

# Mechanism of action of isothiocyanates: the induction of ARE-regulated genes is associated with activation of ERK and JNK and the phosphorylation and nuclear translocation of Nrf2

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## Abstract

The up-regulation of phase II detoxifying and stress-responsive genes is believed to play an important role in cancer prevention, and many natural compounds have been shown to be potent inducers of these genes. Previous studies showed that the antioxidant responsive element (ARE), found in these genes, can be bound by the transcription factor Nrf2, and is responsive to the activation by chemopreventive compounds and by oxidative stress. In the present study, we investigated the roles of extracellular signal-regulated kinase (ERK) and c-Jun-NH<sub>2</sub>-kinase (JNK) in the regulation of phenethyl isothiocyanate (PEITC)-induced and Nrf2-dependent ARE activity and ARE-driven *heme oxygenase-1* (*HO-1*) gene expression in PC-3 cells. ARE activity and HO-1 expression were strongly increased after treatment with PEITC. PEITC also increased the phosphorylation of ERK1/2 and JNK1/2 and caused release of Nrf2 from sequestration by Keap1, and its subsequent translocation into the nucleus. Importantly, Nrf2 was also translocated into the nucleus after transfection with ERK or JNK and that these activated ERK and JNK colocalized with Nrf2 in the nucleus. Activation of ERK and JNK signaling also resulted in the elevation of ARE activity and HO-1 expression. Importantly, PEITC-induced ARE activity was attenuated

by inhibition of ERK and JNK signaling. *In vitro* kinase assays showed that both ERK2 and JNK1 could directly phosphorylate glutathione S-transferase-Nrf2 protein. Taken together, these results strongly suggest a model in which PEITC treatment of PC-3 cells activates ERK and JNK, which, in turn, phosphorylate Nrf2 and induce its translocation to the nucleus. Nuclear Nrf2 activates ARE elements and induces expression of stress-responsive genes, including *HO-1*. [Mol Cancer Ther 2006;5(8):1918–26]

## Introduction

The up-regulation of cellular defense genes, such as phase II detoxifying and stress response genes, is believed to play an important biological function in the protection against carcinogenesis and in the attenuation of cancer development (1, 2). For example, the potent natural chemopreventive compound sulforaphane can strongly induce phase II gene expression *in vivo* and *in vitro* (3–6), and the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis was shown to be correlated with phase II gene regulation (7). The role of phase II conjugation in the metabolism of drugs, xenobiotics, and carcinogens in the human body has been long studied. The mechanism by which induction of phase II genes occurs, however, remains unclear until recently (8). These recent findings suggest a key role for the antioxidant response element (ARE)/electrophile response element in the regulation of some phase II and stress-responsive antioxidant genes, such as *NAD(P)H:quinone oxidoreductase 1*, *glutathione S-transferase (GST)*, *UDP-glucuronosyltransferase*, and *heme oxygenase-1 (HO-1)* by many chemicals, phenolic antioxidants, and other naturally occurring cancer chemopreventive agents (5, 9–12). Recently, several ARE-binding proteins have been proposed and identified, including Nrf1, Nrf2, Nrf3, small Maf proteins, which are members of the basic leucine zipper transcription factor family. The central role of Nrf2 in the transcriptional activation of ARE has been recently confirmed in some ARE-mediated genes, including *γ-glutamylcysteine ligase*, *HO-1*, *NAD(P)H:quinone oxidoreductase 1*, and *GST* (9, 13–18).

Questions remain as to how Nrf2 is activated by such diverse chemical compounds. Several models have been proposed, and the biological reality probably involves the cross-talks and/or the convergence of various signaling pathways, depending on the context of the chemical structures and or the type of cells or tissues (1, 8). Previously, our group have shown, in transient transfection and kinase-specific chemical inhibitors studies, that the mitogen-activated protein kinases (MAPK) are involved

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in the regulation of the ARE in a Nrf2-dependent manner (19, 20). We found that the extracellular signal-regulated kinases 2 and 5 (ERK2 and ERK5), and c-Jun NH<sub>2</sub>-terminal kinase 1 (JNK1), up-regulate the ARE (10, 20, 21), whereas the p38 MAPK seems to suppress it (22). Later studies have suggested that phosphatidylinositol 3-kinase may be involved in Nrf2 nuclear translocation in response to tBHQ-induced oxidative stress in conjunction with cytoplasmic actin rearrangement (23). In addition, protein kinase C can directly phosphorylate Nrf2, potentially at Ser<sup>40</sup> (24). Furthermore, Cullinan et al. (25, 26) have indicated that Nrf2 is directly phosphorylated by PERK, a transmembrane transcription factor, following the accumulation of unfolded proteins in the endoplasmic reticulum. Taken together, these results suggest that multiple kinase signaling pathways are involved in the transcriptional activation of ARE in a Nrf2-dependent manner.

Dinkova-Kostova et al. (27–29) have reported a compelling regulatory mechanism for Nrf2 activation that does not involve its phosphorylation. They showed that phase II inducers, most of which are strong electrophiles, directly cleave the Nrf2-Keap1 complex by modifying Keap1 at cysteine residues through the Michael reaction. In support of this hypothesis, a recent study has shown that two of the 15 cysteine residues (Cys<sup>273</sup> and Cys<sup>288</sup>) in Keap1 could play an important role in releasing Nrf2 in response to electrophiles and oxidative stress *in vitro* via the formation of an intermolecular disulfide bridge (30). Some phase II inducers, including cadmium, tBHQ,  $\beta$ -naphthoflavone, organosulfur compounds, allyl isothiocyanate, and sulforaphane, can also potentially increase Nrf2 accumulation (12, 18, 31, 32).

Cancer initiation and development involves a variety of carcinogens, such as viruses, chemicals, UV light, and irradiation. Cancer can ultimately spread to the other parts of the body from its origin during the metastasis stage (1). Numerous studies indicate that many human cancers could be prevented or their progression could be slowed down by some natural compounds (33). Because the efficacy of current clinical cancer remedies is limited, the prevention of cancer and the interference of its progression especially in its early stages would seem to be important. However, these approaches have not yet been taken seriously in the clinical settings (2). Many epidemiologic studies have shown that consumption of cruciferous vegetables can reduce the risk of different kinds of cancers in humans (34–36). Sulforaphane and some other isothiocyanates that are derived from cruciferous vegetables are highly effective in preventing or reducing the risk of cancer induced by carcinogens in various animal models (33, 34, 36, 37).

Prostate cancer is one of the most prevalent malignancies and the second leading cause of cancer deaths of men in the United States. Because it is usually diagnosed in patients >50 years old, there seems to be ample opportunity for intervention using cancer chemopreventive compounds that prevent or slow down the initiation, promotion, and progression of this disease. Recent studies have indicated that using dietary chemopreventive compounds such as

isothiocyanates could be a promising strategy to decrease the incidence of prostate cancer (38, 39). Studies have shown that sulforaphane and two other isothiocyanates, phenethyl isothiocyanate (PEITC) and allyl isothiocyanate, can reduce the risk of prostate cancer in animal models, inhibit prostate cancer cell growth, induce apoptosis, and retard the growth of prostate cancer cell xenografts *in vivo* (4, 37, 38, 40–44). Recently, it has been shown that p53 may not be essential for PEITC-induced apoptosis and that PEITC-induced apoptosis in PC-3 human prostate carcinoma cell line could be mediated by ERK (40). In addition, our previous studies have indicated that sulforaphane and PEITC can potentially inhibit nuclear factor- $\kappa$ B activity and nuclear factor- $\kappa$ B-regulated genes expression in PC-3 cells through the inhibition of the IKK $\alpha$ / $\beta$ -I $\kappa$ B $\alpha$ -p65 signaling pathway (45).

At the present time, it is still not clear as to how the signaling pathways modulated by isothiocyanates such as PEITC would play a role in the regulation of Nrf2-dependent and ARE-driven defensive gene expression. This lack of understanding despite the fact that many previous studies have indicated that the chemopreventive efficacy of natural compounds is tightly associated with antioxidant signaling and phase II gene induction. In the current study, we investigate the potential roles of MAPKs such as ERK and JNK in the phosphorylation and regulation of PEITC-induced and Nrf2-dependent ARE activity and ARE-driven HO-1 expression in PC-3 cells. Our results show that PEITC treatment of PC-3 cells strongly activates ERK and JNK, and that ERK and JNK can phosphorylate Nrf2, induce the translocation of Nrf2 into the nucleus, transcriptionally activate ARE, and induce the expression of stress-responsive genes including *HO-1*.

## Materials and Methods

### Materials

PEITC was purchased from Sigma (St. Louis, MO). Luciferase assay reagent was purchased from Promega Corp. (Madison, WI). Antibodies against phospho-JNK1/2 (Thr<sup>183</sup>/Tyr<sup>185</sup>), phospho-ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>), ERK1/2 (p42/p44), and JNK1/2 (p46/p54) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies that recognize ERK2, JNK1, and Nrf2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Alexa Fluor 633 goat anti-rabbit IgG and Alexa Fluor 633 goat anti-mouse IgG were purchased from Molecular Probes, Inc. (Eugene, OR). The ARE-luciferase reporter plasmid construct containing ARE consensus binding sites was kindly provided by Dr. William Fahl (University of Wisconsin, Madison, WI). pcDNA3-HA-JNK1 plasmid was kindly provided by Dr. Michael Karin (University of California, San Diego, CA). Constitutively active MAP/ERK kinase1 (MEK1; DNEE-MEK1-pcDNA3) was a gift from Dr. Rony Seger (The Weizmann Institute of Science, Rehovot, Israel). Plasmids of MKK4 and ERK2 were described previously (12, 46).

### Cell Culture

Human prostate cancer PC-3 cells were obtained from American Type Culture Collection (Rockville, MD). Cells

were maintained in MEM supplemented with 10% fetal bovine serum (FBS), 2.2 g/L sodium bicarbonate, and 10 mL/L PSN antibiotic mixture (Life Technologies, Grand Island, NY) in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C. Before treatment, the medium was removed when cells were ~80% confluent, and cells were maintained overnight in MEM containing 0.5% FBS.

#### Stable and Transient Transfection

The ARE luciferase construct was stably transfected into PC-3 cells by Lipofectamine 2000 (Invitrogen Life Technology, Rockville, MD) following the instructions of the manufacturer. Five hours after transfection, the medium was replaced with MEM containing 10% FBS, and the cells were selected with G418 to obtain single cell stable clones. One of the clones, PC-3 C5, which contained the ARE-luciferase reporter construct, was used for all the subsequent experiments. Transient transfections were also done using the same procedure as the stable transfection, only without G418 selection.

#### 3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium, Inner Salt, Assay

The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was done with CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay kit (Promega) according to the instructions from the manufacturer. Briefly, PC-3 cells were plated in 24-well plates, and after 24 hours growth medium was replaced with medium containing 0.5% serum overnight. Cells were then treated with different concentrations of PEITC for 24 and 48 hours. The medium was then removed, and culture medium containing MTS and phenazine methosulfate solution was added. About 30 minutes later, the absorbance was measured at 490 nm with a  $\mu$ Quant ELISA reader (BIO-TEK Instruments, Inc., Madison, WI). The data are expressed as percentage of cell viability compared with that of the control, which was treated with 0.1% ethanol. Data are presented as mean ( $n = 4$ )  $\pm$  SD.

#### ARE-Dependent Reporter Gene Expression Assay

ARE activity was measured by ARE-luciferase reporter gene expression. After PC-3 C5 cells were treated or transiently transfected, the ARE-luciferase activities were measured according to the instructions from the manufacturer (Promega). Briefly, the cells were washed with ice-cold PBS and harvested in 1 $\times$  reporter lysis buffer. After centrifugation, 20  $\mu$ L aliquots of the supernatants were measured for luciferase activity using a Sirius Luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany). The luciferase activity was normalized by protein concentrations or  $\beta$ -galactosidase activity and expressed as fold induction of luciferase activity over the control cells, which were treated with 0.1% ethanol. Data are expressed as mean ( $n = 3$ )  $\pm$  SD.

#### Western Blot Analysis

PC-3 cells were plated in six-well plates and cultured for 24 hours. Growth medium was then replaced with medium containing 0.5% serum overnight. Cells were subsequently

treated with PEITC for different time periods and harvested with MAPK lysis buffer [10 mmol/L Tris-HCl, 50 mmol/L sodium chloride, 30 mmol/L sodium PPI, 50 mmol/L sodium fluoride, 100  $\mu$ mol/L sodium orthovanadate, 2 mmol/L iodoacetic acid, 5 mmol/L ZnCl<sub>2</sub>, 1 mmol/L phenylmethanesulfonyl fluoride, and 0.5% Triton X-100] or whole-cell lysis buffer [10 mmol/L Tris-HCl, 250 mmol/L sodium chloride, 30 mmol/L sodium PPI, 50 mmol/L sodium fluoride, 0.5% Triton X-100, 10% glycerol, 1 $\times$  proteinase inhibitor mixture, 1 mmol/L phenylmethanesulfonyl fluoride, 100  $\mu$ mol/L sodium orthovanadate, 2 mmol/L iodoacetic acid, and 5 mmol/L ZnCl<sub>2</sub>]. Protein extracts were prepared, and the concentrations were determined by Bio-Rad protein assay according to the instructions from the manufacturer. Twenty micrograms of total protein from each sample were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes using a semidry transfer system (Fisher, Pittsburgh, PA). The membranes were then blocked with 5% bovine serum albumin or 5% nonfat milk in TBST buffer [2.42 g/L Tris-HCl, 8 g/L NaCl, 1 mL/L Tween 20 (pH 7.6)] and incubated overnight at 4°C with primary antibodies in TBST buffer. Membranes were then incubated with secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology), and the detection was done with enhanced chemiluminescence Western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

#### Immunofluorescence and Confocal Microscopy

PC-3 cells were seeded into six-well plates containing glass coverslips and cultured for 24 hours. Cells were then transfected with EGFP-Nrf2 and/or DsRed-Keap1, MKK4 + JNK1, DNEE-MEK1 + ERK2 (with 1  $\mu$ g of each plasmid), and cultured in normal MEM for an additional 24 hours. The transfected cells were treated with 5  $\mu$ mol/L PEITC for 2 hours with DMSO as a control. These cells were then fixed with 3.8% formaldehyde in PBS, and phospho-JNK1 and phospho-ERK2 were detected with primary antibodies (Cell Signaling Technology) and stained with second antibodies conjugated with Alexa Fluor 633. Stained cells were viewed by using a Zeiss LSM 510 laser scanning confocal microscope equipped with a  $\times$ 63 water immersion objective (numerical aperture, 1.3).

#### In vitro Phosphorylation of Nrf2

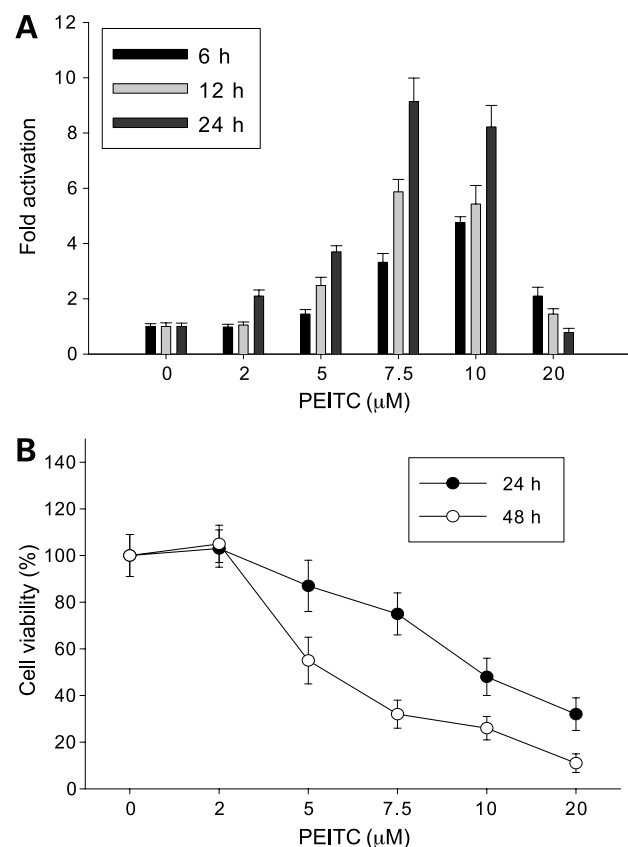
*In vitro* phosphorylation of Nrf2 mediated by JNK1 and ERK2 was assessed by an immunoprecipitation kinase assay, similar to that previously described (45). Briefly, PC-3 cells were cultured on 10-cm Petri dishes for 24 hours. Cells were then transiently transfected with DNEE-MEK1 + ERK2 or MKK4 + JNK1 (1  $\mu$ g each) and cultured in normal MEM for 24 hours. Alternatively, cells were treated with PEITC for 2 hours. Whole-cell lysates were then prepared. ERK2 and JNK1 were immunoprecipitated from 500  $\mu$ g of the lysate using antibodies and Protein A Sepharose beads (Zymed Laboratories, Inc., South San Francisco, CA). After incubation overnight at 4°C, the beads were washed with lysis buffer and kinase assay buffer [20 mmol/L HEPES (pH 7.4), 10 mmol/L MgCl<sub>2</sub>, 2 mmol/L MnCl<sub>2</sub>, 2 mmol/L DTT, 50 mmol/L  $\beta$ -glycerophosphate, 10 mmol/L *p*-nitrophenyl

phosphate, and 0.1 mmol/L sodium orthovanadate]. Kinase reactions were initiated by the addition of 30  $\mu$ L of kinase assay buffer containing 2  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP, 10  $\mu$ mol/L unlabeled ATP, and 10  $\mu$ g of substrate [bacterially purified full-length GST-Nrf2 fusion protein, myelin basic protein (MBP), or GST-C-Jun (1–79)]. After incubation at 30°C for 30 minutes, protein was resolved by 10% SDS-PAGE, the gel was stained and dried, and the phosphorylation of Nrf2, MBP, and GST-C-Jun (1–79) was visualized by autoradiography and quantified with a phosphoimager.

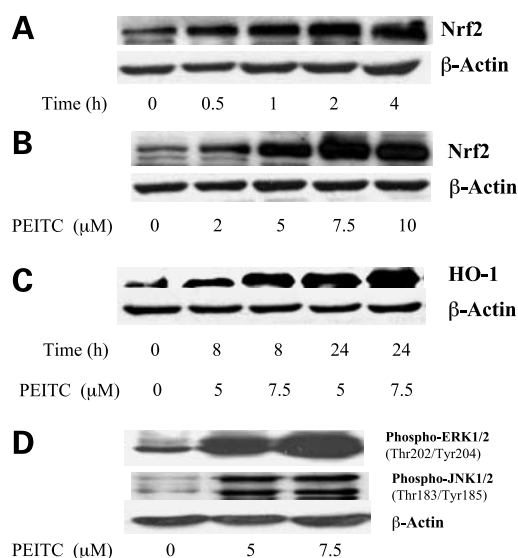
## Results

### PEITC Induces ARE-Luciferase Reporter Gene Activity and Cell Death

To investigate the effects of PEITC on ARE activity, we did dose-response and time course experiments in PC-3 C5 cells, which contain an ARE-luciferase reporter construct. Treatment with PEITC caused a dose-dependent induction of ARE-luciferase activity, with peak activity seen at 7.5 to 10  $\mu$ mol/L (Fig. 1A). A higher dose of 20  $\mu$ mol/L showed



**Figure 1.** Effects of PEITC on ARE-luciferase reporter gene activity and cell viability in PC-3 cells. **A**, ARE-luciferase reporter activity following PEITC treatment. PC-3 C5 cells were grown overnight in 0.5% FBS and then treated with PEITC. The ARE transcriptional activity was measured over time by a luciferase activity assay. *Columns*, mean ( $n = 3$ ); *bars*, SD. **B**, effect of PEITC on the cell viability. PC-3 cells were treated with PEITC following overnight incubation in low-serum 0.5% FBS. Cell viability at 24 and 48 h was determined by MTS assay. *Points*, mean ( $n = 4$ ); *bars*, SD. Each experiment was repeated twice.



**Figure 2.** Effects of PEITC on Nrf2 accumulation, HO-1 expression, and phosphorylation of ERK1/2 and JNK1/2 in PC-3 cells. PC-3 C5 cells were grown overnight in 0.5% FBS and then treated with PEITC. **A**, time course of Nrf2 accumulation after 7.5  $\mu$ mol/L PEITC. Samples were harvested at the indicated times, and whole-cell lysates were analyzed for Nrf2 by Western blotting.  $\beta$ -Actin levels are shown to control for equal loading. **B**, dose response of Nrf2 accumulation induced by PEITC. Samples were harvested with whole-cell lysis buffer after treatment for 2 h. Nrf2 and  $\beta$ -actin levels were determined by Western blot analysis. **C**, dose response and time course of HO-1 expression induced by PEITC. Samples were harvested with whole-cell lysis buffer after the indicated times and doses. HO-1 and  $\beta$ -actin levels were determined by Western blotting. **D**, effect of PEITC on phosphorylation of ERK1/2 and JNK1/2. After treatment with PEITC for 2 h, samples were harvested with MAPK lysis buffer, and Western blots against phospho-ERK1/2 and phospho-JNK1/2 were done.

less induction. ARE-dependent luciferase activity was also time dependent. A stronger response was observed after 24 hours than after 6 or 12 hours. The exception to this time dependence was at the highest dose of 20  $\mu$ mol/L (Fig. 1A).

We predicted that the decrease in ARE activity at 20  $\mu$ mol/L PEITC might be due to cellular toxicity. Therefore, we next examined cell viability after PEITC treatment using an MTS assay. Treatment with PEITC potently killed PC-3 cells at a higher dose level. Cell viability after treatments with 10 and 20  $\mu$ mol/L for 24 hours was  $48 \pm 8\%$  and  $32 \pm 7\%$ , respectively. Cell viability was further decreased when the treatment period was prolonged to 48 hours, with viability after 10 and 20  $\mu$ mol/L PEITC declining to  $26 \pm 5\%$  and  $11 \pm 4\%$ , respectively (Fig. 1B). Treatment with lower concentrations of 5 or 7.5  $\mu$ mol/L PEITC for 48 hours also had potent effects on cell viability (Fig. 1B).

### PEITC Induces Nrf2 Accumulation, HO-1 Expression, and Phosphorylation of ERK1/2 and JNK1/2 in PC-3 Cells

It is known that Nrf2 can bind to the ARE located in the 5'-flanking region of some phase II detoxifying and antioxidant- and stress-responsive genes, such as *HO-1*, and transcriptionally activate their expression (1, 8). Because PEITC could potently activate ARE activity (Fig. 1A), we next investigated the effects of PEITC on Nrf2 accumulation and the induction of endogenous HO-1 in PC-3 cells.

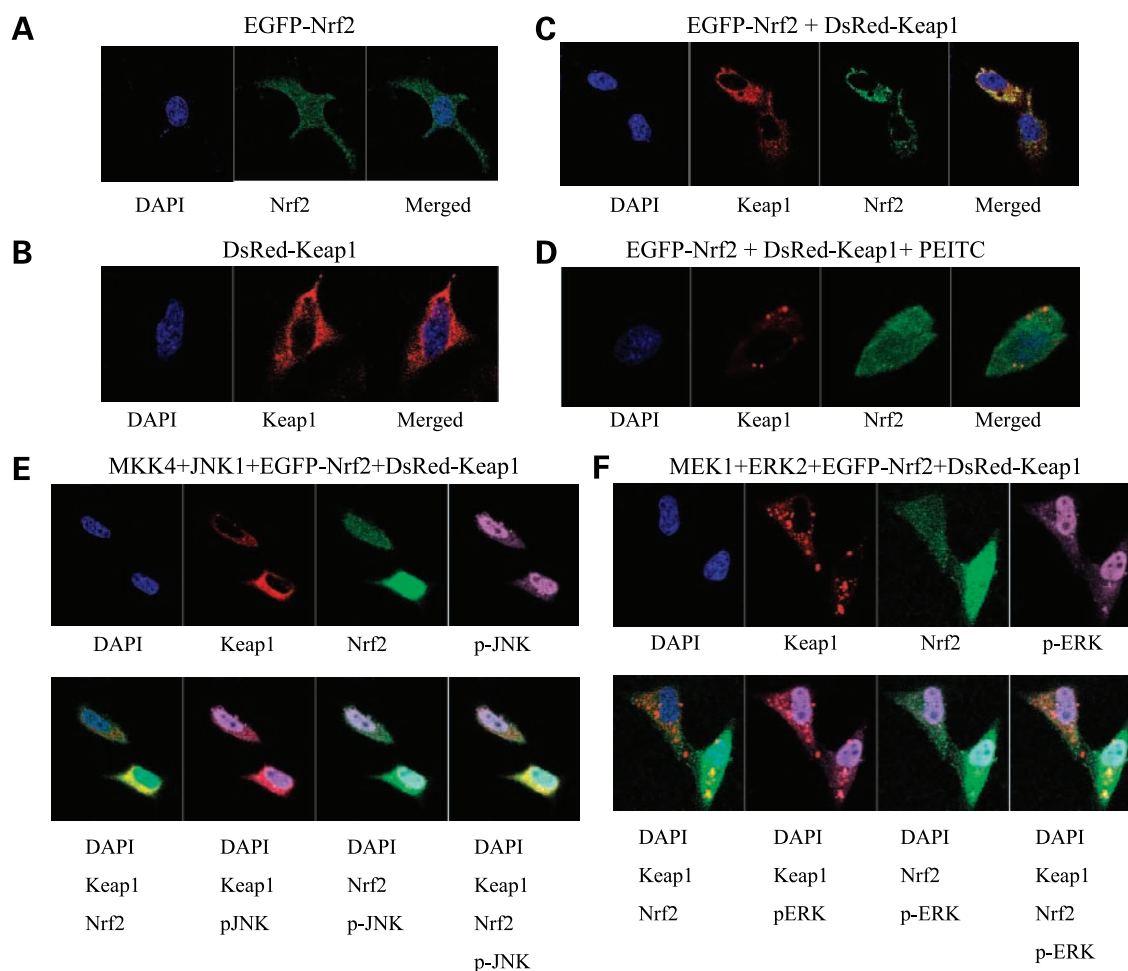
Treatment with 7.5  $\mu\text{mol/L}$  PEITC for 30 minutes up to 4 hours strongly increased the accumulation of Nrf2 protein in whole-cell lysates of PC-3 cells in a time-dependent fashion (Fig. 2A). In addition, in treatment of PC-3 cells with 5, 7.5, or 10  $\mu\text{mol/L}$  PEITC for 2 hours, PEITC also strongly induced Nrf2 accumulation in a dose-dependent manner (Fig. 2B). We next examined the induction of endogenous HO-1 protein after PEITC treatments with 5 and 7.5  $\mu\text{mol/L}$  for longer time periods of 8 and 24 hours, and the results showed that dramatic increases in HO-1 protein levels in PC-3 cells (Fig. 2C). These data indicate that the increase in ARE activity observed after PEITC treatment in Fig. 1 likely occurs through the accumulation and the subsequent transcriptional activity of Nrf2.

In our previous studies, we found that MAPK pathways, especially MEK1-ERK and MKK4-JNK, can positively regulate Nrf2-ARE transcriptional activity (20). Therefore, we examined the effects of PEITC on the activation/phosphorylation of ERK1/2 and JNK1/2 in PC-3 cells

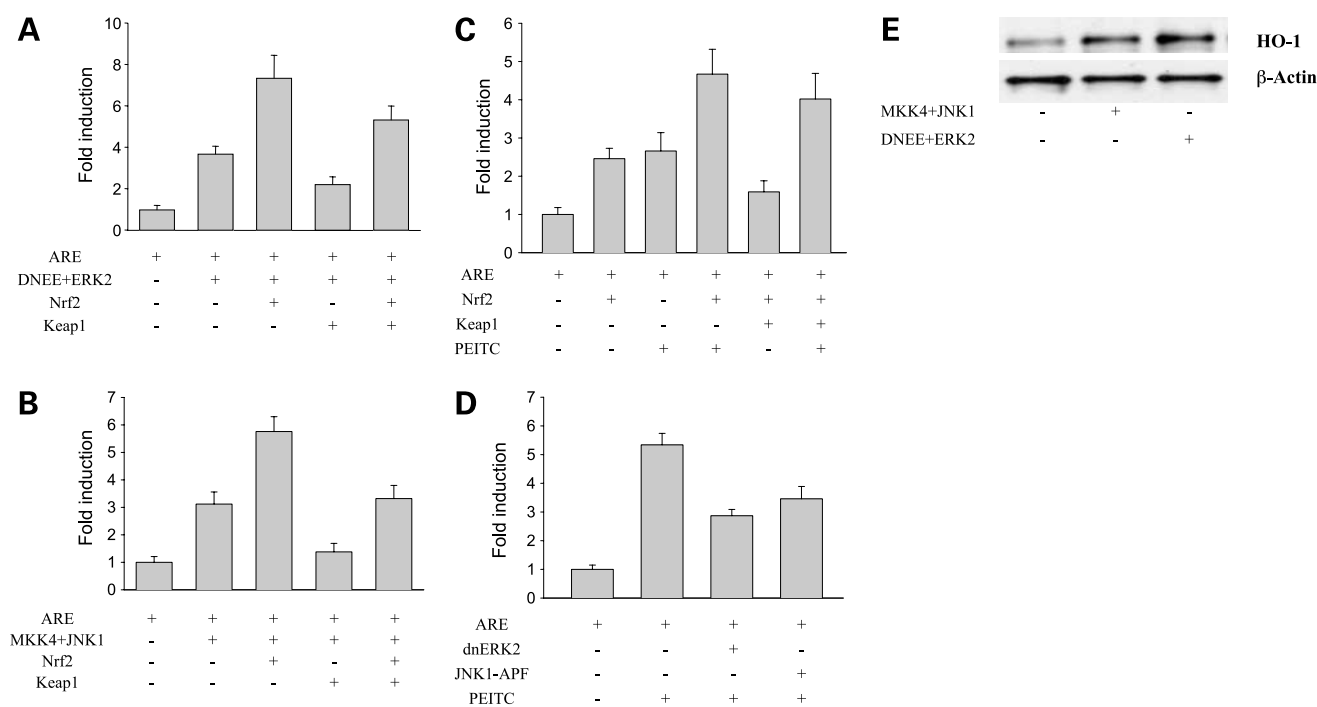
under the same conditions. The phosphorylation of both ERK1/2 and JNK1/2 was strongly elevated following the treatments with 5 and 7.5  $\mu\text{mol/L}$  PEITC for 2 hours (Fig. 2D) and at 4 hours (data not shown). These results suggest that PEITC activates MAPK pathways, which may in turn induce Nrf2 activation of ARE-containing genes.

#### Activated ERK2 and JNK1 Cause Release of Nrf2 from Keap1 and Translocation of Nrf2 into the Nucleus

We showed above that PEITC could induce the phosphorylation of ERK1/2 and JNK1/2 and increase the accumulation of Nrf2 in PC-3 cells (Fig. 2). Under quiescent conditions, Nrf2 is typically sequestered by Keap1 in the cytoplasm, where it is degraded (1). Therefore, the next question we asked was whether the activation of ERK or JNK or treatment with PEITC changes the localization of Nrf2 in PC-3 cells. As shown in Fig. 3A, transfection of EGFP-Nrf2 into PC-3 cells led to a whole-cell distribution (both cytoplasmic and nuclear) of EGFP-Nrf2. Transfected DsRed-Keap1, on the other hand, showed an entirely



**Figure 3.** Localization of EGFP-Nrf2, DsRed-Keap1, and phosphorylated JNK and ERK in treated PC-3 cells. PC-3 cells seeded onto glass coverslips were transiently transfected with EGFP-Nrf2 (A), DsRed-Keap1 (B), EGFP-Nrf2 + DsRed-Keap1 (C), EGFP-Nrf2 + DsRed-Keap1 (D), MKK4 + JNK1 + EGFP-Nrf2 + DsRed-Keap1 (E), or DNEE-MEK1 + ERK2 + EGFP-Nrf2 + DsRed-Keap1 (F), and cultured for 24 h. Cells in D were treated with 5  $\mu\text{mol/L}$  PEITC for 2 h at the end of the culture period. Cells were then fixed and examined for the proteins indicated along the bottom of each frame.



**Figure 4.** Regulation of ARE-luciferase activity and HO-1 expression by ERK and JNK. **A to D**, PC-3 cells were transiently transfected with the indicated plasmids, as well as with pRSV- $\beta$ -galactosidase, to normalize for transfection efficiency. The ARE transcriptional activity was measured by luciferase activity assay and normalized to  $\beta$ -galactosidase activity for each sample. *Columns*, mean ( $n = 3$ ); *bars*, SD. **A and B**, cells were cultured for 24 h before harvesting. **C**, transfected cells were cultured for 24 h and then treated with 5  $\mu$ mol/L PEITC for 12 h as indicated. **D**, transfected cells were cultured for 24 h, and then treated with 7.5  $\mu$ mol/L PEITC as indicated for 12 h. **E**, cells were transfected with plasmids as indicated, cultured for 24 h, and harvested with whole-cell lysis buffer. HO-1 and  $\beta$ -actin protein levels were determined by Western blot analysis.

cytoplasmic distribution in PC-3 cells (Fig. 3B). Notably, when EGFP-Nrf2 and DsRed-Keap1 were cotransfected into PC-3 cells, Nrf2 was sequestered in the cytoplasm (Fig. 3C). Next, we treated the cells with 5  $\mu$ mol/L PEITC for 2 hours after cotransfection of EGFP-Nrf2 and DsRed-Keap1. PEITC induced a significant portion of the EGFP-Nrf2 protein translocated into the nucleus, whereas Keap1 remained in the cytoplasm with the remaining Nrf2 (Fig. 3D). Next, we transfected JNK1 and its activating kinase, MKK4, into PC-3 cells together with EGFP-Nrf2 and DsRed-Keap1 to check for nuclear trafficking of Nrf2. MKK4 and JNK1 expression induced an abundant translocation of Nrf2 into the nucleus. In these cells, the phosphorylated JNK was also mainly localized in the nucleus, whereas Keap1 remained in the cytoplasm (Fig. 3E). Similar results were found when ERK2 and a constitutively activated form of it, activating kinase MEK1 (DNEE-MEK1), were cotransfected into PC-3 cells with EGFP-Nrf2 and DsRed-Keap1 (Fig. 3F). Nrf2 was released from Keap1 cytoplasmic sequestration and was found in both the cytoplasm and the nucleus, whereas Keap1 stayed in the cytoplasm. Furthermore, the majority of phosphorylated ERK was also localized in the nucleus (Fig. 3F).

#### Activated JNK1 and ERK2 Increase ARE-Luciferase Activity and HO-1 Expression in PC-3 Cells

To further corroborate the positive effects of JNK and ERK signaling on the regulation of ARE activity, we

transiently transfected the ARE-luciferase reporter construct with DNEE-MEK1 + ERK2 or MKK4 + JNK1 into PC-3 cells. DNEE-MEK1 + ERK2 increased ARE activity ~3.7-fold. This effect presumably results from activation of endogenous Nrf2. Transfection of exogenous Nrf2 with DNEE-MEK1 + ERK2 further increased the ARE activity to ~7.4-fold (Fig. 4A). Keap1, on the other hand, decreased the ARE-luciferase activity induced by DNEE-MEK1 + ERK2. This latter effect by Keap1 was strongly reversed by cotransfection with Nrf2, with the activity of ARE increased to 5.3-fold above the basal levels (Fig. 4A).

Similar results were seen in experiments with MKK4 and JNK1. Transfection of MKK4 + JNK1 increased ARE-luciferase activity 3.1-fold, whereas cotransfection with Nrf2 increased the ARE activity 5.8-fold (Fig. 4B). As with DNEE-MEK1 + ERK2 transfection, Keap1 decreased the ARE activity induced by MKK4 + JNK1 to 1.4-fold over basal levels, and cotransfection with Nrf2 restored the ARE activity to 3.3-fold (Fig. 4B).

We next investigated the effects of transfection with Nrf2 and Keap1 on PEITC-induced ARE activity. Transfection with Nrf2 alone increased the ARE activity 2.5-fold. Treatment of the cells with 5  $\mu$ mol/L PEITC for 12 hours increased the ARE activity by 2.7-fold (Fig. 4C). The combination of Nrf2 transfection followed by PEITC treatment increased ARE activity to 4.7-fold. Transfection with Keap1 decreased the ARE activity induced by Nrf2 to

1.6-fold above the basal levels, whereas PEITC treatment reversed this decrease to 4.0-fold over the basal activity (Fig. 4C).

Next, we transfected a dominant-negative mutant form of JNK1 (JNK1-APF) and ERK2 (dnERK2) into PC-3 cells. As shown above, treatment with 7.5  $\mu\text{mol/L}$  PEITC for 12 hours induced ARE-luciferase activity by 5.4-fold. Transfection of dnERK2 or JNK1-APF into PC-3 cells decreased the ARE activity induced by PEITC to 2.9- and 3.5-fold, respectively (Fig. 4D).

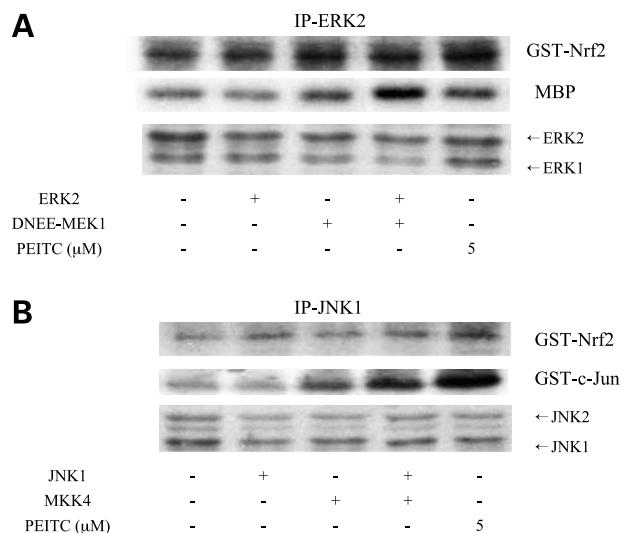
To corroborate the results that we obtained with the ARE reporter gene, we next examined the induction of endogenous HO-1 after transfection with DNEE-MEK1 + ERK2 or MKK4 + JNK1. Both ERK and JNK strongly increased the endogenous HO-1 expression (Fig. 4E). These results further supported the evidence that ERK and JNK signaling pathways play positive roles in the regulation of ARE activity and ARE-mediated HO-1 expression, without the challenge of any electrophilic compounds. Furthermore, treatment with PEITC seems to induce ERK and JNK signaling to activate ARE that can be blocked by its dominant-negative mutants in PC-3 cells.

#### ERK2 and JNK Phosphorylate GST-Nrf2 *In vitro*

From the results presented above, it seems that activated ERK1/2 and JNK1/2 signaling pathways can release Nrf2 from Keap1, causing the translocation of Nrf2 into the nucleus (Fig. 3), and subsequently increasing the transcription of ARE-containing genes in PC-3 cells (Fig. 4). Furthermore, we found that PEITC can activate the phosphorylation of ERK1/2 and JNK1/2 (Fig. 2D) and mimic the function of ERK and JNK signaling (Figs. 3D and 4C). Therefore, we next investigated the potential direct functional effect of activated ERK and JNK on Nrf2. After transfection of PC-3 cells with various plasmids, we immunoprecipitated ERK2 and JNK1 to perform *in vitro* kinase assays using purified GST-Nrf2 as substrate. We found that transfected ERK2 or JNK1 could phosphorylate GST-Nrf2 (Fig. 5A and B), whereas when GST protein alone was used as control, there was no phosphorylation using immunoprecipitated ERK or JNK (data not shown). After cotransfection with DNEE-MEK1 or treatment with PEITC, the phosphorylation level of GST-Nrf2 mediated by transfected ERK2 was increased (Fig. 5A). Cotransfection of MKK4 or treatment with PEITC also increased the phosphorylation of GST-Nrf2 mediated by transfected JNK1, although to a lesser extent than that phosphorylated by activated ERK2 (Fig. 5B).

## Discussion

In the present study, treatment of PC-3 cells with 2 to 10  $\mu\text{mol/L}$  PEITC increased ARE luciferase reporter gene activity (Fig. 1). There was no increase of ARE activity with 20  $\mu\text{mol/L}$  PEITC, likely due to the extreme toxic effects and loss of cell viability at this high dose level (Fig. 1). Our previous studies found that garlic organosulfur compounds (12) and isothiocyanates (32), such as allyl isothiocyanate, can potentially increase the accumulation of



**Figure 5.** *In vitro* phosphorylation of GST-Nrf2 mediated by ERK2 and JNK1. PC-3 cells were transiently transfected with DNEE-MEK1 and/or ERK2 (**A**), and MKK4 and/or JNK1 (**B**). Alternatively, cells were treated with 5  $\mu\text{mol/L}$  PEITC for 2 h without transfection. Whole-cell lysates were prepared and immunoprecipitated (IP) with antibodies against ERK2 (**A**) or JNK1 (**B**). *In vitro* kinase assays were done by using GST-Nrf2 as the substrate. MBP and GST-c-Jun (1–79) were used as positive control substrates for ERK2 and JNK1 kinases, respectively. Equal amounts of lysates were also examined by Western blot against ERK1/2 (**A**) and JNK1/2 (**B**) to determine total protein levels of the kinases (bottom row).

Nrf2 in human hepatoma HepG2 cells as well as increase the expression of HO-1 (32). In the current study, we found that PEITC also strongly induces Nrf2 accumulation and increases the expression of endogenous HO-1 protein in a dose-responsive and time-dependent manner (Fig. 2). It was surprising to find that the total Nrf2 levels were significantly elevated after PEITC treatment for as short as 30 minutes (Fig. 2A and B). This rapid accumulation of Nrf2 has been shown in our previous study on Nrf2 degradation, which showed that PEITC could inhibit Nrf2 degradation (12, 32). We are currently investigating whether this inhibitory effect of Nrf2 degradation elicited by PEITC is related to the activation of ERK and/or JNK.

We have previously observed that both ERK and JNK signaling pathways can stimulate Nrf2 transcriptional activation in HepG2 cells (20). In addition, phase II defensive enzymes induced by natural chemopreventive compounds, such as sulforaphane, are usually mediated by Nrf2 (12, 32). Thus, we would like to understand how PEITC induces ARE activity and increases Nrf2 accumulation, and what the roles of ERK and JNK signaling pathways are in the transcriptional activation of ARE-mediated gene expression. Treatment with PEITC dramatically increased the levels of phospho-ERK1/2 and phospho-JNK1/2 (Fig. 2D), and Nrf2 was released from its sequestration by Keap1 in the cytoplasm and was translocated into the nucleus (Fig. 3C). Transfection with ERK2 or JNK1 with its respective upstream kinase could also mimic the effects of PEITC, leading to Nrf2 translocation

into the nucleus (Fig. 3E and F). The phosphorylated ERK and JNK were mainly localized in the nucleus (Fig. 3E and F). These results suggest that the activated ERK and JNK signaling pathways could play a direct and important role in the activation of Nrf2, i.e., release from Keap1 cytoplasmic sequestration and nuclear import. It is interesting to find the colocalization of phosphorylated ERK and JNK with Nrf2 in the nucleus. However, this colocalization is not sufficient to conclude that ERK or JNK activates Nrf2 in the nucleus, or Nrf2 would need to translocate first into nucleus before being phosphorylated by ERK and JNK. More experiments are underway to elucidate this phenomenon.

In HepG2 cells, pyrrolidine dithiocarbamate induction of the human *glutamate cysteine ligase modulatory* gene is dependent on the activation of ERK, and pretreatment with ERK inhibitor PD98059 blocked the nuclear translocation of Nrf2 induced by pyrrolidine dithiocarbamate (47). In the current study, we found that activated (i.e., phosphorylated) ERK and JNK could colocalize in the nucleus with Nrf2 (Fig. 3E and F). We have also shown, for the first time, that immunoprecipitated ERK2 and JNK1 activated by PEITC-treated PC-3 cells could phosphorylate bacterially purified full-length GST-Nrf2 protein but not GST protein alone *in vitro*, which supported our findings that ERK and JNK signaling pathways could positively regulate Nrf2-mediated transactivation activity in HepG2 cells (20). We are currently performing additional experiments to confirm that, indeed, MAPK can phosphorylate Nrf2. Our preliminary studies thus far showed that (a) in two-dimensional gel, the Nrf2 protein was shifted after kinase assay with immunoprecipitated ERK; (b) we used liquid chromatography-tandem mass spectrometry to identify the phosphorylation sites on Nrf2 after the two-dimensional gel; and (c) we used deletion fragments of Nrf2 as well as site-directed mutations on Nrf2. Our preliminary study also showed that we could locate the MAPK phosphorylation sites on Nrf2. In addition, we have also done experiments using <sup>32</sup>P-orthophosphate labeling and found that Nrf2 can be phosphorylated in the cells (data not shown). Therefore, it is highly conceivable that MAPK can phosphorylate Nrf2 and that our functional data presented in this study pointed in that direction.

In summary, our data presented here show the activation of ERK and JNK by PEITC in PC-3 cells, the phosphorylation of Nrf2 by ERK and JNK, the release of Nrf2 from Keap1 cytoplasmic sequestration by ERK and JNK with the subsequent translocation of Nrf2 into the nucleus, and the induction of ARE transcriptional activity and endogenous HO-1 protein expression. This signaling pathway would likely contribute to the overall beneficial effects of isothiocyanates that are contained abundantly in cruciferous vegetables consumed in our every day diet, which could help to fight various cancers, including prostate cancer.

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