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# Differential Expression and Stability of Endogenous Nuclear Factor E2-related Factor 2 (Nrf2) by Natural Chemopreventive Compounds in HepG2 Human Hepatoma Cells

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**Nuclear factor-E2-related factor 2 (Nrf2) is known as a key regulator of ARE-mediated gene expression and the induction of Phase II detoxifying enzymes and antioxidant enzymes, which is also a common property of many chemopreventive agents. In the present study, we investigated the regulatory role of different chemopreventive agents including sulforaphane (SUL), allyl isothiocyanate (AITC), indole-3-carbinol (I3C), and parthenolide (PTL), in the expression and degradation of Nrf2 and the induction of the antioxidant enzyme HO-1. SUL strongly induced Nrf2 protein expression and ARE-mediated transcription activation, retarded degradation of Nrf2 through inhibiting Keap1, and thereby activating the transcriptional expression of HO-1. AITC was also a potent inducer of Nrf2 protein expression, ARE-reporter gene and HO-1 but had little effect on delaying the degradation of Nrf2 protein. Although PTL and I3C could induce ARE-reporter gene expression and Nrf2 to some extent, they were not as potent as SUL and AITC. However, PTL dramatically induced the HO-1 expression, which was comparable to SUL, while I3C had no effect. In addition, when treated with SUL and PTL, inhibition of proteasome by MG132 did not cause additional accumulation of Nrf2, suggesting the involvement of other degradation mechanism(s) in the presence of these compounds such as SUL and PTL. In summary, the results of our current study indicated that different chemopreventive compounds have different regulatory properties on the accumulation and degradation of Nrf2 as well as the induction of cellular antioxidant enzyme HO-1.**

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## Introduction

Exposure of cells to various stimuli such as oxidative stress, certain antioxidants and chemopreventive agents results in the induction of Phase II detoxifying and/or antioxidant enzymes such as glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase 1 (NQO1), and heme oxygenase 1 (HO-1) (Primiano *et al.*, 1997; Jaiswal, 2000; Kong *et al.*, 2001). Induction of these antioxidant enzymes is sought to be mediated by antioxidant or electrophile response element (ARE/EpRE), which is found at the 5-flanking region of the Phase II and antioxidant genes (Favreau and Pickett, 1991; Rushmore *et al.*, 1991; Alam *et al.*, 2000). The ARE-mediated gene expression and the resulting induction of the antioxidant enzymes has been implicated as a pivotal protection mechanism of cells/tissues against endogenous or exogenous carcinogenic intermediates (Kong *et al.*, 2001). Nuclear factor-E2-related factor 2 (Nrf2), a member of CNC family of bZIP proteins, is known to play a key role in the ARE-mediated gene expression. Over-expression of Nrf2 has been reported to up-regulate ARE-mediated reporter gene expression that was inducible by various antioxidants and chemopreventive agents such as beta-naphthoflavone, tert-butyl hydroquinone, and phenethyl isothiocyanate (Venugopal and Jaiswal, 1996; Keum *et al.*, 2003). The critical role of Nrf2 in the expression of antioxidant genes has been further confirmed by results from studies with *nrf2*-disrupted mice. The *nrf2*-dirupted mice have been found to have not only reduced levels of antioxidant enzymes but also more

susceptibility to carcinogens compared to wild-type mice (Itoh *et al.*, 1997; Ramos-Gomez *et al.*, 2001; Fahey *et al.*, 2002). Therefore, the regulation of Nrf2 is now of great interest. Recently, a cytoskeletal actin binding protein Keap1 (Kelch-like ECH-associated protein 1) or INrf2 (inhibitor of Nrf2) have been identified to play a key role in regulating Nrf2 (Itoh *et al.*, 1999; Dhakshinamoorthy and Jaiswal, 2001). Keap1 has been reported to involve in the liberation of Nrf2 from Keap1 as well as the cytoplasmic-nuclear shuttling and proteasomal degradation of Nrf2 (Itoh *et al.*, 2003; McMahon *et al.*, 2003; Zhang and Hannink, 2003). Treatments of cells with antioxidant and xenobiotics can lead to the release of Nrf2 from Keap1 (Jaiswal, 2004).

Many natural compounds have been found to be potent cancer chemopreventive agents in cell lines and experimental animal models (Yang *et al.*, 2002). Of great interest is the isothiocyanates and indole-3-carbinol found in cruciferous vegetables, and terpenoids including mono-, sesqui-, di-, and tri-terpenoids present in many plants. Despite of significant progress in the basic understanding of carcinogenesis, the molecular and cellular targets for effective chemoprevention by these compounds are poorly characterized, and are currently the subjects of intense investigation. Recent studies have reported the induction of Nrf2 by several chemopreventive compounds including curcumin, caffeic acid phenethyl ester, triterpenoid saponins avicins, sulforaphane (SUL) and its analogue 6-methylsulfinylhexyl isothiocyanate (Morimitsu *et al.*, 2002; Balogun *et al.*, 2003; McMahon *et al.*, 2003; Zhang & Hannink, 2003; Haridas *et al.*, 2004). However, how different classes of natural chemopreventive compounds differentially regulate the expression and stability of Nrf2 and how the regulation of Nrf2 by these compounds influences the expression of the antioxidant enzymes such as heme oxygenase 1 (HO-1) are largely unknown. In addition, the relationship between Nrf2 stability and Keap1 in the presence of chemopreventive compounds remains unclear.

In the present study, using different natural chemopreventive compounds including SUL, allyl isothiocyanate (AITC), indole-3-carbinol (I3C), and a sesquiterpene parthenolide (PTL), we have investigated the effect of these natural chemopreventive compounds on the expression and degradation of endogenous Nrf2 in the presence or absence of a protein synthesis inhibitor cycloheximide (CHX), protease inhibitor MG-132, or Keap1. In addition, the effect of different chemopreventive compounds on the induction of antioxidant enzyme, HO-1 was also studied. In this study, we show differential regulation of these chemopreventive compounds on the expression and stability of endogenous Nrf2 and HO-1.

## Materials and Methods

**Materials** Allyl isothiocyanate (AITC), indole-3-carbinol (I3C), parthenolide (PTL), and cycloheximide (CHX) were purchased from Sigma Chemicals Co. (St Louis, USA). Sulforaphane (SUL) was obtained from LKT Laboratories (St. Paul, USA). MG-132, a proteasome inhibitor, was from Calbiochem (San Diego, USA). Antibodies against Nrf2, HO-1, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Nrf2 protein standard was generated in our laboratory as follows; A high-level expression plasmid of human Nrf2 gene linked at its N terminus to a 6xHis tag was obtained by cloning the human Nrf2 cDNA (Gene Bank accession no. BC011558) into the pQE-30 vector (Qiagen, Chatsworth, USA) using *Bam* HI and *Kpn* I restriction sites. After expression in *Escherichia coli* M15 cells, Nrf2 protein was purified to near homogeneity by Ni-NTA metal chelate affinity chromatography essentially according to the manufacturer's protocols (Qiagen). All other reagents were of analytical grade and were purchased from commercial sources.

**Cell culture and treatments** HepG2 human hepatoma cells were obtained from American Type Culture Collections (Manassas, USA). Cells were maintained in modified F-12 Medium supplemented with 10% FBS, 1.17 g/L sodium bicarbonate, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% essential amino acids, and 0.1% insulin, in a humidified atmosphere of 95%, 5% $CO_2$ at 37°C. Cells were seeded in 6-well plates and allowed to grow for 24 h (about 80 to 90% confluency). Then, the cells were starved overnight with serum-free F-12 media prior to further treatments with either vehicle (DMSO, 0.1%) or chemopreventive compounds. For evaluation of Nrf2 expression, cells were treated with compounds for various time periods. For detection of Nrf2 stability, cells were incubated with either vehicle or compounds for 2 h. Then the media were replaced with media containing CHX (100 μg/ml), a protein synthesis inhibitor, and cells were harvested at various time periods. For assessment of accumulation of Nrf2 using a proteasome inhibitor MG-132, cells were treated with vehicle or compounds with MG-132 (10 μM) for various time periods.

**ARE-luciferase activity assay** HepG2-ARE-C8 (HepG2-C8) cell line was established in our laboratory by stable transfection of HepG2 cells with pARE-TI-luciferase reporter gene and previously described (Yu *et al.*, 1999). The HepG2-C8 cells were treated with compounds for 6, 12 or 24 h. The ARE-luciferase activity was determined using a luciferase kit from Promega (Madison, USA) according to the manufacturer's instructions. Briefly, after treatments, the cells were washed twice with ice-cold phosphate buffered-saline (pH 7.4) and harvested in 1× Reporter lysis buffer. After centrifugation at 13,000 rpm for 10 min, a 10-μl aliquot of the supernatant was assayed for luciferase activity with a SIRIUS luminometer (Berthold Detection System GmbH, Pforzheim, Germany). The luciferase activity was normalized against protein amount, determined by BCA protein assay (Pierce, Rockford, USA), and expressed as fold of induction over the luciferase activity of control vehicle-treated cells. Data were statistically analyzed by ANOVA, followed by unpaired Student's *t*-test.

**Transient transfection with Keap1** To determine the effect of Keap1 on the expression and stability of Nrf2 in the presence of chemopreventive compounds, HepG2 cells were transiently transfected with Ds-Red Keap1 prior to compound treatments. Preparation of Ds-Red Keap1 was previously described (Shen *et al.*,

2004). HepG2 cells were seeded in six-well plates for 24 h and transfected with either 3 μg empty vector (pcDNA3.1) or 50 ng Ds-Red Keap1 with 3 μg empty vector using Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. Briefly, plasmids were diluted with 150 μl OPTI-MEM and mixed with Lipofectamine 2000 reagents in 150 μl OPTI-MEM. The mixture was incubated for 20 min at room temperature to allow DNA-LF2000 reagent complexes to form. The culture media were removed from the cell culture plates and replaced with 2 ml of OPTI-MEM. Then, 300 ml of the DNA-LF2000 reagent mixture was added directly to each plate, mixed gently by rocking the plate, and incubated in a humidified atmosphere of 95%, 5% $CO_2$ at 37°C. After 4-5 hr incubation, the media were replaced with culture media containing 10% FBS. Treatments with compounds and CHX were performed as mentioned above in the Cell culture and treatments section.

**Western blot analysis** After treatments, cells were washed with ice-cold PBS (pH 7.4) and harvested with 200 μl of a whole cell lysis buffer (pH 7.4) containing 10 mM Tris-HCl, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovandate, 2 mM iodoacetic acid, 5 mM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton-X 100. Cell lysates were vigorously vortexed, homogenized in an ultrasonicator for 10 sec and left on ice for 30 min. The homogenates were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatants were collected and equal amounts of total protein of each sample, as determined by BCA protein assay (Pierce), were mixed with 4× loading buffer, and heated at 95°C for 5 min. The samples were then separated in a 10% criterion Tris-HCl precast gel at 200 V and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA) for 1.5 h using a semi-dry transfer system (Fisher). The membranes were blocked with 5% nonfat dry milk in 1× PBST buffer (0.1% Tween 20 in PBS) for 1 h at room temperature and incubated with anti-Nrf2 or anti-HO-1 antibodies in 3% nonfat dry milk (1:500 dilution) overnight at 4°C. After hybridization with primary antibody, membranes were washed three times with PBST, and then incubated with anti-rabbit (for Nrf2) or anti-goat (for HO-1 and actin) antibodies with horseradish peroxidase (Santa Cruz Biotechnology Inc.) for 1 h at room temperature and washed with PBST three times. Final detection was performed with enhanced chemiluminescence (ECL™) Western blotting reagents (Amersham Pharmacia Biotech, Piscataway, USA). Bands were visualized with BioRad ChemiDoc XRS system (Richmond, USA ) and quantitative data normalized against β-actin were obtained by densitometric analyses with the BioRad Quantity One software.

## Results

**Induction of ARE-luciferase activity by ITCs, I3C, and PTL** To evaluate the effect of chemopreventive compounds including ITCs, I3C, and PTL on the ARE-mediated gene expression, ARE-luciferase activity by these compounds was determined in HepG2-C8 cell line that was stably transfected with pARE-TI-luciferase reporter gene. The ARE-luciferase activity of treated group was expressed as a fold-induction over that of vehicle-treated control. As shown in Fig. 2, all compounds tested in this study induced the ARE-luciferase activity in HepG2-C8 cells. The fold induction of the ARE-luciferase activity was greatest by SUL treatment. SUL at 25 μM time-dependently induced the ARE-luciferase activity in the HepG2-C8 cells within 24 h and showed about 40-fold and 55-fold induction after 12 and 24 h incubations, respectively, which is consistent with previously reported results (Kim *et al.*, 2003). On the other hand, the induction of ARE-luciferase activity by AITC, I3C, and PTL was greater at 12 h than at 24 h.

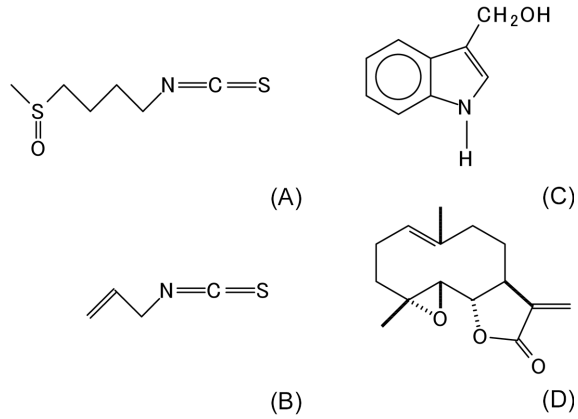


**Fig. 1.** Chemical structures of chemopreventive agents in the present study. A, SUL; B, AITC; C, I3C; D, PTL.

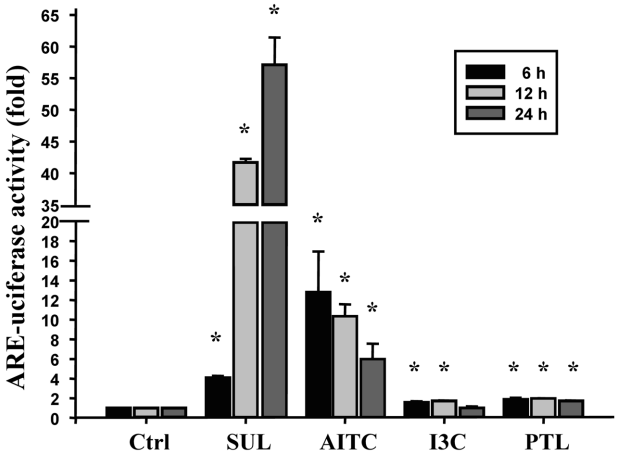


**Fig. 2.** ARE-luciferase activity by SUL, AITC, I3C, and PTL in HepG2-C8 cells. HepG2-C8 cells stably transfected with pARE-TI-luciferase reporter gene were treated with vehicle or chemopreventive agents (25 μM) for the indicated time periods. Luciferase activity was normalized with protein content and expressed as fold induction against vehicle-treated control. Values are means of three independent experiments ± S.D. (*) $p<0.05$ compared to the vehicle-treated control cells, using Student's *t*-test.

**Expression of Nrf2 by ITCs, I3C, and PTL** The chemopreventive compounds that induced ARE-luciferase

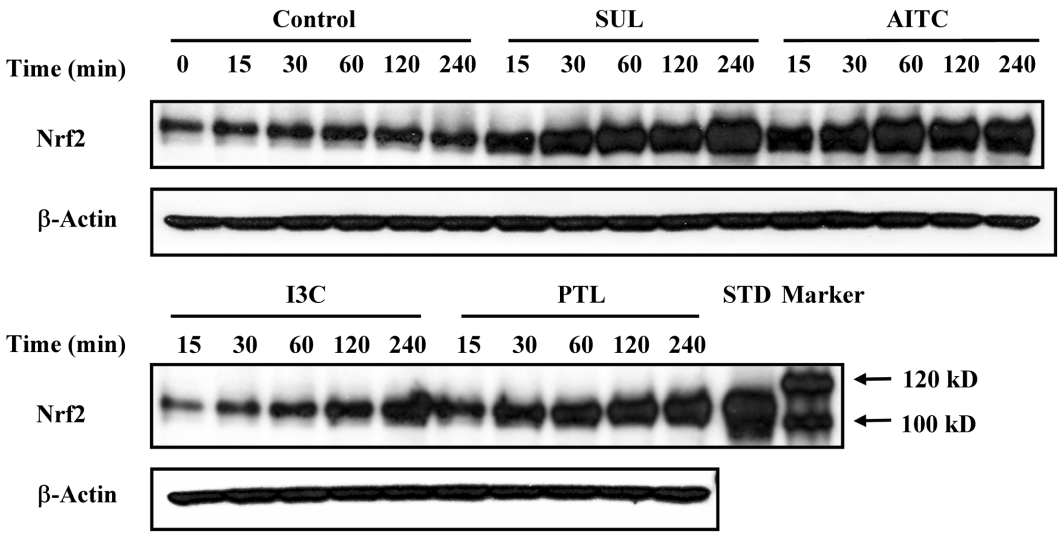


**Fig. 3.** Time dependent expression of Nrf2 by ITCs, I3C and PTL in HepG2 cells. HepG2 cells were treated with vehicle (DMSO, 0.1%) or different chemopreventive agent (25 μM) for the indicated time and then equal amount of proteins from whole cell lysates were analyzed for Nrf2 and β-actin by Western blotting. STD, Nrf2 standard as described in the Materials and methods. The data shown are representative of two independent experiments with similar results.

activity in Fig. 2 were further investigated for their effects on the endogenous expression level of Nrf2 protein that is known to bind to the ARE in the upstream regulatory region of many antioxidant genes. Fig. 3a shows time course (up to 4 h) expressions of Nrf2 protein by 25 μM treatment of chemopreventive compounds. As expected from the results of ARE-luciferase assays in Fig. 2, these compounds enhanced the expression of endogenous Nrf2 protein in HepG2 cells while the protein expression patterns during treatments were not identical between treatments. Treatments with 25 μM of SUL, AITC, and PTL caused a rapid induction of Nrf2 protein expression in 15 min. In murine keratinocyte PE cells, treatment with the synthetic chemopreventive agent D3T (3H-1,2-dithiole-3-thione) has been shown to induce Nrf2 within 20 min both in nuclei and total celluar homogenates (Kwak *et al.*, 2002). Within 4 h, treatments with SUL, I3C, and PTL time-dependently augmented the protein level whereas the Nrf2 expression by AITC treatment reached maximum at 60 min. As observed in ARE-luciferase assay results (Fig. 2), SUL was the most potent enhancer of Nrf2 protein expression showing about 3.3-fold induction at 4 h. Induction of the Nrf2 by AITC reached a plateau at 1 h with about 3-fold induction.

**Effect of chemopreventive compounds on Nrf2 degradation**

In addition to the expression of Nrf2 protein by chemopreventive compounds, their effect on the degradation of Nrf2 protein was evaluated using a protein synthesis inhibitor cycloheximide (CHX). HepG2 cells were treated with either vehicle or chemopreventive compounds for 2 h and washed out before challenged with 100 μg/ml of CHX. The half-life of Nrf2 protein has been recently shown to be about 13 min in unstimulated mouse Hepa cells (Stewart *et al.*, 2003). In the present study, we observed an extended half-life of Nrf2 (estimated to be about 45 min) in vehicle-treated human hepatoma HepG2 cells (Fig. 4a and 4b). Stimulated Nrf2 by 25 μM SUL exhibited more stability (half-life about 75 min) compared to the control Nrf2 after blocking protein synthesis with CHX. However, the degradation rate of the Nrf2 induced by other chemopreventive compounds including AITC, I3C, and PTL was similar to or even faster than that of vehicle-treated control. The reason for this is not clear but it might come from different effects of these compounds on Keap1, a cytoplasmic inhibitor of Nrf2, which will be discussed later in this paper. The stability of HO-1, a antioxidant enzyme, was not affected by CHX treatment during the given time period (data not shown).

Stimulation of Nrf2 expression by treatments of various selective inhibitors of 26 S proteasome such as MG-132, lactacystin, and PI has been reported in mouse hepatoma cells (Stewart *et al.*, 2003). Of these proteasome inhibitors, MG-132 has been found to display the most potent inhibition. In the current study, we examined the effect of chemopreventive compounds on Nrf2 expression in the presence of the proteasome inhibitor MG-132. Treatment of HepG2 cells with MG132 caused a dramatic accumulation of Nrf2 protein in a time-dependent manner (Fig. 5). Co-treatment with MG132 and AITC or I3C also strongly enhanced the accumulation of Nrf2. However, the Nrf2 accumulation by MG132 was more robust than the accumulation by co-treatments with chemopreventive compounds. In addition, the expression level of Nrf2 induced by SUL or PTL in the presence of MG132 was not dramatically increased during the treatment time periods. Therefore, the treatment with MG132 was not sufficient in blocking the degradation of Nrf2 that was induced by SUL or PTL. These results suggest that the dramatic increase in Nrf2 expression by MG132 is probably

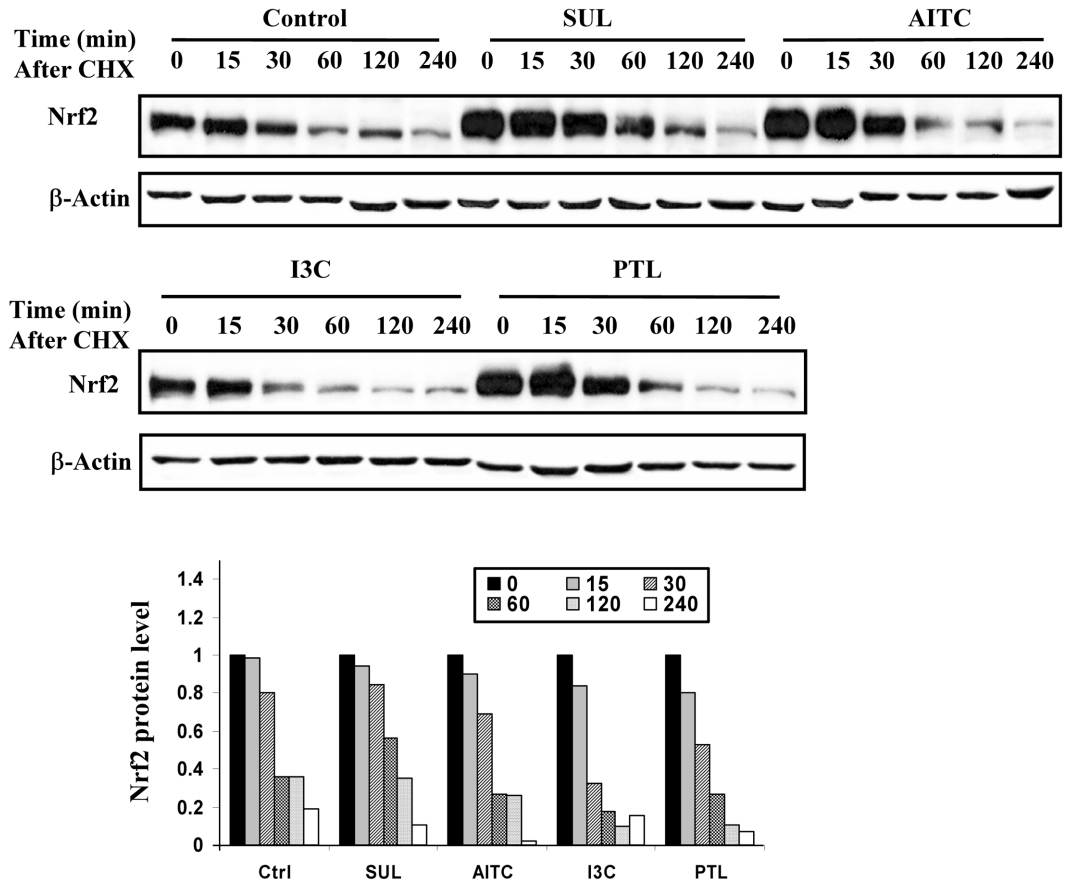


**Fig. 4.** Effects of chemopreventive agents (ITCs, I3C and PTL) on the time dependent degradation of Nrf2 in HepG2 cells. HepG2 cells were treated with vehicle (DMSO, 0.1%) or chemopreventive agents (25 μM) for 2 h and culture media were replaced with media containing CHX (100 μg/ml), followed by harvesting at the indicated times. Equal amount of proteins from whole cell lysates were analyzed for Nrf2 and β-actin by Western blotting. The results were quantitated by densitometry and the Nrf2 protein levels were plotted after normalization with actin. The blots shown are representative of two independent experiments with similar results.

dependent on the type of chemopreventive compounds and there might be other unknown mechanism(s) involved in the degradation of Nrf2 other than the 26S proteasome pathway when cells are exposed to these chemopreventive compounds.

**Effect of Keap1 on the expression and degradation of Nrf2 in the presence of chemopreventive compounds** Nrf2 is sequestered in the cytoplasm, associated with inhibitor protein, Keap1, which also has been reported to target Nrf2 for proteasome-mediated degradation (Itoh *et al.*, 1999; Itoh *et al.*, 2003; McMahon *et al.*, 2003; Zhang and Hannink, 2003). To investigate the role of Keap1 in expression and degradation of Nrf2 by chemopreventive compounds, HepG2 cells were transfected with expression vector for Keap1 prior to vehicle or compounds treatments and the Nrf2 protein levels were analyzed by immunoblot. Transfection with Keap1 expression vector resulted in a decrease in the endogenous Nrf2 expression compared to that with empty vector pcDNA3.1 during 8 h incubation (Fig. 6a). Overall expression levels of Nrf2 induced by chemopreventive compounds in the cells transfected with Keap1 seemed to be attenuated when compared to the result observed in untransfected cells (Compare Fig. 6a and Fig. 3). However, the expression pattern of Nrf2 by chemopreventive compounds in the Keap1 transfected cells was similar to those observed in untransfected cells. Effect of transfection with Keap1 on the degradation of Nrf2 is shown in Fig. 6b. Over-expression of Keap1 in HepG2 cells resulted in a rapid degradation of endogenous Nrf2 compared to the empty vector-transfected control group. Transfection with Keap1 caused about 70% degradation of endogenous Nrf2 in 30 min and almost complete degradation in 60 min. When treated with SUL, however, the degradation of Nrf2 in the HepG2 cells transfected with Keap1 was greatly delayed. The Nrf2 degradation at 60 min was about 50% by SUL treatment, which is close to the inhibition of Nrf2 degradation by SUL in the untransfected cells shown in Fig. 4. This result suggests a potent inhibitory activity of SUL against Keap1-mediated Nrf2 degradation. The enhanced degradation of Nrf2 by Keap1 was also delayed by AITC treatment. However, exposure of the cells to I3C and PTL was not sufficient to delay the enhanced Nrf2 degradation by Keap1.

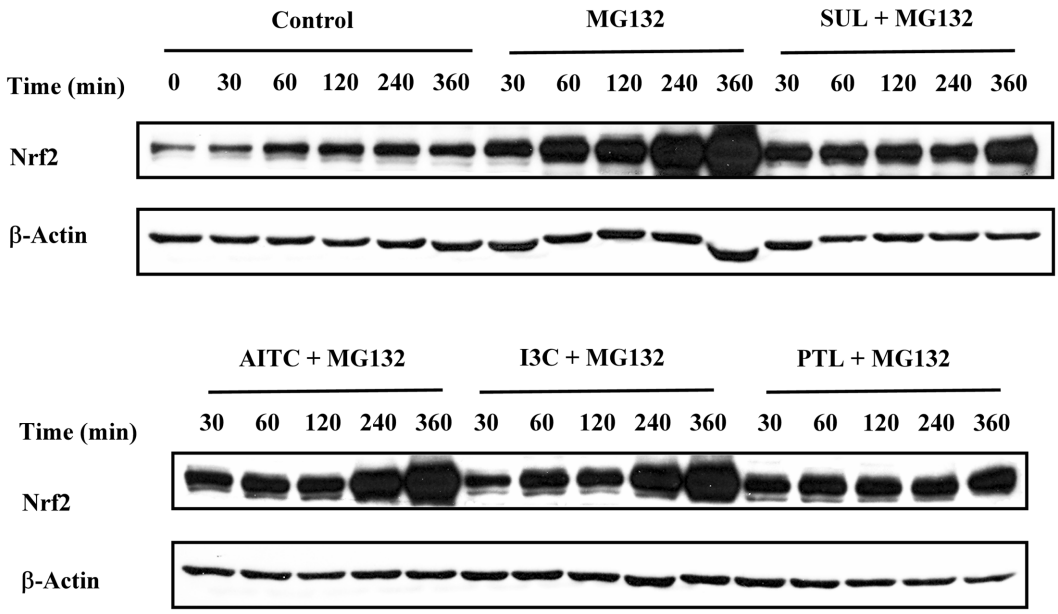


**Fig. 5.** Time-course accumulation of Nrf2 by chemopreventive agents (ITCs, I3C and PTL) in the presence of the proteasome inhibitor MG-132. HepG2 cells were treated with vehicle (DMSO, 0.1%) or chemopreventive agents (25 μM) with MG-132 (10 μM) at the indicated times and equal amount of proteins from whole cell lysates were analyzed for Nrf2 and β-actin by Western blotting. The blots shown are representative of two independent experiments with similar results.

**Expression of HO-1 by ITCs, I3C, and PTL** We further investigated the time-course effect of different chemopreventive compounds on the expression of endogenous HO-1 protein, a antioxidant enzyme that has been known to be under ARE regulation. HO-1 catalyzes degradation of heme to CO, iron and biliverdin (Balogun *et al.*, 2003). It has been implicated to play a protective role in various disease processes such as inflammation, atherosclerosis, and neurodegenerosis (Alcaraz *et al.*, 2003; Durante, 2003). Since most of the tested compounds enhanced the expression of HO-1 protein within 4 h (data not shown) at the given concentration, we extended the treatment time to 12 h and the results are listed in Fig. 7. Induction of HO-1 protein expression was initiated at 8 h after treatments of HepG2 cells with SUL and AITC, and at 4 h with PTL but not with I3C. A strong induction in HO-1 was observed with treatments of SUL (~20 fold) at 12 h, AITC (~6 fold) at 8 and 12 h, and PTL (~20 fold) at 8 and 12 h. The most potent induction of HO-1 occurred 4 h after the strongest induction of Nrf2 by these three compounds. The accumulation of HO-1 protein was time-dependently increased up to 12 h by SUL treatment whereas the protein level by AITC and PTL treatments slightly decreased after the maximum induction at 8 h.

## Discussion

One of the most prominent strategies of cancer chemoprevention might be protecting cells/tissues against various carcinogens and carcinogenic metabolites that could come from exogenous and/or endogenous sources. This could be achieved from induction of antioxidant enzymes such as GST, NQO1 and HO-1, which is mediated in part by ARE in the promoter region of the genes (Kong *et al.*, 2001). Our lab has previously shown transcription activation of ARE-mediated reporter gene through activation of Nrf2 with natural chemopreventive agents such as EGCG, ECG, PEITC, and SUL (Chen *et al.*, 2000; Keum *et al.*, 2003; Kim *et al.*, 2003). Recent studies by other laboratories have also reported the induction of Nrf2 by several chemopreventive compounds including curcumin, caffeic acid phenethyl ester, triterpenoid avicins, SUL and 6-methylsulfinylhexyl isothiocyanate (Morimitsu *et al.*, 2002; Balogun *et al.*, 2003; McMahon *et al.*, 2003; Zhang & Hannink, 2003; Haridas *et al.*, 2004).

In the present study, we have investigated time-dependent regulation of SUL, AITC, I3C and PTL on the induction and degradation of Nrf2 as well as their effects on HO-1 expression. Chemopreventive properties of isothiocyanates and I3C are found elsewhere (Talalay & Fahey, 2001). SUL has been recently reported for its role in the regulation of Nrf2 and antioxidant gene expression (Wu and Juurlink, 2001; Zhang and Hannink, 2003), but the modulatory properties of AITC, I3C and PTL on Nrf2 and HO-1 have been scarcely studied. A sesquiterpene lactone parthenolide, which is found in feverfew (*Tanacetum parthenium*), has been known to have potent anti-inflammatory properties by blocking pro-inflammatory cytokines through inhibiting NF-κB and STATs signaling pathways (Zhang *et al.*, 2004). However, studies on the chemopreventive properties of parthenolide are limited and its role in Nrf2 and antioxidant gene expression has never

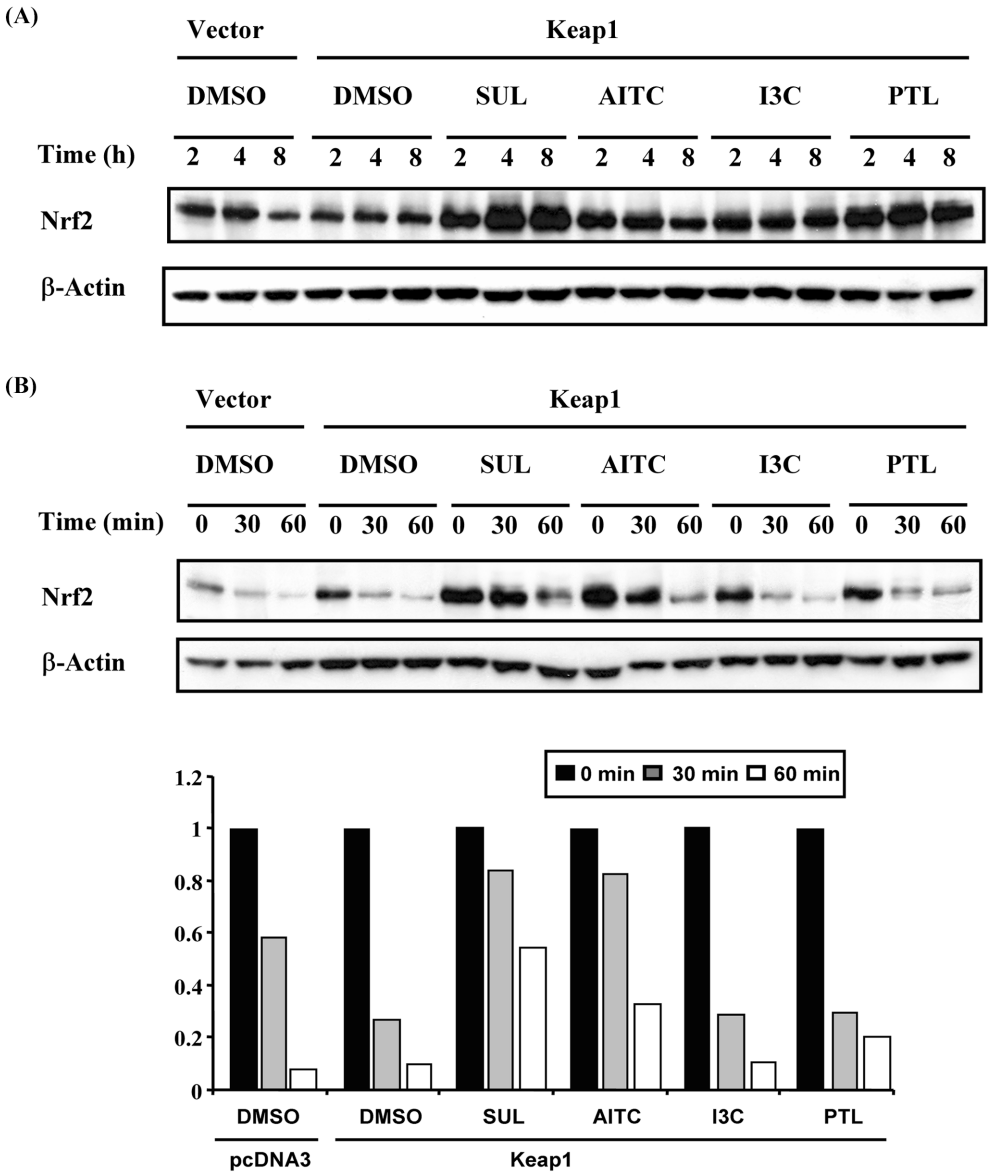


**Fig. 6.** Effect of Keap1 overexpression on the expression and degradation of Nrf2 by ITCs, I3C and PTL. (A) After transfection of HepG2 cells with vector (pcDNA3.1) or Ds-red Keap1 for 24 h, the cells were treated with vehicle (DMSO, 0.1%) or different chemopreventive agent (25 μM) for the indicated time and then equal amount of proteins from whole cell lysates were analyzed for Nrf2 and β-actin by Western blotting. (B) After transfection of HepG2 cells with vector (pcDNA3.1) or Ds-red Keap1 for 24 h, the cells were treated with vehicle (DMSO, 0.1%) or chemopreventive agents (25 μM) for 2 h and culture media were replaced with media containing CHX (100 μg/ml), followed by harvesting at the indicated times. Equal amount of proteins from whole cell lysates were analyzed for Nrf2 and β-actin by Western blotting. The results were quantitated by densitometry and the Nrf2 protein levels were plotted after normalization with actin. The blots shown are representative of two independent experiments with similar results.

been reported.

Recent studies have demonstrated that inducers of Nrf2-dependent transcription inhibit Keap1-dependent Nrf2 degradation mediated by proteasome pathway (Kwak *et al.*, 2002; Nguyen *et al.*, 2003; Stewart *et al.*, 2003; Zhang & Hannink, 2003). In mouse peritoneal macrophage cells, an electrophile diethylmaleate has been shown to facilitate nuclear accumulation of Nrf2 by suppressing Keap1 activity (Itoh *et al.*, 2003). Treatment with D3T in murine keratinocyte PE cells has been reported to increase Nrf2 much greater in nuclear than in cytosol, and the rapid accumulation of Nrf2 within nuclei by D3T has been implicated to upregulate its own expression, leading to a more sustained signaling of antioxidant gene expression (Kwak *et al.*, 2002). However, other study has shown that Nrf2 remains largely cytoplasmic after treatment with SUL or tBHQ in COS1 cells cotransfected with Nrf2 and Keap1 (Zhang and Hannink, 2003). The authors have also indicated that Nrf2 is not

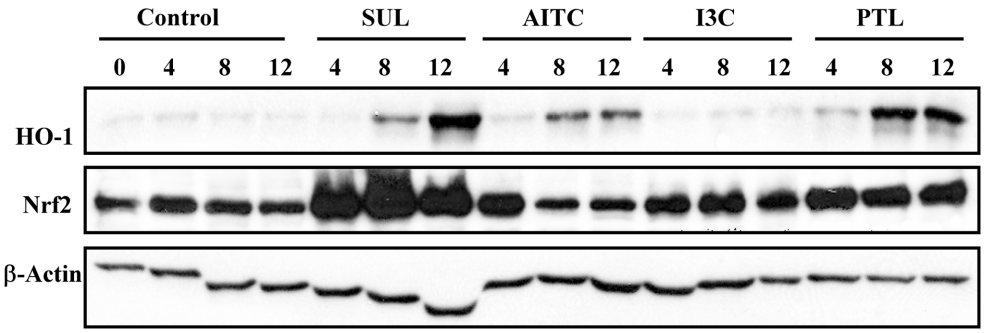


**Fig. 7.** Time-course expression of HO-1 and Nrf2 by ITCs, I3C and PTL. HepG2 cells were treated with vehicle (DMSO, 0.1%) or different chemopreventive agent (25 μM) for the indicated time and then equal amount of proteins from whole cell lysates were analyzed for HO-1, Nrf2 and β-actin by Western blotting. The blots shown are representative of two independent experiments with similar results.

quantitatively released from Keap1 by inducers of Nrf2-dependent transcription, regardless of the inhibition of Keap1-dependent degradation by these compounds. Therefore, activation and degradation mechanism(s) of Nrf2 induced by chemopreventive compounds might be specific for compounds as well as cell types. In the present study, we observed differential induction and degradation mechanism(s) of Nrf2 by different chemopreventive agents. Although all the chemopreventive compounds in the current study were able to time-dependently induce Nrf2, their effects on the degradation and accumulation mechanisms of Nrf2 seemed to be distinct. For example, only SUL was able to prolong the half-life of Nrf2 degradation and other compounds such as AITC, I3C, and PTL displayed similar or even faster degradation of Nrf2 compared to vehicle treatment. Electrophiles such as diethylmaleate and heavy metals including $CdCl_2$ have been shown to delay the rate of Nrf2 degradation by repressing the effect of Keap1 leading to inhibition of proteasome-mediated degradation of Nrf2 (Itoh *et al.*, 2003; Stewart *et al.*, 2003). It has also been reported that co-treatment with diethylmaleate and proteasome inhibitors does not show additive effect on the accumulation of Nrf2, suggesting that the compound stabilize Nrf2 protein by displacing it from proteasome-mediated degradation (Itoh *et al.*, 2003). In our study, co-treatment with chemopreventive compounds with proteasome inhibitor MG-132 did not show any additive effect on Nrf2 accumulation. Interestingly, SUL and PTL seemed to diminish the effect of MG-132. The reason for this is not clear, but it might be a drug-drug interaction or a proteasome-independent degradation pathway of Nrf2 by these compounds.

Although in most case the induction of Nrf2 seems to be essential for the induction of the known Nrf2 targeted antioxidant genes, we observe that the extent of the induced Nrf2 by chemopreventive agents does not always correlate with that of the Nrf2-targeted antioxidant genes. I3C, which was able to induce Nrf2, hardly induced HO-1 protein. In addition, a marked induction of HO-1 was seen by treatment with PTL, which had less potency in the induction of ARE-reporter gene expression as well as endogenous Nrf2 than ITCs. Recent studies have shown the induction of apoptosis by PTL through depleting intracellular thiols and increasing calcium levels (Wen *et al.*, 2002; Zhang *et al.*, 2004). Therefore, besides the ARE-mediated gene expression pathway, other mechanism(s) might be involved in the induction of the antioxidant enzyme expressions by certain chemopreventive compounds. In our study, the initiation of HO-1 induction was detected at 8 h after treatments with ITCs and PTL and the strongest induction of HO-1 in each treatment occurred about 4 h after the maximum induction of Nrf2. Accumulation of HO-1 protein has been reported after 2 h exposure to $CdCl_2$ in mouse hepatoma (Hepa) cells (Stewart *et al.*, 2003), suggesting either a delayed activation of antioxidant gene expression by stimuli in human cells than in mouse cells or a more complex mechanism(s) of HO-1 induction by chemopreventive agents. Haridas et al have observed a delayed induction of HO-1 (at 8 and 16 h) in HepG2 cells by a triterpenoid avicin D compared with other ARE-regulated enzymes (Haridas *et al.*, 2004). The authors have suggested that this delay might come from the net effects of transcription factors, which can bind to the binding sites in the promoter region of the HO-1 gene such as NF-kB, AP-1, AP-2, and IL-6 response element (Haridas *et al.*, 2004).

Taken together, our present study suggests that different chemopreventive compounds have different regulatory properties in the induction and degradation of Nrf2 as well as the antioxidant enzyme HO-1. SUL strongly induces Nrf2 expression and ARE-mediated transcription activation, retards degradation of Nrf2 through inhibiting Keap1, and thereby activates the expression of HO-1. AITC is also a potent inducer of Nrf2, ARE-reporter gene and HO-1 but is not sufficient enough to delay the degradation of Nrf2. Although PTL and I3C could induce ARE-reporter gene expression and Nrf2 to some extent, they were not as potent as SUL and AITC. However, PTL dramatically induced the HO-1 expression, which was as potent as SUL, while I3C had no effect. In addition, when treated with SUL and PTL, inhibiting proteasome by MG132 did not cause additional accumulation of Nrf2, suggesting involvement of other degradation

mechanism(s) in the presence of these compounds. Several kinases such as MAPKs, PKC, PI3K, and PERK have been implicated to involve in the activation of Nrf2 and ARE-mediated transcription through Nrf2 (Huang *et al.*, 2000; Yu *et al.*, 2000; Cullinan *et al.*, 2003; Keum *et al.*, 2003; Nakaso *et al.*, 2003). Recently, our laboratory has demonstrated the up-regulation of Nrf2 transactivation domain by ERK and JNK pathways through coactivator CBP (Shen *et al.*, 2004). Therefore, further studies on the upstream signaling events involved in the regulation of Nrf2 and the antioxidant enzymes by these chemopreventive compounds are needed to elucidate differential role of these compounds in the different chemoprevention mechanisms.

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