The Nrf2 transcription factor contributes both to the basal expression of glutathione S-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants, butylated hydroxyanisole and ethoxyquin

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Abstract

An overview is provided of the cancer chemoprevention actions of phenolic antioxidants and 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (ethoxyquin). These agents principally appear to exert their beneficial effects through induction of phase II drug-metabolizing enzymes such as glutathione S-transferase (GST). The requirement for oxidative metabolism of the synthetic antioxidants to carbonyl-containing compounds, including quinones, in order that they can induce gene expression is discussed. Previous work has shown that the basic leucine zipper transcription factor Nrf2 is involved in induction of GST by the phenolic antioxidant butylated hydroxyanisole (BHA). Evidence is provided from a mouse possessing a targeted disruption of the Nrf2 gene that, in murine liver, the transcription factor regulates basal expression of several class Alpha and class Mu GST subunits, but not class Pi GST. In the Nrf2 knock-out mouse, hepatic induction of class Alpha and class Mu GST by BHA and the synthetic antioxidant ethoxyquin is similarly impaired, suggesting that these agents affect gene activation by a related mechanism. Significantly, residual induction of GST by antioxidants is apparent in the Nrf2 mutant mouse, indicating the existence of an alternative mechanism of gene activation.

Historical perspective on cancer chemoprevention

Cancer chemoprevention is defined as the use of chemical agents or dietary components either to protect against the initiation of carcinogenesis, or to retard the progression of neoplastic disease once it has begun [1]. The original discovery of this phenomenon can be traced back at least 70 years to the report by Berenblum [2] that the mustard gas dichloroethyl sulphide can be used in the prophylaxis of epidermal neoplasia in the mouse produced by painting the skin with carcinogenic tar. Subsequently, Crabtree [3,4] described a range of other compounds, including anhydrides of a,\(\alpha\)-unsaturated dicarboxylic acids and aromatic hydrocarbons, which could also serve to inhibit epidermal carcinogenesis in the mouse skin model. A significant time elapsed before it was recognized that chemicals which prevent tumour development can act systemically, and are therefore applicable to tissues other than the skin [5–8]. However, the majority of the agents described in early studies of chemoprevention of liver, mammary gland and small intestinal neoplasia in the rat were themselves noxious compounds. It was therefore uncertain whether this form of preventive medicine could be of therapeutic use in humans (for reviews, see [9,10]).

The demonstration that relatively non-toxic synthetic antioxidants may be used to prevent chemical carcinogenesis represented a major discovery, since a number of such compounds were, and continue to be, widely consumed as additives in processed food by European and North American populations. The initial report that phenolic antioxidants exhibit chemopreventive properties was provided by Frankfurt et al. [11], who found...
that feeding rats on diets containing butylated hydroxytoluene (BHT) prevented hepatoma initiated by 4-dimethylaminoazobenzene. Interest in synthetic antioxidants as chemopreventive agents was greatly stimulated by the discovery of Wattenberg [12] that, in addition to BHT, butylated hydroxyanisole (BHA) and 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (ethoxyquin) have anticarcinogenic activity in rats and mice. When administered to mice in the diet concomitantly with benzo[a]pyrene or dimethylbenz[a]anthracene (DMBA), all three antioxidants decreased both the number of animals with tumours of the fore-stomach, as well as the number of tumours per animal [12]. Using the rat as an experimental model, it was also shown that dietary administration of BHA, BHT or ethoxyquin protected against the formation of DMBA-initiated mammary tumours [12]. Most significantly, the naturally occurring antioxidant α-tocopherol did not inhibit significantly fore-stomach or mammary carcinogenesis [12]. Following this report, Wattenberg [13,14] proceeded to show that synthetic antioxidants could inhibit the carcinogenic effects of a range of chemicals at a variety of sites. For example, it was found that BHA and ethoxyquin both prevented lung cancer in the mouse caused by either diethylnitrosamine or 4-nitroquinoline-N-oxide [13]. BHA could also prevent lung cancer in the mouse produced by 7-hydroxymethyl-12-methylbenz[a]anthracene, uracil mustard or urethane [14]. Ulland et al. [15] showed BHT could prevent liver cancer in the rat caused by acetylaminofluorene. It was also capable of preventing colon cancer in the rat caused by azoxymethane [16]. While much of the chemoprevention field concerned itself with carcinogens that are environmental pollutants or products of combustion, it should be noted that BHA, BHT and ethoxyquin can protect the rat against hepatocarcinogenesis caused by the mycotoxin aflatoxin B1, which is produced by the mould Aspergillus flavus and is widely encountered in humid regions of the world [17,18]; for reviews, see [19,20].

**Induction of detoxication and antioxidant enzymes as a mechanism of chemoprevention**

The mechanism whereby phenolic antioxidants prevent carcinogenesis was initially assumed to involve their ability to scavenge free radicals. A body of data exists showing that the addition in vitro of BHA, BHT and ethoxyquin to Ames testing of polycyclic aromatic hydrocarbons can inhibit mutagenesis [21], indicating that antioxidants may exert direct anti-carcinogenic effects. However, the fact that Wattenberg [12] found α-tocopherol to be essentially ineffective in prevention of carcinogenesis in models where BHA, BHT and ethoxyquin conferred protection suggests that it is unlikely the synthetic antioxidants act in vitro to prevent chemical carcinogenesis solely by trapping carcinogenic radical species. Also, the fact that human intervention studies with α-tocopherol and β-carotene have been disappointing [22] argues against the scavening of free radicals being a major mechanism of cancer chemoprevention.

Since the mid-1970's, evidence has accumulated that BHA, BHT and ethoxyquin act to enhance detoxification processes. Examination of phase I drug metabolism has revealed that administration of synthetic antioxidants to rodents produces modest changes in the levels of cytochrome P450 (CYP) enzyme activities [21,23-26]. Much emphasis has been placed on the relative inability of BHA to induce aryl hydrocarbon hydroxylase activity [27] (i.e. CYP1A1), because this enzyme is associated with a high capacity to toxify polycyclic aromatic hydrocarbons [28]. However, immunoblotting has shown that synthetic antioxidants increase the hepatic expression of CYP2B and CYP3A members [29-32], and, since these are capable of activating carcinogens [28], it would appear that the balance between the activities of phase I and phase II drug-metabolizing enzymes is of critical importance in determining whether synthetic antioxidants confer protection against neoplasia. Clearly, it is an oversimplification to use aryl hydrocarbon hydroxylase induction as the sole criterion for safety evaluation.

The principal beneficial effect of phenolic antioxidants appears to involve induction of phase II drug-metabolizing enzymes. The first evidence that UDP-glucuronosyl transferase was inducible by these compounds came from the studies of Grantham et al. [33], who showed that rats fed on a BHT-containing diet excreted in the urine an increased portion of a test dose of acetylamino-fluorene as the glucuronic acid conjugate. Using the mouse as an experimental model, the laboratories of Bueding, Cha, Talalay and Wattenberg provided data showing that feeding a diet containing BHA markedly induces hepatic glutathione S-transferase (GST) [34,35], microsomal epoxide hydrolase [35-37] and NAD(P)H:quinone oxidoreductase (NQO) [38] activities. BHT and ethoxyquin were also shown to induce GST...
Induction of gene expression by phenolic antioxidants is not limited to phase II drug-metabolizing enzymes, since the hepatic content of glutathione and glutathione-associated enzymes can be elevated in rodents by this class of compound. Many authors have reported that the acid-soluble, non-protein SH levels (total glutathione) are increased by synthetic antioxidants in many organs of the mouse and rat [21]. This increase in glutathione would appear to be due to enhanced synthesis de novo, because mRNA for the catalytic heavy subunit of γ-glutamylcysteine synthetase is elevated by BHA in mouse liver [45]. Significantly, glutathione reductase is also inducible [26,46], presumably ensuring that GSH predominates over GSSG. It is also interesting to note that γ-glutamyl transpeptidase is inducible by antioxidants [47,48]. Since these enzymes are involved in glutathione homeostasis, the view has evolved that BHA, BHT and ethoxyquin alter the metabolic balance in the cell to favour reduction and conjugation reactions. Antioxidants may also enhance ATP-dependent efflux of detoxified conjugates from the cell by the multidrug resistance-associated proteins (MRPs), since these transporters are functionally interdependent on the phase II drug-metabolizing enzymes [49].

**Pharmacology of gene induction by metabolizable antioxidants**

It is not immediately obvious from a teleological point of view why synthetic antioxidants should be capable of increasing the expression of antioxidant proteins. The answer to this question, at least for BHA, lies in the fact that it is subject to biotransformation reactions. Within the cell, certain BHA metabolites, rather than the parent compound, affect induction. Investigation of the ability of a series of BHA analogues to induce GST and NQO activities, along with prior knowledge of metabolism of the antioxidant (from [50]), led Prochaska et al. [51] to propose that BHA is required to be converted into t-butyldihydroquinone (tBHQ) in order to act as an inducing agent (see Figure 1). It was suggested that the ability of tBHQ to be oxidized to a quinone, which could undergo redox-cycling (and hence cause production of free radicals and oxidative stress), was the crucial factor in gene induction [52]; for reviews of quinone toxicology, see [53,54].

In contrast with BHA, less is known about how BHT and ethoxyquin cause increased expression of enzymes. Although probable, it has not been demonstrated that it is essential for these compounds to be metabolized before they cause induction. Both BHT and ethoxyquin are subject to extensive biotransformation in rats and mice. Figure 1 shows a simplified overview of BHT metabolism in rodents, indicating it can be oxidized by two pathways [55]: one involves hydroxylation of alkyl moieties; the other involves oxidation of the π electron system. Hydroxylation of the methyl substituent of BHT ultimately gives rise to an aromatic aldehyde and, based on the hypothesis that all 'monofunctional' inducers are Michael reaction acceptors [27,56], it seems unlikely that this hydroxylation pathway contributes significantly to enzyme induction. However, oxidation of the π electrons of BHT leads to formation of a quinone, and this product is a good candidate for the function of ultimate inducer. Figure 1 also shows the O-de-ethylation of ethoxyquin to form 6-hydroxy-2,2,4-trimethyl-1,2-dihydroquinone, and its subsequent oxidation to 2,2,4-trimethyl-6-quinolone [57]. The latter oxidation product is an α,β unsaturated carbonyl, which seems likely to represent the active inducing metabolite [43,49,56].

The CYP isoenzymes responsible for metabolizing BHA, BHT or ethoxyquin to the ultimate inducing agent have not been identified. The majority of transformed cell lines employed to study regulation of detoxication genes possess greatly reduced levels of CYP, and this has made it difficult to establish in vitro the metabolic cascade required for induction. To date, it has been shown that the BHA metabolite tBHQ can induce NQO enzyme activity in murine Hepa 1c1c7 cells [58]. In the future, it will be important to reconstitute the metabolic pathways required for induction by the synthetic antioxidants themselves in human cell lines. This will allow an understanding of
BHA, BHT and ethoxyquin are oxidized in vivo to various hydroquinone and carbonyl-containing metabolites \([50,55,57]\). In the case of BHA it has been proposed that tBHQ (the first CYP product shown) is the ultimate inducing agent \([51]\). Although probable, it has not been proven whether biotransformation of BHT and ethoxyquin is essential for induction. Biotransformation of BHT results in hydroxylation of the t-butyl groups \([55]\), but for the sake of simplicity this pathway is not shown because it seems unlikely that it contributes to induction of detoxication enzymes. OX, spontaneous or enzyme-catalysed oxidation.

**Figure 1**
Metabolites of phenolic antioxidants and ethoxyquin

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Factors, such as genetic polymorphisms in CYP and phase II drug-metabolizing enzymes or drug–drug interactions, which might cause inter-individual variation in induction.

**Identification of the antioxidant responsive element**

The data presented above suggest that BHA, BHT and ethoxyquin co-ordinately induce a specific group of genes encoding detoxication enzymes. One of the genes in rat liver that is highly responsive to synthetic antioxidants is that for the class Alpha GSTA2 subunit (originally called Ya) \([43,59]\). Molecular cloning and functional analysis of the promoter of this gene by Pickett and his colleagues showed that it contains a previously unrecognized enhancer, 5'-GTGACAAAGC-3', which responds to \(\beta\)-naphthoflavone (\(\beta\)-NF) and tBHQ \([60–62]\). This enhancer was designated the antioxidant responsive element (ARE), and mutagenesis studies defined the core consensus sequence to be 5'-GTGACNNNGC-3' \([62]\). The sequence context of the ARE influences its function. In particular, the immediate 5'-flanking region of the ARE in the rat GSTA2 gene contains a similar sequence in the same orientation (5'-ATGGCATTGC-3'), located five nucleotides upstream (see \([32]\) for complete sequences), which deletion analyses demonstrated modulates basal expression, but not induction \([62]\). An independent study of the promoter of the mouse GstA1 gene by Friling et al. \([63]\) showed that it contains a similar enhancer called the electrophile responsive element (EpRE), which comprises an ARE and, like the promoter of rat GSTA2, a related upstream sequence in the same orientation; the upstream sequence in the mouse GstA1 gene is 5'-ATGACATTGC-3', and this shares greater sequence identity with the core ARE than the equivalent upstream sequence in rat GSTA2 (see above). Mutational analyses of the murine EpRE, including the upstream ARE-like sequence, showed that, in terms of responsiveness to tBHQ, the proximal ARE (5'-GTGACAAAGC-3') is more important than the upstream sequence \([64]\). This result might have been predicted from the work performed on the rat GSTA2 gene \([62]\). However, mutation of any of the first three bases (5'-TAA-3') within the five-nucleotide linker between the core ARE and the distal ARE-like sequence \([32]\) drastically reduced the level of expression observed with tBHQ treatment \([64]\); whether these mutations influence basal or inducible expression remains to be clarified.

The promoter of the rat NQO1 gene has also been characterized, and it likewise contains a
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functional ARE [65]. In contrast with the regions upstream of the core AREs in the class Alpha GST gene promoters of rat and mouse, the immediate 5'-flanking region of the ARE in rat NQO1 contains a partially conserved sequence in reverse orientation (5'-CTAGAGTCACAGTGACTT-GGC-3', where the core ARE is underlined). As a consequence of this flanking sequence, a 13-bp palindrome can be formed (i.e. 5'-AGTCACAGTGACT-3') that includes six nucleotides within the core enhancer. Mutation of nucleotides in the distal half of this palindromic region reduces basal expression to about 10%, but does not abolish induction [66]. Mutation of nucleotides in the proximal half of the palindrome not only reduces basal expression to about 10%, but also abolishes inducibility [66].

It remains to be established whether the orientation of the distal ARE-related sequence is of functional significance. Favreau and Pickett [66] have shown, however, that, although induction of reporter genes driven by AREs from NQO1 and GSTA2 do not differ substantially, the basal expression from the reporter construct containing the NQO1 ARE is 10-fold greater than that obtained from the GSTA2 ARE.

Identity of transcription factors that bind to the ARE

There has been considerable debate about the identity of the transcription factors that are recruited to the ARE, and mediate gene induction by BHA. The fact that part of this cis-acting element resembles the TPA (PMA)-responsive element (TRE; 5'-TGA(C/G)T(A/C)A-3'), to which activator protein-1 binds, provoked the hypothesis that Jun and Fos may recognize the ARE [67]. Work from several laboratories has led to the conclusion that, although Jun and Fos do not mediate transcriptional activation via the ARE [68–70], other basic-region leucine zipper (bZIP) transcription factors are involved. In this context, it is important to note that, because of the redundancy in nucleotides 6–8 of the core enhancer described by Rushmore et al. [62], it is possible for the ARE to embody a TRE. As a consequence, certain AREs, such as that in the human NQO1 promoter [71], may serve dual functions, whereas those in other genes may not. Indeed, mutagenesis can create an enhancer that responds to both tBHQ and TPA by conversion of the core ARE (5'-GTGACAAAGC-3') found in rat GSTA2 into 5'-GTGACTCAGC-3' [68].

On the basis of the realization that the ARE represents an extended, TRE-like, binding site, Venugopal and Jaiswal [72] tested whether the nuclear factor-erythroid 2-related factors 1 and 2 (Nrf1 and Nrf2), which recognize such enhancers, might stimulate transcription from an ARE-linked reporter gene. These proteins are part of a family of transcription factors, the founding member of which is the p45 subunit in nuclear factor-erythroid 2 (NF-E2-p45) [73]; family members are characterized by the fact they contain the cap n' collar (CNC)-type bZIP domain, and heterodimerize with small Maf proteins [74] (i.e. MafF, MafG and MafK [75,76]) to bind to their DNA sequence motif. Venugopal and Jaiswal [72] demonstrated that transfection of human HepG2 cells with increasing amounts of expression constructs for Nrf1 and Nrf2 resulted in a dose-dependent activation of ARE-driven transcription. They also showed that treatment with either tBHQ or β-NF enhanced the ability of both Nrf1 and Nrf2 to activate ARE-driven gene expression. The involvement of other CNC-bZIP factors, e.g.

<table>
<thead>
<tr>
<th>Antibodies employed to probe expression of GST in mouse liver</th>
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<tbody>
<tr>
<td>Murine GST subunit(s)* which cross-react(s) in Western blotting</td>
</tr>
<tr>
<td>Antibody (i.e. original immunogen)</td>
</tr>
<tr>
<td>GSTA1/2 Anti-(mouse GST Ya,Ya)</td>
</tr>
<tr>
<td>GSTA3 Anti-(mouse GST YcYc)</td>
</tr>
<tr>
<td>GSTA4 Anti-(rat GST YkYk)</td>
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<tr>
<td>GSTM1 Anti-(mouse GST Yb,Yb)</td>
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<tr>
<td>GSTM5 Anti-(rat GST YoYo)</td>
</tr>
<tr>
<td>GSTP1/2 Anti-(rat GST YYY)</td>
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*The nomenclature for GST in the mouse is defined in [43].

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NF-E2-p45 [73], Bach1 and Bach2 [77], and the more recently identified Nrf3 [78], in mediating gene induction has not been reported to date.

Positive evidence that Nrf proteins are involved \textit{in vivo} in the regulation of phase II drug-metabolizing enzymes has come from gene knock-out experiments. The Nrf1 knock-out mouse is phenotypically abnormal, and mutation of this gene has been reported either to be embryonically lethal or to cause loss of definitive haematopoiesis [79,80]. Its role in induction \textit{in vivo} is, at present, unknown. Two groups have, however, reported an Nrf2 knock-out mouse that is developmentally normal and phenotypically healthy [81,82]. Itoh et al. [82] reported that the Nrf2-nulled mouse possessed a modest decrease in the basal hepatic expression of at least one class Mu GST subunit, and a severe attenuation of induction of class Alpha, Mu and Pi GST by BHA in liver and intestine.

In the present study, the ability of ethoxyquin to induce hepatic class Alpha, Mu and Pi GST is compared with that of BHA in wild-type and the Nrf2 mutant mouse. Western blotting was performed to monitor the levels of GST; Table 1 provides details of antibodies used [83–87]. As expected from previous work, BHA markedly induced class Alpha and Mu GST in the livers of male mice possessing intact Nrf2 [83,84,88,89]. Figure 2 shows that, in comparison with the wild-type mouse, expression of the GSTA1 and/or GSTA2, GSTA4, GSTM1 and GSTM5 subunits is decreased substantially in male Nrf2 knock-out mice fed on a control RM1 diet (supplied by SDS Ltd., Witham, Essex, U.K.). The levels of these proteins were estimated from visual inspection to be between 25% and 50% of that in the wild-type, whereas no significant changes were observed in the GSTA3 and GSTP1 and/or GSTP2 subunits. It is anticipated that the class Pi GST subunits are inducible in the female mouse (see [83]). Only male mice, which express substantially greater basal levels of class Pi GST in the liver than do female mice [87], were investigated in the present study.

Feeding the wild-type mice \textit{ad lib.} on a diet containing 0.5% (w/w) ethoxyquin resulted in quantitatively similar induction of GSTA1/2, GSTA4, GSTM1 and GSTM5, as was obtained in wild-type mice fed on diet containing 0.5% (w/w) BHA. The levels of these GST subunits observed in the Nrf2 knock-out mouse fed on a diet containing either BHA or ethoxyquin was significantly lower than was observed in the wild-
pression via a mechanism related to that utilized by BHA. However, if allowance is made for the reduced basal expression of these transferases, it is apparent that inducibility by ethoxyquin and BHA is not completely abolished in the Nrf2 knock-out mouse (Figure 2). Thus, in the case of GSTA1/2, GSTA4 and GSTM5, BHA was still found to exert significant induction in the Nrf2 mutant mouse. This residual inducibility is possibly less obvious for the GSTM1 subunit than those mentioned above, and in this case induction appears to be severely impaired.

**Activation of the Nrf2 transcription factor and its recruitment to the ARE**

Evidence suggests that, under basal conditions, the Nrf2 transcription factor is not present in the nucleus, but is tethered in the cytoplasm to an actin cytoskeleton-binding protein Keap1 (i.e. Kelch-like ECH-associated protein 1, where ECH represents chicken Nrf2) [90,91]. The region of Nrf2 that interacts with Keap1 comprises a stretch of hydrophilic amino acids within the N-terminal Neh2 domain, i.e. the Nrf2-ECH-homology 2 domain, and probably includes residues 33-73 of the bZIP protein. Keap1 binds to the Neh2 domain of Nrf2 via a portion of its C-terminus, which contains double glycine repeats [90], and is itself located to the cytoskeleton via the same domain. Immunocytochemistry experiments involving co-transfection of Nrf2 and Keap1 into 293T cells have shown that, under normal culture conditions, the CNC-bZIP factor is located in the cytoplasm [90]. However, exposure of the transfected 293T cells to the electrophile diethylmaleate (a compound which induces GST in rat liver [92]) results in translocation of Nrf2 to the nucleus [90]; presumably, Nrf2 contains a cryptic nuclear-import signal that allows its immediate translocation upon release from Keap1.

The mechanism by which cells sense the presence of inducing agents in order to effect transcriptional activation through the ARE is unknown. Because metabolites of phenolic antioxidants are thiol-active agents (as is diethylmaleate), it has been postulated that these compounds are required to react with certain 'critical' protein cysteine residues in order to bring about transcriptional activation of phase II drug-metabolizing genes [43]. The identity of the proteins that serve as sensors is unknown. In view of the broad spectrum of chemicals which can act as inducing agents, it seems improbable that a single protein serves this role. Clearly, the Keap1–Nrf2 complex represents a good candidate for one of the sensors of electrophile and/or oxidative stress [90,91]. It is not known whether both, or only one, of these proteins are capable of interacting with inducing agents. Nrf2 contains only five cysteine residues, whereas Keap1 contains 25 of them. Interestingly, in the latter protein, three of the cysteines are located in the 12 C-terminal amino acids, and the C-terminal tripeptide is Cys-Thr-Cys [90].

A series of papers from Kong and his colleagues [93-97] have shown that several protein kinase pathways are activated by cancer chemopreventive agents. Significantly, data presented by Yu et al. [96] demonstrate that extracellular signal-regulated protein kinase 2 (ERK2) positively regulates induction by tBHQ of both NOQ enzyme activity and ARE-linked reporter gene expression. Induction by the isothiocyanate sulforaphane was similarly found to be influenced by ERK2. Use of the mitogen-activated protein (MAP) kinase kinase inhibitor PD98059 to block ERK2 activity diminished induction by tBHQ and sulforaphane [96]. Likewise, overexpression of a dominant-negative mutant of ERK2 in HepG2 cells impaired induction of ARE-driven expression by tBHQ and sulforaphane. Importantly, in addition to attenuating induction, PD98059 also diminished basal NOQ enzyme activity in mouse Hepa 1c1c7 cells. Yu et al. [96] showed that the Raf-1 serine/threonine kinase activity is stimulated by tBHQ and sulforaphane in vitro, and proposed that Raf-1 is activated directly by inducing agents in vivo. Whether activation of Raf-1 involves modification of cysteine residues is not known. However, Raf-1 contains a cysteine-rich domain that, if mutated, results in increased kinase activity through failure of auto-inhibition by the N-terminal regulatory region of the protein [98]. The exciting possibility exists that modification of cysteines within this domain will activate the ERK pathway. Although ERK2 appears to enhance induction of gene expression affected via the ARE, it remains to be established whether ERK2 phosphorylates Nrf2 and/or Keap1, or another factor. This issue can be addressed using primary cell culture of hepatocytes from the Nrf2 knock-out mouse.

Additional work has shown that tBHQ also activates p38, a member of the MAP kinase family, but in this case stimulation of the kinase negatively regulates induction of phase II drug-metabolizing enzymes [97]. Interestingly, both basal expression and inducibility of NOQ enzyme activity and an ARE-reporter gene product were enhanced by
SB203580, an inhibitor of p38 [97]. This dual effect on basal, as well as inducible, expression is reminiscent of our observations in the knock-out mouse (Figure 2), suggesting that p38 might act directly on Nrf2.

**Concluding comments**

It is becoming clear that the Nrf2 transcription factor is part of a cytoprotective adaptive response mechanism to oxidative and chemical stress. As such, there are many areas of toxicology and pharmacology in which Nrf2 is likely to be important in conferring resistance to environmental agents. Much remains to be discovered about the role of this protein in mediating induction by different cancer chemopreventive agents; for example, is it required for the chemopreventive effects of coumarins, dithiolethiones, flavones, indoles, isothiocyanates and terpenes? An interesting question raised by the present study concerns the identity of the factors responsible for residual induction of GST by BHA and ethoxyquin in the Nrf2 mutant mouse. Furthermore, it will be important to establish which small Maf protein Nrf2 dimerizes with in vitro, and whether either are phosphorylated by MAP kinases.

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