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REGULATORY MECHANISMS CONTROLLING GENE EXPRESSION MEDIATED BY THE ANTIOXIDANT RESPONSE ELEMENT

Truyen Nguyen, Philip J. Sherratt, and Cecil B. Pickett
Schering-Plough Research Institute, Kenilworth, New Jersey 07033;
email: truyen.nguyen@spcorp.com, philip.sherratt@spcorp.com,
cecil.pickett@spcorp.com

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■ **Abstract** The expression of genes encoding antioxidative and Phase II detoxification enzymes is induced in cells exposed to electrophilic compounds and phenolic antioxidants. Induction of these enzymes is regulated at the transcriptional level and is mediated by a specific enhancer, the antioxidant response element or ARE, found in the promoter of the enzyme's gene. The transcription factor Nrf2 has been implicated as the central protein that interacts with the ARE to activate gene transcription constitutively or in response to an oxidative stress signal. This review focuses on the molecular mechanisms whereby the trancriptional activation mediated by the interaction between the ARE and NF-E2-related factor 2 (Nrf2) is regulated. Recent studies suggest that the sequence context of the ARE, the nature of the chemical inducers, and the cell type are important for determining the activity of the enhancer in a particular gene.

INTRODUCTION

The antioxidant response element (ARE) is a *cis*-acting enhancer sequence that mediates transcriptional activation of genes in cells exposed to oxidative stress (1). The term oxidative stress encompasses a broad spectrum of circumstances that cause a change in the cellular redox status such as an increased production of free radical species within the cell or by pro-oxidant xenobiotics that are thiol reactive and mimic an oxidative insult. In keeping with this, genes that are regulated by the ARE encode proteins that help control the cellular redox status and defend the cell against oxidative damage (2). Proteins that are encoded by the ARE gene battery include enzymes associated with glutathione biosynthesis (3, 4), redox proteins with active sulfhydryl moieties (5, 6), and drug-metabolizing enzymes (7–10).

The first evidence for the existence of the ARE pathway for gene regulation came from the observation that certain xenobiotics could modulate the regulation of Phase I and Phase II drug-metabolizing enzymes in different ways (11). The Phase I drug-metabolizing enzyme cytochrome P450 (CYP) 1A1 is induced in cells following exposure to endogenous and xenobiotic compounds that are ligands for the aryl-hydrocarbon receptor (AhR) (12). This well-characterized pathway involves the AhR-ligand complex dimerizing with the AhR-nuclear translocator protein (ARNT) and then binding to a DNA enhancer region known as the xenobiotic response element (XRE) (13, 14). Certain AhR ligands are also capable of inducing Phase II drug-metabolizing enzymes such as the glutathione *S*-transferases (GST) (15) and NAD(P)H:quinone oxidoreductase (NQO1) (8). Compounds that can induce both Phase I and Phase II drug-metabolizing enzymes were designated bifunctional inducers. In addition, a number of compounds were identified that could only regulate the expression of Phase II enzymes; these chemicals were referred to as monofunctional inducers (16).

Further experiments using cell lines with a low level of AhR expression and mice that expressed high- or low-affinity AhR showed that the induction of Phase II drug-metabolizing enzymes following exposure to bifunctional inducers was impaired. However, the compounds that were classed as monofunctional inducers were still capable of increasing the expression of Phase II enzymes. For the bifunctional inducers to increase Phase II detoxification enzyme expression, the inducer has to be metabolized by the monooxygenase system to compounds that are chemically similar to the monofunctional inducers. This is facilitated by their ability to induce *CYP 1A1*, thus increasing their own metabolism (7). This work demonstrated the existence of a distinct pathway for Phase II enzyme induction that was independent of the AhR.

Following these studies, a novel *cis*-acting element was discovered within the 5′ flanking region of the rat *GSTA2* subunit gene that was responsive to β-naphthoflavone (β-NF), a bifunctional inducer, in the presence of a functional AhR (17). Promoter analysis showed that the DNA sequence of this 41-bp regulatory region was distinct from the XRE sequence (15). This unique enhancer region was later designated the ARE, as it was not only responsive to β-NF but also to phenolic antioxidants (7). Thus, the ARE can mediate a transcriptional response to a broad spectrum of structurally diverse chemicals (Figure 1).

The core DNA sequence essential for the response to these chemicals was determined through deletion and mutational analysis. The ARE consensus sequence is defined as 5′-TGACnnnGC-3′ (1). Subsequent to these studies, other genes have been identified that are induced by monofunctional and bifunctional agents possessing this *cis*-acting enhancer, including the mouse *Gsta1* subunit gene (18) and the rat and human *NQO1* genes (8, 19–21).

This review intends to provide an account of the current knowledge on the ARE gene battery and focuses on the most recent topics of research to determine the transcription factors and signaling events that are important in regulating this enhancer.

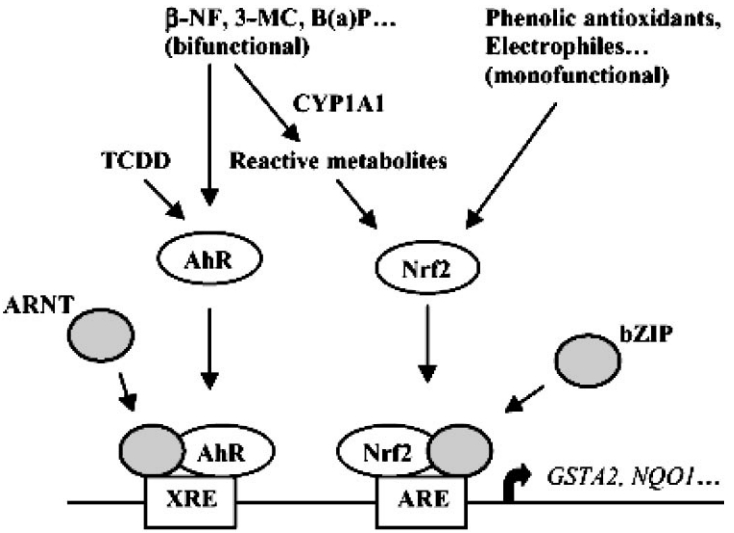


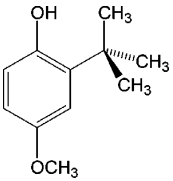
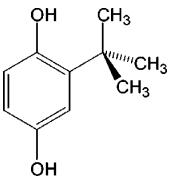
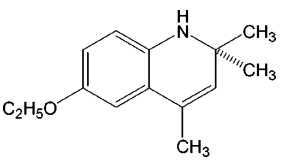
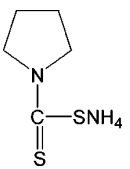
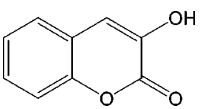
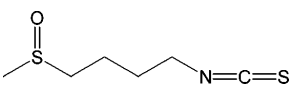
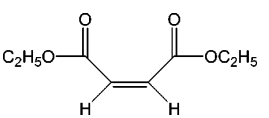
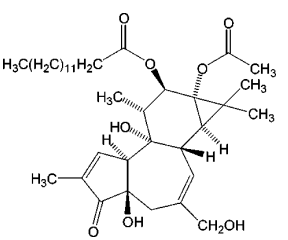
Figure 1 Transcriptional regulation of the rat *GSTA2* and *NQO1* genes by bifunctional and monofunctional inducers. The bifunctional inducers and the dioxin TCDD bind to and activate the AhR, which then translocates into the nucleus and associates with ARNT to activate transcription through the XRE. The bifunctional inducers can also activate transcription through the ARE via a separate pathway following their biotransformation into reactive metabolites that have characteristics of the monofunctional inducers. The monofunctional inducers can only act through the ARE-mediated pathway. 3-MC, 3-methylcholanthrene; B(a)P, benzo(a)pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

ENZYME INDUCTION BY XENOBIOTICS MEDIATED BY THE ARE

A wide range of structurally diverse compounds can activate the ARE. Classes of xenobiotics that can stimulate ARE-driven transcription include large planar compounds such as flavonoids and phenolic antioxidants (1), thiol-containing compounds such as isothiocyanates (22, 23) and 1,2-dithiole-3-thiones (24), heavy metals (25), and heme complexes (26, 27). Table 1 shows classes and examples of xenobiotics that are known to stimulate ARE-driven transcription.

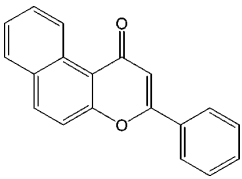
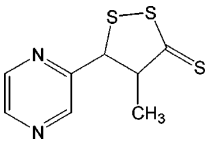
Monofunctional inducers share certain common chemical properties. They are electrophilic compounds that are capable of reacting with sulfhydryl groups; in addition, certain compounds can also undergo oxidation-reduction reactions (28). The bifunctional inducers acquire these properties following oxidative metabolism. Compounds such as the isothiocyanates and diethyl maleate do not require metabolism and can directly react with sulfhydryl groups, oxidizing cysteine

TABLE 1 Types of compounds that can stimulate ARE-driven transcription

Compound	Structure	Class	Nature of compound
Butylated hydroxyanisole		Synthetic phenolic antioxidant	A compound used as a food preservative and antioxidant in dermatological creams
tert-butyl-hydroquinone		Synthetic phenolic antioxidant	Metabolite of BHA used as a model inducing agent for ARE-driven transcription
Ethoxyquin		Synthetic antioxidant	A compound used as a pesticide and insecticide. Currently used as a pet food preservative
Pyrrolidin-edithiocarbamate		Synthetic antioxidant	A molecule that blocks activation of NF-κB and induction of iNOS
3-hydroxycoumarin		Coumarin	A naturally occurring chemopreventive agent found in leguminous vegetables
Sulforaphane		Isothiocyanate	A naturally occurring chemopreventive agent found in cruciferous vegetables
Diethyl maleate		GSH-depleting agent	A synthetic compound used as a model inducer of drug-metabolizing enzymes
Phorbol 12-myristate 13-acetate		Phorbol Ester	A potent tumor-promoting agent in mouse skin and has numerous biochemical effects such as activating PKC isoenzymes and stimulating expression of iNOS and COX-2

(*Continued*)

TABLE 1 (*Continued*)

Compound	Structure	Class	Nature of compound
β-naphthoflavone		Flavonoid	Synthetic compound used as a model inducer of drug-metabolizing enzymes
Oltipraz		1,2-dithiole-3-thione	Antischistosomal drug and cancer chemopreventive agent

residues and depleting reduced cellular glutathione (GSH); in this way, they mimic an oxidative insult. Compounds such as the phenolic antioxidant butylated hydroxyanisole (BHA) are metabolized to *tert*-butylhydroquinone (*t*BHQ), which following a dealkylation step can undergo oxidation-reduction reactions within cells. In this case, BHA is acting as a pro-oxidant. A model bifunctional inducer is β-NF. This synthetic flavonoid is metabolized by CYP 1A1 to a quinone intermediate that can undergo redox cycling (7).

In correlation with the nature of compounds that can induce ARE-driven transcription, many of the proteins whose expression is mediated by the ARE have an endogenous role in regulating cellular redox status and protecting the cell from oxidative damage. Enzymes such as GST, NQO1, and HO-1 function to detoxify harmful by-products of oxidative stress, including lipid and DNA base hydroperoxides (29, 30), quinones (31), and heme-containing molecules (32). The induction of enzymes involved in GSH biosynthesis leads to an increase in cellular GSH levels that provides a buffer against oxidative insult (2).

Apart from protecting the cell against endogenous toxic compounds, proteins associated with Phase II of drug metabolism are important for detoxifying genotoxic compounds that may be produced as a consequence of Phase I xenobiotic metabolism (33). For this reason, the use of monofunctional inducers to increase the capacity to detoxify chemical carcinogens during Phase II of drug metabolism has some therapeutic potential in cancer chemoprevention (34).

A number of studies have shown that synthetic and naturally occurring compounds, when included in the diet of rodents, can induce genes under the control of an ARE that provides protection against chemical carcinogens. For example, the inclusion of ethoxyquin or coumarin in the diet of rats can protect against the hepatocarcinogen aflatoxin B1 (35, 36). This compound is converted into the genotoxic intermediate aflatoxin B1 8,9-*exo*-epoxide by the action of CYP isoenzymes. The highly inducible GST A5-5 and aflatoxin-aldehyde reductase (AFAR) enzymes in rat liver that are regulated by the ARE detoxify this intermediate. Another

chemopreventive agent, oltipraz, which is a member of the 1,2-dithiole-3-thione class of compounds, is currently being used in a clinical trial in a region of China where aflatoxin B1-induced hepatocarcinogenesis is a real problem (37). There is also a good correlation between consuming a diet high in fruits and vegetables and a decreased risk in developing cancer (38). Many fruits and vegetables are natural sources of compounds such as isothiocyanates and coumarins that possess chemopreventive properties (39, 40).

THE ANTIOXIDANT RESPONSE ELEMENT

The existence of mono- and bifunctional inducers as proposed by Talalay et al. (11, 16) suggests that expression of many Phase II drug-metabolizing enzymes may be regulated by an additional pathway that is distinct from that mediated by the AhR via the XRE. Following the isolation and characterization of the structural gene encoding the rat GSTA2 subunit (17), a reporter construct was generated in which the 5′ regulatory sequence of this gene was fused to that encoding the bacterial enzyme chloramphenicol acetyl transferase (CAT). Analysis of this construct in transient transfection experiments showed that HepG2 cells containing this plasmid exhibited elevated CAT activity when exposed to β-NF (17). These observations provided evidence that expression of rat *GSTA2* is regulated at the transcriptional level in response to xenobiotics. A series of deletion mutants were then generated from the original reporter construct and tested in transient transfection assays in order to identify *cis*-acting sequences responsible for the responsiveness to β-NF. Results obtained from these experiments eventually revealed the presence of a novel *cis*-acting element that is distinct from and in addition to the XRE (15). When a reporter construct under the control of this *cis*-acting element was transfected into cells, induction of CAT activity was observed not only in response to β-NF but also to the phenolic antioxidant *t*BHQ, a compound unable to elicit a response via the XRE (1, 7). Because of its responsiveness to *t*BHQ, this enhancer was therefore referred to as the ARE. At the same time, an identical enhancer element was also discovered in the mouse *Gsta1* subunit gene, which is the orthologue of the rat *GSTA2* subunit gene, and was referred to as the electrophile response element or EpRE (18). Another feature of the ARE that distinguishes it from the XRE is its ability to mediate a response to *t*BHQ in mutant cell lines lacking either a functional AhR or CYP 1A1 (7) (Figure 1). Thus, these data strongly confirm the proposal that induction of Phase II enzymes may also be mediated by an AhR-independent mechanism (11, 16). Further characterization of the ARE in the rat *GSTA2* gene by point mutation analysis led to the identification of a core sequence 5′-TGACnnnGC-3′, which was shown to be essential for both basal and/or inducible activity (1).

In addition to the genes that encode the rat GSTA2 and mouse GSTA1 polypeptides, genes encoding the rat and human NQO1 proteins (8, 9), γ-glutamylcysteine catalytic (γ-GCS_h) and regulatory (γ-GCS_l) subunits (3, 4, 41), and HO-1 (27) were also found to be transcriptionally regulated via the ARE. Through computer

searches of various databases, a number of other genes, including those encoding human phenolic sulfotransferases, were found to contain ARE-like sequences (42, 43), though they are yet to be functionally characterized.

As discussed in the previous section, the ARE mediates gene expression in response to a wide variety of compounds. The observations that these compounds affect the cellular redox status along with the similarity in sequence identified between the ARE and the TPA-response element (TRE) raised the possibility that the ARE may be regulated by members of the AP-1 protein family. These transcription factors are known to activate gene transcription in response to numerous signals, including phorbol esters, growth factors, UV irradiation, and oxidative stress (44). However, despite their close similarity in sequence, the ARE has unique features that distinguish it from the TRE. The main difference between these two enhancers is the presence of the -GC- box at the 3′ end of the ARE core sequence, which has been shown to be critical for its inducibility (1, 19, 45). Thus, cells transfected with an ARE/TRE-CAT construct in which the ARE sequence had been mutated at noncritical nucleotides so as to also contain a consensus TRE motif (5′-TGACAAAGC-3′ to 5′-TGACTCAGC-3′) were found to be responsive to both *t*BHQ and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Mutation of the -GC- box in this construct resulted in a failure to respond to *t*BHQ; however, the reporter construct was still responsive to phorbol ester treatment (45). Furthermore, induction of CAT activity was only observed in HepG2 cells transfected with an ARE-CAT reporter construct, but not with a TRE-CAT reporter construct, in response to *t*BHQ treatment (45). These data correlate with those from other studies that suggest that activation of gene expression via the ARE and TRE are mediated by different signaling pathways (25, 46).

It should be noted, however, that the ARE found in some of the genes contains an embedded TRE sequence (see Table 2), and it is possible that these genes are

TABLE 2 Sequences of ARE found in different genes

GSTA2 (rat)	5′-AAATGGCATTGCTAATGG **TGACAAAGC** AACT-3′
Gsta1 (mouse)	5′-AAATGACATTGCTAATGG **TGACAAAGC** AACT-3′
GSTP (rat)	5′-AGTAGTCAGTCACTA **TGATTCAGC** AACA-3′
NQO1 (rat)	5′-AGTCTAGAGTCACAG **TGACTTGGC** AAAA-3′
NQO1 (human)	5′-AAATCGCAGTCACAG **TGACTCAGC** AGAA-3′
GCS_h (human)	5′-AGC **GCTGAGTCA** CGGGG-. . . .-CCCGCGCAGTCAC-3′
GCS_l (human)	5′-GGATGAGTAACGG-. . . .-TTCT **GCTTAGTCA** TTG-3′
Ho-1 (mouse)	5′-TCT **GCTGAGTCA** AGGTCCG-3′
NF-E2 (consensus)	5′-TGT **GCTGAGTCA** CTG-3′
TRE (consensus)	5′-GCA **TGAGTCA** GAC-3′

The core ARE sequence is indicated by the nucleotides in boldface type. Underlined nucleotides represent the AP-1-like sequence required for full basal expression for certain genes.

under the control of both enhancers. In fact, in vitro DNA binding studies using antibody supershift assays have detected a number of transcription factors that can bind to the ARE, notably Nrf1 and Nrf2 (NF-E2-related factor), members of the AP-1 family, and small Maf proteins (discussed below). This is not surprising as many of these basic region-leucine zipper (bZIP) proteins can potentially dimerize with each other to generate a diverse array of functional protein complexes that bind to DNA with unique and/or overlapping specificity.

TRANSCRIPTION FACTORS ACTING ON THE ARE

The ARE contains the 5′-TGAC-3′ tetranucleotide within its core sequence that resembles the half-site recognized by members of the AP-1 (consensus: 5′-TGACTCA-3′) and the ATF/CREB (consensus: 5′-TGACGTCA-3′) families of transcription factors. All members of these two families of proteins belong to a class of transcription factors collectively known as bZIP proteins, which can bind to DNA as part of a homodimer and/or heterodimer complex. These observations suggest that bZIP proteins may be involved in regulating the ARE. In earlier studies, members of the AP-1 protein family were believed to act on the ARE on the basis that these proteins are known to be activated by various signals that cause cellular stress (20, 21, 47). In addition, the presence of a consensus TRE sequence within the ARE of the human *NQO1* gene further strengthened this belief (20, 21). However, a number of studies have failed to confirm that AP-1 proteins play a major role in mediating the ARE response, largely based on data showing that *t*BHQ, a model inducing agent for the ARE, could not elicit a response through the TRE (25, 45, 46). Furthermore, in vitro DNA binding assays have shown that the AP-1 proteins cannot bind to the ARE with high affinity and specificity (19, 45). In more recent studies, compelling evidence has been obtained from in vitro and in vivo studies suggesting that the bZIP transcription factor Nrf2 is intimately involved in mediating the ARE-driven response to oxidative stress and xenobiotics (10, 48–52).

The Nrf2 Protein

The Nrf2 protein was first isolated by an expression cloning procedure using an oligonucleotide containing the NF-E2 DNA binding motif as probe to screen for closely related proteins (53). NF-E2 is a dimeric protein originally identified as involved in the regulation of globin gene expression in hematopoietic cells (54, 55). The NF-E2 protein activates gene transcription following binding to its consensus DNA binding motif 5′-TGCTGAGTCAC-3′ as a heterodimer consisting of a 45-Kda and an 18-Kda subunit (56). The p45 subunit is a bZIP protein consisting of a transactivation domain within the N-terminal region and a basic DNA binding region/leucine zipper structure in the C-terminal region of the protein (54, 55). The p18 subunit was subsequently identified as a member of the small Maf proteins containing the basic region/leucine zipper but lacking any apparent transactivation

domain (56). Whereas expression of the p45 NF-E2 subunit is restricted to hematopoietic tissues (54, 55), two other related bZIP family members, Nrf1 and Nrf2, are ubiquitously expressed in a wide range of tissues and cell types (10, 53, 57). The tissue distribution profile of these proteins combined with their DNA binding motif being similar in comparison to that of the ARE led to the suggestion that ARE-responsive genes may be regulated by Nrf1 and/or Nrf2 (48). Their involvement was confirmed in transfection experiments where ectopic expression of either Nrf1 or Nrf2 in HepG2 cells was found to increase CAT activity from an ARE-CAT reporter construct derived from the human *NQO1* gene but was unable to induce CAT activity from a mutant ARE-CAT construct (48). Nrf2 was subsequently demonstrated to be involved in the transcriptional activation of other ARE-responsive genes including those encoding the human γ-GCS_h and γ-GCS_l subunits (49), mouse HO-1 (50), and rat NQO1 and GSTA2 subunits (51). More significantly, data from in vivo studies using gene knockout mice clearly implicated Nrf2 as a critical protein in regulating the expression of the *Gsta1* and *Nqo1* genes (52). Immunoblot analysis demonstrated that the expression level of these enzymes was reduced in Nrf2 (−/−) mice as compared to heterozygous animals. Of particular importance was the finding that induction of these enzymes by BHA, an inducer of ARE activity, was greatly attenuated in the null mice (52). These data suggest that Nrf2 mediates both the basal and inducible activity of the ARE. Further detailed characterization of these knockout mice demonstrated that the loss of Nrf2 resulted in a profound reduction in the expression and enzyme activities of NQO1, certain GST isoenzymes, and the γ-GCS_h subunit (10, 58).

Of particular interest from these studies were observations demonstrating the different effects Nrf2 may have on basal as well as inducible expression of these enzymes. For instance, the basal expression of NQO1 and certain GST subunits was significantly lower in Nrf2 (−/−) mice than in the wild-type animals. Whereas the inducible expression of NQO1 was mostly abolished in knockout mice, that of GSTs, particularly the GSTA1 subunit, was unaffected in the same animals in response to known ARE inducers such as BHA, ethoxyquin, and coumarin. By contrast, in the same studies the basal expression of the γ-GCS_h subunit was found to be similar in Nrf2 (−/−) and wild-type mice; however, its induction by the ARE inducers was abolished with the loss of Nrf2 (10). These data provide evidence suggesting the differential mode of regulation by Nrf2 for different genes. The biochemical basis for the differences observed in the involvement of Nrf2 in basal and/or inducible expression of these enzymes is currently not known.

The differential regulation of these genes may be explained at least in part by the sequence context of their ARE. Data from in vitro DNA footprinting analysis of the rat *GSTA2* and *NQO1* gene promoters showed that the DNase I–protection patterns produced by nuclear extracts isolated from human HepG2 or rat H4IIE cells were different for the two genes (51). Furthermore, in vitro synthesized Nrf2/MafK was found to exhibit a DNA binding activity with a higher affinity for the rat *NQO1*

ARE than the rat *GSTA2* subunit gene ARE by both DNA footprinting analysis and gel mobility shift assays (51). These results suggest that the context of the ARE, outside its core sequence, of each particular gene contributes an important role in its regulation. This was also confirmed by previous transfection experiments, which showed that mutating the rat *GSTA2* subunit ARE to contain a perfect TRE (i.e., ARE/TRE, see above) sequence significantly increased its basal activity and induction in response to β-NF, *t*BHQ, and TPA (45). The increased responsiveness is possibly due to the enhancer representing a higher-affinity binding site for Nrf2 as well as a site for AP-1 proteins. The ARE of the human *NQO1* gene, which contains an embedded TRE consensus sequence, may represent this type of dual enhancer. It has been shown by cotransfection experiments that Nrf1/Nrf2 and Jun proteins can synergize to potentiate the reporter gene expression under control of this ARE sequence as well as that of the rat *GSTA2* gene (59). Although DNA binding assays using nuclear extracts from mouse hepa1c1c7 cells and transcription factor antibodies showed that Nrf1, c-Fos, and JunD interacted with the *NQO1* ARE (48), it is not clear from these experiments whether the Nrf and Jun proteins acted independently or interacted together to enhance the ARE-mediated response because overexpression of these proteins individually also led to an increase of expression (59). Unexpectedly, overexpression of c-Jun and c-Fos proteins together failed to activate CAT gene transcription and caused a decrease in CAT activity in transfected cells, suggesting that AP-1 complexes may have a negative effect upon the ARE (48). Interestingly, an independent study demonstrated that phenolic antioxidants can inhibit AP-1 activity (46). The physiological role of members of the AP-1 protein family in regulating the expression of the human *NQO1* gene therefore remains to be established.

In the case of genes encoding the human γ-GCS_h and γ-GCS_l subunits, full basal and inducible expression has been shown to be controlled by two separate enhancers in close proximity to each other. One enhancer contains an AP-1 binding site, whereas the second is an ARE with an embedded AP-1 site (see Table 2). For the gene encoding the γ-GCS_h subunit, the presence of both enhancers is required for full basal activity, but only the ARE/AP-1 enhancer was important for β-NF induction (41). The promoter region responsible for the induction of the γ-*GCS*$_l$ subunit gene by β-NF also contains two enhancers that appear to act in a cooperative manner to produce a full inducible response (3). In addition to Nrf2, overexpression of JunD and small Maf was found to have an effect on the expression of these subunits (49). These data suggest that interactions may occur between other signaling pathways and the ARE to regulate the expression of certain genes. This may explain in vivo data that indicates that full basal expression of the γ-GCS_h subunit was unaffected in Nrf2 ($-/-$) mice, whereas its upregulation by known ARE inducers was essentially abolished (discussed above; 10).

Current data from in vitro DNA binding assays and transfection experiments point to Nrf2 as the most important protein involved in stimulating ARE-driven transcription. Using the chromatin immunoprecipitation assay (ChIP) studies from

this laboratory, we have observed that Nrf2 does interact directly with the rat *GSTA2* ARE in the nuclear compartment of rat H4IIE cells (unpublished observation). These data support the notion provided by in vivo studies in Nrf2 (−/−) mice that Nrf2 is important in basal as well as inducible gene expression. The role of Nrf2 has been demonstrated to be critical in recent studies that report that the reduced expression of Phase II drug-metabolizing enzymes in Nrf2 (−/−) mice confers a sensitive phenotype to the toxic effects of carcinogens and inflammatory drugs compared to wild-type animals (60–63).

Small Maf Proteins

As mentioned above, the bZIP proteins function as dimers in order to bind to DNA. Presently, there is no conclusive evidence to indicate whether Nrf2 may activate gene expression as a homodimer or whether it is obligated to form heterodimeric complexes. Because p45 NF-E2, the first family member of these proteins, was found to dimerize with MafK in order to bind DNA and activate transcription (56), it was hypothesized that small Maf proteins may represent a partner for Nrf2 in effecting transcriptional activation (52).

There are three known small Maf proteins, designated MafF, G, and K, each consisting of a basic DNA binding region and a leucine zipper; however, they lack a transactivation domain (64). These proteins also possess a cap 'and' collar (CNC) homology domain that has been shown to be important for recognition of an extended AP1-like binding motif (65). Small Mafs are present in the nucleus where they can form heterodimers with all members of the CNC protein family including the p45 NF-E2 and related factors (56, 66–68), the BACH proteins (69), and some of the AP-1 transcription factors (70).

The physiological role of small Maf proteins in terms of ARE regulation remains unresolved. Results from in vitro DNA binding experiments clearly demonstrated that Nrf2 only binds to DNA with a high affinity and specificity in the presence of small Maf proteins (51, 68). Nrf2 has not been shown to bind to DNA independently. This is presumably because it cannot either form a homodimer (53) or bind to DNA with a high affinity as a homodimer. Although small Maf proteins facilitate the ability of Nrf2 to bind to its target DNA sequence with a high affinity, this does not appear to effect an increase in transcriptional activity. In transfection experiments, the converse appears to be the case; MafK acts as a repressor of ARE-driven transcription. An increase in the amount of a plasmid that expresses the rat MafK inherently leads to decreasing levels of rat *GSTA2* and *NQO1* ARE-CAT activity in the presence of a constant amount of Nrf2 (51). Independent studies have also reported similar repressive effects associated with small Maf proteins on the human *NQO1* gene (71) and the human γ-GCS_h subunit gene (49). Interestingly, experiments employing a reporter gene construct containing the promoter region of the human γ-GCS_l subunit gene when transfected into HepG2 cells with a plasmid expressing Nrf2 and approximately a 10-fold lower molar amount of a plasmid expressing MafG showed a 1.4-fold increase in reporter gene activity

compared to cells overexpressing Nrf2 alone. However, increasing the amount of MafG plasmid in the transfection caused a decrease in the reporter gene activity (49). In the same studies, overexpression of a dominant-negative form of MafK (MafK DN), which cannot bind to DNA, also led to negative effects on the expression of both the catalytic and regulatory subunit genes. It is interesting that similar effects were observed for both wild-type MafG and MafK DN; therefore, further studies are required to determine the role of small Maf in the regulation of the γ-*GCS*$_l$ subunit gene expression.

In an independent study, in vitro gel mobility shift assays showed that MafG was present in a nuclear protein complex from CHO cells that interacted with an ARE-like enhancer in the mouse *Ho-1* gene, designated the StRE. In transfection experiments, overexpression of MafK DN was shown to block activity of this enhancer in response to cobalt (72). Although overexpression of MafK DN may have sequestered Nrf2 away from the StRE, these data alone were not sufficient to demonstrate a direct role for small Maf in transcriptional activation of *Ho-1* gene. The only evidence of small Maf proteins participating in transcriptional activation is when they act in concert with p45 NF-E2 (56, 66, 67, 73).

Unlike p45 NF-E2 and related factors, small Maf proteins are capable of binding DNA either as homodimers or heterodimers formed among other small Maf family members. It has therefore been postulated that small Maf dimers may bind to DNA and act as transcriptional repressors because they lack any transactivation domain (64). This would suggest that there is competition within the nucleus between small Mafs and transcriptional activators for the same binding site that determines a balance between repression and active (or derepressed) gene transcription. This was demonstrated in transfection experiments in Cos-1 cells, where at a low concentration MafK activated reporter gene transcription in concert with p45 NF-E2, whereas at higher concentrations its repression activity overcame the transactivation potential of p45 NF-E2 (74). In similar experiments employing the rat *GSTA2* and *NQO1* ARE, as described earlier, at no time did MafK elicit a positive effect on reporter gene expression even when cotransfected with Nrf2.

To assess whether endogenous levels of small Maf proteins are affected by known ARE inducers, various cell lines were treated with *t*BHQ, pyrrolidinedithiocarbamate (PDTC), and phenylethyl isothiocyanate (PEITC) (75). In these studies, all three small Maf proteins were constitutively expressed at various levels in different cell lines. Interestingly, the expression of the three proteins increased following exposure of the cells to PDTC and PEITC. However, treatment with *t*BHQ did not appear to induce expression levels of these proteins, suggesting that their induction may not be essential for ARE-mediated gene expression (75). These findings show some of the subtleties involved in regulation of the ARE and infer a possible feedback mechanism involving small Maf proteins in responses to certain stimuli.

All of the in vitro DNA binding studies to date have shown that small Maf binds to DNA more readily as a heterodimer with either p45 NF-E2, Nrf1, Nrf2,

or Nrf3 than as a homodimer or heterodimer formed between themselves. Thus, for small Maf dimers to compete with p45-small Maf complexes for the same binding site, the level of small Maf proteins would likely have to be greater than that of p45, assuming that the dimerization kinetics are similar among these proteins. Therefore, these observations raise the question of how a slight increase in the abundance of small Maf may tip the balance from enhanced to repressed gene expression, and this was shown in transfection studies (74) as well as by in vivo studies (76) that examined the transcriptional activation of genes regulated by NF-E2. A potential mechanism that may account for this effect is the association of small Maf with other bZIP proteins that may result in the formation of a repression complex that can effectively compete with NF-E2 for their binding site. MafK can dimerize with c-Fos to form a high-affinity DNA binding complex that acts as transcriptional repressor of NF-E2-mediated gene expression (70). However, this mechanism of repression does not explain the lack of any activation potential of small Maf proteins together with Nrf2 on ARE-mediated gene transcription.

Currently, the precise role of small Maf proteins in the regulation of ARE-driven transcription is unresolved. Further experiments using cell-based studies combined with in vivo gene knockout studies similar to those examining the basal and inducible expression of ARE-responsive genes in Nrf2 (−/−) mice (10, 52) involving individual and multiple Maf proteins would provide a better insight on this question. For instance, is BHA induction of *Gsta1* and *Nqo1* lost in small Maf (−/−) mice or mutant cell lines?

Other Proteins

Nrf2 is established as having a central role in the regulation of ARE-mediated gene expression; however, questions remain as to whether other related bZIP proteins may also be involved. There is some evidence indicating that Nrf1 may contribute to the expression of ARE-responsive genes. Ectopic expression of Nrf1 activates the human *NQO1* ARE reporter gene in HepG2 cells (48). Nrf1 also activates a reporter gene linked to the promoter of the γ-*GCS*$_h$ subunit gene and increases the intracellular level of GSH (77). A role for Nrf1 has been demonstrated further in studies employing cells containing a targeted disruption of the *Nrf1* locus. Although the loss of Nrf1 is lethal in early embryonic development (78), an analysis of mouse fibroblasts derived from Nrf1 (−/−) mouse embryonic tissue showed reduced levels of GSH and of γ-GCS$_l$ subunit expression compared to wild-type cells. Interestingly, induction of this enzyme was not affected by the loss of Nrf1, as treatment of the Nrf1-deficient fibroblasts with paraquat resulted in an increased level of its mRNA (79). These data and those obtained from studies involving Nrf2 (−/−) mice suggest that the expression of γ-*GCS* genes is mediated by both Nrf1 and Nrf2, with Nrf1 being involved in basal expression and Nrf2 in the inducible expression. It is not known whether Nrf1 plays a similar role, if any, on the ARE of other genes.

The p45 NF-E2 is unlikely to have any role, as its expression is restricted to hematopoietic cell lineages. Ectopic expression of p45 NF-E2, unlike Nrf2, did not increase reporter gene activity linked to the rat *GSTA2* and *NQO1* ARE in HepG2 cells (our unpublished observation), presumably because HepG2 cells as well as other nonhematopoietic cells lack specific cofactors required for full NF-E2 transcriptional activity. These observations are supported by a recent study that demonstrated in transient transfection experiments that overexpression of p45 NF-E2 could activate a reporter gene linked to an NF-E2 binding site in the erythroid cell line K562 but not in Cos-1 cells (73). The role of another member of the CNC protein family, Nrf3, in the regulation of ARE-responsive genes is not known, although overexpression of this protein activates a reporter gene linked to an enhancer containing the NF-E2 binding site (80).

In other recent studies, yeast two-hybrid screening experiments were employed to identify potential proteins that interact with Nrf2. In one such study, the bZIP protein ATF4 was identified and presumed to associate with Nrf2 by dimerization via its leucine zipper. The ATF4 protein bound and activated the mouse *Ho-1* ARE-reporter gene only in the presence of Nrf2 (81). At present, it is not known whether ATF4 has any function in the expression of other ARE-responsive genes. Using the same screening procedure, the polyamine-modulated factor-1 protein (PMF-1) has also been reported to be able to interact with Nrf2 and may contribute to the transcriptional regulation of the *spermidine/spermine N^1-acetyltransferase* gene via the polyamine response element (PRE, an ARE-like enhancer). Because overexpression of Nrf2 did not transcriptionally activate a reporter gene linked to the PRE, the significance of the interaction between Nrf2 and PMF-1 on ARE-responsive genes is not apparent (82, 83).

CELL SIGNALING PATHWAYS ASSOCIATED WITH THE ARE

Another major question is how does the cell sense and respond to the appropriate signals to initiate the cascade of signaling events leading to the activation of the ARE? Recent studies have implicated protein phosphorylation as being of major importance in the response to xenobiotics and oxidative stress that stimulates ARE-driven transcription. In addition to the action of kinases, a protein designated Kelch-like-ECH-associated protein 1 (Keap1) has been shown to repress Nrf2 activity within cells. It has been postulated that sulfhydryl group chemistry may also play an important part in the regulation of cell signaling pathways and in protein-protein interaction such as between Keap1 and Nrf2.

Signaling Pathways

To date, three major signal transduction pathways have been implicated in regulation of the ARE, which include those mediated by the MAPK cascades, PI3K, and PKC (Figure 2).

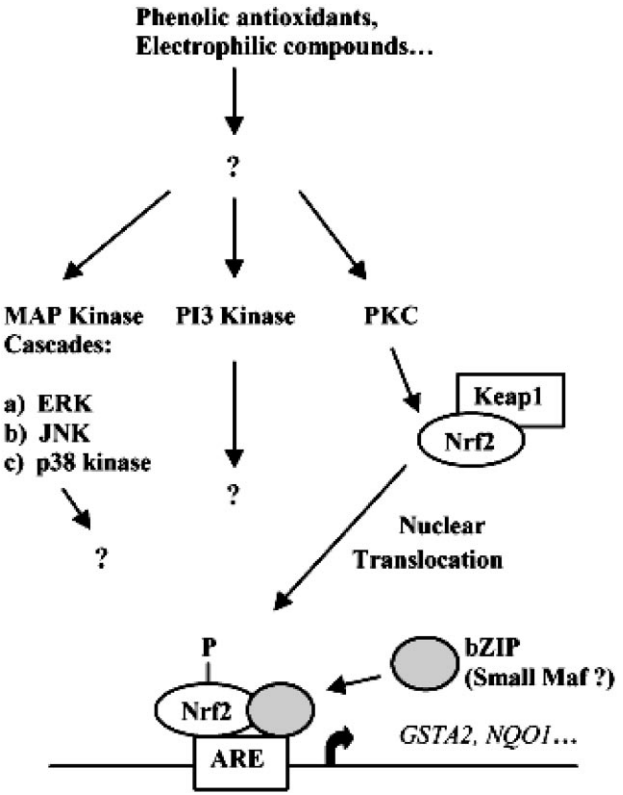


Figure 2 Signaling events involved in the transcriptional regulation of gene expression mediated by the ARE. Three major signaling pathways have been implicated in the regulation of the ARE-mediated transcriptional response to chemical stress. In vitro data suggest that direct phosphorylation by PKC may promote Nrf2 nuclear translocation as a mechanism leading to transcriptional activation of the ARE. Nrf2 has been proposed to be retained in the cytoplasm through an interaction with Keap1 and it is possible that phosphorylation of Nrf2 may also cause the disruption of this interaction. As a bZIP protein, Nrf2 binds to the ARE as a dimer. Although small Maf proteins have been proposed to represent the dimerizing partners for Nrf2 in the activation complex, this has not been conclusively demonstrated. The molecular mechanisms controlling the ARE-mediated transcription by the MAP kinase and PI3 kinase pathways remain to be determined (see text for detailed discussion).

MAPK SIGNALING PATHWAYS The first signal transduction pathways to be investigated in relation to the ARE were the mitogen activated protein kinase (MAPK) cascades. The first studies investigating their involvement reported that the classical extracellular regulated kinase (ERK) pathway made a positive contribution to ARE-driven gene expression (84). Experiments showed that ARE reporter gene

activity stimulated by the monofunctional inducers *t*BHQ and the isothiocyanate sulforophane (Sul) was mediated at least in part by ERK2 in human and mouse hepatoma cell lines. These compounds were found to cause an increase in the phosphorylation of ERK2 in hepatoma cells, and the MEK1 inhibitor PD 98059 could attenuate this activation. This was supported by data from reporter gene transfection experiments where the overexpression of a dominant-negative form of ERK2 also attenuated induction by *t*BHQ and Sul. In the mouse hepatoma cell line hepa1c1c7 cells, the induction of NQO1 activity by *t*BHQ and Sul could also be impaired by PD 98059. Interestingly, through the use of dominant-negative mutants of kinases upstream of ERK2, it was demonstrated that activation required raf-1 and occurred in a ras-independent manner.

In a parallel study, *t*BHQ and the bifunctional inducer β-NF, but not Sul, were shown to activate the p38 MAPK pathway in the same cell lines (85). The involvement of this pathway was identified by the use of SB 203580, an inhibitor of p38 kinase activity. Contrary to findings with the ERK pathway, the activation of p38 was found to be correlated with a down-regulation of basal and inducible ARE-reporter gene activity by this MAPK pathway. This was supported by transfection experiments showing that overexpression of dominant-negative mutants of other protein kinases that are components of the p38 pathway also effected a decrease in basal and inducible reporter gene activity in transfected cells. These data led to the suggestion that p38 exerts a general repressive effect upon the ARE and thus acts in opposition to the ERK pathway in response to *t*BHQ and β-NF in hepatoma cells.

A follow-up study investigating the third well characterized MAPK pathway showed that the c-jun N-terminal kinase (JNK) pathway may be activated in response to sodium arsenite and mercury chloride (86). This activation had a positive effect on the ARE and was dependent upon Nrf2, but not the c-jun transcription factor, a down-stream target of JNK. It was noted that JNK activity had no bearing on the activation of the ARE by *t*BHQ. Similar to ERK, the actions of JNK were also antagonized by the p38 pathway. These studies surmise that contribution of the MAPK signaling pathways in regulating the ARE depends upon the nature of the inducing agent.

A study to deduce the upstream signaling events leading to induction of the γ-GCS_h and γ-GCS_l subunits by PDTC reported slightly different findings (87). The treatment of human hepatoma cells with PDTC activated both the ERK and p38 MAPK pathways. It was discovered that these two pathways were acting synergistically to increase the transcription of the γ-*GCS* subunit genes. These conclusions were based upon the observation that a complete inhibition of PDTC induction could only be achieved following cotreatment with both PD 98059 and SB 202190. Although in partial agreement with previous studies as to the role of ERK, the role of p38 in the response to PDTC is different from that observed for *t*BHQ or β-NF induction.

So what factors may explain the differences observed for the contribution of the p38 pathway? The two studies do employ chemically distinct xenobiotics to

stimulate the ARE, and it may be that cell signaling pathways are tailored to specific stimuli. The genes of interest were also different between the two bodies of work. The endogenous gene of choice for the first set of data was the mouse *Nqo1* gene for which the promoter has not been characterized; the orthologous rat *NQO1* gene contains a putative ARE sequence, whereas the orthologous human *NQO1* gene contains an ARE with an embedded TRE (see Table 2). It is likely that the mouse *Nqo1* gene is regulated by an ARE, but it is not known whether AP-1 factors may also be important. These workers also used a reporter gene construct that contained the mouse *Gsta1* ARE sequence, which does not contain a TRE. The second study focused on the human γ-*GCS* subunit genes. The ARE sequence within theses genes contains an embedded AP1 site, like the human *NQO1* gene ARE, and also a farther AP1 site in close proximity to this ARE (Table 2). In addition, the ARE used in the respective reporter gene construct was from the human γ-GCS_l subunit gene that contains these ARE and AP-1 enhancers. It has been shown that the p38 MAPK cascade can elicit a positive effect upon the TRE, such as in the case of the MMP-9 collagenase gene (88). Thus, the differences in the role of p38 may be accounted for by the context of the ARE and how AP-1 transcription factors may interact with Nrf2.

In addition, there were differences in the finer details of the experimental conditions employed. The implication of p38 as a negative effector on the regulation of the ARE was demonstrated employing SB 203580 at a concentration of 5 μM, which is sufficient to inhibit p38 activity in cell lines. These data were supported by the use of dominant-negative mutant kinases. The implication of p38 as a positive regulator of the ARE was demonstrated using the pharmacological inhibitor SB 202190, which has a very similar specificity to SB 203580 but is more potent (89), at a concentration of 25 μM. The latter study did not employ the use of dominant-negative kinase mutants to support the findings from the inhibitor data. It has been noted that SB 203580 may have inhibitory effects on ARE-driven transcription at higher doses above 5 μM (our unpublished data), thus the differences observed could also be accounted for by the dose of compound used. Whether or not the inhibitory effects of high dose SB 202190 on γ-*GCS* induction are directly associated with the p38 pathway are therefore uncertain. There were also differences in cell culture conditions; the initial studies used serum-starved culture conditions, which is thought to be important in reducing basal MAPK activity within cells, particularly the ERK pathway. The second body of work carried out experiments in media containing high serum (10% v/v). The effects of serum upon the ARE have not been rigorously tested.

From other work, it appears possible that cell-specific effects could impact on the importance of certain transduction pathways that are associated with the ARE. Two independent studies that looked at the MAPK pathways involved in the regulation of human *HO-1* induction in response to cadmium reported different findings. In MCF-7 human mammary epithelial cells, cadmium activated the ERK, JNK, and p38 MAPK pathways (90). These experiments employed pharmacological inhibitors and dominant-negative mutants to deduce that basal and

cadmium- inducible HO-1 activity was dependent upon the p38 pathway and not the ERK or JNK pathways. However, in an alternative study, induction of the *HO-1* gene by cadmium was found to utilize the ERK pathway as opposed to the p38 pathway in HeLa cells (91). These conclusions were made employing the kinase inhibitors PD 98059 and SB 203580. It was not determined whether the p38 pathway had any repressive effects in either of the studies on *HO-1* regulation.

It appears that the many factors leading to the signaling context for a particular ARE may determine which of the MAPK signaling pathways are important in a given circumstance.

THE PI3K SIGNALING PATHWAY Separate from studies on the association between the ARE and the MAPK pathways, other studies have suggested that phosphatidylinositol 3-kinase (PI3K) is important in transducing a response to phenolic antioxidants and oxidative stress through to the ARE. This kinase is an integral component of the insulin signaling pathway. Experiments have shown that pretreatment of the H4IIE rat hepatoma cell line with the PI3K inhibitors, Wortmannin, and LY 294002 abolishes the induction of rat *GSTA2* mRNA by *t*BHQ and sulfur amino acid deprivation (SAAD) (92). In addition, it was shown that *t*BHQ treatment caused an increase in phosphorylation of Akt/PKB, a kinase downstream of PI3K in the insulin signaling pathway (93). No direct evidence was provided to implicate Akt/PKB as having an effect on the ARE. This report also suggested that ERK activity antagonizes ARE-mediated transcriptional activation from experimental data using PD 98059. The protein kinases, p38 and JNK1, did not affect induction of rat *GSTA2* by *t*BHQ in these experiments. It should be noted that the rat *GSTA2* subunit gene also contains other regulatory elements within its 5′ flanking region that includes an XRE (15). Whereas *t*BHQ does not affect the XRE (23), the pharmacological inhibitor PD 98059, which is a flavonoid, may do so. This compound has been shown to be a ligand for the AhR (94) so the clarity of findings with this compound on the role of ERK in H4IIE cells is uncertain. Flavonoids may be metabolized to inducing agents that activate the ARE; such is the case for β-NF. This emphasizes the importance of using more than one chemically distinct kinase inhibitor where possible to support findings using such compounds.

In a second body of work, there is further support for the notion that PI3K is important for enhancement of transcription at the ARE. These workers utilized the IMR-32 neuroblastoma cell line and demonstrated that pharmacological inhibitors of PI3K attenuated ARE-driven transcription in response to *t*BHQ (95). In experiments where the expression of a reporter gene is controlled by the human *NQO1* ARE, induction by *t*BHQ was attenuated by the PI3K pharmacological inhibitor LY 294002. In addition to chemical inhibitors, these studies employed a constitutively active PI3K, which increased the activity of a reporter gene construct containing the human *NQO1* ARE. However, contrary to the studies discussed above, there appeared to be no activation of PKB downstream of PI3K in cells exposed to *t*BHQ. In addition, these workers also provided evidence that activation of ERK is not important for *t*BHQ induction of *NQO1* in these cells based upon experiments

employing PD 98059. It is not known whether insulin can stimulate ARE-driven transcription.

In a follow-up study, a microarray approach was applied to identify *t*BHQ-inducible genes in the neuroblastoma cell line (96). IMR-32 cells were also cotreated with LY 294002 to determine which of these genes were regulated in a PI3K-dependent manner. The results highlighted 63 genes that were induced by *t*BHQ; the PI3K inhibitor attenuated the induction of 43 of these genes. Among the genes induced by *t*BHQ were those encoding NQO1, HO-1, and the γ-GCS subunits. Of these, *NQO1* and *HO-1* were induced in a PI3K-dependent manner, but the *γ-GCS* subunit genes were not. Interestingly, the gene for the human homologue of the Keap1 protein (discussed later) was also induced by *t*BHQ in a PI3K-dependent manner; it is not known what regulatory elements may be involved in controlling the expression of this gene. These data make a strong case for the importance of PI3K in the neuroblastoma cell line, although its importance in other cell lines is not clear.

THE PKC SIGNALING PATHWAY Of the investigations into regulation of the ARE by signal transduction pathways discussed so far, none have shown that phosphorylation of the transcription factor Nrf2 itself correlates with an increase in ARE activity. The first evidence for a direct effect of a kinase upon the transcription factor Nrf2 came from studies into the role of the PKC signaling pathways (97). Early reports characterizing the ARE demonstrated that phorbol esters as well as redox cycling compounds could stimulate transcription (45). From this fact, it was hypothesized that protein kinase C (PKC) isoenzymes may be involved in regulating the ARE.

The involvement of PKC in the regulation of the ARE was studied in the human hepatoma cell line HepG2 and the rat hepatoma cell line H4IIE (97). In transfection experiments, ARE reporter gene constructs were employed that contained either the rat *GSTA2* or the rat *NQO1* ARE. The use of staurosporine and RO-32-0432, which are broad-spectrum PKC inhibitors, impaired ARE reporter gene activity stimulated by TPA, *t*BHQ, and β-NF. In vivo labeling experiments were used to demonstrate that the Nrf2 protein became phosphorylated in response to *t*BHQ. In addition, it was shown that the translocation of endogenous Nrf2 from the cytoplasm to the nucleus and phosphorylation of the transcription factor were inhibited by pharmacological inhibitors of PKC. These events were not inhibited by the MEK1 and p38 kinase inhibitors U0126 or SB 203580, respectively. Importantly, it was subsequently shown that the PKC-catalytic subunit and PKC immunoprecipitated from cells could directly phosphorylate Nrf2 in vitro. The activity of immunoprecipitated PKC toward Nrf2 was higher in cells that had been stimulated with TPA, *t*BHQ, or β-NF. These experiments provide the first evidence that a kinase may have a direct effect on activation of the ARE in an Nrf2-dependent mechanism.

There is certain evidence that protein phosphorylation is important for stimulating and also possibly repressing ARE-driven transcription. Factors that appear

to determine which of the signal transduction pathways are important include the nature of the inducing agent, the cell type, and the context of the ARE in the gene of study. A heavy reliance has been placed upon information obtained from the use of pharmacological inhibitors of signaling pathways. Due to the frequency of contrasting data, in particular surrounding the MAPK cascades, more rigorous experiments are required using multiple approaches when deducing the signaling events leading to a specific ARE response. It is possible, however, that all of the signaling pathways discussed may play some role in regulating the ARE because there is the capacity for cross-talk to occur between these kinases. For example, it has been demonstrated that PI3K can activate PKC isoenzymes (98), and in turn that PKC isoenszymes can activate the MAPK cascades (99, 100). There is likely to be many complexities surrounding the association of protein kinases with the ARE.

The Importance of Other Cell Signaling Mechanisms and ARE Gene Expression

Apart from the phosphorylation events that occur within cells in response to xenobiotics, it has been suggested that sulfhydryl group chemistry is also an important factor in regulation of the ARE. There are a number of instances where the redox status of cysteine residues plays a crucial role in the activity of signal transduction pathways and transcription factors. As mentioned previously, the common factor linking compounds that induce ARE-driven transcription is that they can either directly or following metabolism give rise to thiol-reactive compounds. It has been hypothesized that activation of the ARE involves an intracellular redox sensor that possesses reactive cysteine residues that are sensitive to modification. One candidate for this redox sensor comes in the form of the Keap1 protein (101). Experiments have shown that Keap1 functions as a cytoplasmic effector for Nrf2 and thus inhibits its ability to transactivate the ARE (101, 102) (Figure 2). The two proteins associate through the double glycine-rich domains of Keap1 and a hydrophilic region in the Neh2 domain of Nrf2. The Keap1 protein contains a number of potential reactive cysteine residues. It has been postulated therefore that oxidative stress leads to modification of these cysteine residues, which results in the release of Nrf2, allowing it to translocate to the nucleus and transactivate the ARE. Electrophilic compounds such as diethyl maleate and catechol that are thiol reactive have been shown to promote the dissociation of Nrf2 from Keap1. It is also possible, however, that Keap1 may be a target for phosphorylation; computer-aided analysis shows that the protein does contain potential phosphorylation sites. It is not currently known whether Keap1 is phosphorylated. What is certain is that Keap1 along with Nrf2 is expressed in most tissues and cell lines studied so far and that it is a pivotal component of the ARE pathway.

There is also the potential for some feedback mechanism in the signaling pathways that involve proteins that are products of ARE-containing genes. Examples of these include thioredoxin, which is an endogenous inhibitor of ASK1, an

upstream kinase of both the JNK and p38 MAPK pathways (103). It has been shown that GSTs may also directly attenuate ASK1 (104) or JNK activity within cells (105). When cells become chemically stressed, thioredoxin and GST dissociate from ASK1, and JNK in the case for GST, to leave the kinases susceptible to activation. The inhibition of these kinases by thioredoxin and GST is suggested to be dependent upon interaction of these proteins through reactive cysteine residues.

There is likely to be a highly ordered network of signaling mechanisms that act to regulate the ARE in an appropriate manner. Many studies are required to determine the moment where each component of the signaling network, whether it be a kinase, Keap1, or other associated factors, impact to either promote or repress ARE-driven transcription. There appears likely to be a high degree of communication and cross-talk between signaling pathways associated with such an important response mechanism that we are beginning to elucidate.

CONCLUDING REMARKS

In this review, we have provided an overview of the link between detoxification enzyme monofunctional inducers and the discovery and initial characterization of the ARE. This has created an area of research that has given insights into cellular function and potential for the development of therapeutics that will benefit human health. Apart from the expanding field of cancer chemoprevention, enzymes that are regulated by the ARE may also have a protective role in other diseases, such as Alzheimer's disease and arthritis, where oxidative stress has been linked to their pathology. The number of genes that constitute the ARE gene battery is likely to increase with the use of modern genomic technology such as microarray analysis. This will provide further information as to the importance of this gene regulatory pathway within cells.

The focus of this review has centered upon the most recent studies into the regulation of the ARE at the molecular level. Currently, researchers are working to characterize the specific transcription factors and cell signaling pathways that are responsible for eliciting a transcriptional response. Where it is certain that proteins such as Nrf2 and Keap1 are critical in this response, the role of small Maf proteins, which to date are the only potential partners for Nrf2 in binding to the ARE, and other transcription factors requires further clarification. It is also certain that protein phosphorylation is important in regulating the ARE. The participation of protein kinase signaling pathways is complicated and appears to vary according to the nature of the inducing agent, cell type, and sequence context of the ARE. How the MAPK, PI3K, and PKC function and interact within cells to regulate the ARE requires careful dissection. What is not known is how the ARE response is initiated. It would be most valuable to identify the sensing mechanism within cells that leads to oxidative stress activating the ARE pathway. It is hoped that these studies will lead to the design of safe therapeutic agents that may help to prevent the progression of cancer as well as inflammatory and neurodegenerative disease.

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