Effect of sulforaphane on metallothionein expression and induction of apoptosis in human hepatoma HepG2 cells

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The molecular mechanism of sulforaphane on the induction of metallothionein (MT) genes in HepG2 cells and the antiproliferative effects of sulforaphane were investigated in this study. Treatment of the cells with sulforaphane at non-toxicity concentration (0–20 μM) resulted in coordinate increases in the induction of MT-I and MT-II mRNA, followed by corresponding increases in MT protein expression. Western blot analysis revealed the increased level of the transcription factor, Nrf2 in a time-dependent manner from sulforaphane-treated cells. Furthermore, sulforaphane activated the extracellular signal-regulated protein kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) pathways, SB203580, a specific inhibitor of p38 and PD98059, a specific inhibitor of ERK, abolished sulforaphane-induced MT protein expression, whereas SP600125, a specific inhibitor of JNK, had no significant effect. At relatively high concentration (30–100 μM), sulforaphane is a cell growth modulator, as it induced apoptotic cell death characterized by internucleosomal DNA fragmentation and caused a rapid induction of caspase 3 activity, according to the appearance of the caspase 3 fragments and stimulated proteolytic cleavage of poly (ADP-ribose) polymerase in a time-dependent manner. Moreover, sulforaphane-induced apoptotic cell death was accompanied by upregulation of Bax and downregulation of Bcl-2 and Bcl-XL protein. Sulforaphane-induced DNA fragmentation was blocked by the N-acetyl-L-cysteine and catalase, suggesting that the death signaling was triggered by oxidative stress. Taken together these results strongly suggest that at low concentrations of sulforaphane, activation of MAPKs, such as ERK and p38 pathway, lead to Nrf2-mediated MT expression. Whereas at a higher concentration, sulforaphane is an effective apoptosis inducer in HepG2 cells through regulation of Bcl-2 family molecular and activation of ICE/Ced-3 protease (caspase 3) cascade. The results from this study may provide more evidence for its chemopreventive function.

Introduction

Metallothioneins (MTs) are a family of low molecular mass (6–7 kDa), cysteine-rich, inducible, intracellular proteins that bind heavy metals with high affinity (1). The physiological functions of MTs have not been fully resolved. However, it is generally agreed that MTs play an important role in the homeostasis of essential metals and in the detoxification of heavy metals (2). In addition, MTs have been suggested to play an antioxidant role, since they contain sulfhydryl groups (3). There are four isoforms of MTs, namely MT-I, MT-II, MT-III and MT-IV. MT-I and MT-II are widely expressed in all tissues, whereas MT-III and MT-IV are expressed mainly in the central nervous system and the squamous epithelia, respectively (4,5). Protection against metal toxicity has been attributed to MT-I and MT-II, although MT-III is thought to play a role in Zn homeostasis in neurons (6). The function of MT-IV remains unknown.

Different types of factors, such as heavy metals (Zn, Cd, Co, Ni, Ag, Hg and Bi), glucocorticoids (e.g. dexamethasone), some alkylating agents (e.g. iodoacetate), oxidants/antioxidants [e.g., H2O2, tert-hydroquinone (t-BHQ)] and inflammatory signals (lipopolysaccharide) (7) can induce MTs. Induction by any of these compounds (except heavy metals) results in the accumulation of zinc bound to the newly synthesized apo-MT. The functions of MTs are uncertain, but they can detoxify heavy metals, provide a reserve of zinc and protect against oxidative stress (8). Induction of MT genes by metals requires multiple metal response element (MRE) sequences located in the promoter region and the zinc-finger transcription factor, MTF-1 (9). Treatment of cells with metals results in translocation of MTF-1 to the nucleus and binding to MREs (10). Direct binding of zinc to regulatory sites on MTF-1, activation of signal transduction pathways leading to covalent modification of MTF-1 and release of MTF-1 from regulatory molecules have all been proposed (11). The induction of MT-I and MT-II by glucocorticoids is mediated by the glucocorticoid receptor binding to a pair of glucocorticoid-response element (GRE) sequences upstream of the MT-II gene (12). The MT-I gene contains an antioxidant response element (ARE) sequence similar to those found in rat phase II genes NAD(P)H:quinone oxidoreductase (NQO1) and glutathione S-transferase A1 (GSTA1). The mechanisms by which other inducers activate MT gene transcription are less well understood.

Sulforaphane, an isothiocyanate first isolated from broccoli, has received intense attention for its chemopreventive potential of cancer because it is one of the most potent inducers of phase II detoxifying enzymes among many natural compounds (13). The phase II detoxifying enzymes that can be induced by
sulforaphane contain the ARE sequence in the promoter region of their gene (14). Direct linkage has been made between the activity of sulforaphane and the cellular molecular sensor, Nrf2–KEAP1 complex that regulates the induction of phase II enzymes. Nrf2, a member of the NF-E2 transcription factor family, induces phase II enzymes by binding to the ARE region of the promoter. Under basal condition, Nrf2 is suppressed by binding to Keap1, a cytoplasmic protein anchored to the actin cytoskeleton. It is consistent that although sulforaphane is chemoprotective in wild-type animals, it loses its efficacy in the reduction of benzo[a]pyrene-induced gastric tumors in Nrf2 deficient mice (15). Chemoprotection with sulforaphane also resulted in the delayed appearance of tumors. In HT29, a human colon cancer cell line, sulforaphane induces apoptotic cell death with the appearance of proapoptotic protein bax, release of cytochrome c into the cytosol and cleavage of poly(ADP-ribose) polymerase (PARP) (16). Enzyme induction has been observed in cell lines including murine hepatoma (HepG2), the BPrecl p450-deficient mutant and human adult retinal pigment epithelial cells (ARPE-19) as well as in organs (liver, stomach, small intestine and lung) of sulforaphane fed mice (17). A recent study in the rat reported plasma concentration of sulforaphane of the order of 20 μM, and the most robust cluster of genes is the MT-like genes (MT-1/2 and MT-1a), which increased up to 10-fold, by 2–4 h after sulforaphane dosing (18). However, the regulated mechanism of the induction of MT-like genes by sulforaphane is not very clear. Therefore, measuring the induction of MT genes may provide an efficient approach to understanding the chemopreventive mechanisms of sulforaphane compounds.

The hepatoma cell line, HepG2, not only resembles morphologically normal hepatocytes (19), but has also been shown to retain many of the enzymes involved in xenobiotic metabolism, including a functional Ah receptor (20), an inducible sulforotranserase (21) and an inducible NQO1 (22). In this cell line MT is also inducible, the predominant isofoms present in the control cells, being the MT-1 and MT-II forms of human MT. To experimentally test the hypothesis that sulforaphane may induce the expression of phase II MT genes, we used this highly differentiated human hepatoma cell line, HepG2, as a model to assess the effects of sulforaphane, on the induction of MT gene. In addition, the antiproliferative effects of sulforaphane in human hepatoma cell lines, HepG2, were also determined.

Materials and methods

**Chemicals**

Dubecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and trypsin-EDTA (T/E) were obtained from Gibco BRL (Grand Island, NY); d-l-sulforaphane (1-isothiocyanato-4-(methylsulfonyl)butane, >99% pure) was obtained from LKT Laboratories (St Paul, MN); N-acetyl-l-cysteine (NAC), catalase (CAT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the anti-rabbit IgG polyclonal antibody conjugated to peroxidase were obtained from Sigma Chemical (St Louis, MO); antibody against MT antibody was obtained from Calbiochem (San Diego, CA); antibodies against Nrf2 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against c-Jun N-terminal kinase (JNK), phospho-JNK (p-JNK), extracellular signal-regulated protein kinase (ERK), phospho-ERK (p-ERK), p38 MAPK, phospho-p38 MAPK (p-p38 MAPK), PARP and caspase 3 were obtained from Cell Signaling Technology (Beverly). Antibodies against Bcl-2, Bcl-X, and Bax were obtained from Pharmingen (San Diego). The inhibitors of mitogen-activated protein kinase (MAPKs), PD98059, SB203580, and SP600125 were obtained from Biosoarc (Camarillo, CA); polyvinilidifluoride (PVDF) membrane for western blotting was obtained from Millipore (Bedford, MA); a TRIzol RNA isolation kit was obtained from Life Technologies (Rockville, MD); 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.

**Cell culture**

Human hepatoma cells (HepG2 cells) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were grown in DMEM, supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.37% (w/v) NaHCO3, 0.1 mM NEAA, 1 mM sodium pyruvate and 0.03% l-glutamine at 37°C, in a humidified atmosphere of 95% air and 5% CO2. The culture medium was renewed each day. Cells were detached weekly, for transfer with 0.1% trypsin and 10 μM ethylenediaminetetraacetic acid (EDTA) in phosphate buffer saline (PBS).

**Cell survival assays**

Cell viability was determined by the MTT assay. HepG2 cells were seeded onto 96-well plates at a concentration of 1 × 105 cells/well in DMEM plus 10% FBS. After incubating for 24 h, the cells were treated with various concentrations of sulforaphane in 0.1% dimethylsulfoxide (DMSO) for 24 h. The percent viability of the treated cells was calculated as follows: (A570 mm − A530 mm/sample)/A570 mm − A530 mm/control × 100.

**Western blotting**

The sulforaphane-treated and untreated cells were rinsed twice with PBS. 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 for transfer with 0.1% trypsin and 10% (w/v) sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol and 0.1% bromophenol blue. 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 ultraviolet 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 MT signal, relative to the corresponding β-actin signal, from the same sample, expressing the data as the MT/β-actin ratio.
The mixtures were boiled at 95°C for 5 min, and then subjected to 12% SDS–polyacrylamide minigels at a constant current of 20 mA. Electrophoresis was ordinarily carried out on SDS–polyacrylamide gels (SDS-PAGE). Following electrophoresis, proteins on the gel were electrotransferred onto an immobile membrane (PVDF; Millipore) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine and 20% methanol. The membrane was then washed with Tris-buffered saline (TBS) (10 mM Tris and 150 mM NaCl) containing 0.05% Tween-20 (TBST) and blocked in TBST containing 5% non-fat dried milk. The membrane was further incubated overnight at 4°C with respective specific antibodies, such as MT1 (1:2000), Nrf2 (1:2000), p-ERK (1:2000), ERK (1:2000), p-JNK (1:1000), JNK (1:2000), p-p38 MAPK (1:2000), p38 MAPK (1:2000), caspase 3 (1:2000), PARP (1:2000), Bcl-2 (1:2000), Bcl-XL (1:2000), Bax (1:2000) and β-actin (1:5000). After hybridization with primary antibodies, the membrane was washed with TBST three times, then incubated with horseradish peroxidase-labeled secondary antibody for 45 min at room temperature, and washed with TBST three times. Final detection was performed with ECL™ (Enhanced Chemiluminescence) western blotting reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK).

DNA fragmentation analysis

HepG2 cells (2 x 10⁵ cells/ml) under different treatments were collected, washed with PBS twice and then lysed in 100 µl of lysis buffer [50 mM Tris (pH 8.1); 10 mM EDTA; 0.5% sodium sarkosinate and 1 mg/ml protease K] for 3 h at 56°C and treated with RNase A (0.5 µg/ml) for another hour at 37°C. The DNA was extracted by phenol:chloroform:isoamyl alcohol (v/v/v, 25:24:1) before loading and analyzed by 1.8% agarose gel electrophoresis. The agarose gels were run at 50 V for 90 min in Tris–borate/EDTA electrophoresis buffer (TBE). Approximately 30 µg of DNA was loaded in each well and visualized under UV light and photographed.

Measurement of caspase 3 activity

After treatment with sulforaphane, cells were collected and washed with PBS and lysed in lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris–HCl pH 8, 2 mM dithiothreitol, 2 mM phenylmethanesulfonyl fluoride, 10 µg/ml pepstatin A and 10 µg/ml leupeptin) for 20 min at 4°C followed by centrifugation (10000 x g) for 30 min. Caspase 3 activity was assayed in 50 µl reaction mixtures with fluorogenic report substrate peptide specific for caspase 3. The substrate peptide (200 µM) was incubated at 37°C with cysteolic extracts (50 µg of total protein) in reaction buffer [100 mM HEPES, 10% sucrose, 10 mM dithiothreitol, 0.1% 3-(3-chloroanilidopropyl(dimethylamino)-1-propanesulfonate)]. Fluorescence was measured after 2 h (excitation wavelength, 400 nm; emission wavelength, 505 nm) with a FLUOSTAR galaxy fluorescence plate reader (BMG LabTechnologies).

Statistical analysis

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

Results

Cytotoxicity of sulforaphane on HepG2 cells

To determine the appropriate concentration range of sulforaphane required to induce MT gene expression, the cytotoxicity of sulforaphane to HepG2 cells was investigated by an MTT assay. As shown in Figure 1, no significant effect on the cell growth of HepG2 cells was observed in the treatment <20 µM of sulforaphane. However, significant inhibitory effects were observed when the cells were treated with >50 µM of sulforaphane. The estimated IC₅₀ value (concentration causing 50% cell death) of sulforaphane was 65.2 µM. Our data showed that 20 µM is an appropriate concentration for MT induction in vitro.

Transcriptional induction of the MT gene by sulforaphane

To further investigate the MT mRNA expression induced by sulforaphane, we first evaluated MT-I and MT-II mRNA expressions in response to sulforaphane in HepG2 cells, which have been widely used in drug metabolism and chemoprevention studies. RT–PCR analyses were performed to examine the steady-state levels of MT-I and MT-II mRNA in HepG2 cells after exposure to the indicated concentration range of sulforaphane for 24 h. As shown in Figure 2, MT-I and MT-II mRNA expressions are coordinately increased by sulforaphane in a dose–response manner, as evidenced by a linear increase in mRNA expression by doses of sulforaphane.
ranging from 2.5 to 20 μM. MT-I and MT-II mRNA levels showed an initial rise at 2.5 μM and a peak induction at 20 μM. A maximum of 11.5-fold MT-I mRNA expressions was observed at 20 μM. This result indicates that MT-I and MT-II mRNA expression were upregulated after a 24 h exposure to the sulforaphane. Therefore, we believe that increased MT production by sulforaphane in HepG2 cells is regulated through transcriptional activation. Because the concentration of sulforaphane at 20 μM was capable of inducing maximal expression of MT in HepG2 cells, all subsequent experiments involving sulforaphane were performed using a concentration of 20 μM of sulforaphane.

Induction of MT protein expression in HepG2 cells by sulforaphane

To further explore the induction of MT protein expression by sulforaphane, dose-dependent experiments were performed with the HepG2 cells, using western blotting techniques. As shown in Figure 3A, MT protein was markedly induced by sulforaphane, in a dose–response manner, in concentrations of 2.5–20 μM. A maximum of 10.3-fold induction was observed at 20 μM. To know the induction time of MT protein by sulforaphane, a time-course study was also undertaken. Significant induction of MT protein was observed 24 h after the addition of 20 μM of sulforaphane (Figure 3B). The increased MT gene transcription, observed in HepG2 cells after exposure to sulforaphane, corresponded to increased protein expression (Figures 2 and 3). This may be a specific effect of sulforaphane on MT protein translation since β-actin protein levels were not affected by 20 μM of sulforaphane.

Sulforaphane stimulates Nrf2 protein expression

As a DNA-binding protein that recognizes the ARE enhancer sequence, Nrf2 functions as an important mediator in the expression of several phase II enzyme genes. The MT-I gene contains an ARE sequence similar to those found in rat phase II genes NQO1 and GSTa1. Since MT-I gene transcription induced by sulforaphane is dependent on protein expression (Figure 3), we examined whether sulforaphane has any effect on the levels of this transcription factor. HepG2 cells were treated with 20 μM of sulforaphane for 0.5, 1, 2 and 4 h, and Nrf2 protein levels were determined by western blot. As shown in Figure 4, Nrf2 protein expression was induced in a time-dependent manner. Nrf2 protein expression levels showed an initial rise at 0.5 h, and a peak induction at 4 h. The increase in Nrf2 protein by sulforaphane (3 h) occurs prior to the increase of MT protein (none at 3 h, Figure 3B).
These observations were consistent with the results from the MT protein expression (Figure 3), suggesting that the Nrf2 protein accumulation might contribute to the induction of ARE-mediated MT gene expression after sulforaphane treatment.

Involvement of the ERK and p38 pathway in the induction of MT expression by sulforaphane in HepG2 cells

Recently, studies on MT induction by oxidative stress stimuli have shown that pathways involving MAPKs are responsible for the transduction of signals to initiate gene activation (24). To determine whether a similar signal mechanism is responsible for the upregulation of MT expression by sulforaphane in HepG2 cells, we examined the activation states of three MAPK subfamilies, JNK, ERK and p38, in HepG2 cells. Cells were exposed to sulforaphane and then immunoblots were performed using antiphospho JNK, ERK and p38. As shown in Figure 5A, phosphorylated JNK, ERK and p38, indicating activation, were all increased by sulforaphane. The same blots were probed with antibody to total JNK, ERK or p38 as protein loading controls. The sulforaphane-mediated increase in MT protein expression was completely blocked by SB203580 (a specific inhibitor of p38) and PD98059 (a specific inhibitor of ERK), whereas similar concentration of SP600125 (a specific inhibitor of JNK) had no significant effect (Figure 5B). These results indicated that kinases of the ERK and p38 pathway might be involved in the regulation of MT expression by sulforaphane.

DNA fragmentation induced by sulforaphane in HepG2 cells

As described previously, the cytotoxicity of sulforaphane is possibly the result of apoptosis (Figure 1). To determine whether the inhibition of cell growth by high dose sulforaphane resulted from the induction of apoptosis, DNA fragmentation, a hallmark of apoptosis, was demonstrated by incubating HepG2 cell with different concentrations of sulforaphane for 24 h. After treatment with different concentrations of sulforaphane for 24 h, the genomic DNA from cells was subjected to 1.8% agarose gel electrophoresis. As shown in
Figure 6, the intact genomic DNA was found in control. DNA fragmentation became apparent at 30 μM of sulforaphane treatment, and these DNA fragmentation responses were dose dependent (Figure 6A). When cells were treated with 100 μM of sulforaphane, DNA ladders were just visible as early as 6 h in HepG2 cells after treatment, and gradually increasing DNA fragmentation was observed from 6 to 24 h (Figure 6B). DNA fragmentation was observed at 24 h in a dose-dependent manner with 50–250 μM, and at 50 μM in a time-dependent manner for 6–48 h. The efficacious induction for apoptosis was observed at 50 μM for 24 h, suggesting that the cell death induced by the sulforaphane treatment was mainly caused by apoptosis.

**Sulforaphane induced the caspase 3 activation**

Caspase 3 has been shown to play a pivotal role in the terminal execution phase of apoptosis induced by diverse stimuli. To monitor the enzymatic activity of caspase 3 during sulforaphane-induced apoptosis, we used the specific fluorogenic peptide substrate (Ac-DEVD-MCA) for the detection of caspase 3 activity. As illustrated in Figure 7, sulforaphane (100 μM) induced a dramatic increase of DEVD-specific caspase activity in treated HepG2 cells. The induction of DEVD-specific activity was rapid. As described previously, the cell death response induced by sulforaphane was mainly time dependent (Figure 6B), so we examined the time-dependent activation of caspase 3 as well. HepG2 cells were treated with 100 μM of sulforaphane for different periods (hours). Cytosolic proteins were extracted and assayed for caspase 3 activity by incubation with a fluorogenic peptide substrate (Ac-DEVD-MCA). As shown in Figure 7A, in HepG2 cells, sulforaphane treatment caused a significant time-dependent increase in the caspase 3 proteolytic activities (P < 0.05). Additional evidence of caspase 3 activation is the proteolysis of procaspase 3 into small active fragments and cleavage of PARP. Sulforaphane induced PARP cleavage, as evidenced by the accumulation of an 85 kDa fragment compared with the 115 kDa intact protein (Figure 7B). Furthermore, sulforaphane treatment caused the degradation of 32 kDa procaspase 3 which generates 20/11 or 17/11 kDa fragment in a time-dependent manner (Figure 7B) corresponding to those at which sulforaphane induces DNA fragmentation (Figure 6B). The control protein β-actin showed no change in the experiments. These results indicate that sulforaphane-induced HepG2 cell apoptosis involves a caspase 3 activation.

**Preventive effects of free radical scavengers on sulforaphane-induced apoptotic responses**

To examine whether the generation of intracellular reactive oxygen species (ROS) is a crucial step in sulforaphane-induced apoptosis, we investigated the preventive effects of free radical scavengers on sulforaphane-induced apoptosis. The cells were pretreated with free radical scavengers including NAC and CAT for 2 h at different concentrations, and then exposed to 100 μM sulforaphane for 24 h to determine DNA fragmentation. NAC or CAT at 2 mM and 100 U/ml respectively, did not induce DNA fragmentation and affect cell viability (data not shown). However, NAC and CAT at 2 mM and 100 U/ml respectively, completely inhibited sulforaphane-induced DNA fragmentation, suggesting that sulforaphane-induced apoptosis involves activation and ROS, which can be blocked by free radical scavengers including NAC and CAT (Figure 8).

**Regulation of Bcl-2 family proteins during sulforaphane-induced apoptosis**

The imbalance of expression of antiapoptotic and proapoptotic proteins after the stimulus is one of the major mechanisms underlying the ultimate fate of cells in the apoptotic process. We examined the time-dependent effects of sulforaphane on the constitutive protein levels of Bcl-2, Bcl-XL and Bax in the HepG2 cells. The western blot analysis exhibited a significant increase in the protein expression of Bax at a concentration of 100 μM of sulforaphane, which was observed up to 6 h post-treatment compared with the basal levels (Figure 9A). In contrast, the protein expression of Bcl-2 and Bcl-XL was significantly decreased by sulforaphane treatment at 100 μM. Similar decrease in Bcl-2 and Bcl-XL protein expressions with 100 μM sulforaphane was observed to be time-dependent (6, 12 and 24 h). A significant time-dependent shift in the ratio of Bax and Bcl-2 was observed after the sulforaphane treatment, indicating the induction of apoptotic process (Figure 9B).

**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 the...
isothiocyanates (25). Sulforaphane is one member of the isothiocyanate class of cancer chemopreventive compounds that has been shown to be effective in blocking initiation and progression of carcinogenesis (13). Several studies have demonstrated that sulforaphane can potently induce phase II detoxifying enzymes, which contributes to its chemopreventive functions (26). Furthermore, sulforaphane also effectively induced phase II MT gene (MT-I and MT-II) in rat livers in vivo by using 4967 oligonucleotides microarray (18). However, the molecular signaling mechanism by MT gene upregulation was not completely understood.

In this study, we investigated the signaling mechanisms of MT gene (MT-I and MT-II) upregulation following sulforaphane stimulation in HepG2 cells. The present work clearly demonstrates that sulforaphane at high concentrations (30–100 μM) were found to be antiproliferative. The estimated IC₅₀ value of sulforaphane was 65.2 μM. No significant effects on cell growth were observed in the treatment with sulforaphane at low concentration (2.5–20 μM) (Figure 1). This is in good agreement with the previous work of Rose et al. (27) showing beta-phenylethyl isothiocyanate (PEITC) induction of apoptosis and cell-cycle arrest in hepatoma HepG₂ cells. Thus, the cytotoxicity of sulforaphane was possibly the result of apoptosis. Our results indicate that sulforaphane at a higher concentration showed, in general, higher growth-inhibition. Since the concentration of sulforaphane <20 μM had no significant effects on cell growth, all subsequent experiments involving sulforaphane were performed using a concentration of 20 μM sulforaphane to avoid cytotoxicity.

Chemoprevention is one of the most promising areas in cancer research. Potential chemopreventive agents may function by a variety of mechanisms directed at all major stages of carcinogenesis. One proposed mechanism for cellular protection, against the chemical and neoplastic effects of carcinogens, involves the induction of phase II detoxification enzymes (28). There is considerable evidence that induction of phase II

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**Fig. 8.** Suppression of sulforaphane-induced DNA fragmentation by CAT and NAC. HepG₂ cells were pretreated for 1 h with CAT (50 U/ml, 100 U/ml) and NAC (1 mM, 2 mM), and then challenged with 100 μM sulforaphane for 24 h, and agarose gel analysis of DNA fragmentation was performed. This experiment was repeated three times with similar results. M, DNA ladder marker.

**Fig. 9.** (A) Effect of sulforaphane on protein expression of Bcl-2, Bcl-X₁, and Bax in HepG₂ cells. The cells were treated with vehicle (0.05% DMSO) only or sulforaphane (100 μM) for the specified time and then harvested. (B) Time-dependent effect of sulforaphane on Bax/Bcl-2 ratio. Data from three independent experiments are presented as mean ± SD. Statistically significant differences from the control are indicated as *P < 0.05.
detoxification enzymes can modulate the threshold for chemical carcinogenesis, increasing cellular resistance to carcinogen exposure (29). Sulforaphane has been shown to inhibit phase I enzymes (30), activate phase II enzymes (31), increase reduced glutathione concentration (32) and to induce apoptosis in many kinds of cancer cells (33). The present study demonstrates, for the first time, that sulforaphane is a potent inducer of MT gene (MT-I and MT-II) expression in HepG2 cells. We show that the levels of both MT-I and MT-II mRNA expressions are increased in a concentration-dependent manner by the treatment of the cells with sulforaphane (Figure 2). This result is consistent with previous report concerning treatment of rat with sulforaphane inducing MT gene expression (18). The present study also shows that both MT-I and MT-II mRNAs are coordinately increased following treatment of sulforaphane. This is in keeping with the previous reports that in rodents, MT-I and MT-II genes are coordinately expressed (7). In the present study, we have not determined whether the increase in MT mRNAs following sulforaphane treatment is the result of increased transcription and/or increased stabilization of MT mRNAs. However, accumulation of MT mRNAs following treatment by various inducers is predominantly controlled at the transcriptional level (7). Therefore, it can be inferred that the increase in MT mRNAs by sulforaphane might also be regulated mainly at the transcription level.

Next, we tested the ability of sulforaphane to increase MT protein expression, using the western blotting technique. The increased MT gene transcription, observed in HepG2 cells after exposure to sulforaphane, corresponded to increased protein expression. Densitometric quantification of the visualized bands of MT protein, revealed by western blot analysis, showed a significant induction in MT protein expression by sulforaphane, in a dose- and time-dependent manner (Figure 3). To our knowledge, this is the first reported western blot analysis of MT protein expression, in response to in vitro exposure to sulforaphane. The enhanced MT protein expression in HepG2 cells after treatment with sulforaphane corresponded to the induction of MT gene expression, suggesting that the observed induction of MT protein synthesis by sulforaphane is owing to activation of MT gene transcription (Figures 2 and 3). It has been reported that MT-I and MT-II are induced in a wide range of cell types by different classes of inducers, consistent with their roles in zinc and copper homeostasis, as well as defense against metal overload and oxidative stress (34). Also, it has a strong effect in scavenging free radicals because of its high thiol content (35). The regulation of MT gene expression is complex, involving a number of different transcription factors and signaling pathways. The ARE in the upstream promoter region of the MT gene and the transcription factor Nrf2 play a key role in the transcriptional regulation of the MT gene by multiple inducers (36). Nrf2 is usually co-located with the Keap1 protein in the cytoplasm. After activation by inducer, activated Nrf2 is dissociated from Keap1 and translocated into the nucleus. Once in the nucleus, activated Nrf2 dimerizes with other cofactors, such as mafG and binds to the AREs, activating MT gene expression (37). Although the transcription factors and cis-acting elements required for induction by antioxidants and glucocorticoids have been identified (38), the factors and binding sites mediating induction by sulforaphane stimuli are not established. The result of current study showed that sulforaphane can activate Nrf2 (Figure 4). The amount of Nrf2 protein expression is significantly increased by sulforaphane treatment in HepG2 cells, suggesting that increased expression of Nrf2 protein may play a key role in sulforaphane-induced MT gene activation.

MAPK pathways, including ERK and/or p38 MAPK have been reported to participate in the activation of the MT gene by inducing xenobiotics (24). Previous studies have shown that overexpression or activation of MAPKs differentially affects Nrf2 activity and phase II detoxifying enzymes (39). In addition, it has been shown that induction of phase II detoxifying enzymes by PEITC is dependent on c-JNK activation (40,41), and the induction of NQO1 by isothiocyanates treatments is mediated by ERK pathways (42). To identify other upstream regulatory mechanisms involved in sulforaphane-induced signaling events, MAPK pathways were also examined in the present study. Both ERK and p38 MAPK pathways are involved in the induction of MT expression by sulforaphane in HepG2 cells. ERK1/2 is activated by sulforaphane. Compared with the untreated HepG2 cells, sulforaphane-treated cells have higher levels of p-ERK and p38 MAPK, whereas the levels of p-JNK are not changed. Inhibition of the ERK1/2 MAPK pathway by PD98059 or inhibition of the p38 MAPK pathway by SB203580 partially blocked the increase of MT protein expression, whereas inhibition of both pathways almost completely blocked the sulforaphane-induced MT protein expression, suggesting the both ways are important for MT gene induction by sulforaphane. In a previous study, Shen et al. (43), reported that activation of MAPK pathways induces ARE-mediated gene expression via the Nrf2-dependent mechanism.

Apoptosis is one of the major mechanisms of cancer suppression. It is a highly regulated process that involves activation of a series of molecular events, leading to cell death that is characterized by cellular morphological change, chromatin condensation, and apoptotic bodies which are associated with DNA cleavage into ladders (44). Recently, apoptosis was suggested as a novel target for cancer chemoprevention (45). Fimognari et al. (46) reported that sulforaphane arrested cell-cycle progression in G1 phase by a significant downmodulation of cyclin D3 in transformed human T lymphocytes. Moreover, sulforaphane mediated growth arrest and apoptosis in human pancreatic cancer cell lines MIA PaCa-2 and PANC-1, and the production of ROS is involved in the process of sulforaphane action (33). As described previously, our results indicated that sulforaphane showed a strong growth-inhibitory effect with doses of sulforaphane ranging from 30 to 100 μM (Figure 1). The estimated IC50 value of sulforaphane was 65.2 μM. However, the mechanisms involved in these observations remain unclear. On the basis of the estimated IC50 values for HepG2 cells, we used the concentration range of 30–100 μM, which showed the most potent inhibitory periods, to investigate the mechanism of apoptosis induced by sulforaphane in HepG2 cells. The induction of apoptosis will stimulate endonuclease that involves double-strand DNA breaks into oligonucleosome length fragments, resulting in a typical ladder in DNA electrophoresis, and is one of the hallmarks of apoptotic cell death. As evidenced by DNA fragmentation, it appears that apoptosis is the main mechanism for cell killing in the presence of sulforaphane. The apoptosis-inducing effect of sulforaphane in HepG2 cells appeared in a concentration- and time-dependent manner (Figure 6). This efficacy of sulforaphane was found to be similar to their cytotoxic activity in HepG2 cells (Figure 1). Similar to a previous report (47), our
observations suggest treatment sulforaphane induced apoptotic cell death in human hepatoma cells.

Multiple lines of evidence indicated that apoptosis could be triggered by the activation of a set of death effector cysteine proteases called caspases with specificity for Asp-X bonds and their activations play important roles during apoptosis. In most of the apoptotic processes, caspase 3 has been shown to play a pivotal role in the terminal execution phase of apoptosis induced by diverse stimuli (48). We next examined whether the caspase 3 protease was involved in the sulforaphane-induced cell death response. Sulforaphane treatment induced the proteolytic processing of caspase 3 precursor in a time-dependent manner (Figure 7). Caspase can be grouped into ‘apoptotic initiator’, such as caspase 8, and ‘apoptotic effector’, such as caspase 3, according to their substrate specificities and target proteins (49). Caspase 3 is a cysteine protease that exists as an inactive zymogen in cells and becomes activated by some proteolytic events that cleave the 32 kDa precursor at aspartic acid residues to generate an active heterodimer comprising of 20 and 12 kDa sub-units (50). Furthermore, activation of caspase 3 leads to the cleavage of a number of proteins, including PARP. Although PARP is not essential for cell death, the cleavage of PARP is another hallmark of apoptosis. Gingras et al. (51) found that sulforaphane induced medulloblastoma cell death by apoptosis, as determined by DNA fragmentation and chromatin condensation. Medulloblastoma apoptosis correlates with the induction of caspases 3 and 9 activities, resulting in the cleavage of PARP and vimentin. In addition, sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards the growth of PC-3 xenografts in vivo (52). The results presented in the present study comprise the first report to show that sulforaphane inhibited the growth of human hepatoma cells and induced apoptosis, which is associated with the proteolytic degradation of PARP via the activation of caspase 3 (Figure 7).

Recent studies have reported that the generation of ROS and disruption of redox homeostasis could induce apoptosis. ROS have been demonstrated to play an essential role in G2/M phase cell-cycle arrest involving checkpoint kinase 2-mediated phosphorylation of cell division cycle 25C induced by sulforaphane in PC-3 human prostate cancer cells (53). Therefore, we investigated the role of ROS as the upstream signal to induce apoptosis in sulforaphane-treated HepG2 cells. Numerous investigations have indicated the involvement of ROS in apoptosis induced by various stimuli (54). In our study, the free radical scavengers including NAC and CAT can protect cells against the sulforaphane-induced DNA fragmentation apoptosis (Figure 8). NAC is a free radical scavenger and glutathione precursor, which can protect cells against oxidative damage. CAT is an antioxidative enzyme, which converts H2O2 to H2O and O2, and protects cells from ROS-mediated damage. These data suggested that ROS might be present in sulforaphane-induced cells.

Members of the Bcl-2 family of proteins are critical regulators of the apoptotic pathways. Additionally, Bcl-2 family proteins also have been demonstrated to be involved in the process of apoptosis (proapoptotic and antiapoptotic). Bcl-2 family proteins including Bax, Bak and Bcl-Xs for proapoptosis and Bcl-2, Bcl-XL and Mcl-1 for antiapoptosis have been identified. Previous studies indicated that an increase in proapoptotic Bcl-2 family proteins and a decrease in antiapoptotic Bcl-2 family proteins participated in the process of apoptosis (55).

Wang et al. (56) found that sulforaphane downregulated the expression of Bcl-2, a suppressor of apoptosis, and activated caspases to execute apoptosis in the prostate cancer cells. Furthermore, upregulation of Bcl-2 and activation of caspases 3, 9 and 8 were involved in sulforaphane-induced cell apoptosis. Bcl-2 has been shown to form a heterodimer with the proapoptotic member Bax and might thereby neutralize its proapoptotic effects (57). Therefore, alterations in the levels of Bax and Bcl-2, i.e. the ratio of Bax/Bcl-2, is a decisive factor and plays an important role in determining whether the cell will undergo apoptosis under experimental conditions that promote cell death. In our study, a decrease in Bcl-2 and Bcl-Xq expression was observed in sulforaphane-treated cells (Figure 9). Importantly, the protein expression of Bax, however, was upregulated in sulforaphane-treated cells after the treatment up to 24 h; hence the ratio of Bax to Bcl-2 was altered in favor of apoptosis. Our results suggested that the upregulation of Bax and the downmodulation of Bcl-2 and Bcl-Xq might be another molecular mechanism through which sulforaphane induced apoptosis in HepG2 cells.

Recent studies concerning bioavailability of glucosinolates and isothiocyanates are in agreement with their potential therapeutic effects (58). Sulforaphane, an isothiocyanate 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 (13). Related analogs of sulforaphane, the allyl isothiocyanate (AITC), 1-isothiocyanato-3-(methylthio)propane (ibvererin), 1-isothio- cyanato-4-(methylthio) butane (erucin), 1-isothiocyanato-3-(methylsulfinyl)propene (iberin), and 1-isothiocyanato-3-(methylsulfonyl)propene (cheirolin) were also active inducers of phase II detoxification enzymes in murine hepatoma cells (59). Sulforaphane, erucin (ERN, C4 methyl sulfide analog and erysolin (C4 methyl sulfone analog) induced GST and QR in various tissues of female CD-1 mice (60). The relative inductive activities of the various isothiocyanates in rat tissues are in accord with those determined in cells in vitro. In isolated cells, sulforaphane was far more active than AITC (61), but the two compounds were of similar activity in vivo, in terms of both degree of enzyme induction and site of effect. In vitro, there is a clear correlation between the inductive ability of an isothiocyanate and the degree to which it is accumulated within cells (62), and this is probably also true in vivo. A recent study in the rat reported plasma concentration of sulforaphane of the order of 20 μM and the most robust cluster of genes is the MT-like genes (MT-1/2 and MT-1a), which increased up to 10-fold, 2–4 h after sulforaphane dosing (18). Moreover, in a human intervention study, following a single dose of 200 μmol of isothiocyanates (largely, sulforaphane, with lesser amount of iberin and erucin), the total plasma isothiocyanate levels reached 0.94–2.27 μM after 1 h of feeding (63). Our study shows that in this concentration range, sulforaphane significantly causes elevation of phase II MT gene, constituting a very effective mechanism of elimination of potential carcinogens. Furthermore, apoptosis in the HepG2 cells is induced at a higher sulforaphane concentration (>30 μM). Our study shows that sulforaphane in physiological ranges of concentration causes phase II MT gene induction and does not induce apoptosis. Programmed cell death appears only after treatment of cells with sulforaphane concentrations higher than those occurring in human blood after ingestion of isothiocyanates.
In conclusion, the present studies show that at low concentrations, sulforaphane may activate the ERK and/or p38 MAPKs pathway which may lead to the induction of phase II MT gene expression through the Nrf2 transcription factor, resulting in protection and/or survival mechanism. However, when the cells are exposed to a high concentration, sulforaphane might generate an oxidant signal to stimulate ICE/Ced-3 protease (caspase 3) pathway activation, and DNA fragmentation leading to the decreased cell viability. Free radical scavengers including NAC and CAT, effectively inhibited apoptosis induced by sulforaphane. Furthermore, these effects were found to be correlated with a shift in Bax/Bcl-2 ratio more toward apoptosis. Our result is the first evidence that sulforaphane mediated induction of a phase II detoxifying enzyme MT and apoptosis in human hepatoma HepG2 cells. Taken together, our study provides evidence that sulforaphane may be useful in the chemopreventive treatment of human hepatoma.

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