# Involvement of Nrf2 and JNK1 in the Activation of Antioxidant Responsive Element (ARE) by Chemopreventive Agent Phenethyl Isothiocyanate (PEITC)

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***Purpose.*** Phenethyl isothiocyanate (PEITC) has been of great interest as a promising cancer chemopreventive agent. To better understand its chemopreventive activity, we examined the effect of PEITC on the antioxidant responsive element (ARE), which is an important gene regulatory element of many phase II drug-metabolizing/detoxification enzymes as well as cellular defensive enzymes.

***Methods.*** HeLa cells were transiently transfected with different cDNA plasmids using calcium phosphate precipitation. Subsequently, the cells were maintained in fresh media, and various concentrations of PEITC were added to the transfected cells. After harvesting and lysing of the cells, ARE-luciferase reporter gene activity was measured and normalized against β-galactosidase activity.

***Results.*** Treatments of HeLa cells with PEITC transiently stimulated ARE-reporter gene expressions in a dose-dependent manner. Overexpression of wild-type NF-E2 related factor-2 (Nrf2) dramatically increased ARE-reporter gene expression in a dose-dependent manner. Similar effects were seen when wild-type c-Jun N-terminal kinase 1 (JNK1) was transfected, although the transactivating potential of JNK1 was much less than that of Nrf2. Cotransfection of Nrf2 and JNK1 showed additional enhancement of ARE reporter gene expression, implying that JNK1 might be an upstream activator of Nrf2. To support this, overexpression of dominant-negative JNK1 suppressed Nrf2-induced ARE reporter gene expression in a dose-dependent manner. When PEITC was added, slight enhancement of ARE reporter gene expression was observed in either Nrf2- or JNK1-transfected cells. Finally, ARE reporter activity induced by PEITC was substantially attenuated by transfection of either dominant-negative mutant of Nrf2 or dominant-negative mutant of JNK1.

***Conclusion.*** Taken together, these data suggest that JNK1 acts as an upstream activator of Nrf2 and that PEITC activates ARE-mediated phase II drug metabolism gene expressions via the JNK1- and Nrf2-dependent pathways.

**KEY WORDS:** chemoprevention; isothiocyanate (ITC); phenethyl isothiocyanates (PEITC); antioxidant response element (ARE); Nrf2; JNK1; signal transduction.

## INTRODUCTION

Investigations of the anticarcinogenic properties of various plant families have found cruciferous vegetables to have chemopreventive potentials superior to those of any other plant family (1). The plants of Cruciferae (also known as the Brassicaceae) family include broccoli, horseradish, cabbage, cauliflower, and watercress. Cruciferous vegetables are rich in glucosinolates, which can be degraded nonenzymatically by physical factors or enzymatically by myrosinases to isothiocyanates (ITCs) during food preparation, cooking, and chewing (2). Numerous epidemiologic reports showed that consumption of cruciferous vegetables is inversely related to the risk of developing various types of cancer (3). Mechanistic studies have indicated that remarkable chemopreventive activity of ITCs stems from their biological effects on carcinogen metabolism and detoxification. Thus, phenethyl isothiocyanate, the most predominant ingredient among isothiocyanates, has been investigated extensively as a promising chemopreventive compound.

The concept of metabolism modulation as a possible strategy of protection against carcinogenesis originated from the fact that many chemical carcinogens are not chemically reactive *per se* but must undergo bioactivation to electrophiles by certain enzymes (4). Careful observation of drug-metabolizing enzymes led to the recognition of separate enzyme systems, phase I (cytochrome P450s) and phase II drug-metabolizing or detoxifying enzymes. These enzymes are not separate entities but closely linked in action. When exposed to chemical carcinogens, phase I enzymes convert potential carcinogens by oxidation or reduction. Phase II enzymes then promote the conjugation of phase I products with endogenous cofactors such as glutathione (by glutathione *S*-transferase; GST) and glucuronic acid (by UDP-glucuronosyltransferases; UGT), resulting in more water-soluble products, which can be easily excreted.

Many investigators have shown that induction of several phase II and cellular defensive enzymes is under the transcriptional control of antioxidant responsive element (ARE) (5). The promoters of many of these genes, including GST, NAD(P)H: quinone oxidoreductase (NQO), γ–glutamylcysteine synthetase (γ-GCS), and heme oxygenase I (HO-1), indeed possess the ARE DNA sequence. The core sequence of ARE as obtained by mutational analyses is GTGACNNNGC (6,7). This sequence bears a resemblance to the nuclear factor E2 (NF-E2) consensus sequence, which is positively controlled by a transcriptional factor, NF-E2 related factor 2 (Nrf2). There is much evidence to show that Nrf2 is a key transcription factor of ARE activation. First, many of Nrf2 target genes encode proteins that play an important role in the adaptive response to oxidative stress with association of other cofactors, such as Maf family proteins (8). Second, exogenous induction of either wild-type or dominant-negative Nrf2 can positively or negatively regulate ARE expression (9). Third, homozygous Nrf2 knock-out mice are deficient in their ability to induce the phase II-related genes and, as a result, are highly sensitive to oxidative stress (10). The mode of transcriptional activation of Nrf2 has been debated, with several mechanisms [reviewed by Kong *et al.* (11)]. One of the mechanisms is postulated to be similar to that of NF-κB. Nrf2 appears to be sequestered in the cytoplasm as an inactive complex with Keap1. On exposure to oxidative stresses or chemicals, Nrf2 dissociates from Keap1 and translocates into the nucleus, thereby activating ARE (12). The other mechanisms can be via the signaling kinases including mitogen-activated protein kinase (MAPK) (9,13), protein kinase C (PKC) (14), or phosphatidylinositol 3-kinase (PI3K) (15).

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The MAPKs are evolutionarily conserved enzymes that transfer regulatory information from the cell surface to the nucleus. By now, three major classes of MAPKs have been identified, which differ in their substrate specificity and regulation. These include the extracellular signal-regulating kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 kinases. Our previous studies have shown that MAPK is involved in ARE activation and that ARE activation driven by MAPK is Nrf2 dependent (9,13). However, in spite of these results, it is still unclear which MAPK module among ERKs, JNKs, and p38 acts as an upstream mediator of Nrf2. Recently, Jeyapaul *et al.* have shown that overexpression of wild-type c-Jun could induce ARE activation by executing the translocation of Nrf2 protein into the nucleus (16). Accordingly, we postulated that JNK1, which is mainly responsible for c-Jun phosphorylation might be localized in the upstream signaling pathway of Nrf2. In the present study, we performed transient transfection assays to address the involvement of Nrf2 and JNK1 in the transcriptional activation of ARE by PEITC.

## MATERIAL AND METHODS

### Reagents

PEITC and ONPG (O-nitrophenyl-β-D-galactopyranoside) were purchased from Sigma Chemical Co. (St. Louis, MO). The luciferase assay kit was purchased from Promega (Madison, WI).

### Cell Culture and Plasmids

HeLa cells (human cervical squamous carcinoma) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured at 37°C and 5% $CO_2$ in minimum essential medium supplemented with 10% fetal bovine serum, 2.2 mg/ml sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin. ARE-luciferase reporter construct was generously provided by Dr. William Fahl (University of Wisconsin, Madison, WI). pcDNA1.1-Nrf2 construct was kindly provided by Drs. Yuet W. Kan and Jefferson Y. Chan (University of California, San Francisco, CA). Generation of Nrf2(M2), a dominant-negative Nrf2, was described previously (13). pcDNA3-HA-JNK1 was kindly provided by Dr. Michael Karin (University of California, San Diego, CA). Expression vector of kinase mutant, pcDNA3-Flag-JNK1(APF1) was kindly provided by Dr. Roger Davis (University of Massachusetts, Worcester, MA).

### Transient Transfection and Reporter Gene Activity Assays

HeLa cells were plated in six-well plates at a density of $2.0 \times 10^5$ cells/well and transfected with 1 μg of ARE-luciferase reporter construct alone or together with the expression plasmids such as wild-type Nrf2 (pcDNA 1,1-Nrf2), mutant of Nrf2 [Nrf2(M2)], wild-type JNK1 (pcDNA3-HA-JNK1), and mutant JNK1 [(pc-DNA3-Flag-JNK(APF)] using the calcium-phosphate precipitation method as previously described (9,13). One microgram of β-galactosidase plasmid was used in each individual experiment to normalize transfection efficiency. Total amount of plasmid DNA in each well was adjusted to 5 μg with an empty vector, pcDNA3.1. Twelve hours after transfection, media were aspirated, and cells were replaced with fresh media for 24 h. Cells were then treated with various concentrations of PEITC for 6 h, and subsequently cells were harvested and lysed with luciferase buffer. Luciferase activity was measured according to the manufacturer's instructions (Promega, Madison, WI), and β-galactosidase activity was measured using ONPG as a substrate. In brief, cells were washed twice with 1× ice-cold phosphate-buffered saline. Cells were then incubated with 1× reporter lysis buffer for 30 min and scraped off the plate. After brief centrifugation at 13,000*g*, 10 μl of aliquot of the supernatant was analyzed for luciferase activity with a luminometer. 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. All experiments were performed in duplicate unless indicated otherwise.

## RESULTS

### PEITC Induces ARE in a Dose-Dependent Manner

HeLa cells were transiently transfected with the ARE-reporter plasmid. After transfection, the cells were treated with different concentrations of PEITC for 6 h and harvested. Luciferase activity was then determined. As shown in Fig. 1, treatments of HeLa cells with PEITC resulted in a dose-dependent stimulation of luciferase activity and reached a maximum at 5 μM. Subsequently, decreasing luciferase activity was observed with higher concentrations of PEITC (20 and 50 μM). When the cells were checked under the microscope at these concentrations, cell death was observed, suggesting that PEITC are cytotoxic at these concentrations.

### Nrf2 and JNK1 are Positive Regulators of the ARE

Numerous studies have illustrated that Nrf2 is a transcriptional regulator of ARE. However, so far no attempts have been made to directly link JNK1 to ARE- and/or Nrf2-

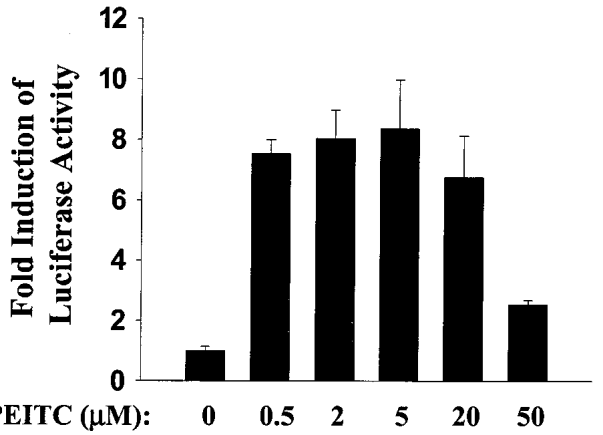



**Fig. 1.** Effects of PEITC on the activation of ARE-luciferase reporter activity in HeLa cells. HeLa cells were transfected with both 1 μg of β-galactosidase plasmid and 1 μg of ARE-luciferase reporter construct overnight. Transfected cells were washed, replaced with fresh medium containing 0.5% fetal bovine serum, and incubated for additional 24 h. Cells were then treated with PEITC at concentrations as indicated above for 6 h. The cells were harvested, and luciferase activity was quantified as described in detail under Material and Methods.

ARE transcription activation. As seen in Fig. 2a, transient transfection of wild-type JNK1 caused a dose-dependent activation of the ARE activation. Overexpression of wild-type Nrf2 resulted in a large induction of ARE-luciferase activity, analogous to that reported in other cell line such as HepG2 (13,16). The transactivating potential of Nrf2 is much higher than that of JNK1 (Fig. 2b). To determine whether Nrf2 and JNK1 can cause synergistic ARE activation, transient cotransfection of wild-type Nrf2 and wild-type JNK1 was performed. Cotransfection of Nrf2 and JNK1 showed substantial synergistic induction of the ARE (Fig. 2b) and reached a plateau at 1 μg of transfected Nrf2, implying that signaling saturation might have occurred at this concentration of Nrf2. To further support the linkage between Nrf2 and JNK1, wild-type Nrf2 and dominant negative JNK1 (JNK-APF) were cotransfected. As shown in Fig. 2c, ARE reporter gene activity decreased in a dose-dependent manner as the amount of transfected JNK-APF increased up to 0.5 μg. Total inhibition of ARE was not achieved even when up to 2 μg of JNK1-APF was introduced, suggesting that signaling pathways other than JNK1 might exist in the Nrf2-mediated ARE induction. Collectively, these data imply that JNK1 may exist as a regulator located in the upstream signaling of Nrf2.

### PEITC Enhances the ARE Activation by Nrf2 and JNK1

As seen in Fig. 3, treatments of Nrf2-transfected cells with PEITC resulted in further increases of ARE-luciferease activity. However, at concentrations of 20 and 50 μM of PEITC, the induction of ARE-reporter activity was decreased. Again, these were the result of cytotoxicity, as confirmed by trypan blue exclusion methods (data not shown). In Fig. 4, treatments of JNK1-transfected cells with PEITC also resulted in further increases in ARE-luciferase activity. At higher concentrations of PEITC (50 μM), the ARE luciferase activity was decreased, again because of cytotoxicity. To exclude the possibility that the low enhancement of ARE activity induced by PEITC could be related to the short period of incubation of PEITC (6 h), kinetic time course studies were performed. No remarkable difference in ARE activation difference was observed up to 48 h (data not shown). These data suggest that Nrf2 and JNK1 are positive regulators of the PEITC-induced ARE-mediated gene transcription machinery.

### Interfering with the Dominant-Negative Nrf2 and the Dominant-Negative JNK Attenuates the Induction of ARE by PEITC

To further corroborate the roles of Nrf2 and JNK1 in ARE activation by PEITC, either dominant-negative Nrf2 or dominant-negative JNK1 was transfected in HeLa cells, and the cells were treated with PEITC for 6 h. As shown in Fig. 5, transient transfection with either dominant-negative mutant of Nrf2 or dominant-negative JNK1 substantially attenuated the induction of ARE-luciferase activity by PEITC, as compared to the wild-type control and to treatments with PEITC. At 50 μM of PEITC, no measurable ARE-luciferase activity was observed because of cytotoxicity.

## DISCUSSION

The chemopreventive activity of isothiocyanates (ITCs) has been quite extensively studied and documented (3). As

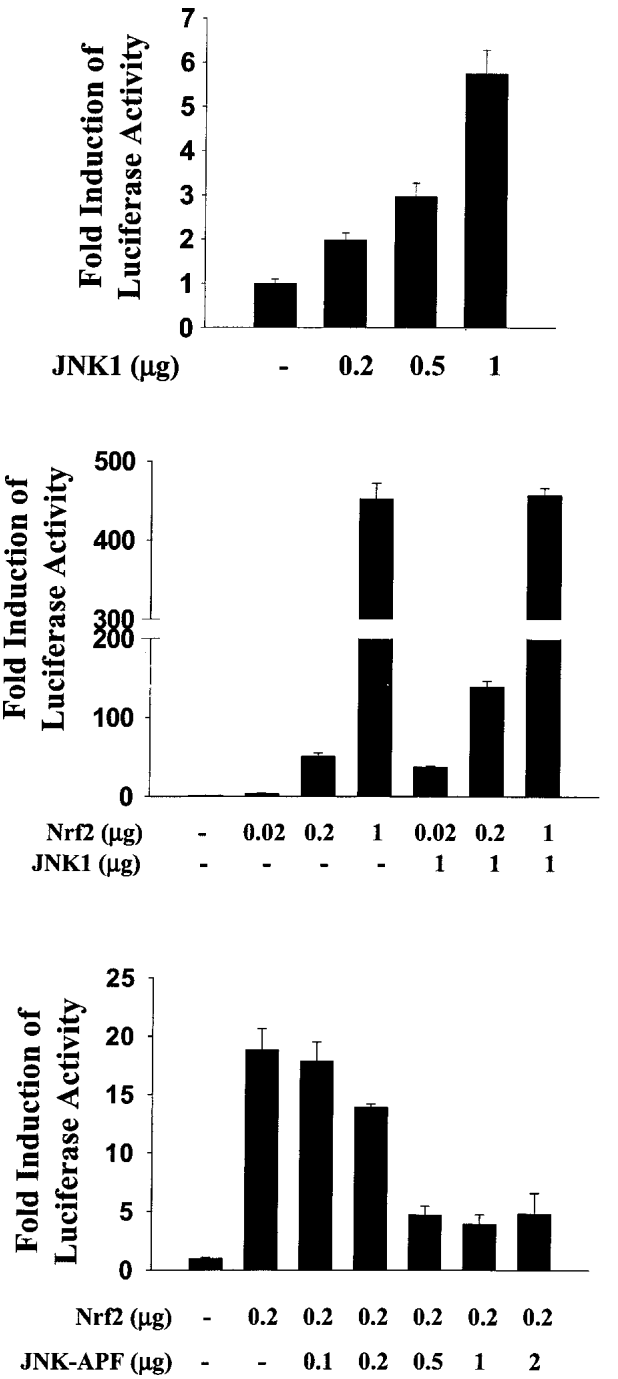



**Fig. 2.** A, JNK1-induced ARE activation. HeLa cells were transfected with 1 μg of β-galactosidase plasmid and 1 μg of ARE-luciferase reporter construct plus various amounts of wild-type JNK1 plasmid. Transfected cells were washed and replaced with fresh medium containing 0.5% fetal bovine serum for 24 h. The cells were harvested, and luciferase activity was quantified as described above. B, Synergistic effect of JNK1 on Nrf2-induced ARE activation. HeLa cells were transfected with 1 μg of β-galactosidase plasmid and 1 μg of ARE-luciferase reporter and wild-type of Nrf2 alone or together with wild-type JNK1. After 24 h, transfected cells were harvested, and luciferase activity was measured as described above. C, Attenuation of Nrf2-induced ARE activation by dominant-negative JNK1. HeLa cells were transfected with 1 μg of β-galactosidase plasmid and 1 μg of ARE-luciferase reporter and wild-type of Nrf2 together with dominant-negative JNK1 (JNK-APF). After 24 h, transfected cells were harvested, and luciferase activity was measured as described above.

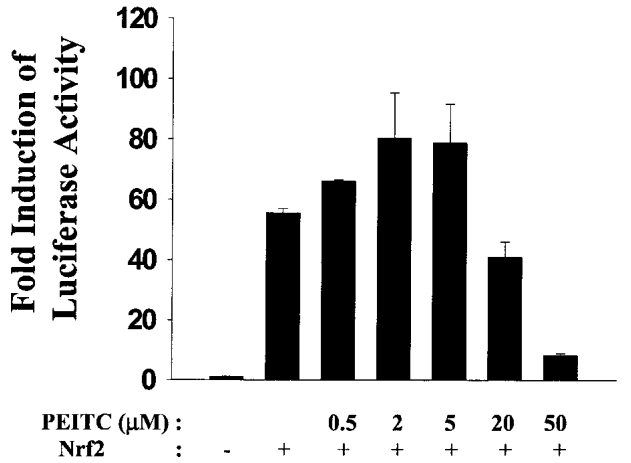



**Fig. 3.** Effects of PEITC on the activation of ARE luciferase reporter activity in Nrf2-overexpressed HeLa cells. HeLa cells were transfected with 1 μg of β-galactosidase plasmid and 1 μg of ARE-luciferase reporter construct plus 0.2 μg of wild-type Nrf2 plasmid. Transfected cells were washed and replaced with fresh medium containing 0.5% fetal bovine serum for 24 h. Cells were then treated with PEITC at concentrations as indicated above for 6 h and harvested.

discussed in the Introduction, potential chemoproventive activity by ITCs can be ascribed to either blocking carcinogen metabolism (phase I) or enhancing carcinogen detoxification (phase II). ITCs can possess either type of activity or both. Prochaska and Talalay have designated the former as monofunctional inducers and the latter as bifunctional inducers (17). Most of the ITCs including PEITC act as monofunctional inducers, especially with respect to induction of phase II detoxifying enzymes, with little or no induction of phase I enzymes. In the current study, treatment of HeLa cells with PEITC leads to activation of ARE in a dose-dependent manner (Fig. 1). This result suggests that the chemopreventive effect of PEITC can, at least in part, be attributed to the increased carcinogen detoxification by the induced phase II enzymes. Numerous studies have shown that other structurally related isothiocyanate chemicals are also potent inducers

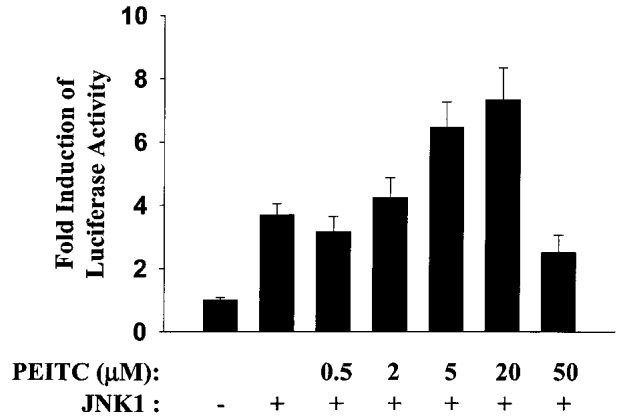
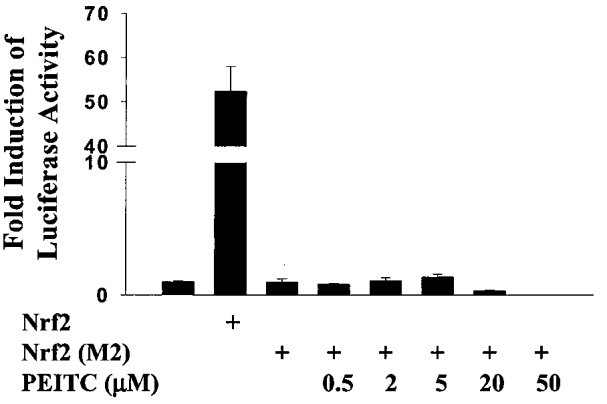



**Fig. 4.** Effects of PEITC on the activation of ARE-luciferase reporter in JNK1-overexpressed HeLa cells. HeLa cells were transfected with 1 μg of β-galactosidase plasmid, 1 μg of ARE-luciferase reporter construct, and 1 μg of JNK1. Transfected cells were incubated in fresh medium containing 0.5% fetal bovine serum for 24 h. Cells were then treated with PEITC at concentrations as indicated above for 6 h and harvested.



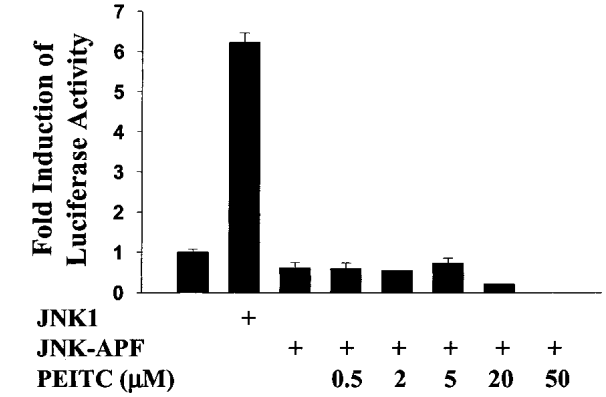




**Fig. 5.** A, Effect of PEITC on ARE activation in HeLa cells transfected with dominant-negative of Nrf2. HeLa cells were transfected with both 1 μg of β-galactosidase plasmid and 1 μg of ARE-luciferase reporter construct plus 0.2 μg of dominant-negative Nrf2 (Nrf2-M2). After washing, cells were treated with PEITC at concentrations as indicated above for 6 h and harvested. B, Effect of PEITC on ARE activation in HeLa cells transfected with dominant-negative JNK1. HeLa cells were transfected with both 1 μg of β-galactosidase plasmid and 1 μg of ARE-luciferase reporter construct plus 1 μg of dominant-negative JNK1 (JNK-APF). After washing, cells were treated with PEITC at concentrations as indicated above for 6 h and harvested.

of phase II enzymes. For example, 4-methylsulfinylbutyl isothiocyanate (sulforaphane) (18), benzyl isothiocyanate (BITC) (19), 7-methylfulfinylheptyl isothiocyanates, and 8-methylsulfinyloctyl isothiocyanates (20) exhibited significant phase II enzyme inductions. Thus, it appears that ARE transcriptional activation by PEITC may not be a unique property but rather a general event exerted by the ITC class of compounds. The other possibility of chemopreventive actions could be via the inhibition of the metabolic activation of carcinogens by phase I enzymes, as described earlier (21), or more recently via the induction of apoptosis of malignant cells (22).

It is apparent that transcription activation of ARE required Nrf2. However, the upstream signaling events governing Nrf2 transcription activation is currently not clear. Previ-

ously, we provided evidence that MAPK may be responsible for the regulation of ARE. ERK was shown to positively control ARE (9), whereas p38 could negatively modulate ARE (23). In contrast, Zipper and Mulcahy have provided slightly different results, demonstrating that both p38 and ERK could positively regulate ARE expression (24). The reason for this difference is unclear and probably depends on the cell type and/or other currently unknown conditions. Distinct regulatory mechanisms of Nrf2 other than MAPK have also been reported. Lee *et al*. demonstrated that PI3K, and not ERK, regulates ARE in IMR-32 human neuroblastoma cell lines (15). Similarly, Huang *et al.* reported that the potential phosphorylation of Nrf2 by PKC may be important for Nrf2 activation (14). On the other hand, Dinkova-Kostova *et al.* have recently postulated that the dissociation of Nrf2-Keap1 complex could result from the direct interaction of phase II inducers, most of which are electrophilic agents with reactive thiol residues in either of the two proteins, and may contribute to Nrf2 activation (25). Most recently, Kwak *et al.* showed the existence of an ARE-like sequence in the promoter region of Nrf2, which was responsible for sustaining the duration of ARE activation by providing the binding site of Nrf2 itself (26). Taken together, all of these studies suggested the possibility that multiple mechanisms are involved in Nrf2-mediated transcription activation.

It is commonly known that JNK1 signal transduction plays an important role in apoptosis. However, to date there is no report on the role of JNK1 as a potential regulator of phase II genes. Our studies described here provide the first clue that JNK1 can positively affect ARE transcription. However, how PEITC induces the ARE activation by way of JNK1 is still unclear. Zhang and Talalay suggested that ITCs might induce oxidative stress, thereby stimulating a variety of signal transduction pathways including JNK (27). In support of this notion, they showed that treatment of cells with ITC-class chemicals resulted in a direct interaction of ITCs with GSH and formed a dithiocarbamate conjugate [R-NH-C(=S)-SG], which may cause a depletion of the intracellular GSH level and subsequent oxidative stress (27). Chaudhary *et al.* also showed that the caspase inhibitor, Z-VAD-fmk did not inhibit PEITC-induced depletion of GSH while preventing PEITC-induced apoptosis (28). Because caspase-8 is potentially localized downstream of the JNK signaling pathway (28), it is tempting to speculate that oxidative stress generated by PEITC-induced intracellular GSH depletion may lead to activation of JNK1, which in turn transcriptionally activates Nrf2-ARE-mediated phase II enzyme induction.

In conclusion, the following results were demonstrated in the present study. (a) PEITC can transcriptionally activate ARE in a dose-dependent manner. (b) Transfection of wild-type Nrf2 or JNK1 induced ARE. Importantly, cotransfection of JNK1 and Nrf2 showed additional enhancement of ARE, and cotransfection of dominant-negative JNK1 (JNK-APF) with Nrf2 led to a dose-dependent decrease of ARE. (c) PEITC enhanced ARE activation in a dose-dependent manner in the presence of either wild-type Nrf2 or wild-type JNK1. (d) Transient expression of either dominant-negative Nrf2 or dominant-negative JNK1 attenuated PEITC-induced ARE expression. Collectively, these data suggest that JNK1 could be an upstream activator of Nrf2 and that PEITC-induced ARE activation may be dependent on Nrf2 and JNK1 upstream signaling pathways.

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