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Research Paper

Structural Influence of Isothiocyanates on the Antioxidant Response Element (ARE)-Mediated Heme Oxygenase-1 (HO-1) Expression

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Purpose. Isothiocyanates (ITCs), existing abundantly in cruciferous vegetables, is one class of promising dietary cancer chemopreventive agents that possess strong cancer protective effects by modulation of phase II detoxifying/antioxidant enzyme activities. However, limited studies regarding to the structure-activity relationship (SAR) of ITCs on the induction of phase II detoxifying/antioxidant enzymes are reported. In this study, the effects of ten structurally related isothiocyanates on the antioxidant response element (ARE)-mediated antioxidant enzyme heme oxygenase-1 (HO-1) induction in human hepatoma HepG2-C8 cells were evaluated.

Materials and Methods. After exposure of HepG2-C8 cells to ITCs, cell viability, luciferase reporter assay, Western blot analysis and quantitative real-time PCR were conducted.

Results. Treatments with most ITCs significantly activated ARE-mediated luciferase activity with different maximal degree of ARE induction. In addition, ITCs caused a substantial induction of HO-1 protein, which was closely correlated with inductive level of Nrf2 protein. Real-time PCR revealed that the expression of HO-1 mRNA and protein was significantly increased after treatments with ITCs, although not directly correlated. HO-1 induction by ITCs was attenuated in HepG2-C8 cells transiently transfected with a dominant negative mutant of Nrf2 (Nrf2-M4), whereas it was totally absent in *Nrf2*$^{-/-}$ mouse embryonic fibroblasts. In addition, ARE activation by ITCs was associated with the depletion of intracellular glutathione.

Conclusion. Collectively, our results demonstrate that the ITC class of compounds activates ARE-mediated HO-1 gene transcription through Nrf2/ARE signaling pathway, however, their inductive effects are quite specific, depending on the chemical structure. These results suggest the possibility that some synthetic ITCs might have superior chemopreventive activity than natural ITCs.

KEY WORDS: heme oxygenase-1 (HO-1); isothiocyanates (ITCs); Nrf2; phase II drug metabolism; structure-activity relationship.

INTRODUCTION

The cancer protective effects of daily-consumed dietary compounds against chemical-induced carcinogenesis and genetically derived tumor models have been repeatedly reported in many epidemiological or population studies as well as experimental models (1–4). Based on the relatively nontoxic properties, many dietary chemopreventive agents derived from human diet have gained much attention recently. It is well recognized that the induction of phase II detoxifying/antioxidant enzymes provides significant biological mechanisms for the protection against toxic effects of endogenous reactive oxygen species (ROS), exogenous carcinogens and/or their reactive intermediates (5). Therefore, identification of new and more potent cancer chemopreventive agents which can enhance cellular defense system via the induction of detoxifying/antioxidant enzymes has become an area of great interest.

Isothiocyanates (ITCs), existing abundantly in cruciferous vegetables, is one type of promising dietary chemopreventive agents that possess strong cancer chemoprotective effects, both

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ABBREVIATIONS: ARE, antioxidant response element; HO-1, heme oxygenase-1; ITCs, isothiocyanates; ITC-1, 2-methoxyethyl isothiocyanate; ITC-2, 3-methoxypropyl isothiocyanate; ITC-3, furfuryl isothiocyanate; ITC-4, tetrahydrofurfuryl isothiocyanate; ITC-5, methyl-3-isothiocyanatopropionate; ITC-6, 3-(diethylamino)propyl-isothiocyanate; ITC-7, 2-(4-morpholino)ethyl isothiocyanate; ITC-8, 3-(4-morpholino)propyl isothiocyanate; ITC-9, 4-cyanophenyl isothiocyanate; ITC-10, 3,4-methyelenedioxybenzyl isothiocyanate; Keap1, kelch-like ECH-associated protein 1; Nrf2, Nuclear E2-factor related factor 2; SAR, structure-activity relationship; SFN, sulforaphane.

in vivo and *in vitro*. This ITC class of compounds is noticeable for the beneficial modulation of detoxifying/antioxidant enzymes, and therefore ITCs class of compounds appear to possess strong anti-oxidative stress effects. Accumulating evidence indicates that coordinated induction of phase II detoxifying/antioxidant enzymes is under the transcriptional control of a key transcription factor nuclear E2-factor related factor 2 (Nrf2) and the *cis*-acting element called the antioxidant response element (ARE), located in the promoter region of those genes. Briefly, the molecular events of ARE activation include dissociation of Nrf2 from a cytosolic repressor Kelch-like ECH-associated protein 1 (Keap1), nuclear translocation of Nrf2, heterodimerization with small Maf (sMaf) protein, binding of Nrf2/sMaf dimer to ARE and initiation of the transcription of ARE-driven genes. Because ITCs can react rapidly but reversibly with thiols, thus, modulation of cysteine thiols of Keap1 and/or coupled with activation of signaling kinases by ITCs are believed to be some of the important events triggering the release of Nrf2 from the Nrf2/Keap1 complex and mediating up-regulation of ARE-mediated gene (6–8).

To date, several naturally occurring ITCs such as sulforaphane (SFN; 4-methylsulfinylbutyl isothiocyanate), phenethyl isothiocyanate (PEITC), benzyl isothiocyanate (BITC; 9,10), have been shown to have beneficial bioactive effects. In addition, their effects appear to be quite specific, depending on the chemical structure of the ITCs, carcinogens and the experimental systems (9). However, more effective and more potent isothiocyanate(s) for future cancer intervention study would clearly be needed to enhance the chances of success. Therefore, better understanding of the structural relationship of ITCs on Nrf2/ARE-dependent gene expression would be valuable basic knowledge to optimize cancer-protective effect of ITCs. SFN, one of the most commonly investigated natural occurring ITCs, which is particularly abundant in Broccoli, has been demonstrated to exert the cancer protective effects by inducing ARE-driven phase II detoxifying/antioxidant enzyme expression through activation of Nrf2 (11) and also modulating apoptosis and cell cycle arrest (12–14). In our present study, we investigated ten structurally related synthetic ITCs for their biological activities to induce ARE-mediated luciferase reporter gene as well as endogenous HO-1 expression. For comparison purposes, SFN was used as a positive standard, to verify whether the inductive effects are mediated through the induction of Nrf2 protein itself, and/or involved the intracellular glutathione (GSH) level.

MATERIALS AND METHODS

Chemicals and Antibodies

Isothiocyanate compounds used in this study were purchased from Lancaster Synthesis Inc. (Pelham, NH). The purities of these ITCs (2-methoxyethyl isothiocyanate (CAS: 38663-85-3); 3-methoxypropyl isothiocyanate (CAS: 17702-11-3); furfuryl isothiocyanate (CAS: 4650-60-6); tetrahydrofurfuryl isothiocyanate (CAS: 36810-87-4); methyl-3-isothiocyanatopropionate (CAS: 18967-35-6); 3-(diethylamino)propyl-isothiocyanate (CAS: 2626-52-0); 2-(4-morpholino)ethyl isothiocyanate (CAS: 63224-35-1); 3-(4-morpholino)propyl isothiocyanate (CAS: 32813-50-6); 4-cyanophenyl isothiocyanate (CAS: 2719-32-6); 3,4-methyelenedioxybenzyl isothiocyanate (CAS: 4430-47-1)) were more than 96% purity. Sulforaphane was from LKT laboratories (St. Paul, MN). Luciferase assay system and CellTiter 96 aqueous non-radioactive cell proliferation assay were from Promega (Madison, WI). Antibodies against HO-1, Nrf2, β-actin, and GADPH were from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). TRIzol and SuperScript III first-strand cDNA synthesis system were purchased from Invitrogen (Carlsbad, CA).

Cell Culture

HepG2-C8 cell generated in our laboratory by stable transfection of ARE-luciferase construct (15) were cultured and maintained in modified F-12 medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids, and 0.1% insulin at 37°C in humidified incubator with 5% CO_2. Cells were allowed to grow up for 24 h to reach 70% confluency before replacing with modified F-12 medium containing 0.5% FBS for 12 h prior to treatment with isothiocyanate compounds.

Preparation of mouse embryonic fibroblasts from wild-type ($Nrf2^{+/+}$) and *Nrf2* knockout ($Nrf2^{-/-}$) C57BL/6J mice were conducted as described previously (16). Cells were cultured and maintained at 37°C and 5% CO_2 in MEM medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. In all experiments, ethanol was used as a vehicle and the final concentration of ethanol in control vehicle-treated cells was 0.1%.

Transient Transfection of Mutant Nrf2

Mutant Nrf2 DNA (Nrf2-M4) construct, encoding Nrf2 protein (amino acids 401–589) that contains DNA binding domain but lacks transactivation domain, was generated in our laboratory by cloning into mammalian expression pHM6 vector (Invitrogen; 16). HepG2-C8 cells were plated in six-well plates and transfected, according to the manufacturer's instructions, with 4 μg of Nrf2-M4 using Lipofectamine 2000 (Invitrogen). After transfection, cells were cultured for an additional 24 h with fresh medium, treated with ITCs and further harvested for Western blot analysis.

Cell Viability (MTS Assay)

HepG2-C8 cells were cultured in 96-well plate and treated with different isothiocyanate compounds at vary concentrations for additional 24 h. Cell viability was determined with the CellTiter 96 aqueous non-radioactive cell proliferation assay according to the manufacturer's instructions. Briefly, cells were treated with combined solution of a tetrazolium compound, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt], and an electron coupling reagent, phenazine methosulfate (PMS), for 2 h at 37°C. The absorbance of the formazan product at 490 nm was measured directly with a μQuant Biomolecular Spectrophotometer from Bio-Tek Instruments Inc. (Winooski, VT).

Luciferase Reporter Assay

The ARE-luciferase activities in HepG2-C8 cells were determined using a luciferase assay system according to the

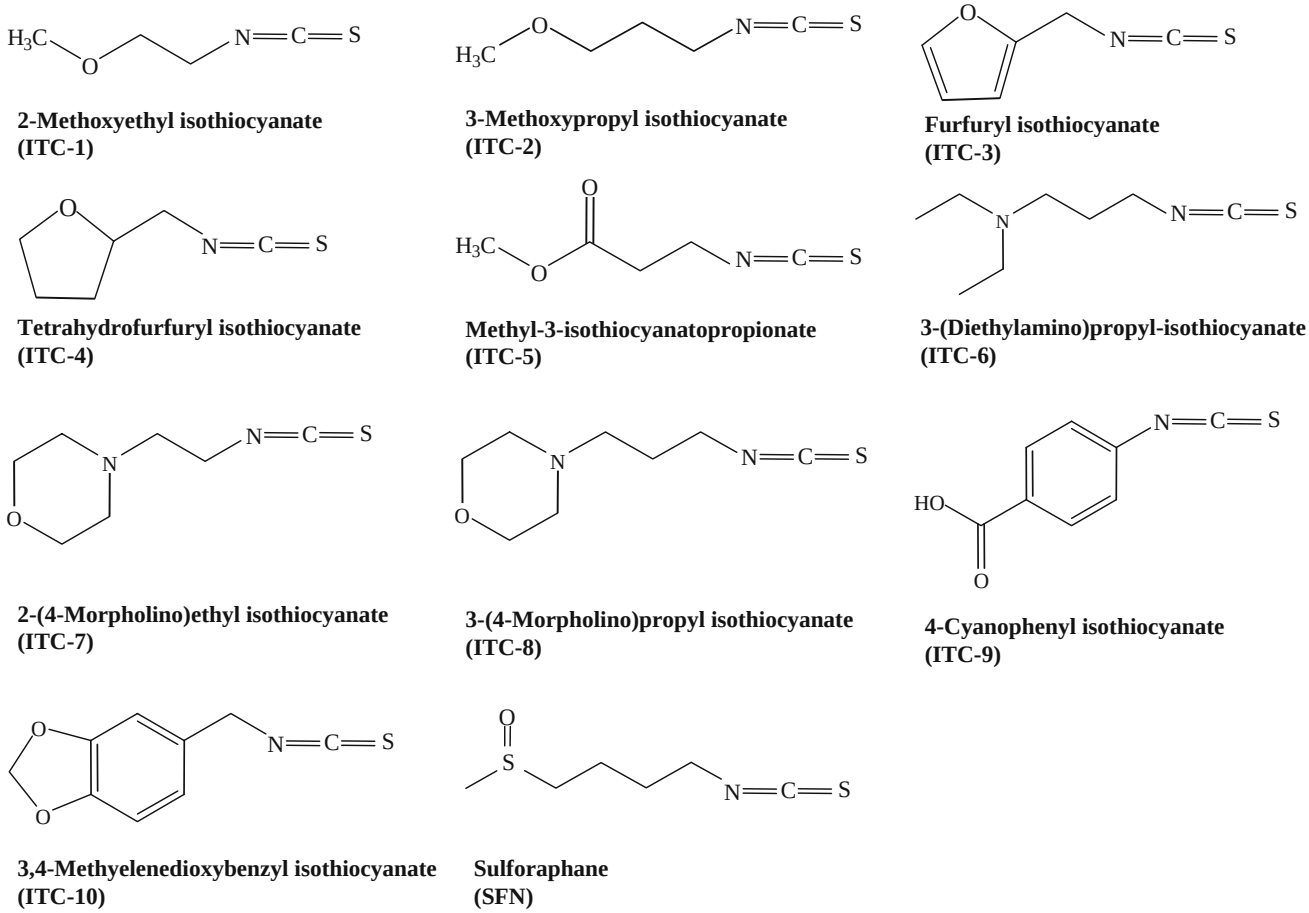

Fig. 1. Chemical structures of isothiocyanate compounds used in this study.

manufacturer's instructions. Briefly, after treatment, cells in six-well plates were washed twice with ice-cold phosphate-buffered saline (PBS; pH 7.4) and were lysed by adding 400 µl of 1× reporter lysis buffer (Promega). After centrifugation at 12,000×*g* for 15 s at room temperature, 10 µl-aliquot of supernatant was analyzed for luciferase activity by reading with a SIRIUS luminometer (Berthold Detection System, Germany). Normalization of the luciferase activity was done based on the protein concentration, which was determined by using BCA protein assay kit from Pierce Biotechnology (Rockford, IL). The luciferase activity was then expressed as fold of induction over the activity of control vehicle-treated cells (0.1% ethanol).

RNA Extraction and Quantitative Real-Time PCR

HO-1 mRNA expression was evaluated by quantitative real-time Polymerase Chain Reaction (real-time PCR). Total RNA from HepG2-C8 cells were isolated by using a method of

Table I. Comparative Effect of Individual Isothiocyanate Compound on the Cell Viability (MTS) Assay

	Concentration in µM (% Cell Viability)					
	1 µM	2 µM	5 µM	10 µM	15 µM	20 µM
Sulforaphane	110.20±2.82	127.15±20.21	131.78±25.00	108.50±13.87	112.60±12.92	79.04±5.96
ITC-1	114.89±7.16	122.23±1.57	123.05±16.31	106.02±8.86	94.38±9.23	102.54±10.88
ITC-2	119.32±4.07	121.24±1.21	124.60±5.33	118.76±21.77	104.41±6.49	102.60±6.50
ITC-3	116.16±2.26	122.54±3.23	128.73±4.93	128.64±6.54	127.29±10.54	115.28±6.70
ITC-4	118.84±4.75	116.47±6.53	108.73±11.93	101.89±10.15	92.46±4.04	82.09±7.77
ITC-5	117.29±16.60	119.44±16.20	125.65±19.11	116.53±15.98	117.26±4.10	98.05±18.88
ITC-6	119.86±8.22	132.26±3.01	147.43±5.62	130.62±0.43	134.27±3.94	95.03±5.48
ITC-7	121.16±8.46	138.42±2.16	150.62±2.68	147.66±0.93	143.02±5.32	104.18±6.02
ITC-8	110.54±8.79	120.65±15.63	120.71±19.76	113.08±18.65	96.44±17.45	75.68±11.01
ITC-9	114.58±16.75	122.23±22.82	120.56±16.37	104.83±26.00	100.48±19.31	93.16±11.87
ITC-10	111.53±7.02	128.11±8.01	123.59±7.37	93.31±9.33	87.54±10.17	57.09±8.62*

Each point represents the mean±SD of three independent assays
*$p<0.01$ *versus* control

Table II. Concentration-Dependent Induction of ARE-Luciferase Activity by Individual Isothiocyanate Compounds

	Concentration in μM (Fold Induction Compared Control)						EC50 (μM)[a]
	1 μM	2 μM	5 μM	10 μM	15 μM	20 μM	
Sulforaphane	1.27±0.05	1.88±0.58	4.76±0.86*	6.32±1.22*	15.31±2.96*	20.46±0.45*	12.69±0.68
ITC-1	3.05±0.38**	4.65±0.81*	5.18±1.95*	6.96±0.95*	12.93±3.37*	15.40±1.62*	11.77±0.35
ITC-2	1.66±0.14	1.37±0.15	1.81±0.42	3.37±0.08*	3.10±0.20*	5.30±2.18*	10.02±3.20
ITC-3	1.24±0.19	1.38±0.27	1.28±0.38	2.42±0.61*	2.00±0.80**	2.02±0.23**	8.64±1.61
ITC-4	2.38±0.09	2.34±0.23	4.11±0.44*	9.20±2.73*	12.81±0.03*	14.60±0.66*	9.64±2.23
ITC-5	1.93±1.09	1.83±0.31	2.91±0.35*	2.97±0.79*	4.75±0.08*	10.28±1.42*	15.96±0.19
ITC-6	2.12±0.53**	1.87±0.05	2.47±0.12*	5.73±0.26*	4.62±1.18*	8.51±0.05*	9.15±0.26
ITC-7	1.39±0.01	1.46±0.04	1.73±0.08**	2.50±0.02*	2.44±0.99*	3.28±0.20*	8.95±0.65
ITC-8	1.84±0.23	3.22±0.83	4.60±1.13**	9.29±0.03*	17.76±0.47*	34.35±3.97*	14.85±0.98
ITC-9	1.31±0.19	1.29±0.38	0.78±0.88	2.64±0.34*	2.65±0.33*	3.63±0.32*	8.70±0.09
ITC-10	1.47±0.24	2.33±0.34*	5.00±1.11*	24.59±0.63*	0.87±0.00	1.16±0.01	6.97±0.19

Each point represents the mean±SD of three independent assays. HepG2-C8 cells were treated with different concentrations of ITCs for 24 h, then the luciferase activity was measured and normalized against the respective protein concentrations. The luciferase activity in control HepG2-C8 cells (0.1% ethanol) was set arbitrarily at 1.

[a] EC50 (μM) of individual compounds in the ARE-luciferase assay was calculated from a concentration response tested with each isothiocyanate.

*$p<0.01$ *versus* control

**$p<0.05$ *versus* control

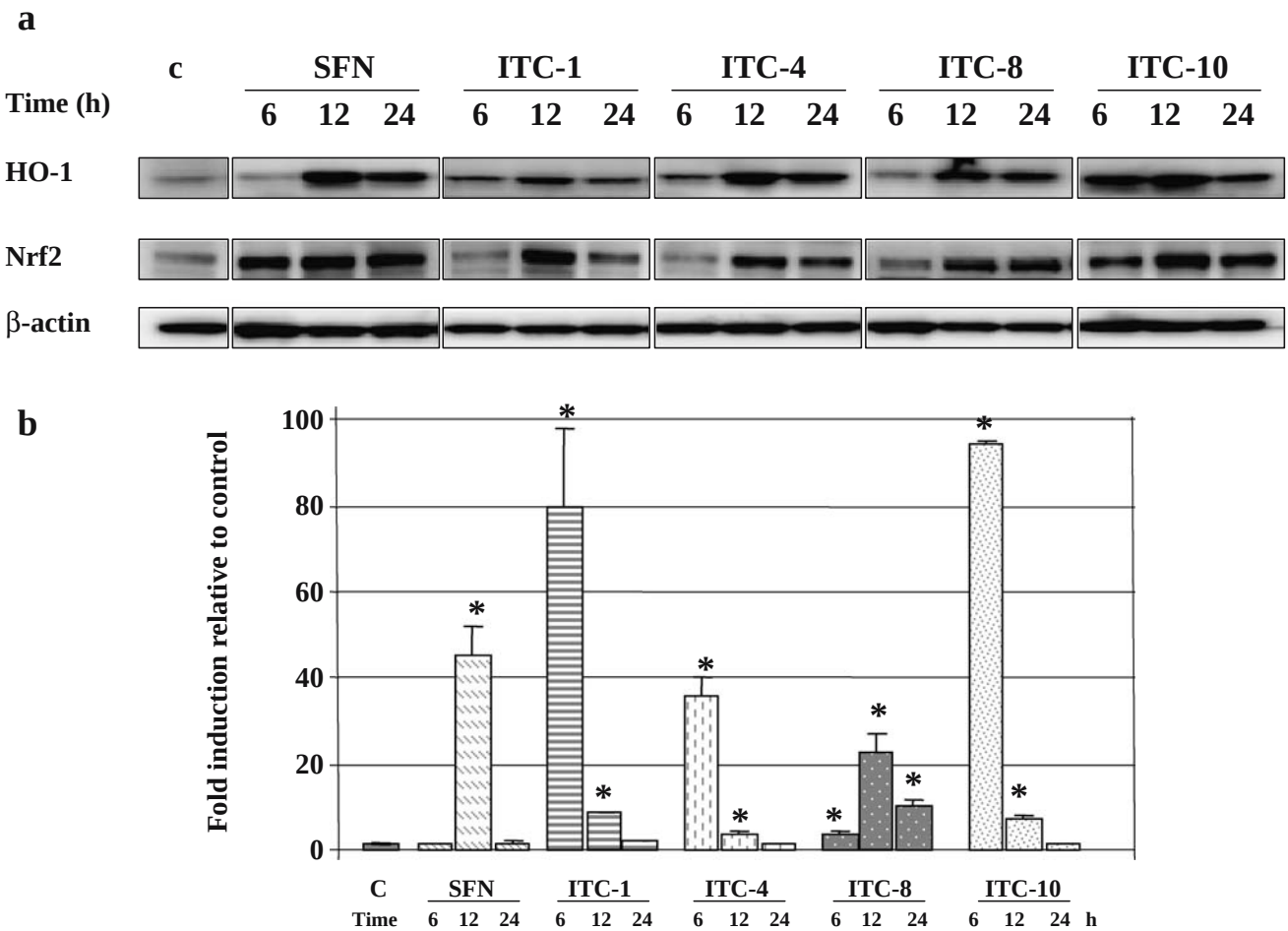

Fig. 2. The induction of HO-1 and Nrf2 protein (**a**) and HO-1 mRNA expression (**b**) by isothiocyanate compounds. HepG2-C8 cells were treated with SFN (20 μM), ITC1 (20 μM), ITC4 (20 μM), ITC8 (20 μM), and ITC10 (10 μM) for a period of times as indicated. The protein expression of HO-1, Nrf2, and β-actin was determined by Western blotting. The mRNA expression of HO-1 and β-actin was quantified by real-time PCR analysis. *Asterisks* indicate $p<0.05$ *versus* control.

TRIzol extraction coupled with the RNeasy kit from Qiagen (Valencia, CA). RNA integrity was examined by electrophoresis, and concentrations were determined by UV spectrophotometry (DU 530 Life Science UV/Visible Spectrophotometer, Fullerton, CA). Total RNA was then reverse transcribed to single-stranded cDNA by the SuperScript III first-strand cDNA synthesis system. Levels of quantitative reverse transcription product were measured using SYBR Green fluorescence collected during real-time PCR on an Applied Biosystems PRISM 7900HT system (Foster City, CA). A control cDNA dilution series was constituted to establish a standard curve. Each reaction was subjected to melting point analysis to confirm single amplified products. The specific primers for HO-1 and β-actin genes were designed by using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). The 5′ and 3′ primers used for amplifying HO-1 were 5′-GGT GAC CCG AGA GGG CTT-3′ and 5′-CGA AGA CTG GGC TCT CCT TGT-3′. β-actin was used as an internal control and was amplified with the 5′ and 3′ primers: 5′-CAG TGT GGG TGA CCC CGT-3′ and 5′-CCC AGC CAT GTA CGT TGC TA-3′. The *HO-1* gene expression levels were determined by normalization with control gene *β*-actin levels.

Western Blot Analysis

Following experimental treatments, cells were washed twice with ice-cold PBS (pH 7.4), and lysed with 1× cell lysis buffer (Cell Signaling Technologies, Beverly, MA). Cell lysates were then centrifuged at 10,000 *g* for 10 min at 4°C. Of the protein samples, 15 μg were subjected to 10% SDS-polyacrylamide gel electrophoresis and the resolved proteins were then transferred on to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA) using a semi-dry transfer system (Fisher Scientific, Pittsburgh, PA). The nonspecific binding of antibodies were blocked by 5% nonfat dried milk in PBST buffer (0.1% Tween 20 in PBS). Immunodetection of HO-1, Nrf2, β-actin and GADPH proteins was carried out using respective primary antibodies (1:1,000 in 3% nonfat dried milk in PBST buffer) and horseradish peroxidase (HRP) conjugated secondary antibodies (1:3,000 in 3% nonfat dried milk in PBST buffer). The immunocomplexes were determined by using the enhanced chemiluminescent system for detecting HRP on immunoblots (Amersham Pharmacia, Piscataway, NJ) and the bands were visualized and quantified by BioRad ChemiDoc XRS system (Hercules, CA).

Statistical Analyses

Experiments were performed at least three times. Values were presented as means±standard error of mean (SEM). Statistical analysis of the data was performed by Student's *t* test. *p* Values lower than 0.05 were considered significant.

RESULTS

Structural Relationships of ITCs to Their Activities to Induce the Protein Expression of HO-1 by Activating ARE-mediated Gene Expression Pathway

To better understand the chemical structural relationships of ITCs on ARE-mediated gene expression, HepG2-C8 cells generated in our laboratory by stable transfection of ARE-luciferase construct were treated with various concentrations of ten structurally related ITCs (Fig. 1) and SFN was used as a positive control. Those synthetic ITCs included seven aliphatic ITCs (ITC-1, ITC-2, ITC-4, ITC-5, ITC-6, ITC-7, and ITC-8) and three aromatic ITCs (ITC-3, ITC-9, and ITC-10) with different physico-chemical properties such as solubility and acid-base properties. We found that treatments with most ITCs, if not all, significantly induced ARE-luciferase activities and the half-maximal induction concentration (EC_{50}) values of those ITCs were between 7 and 16 μM as summarized in Table II. When comparing the ARE-mediated luciferase activity of ten ITCs tested in this study, each compound has different maximal degree of luciferase induction (efficacy). Among the aliphatic ITCs, the degree of maximal induction on ARE-mediated luciferase activity from high to low were ITC-8 (3×(-CH_2-) and -morpholine), SFN (4×(-CH_2-) and -methylsulfinyl), ITC-1 (2×(-CH_2-) and -methoxy), ITC-4 (1×(-CH_2-) and -tetrahydrofuran), ITC-5 (methyl-3-isothiocyanato-propionate; 2×(-CH_2-)), ITC-6 (3×(-CH_2-) and -diethylamino), ITC-2 (3×(-CH_2-) and -methoxy), and ITC-7 (2×(-CH_2-) and morpholine), respectively. Whereas the order for aromatic ITCs were ITC-10 (1×(-CH_2-) and - 3,4-(methylenedioxy-benzene)>ITC-3 (1×(-CH_2-) and -furan)>ITC-9 (4-cyanophenyl isothiocyanate; 0×(-CH_2-)). We noticed that, in this study, there was no ITCs that possessed inhibitory effect on ARE-mediated luciferase activity. Although, most ITCs had no cytotoxicity effect at all concentrations tested (1–20 μM; Table I), we found that ITC-10 exhibited significant cytotoxicity on HepG2-C8 cells at high concentration (>15 μM). Overall, ITC-8, had the highest maximum induction activity on ARE-mediated luciferase reporter gene, in addition ITC-8 and ITC-10 had better ARE activation than SFN. However, the ARE activation effect of ITC-10 declined at higher concentration (>15 μM), possibly because of its cytotoxicity. Comparing to the positive SFN, ITC-1 and ITC-4 had similar dose–response kinetics with moderate ARE activation activity, whereas the other ITCs had either lower efficacy or almost no effect. In summary, it appears that the ARE inductive potency of ITCs was influenced by either the number of methylene (-CH_2-) groups in the bridge linking the functional group, the isothiocyanate moieties and/or the type of functional group (Table II).

It is known that ITCs elicit their cancer-protective effects by increase cellular defense system via induction of detoxifying/antioxidant enzymes through Nrf2/ARE pathway. To address whether ARE activation by selected ITCs is translated into the induction of ARE-dependent protein expression, we have evaluated four ITCs, which have high to moderate ARE-mediated luciferase activity, including ITC-1 (20uM), ITC-4 (20 μM), ITC-8 (20 μM), and ITC-10 (10 μM) on the ARE-mediated antioxidant enzyme HO-1 induction in HepG2-C8 cells at different time points. As shown in Fig. 2a, exposure of HepG2-C8 cells to these ITCs significantly induced the HO-1 protein expression in a time-dependent manner. Overall, HO-1 protein induction was significantly observed after 12 h and declined at 24 h, except for ITC-10 which could upregulate HO-1 protein expression earlier at 6 h (Fig. 2a). In addition, real-time PCR (Fig. 2b) showed that HO-1 mRNA was also upregulated by the selected ITCs treatment. HO-1 mRNA induction was observed maximally at 6 h for ITC-1, ITC-4 and ITC-10 and 12 h for ITC-8 and

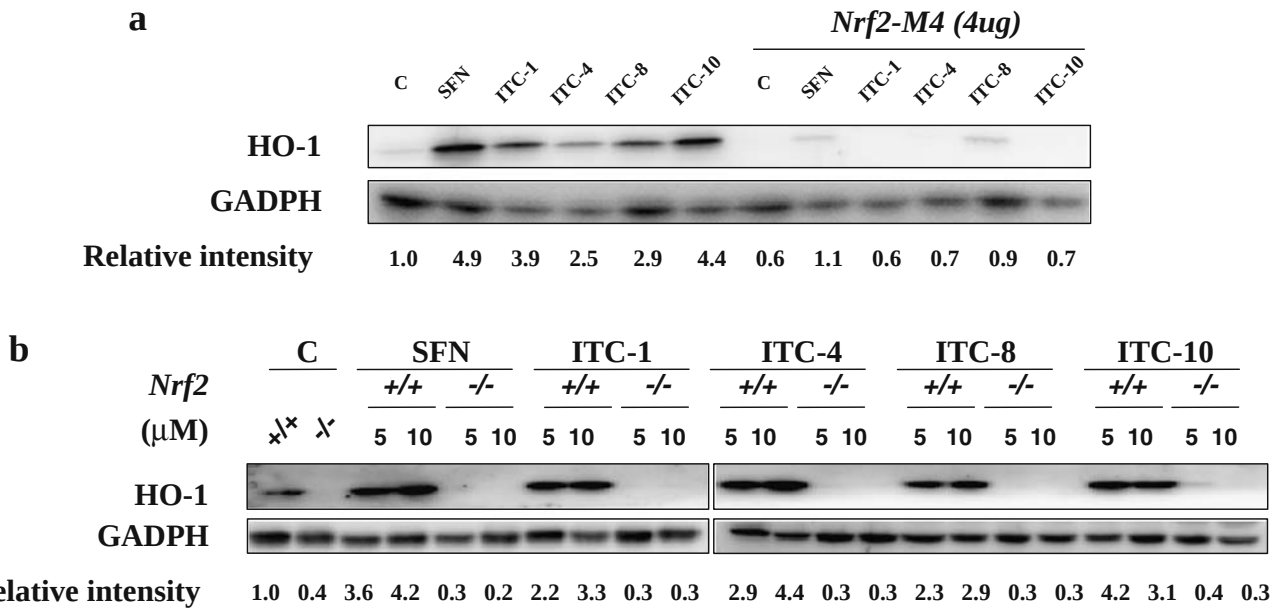

Fig. 3. Nrf2 is required for the induction of HO-1 by isothiocyanate compounds. **a** After transient transfection with mutant Nrf2 plasmid (*Nrf2-M4*), HepG2-C8 cells were treated with SFN (20 μM), ITC1 (20 μM), ITC4 (20 μM), ITC8 (20 μM), and ITC10 (10 μM) for 24 h and the protein expression of HO-1 and GADPH was measured by Western blotting. **b** $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mouse embryonic fibroblasts were exposed to SFN (5, 10 μM), ITC1 (5, 10 μM), ITC4 (5, 10 μM), ITC8 (5, 10 μM), and ITC10 (5, 10 μM) for 12 h prior to determination of the HO-1 and GADPH protein expression by Western blot analysis. The protein bands were quantitated by densitometry and represented as the HO-1:GAPDH ratio.

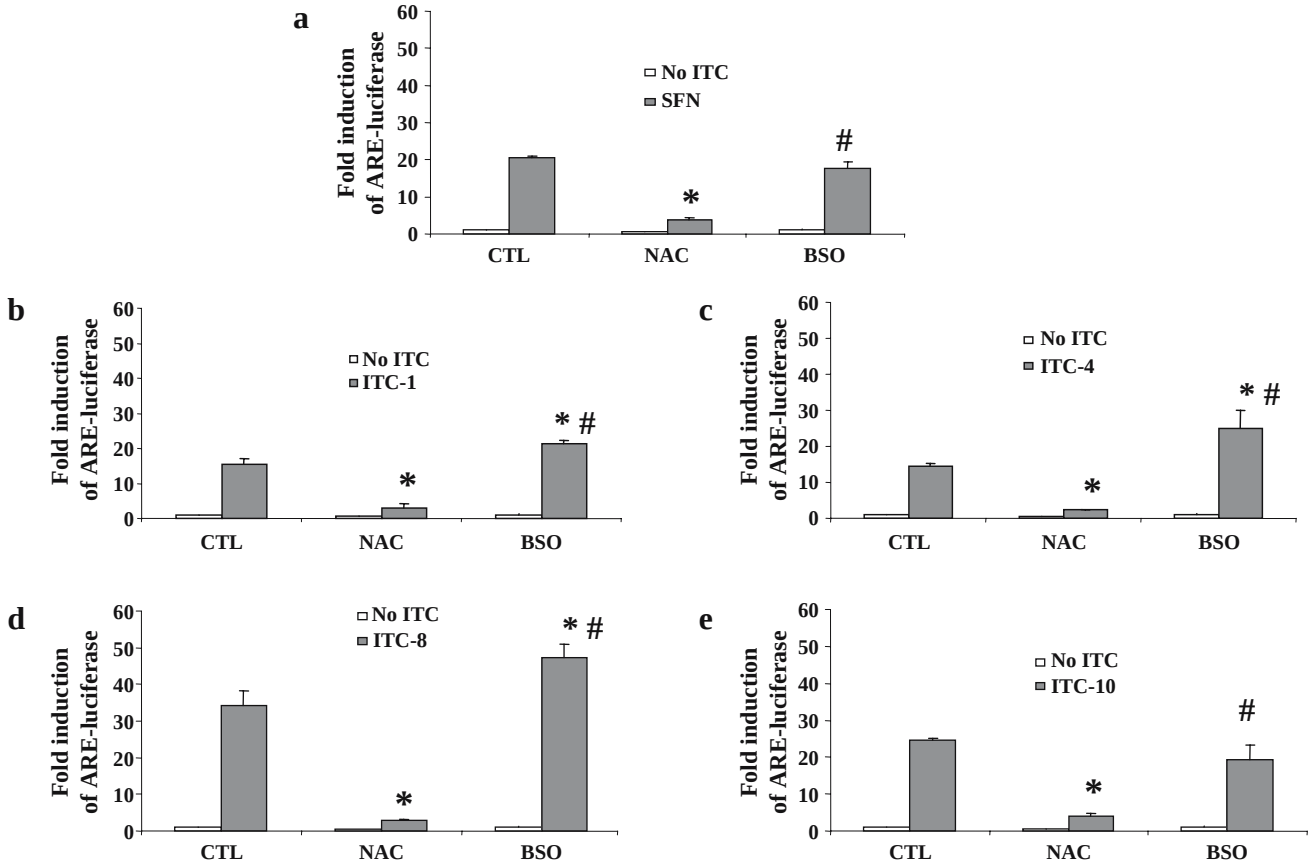

Fig. 4. ARE activation by isothiocyanate compounds is dependent on the depletion of intracellular GSH. HepG2-C8 cells were incubated with SFN (20 μM; **a**), ITC1 (20 μM; **b**), ITC4 (20 μM; **c**), ITC8 (20 μM; **d**), and ITC10 (10 μM; **e**) in the absence or presence of either *N*-acetylcysteine (*NAC*, 1 mM) or buthionine sulfoximine (*BSO*, 200 μM) for 24 h and the resulting luciferase activity was measured. *Asterisks* indicate $p<0.05$ *versus* ITC treatment alone, *pound signs* indicate $p<0.05$ *versus* co-treatment of ITC and NAC.

SFN. Since, the HO-1 mRNA pattern of some ITCs did not clearly correlate with that of HO-1 protein. It's possible that ITCs could modulate HO-1 expression by both transcriptional and translation regulation mechanism.

Nrf2 is an Indispensable Transcription Factor That Is Required for the Induction of HO-1 Protein by ITCs

In an earlier report, induction of HO-1 protein expression by SFN was mediated through Nrf2/ARE-dependent pathway (16). Supporting this idea, we have examined the effects of selected ITCs on the protein expression of Nrf2, the key regulatory protein of ARE activation. As seen in Fig. 2a (lower panel), treatments with selected ITCs could induce protein expression of Nrf2 in a time-dependent manner and this inductive level closely correlated with that of HO-1 protein. This suggests that, in HepG2-C8 cells, the induction of HO-1 protein by ITCs is due to the activation of ARE-driven gene expression via the induction of Nrf2.

Given the fact that the putative binding sites for many transcription factors other than Nrf2 are found within the upstream human HO-1 promoter, we next tested whether Nrf2 is necessary for the induction of HO-1 protein by ITCs. As presented in Fig. 3a, we found that overexpression of mutant Nrf2 plasmid (Nrf2-M4) significantly attenuated ITC-mediated induction of HO-1 protein in HepG2-C8 cells. In addition, when $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mouse embryonic fibroblasts were exposed to selected ITCs at 5 and 10 μM for 12 h, the induction of HO-1 protein was seen only in $Nrf2^{+/+}$ mouse embryonic fibroblasts with a dose-dependent pattern, whereas it was totally abolished in $Nrf2^{-/-}$ mouse embryonic fibroblasts (Fig. 3b). Taken together, these data imply that Nrf2 is an indispensable transcription factor required for the induction of HO-1 protein by ITCs.

ARE Activation by ITCs is Dependent on the Depletion of Intracellular Glutathione

It has been shown that ITCs can generate cellular oxidative stress by directly reacting with and depleting the intracellular level of reduced glutathione (GSH; 17). To verify the role of intracellular GSH level in ARE-dependent gene activation by ITCs, we determined whether an antioxidant *N*-acetylcysteine (NAC), a precursor of GSH synthesis, and a pro-oxidant buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis, could have an impact on ITC-mediated ARE activation. As seen in Fig. 4a–e, treatments of NAC (1 mM) alone did not affect the basal level of ARE-dependent luciferase activity (0.87±0.03-fold induction *versus* control vehicle-treated cells 1.00±0.10). While treatment with BSO (200 μM) alone could significantly activate endogenous ARE-mediated gene expression (1.92±0.03-fold induction *versus* control 1.00±0.10), suggesting that depletion of intracellular GSH by inhibiting GSH synthesis could drive the ARE activation. Interestingly, co-incubation of NAC significantly suppressed ARE activation by ITCs, while that with BSO tend to augment the ARE activation by ITCs, implying that ARE-dependent gene activation by ITCs is responsible at least in part by the depletion of intracellular GSH.

DISCUSSION

Several dietary ITCs from a variety of cruciferous vegetables such as *Brussica* species, are considered to be promising chemopreventive agents. They are known to have cancer protective activity against the carcinogenesis *in vivo* and *in vitro*. SFN, one of the most commonly investigated ITCs, is initially recognized as a principle inducer of phase II detoxifying enzymes including NAD(P)H:(quinone-acceptor) oxidoreductase (NQO) and glutathione *S*-transferases (GST; 18). Subsequently, several studies demonstrated that SFN activated ARE-driven phase II detoxifying/antioxidant enzyme expression through activation of Nrf2 as a potential mechanism (11,16). In addition to its modulatory effects on endogenous reactive oxygen species and exogenous carcinogens and/or their reactive intermediates metabolism, it was also identified as an effective inducer of apoptosis and/or cell cycle arrest especially at high dosages (19).

The current study was undertaken to shed light on the structure-activity relationships of ITCs on Nrf2/ARE-dependent gene expression. We found that synthetic ITCs had different maximal induction effect of ARE-mediated luciferase activity. Comparing their structures, the induction potency and/or efficacy was influenced by either the type of functional group or the number of methylene (-CH_2-) groups in the bridge linking the functional group and isothiocyanate moieties. Our results also suggested that differences of the inducing ARE activity might be more affected by the type of the functional group than the number of methylene groups. This observation is in accordance with Morimitsu *et al.* (20) that the GST inducer potency of sulforaphane and its analogues was influenced by the oxidation state of sulfur and the number of methylene (-CH_2-) groups. Data from our current study suggested that, in addition to methylsulfinyl group, morpholine, methylenedioxybenzene, tetrahydrofuran and methoxy groups appear to be suggestive as well to be involved in the inductive ARE-mediated luciferase activity. These results might suggest that ITCs with six-member ring structure (such as morpholinolene) are more susceptible to induce ARE activity. However, methylenedioxybenzene group was noticed to associate with cytotoxicity effect at higher concentration, while other ITCs had no toxic effect. Talalay *et al.* (21) suggested that the inductive ability of various alkyl and aromatic isothiocyanates depended on the presence of at least one hydrogen on the carbon adjacent to the isothiocyanate moiety and that tautomerization of the methylene-isothiocyanate moiety to a structure resembling an α, β-unsaturated thioketone may be important for inductive activity. Consistently, we found that ITC-9 (4-cyanophenyl isothiocyanate), which had no hydrogen on the carbon adjacent to the isothiocyanate moiety, had very low activity of ARE activation. In addition, differences of physicochemical properties such as solubility and acid-base properties may influence the induction efficacy of ITCs as well.

According to earlier studies, SFN had been reported to increase HO-1 expression by activating ARE through induction of Nrf2. The potential mechanism was hypothesized to be posttranslational stabilization of Nrf2 protein rather than increased transcriptional activation of Nrf2 (16). Data presented here indicate that HO-1 protein/mRNA induction by selected synthetic ITCs treatments in this study

required Nrf2 as an indispensable transcription factor. However, the correlation between the induced levels of HO-1 protein and that of HO-1 mRNA of some ITCs do not appear to be correlative, implying the possibility of other mechanism, such as posttranslational stabilization of HO-1 protein, might be involved.

Previous report showed that multiple signaling pathways, including MAPK cascades, PI3K and PKC, are involved in the regulation of ARE-dependent gene expression (16,22). Phosphorylation of these signaling kinases has been suggested to play important role in Nrf2-mediated ARE activation. Thus, there is possibility that synthetic ITCs tested here could exert the ARE activation through these upstream signaling pathways. Although the primary target of tested ITCs in this study is still unknown, there is evidence that the intracellular GSH, which is responsible for regulating the redox state of the cells, may be an important sensor to initiation of the cellular response to ITCs. Consistent with this idea, we found that combination treatments of NAC significantly decreased ARE activation by ITCs, thus, lower cellular stress by NAC could lead to lower cellular response to ITCs. In fact, it is also recognized that an adequate amount of oxidative stress is needed for stimulation of a variety of signal transduction events including gene expression. In addition to HO-1 (17,23), oxidative stress has been reported to enhance the expression of other phase II detoxifying/antioxidant enzymes such as GST (20) and γ-GCS genes (24).

In conclusion, our data indicate that synthetic ITCs activate ARE-mediated gene transcription through Nrf2/ARE signaling pathway, however, their inductive effects are quite specific, depending on the chemical structure. Our results demonstrate that, in addition to methylsulfinyl (SFN) functional group, the morpholine (ITC-8) and methylenedioxybenzene (ITC-10) groups, are also suggestive to have superior ARE activation than other functional groups. Therefore, these synthetic ITCs may have potential to be chemopreventive agents that might be superior than that of the natural ITCs. However, further studies of the chemopreventive activities and toxicological profiles of these ITCs using *in vivo* carcinogenesis animal models to assess their efficacy and safety are needed in the future.

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