# Mechanism of Action of Sulforaphane: Inhibition of p38 Mitogen-Activated Protein Kinase Isoforms Contributing to the Induction of Antioxidant Response Element–Mediated Heme Oxygenase-1 in Human Hepatoma HepG2 Cells

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## Abstract

**Exposure of sulforaphane to HepG2 cells increased heme oxygenase-1 (HO-1) expression by activating antioxidant response element (ARE) through induction of Nrf2 and suppression of Kelch-like ECH-associated protein 1 (Keap1). Using human HO-1 promoter reporter plasmids and ChIP assay, we have identified that sulforaphane transcriptionally activated the upstream ARE-rich enhancer region, located at −9.0 kb upstream human HO-1 promoter. Induction of HO-1 by sulforaphane was attenuated by overexpression of mutant Nrf2 plasmid in HepG2 cells and totally abolished in Nrf2 knockout mouse embryonic keratinocytes and fibroblasts. Overexpression of individual p38 mitogen-activated protein (MAP) kinase (MAPK) isoforms also suppressed constitutive as well as sulforaphane- or Nrf2-induced ARE-dependent gene expression. Among the upstream kinases, although MKK3 was not involved in suppression of ARE by any of p38 MAPK isoforms, MKK6 selectively suppressed ARE by p38γ or p38δ, but not by p38α or p38β. Importantly, sulforaphane not only activated MAP/extracellular signal-regulated kinase (ERK) kinases 1/2 and ERK1/2, but also strongly suppressed anisomycin-induced activation of p38 MAPK isoforms by blocking phosphorylation of upstream kinases, MKK3/6. Finally, we found that stimulation of p38 MAPK isoforms phosphorylated purified Nrf2 protein and caused an increase in the interaction between Nrf2 and Keap1 *in vitro* and the suppression of Nrf2 translocation into the nucleus. Collectively, our results indicate that transcriptional activation of Nrf2/ARE is critical in sulforaphane-mediated induction of HO-1, which can be modulated in part by the blockade of p38 MAPK signaling pathway. In addition, our study shows that p38 MAPK can phosphorylate Nrf2 and promotes the association between Nrf2 and Keap1 proteins, thereby potentially inhibiting nuclear translocation of Nrf2.** (Cancer Res 2006; 66(17): 8804-13)

## Introduction

Epidemiologic studies have shown that consumption of cruciferous vegetables can protect against carcinogenesis in human (1). Cruciferous vegetables are rich in glucosinolates, which are metabolized into isothiocyanates in the body by the enzymatic action of plant-specific myrosinase or intestinal microflora (2). Sulforaphane (4-methylsulfinylbutyl isothiocyanate) is a naturally occurring isothiocyanate, which was first identified from broccoli extracts as a principal inducer of the quinone reductase activity (3). Subsequently, a number of experimental animal studies have shown the strong chemopreventive effects of sulforaphane. The administration of sulforaphane blocked 7,12-dimethylbenz(*a*)anthracene–induced mammary tumor formation in rats (4), retarded the growth of PC-3 xenografts in athymic nude mice (5), and reduced benzo(*a*)pyrene-induced murine forestomach tumor formation (6). At present, two possible biochemical mechanisms have been postulated to explain the chemoprotective effects of sulforaphane. The first includes the deletion of preinitiated cells from damaged tissues through cell cycle arrest and apoptosis. Biochemical studies have shown that cell cycle arrest by sulforaphane occurred through an irreversible $G_2$-M phase arrest with a reduction of key $G_2$-M–regulating proteins (7), whereas overexpression of Bax, down-regulation of Bcl-2, and activation of caspase-8 and caspase-9 were implicated in sulforaphane-mediated apoptosis (8). On the other hand, beneficial modulation of metabolizing enzymes has been suggested as another set of chemopreventive mechanism that can block carcinogen activation (inhibition of cytochrome P450s) or enhance the detoxification of activated carcinogen (activation of phase II detoxifying enzymes).

It is well recognized that the induction of phase II detoxifying enzymes provides significant biological mechanisms for protection against toxic effects of endogenous reactive oxygen species and exogenous carcinogens and/or their reactive intermediates (9). Coordinated expression of these enzymes, such as glutathione *S*-transferase (GST), NAD[P]H:quinone oxidoreductase 1 (NQO1 or quinone reductase), γ-glutamylcysteine synthetase (γ-GCS), and heme oxygenase-1 (HO-1), is under the transcriptional control of antioxidant response element (ARE), a *cis*-regulatory DNA sequence located in the promoter region of these genes (10). NF-E2-related factor 2 (Nrf2), a member of the cap'n'collar family of basic leucine zipper (bZIP) proteins, was identified as a key transcriptional factor of ARE (11). Under basal condition, Nrf2 is tethered to a cytosolic repressor protein, Kelch-like ECH-associated protein 1 (Keap1), in the cytoplasm. Oxidative or chemical stresses, however, can disrupt Nrf2/Keap1 complex and permit Nrf2 to translocate into the nucleus, where it enhances the transcription of phase II detoxifying genes by binding to ARE sequences (12). To date, multiple signaling kinases have been reported to regulate ARE, which include p38 mitogen-activated protein kinase

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doi:10.1158/0008-5472.CAN-05-3513

(p38 MAPK), extracellular signal-regulated kinase (ERK), c-jun $NH_2$-terminal kinase (JNK), phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), and the pancreatic endoplasmic reticulum kinase (PERK; ref. 13). All of these kinases have been reported to positively regulate ARE-mediated gene expression with the exception of p38 MAPK.

p38 MAPK was first identified for its activation in response to hyperosmolarity (14) and endotoxic lipopolysaccharide (15). p38 MAPK consists of four isoforms: p38α (also called CSBP or SAPK2a), p38β (also called SAPK2b), p38γ (also called SAPK3 or ERK6), and p38δ (also called SAPK4). All of the p38 MAPK isoforms are reported to have a canonical tripeptide sequence (Thr-Gly-Tyr) in the activation loop, where their activation is associated with the dual phosphorylation of both threonine (Thr) and tyrosine (Tyr) residues (16). The recognition and phosphorylation of p38 MAPK isoforms are mediated by upstream MAPK kinases (MKKs or MEKs), i.e., MKK3, MKK4, and MKK6, which are in turn phosphorylated by several different and overlapping sets of MAPK kinase kinases (MAPKKKs or MEKKs; ref. 17). Once p38 MAPK is phosphorylated and activated, it phosphorylates and/or activates downstream substrates, kinases, or transcriptional factors, including myelin basic protein, MAPK-activated protein kinase 2/3, heat shock protein 27, and activated transcription factor-2 (ATF-2), resulting in various cellular responses, such as proliferation, apoptosis, cell cycle arrest, and inflammation (18).

Although many studies have consistently shown that sulforaphane is a strong inducer of phase II detoxifying enzymes, the detailed upstream signaling mechanisms are still somewhat unclear. In the present study, we have examined the roles of p38 MAPK isoforms (p38α, p38β, p38γ, and p38δ) on Nrf2/ARE function and their modulation by chemopreventive isothiocyanate sulforaphane on the regulation of ARE-mediated cellular defensive enzyme, HO-1.

## Materials and Methods

**Reagents and antibodies.** Sulforaphane (>99% purity) was purchased from LKT Laboratories (St Paul, MN). SB203580 and phosphospecific MAP/ERK kinase 1/2 (MEK1/2) antibody were purchased from Calbiochem (San Diego, CA). Phenethyl isothiocyanate (PEITC), curcumin, anisomycin, sodium bicarbonate, *o*-nitrophenyl-β-D-galatopyranoside (ONPG), anti-FLAG monoclonal antibody, and agarose-conjugated anti-FLAG M2 monoclonal antibody were purchased from Sigma (St. Louis, MO). F-12 medium, MEM, fetal bovine serum (FBS), penicillin and streptomycin, essential amino acids, and insulin were purchased from Invitrogen (Carlsbad, CA). Antibodies against β-actin, Nrf2, Keap1, total p38 MAPK, MKK3, MKK6, and green fluorescent protein (GFP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ATF-2 fusion protein and antibodies against phosphospecific ATF-2, ERK1/2, p38 MAPK, and MKK3/6 were purchased from Cell Signaling Technology (Beverly, MA). [$\gamma$-$^{32}$P]ATP was purchased from Perkin-Elmer Life and Analytical Science, Inc. (Boston, MA). Other chemicals and solvents used were of highest analytic grade.

**Cell culture and plasmids.** HepG2 cells were cultured at 37°C and 5% $CO_2$ in modified F-12 medium supplemented with 10% FBS, 1.7 mg/mL sodium bicarbonate, 100 units/mL penicillin and 100 units/mL streptomycin, essential amino acids, and insulin. HeLa cells were cultured at 37°C and 5% $CO_2$ in MEM supplemented with 10% FBS, 2.2 mg/mL sodium bicarbonate, 100 units/mL penicillin, and 100 units/mL streptomycin. Preparation and culture of mouse embryonic keratinocytes and fibroblasts from wild-type and Nrf2 knockout C57BL/6J mice were conducted as described (19). Human HO-1 promoter reporter plasmids, −9.1 kb (pHOGL3/9.1), −4.5 kb (pHOGL3/4.5), and −4.0 kb (pHOGL3/4.0), were generous gifts from Dr. Anupam Agarwal (University of Alabama, Birmingham, AL). ARE-luciferase reporter construct was a generous gift from Dr. William Fahl (University of Wisconsin, Madison, WI). pcDNA1.1-Nrf2 was kindly provided by Drs Yuet W. Kan and Jefferson Y. Chan (University of California, San Francisco, CA). Mutant Nrf2 DNA (Nrf2-M4), encoding Nrf2 protein (amino acids 401-589) that contains DNA binding domain but lacks transactivation domain, was amplified by PCR and cloned into mammalian expression pHM6 vector (Invitrogen). Full-length Nrf2 was amplified by PCR and cloned into the fluorescent EGFP vector (Clontech, Palo Alto, CA). Wild-type FLAG-p38α, FLAG-p38β, FLAG-p38γ, and FLAG-p38δ were kind gifts from Dr. Jiahuai Han (Scripps Research Institute, CA). Wild-type MKK6 (pcDNA3-HA-MKK6) and MKK3 (pRSV-FLAG-MKK3) cDNA constructs were kind gifts from Dr. Zhengbin Yao (Amgen, Inc., Boulder, CO) and Dr. Roger J. Davis (University of Massachusetts, Worcester, MA), respectively.

**Transient transfection and measurement of luciferase activity.** Cells were plated in six-well plates, grew to around 70% confluency, and transfected with LipofectAMINE 2000 (Invitrogen). After transfection, cells were cultured for an additional 24 hours with fresh medium and harvested or further treated with chemicals, when necessary. In every luciferase activity measurement, 0.5 μg of pRSV-β-galactosidase plasmid was used to normalize transfection efficiency. Luciferase activity was measured according to the method provided by the manufacturer (Promega, Madison, WI) and galactosidase activity was measured with ONPG as a substrate. Briefly, cells were washed twice with 1× ice-cold PBS. Cells were then incubated with 200 μL of 1× reporter lysis buffer (Promega) for 30 minutes and scraped off from the plate. After brief centrifugation at 13,000 × *g*, 10 μL of aliquot of the supernatant was analyzed for luciferase activity with a Sirius luminometer (Berthold Detection System, Pforzheim, Germany) and the same amount of supernatant was used to measure β-galactosidase activity. The luciferase activity was normalized against β-galactosidase activity and expressed as fold induction over the control cells. Values are expressed as mean ± SD of experiments and all experiments were done in duplicate at least thrice.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChIP) assays were done using the ChIP kit (Upstate Biotechnology, Lake Placid, NY) with a slight modification. Briefly, $1 \times 10^7$ HepG2 cells were cross-linked by addition of formaldehyde into the medium at a final concentration of 1% and incubated for 10 minutes at 37°C. Cells were washed with 1× PBS and resuspended in 200 μL ChIP lysis buffer [1% SDS, 10 mmol/L EDTA, and 50 mmol/L Tris-HCl (pH 8.0)] with protease inhibitors (Roche, Indianapolis, IN) and incubated on ice for 10 minutes. After sonication, lysates were diluted to 2 mL with ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl (pH 8.0), and 167 mmol/L NaCl] and 10% of the chromatin solution (200 μL) was reserved for total input. Diluted lysates were precleared with 80 μL protein A-agarose beads for 1 hour at 4°C with agitation. The precleared lysates were immunoprecipitated using anti-GFP antibody at 4°C overnight and immune complexes were collected with 60 μL protein A-agarose and washed once with 1 mL each of the following buffers: low salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.0), and 150 mmol/L NaCl], high salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.0), and 500 mmol/L NaCl], LiCl wash buffer [250 mmol/L LiCl, 1% NP40, 1% sodium deoxycholate, 1 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 8.0)] and twice with 1× TE buffer [10 mmol/L Tris-HCl (pH 8.0), and 1 mmol/L EDTA]. Immune complexes were next eluted using freshly prepared elution buffer (1% SDS and 0.1 mol/L $NaHCO_3$). Cross-links were reversed by heating at 65°C in the presence of NaCl overnight, followed by proteinase K treatment. The DNA was recovered by phenol/chloroform extraction followed by ethanol precipitation and resuspended in 20 μL distilled water. PCR was done with 1 μL of ChIP samples, using the following primers (forward: 5′-CCCTGCTGAG-TAATCCTTTCCCGA-3′ and reverse: 5′-ATGTCCCGACTCCAGACTCCA-3′) that span three AREs in B region of HO-1 promoter.

**Preparation of cell lysates and Western blotting.** For preparation of whole cell lysates, cells were harvested in whole cell lysis buffer [10 mmol/L Tris-HCl (pH 7.9), 250 mmol/L NaCl, 30 mmol/L sodium PPi, 50 mmol/L sodium fluoride, 0.5% Triton X-100, 10% glycerol, 1× proteinase inhibitor mixture, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 100 μmol/L

$Na_3VO_4$, 5 μmol/L $ZnCl_2$, 2 mmol/L indole acetic acid] for 30 minutes on ice. Lysates were then collected by centrifugation at 14,800 × *g* for 30 minutes. Nuclear and cytoplasmic extracts of HepG2 cells were prepared, using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined by the BCA protein assay kit (Pierce Biotechnology). Aliquots of supernatant, containing 20 μg protein, were boiled in 1× SDS sample loading buffer for 2 minutes and resolved using 12% SDS-PAGE. Proteins in SDS-polyacrylamie gel were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) using a semidry transfer system (Fisher Scientific, Pittsburgh, PA). The membrane was blocked with 5% fat-free milk in PBS-Tween 20 (PBST, 0.1% Tween 20) at room temperature for 2 hours. The membrane was then probed with primary antibodies (1:1,000) in 3% nonfat milk in PBS overnight at 4°C. Blots were rinsed with PBST thrice and then incubated with 1:5,000 dilution of horseradish peroxidase–conjugated second antibody at room temperature for 1 hour. The blots were washed in PBST buffer for 5 minutes thrice and the transferred protein was visualized, using the enhanced chemiluminiscence detection system (Amersham Pharmacia, Piscataway, NJ).

**Immunoprecipitation and *in vitro* kinase assay of ATF-2 and Nrf2.** After transfection of plasmids, cells were washed twice with 1× ice-cold PBS and lysed in 0.5 mL of chilled 1× lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium PPi, 1 mmol/L glycerolphosphate, 1 mmol/L $Na_3VO_4$, and 1 μg/mL leupeptin]. Cell lysates were collected in microcentrifuge tubes and centrifuged at 12,000 × *g* for 15 minutes. The supernatants were transferred to new tubes. Active p38 MAPK isoforms were immunoprecipitated with 100 μL agarose-conjugated Anti-FLAG M2 monoclonal antibody at 4°C overnight and GST-Nrf2 protein was purified by using reduced glutathione (GSH)–conjugated beads. Measurement of the kinase activity by p38 MAPK isoforms was carried out by using a nonradioactive p38 MAPK assay kit (Cell Signaling Technology). In brief, the immunoprecipitate were centrifuged and washed twice with 500 μL of 1× lysis buffer and twice with of 1× kinase assay buffer solution [25 mmol/L Tris-HCl (pH 7.5), 5 mmol/L glycerolphosphate, 2 mmol/L DTT, 0.1 mmol/L $Na_3VO_4$, and 10 mmol/L $MgCl_2$]. Purified p38 MAPK isoforms were incubated with purified ATF-2 (2 μg) as a substrate in the presence of 100 μmol/L ATP and 1× kinase assay buffer, which will allow immunoprecipitated active p38 MAP kinase isoforms to phosphorylate ATF-2. Phosphorylation of ATF-2 was then measured by immunoblot analysis with phosphospecific ATF-2 antibody at $Thr^{71}$. Similarly, measurement of Nrf2 phosphorylation by p38 MAPK isoforms was carried out using [$\gamma$-$^{32}$P]ATP. In brief, the immunoprecipitate was incubated with purified Nrf2 (2 μg) as a substrate in the presence of 100 μmol/L ATP and 1× kinase assay buffer for 30 minutes, which will allow immunoprecipitated active p38 MAP kinase isoforms to phosphorylate ATF-2. Phosphorylated Nrf2 protein was resolved in 12% SDS-polyacrylamide gels and visualized by autoradiography.

**GST pull-down assay using p38-phosphorylated GST-Nrf2.** GST-Nrf2 protein was purified after phosphorylation by immunoprecipitated active p38δ kinase as described above; the control GST-Nrf2 protein was purified without phosphorylation by p38δ kinase. Cells were harvested in modified radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 150 mmol/L NaCl, 0.25% sodium deoxycholate, 1 mmol/L $Na_3VO_4$, 1 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L PMSF, and 1× protease inhibitors cocktail] on ice and centrifuged at 14,800 × *g* for 30 minutes. The protein concentration in the supernatant was determined by BCA protein assay kit (Pierce Biotechnology) and aliquots containing 500 μg proteins of each sample were used for pull-down assay. The lysates were precleared by incubating with glutathione-conjugated resin (Calbiochem) for 1 hour at 4°C with rotation and 10 μg phosphorylated or nonphosphorylated GST-Nrf2 and glutathione-conjugated resin were added into the lysates and incubated at 4°C overnight with rotation. The resins were collected by centrifugation and washed using modified RIPA buffer thrice. The proteins bound to resin were eluted by boiling in SDS-PAGE loading buffer for 3 minutes, and Western blotting was carried out.

**Statistics.** Values are expressed as mean ± SD of experiments. Statistical analysis was done by the two-tailed Student's *t* test for unpaired data, with $P < 0.05$ considered statistically significant.

## Results

**Sulforaphane induces the protein expression of HO-1 by activating ARE-dependent gene expression through Nrf2 induction and Keap1 repression.** HO-1 is a ubiquitous and redox-sensitive enzyme, which catabolizes heme into carbon monoxide (CO), iron, and biliverdin (20). HO-1 is usually expressed at low levels under basal conditions, but highly inducible in response to various agents causing oxidative stress, including UV irradiation, hydrogen peroxide, heavy metals, and hypoxia. Because CO and bilirubin may act as adaptors to provide cytoprotection against various environmental stresses, induction of HO-1 by some chemopreventive agents, such as curcumin and caffeic acid phenethyl ester, has been postulated as a part of the chemoprotective mechanisms (21). As seen in Fig. 1*A*, treatment of isothiocyanates (PEITC and sulforaphane) and curcumin for 24 hours strongly induced the protein expression of HO-1 in HepG2 cells. The degree of HO-1 induction by sulforaphane was notably higher than that induced by PEITC or by curcumin. Next, HepG2 cells were transfected with ARE-luciferase reporter plasmid, exposed to PEITC, sulforaphane, and curcumin for 24 hours and

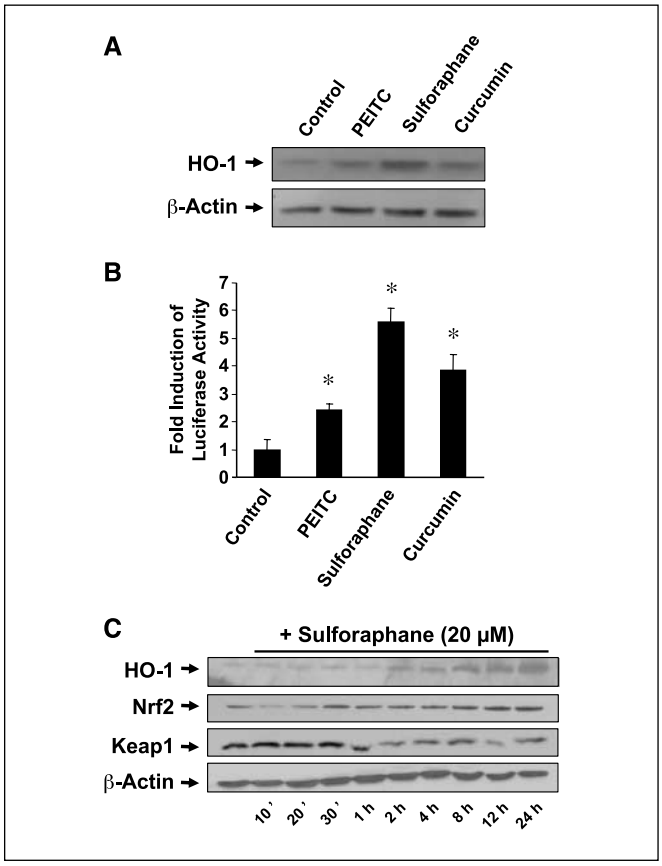


**Figure 1.** Sulforaphane treatment induces the expression of HO-1 by ARE-dependent gene activation via induction of Nrf2 and repression of Keap1 in HepG2 cells. *A,* HepG2 cells were treated with 20 μmol/L PEITC, sulforaphane, and curcumin for 24 hours, and the protein expression of HO-1 and β-actin (used to normalize equal protein loading) was measured by Western blotting. *B,* HepG2 cells were transfected with 0.5 μg ARE-luciferase reporter plasmid and treated with PEITC, sulforaphane, and curcumin (20 μmol/L) for 24 hours. Cells were harvested and the luciferase activity was measured. *, $P < 0.05$, significantly different from the value observed in HepG2 cells, treated with DMSO only. *C,* HepG2 cells were treated with 20 μmol/L sulforaphane at different times indicated (10′, 10 minutes, and accordingly) and the protein expression of Nrf2, Keap1, and β-actin was measured by Western blotting.

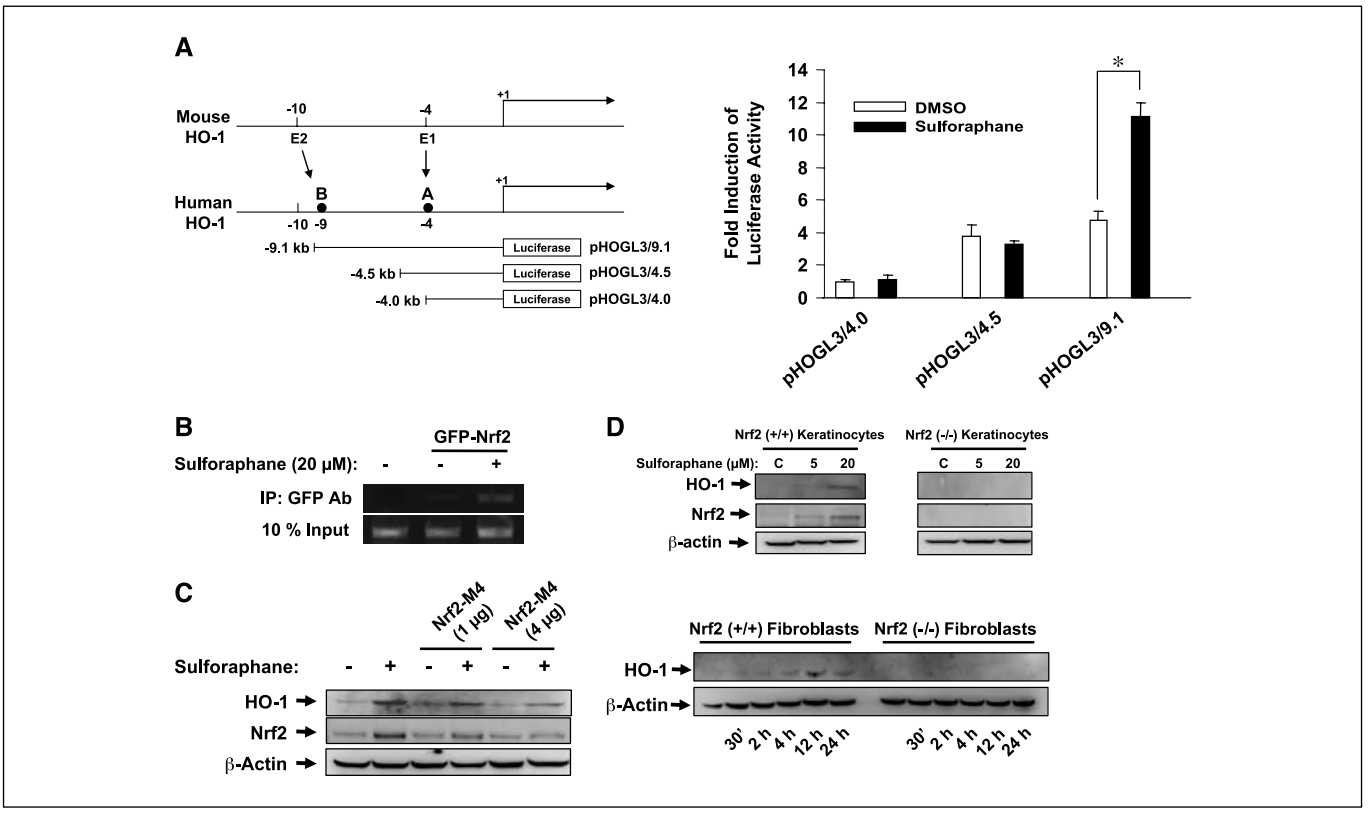


**Figure 2.** Sulforaphane mediates Nrf2-induced activation of ARE-rich regions (B region), located at −9.0 kb upstream the human HO-1 promoter, and Nrf2 is an indispensable transcriptional factor required for the induction of HO-1 by sulforaphane. *A,* HepG2 cells were transfected with 0.5 μg human HO-1 promoter reporter constructs (pHOGL3/4.0, pHOGL3/4.5, and pHOGL3/9.1). After transfection, cells were treated with 20 μmol/L sulforaphane for 24 hours and the luciferase activities were measured. *, $P < 0.05$, significantly different from the value observed in HepG2 cells, transfected with pHOGL3/9.1 and treated with DMSO. *B,* after transfection of 5 μg GFP-Nrf2 plasmid in HepG2 cells and treatment of 20 μmol/L sulforaphane for 18 hours, ChIP assay was conducted as described in Materials and Methods. *C,* after transfection with mutant Nrf2 plasmid (Nrf2-M4), HepG2 cells were treated with 20 μmol/L sulforaphane for 24 hours and the protein expression of HO-1, Nrf2, and β-actin was measured by Western blotting. *D,* Nrf2(+/+) and Nrf2(−/−) mouse embryonic keratinocytes were exposed to sulforaphane (5 and 20 μmol/L) for 24 hours and the protein expression of HO-1, Nrf2, and β-actin was measured by Western blotting (*top*). Nrf2(+/+) and Nrf2(−/−) mouse embryonic fibroblasts were exposed to 20 μmol/L sulforaphane at various time points and the protein expression of HO-1 and β-actin was measured by Western blotting (*bottom*).

the resulting luciferase activities were measured. As shown in Fig. 1*B*, the activation of ARE-dependent gene expression by sulforaphane was higher, compared with that activated by PEITC and curcumin, suggesting that a stronger induction of HO-1 protein by sulforaphane can be attributable to ARE-dependent gene activation. Supporting this idea, we have made similar observations when these chemicals were treated in HepG2C8 cells, established in our laboratory by stable transfection with ARE-luciferase reporter plasmid and the luciferase activity was measured (data not shown). In addition, prolonged exposure of HepG2 cells to sulforaphane not only induced protein expression of Nrf2 but also suppressed that of Keap1, a cytosolic inhibitor of Nrf2 (Fig. 1*C*). Together, these data imply that sulforaphane treatment induces HO-1 protein by activating ARE-dependent gene expression through Nrf2 induction and Keap1 suppression in HepG2 cells.

**Sulforaphane induces Nrf2-mediated activation of the distal ARE-rich region, located at −9.0 kb upstream of human HO-1 promoter, and Nrf2 is an indispensable transcription factor for the induction of HO-1 protein by sulforaphane.** It is known that the induction of mouse *HO-1* gene in response to most of the inducers is exclusively mediated by two distal promoter regions, named E1 and E2 (previously known as SX2 and AB1, respectively), and human HO-1 promoter contains the analogous E1 (A) and E2 (B) regions at −4.0 and −9.0 kb, respectively (Fig. 2*A, left*), in which E1 contains two AREs and a cadmium response element and region B contains three AREs (22). To address whether activation of E1 and/or E2 region(s) might be responsible for the induction of HO-1 by sulforaphane, HepG2 cells were transfected with a series of human HO-1 promoter-reporter constructs (pHOGL3/9.1, pHOGL3/4.5, and pHOGL3/4.0), treated with sulforaphane for 24 hours and the resulting luciferase activities were measured. As a result, we found that sulforaphane treatment significantly activated luciferase activity when cells were transfected with pHOGL3/9.1 reporter plasmid, whereas it failed to affect the luciferase activity when transfected with pHOGL3/4.0 or pHOGL3/4.5 reporter plasmids (Fig. 2*A, right*). To test whether Nrf2 protein is involved in the stimulation of B region by sulforaphane, HepG2 cells were treated with sulforaphane and the ChIP assay was done with the use of polyclonal anti-Nrf2 antibody. In this experiment, HepG2 cells were treated with sulforaphane for 18 hours because we found that nuclear translocation of Nrf2 was maximal at this time point (data not shown). However, in spite of the repeated ChIP experiments, we failed to come up with any convincing results because of a weak binding affinity of polyclonal Nrf2 antibody. To circumvent this problem, we transfected HepG2 cells with GFP-Nrf2 plasmid, exposed them to sulforaphane, and conducted ChIP assay with the use of monoclonal anti-GFP antibody. As a result, we found that exposure of sulforaphane to HepG2 cells resulted in a significant increase of GFP-Nrf2 protein binding to AREs in B region (Fig. 2*B*), suggesting that transcriptional

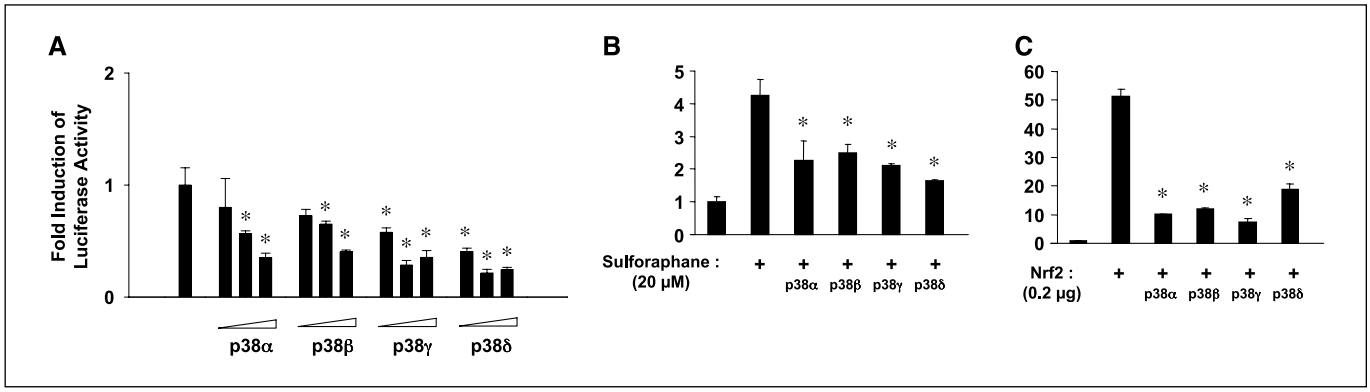


**Figure 3.** Overexpression of p38 MAPK isoforms inhibit constitutive and inducible ARE-mediated gene expression by attenuating the nuclear translocation of Nrf2. *A,* HepG2 cells were transfected with ARE-reporter luciferase plasmid alone or together with various amounts (0.1, 0.5, and 1 μg) of plasmids, encoding FLAG-p38 MAPK isoforms and the luciferase activities were measured. *, $P < 0.05$, significantly different from the value observed in HepG2 cells transfected with ARE-luciferase construct. *B,* HepG2 cells were transfected with ARE-reporter luciferase plasmid alone or together with plasmid encoding 1 μg FLAG-p38 MAPK isoforms. After transfection, cells were treated with 20 μmol/L sulforaphane for 24 hours and the luciferase activities were measured. *, $P < 0.05$, significantly different from the value observed in HepG2 cells, transfected with ARE-luciferase construct and treated with sulforaphane. *C,* HepG2 cells were cotransfected with ARE-luciferase reporter construct and Nrf2 plasmid, alone or together with 1 μg FLAG-p38 MAPK isoforms, and the luciferase activities were measured. *, $P < 0.05$, significantly different from the value observed in HepG2 cells transfected with ARE-luciferase and Nrf2 plasmids.

activation of B region by sulforaphane occurs through an increased recruitment of Nrf2 protein.

Because the putative binding sites for many transcription factors other than Nrf2 are found within the upstream human HO-1 promoter, we next asked whether Nrf2 is necessary for the induction of HO-1 protein by sulforaphane. As seen in Fig. 2*C*, we found that overexpression of increasing amounts of mutant Nrf2 plasmid (Nrf2-M4) substantially attenuated sulforaphane-mediated induction of Nrf2 and HO-1 proteins in HepG2 cells (Fig. 2*C*). In addition, when mouse embryonic keratinocytes were exposed to sulforaphane for 24 hours, the induction of Nrf2 and HO-1 proteins was evident in Nrf2(+/+) mouse embryonic keratinocytes by treatment with 20 μmol/L sulforaphane, but not with 5 μmol/L sulforaphane, whereas it was totally abolished in Nrf2(−/−) mouse embryonic keratinocytes (Fig. 2*D, top*). Similar result was obtained when Nrf2(+/+) and Nrf2(−/−) mouse embryonic fibroblasts were exposed to 20 μmol/L sulforaphane at different time points and the induction of HO-1 protein was measured (Fig. 2*D, bottom*). Together, these data imply that Nrf2 is an indispensable transcription factor, required for the induction of HO-1 protein by sulforaphane.

**Overexpression of p38 MAPK isoforms inhibits constitutive and inducible ARE-dependent gene expression by suppressing the nuclear translocation of Nrf2.** Previously, we have shown that p38α exerted a negative effect on ARE-mediated gene expression (23). However, the roles of other p38 MAPK isoforms (p38β, p38γ, and p38δ) on Nrf2/ARE function have not yet been clarified. So, we first determined to examine the effects of p38 MAPK isoforms on ARE-dependent gene expression. To address this question, we cotransfected plasmids encoding all p38 MAPK isoforms with ARE-reporter luciferase plasmid in HepG2 cells and analyzed the resulting luciferase activities. As seen in Fig. 3*A*, overexpression of individual p38 MAPK isoforms suppressed the transfected ARE-reporter activity in a dose-dependent manner, suggesting that all p38 MAPK isoforms possess negative effects on ARE-mediated gene expression.

Increasing evidence illustrates that p38 MAPK pathway is involved in the regulation of HO-1 induction by various extracellular stimuli. In particular, Wu et al. (24) have shown that p38 MAPK is involved in HO-1 induction by proteosomal inhibitors. In view of the fact that the amount of cellular Nrf2 is controlled by proteosomal degradation (25), we envisioned that p38 MAPK might be also involved in the induction of HO-1 by sulforaphane. As seen, we found that overexpression of p38 MAPK isoforms substantially attenuated ARE-dependent gene activation, induced by sulforaphane treatment or by ectopic expression of Nrf2 (Fig. 3*B* and *C*), implying that p38 MAPK pathway negatively regulates not only constitutive but also inducible ARE-dependent gene expression.

**MKK6 selectively regulates p38γ and p38δ-mediated ARE suppression, whereas MKK3 is not involved in ARE suppression by any of p38 MAPK isoforms.** It is generally known that the p38 MAPK is phosphorylated and activated by upstream kinases, MKK3 and MKK6 (26). Hence, we tested whether MKK3 and MKK6 are involved in suppression of ARE by p38 MAPK isoforms. It would be noted that MKK4 could also phosphorylate and activate p38 MAPK isoforms *in vitro*. However, MKK4 was excluded from our study, because it has a dual-kinase activity, which can activate JNK and its implication *in vivo* has been questioned (27). To address whether MKK3 is implicated in suppression of ARE by p38 MAPK isoforms, MKK3 plasmid was cotransfected with p38 MAPK isoforms and the resulting luciferase activity was measured. As shown in Fig. 4*A*, overexpression of MKK3 slightly reduced ARE-reporter luciferase activity. However, cotransfection of p38 MAPK isoforms failed to affect MKK3-induced ARE suppression, indicating that suppression of ARE by p38 MAPK isoforms may not be mediated by MKK3. Therefore, we next explored whether MKK6 could mediate suppression of ARE by p38 MAPK isoforms. In contrast to MKK3, overexpression of MKK6 alone led to a substantial stimulation of ARE-reporter gene activity (Fig. 4*B*). Interestingly, coexpression of p38α or p38β failed to affect the induction of ARE-reporter activity by MKK6, but that of p38γ and p38δ significantly suppressed MKK6-induced ARE-reporter activation. This fact implies that MKK6 is selectively involved in ARE suppression by p38γ and p38δ, although the effect of MKK6 on the ARE *per se* is stimulatory, presumably through other kinases, such as the MEK5-ERK5 signaling pathway.[4]

[4] Unpublished results.

To ascertain that both MKK3 and MKK6 are not involved in suppression of ARE by p38α and p38β, HepG2 cells were transfected with MKK3 or MKK6 and treated with SB203580, a selective inhibitor of p38α and p38β (28). As shown in Fig. 4*C*, treatment of SB203580 for 24 hours increased ARE-reporter activity in HepG2 cells, suggesting that p38α and p38β indeed negatively regulated ARE-mediated gene expression. However, overexpression of MKK3 failed to affect ARE-reporter induction by SB203580. In addition, SB203580 had no effect on ARE activation, caused by overexpression of MKK6. These data corroborate our above results that both MKK3 and MKK6 are not involved in ARE suppression by p38α and p38β MAPK.

**Sulforaphane not only activates ERK1/2 by phosphorylation of MEK1/2, but also inhibits the kinase activities of p38 MAPK isoforms by preventing phosphorylation of MKK3/6.** It is widely believed that multiple signaling cascades are implicated in the induction of ARE-dependent phase II detoxifying enzymes. In particular, the pharmacologic effects of sulforaphane largely stem from its ability to modulate MAPK signaling cascades, consisting of ERK, JNK, and p38 MAPK. Previous studies from our laboratory have shown that both ERK and JNK are positively involved in ARE-driven gene expression (13). In particular, ERK has been shown to

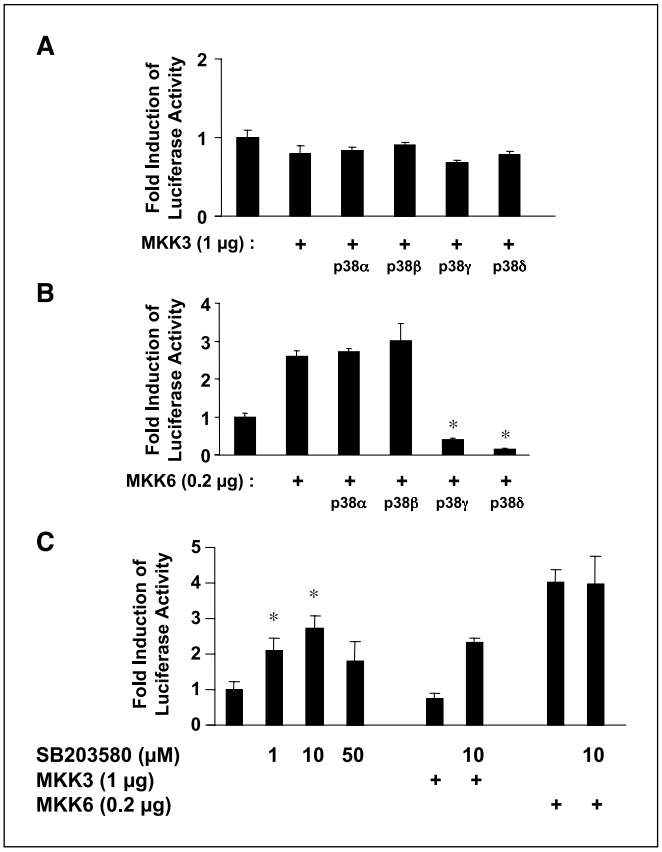


**Figure 4.** Effects of MKK3 and MKK6 on ARE-mediated gene suppression by p38 MAPK isoforms. HepG2 cells were cotransfected with plasmids encoding 1 μg MKK3 (*A*) or 0.2 μg MKK6 (*B*) alone or together with 1 μg FLAG-p38 MAPK isoforms. After transfection, cells were harvested and the resulting luciferase activities were measured. *, $P < 0.05$, significantly different from the value observed in HepG2 cells transfected with MKK6 plasmid. *C,* HepG2 cells were transfected with ARE-reporter construct alone or together with plasmids encoding MKK3 (1 μg) or MKK6 (0.2 μg) and incubated with 10 μmol/L SB203580 for 24 hours. Cells were harvested and the luciferase activities were measured. *, $P < 0.05$, significantly different from the value observed in HepG2 cells transfected with ARE-luciferase construct.

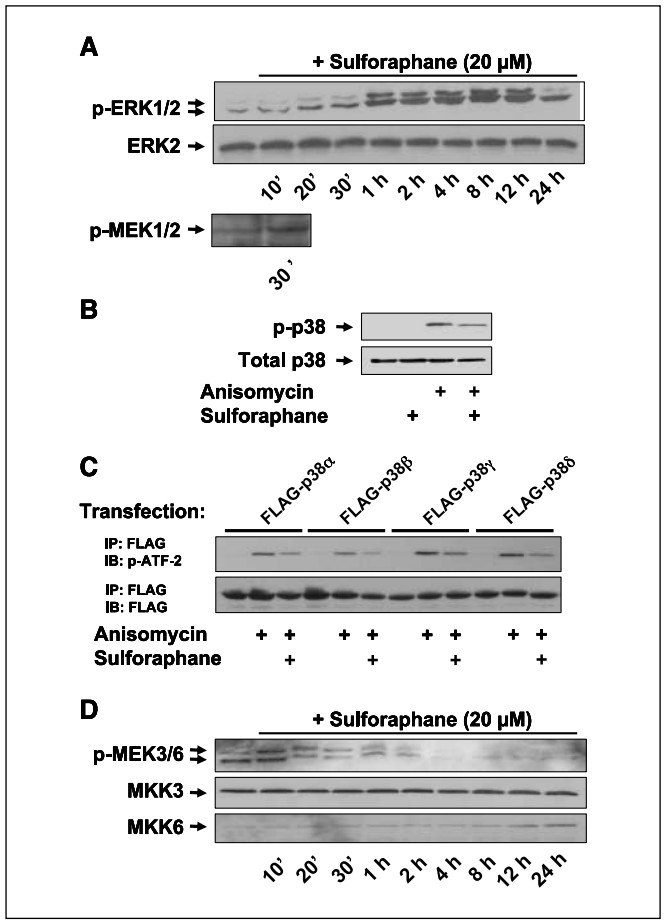


**Figure 5.** Sulforaphane not only causes phosphorylation of ERK1/2 by activating MEK1/2, but also suppresses activation of anisomycin-induced p38 MAPK isoforms by inhibiting MKK3/6. *A,* phosphorylation level of ERK1/2 and MEK1/2 were measured by Western blotting with the same cell lysates in Fig. 1*C*, using phosphospecific ERK1/2 and MEK1/2 antibodies. Endogenous expression of ERK2 was also measured by Western blotting using a polyclonal ERK2 antibody. *B,* HepG2 cells were pretreated with 20 μmol/L sulforaphane for 30 minutes before the challenge with anisomycin (10 μg/mL) for 30 minutes. Cell lysates were blotted against phosphospecific p38 MAPK antibody. Endogenous expression of total p38 MAPK was also measured by Western blotting using a polyclonal p38 MAPK antibody. *C,* after transfection with individual FLAG-p38 MAPK isoforms, HepG2 cells were pretreated with 20 μmol/L sulforaphane for 30 minutes, if necessary, and challenged with anisomycin (10 μg/mL) for 30 minutes. Cells were immunoprecipitated using agarose-conjugated anti-FLAG M2 monoclonal antibody. The kinase activity of p38 MAPK isoforms was measured by Western blotting using the phospho-ATF-2 antibody as described in Materials and Methods. The expression of epitope-tagged proteins was determined by Western blotting using anti-FLAG monoclonal antibody M2. *D,* phosphorylation level of MKK3 and MKK6 were measured by Western blotting with the same cell lysates in Fig. 1*C*, using phosphospecific MKK3/6. Endogenous expression of MKK3 and MKK6 was also measured by Western blotting using polyclonal MKK3 and MKK6 antibodies.

the most efficiently stimulated and critically involved in ARE activation by multiple extracellular stimuli. As seen in Fig. 5*A*, exposure of sulforaphane to HepG2 cells resulted in a strong phosphorylation of ERK1/2, where it increased up to 12 hours after treatment but slightly decreased after 24 hours. In addition, we found that treatment of sulforaphane for 30 minutes substantially phosphorylated MEK1/2, an upstream kinase of ERK1/2, implying that phosphorylation of upstream signaling kinase, i.e., MEK1/2, by sulforaphane might be responsible for ERK1/2 activation.

Unlike ERK and JNK, we showed that all p38 MAPK isoforms were negatively involved in ARE-dependent gene expression

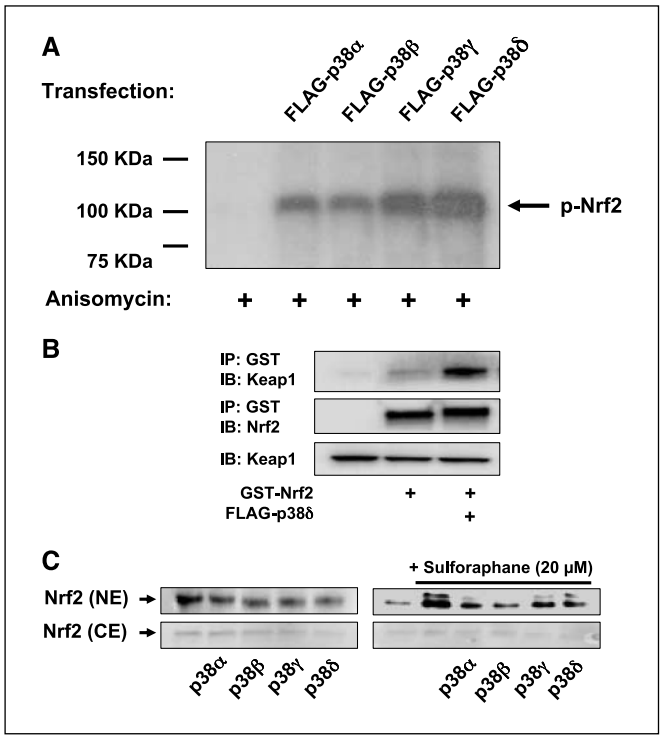


**Figure 6.** Phosphorylation of purified Nrf2 protein by p38 MAPK promotes the interaction between Nrf2 and Keap1 proteins *in vitro* and overexpression of p38 MAPK isoforms attenuates constitutive and inducible Nrf2 translocation into the nucleus. *A,* after transfection with 1 μg FLAG-p38 MAPK isoform plasmids, HeLa cells were stimulated with anisomycin (10 μg/mL) for 30 minutes and FLAG-p38 MAPK isoforms were immunoprecipitated using agarose-conjugated anti-FLAG M2 monoclonal antibody. Purified GST-Nrf2 protein was immunoprecipitated with FLAG-p38 MAPK isoforms and was incubated with $^{32}$P-labeled ATP in the presence of the precipitated p38 MAPK isoforms. Phosphorylation of Nrf2 protein by activated p38 MAPK isoforms was visualized by autoradiography. *B,* HeLa cells were transfected with 1 μg FLAG-p38δ plasmid and treated with 0.1% DMSO or anisomycin (10 μg/mL) for 30 minutes. FLAG-p38δ protein was immunoprecipitated using agarose-conjugated anti-FLAG M2 monoclonal antibody and incubated with purified GST-Nrf2 protein and protein lysates of MEFs. GST-Nrf2 protein was immunoprecipitated with GSH-conjugated beads and the amount of endogenous Keap1 protein in MEFs that was bound to immunoprecipitated GST-Nrf2 was measured by Western blotting (*top*). The presence of immunoprecipitated GST-Nrf2 (*middle*) in the reaction mixture and endogenous Keap1 protein in protein lysates of MEFs (*bottom*) was verified by Western blotting. *C,* HepG2 cells were transfected with 1 μg individual FLAG-p38 MAPK isoforms (*left*) and further treated with 20 μmol/L sulforaphane for 24 hours (*right*). The nuclear (*NE*) and cytosolic extracts (*CE*) were prepared as described in Materials and Methods and the localization of Nrf2 protein was measured by Western blotting.

(Fig. 3). Thus, we speculated that, if sulforaphane inhibits the kinase activities of p38 MAPK isoforms, modulation of p38 MAPK activities might contribute to sulforaphane-induced HO-1. As seen in Fig. 5*B*, we found that exposure of sulforaphane to HepG2 cells failed to affect the phosphorylation of total p38 MAPK (*lane 2*), but pretreatment of sulforaphane significantly attenuated anisomycin-induced phosphorylation of p38 MAPK (*lane 3* and *4*). To examine the possibility that p38 MAPK isoforms might be selectively inhibited, the individual FLAG-tagged p38 MAPK isoforms were transiently overexpressed in HepG2 cells and each p38 MAPK isoforms were immunoprecipitated using agarose-conjugated anti-FLAG M2 monoclonal antibody. Then, the kinase activities of individual p38 MAPK isoforms were measured, using ATF-2 as a substrate. As a result, we observed that pretreatment of sulforaphane significantly inhibited anisomycin-induced phosphorylation of all p38 MAPK isoforms (Fig. 5*C*). Because selective inhibition of p38 MAPK isoforms was not observed, we assumed that sulforaphane did not inhibit p38 MAPK isoforms themselves, but rather suppressed their kinase activities by acting on the upstream signaling pathways. Supporting this hypothesis, exposure of sulforaphane to HepG2 cells inhibited phosphorylation of MKK3/6 in a time-dependent manner, with a total inhibition after 2 hours treatment (Fig. 5*D*).

**Stimulation of p38 MAPK isoforms phosphorylates purified Nrf2 protein and promotes its interaction with Keap1 protein, and overexpression of p38 MAPK isoforms suppresses Nrf2 translocation into the nucleus.** As protein modification by phosphorylation is one of the major posttranslational mechanisms in signaling processes, it is believed to have a central role in regulating the activity of Nrf2 protein. To test the possibility whether p38 MAPK isoforms can directly phosphorylate Nrf2 protein, the individual FLAG-tagged p38 MAPK isoforms were transfected and stimulated with anisomycin for 30 minutes. Then, each p38 MAPK isoforms were immunoprecipitated, using agarose-conjugated anti-FLAG M2 monoclonal antibody. In this experiment, we used HeLa cells instead of HepG2 cells for the ease of transfection efficiency. Next, we constructed a high-level expression plasmid of human *Nrf2* gene linked at its $NH_2$ terminus to GST-tag and purified to near homogeneity by GSH-conjugated beads. Purified GST-Nrf2 was used as a substrate to measure the *in vitro* kinase activities of each of p38 MAPK isoforms. As shown in Fig. 6*A*, stimulation of all p38 MAPK isoforms by anisomycin strongly phosphorylated Nrf2 protein (*lanes 2-5*), whereas no phosphorylation was detected in the control immunoprecipitates (*lane 1*). We next speculated that phosphorylation of Nrf2 protein by p38 MAPK isoforms might contribute to the association between Nrf2 and Keap1 proteins, because p38 MAPK acted as a negative signaling kinase pathway on ARE-dependent gene expression (Figs. 3 and 4). GST pull-down assay using purified GST-Nrf2 protein and whole cell lysates was done to address this issue. As seen in Fig. 6*B*, we found that the association between GST-Nrf2 and Keap1 proteins was significantly increased (*lanes 2* and *3, top*), when GST-Nrf2 protein was phosphorylated by p38δ protein (note that GST-Nrf2 band was shifted due to its phosphorylation, *lane 3, middle*). Supporting this observation, Western blot analysis, using nuclear and cytosolic extracts, showed that nuclear translocation of constitutive and sulforaphane-induced Nrf2 protein was significantly abrogated by overexpression of p38 MAPK isoforms (Fig. 6*C*). Together, these results suggest that p38 MAPK isoforms might phosphorylate Nrf2 protein and stabilize the interaction between Nrf2 and Keap1 proteins, thereby contributing to a suppression of Nrf2 nuclear translocation.

## Discussion

Sulforaphane has pleiotropic pharmacologic effects. Here, we show that sulforaphane induces HO-1 by activating ARE through the induction of Nrf2 protein in HepG2 cells (Fig. 1). It seems that the induction of Nrf2 by sulforaphane is not regulated by transcriptional activation but by posttranslational stabilization,[5] which is analogous to the observation that treatment of *tert*-butylhydroquinone failed to affect Nrf2 mRNA but strongly

[5] Unpublished data.

attenuated ubuiquitinylation and proteosomal degradation of Nrf2 (25). In addition to the induction of Nrf2, we found that sulforaphane strongly decreased the protein expression level of Keap1 (Fig. 1*C*). Because Keap1 does not simply retain Nrf2 in the cytoplasm but actively targets Nrf2 to the ubiquitin-mediated proteolysis by Cul3-based E3 ligases (29), it seems that suppression of Keap1 by sulforaphane may also contribute to the attenuation of Nrf2 polyubiquitination, resulting in an overall increase of Nrf2 protein and Nrf2 nuclear accumulation followed by the subsequent induction of HO-1.

The expression of multiple p38 MAPK isoforms suggests that the physiologic function of these kinases may be overlapping but may not necessarily be redundant and/or identical. Supporting this notion, Pramanik et al. (30) have shown that p38β increases the activation of activator protein 1 (AP-1) transcriptional activities, whereas p38γ/p38δ inhibits and/or has no effect on the stimulation of AP-1 transcriptional activities. Dashti et al. (31) have shown that p38α enhanced MEK6 (MKK6)–dependent keratinocyte differentiation, but p38δ inhibited it. However, this does not seem to be the case in our study, because overexpression of all four p38 MAPK isoforms was found to negatively regulate constitutive and inducible ARE-dependent gene expression (Fig. 3*A-C*). In addition to the existence of multiple isoforms, selective regulation of p38 MAPK isoforms is another biological strategy to create appropriate biological responses against a variety of extracellular stimuli. For example, transforming growth factor-β selectively activated p38α and p38δ in murine mesangial cells (32). Hypoxia selectively activated p38α and p38γ in PC-12 cells (33). Lipopolysaccharide exclusively activated p38α in neutrophils (34). Furthermore, not only extracellular stimuli but also intracellular upstream kinases of p38 MAPK isoforms (MKK3 and MKK6) can selectively regulate the different p38 MAPK isoforms. Although MKK6 activates all the p38 MAPK isoforms, MKK3 activates p38α, p38γ, and p38δ, but not p38β (35). Enslen et al. (36) have identified two important structural requirements for selective activation of p38 MAPK isoforms by MKK3, i.e., (*a*) common docking sequences in the $NH_2$-terminal of MKK3 and (*b*) isoform-specific sequences of p38 MAPK isoforms within the activation loop (T-loop). However, Alonso et al. (37) have contradicted their hypothesis by showing that both of these sequences are not responsible for the selective regulation of p38 MAPK isoforms by MKK3. Instead, they have postulated that the on- and off-binding rates of p38 MAPK isoforms with upstream kinases may play a critical role in the selective regulation of p38 MAPK isoforms. This implies that selective activation of p38 MAPK isoforms might be variable, depending on the stimulus and/or possibly on the cell types. In line with this idea, we have observed that negative regulation of ARE-mediated gene expression by p38α and p38β was not dependent on MKK3 or MKK6 (Fig. 4*A-C*). The molecular basis of this selectivity is not clear, but it is possible that distinct signaling pathways converging on p38α and p38β other than MAPKK can also exist. For example, Ge et al. (38) have shown that the adaptor protein TAB1 represents such an example of MAPKK-independent mechanism. TAB1 usually binds and activates TAK1, a MAPKKK that can activate both JNK and p38 MAPK pathway. However, TAB1 was also found to bind to p38α and cause MAPKK-independent activation of p38α through autophosphorylation. Therefore, the possibility that autophosphorylation of p38α or p38β contributes to ARE suppression might exist, although it seems that autophosphorylation is less efficient than activation through upstream signaling cascades.

Contrasting our results, a couple of investigators have reported that p38 MAPK pathway plays a positive role in ARE-dependent phase II detoxifying enzymes, such as HO-1 (39), GSTA1/2 (40), and γ-GCS (41). To reach this conclusion, they have adopted pharmacologic inhibitors of p38α and p38β (SB203580 or SB212090) and showed that treatment with these inhibitors suppressed the transcriptional or translational expression of these proteins. However, it should be pointed out that drawing conclusion solely based on using these chemical inhibitors may sometimes be misleading because these chemical inhibitors may not exclusively inhibit p38 MAPK activity but may also activate other kinases or functional proteins. Another dilemma is that these inhibitors cannot address the roles of p38γ and p38δ. Although the biological importance of p38γ and p38δ has been neglected because of their limited tissue expression and substrate specificity, it has become increasingly apparent that p38γ and p38δ exert important physiologic effects as well. Consistent with this notion, our current study has illustrated that overexpression of p38γ or p38δ significantly inhibited ARE-mediated gene expression (Fig. 3*A*). Furthermore, the possibility that SB203580 mediated regulation of phase II detoxifying enzymes through additional promoter elements other than ARE also exists, as shown by Kang et al. (42). According to this study, treatment of PD98059, a chemical inhibitor of ERK1/2, led to a significant induction of GSTA2 in H4IIE rat hepatoma cells, suggesting that ERK1/2 pathway could play a negative role in phase II enzyme regulation, which lies in a sharp contrast with our previous observation that ERK1/2 pathway plays a positive role in the regulation of phase II enzymes through enhancing ARE-mediated gene expression (43). However, they found that PD98059 did not induce GSTA2 in ARE-dependent manner. Instead, PD98059 induced GSTA by activation of CCAAT/enhancer–binding protein and enhanced its binding to the cognate promoter region through the PI3K, irrespective of the inhibition of MEK(MKK)1/ERK activity by PD98059. Hence, it is possible that induction of phase II detoxifying enzymes by SB203580 or SB212090 might occur through unknown but distinct signaling pathways other than via ARE.

The possibility that Nrf2 could be directly phosphorylated by kinases have been raised and tested by numerous investigators. Huang et al. (44) have reported that direct phosphorylation of Nrf2 at $Ser^{40}$ by PKC play a positive role in Nrf2-mediated ARE activation by interfering with the interaction of Nrf2 with Keap1. Cullinan et al. (45) have also shown that Nrf2 could be directly phosphorylated by PERK, although its target sites are not yet identified. In the present study, we observed that stimulation of p38 MAPK isoforms directly phosphorylated Nrf2 protein (Fig. 6*A*) and phosphorylation of Nrf2 protein by activated p38δ promoted the association between Nrf2 and Keap1 proteins (Fig. 6*B*). Based on these data, we speculate that phosphorylation of Nrf2 by p38 MAPK could contribute to inhibition of ARE-dependent gene expression by increasing the protein-protein interaction between Nrf2 and Keap1. In the preliminary studies, we have identified a unique phosphorylation site by p38δ, but not by ERK2 and JNK1, at the COOH terminus of Nrf2 in the preliminary studies (data not shown). In addition, we have also uncovered that other MAPKs (ERK2 and JNK1) could directly phosphorylate purified Nrf2 under similar *in vitro* conditions. More importantly, we found that endogenous Nrf2 was phosphorylated using $[^{32}P]P_i$

labeling followed by immunoprecipitation against Nrf2 (data not shown). Because Nrf2 protein is a cap'-n'-collar family of transcription factors that share a highly conserved bZIP structure, it is tempting to speculate that phosphorylation of Nrf2 at different residues by MAPKs might result in differential ARE-dependent gene expression, presumably via positive or negative interactions of Nrf2 with nuclear coactivators/corepressors and/or other bZIP proteins (46). In line with this idea, we have reported that overexpression of increasing amount of nuclear transcriptional coactivator, cAMP-responsive element binding protein–binding protein, activated transfected ARE-luciferase reporter activity (47). On the other hand, Venugopal et al. (48) have shown that overexpression of Fos family proteins significantly inhibited Nrf2-induced ARE activation, whereas Jun family members did not significantly affect ARE activity. Similarly, it was reported that overexpression of MafG and MafK resulted in a significant inhibition of ARE activation (49). Therefore, future studies focusing on how phosphorylation of Nrf2 by p38 MAPK isoforms dictates the association of Nrf2 with coactivators/repressors, signaling kinases, and transcription factors in the nucleus will be needed to account for the negative effects of p38 MAPK signaling pathway on ARE-dependent gene expression.

## Acknowledgments

Received 9/29/2005; revised 5/19/2006; accepted 6/22/2006.

**Grant support:** NIH grant R01-CA094828.

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We thank all the members in Dr. Tony Kong's laboratory for their help in the discussion and preparation of the manuscript and Dr. Michael Shakarjian (Department of Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, NJ) for the help in the preparation of mouse embryonic keratinocytes and fibroblasts.



## References

1. Conaway CC, Yang YM, Chung FL. Isothiocyanates as cancer chemopreventive agents: their biological activities and metabolism in rodents and humans. Curr Drug Metab 2002;3:233–55.
2. Fahey JW, Zalcmann AT, Talalay P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 2001;56:5–51.
3. Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci U S A 1992;89:2399–403.
4. Zhang Y, Kensler TW, Cho CG, Posner GH, Talalay P. Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. Proc Natl Acad Sci U S A 1994;91:3147–50.
5. Singh AV, Xiao D, Lew KL, Dhir R, Singh SV. Sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards growth of PC-3 xenografts *in vivo*. Carcinogenesis 2004;25:83–90.
6. Fahey JW, Haristoy X, Dolan PM, et al. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of *Helicobacter pylori* and prevents benzo(*a*)pyrene-induced stomach tumors. Proc Natl Acad Sci U S A 2002;99:7610–5.
7. Singh SV, Herman-Antosiewicz A, Singh AV, et al. Sulforaphane-induced G2/M phase cell cycle arrest involves checkpoint kinase 2-mediated phosphorylation of cell division cycle 25C. J Biol Chem 2004;279:25813–22.
8. Gamet-Payrastre L, Li P, Lumeau S, et al. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res 2000;60:1426–33.
9. Kwak MK, Egner PA, Dolan PM, et al. Role of phase 2 enzyme induction in chemoprotection by dithiolethiones. Mutat Res 2001;480–1:305–15.
10. Chen C, Kong AN. Dietary chemopreventive compounds and ARE/EpRE signaling. Free Radic Biol Med 2004;36:1505–16.
11. Nguyen T, Yang CS, Pickett CB. The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. Free Radic Biol Med 2004;37:433–41.
12. Itoh K, Chiba T, Takahashi S, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. 2 1997;236:313–22.
13. Keum YS, Jeong WS, Tony Kong AN. Chemoprevention by isothiocyanates and their underlying molecular signaling mechanisms. Mutat Res 2004;555:191–202.
14. Lee JC, Laydon JT, McDonnell PC, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 1994;372:739–46.
15. Han J, Lee JD, Bibbs L, Ulevitch RJ. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 1994;265:808–11.
16. Raingeaud J, Gupta S, Rogers JS, et al. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 1995;270:7420–6.
17. New L, Han J. The p38 MAP kinase pathway and its biological function. Trends Cardiovasc Med 1998;8:220–8.
18. Ono K, Han J. The p38 signal transduction pathway: activation and function. Cell Signal 2000;12:1–13.
19. Hager B, Bickenbach JR, Fleckman P. Long-term culture of murine epidermal keratinocytes. J Invest Dermatol 1999;112:971–6.
20. Choi AM, Alam J. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Respir Cell Mol Biol 1996;15:9–19.
21. Scapagnini G, Foresti R, Calabrese V, Giuffrida Stella AM, Green CJ, Motterlini R. Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers. Mol Pharmacol 2002;61:554–61.
22. Sikorski EM, Hock T, Hill-Kapturczak N, Agarwal A. The story so far: molecular regulation of the heme oxygenase-1 gene in renal injury. Am J Physiol Renal Physiol 2004;286:F425–41.
23. Yu R, Mandlekar S, Lei W, Fahl WE, Tan TH, Kong AT. p38 mitogen-activated protein kinase negatively regulates the induction of phase II drug-metabolizing enzymes that detoxify carcinogens. J Biol Chem 2000;275:2322–7.
24. Wu WT, Chi KH, Ho FM, Tsao WC, Lin WW. Proteasome inhibitors up-regulate heme oxygenase-1 gene expression: requirement of p38 MAPK (mitogen-activated protein kinase) activation but not of NF-κB (nuclear factor κB) inhibition. Biochem J 2004;379:587–93.
25. Nguyen T, Sherratt PJ, Huang HC, Yang CS, Pickett CB. Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26S proteasome. J Biol Chem 2003;278:4536–41.
26. Raingeaud J, Whitmarsh AJ, Barrett T, Derijard B, Davis RJ. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Mol Cell Biol 1996;16:1247–55.
27. Brancho D, Tanaka N, Jaeschke A, et al. Mechanism of p38 MAP kinase activation *in vivo*. Genes Dev 2003;17:1969–78.
28. Lee JC, Kassis S, Kumar S, Badger A, Adams JL. p38 mitogen-activated protein kinase inhibitors—mechanisms and therapeutic potentials. Pharmacol Ther 1999;82:389–97.
29. Kobayashi A, Kang MI, Okawa H, et al. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol 2004;24:7130–9.
30. Pramanik R, Qi X, Borowicz S, et al. p38 isoforms have opposite effects on AP-1-dependent transcription through regulation of c-Jun. The determinant roles of the isoforms in the p38 MAPK signal specificity. J Biol Chem 2003;278:4831–9.
31. Dashti SR, Efimova T, Eckert RL. MEK6 regulates human involucrin gene expression via a p38α- and p38δ-dependent mechanism. J Biol Chem 2001;276:27214–20.
32. Wang L, Ma R, Flavell RA, Choi ME. Requirement of mitogen-activated protein kinase kinase 3 (MKK3) for activation of p38α and p38δ MAPK isoforms by TGF-β1 in murine mesangial cells. J Biol Chem 2002;277:47257–62.
33. Conrad PW, Rust RT, Han J, Millhorn DE, Beitner-Johnson D. Selective activation of p38α and p38γ by hypoxia. Role in regulation of cyclin D1 by hypoxia in PC12 cells. J Biol Chem 1999;274:23570–6.
34. Nick JA, Avdi NJ, Young SK, et al. Selective activation and functional significance of p38α mitogen-activated protein kinase in lipopolysaccharide-stimulated neutrophils. J Clin Invest 1999;103:851–8.
35. Enslen H, Raingeaud J, Davis RJ. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. J Biol Chem 1998;273:1741–8.
36. Enslen H, Brancho DM, Davis RJ. Molecular determinants that mediate selective activation of p38 MAP kinase isoforms. EMBO J 2000;19:1301–11.
37. Alonso G, Ambrosino C, Jones M, Nebreda AR. Differential activation of p38 mitogen-activated protein kinase isoforms depending on signal strength. J Biol Chem 2000;275:40641–8.
38. Ge B, Gram H, Di Padova F, et al. MAPKK-independent activation of p38α mediated by TAB1-dependent autophosphorylation of p38α. Science 2002;295:1291–4.
39. Alam J, Wicks C, Stewart D, et al. Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells. Role of p38 kinase and Nrf2 transcription factor. J Biol Chem 2000;275:27694–702.
40. Kang KW, Ryu JH, Kim SG. The essential role of phosphatidylinositol 3-kinase and of p38 mitogen-activated protein kinase activation in the antioxidant response element-mediated rGSTA2 induction by decreased glutathione in H4IIE hepatoma cells. Mol Pharmacol 2000;58:1017–25.
41. Zipper LM, Mulcahy RT. Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of

GCS genes. Biochem Biophys Res Commun 2000;278:484–92.
42. Kang KW, Park EY, Kim SG. Activation of CCAAT/enhancer-binding protein β by 2′-amino-3′-methoxyflavone (PD98059) leads to the induction of glutathione *S*-transferase A2. Carcinogenesis 2003;24:475–82.
43. Owuor ED, Kong AN. Antioxidants and oxidants regulated signal transduction pathways. Biochem Pharmacol 2002;64:765–70.
44. Huang HC, Nguyen T, Pickett CB. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. J Biol Chem 2002;277:42769–74.
45. Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Mol Cell Biol 2003;23:7198–209.
46. Wu RC, Smith CL, O'Malley BW. Transcriptional regulation by steroid receptor coactivator phosphorylation. Endocr Rev 2005;26:393–9.
47. Shen G, Hebbar V, Nair S, et al. Regulation of Nrf2 transactivation domain activity. The differential effects of mitogen-activated protein kinase cascades and synergistic stimulatory effect of Raf and CREB-binding protein. J Biol Chem 2004;279:23052–60.
48. Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc Natl Acad Sci U S A 1996;93:14960–5.
49. Dhakshinamoorthy S, Jaiswal AK. Small maf (MafG and MafK) proteins negatively regulate antioxidant response element-mediated expression and antioxidant induction of the NAD(P)H:Quinone oxidoreductase1 gene. J Biol Chem 2000;275:40134–41.