DOI 10.1002/mnfr.200800558 Mol. Nutr. Food Res. 2009, *53*, 1166 – 1176

Research Article

Protective effect of sulforaphane on indomethacin-induced cytotoxicity *via* heme oxygenase-1 expression in human intestinal Int 407 cells

Chi-Tai Yeh[1], Hsiang-Fan Chiu[2] and Gow-Chin Yen[2]

[1] National Institute of Cancer Research, National Health Research Institutes, Miaoli County, Taiwan
[2] Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan

Sulforaphane is known to be an indirect antioxidant that acts by inducing NF-E2-related factor 2 (Nrf2)-dependent phase II enzymes. In the present study, we investigated the effect of sulforaphane on the expression of heme oxygenase-1 (HO-1) in human intestinal Int 407 cells. RT-PCR and Western blot data revealed that sulforaphane induced an increase in HO-1 expression at the mRNA and protein levels, respectively. This induction was also marked by an increase in HO-1 activity. Actinomycin D (an RNA synthesis inhibitor) and cycloheximide (a protein synthesis inhibitor) inhibited sulforaphane-responsive HO-1 mRNA expression, indicating that sulforaphane is a requirement for transcription and *de novo* protein synthesis. Moreover, sulforaphane increased the nuclear levels of Nrf2 and increased the binding activity of nuclear proteins to the antioxidant responsive element consensus sequence. We also found that U0126, an ERK kinase inhibitor, suppressed the sulforaphane-induced HO-1 expression and nuclear translocation of Nrf2. Moreover, the cytoprotective effect of sulforaphane on indomethancin-induced cytotoxicity was partially blocked by ERK and HO-1 inhibitors, further demonstrating that sulforaphane attenuated oxidative stress through a pathway that involved ERK and HO-1. Taken together, this study gives additional support to the possible use of sulforaphane as a dietary preventive agent against oxidative stress-induced intestinal injury.

Keywords: Heme oxygenase / Indomethacin / Intestinal Int 407 cells / Nonsteroidal anti-inflammatory drugs / Sulforaphane

Received: December 7, 2008; revised: January 28, 2009; accepted: January 29, 2009

1 Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin are the most widely used analgesic, antipyretic, and anti-inflammatory agents in clinical medicine. However, epidemiologic studies indicate that NSAID users have a higher risk of gastrointestinal ulceration than patients who are not using NSAIDs [1]. NSAID-induced inhibition of cycloxygenase, oxidative stress, and mitochondrial dysfunction are known to contribute to these effects [2]. Oxidative stress is the most relevant in this respect, as such damage at the cellular level is considered to be an important event in the initiation of inflammatory diseases, carcinogenesis, and ageing [3]. The involvement of oxidative stress in gastrointestinal injury observed after ischemia-reperfusion has also been reported [4]. Therefore, the use of exogenous compounds with antioxidative properties may help in the preventive and/or therapeutic intervention of oxidative gastrointestinal disorders [5]. Another strategy for protecting against oxidative gastrointestinal injury may be *via* chemically mediated upregulation of endogenous antioxidant and phase II enzymes in tissues and cells of the gastrointestinal tract.

Among the various cytoprotective enzymes, heme oxygenase-1 (HO-1) was recently highlighted by virtue of its gastrointestinal protective role [6]. Increased HO-1 expression leads to heme degradation and accumulation of iron, bilirubin, and carbon monoxide (CO), followed by reduced sensitivity of the gastrointestinal tissues/cells to oxidant damage [7]. Of these metabolites, bilirubin acts as a direct antioxidant, whereas CO may exert tissue-protective actions primar-

Correspondence: Dr. Gow-Chin Yen, Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan
E-mail: gcyen@nchu.edu.tw
Fax: +886-4-22854378

Abbreviations: ARE, antioxidant response element; **BME/EBSS,** Basal Medium Eagle with Earle's balanced salts; **CS,** calf serum; **EMSA,** electrophoretic-mobility shift assay; **HO-1,** heme oxygenase-1; **ITCs,** isothiocyanates; **MAPKs,** mitogen-activated protein kinase; **Nrf2,** NF-E2-related factor 2; **NSAIDs,** nonsteroidal anti-inflammatory drugs; **ZnPP,** zinc protoporphyrin

ily through its vasodilation and antiplatelet effects [8]. A large body of evidence suggests that HO-1 is a novel enzyme with antioxidant, anti-inflammatory, and antiproliferative effects [9]. The HO-1 gene is primarily regulated at the transcriptional level, and its inducibility is linked to the transcription factor NF-E2-related factor 2 (Nrf2) [10]. Under normal conditions, Nrf2 is sequestered in the cytoplasm by binding to Keap1, an actin-binding protein. However, recent studies have suggested that the nuclear translocation of Nrf2 requires the activation of several signal transduction pathways, such as mitogen-activated protein kinases (MAPKs) [11], protein kinase C (PKC) [12], and phosphatidylinositol 3-kinase (PI3K) [13]. As increasing evidence indicates that HO-1 provides gastroprotection [14], inducing HO-1 expression by a pharmacological modulator may represent a novel target for therapeutic intervention. In particular, a noncytotoxic pharmacological inducer of HO-1 may maximize the intrinsic antioxidant potential of cells. Several antioxidants from plant origins have been reported to induce HO-1 expression in a variety of cells and hence to confer resistance to oxidative stress [15].

Sulforaphane, an isothiocyanate (ITC) first isolated from broccoli, has received intense attention for its chemopreventive potential in cancer because it is one of the most potent inducers of phase II detoxifying enzymes among many natural compounds [16]. The phase II detoxifying enzymes that can be induced by sulforaphane contain the antioxidant response element (ARE) sequence in the promoter region of their genes [17]. Direct linkage has been made between the activity of sulforaphane and the molecular sensor Nrf2 – Keap1, the complex that regulates the induction of phase II enzymes. However, the mechanisms underlying the cytoprotective effects of sulforaphane on intestinal cells have not been fully addressed. In the present study, we utilized a highly differentiated intestinal epithelial cell line (Int 407) which not only resembles epithelial cells morphologically but has also been shown to retain many of the receptors involved in intestinal secretagogues [18]. Moreover, the cells were found to posses many functional properties such as transporters (for glutathione, glucose, fructose, amino acids, taurine, dipeptides, *etc*.) and binding proteins (for vitamin A, fatty acids, calcium, *etc*.) [19]. The objective of this study was to investigate the potency of sulforaphane as a gastrointestinal inducer of HO-1 and its regulation in Int 407 cells. In addition, we also examined the cytoprotective significance of sulforaphane-induced HO-1 against indomethacin-induced oxidative damage in Int 407 cells.

2 Materials and methods

2.1 Chemicals

Basal Medium Eagle with Earle's balanced salts (BME/EBSS), calf serum (CS), trypsin – EDTA (TE), L-glutamine, and PSN antibiotic solution (penicillin – streptomycin – neomycin) were obtained from Gibco BRL (Grand Island, NY, USA). D,L-sulforaphane (1-isothiocyanato-4-(methylsulfinyl)-butane, >99% pure) was obtained from LKT Laboratories (St. Paul, MN, USA). Anti-HO-1 and anti-Nrf2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ERK, antiphospho-ERK, anti-p38, antiphospho-p38, anti-JNK, antiphospho-JNK, antilamin B1, and anti-β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). IgG polyclonal antibody conjugated to peroxidase, Zn (II) protoporphyrin IX (ZnPP IX), and 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical (St. Louis, MO, USA). Molecular mass markers for proteins were obtained from Pharmacia Biotech (Saclay, France). Poly-vinylidene fluoride (PVDF) membranes for Western blotting were obtained from Millipore (Bedford, MA, USA). The inhibitors of MAPKs, U0126, SB203580, and SP600125 were obtained from Biosource (Camarillo, CA, USA); a TRIzol RNA isolation kit was obtained from Life Technologies (Rockville, MD, USA); and primers for RT-PCR, dNTP, reverse transcriptase and Taq polymerase were obtained from Gibco BRL (Cergy Pontoise, France). All other chemicals were of the highest pure grade available.

2.2 Cell culture

Human intestinal epithelial cell line 407 (Int 407) cells (BCRC 60022) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). Cells were grown in BME/EBSS and supplemented with 10% CS at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was renewed each day. Cells were detached weekly, for transfer with 0.1% trypsin and 10 mM EDTA in PBS.

2.3 Cell survival assay

Cell viability was determined by the MTT assay. Int 407 cells were seeded onto 96-well plates at a concentration of 1×10^5 cells/well in BME/EBSS plus 10% CS. After growing to confluence, the cells were then preincubated with 7.5 μM sulforaphane for 16 h. After changing the medium, cells were exposed to different concentrations of indomethacin (0 – 400 μM) for 12 h, then the supernatant was discarded and cells were rinsed with PBS. Dye solution (10 μL), specific for the MTT assay, was added to each well for the additional 4 h incubation at 37°C. After the addition of DMSO (100 μL/well) the absorbance at 570 nm (formation of formazan) and 630 nm (reference) were recorded with a Fluostar Galaxy plate reader (BMG Lab Technology, Offenburg, Germany). The percent viability of the treated

cells was calculated as follows: (A570 nm – A630 nm) sample/(A570 nm – A630 nm) control × 100.

2.4 Immunofluorescence analysis

For immunofluorescence analysis, cells were plated in six-well chamber slides (Nunc, Rochester, NY, USA) for 24 h before treatment with 7.5 μM sulforaphane for 24 h. Afterwards, cells were fixed in 2% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in 0.01 M PBS (pH 7.4) containing 0.2% BSA, air-dried, and rehydrated in PBS. Then, cells were incubated with a rabbit polyclonal antibody against Nrf2 (Santa Cruz Biotechnology), diluted 1:500 in PBS containing 3% normal goat serum for 2 h at room temperature. Negative controls were performed by omitting the primary antibody. After two washings in PBS for 10 min, an antirabbit IgG PE-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:500 in PBS was added for 1 h at room temperature. Cells were then washed in PBS and mounted in Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) to counterstain DNA. Cells were observed using a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany). Microphotographs were acquired using a digital video camera (AxioCam MRc, Zeiss, Germany) and Axiovision Zeiss software.

2.5 RNA extraction and RT-PCR

The expression values of HO-1 were quantified by semi-quantitative reverse transcription-PCR (RT-PCR) analysis, using GAPDH mRNA as an internal standard. Int 407 cells (1×10^6 in 10 mL medium) were plated in 100 mm tissue culture dishes. After preincubation for 24 h, Int 407 cells were subjected to a dose-course, using sulforaphane in 0.05% DMSO. Cellular RNA was extracted with a TRIzol RNA isolation kit (Life Technologies) as described in the manufacturer's manual. RNA was quantified spectrophotometrically. For quality control, RNA concentration and purity were estimated using the OD260/280 ratio. RNA integrity was tested by loading 1 μg of total RNA in a denaturing gel and visualizing the 2:1 ratio of 28S and 18S band intensities with ethidium bromide and checking for RNA degradation. Only samples with OD260/280 ratios from 1.8 to 2.1 and with tight bands with approximate 2:1 ratios of 28S/18S band intensities were used. After adding RNase inhibitor (20 U) the total RNA was stored at −70°C. The sense and antisense primer sequences used were: HO-1: 5′-CTG TGT AAC CTC TGC TGT TCC-3′, 5′-CCA CAC TAC CTG AGT CTA CC-3′; and GAPDH: 5′-TGA AGG TCG GTG TGA ACG GAT TTG GC-3′, 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′. These primer sets yield PCR products of 667 and 983 bp for HO-1 and GAPDH, respectively. Briefly, from each sample, 250 ng of RNA was reverse-transcribed, using 200 U of Superscript II reverse transcriptase, 20 U of RNase inhibitor, 0.6 mM of dNTP and 0.5 μg/μL of oligo (dT) 12–18. Then, PCR analyses were performed on the aliquots of the cDNA preparations to detect HO-1 and GAPDH (as an internal standard) gene expression, using the FailSafe PCR system (Epicenter Technologies, Madison, WI, USA). The reactions took place in a volume of 50 μL, containing (final concentrations): 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MnCl2, 0.2 mM dNTP, 2 U of Taq DNA polymerase, and 50 pmol of 5′ and 3′ primers. After initial denaturation for 2 min at 95°C, 30 cycles of amplification (at 95°C for 1 min, 54°C for 30 s, and 72°C for 45 s) were performed, followed by a 7 min extension at 72°C.

2.6 Analysis of PCR products

A 10 μL aliquot from each PCR reaction was electrophoresed in a 1.8% agarose gel, containing 0.2 μg/mL ethidium bromide. The gel was then photographed under UV transillumination. For quantification, the PCR bands on the photograph of the gel were scanned using a densitometer, linked to a computer analysis system. We normalized the HO-1 signal, relative to the corresponding GAPDH signal, from the same sample, expressing the data as the HO-1/GAPDH ratio.

2.7 Western blot

The cytosolic proteins were isolated from Int 407 cells (2×10^6 cells/mL) after different doses of sulforaphane treatment. The total proteins were extracted by adding 800 μL of cold lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethylesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at $12\,000 \times g$ for 10 min at 4°C. The supernatant protein concentration was measured by BioRad DC protein assay kit with BSA as the standard. The cell lysate were mixed with 4 × sample buffer (8% SDS; 0.04% CBB R-250; 40% glycerol; 200 mM Tris, pH 6.8; and 10% 2-mercaptoethanol) and boiled for 10 min. Samples were electrophoresed in a 12% SDS-PAGE minigel and then transferred onto polyvinylidenedifluoride membranes (PVDF; Millipore) with transfer buffer (48 mM Tris; 39 mM glycine; 0.0037% SDS; and 20% methanol) at 400 mA for 60 min. The membranes were blocked with 5% nonfat milk in PBS solution containing 0.1% Tween-20 (PBST) for 1 h. The membrane was immunoblotted with primary antibodies containing 5% BSA overnight at 4°C. After consecutively washes with PBST for 30 min, the membrane was incubated with horseradish peroxidase-labeled secondary antibody for 60 min at room temperature and washed with PBST for 30 min. Final detection was performed with enhance chemiluminescence (ECL™ kit) Western blotting reagents (Amersham Pharmacia Biotech, New Jersey, USA).

2.8 HO activity assay

Confluent Int 407 cells were incubated in 100 mm culture dishes for 16 h with or without sulforaphane. Some experiments were done in the presence of an HO enzyme inhibitor, ZnPP. A heme oxygenase activity assay was performed as described previously by Kutty and Maines [20]. Briefly, after the incubation, cells were washed twice with PBS, gently scraped off the dishes, and centrifuged (13 000 × *g*, 5 min, 4°C). The cell pellet was resuspended with PBS and then subjected to three cycles of freeze – thawing. Finally, it was sonicated on ice before centrifugation at 13 000*g* for 15 min at 4°C. The reaction mixture used for measurement of HO activity was composed of protein (1 mg), the cytosolic fraction of rat liver (1 mg of protein) as a source of biliverdin reductase, 33 μM hemin, and 333 μM NADPH in 1 mL of 90 mM potassium phosphate buffer (pH 7.4). The reaction was incubated for 1 h at 37°C in the dark and terminated by the addition of 600 μL chloroform. The extracted bilirubin was determined spectrophotometrically using the difference in absorbance at wavelength from 460 to 530 nm with an absorption coefficient of 40 mM^{-1} and cm^{-1}. HO activity was measured as picomoles of bilirubin formed *per* milligram of cell protein *per* hour.

2.9 Preparation of nuclear extracts

To obtain nuclear extracts lysates, the sulforaphane-treated and untreated cells were washed twice with ice-cold 1 × PBS and lysed using NE-PER Nuclear Extraction Reagents (Pierce, Rockford, IL, USA). Protein concentrations of cellular extracts were determined by the bicinchoninic assay with a commercial protein reagent kit (Pierce).

2.10 Electrophoretic-mobility shift assay (EMSA)

Nuclear extract (2 – 5 μg) was used for EMSA according to the manufacturer's instructions (EMSA gel-shift kit, Panomics, CA, USA). Briefly, nuclear extracts containing equal amounts of protein for each sample were incubated with poly(dI-dC) (1 μg/μL) for 5 min, followed by the addition of binding buffer (20 mM HEPES, pH 7.9, 1 mM DTT, 0.1 mM EDTA, 50 mM KCl, 5% glycerol, and 200 μg/mL BSA) and biotinylated oligo (10 ng/μL). To control for specificity of binding in selected samples, a five-fold excess of nonlabeled oligo was added prior to the addition of the biotinylated probe. Binding reaction mixtures were incubated for 30 min at room temperature. Protein – DNA complexes were separated on 5% nondenaturing polyacrylamide gels in Tris-borate/EDTA buffer (0.1 M Tris, 0.09 M boric acid containing 1 mM EDTA) at 4°C. After electrophoresis, gels were transferred to nylon membranes. Transferred oligos were immobilized by UV crosslinking for 3 min. For detection of bound oligos, membranes were blocked using blocking buffer (Panomics EMSA gel-shift kit) followed by the addition of streptavidin – HRP, and blots were developed by ECL according to the manufacturer's instructions (Amersham, Arlington Heights, IL, USA). A commercially available biotinylated oligonucleotide encoding the ARE motif (5′-CTACGATTTCTGCTT-AGTCATTGTCTTCC-3′) was used as a canonical probe (Panomics, AY1062).

2.11 Statistical analysis

Each experiment was performed in triplicate and repeated three times. The results are expressed as means ± SD. Statistical comparisons were made by one-way analysis of variance (ANOVA), followed by a Duncan multiplecomparison test. Differences were considered significant when the *p*-values were < 0.05.

3 Results

3.1 Effect of sulforaphane on HO-1 expression and activity

To examine whether sulforaphane could affect HO-1 activity and HO-1 expression in Int 407 cells, we treated the cells for 24 h with various concentrations of sulforaphane. The effects of various concentrations of sulforaphane (0 – 7.5 μM) on HO-1 activity and HO-1 protein expression in Int 407 cells are shown in Fig. 1. Exposure of Int 407 cells to sulforaphane for 24 h resulted in a dose-dependent increase in HO-1 mRNA levels. A marked induction of HO-1 mRNA expression was observed after a 2.5 μM exposure of sulforaphane; maximum induction of HO-1 mRNA expression was observed at 7.5 μM treatment (Fig. 1A). Western blot analyses using monoclonal anti-HO-1 antibodies confirmed that the increase in HO-1 mRNA levels by sulforaphane was accompanied by an increase in HO-1 protein expression (Fig. 1B). In addition, exposure of Int 407 cells to sulforaphane for 24 h resulted in a dose-dependent increase in HO-1 activity (Fig. 1C). The increase was significantly different from the control (untreated cells, $p < 0.05$), with a maximal enzymatic activity at 7.5 μM of sulforaphane. In Int 407 cells treated with 7.5 μM of sulforaphane, HO-1 expression was increased in a time-dependent manner, reaching a minimum at 8 h, followed by a maximum at 24 h (Fig. 1D). Because the concentration of sulforaphane at 7.5 μM was capable of inducing maximal expression of HO-1 in Int 407 cell line, all subsequent experiments involving sulforaphane were performed using a concentration of 7.5 μM of sulforaphane.

3.2 Transcriptional and translational regulation of HO-1 induction by sulforaphane

To determine whether sulforaphane-induced HO-1 mRNA expression required transcription or translation, we first examined whether HO-1 mRNA expression in Int 407 cells

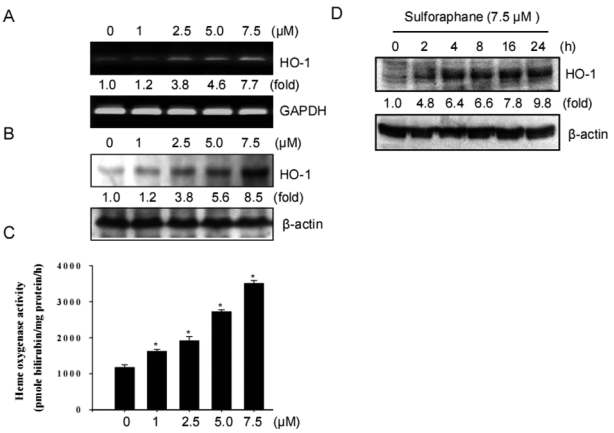

Figure 1. Effect of sulforaphane on HO-1 mRNA, protein expression and enzyme activity in Int 407 cells. (A) Cells were exposed to various concentrations of sulforaphane for 24 h, and total RNA was extracted with the Invitrogen RNA isolation kit. RT-PCR was performed as described in Section 2. Expression of HO-1 mRNA was analyzed by RT-PCR. GAPDH, the housekeeping gene, was used as an internal control. (B) Whole cell lysate was subjected to Western blot analysis using a monoclonal HO-1 antibody. (C) HO-1 activity was measured 24 h after exposure to various concentrations of sulforaphane. (D) Cells were also incubated with 7.5 μM sulforaphane for the indicated times. Relative expression was quantified densitometrically using LabWorks 4.5 software and normalized against β-actin reference bands. Reported values are the means ± SD ($n = 3$). * Significantly different from control ($p < 0.05$).

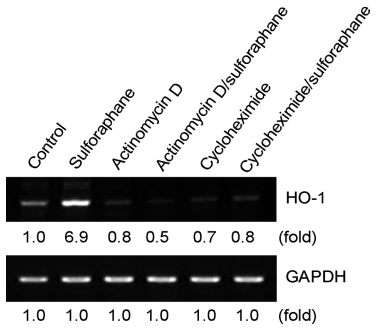

Figure 2. Effects of actinomycin D and cycloheximide on HO-1 mRNA in Int 407 cells after sulforaphane treatment. Int 407 cells were pretreated with either actinomycin D (5 μg/mL) or cycloheximide (10 μg/mL) for 4 h and were then treated with 7.5 μM sulforaphane for 24 h. Total RNA was extracted and analyzed for HO-1 mRNA expression by RT-PCR. GAPDH, the housekeeping gene, was used as an internal control. The intensity of the indicated HO-1 mRNA was detected by densitometric analysis and expressed as fold change relative to control. Data are representative of three independent experiments.

after sulforaphane treatment was dependent on the absence or presence of RNA synthesis inhibitors actinomycin D and the protein synthesis inhibitors cycloheximide, followed by RT-PCR analyses. As shown in Fig. 2, sulforaphane-mediated induction of HO-1 mRNA was completely abolished in the presence of actinomycin D (5 μg/mL). These data suggest that sulforaphane increased HO-1 gene expression by enhancing gene transcription. To further investigate the regulation of HO-1 expression by sulforaphane, Int 407 cells were pretreated with the protein synthesis inhibitor cycloheximide (10 μg/mL) prior to treatment with sulforaphane and were then analyzed for HO-1 mRNA expression. Cycloheximide significantly attenuated the upregulation of HO-1 mRNA steady-state levels in response to sulforaphane treatment, suggesting that synthesis of new proteins is also required for sulforaphane-induced HO-1 mRNA expression (Fig. 2).

3.3 Sulforaphane increased the protein expression, ARE-binding, and nuclear translocation of Nrf2

Most of the genes encoding phase II detoxifying and antioxidant enzymes have an ARE sequence in their promoter region. Nrf2 is an important transcription factor that regulates ARE-driven HO-1 gene expression [10]. Subsequently, we examined whether sulforaphane could activate Nrf2 in association with HO-1 upregulation in Int 407 cells. The EMSA revealed that sulforaphane at a concentration of 7.5 μM increased ARE binding activity in a time-dependent manner. ARE binding activity began to increase 4 h after exposure of cells to sulforaphane and extended to 16 h (Fig. 3A). Competition experiments using excess amounts of either unlabeled ARE or SP-1 oligonucleotides confirmed the specificity of protein binding to the ARE (data not shown).

In the mechanism of HO-1 expression, nuclear translocation of activated Nrf2 is an important upstream step [10]. To investigate whether sulforaphane could induce Nrf2 translocation in Int 407 cells, an immunofluorescence assay was used to detect the distribution of Nrf2 in the cells treated with sulforaphane. In untreated Int 407 cells, Nrf2 fluorescence was found to be distributed throughout the cells, including the cytoplasm and nucleus. After treatment with sulforaphane for 6 h, Nrf2 fluorescence was primarily concentrated in the nuclei (Fig. 3C). To further confirm the Nrf2 nuclear translocation by sulforaphane, cells were incubated with sulforaphane for about 6 h. Using Western blot, Nrf2 proteins in the nuclear compartments of the cells were analyzed. After treatment with sulforaphane, Nrf2 protein in the nucleus was markedly increased (Fig. 3B). These results suggest that induction of HO-1 by sulforaphane involves Nrf2-mediated ARE activation.

3.4 Involvement of the ERK/p38 pathway in the induction of HO-1 expression by sulforaphane in Int 407 cells

Many studies have demonstrated that several MAPKs, including JNK, ERK, and p38, are involved in regulating

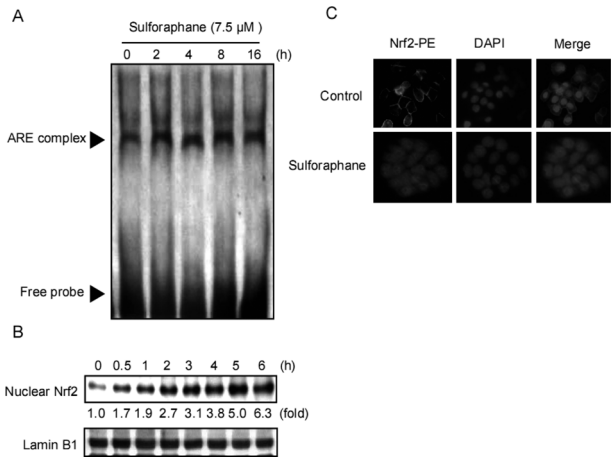

Figure 3. Effect of sulforaphane on the activation of nuclear factor Nrf2 binding to the ARE and translocation into nucleus in Int 407 cells. (A) EMSA analysis of the ARE transcription complex. Nuclear extracts were prepared from Int 407 cells treated with sulforaphane (7.5 μM) for 0 – 16 h. (B) Immunoblot analysis of the levels of Nrf2 in the nuclear fraction of Int 407 cells treated with sulforaphane (7.5 μM) for 0 – 6 h. The immunoreactive protein bound to rabbit anti-Nrf2 antibody was visualized by an ECLTM kit after incubation with HRP-conjugated secondary antibody. Antilamin B1 antibody was used for normalization. The intensity of the indicated Nrf2 proteins was detected by densitometric analysis and expressed as fold change relative to control. Results were confirmed by three separate experiments, and a representative immunoblot is shown. (C) Effect of sulforaphane on nuclear localization of Nrf2. The Confocal image shows that PE-conjugated secondary antibody staining indicated the location of Nrf2 (red) by anti-Nrf2 antibody, DAPI staining indicates the location of the nucleus (blue), and the merged image in sulforaphane-treated cells indicates the nuclear location of the Nrf2 protein.

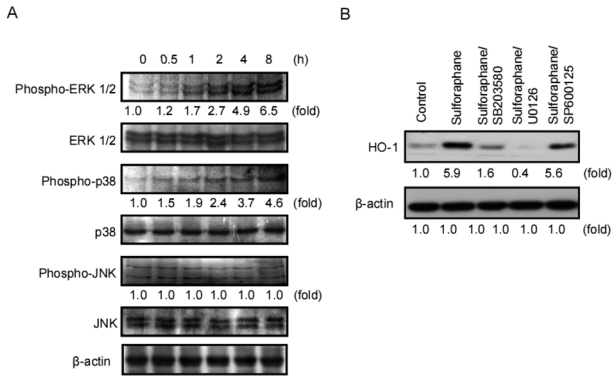

Figure 4. Western blotting of sulforaphane-induced HO-1 expression *via* ERK/p38 MAPK activation in Int 407 cells. (A) The cells incubated in the absence or presence of sulforaphane (7.5 μM) for the indicated time were subjected to Western blot analysis using phospho-specific antibodies to ERK, p38, or JNK. As controls, the same cell lysates were subjected to Western blot analysis using the corresponding nonphospho-specific antibodies to detect total ERK1/2, p38, or JNK. (B) Cells were pretreated with SB203580 (20 μM), U0126 (10 μM), or SP600125 (10 μM) for 2 h and were then exposed to 7.5 μM sulforaphane for 12 h in the presence of inhibitor. Western blot analysis was performed using antibodies specific for HO-1 and β-actin. The intensity of the indicated HO-1 proteins was detected by densitometric analysis and expressed as folds of control. Data shown are representative of three independent experiments.

the phosphorylation of Nrf2 and ARE-mediated phase II gene expression [11]. Thus, we examined whether the MAPK pathway is involved in the process by which sulforaphane causes Nrf2 nuclear translocation. As shown in Fig. 4A, sulforaphane increased the levels of phosphorylated ERK and p38. The same blots were probed with the antibody to total JNK, ERK, and p38 as protein loading controls. The sulforaphane-mediated increase in HO-1 protein expression was completely blocked by U0126 (a specific inhibitor of ERK) and moderately blocked by SB203580 (a specific inhibitor of p38), whereas a similar concentration of SP600125 (a specific inhibitor of JNK) had no significant effect (Fig. 4B). These results indicate that a kinase in the ERK pathway might be involved in the regulation of HO-1 expression by sulforaphane.

3.5 Activation of Nrf2 by sulforaphane via phosphorylation of ERK/p38 MAPK pathway

It has been previously reported that the MAPK signaling pathway may be involved in regulation of the ARE response. We sought to determine whether phosphorylation of MAPK mediated by this pathway may play a role in promoting Nrf2 stability. In this experiment, Int 407 cells were pretreated with either U0126 or SB203580 for 2 h, followed by sulforaphane treatment for 6 h. The nuclear lysate was analyzed by immunoblotting with both anti-Nrf2 and antilamin B1 antibodies. The results of this experiment show that both of these compounds attenuated the effects of sulforaphane on inducing the Nrf2 protein level, by 80 and 70%, respectively (Figs. 5A and B). Furthermore, nuclear accumulation of Nrf2 was reduced by treatment with U0126 (ERK inhibitor) and SB203580 (p38 inhibitor) (Fig. 5C). These data demonstrate a link between phosphorylation and Nrf2 stability, suggesting a role for the ERK/p38 MAPK pathway in Nrf2 stability and localization.

3.6 Sulforaphane protection of Int 407 cells from indomethacin-induced cell death

We further analyzed the protective effects of sulforaphane on indomethacin-induced cytotoxicity in Int 407 cells. In the presence of indomethacin, a dose-dependent decrease in the viability of cells was observed by the MTT assay in Int 407 cells, with an IC_{50} value of around 400 μM (Fig. 6A). Interestingly, when cells were pretreated with sulforaphane at the optimal conditions (7.5 μM and 16 h), the viability of Int 407 cells was significantly higher at all concen-

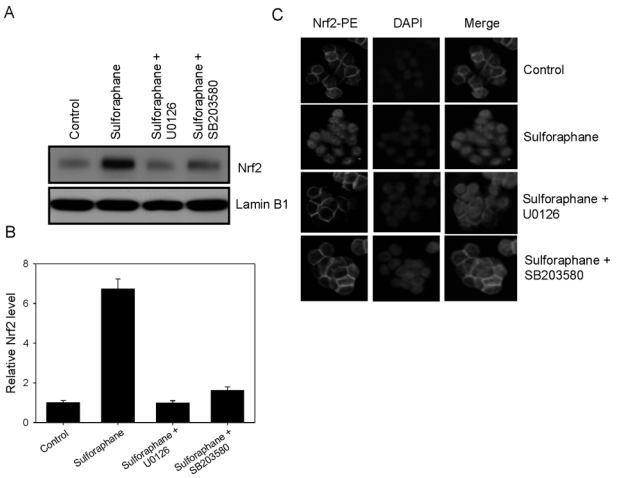

Figure 5. Phosphorylation of Nrf2 mediated by ERK/p38 MAPK pathway. (A) Int 407 cells were treated with control (lane 1), or 7.5 μM sulforaphane (lane 2) for 6 h, 10 μM U0126 (lane 3), or 10 μM SB203580 (lane 4) for 2 h, followed by sulforaphane for 6 h. Nuclear extracts were subjected to Western blotting analysis with anti-Nrf2 and antilamin B1 antibodies. (B) The result in (A) was quantified using a densitometer, and the Nrf2 value was plotted after normalization with values of antilamin B1. The blots shown are representative of three independent experiments with similar results. (C) Int 407 cells were treated with 7.5 μM sulforaphane in the absence or presence of U0126 or SB203580 for 2 h, then immunostained to detect the nuclear localization of Nrf2.

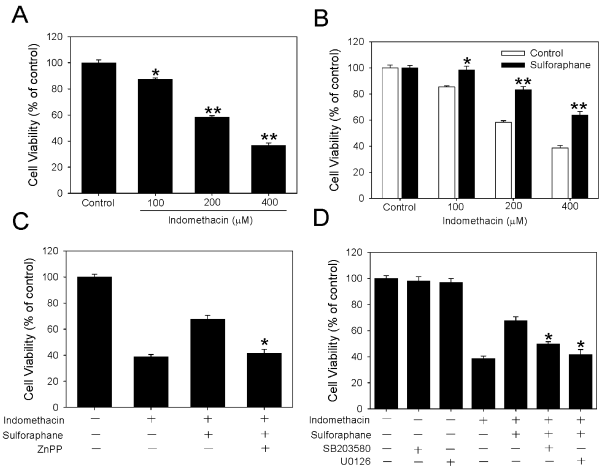

Figure 6. Protective effect of sulforaphane against indomethacin-induced cell death in Int 407 cells. (A) Dose-dependent reduction of the viability of Int 407 by indomethacin using the MTT assay. Cells were treated with different doses of indomethacin (100, 200, and 400 μM) for 24 h, and the viability of Int 407 cells was detected by MTT assay as described in Section 2. (B) Int 407 cells were either untreated with sulforaphane or treated with 7.5 μM sulforaphane for 16 h. After changing the medium, cells were exposed to different concentrations of indomethacin (0–400 μM) for 24 h. Cell viability was determined by MTT assay. *$p < 0.05$ *versus* the corresponding group without sulforaphane pretreatment. (C) The HO-1 enzyme inhibitor ZnPP reversed the protective effect of sulforaphane against indomethacin-induced cell death. Int 407 cells were treated with sulforaphane (7.5 μM) for 16 h and then incubated with different doses of ZnPP (3 μM) for 30 min. Indomethacin (400 μM) was then added to cells for a further 24 h. (D) Int 407 cells were pretreated for 30 min with U0126 and SB203580 prior to addition of sulforaphane. Following 16 h of incubation with sulforaphane, cells were treated with indomethacin for 24 h. Cell viability was assessed by the MTT assay after indomethacin treatment. *Differs from sulforaphane plus indomethacin-treated cells.

trations of indomethacin compared with cells untreated with sulforaphane (Fig. 6B). Zinc protoporphyrin (ZnPP) has been used as an HO-1 specific inhibitor. To determine whether the increased HO-1 activity induced by sulforaphane could confer cytoprotection against oxidative injury, Int 407 cells were pretreated with the HO-1 specific inhibitor ZnPP. ZnPP attenuated the cytoprotective effect of sulforaphane on indomethacin-induced cytotoxicity (Fig. 6C), suggesting that the cytoprotective effect of sulforaphane is mostly mediated through HO-1 induction. Moreover, inhibitors of ERK and p38 also attenuated the protective effect of sulforaphane on indomethacin-induced cytotoxicity (Fig. 6D). These results indicate that the induction of HO-1 by sulforaphane through the ERK/p38 MAPK pathway is essential for the cytoprotective effect of sulforaphane against oxidative stress.

4 Discussion

Epidemiological studies have demonstrated that the consumption of cruciferous vegetables is associated with a lower incidence of cancer. An important group of compounds that have this property are organosulfur compounds, such as ITCs [21]. They are known to have cancer-protective activities against carcinogenesis *in vivo* and *in vitro*. Sulforaphane is one of the most commonly investigated ITCs; it was initially recognized as a principle inducer of phase II detoxifying enzymes, including NAD(P)H:(quinone-acceptor) oxidoreductase and glutathione S-transferases. Subsequently, several studies demonstrated that sulforaphane activates ARE-driven phase II detoxifying/antioxidant enzyme expression through activation of Nrf2 as a potential mechanism [22]. In addition to its modulatory effects on endogenous reactive oxygen species and exogenous carcinogens and/or their reactive metabolic intermediates, it was also identified as an effective inducer of apoptosis and cell cycle arrest especially at high dosages [23]. In this study, we tried to elucidate the cytoprotective effect of sulforaphane against oxidative damage in intestinal cells and the mechanism of its cytoprotective action.

The results of our study show that sulforaphane is capable of strongly inducing HO-1 activity and HO-1 protein expression in dose- and time-dependent manners (Fig. 1). To the best of our knowledge, this is the first report of HO-1

mRNA induction in intestinal epithelial cells in response to sulforaphane. This observation is in agreement with a previous finding [24] that exposure of sulforaphane to $HepG_2$ cells efficiently increased HO-1 expression by activating the ARE through the induction of Nrf2 and the suppression of the Kelch-like ECH-associated protein 1. Thus, our data provide clear evidence that sulforaphane is a potent inducer of HO-1 in human intestinal Int 407 cells. Furthermore, the stimulation of HO-1 expression by most inducers has been shown to occur primarily as a consequence of transcriptional regulation of the HO-1 gene. In this study, we have demonstrated that both actinomycin D (a transcriptional inhibitor) and cycloheximide (a translation inhibitor) eliminated sulforaphane-mediated HO-1 mRNA expression (Fig. 2). Based on our finding, we suggest that HO-1 gene induction by sulforaphane is primarily regulated at the transcriptional level.

NF-E2-related factor-2 (Nrf2), a basic leucine zipper transcription factor, forms heterodimers with the small Maf proteins and binds to the ARE. Through the activation of the ARE in the regulatory region of the genes, Nrf2 plays a critical role in the constitutive and inducible expression of numerous detoxifying and antioxidant genes, including HO-1. The cytoplasmic protein Keap1 sequesters stimuli, including electrophilic agents and compounds that possess the ability to modify thiol groups, and the liberation of Nrf2 from Keap1 allows Nrf2 to translocate into the nucleus and bind to the ARE. This mechanism of gene activation leads to the synthesis of highly specialized proteins that consequently reduce the propensity of tissues and organisms to develop disease or malignancy [25]. Hence, activation of Nrf2, which controls both the constitutive and inducible expression of Phase II detoxifying genes, may be one of the protective mechanisms against xenobiotics.

Recently, the antioxidants curcumin and carnosol have also been shown to induce HO-1 expression [26]. We recently found that sulforaphane induces Nrf2 activation in human hepatoma $HepG_2$ cells [27]. Together, these studies suggest that Nrf2 plays a key role in antioxidant-induced HO-1 expression.

The most significant finding in our current study is the demonstration of the involvement of the Nrf2 pathway in sulforaphane-mediated HO-1 gene induction. The electrophoretic mobility shift assay revealed that the nuclear ARE binding activity was significantly increased by sulforaphane treatment in Int 407 cells. Additionally, sulforaphane also increased the Nrf2 nuclear translocation (Fig. 3), suggesting that the increased expression of the Nrf2 protein may play a key role in sulforaphane-induced HO-1 gene activation. Dinkova-Kostova *et al*. [28] have demonstrated that phase II gene inducers react much more avidly with Keap1 than with Nrf2. Both Nrf2 and Keap1 contain multiple cysteine residues and all cysteines on murine Keap1 were found to react with the phase 2 inducers, including dexamethasone mesylate, sulforaphane and bis(2- and 4-hydroxybenzylidene) acetones (Michael reaction acceptors). Specifically, C257, C273, C288, and C297 were shown to be the most reactive cysteine residues of Keap1. Wakabayashi *et al*. [29] have suggested that the cysteines C273 and C288 in the intervening region of Keap1 may sequester one molecule of Nrf2 between two DGR (double-glycine repeat) domains in the cytosol and ensure its rapid turnover by targeting it for proteasomal degradation. Upon exposure to inducers, the reactive C273 and C288 residues form intermolecular disulfide bonds, covalently linking two monomers of Keap1 and changing its conformation accordingly. The DGR domains are then separated to release Nrf2, which translocates to the nucleus, activating the expression of phase 2 genes [29]. Sulforaphane was shown in this study to stimulate Nrf2 translocation, possibly by dissociating the Nrf2–Keap1 complex. Details of the likely interaction between sulforaphane and Keap1 (or indeed Nrf2) in Int 407 cells require further investigation.

MAPK signaling cascades are stimulated by many extracellular stimuli, such as growth factors, cytokines, and various environment stresses, and they serve as a common signal transduction pathway for signals involved in proliferation, differentiation, functional activation, and stress responses. Many studies have demonstrated that several MAPKs including JNK, p38, ERK, and PI3K, are involved in regulating the phosphorylation of Nrf2 and ARE-mediated HO-1 gene expression [30]. The MAPK pathway was also examined in the present study to further explore the upstream regulatory mechanisms involved in sulforaphane-induced Nrf2 activation and induction of HO-1. Interestingly, we found ERK and p38 MAPK were phosphorylated by sulforaphane (Fig. 4). Compared with the untreated Int 407 cells, sulforaphane-treated cells had higher levels of phosphor-ERK and phosphor-p38, whereas the levels of phosphor-JNK were not changed. Inhibition of the phosphor-ERK and phosphor-p38 pathway by U0126 and SB203580 inhibitors almost completely blocked sulforaphane-induced HO-1 protein expression, suggesting that sulforaphane-induced HO-1 expression occurs *via* the ERK and p-38 MAPK pathway. Furthermore, phosphorylation of Nrf2 and the nuclear accumulation of Nrf2 were reduced by U0126 (an ERK inhibitor) and SB203580 (a p38 inhibitor) (Fig. 5). In a previous study, Shen *et al*. [31] reported that activation of MAPKs induced ARE-mediated gene expression *via* an Nrf2-dependent mechanism. Curcumin induced HO-1 expression by promoting dissociation of the Nrf2–Keap1 complex in a p38-dependent manner [32]. Resveratrol activated Nrf2-driven ARE activation and HO-1 expression *via* the ERK pathway [33]. Our results indicate a requirement for ERK and p38 activation in the induction of HO-1 expression through nuclear translocation of Nrf2.

In gastrointestinal tissue both *in vitro* and *in vivo*, indomethacin has been demonstrated to cause damage *via* cellular oxidative phosphorylation. A number of studies have suggested that the gastrointestinal-toxic effects of indome-

thacin in the rat are due not only to their inhibitory effects on cycloxygenases but also to their direct cytotoxic effects on gastric mucosal lesions [34]. In our study, we observed the acute toxic effects of indomethacin on cultured Int 407 cells, as short-term exposure of the cells to high concentrations of indomethacin rapidly decreased cell viability in a dose-dependent manner (Fig. 6A). These adverse effects are largely responsible for the cytotoxicity mediated by indomethacin in target tissues and cells [35]. We focused on the cytotoxic effects of indomethacin on the Int 407 cells, since they are important in relation to the serious intestinal complications including ulceration and bleeding from the stomach and small intestine *in vivo* [36]. However, whether upregulation of HO-1 also affords gastroprotection against indomethacin-mediated cytotoxicity in Int 407 cells has not been investigated.

Our results clearly show that incubation of Int 407 cells with sulforaphane effectively prevents the indomethacin-induced cell death (Fig. 6B). The involvement of HO-1 in the cytoprotective action of sulforaphane was examined using the HO-1 and ERK inhibitor of ZnPP and U0126, respectively. ZnPP attenuated the protective effect of sulforaphane on indomethacin-induced cytotoxicity (Fig. 6C), suggesting that the cytoprotective effect of sulforaphane is partly mediated through HO-1 induction. Moreover, inhibitors of ERK and p38 MAPKs also attenuated the protective effect of sulforaphane on indomethacin-induced cytotoxicity (Fig. 6D), suggesting the involvement of ERK and p38 MAPKs in sulforaphane-mediated HO-1 gene induction and cytoprotection. These results are consistent with recent evidence that flavonoids in medicinal herbs and dietary plants exert potent antioxidative and anti-inflammatory activities through the induction of HO-1 [37]. These observations strongly suggest that intracellular HO-1 is an important factor in sulforaphane-mediated cytoprotection against indomethacin toxicity in Int 407 cells.

Recently, studies concerning the bioavailability of glucosinolates and ITCs are in agreement with their potential therapeutic effects [38]. Sulforaphane, an ITC first isolated from broccoli, has received intense attention for its cancer chemopreventive potential because it is one of the most potent inducers of phase II detoxifying enzymes among many natural compounds. Related analogs of sulforaphane, the allyl isothiocyanate (AITC), 1-isothiocyanato-3-(methylthio)propane (iberverin), 1-isothio-cyanato-4-(methythio)butane (erucin), 1-isothiocyanato-3-(methysulfinyl)-propane (iberin), and 1-isothiocyanato-3-(methylsulfonyl)-propane (cheirolin) were also active inducers of phase II detoxification enzymes in murine hepatoma cells [39]. Substantial quantities of ITCs (up to 100 mg daily) and even greater quantities of their glucosinolate precursors are widely consumed by humans [40]. Following treatment of mice by gavage with 9 μM sulforaphane *per* day for 1 wk, several classes of genes were identified as targets of sulforaphane in a transcriptional microarray, including cellular NADPH regenerating enzymes, xenobiotic metabolizing enzymes, antioxidant enzymes, and biosynthetic enzymes of glutathione and glucuronidation conjugation pathways [41]. Furthermore, mice treated by gavage with 15 μM sulforaphane *per* day for 5 days had an increase in quinone reductase and glutathione *S*-transferase activities in the liver, forestomach, glandular stomach, proximal small intestine, and lungs [42], establishing that sulforaphane also induced phase II enzymes *in vivo* following oral administration. Moreover, in a human intervention study, following a single dose of 200 μmol of ITCs (largely, sulforaphane, with lesser amount of iberin and erucin), the total plasma ITC levels reached 0.94 – 2.27 μM after 1 h of feeding [43]. The effect of increased sulforaphane levels in the plasma on HO-1 remains to be proven. In the present study, the ability of sulforaphane to modulate HO-1 was found at concentrations that may well be achievable in human plasma. Our results demonstrate an induction of HO-1 in response to sulforaphane in human intestinal cells, and this finding suggests that sulforaphane may be an important chemopreventive agent for use in intestinal protection.

In conclusion, our findings suggest that sulforaphane effectively prevents indomethacin-induced intestinal epithelial cell injury, and HO-1 induction by sulforaphane *via* ERK and Nrf2 pathways plays a key role in this cytoprotection. The present study provides insight into the mechanisms of sulforaphane-induced cytoprotection *via* HO-1 expression and suggests that sulforaphane may be effectively utilized as an alternative drug in the prevention of gastrointestinal complications of NSAIDs.

This research work was partially supported by the National Science Council, NSC97-2321-B005-002, Taiwan, Republic of China.

The authors have declared no conflict of interest.

5 References

[1] Rainsford, K. D., Profile and mechanisms of gastrointestinal and other side effects of nonsteroidal anti-inflammatory drugs (NSAIDs), *Am. J. Med*. 1999, *107*, 27S – 35S.

[2] Basivireddy, J., Vasudevan, A., Jacob, M., Balasubramanian, K. A., Indomethacin-induced mitochondrial dysfunction and oxidative stress in villus enterocytes, *Biochem. Pharmacol.* 2002, *64*, 339 – 349.

[3] Hiraishi, H., Shimada, T., Terano, A., Involvement of oxidative stress in the pathogenesis of NSAID-induced gastric mucosal damage, *J. Gastroenterol*. 2000, *35*, 567 – 569.

[4] Sasaki, M., Joh, T., Oxidative stress and ischemia-reperfusion injury in gastrointestinal tract and antioxidant, protective agents, *J. Clin. Biochem. Nutr*. 2007, *40*, 1 – 12.

[5] Koo, M. W., Cho, C. H., Pharmacological effects of green tea on the gastrointestinal system, *Eur. J. Pharmacol*. 2004, *500*, 177 – 185.

[6] Guo, X., Shin, V. Y., Cho, C. H., Modulation of heme oxygenase in tissue injury and its implication in protection against gastrointestinal diseases, *Life Sci*. 2001, *69*, 3113 – 3119.

[7] Gibbons, S. J., Farrugia, G., The role of carbon monoxide in the gastrointestinal tract, *J. Physiol*. 2004, *556*, 325 – 336.

[8] Ryter, S. W., Morse, D., Choi, A. M., Carbon monoxide and bilirubin: Potential therapies for pulmonary/vascular injury and disease, *Am. J. Respir. Cell Mol. Biol*. 2007, *36*, 175 – 182.

[9] Kirkby, K. A., Adin, C. A., Products of heme oxygenase and their potential therapeutic applications, *Am. J. Physiol. Renal. Physiol*. 2006, *290*, F563 – F571.

[10] Srisook, K., Kim, C., Cha, Y. N., Molecular mechanisms involved in enhancing HO-1 expression: De-repression by heme and activation by Nrf2, the "one-two" punch, *Antioxid. Redox. Signal*. 2005, *7*, 1674 – 1687.

[11] Shih, P. H., Yen, G. C., Differential expressions of antioxidant status in aging rats: The role of transcriptional factor Nrf2 and MAPK signaling pathway, *Biogerontology* 2007, *8*, 71 – 80.

[12] Pi, J., Bai, Y., Reece, J. M., Williams, J., *et al*., Molecular mechanism of human Nrf2 activation and degradation: Role of sequential phosphorylation by protein kinase CK2, *Free Radic. Biol. Med*. 2007, *42*, 1797 – 1806.

[13] Li, M. H., Cha, Y. N., Surh, Y. J., Peroxynitrite induces HO-1 expression via PI3K/Akt-dependent activation of NF-E2-related factor 2 in PC12 cells, *Free Radic. Biol. Med*. 2006, *41*, 1079 – 1091.

[14] Becker, J. C., Grosser, N., Boknik, P., Schröder, H., *et al*., Gastroprotection by vitamin C – A heme oxygenase-1-dependent mechanism? *Biochem. Biophys. Res. Commun*. 2003, *312*, 507 – 512.

[15] Hwang, Y. P., Jeong, H. G., The coffee diterpene kahweol induces heme oxygenase-1 via the PI3K and p38/Nrf2 pathway to protect human dopaminergic neurons from 6-hydroxydopamine-derived oxidative stress, *FEBS Lett*. 2008, *582*, 2655 – 2662.

[16] Ye, L., Zhang, Y., Total intracellular accumulation levels of dietary isothiocyanates determine their activity in elevation of cellular glutathione and induction of Phase 2 detoxification enzymes, *Carcinogenesis* 2001, *22*, 1987 – 1992.

[17] Morimitsu, Y., Nakagawa, Y., Hayashi, K., Fujii, H., *et al*., A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway, *J. Biol. Chem*. 2002, *277*, 3456 – 3463.

[18] Yada, T., Oiki, S., Ueda, S., Okada, Y., Intestinal secretagogues increase cytosolic free Ca2+ concentration and K+ conductance in a human intestinal epithelial cell line, *J. Membr. Biol*. 1989, *112*, 159 – 167.

[19] Iantomasi, T., Favilli, F., Marraccini, P., Magaldi, T., *et al*., Glutathione transport system in human small intestine epithelial cells, *Biochim. Biophys. Acta*. 1997, *1330*, 274 – 283.

[20] Kutty, R. K., Maines, M. D., Oxidation of heme c derivatives by purified heme oxygenase. Evidence for the presence of one molecular species of heme oxygenase in the rat liver, *J. Biol. Chem*. 1982, *257*, 9944 – 9952.

[21] Keum, Y. S., Jeong, W. S., Kong, A. N., Chemopreventive functions of isothiocyanates, *Drug News Perspect*. 2005, *18*, 445 – 451.

[22] Myzak, M. C., Dashwood, R. H., Chemoprotection by sulforaphane: Keep one eye beyond Keap1, *Cancer Lett*. 2006, *233*, 208 – 218.

[23] Gamet-Payrastre, L., Signaling pathways and intracellular targets of sulforaphane mediating cell cycle arrest and apoptosis, *Curr. Cancer Drug Targets* 2006, *6*, 135 – 145.

[24] Keum, Y. S., Yu, S., Chang, P. P., Yuan, X., *et al*., Mechanism of action of sulforaphane: Inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element-mediated heme oxygenase-1 in human hepatoma HepG2 cells, *Cancer Res*. 2006, *66*, 8804 – 8813.

[25] Yu, X., Kensler, T., Nrf2 as a target for cancer chemoprevention, *Mutat. Res.* 2005, *591*, 93 – 102.

[26] Martin, D., Rojo, A. I., Salinas, M., Diaz, R., *et al*., Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol, *J. Biol. Chem*. 2004, *279*, 8919 – 8929.

[27] Yeh, C. T., Yen, G. C., Effect of sulforaphane on metallothionein expression and induction of apoptosis in human hepatoma $HepG_2$ cells, *Carcinogenesis* 2005, *26*, 2138 – 2148.

[28] Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., *et al*., Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants, *Proc. Natl. Acad. Sci. USA* 2002, *99*, 11908 – 11913.

[29] Wakabayashi, N., Dinkova-Kostova, A. T., Holtzclaw, W. D., Kang, M. I., *et al*., Protection against electrophile and oxidant stress by induction of the phase 2 response: Fate of cysteines of the keap1 sensor modified by inducers, *Proc. Natl. Acad. Sci. USA* 2004, *101*, 2040 – 2045.

[30] Yuan, X., Xu, C., Pan, Z., Keum, Y. S., *et al*., Butylated hydroxyanisole regulates ARE-mediated gene expression via Nrf2 coupled with ERK and JNK signaling pathway in $HepG_2$ cells, *Mol. Carcinog*. 2006, *45*, 841 – 850.

[31] Shen, G., Hebbar, V., Nair, S., Xu, C., *et al*., Regulation of Nrf2 transactivation domain activity. The differential effects of mitogen-activated protein kinase cascades and synergistic stimulatory effect of Raf and CREB-binding protein, *J. Biol. Chem*. 2004, *279*, 23052 – 23060.

[32] Balogun, E., Foresti, R., Green, C. J., Motterlini, R., Changes in temperature modulate heme oxygenase-1 induction by curcumin in renal epithelial cells, *Biochem. Biophys. Res. Commun*. 2003, *308*, 950 – 955.

[33] Chen, C. Y., Jang, J. H., Li, M. H., Surh, Y. J., Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells, *Biochem. Biophys. Res. Commun*. 2005, *331*, 993 – 1000.

[34] Othman, A. I., El-Missiry, M. A., Amer, M. A., The protective action of melatonin on indomethacin-induced gastric and testicular oxidative stress in rats, *Redox Rep*. 2001, *6*, 173 – 177.

[35] Ou, Y. C., Yang, C. R., Cheng, C. L., Raung, S. L., *et al*., Indomethacin induces apoptosis in 786-O renal cell carcinoma cells by activating mitogen-activated protein kinases and AKT, *Eur. J. Pharmacol*. 2007, *563*, 49 – 60.

[36] Bertrand, V., Guimbaud, R., Tulliez, M., Mauprivez, C., *et al*., Increase in tumor necrosis factor-alpha production linked to the toxicity of indomethacin for the rat small intestine, *Br. J. Pharmacol*. 1998, *124*, 1385 – 1394.

[37] Yao, P., Nussler, A., Liu, L., Hao, L., *et al*., Quercetin protects human hepatocytes from ethanol-derived oxidative stress by inducing heme oxygenase-1 via the MAPK/Nrf2 pathways, *J. Hepatol*. 2007, *47*, 253 – 261.

[38] Johnson, I. T., Glucosinolates: Bioavailability and importance to health, *Int. J. Vitam. Nutr. Res*. 2002, *72*, 26 – 31.

[39] Rose, P., Faulkner, K., Williamson, G., Mithen, R., 7-Methylsulfinylheptyl and 8-methylsulfinyloctyl isothiocyanates from watercress are potent inducers of phase II enzymes, *Carcinogenesis* 2000, *21*, 1983 – 1988.

[40] Shapiro, T. A., Fahey, J. W., Wade, K. L., Stephenson, K. K., Talalay, P., Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables, *Cancer Epidemiol. Biomarkers Prev.* 1998, *7*, 1091 – 1100.

[41] Thimmulappa, R. K., Mai, H., Srisuma, S., Kensler, T. W., *et al*., Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray, *Cancer Res*. 2002, *62*, 5196 – 5203.

[42] Zhang, Y., Talalay, P., Cho, C. G., Posner, G. H., A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure, *Proc. Natl. Acad. Sci. USA* 1992, *89*, 2399 – 2403.

[43] Ye, L., Dinkova-Kostova, A. T., Wade, K. L., Zhang, Y., *et al*., Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: Pharmacokinetics of broccoli sprout isothiocyanates in humans, *Clin. Chim. Acta* 2002, *316*, 43 – 53.

