Sulforaphane has since been shown to be highly effective in several animal models of cancer prevention [9, 67, 68], including carcinogen-induced neoplasia of the forestomach [69] and preneoplastic lesions [70] in mice. Second, studies with the Nrf2 knock-out mouse, whose cytoprotective enzymes are no longer inducible, show that cytoprotective enzyme induction leads to cancer prevention, as these knock-out mice showed a greater propensity to develop carcinogen-induced tumors in both the bladder [71] and the forestomach [72]. Third, the well-characterized chemopreventive agent and ARE activator oltipraz was no longer able to prevent carcinogenesis in these Nrf2 knock-out mouse studies [71, 72], illustrating more directly the importance of Nrf2 in cancer prevention. Similarly, neoplasia of the forestomach was no longer prevented by sulforaphane in Nrf2 knock-out mice [69].

Evidence in humans that increased expression of cytoprotective enzymes, induced by natural products acting by Nrf2 pathway, can lead to cancer prevention is understandably more difficult to obtain but is accumulating. Cytoprotective enzymes are inducible in humans; individuals consuming broccoli were found to have higher levels of cytoprotective enzymes in their saliva after consumption [73]. Importantly, consumption of broccoli in particular, and cruciferous vegetables in general, correlates with reduced cancer risk in the prostate [74], breast [75], colon and rectum [76], and lung [77, 78]. Cytoprotective enzymes are upregulated in human cell lines in response to chemopreventive agents, including by sulforaphane in MCF-7 cells [79], by genistein in MCF-10A cells [80], and by quercetin in HepG2 cells [27]. In this study, using siRNA against Nrf2, Nrf2 was shown to be critical for the upregulation of cytoprotective enzymes by quercetin [27].

Interestingly, recent studies have found somatic mutations in the KEAP1 gene in human lung cancer cells, which result in increased activity of Nrf2 and higher levels of ARE-regulated genes [81, 82]. These findings indicate that lung tumor cells hijack the Nrf2 pathway to increase their survival, likely to combat the high oxygen environment of the lung as well as chemotherapeutic agents [81, 82]. While these findings could be interpreted to mean that upregulation of the Nrf2 pathway using ARE inducers could contribute to carcinogenesis, this seems unlikely. Cumulatively, the data indicate that induction of cytoprotective enzymes by certain chemopreventive agents will be an important tool in cancer prevention, and that Nrf2 plays a key role in cytoprotective enzyme upregulation.

6 Summary

Our understanding of the molecular basis for the upregulation of cytoprotective enzymes by the Keap1 – Nrf2 pathway has been greatly enhanced by a large number of studies from numerous labs over the past decade. The major contributing mechanisms of Nrf2 activation by ARE inducers identified thus far are modification of Keap1 cysteines, which leads to downregulation of Nrf2 ubiquitination, and activation of Nrf2 phosphorylation, which alters Nrf2 cellular localization.

However, as described in this review, the vast majority of ARE inducers used in these studies are non-natural products that tend to create oxidative stress in the cellular environment, as opposed to food-based natural products which generally tend to have more antioxidant and nontoxic effects on the cell. To date, only three natural product molecules, sulforaphane, carnosol, and quercetin, have been investigated in terms of their molecular basis for Nrf2 nuclear accumulation. Moreover, only sulforaphane has been studied for its roles in multiple mechanisms. In this review, we have chosen to not include the results for natural inducers shown to activate phosphorylation pathways if the mechanistic basis of Nrf2 nuclear accumulation has not yet been elucidated. It seems highly likely that natural product inducers will lead to downregulation of Nrf2 ubiquitination as a growing number of these have been shown to increase Nrf2 protein levels. These include diallyl trisulfide [83], the most potent ARE inducer of cytoprotective enzymes found in garlic oil, caffeic acid phenethyl ester from honey [84], and curcumin from turmeric [84] (Table 1). It also seems likely based on their electrophilic nature that the vast majority of cytoprotective-enzyme inducing natural products [65] will act on the Keap1 – Nrf2 pathway through modification of Keap1 or Nrf2 cysteines (Table 1). As an example, isoliquiritigenin, a chalcone found in shallots, licorice and tonka bean seeds, was identified as a potent inducer of QR1 and also showed a significant response in the mouse mammary organ culture assay [85]. Isoliquiritigenin contains a Michael-reaction acceptor moiety that readily modifies Keap1 cysteines, with C151 being the most reactive toward isoliquiritigenin [32].

Further mechanistic studies of promising, cancer preventive, natural product inducers are required to determine whether the molecular mechanisms of Nrf2 activation utilized by these natural agents are the same as or different from those used by the more widely studied non-natural inducers, many of which cause oxidative stress. An interesting possibility is that natural product ARE inducers will utilize somewhat different mechanisms to activate Nrf2 compared to agents that tend to cause oxidative stress as well as upregulate cytoprotective enzymes. This type of ARE inducer "fingerprint" could facilitate the identification of the most promising cancer preventive agents.

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