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Effects of Isothiocyanates on Antioxidant Response Element-mediated Gene Expression and Apoptosis

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ABSTRACT

The pro-apoptotic effect of phenethyl isothiocyanate (PEITC) and the role of glutathione (GSH) in sulforaphane (SFN)-induced antioxidant response element-dependent gene expression were investigated. The caspase-3 and caspase-9 activities were stimulated by PEITC. The release of cytochrome c was time- and dose- dependent. SP600125 suppressed apoptosis induced by PEITC. Similarly, this JNK inhibitor attenuated both cytochrome c release and caspase-3 activation induced by PEITC. SFN is converted to the glutathione conjugate by glutathione S-transferases (GSTs). It was accumulated in mammalian cells by up to several hundred-fold over the extracellular concentration, by conjugation with intracellular GSH. The induction of ARE by SFN was 8.6-fold higher than by SFN-NAC. The decrease in ARE expression at higher concentrations of SFN and SFN-NAC was correlated with the accelerated apoptotic cell death, with a dose-dependent activation of caspase 3 activity by SFN. Upon addition of extracellular GSH within 6 hr of treatment with SFN, the effect on ARE expression was blocked almost completely.

INTRODUCTION

Isothiocyanates (ITCs), PEITC and SFN, are found in a number of cruciferous vegetables. Numerous epidemiological studies showed the diet containing vegetables reduces the risk of many types of cancers in human and ITCs has been proved to have chemopreventive effects in laboratory animals (Zhang et al., 1994; Chung et al., 2000). The main protective effects of ITCs against tumorigenesis is attributed to activities as inducers of phase 2 detoxifying enzymes (Brooks et al., 2001) and inhibiting cell growth by cell cycle arrest and activating apoptosis (Chiao et al., 2002). The inhibitory effects on phase 1 enzymes responsible for the bioactivation of carcinogens is also a reason for this protection (Maheo et al., 1997; Hecht, 1995). It was reported that PEITC induced JNK activation in HeLa and HT-1080 cells (Yu et al., 1996). In Jurkat cells, PEITC was found to induce a sustained activation of JNK1, and this was associated with activation of MEKK1 (Chen et al., 1998). It was also reported that in p53-deficient PC-3 cells PEITC induced the activation of ERK and p38 but not JNK (Xiao and Singh., 2002). In addition, Yang et al. (2002) showed that N-acetylcysteine conjugate of PEITC activated JNK, p38 and ERK in benzopyrene treated A/J mice. To better understand the anticancer mechanism of isothiocyanates, we examined the effect of PEITC on HT-29 human colon carcinoma cells. Here, we report that PEITC is capable of inducing apoptosis by releasing of cytochrome c from the mitochondria and activation of caspase-9 and caspase-3, and this mitochondrial caspase mediated apoptosis is related with activation of JNK.

SFN has received intense attention for its cancer chemopreventive activities because it is one of the most potent inducer of phase 2 detoxifying enzymes among natural compounds. The phase 2 enzymes which can be induced

by SFN, contain the antioxidant response element (ARE) sequence in the promoter region of their genes (Prestera and Talalay, 1995). Quinone reductase, glutathione S-transferase (GST) Ya, and γ-glutamylcysteine synthetase are the most common enzymes related with ARE. Furthermore, SFN was capable of inducing expression of phase 2 enzymes known to lack ARE, namely GST-a and microsomal GST (Brooks et al., 2001). In this report, we describe whether the intracellular levels of GSH play a critical role in the induction of phase 2 enzymes by SFN in HepG2 C8 cells. To modulate the levels of GSH in cells, SFN-NAC was used in the present study because SFN-NAC does not conjugate with intracellular GSH. Therefore, SFN-NAC has no effect on the transient decreasing of GSH levels shown in SFN-treated cells. We conclude that the transient decrease of intracellular GSH levels may be very important to induce ARE-related phase 2 enzymes.

MATERIALS AND METHODS

Cells and chemicals

HT-29 cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum. HepG2-ARE-C8 cell line was transfected cell with pARE-TI-luciferase construct (provided by Dr. William Fahl, University of Wisconsin) using a FuGENE™ 6 method (Yu et al., 2000). PEITC was obtained from Sigma (St. Louis, MO), SFN was from LKT Laboratories (St. Paul, MN). Mouse anti-cytochrome c monoclonal antibody was obtained from Pharmingen (San Diego, CA). SP600125, PD98059 and SB203580 were purchased from Calbiochem Technology (San Diego, CA). The fluorogenic tetrapeptide substrates of caspases (Ac-DEVD-MCA and Ac-LEHD-MCA) were purchased from Peptides International (Louisville, KY).

Assay of reporter gene activity

HepG2 C8 cells were cultured in fresh F-12 containing 0.5% FBS for 12 hr prior to drug treatment. The luciferase activity was determined according to the protocol provided by the manufacturer (Promega Corp., Madison, WI).

Glutathione assay

After treatment of drug for 24 hr, HepG2 C8 cells were harvested in 600 μL of 0.5% picric acid. The concentrations of total glutathione and oxidized glutathione (GSSG) were assayed by the methods of Griffith (1980). The levels of GSH was calculated from the difference between concentrations of total glutathione (GSH+GSSG) and GSSG. The intracellular levels of GSH was calculated based on cellular protein concentration.

Measuring of cytochrome c release

Cells in 100-mm culture dishes at confluence were treated with PEITC and harvested. Cells were homogenized with a dounce homogenizer for 10 strokes in 500 μL of lysis buffer. The cytosolic fractions (20 μg) were resolved by 10% SDS PAGE and transferred onto PVDF membrane (Immobilone P, Millipore). Cytochrome c on the membrane was detected immunochemically using anti-cytochrome c primary antibody, followed by HRP (Horseradish Peroxidase)-labeled secondary antibody (1 : 10,000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and by the ECL™ (Enhanced Chemiluminescence) western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions.

Caspase activity assay

After treatments with SFN, HepG2-C8 cells were washed twice with ice-cold PBS and lysed as described previously (Yu et al., 1998). The enzymatic activity of caspase 3 was assayed with 200 μM Ac-DEVD-MCA

fluorogenic substrates in assay buffer (100 mM HEPES, 10% sucrose, 10 mM DTT and 0.1% CHAPS) as described previously (Yu et al., 1998). The fluorescence was measured with a FLx 800 microplate fluorescence reader (Winooski, VT), with excitation at 360 nm and emission at 460 nm.

RESULTS AND DISCUSSION

PEITC induced caspase activities

To measure the activation of caspases in PEITC-treated cells, the specific fluorogenic tetrapeptide substrates for each caspase were used. As shown in Fig. 1A, PEITC strongly induced caspase-3 like activity at the concentration of 25 μM. And at the concentration of 50 μM, the activation of caspase-3 can be detected at about 7 h and peaks at 12 h. Caspase-9, the mitochondria damage related caspase, was also activated (Fig. 1B, the 12 hr treatment is significantly different from control (Student t-test, $p<0.05$)). This implied the possible involvement of mitochondrial damage in PEITC-induced apoptosis.

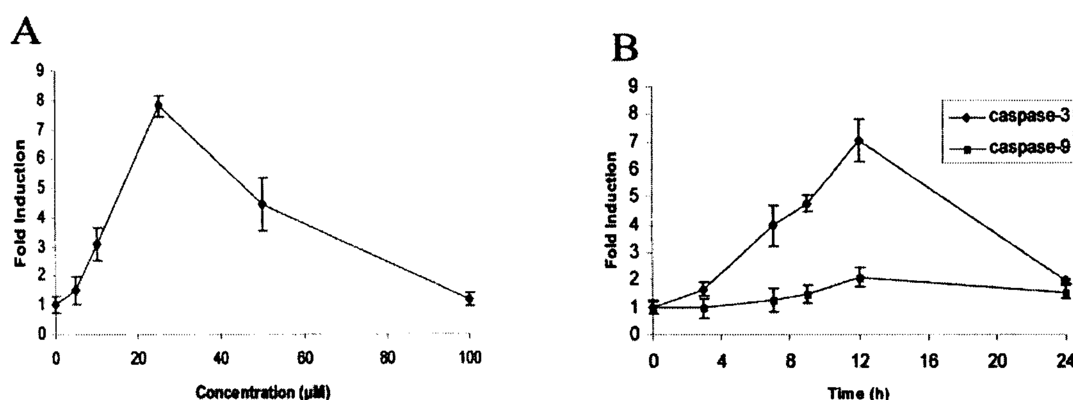

Fig. 1. Activation of caspases by PEITC. (A) Dose-dependent activation of capase-3. Cells were treated with different concentrations of PEITC for 12 h, and the caspase-3 activity was determined. (B) Kinetics of caspase-3 and 9 activation. Cells were treated at the indicated times with 50 μM PEITC. Enzymes activity is expressed as fold induction relative to vehicle treated control. The data shown are means±SD of three separate experiments done in duplicate.

The specific inhibitor of JNK, SP600125, attenuated PEITC-induced apoptosis

Studies using MAPK inhibitors were carried out to determine whether the activation of MAPKs contribute to PEITC-induced apoptosis in HT-29 cells. At 10 μM, SP600125 abolished the release of cytochrome c from mitochondria induced by PEITC, while the ERK and p38 inhibitors had no effect on PEITC-induced cytochrome c release or caspase-3 activity (Fig. 2). The caspase inhibitor zVAD-fmk failed to block the release of cytochrome c, indicating that JNK-mediated activation of the mitochondrial apoptosis pathway is not a secondary consequence of caspase activation.

We tested the involvement of the caspase pathway, by examining the effect of JNK inhibitor on caspase-3 activation. When cells were treated with PEITC for 12 h, caspase-3 activity was induced starting with 10 μM of PEITC treatment (Fig. 3). The effect was attenuated with the pretreatment of the JNK inhibitor SP600125 at 10 μM concentration. Fig. 2 and 3 showed that inhibition of the JNK pathway with SP600125 attenuated the effects of PEITC on mitochondrial cytochrome c release and caspase-3 activation. However, since inhibition of ERK by PD98059 and p38 by SB203580 did not prevent cytochrome c release or cell death induced by PEITC.

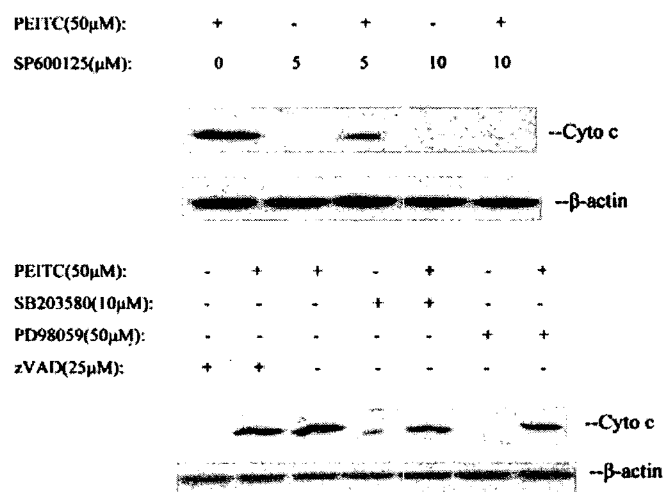

Fig. 2. The effect of MAPK inhibitors SP600125, SB203580 and PD98059, and caspase inhibitor zVAD, on the cytochrome c release from mitochondria induced by PEITC. SP600125, SB203580, PD98050 and zVAD were added as 1 h pre-treatment at the indicated concentrations. Cells were then treated with 50 μM PEITC for 10 h.

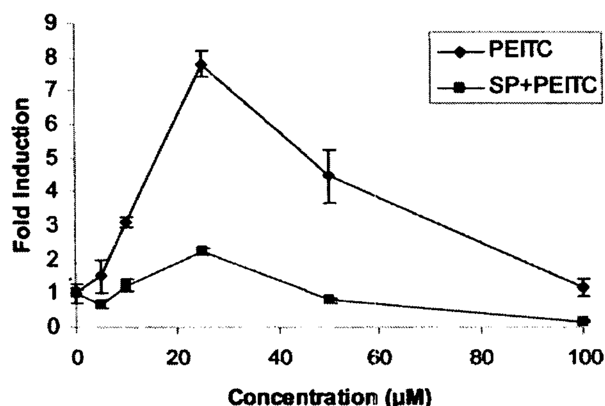

Fig. 3. The activation of caspase-3 by PEITC is blocked by SP600125. Cells were pre-treated with 10 μM of SP600125 and then treated with PEITC for 12 h. The data shown are means±SD of three separate experiments done in duplicate.

Dose-response of SFN and SFN-NAC on the expression of pARE-TI-luciferase

Biochemical and molecular biological studies show that the induction of phase 2 detoxifying enzymes is primarily due to the transcriptional activation of genes and is regulated by a cis-acting regulatory element, called ARE. ARE was first detected in 5'-flanking region of rat and mouse GST Ya subunit gene. HepG2 C8 cells were stably transfected with pARE-TI-luciferase gene into HepG2 cells. The cells were proved as a convenient tool for the monitoring the induction of ARE-mediated phase 2 enzymes. Because the expression of ARE-driven reporter gene, luciferase activity or green fluorescence protein (Ye and Zhang, 2001), was closely correlated with the induction of endogenous phase 2 enzyme activities by ITCs in HepG2 cells.

HepG2 C8 cells was incubated with several doses of SFN and SFN-NAC conjugate for 24 hr. The induction of luciferase activity was increased in a dose-dependent manner and peaked at 35 μM of SFN and 75 μM of SFN-NAC, respectively (Fig. 4A and 4B). This optimal concentration of SFN on the induction of luciferase was coincident with other studies for GST A1 and UDP-glucuronosyl transferase (UGT1A1) inductions in HT29 cells (Griffith, 1980). The highest induction of luciferase by SFN was about 8.6-fold of the highest induction by SFN-NAC.

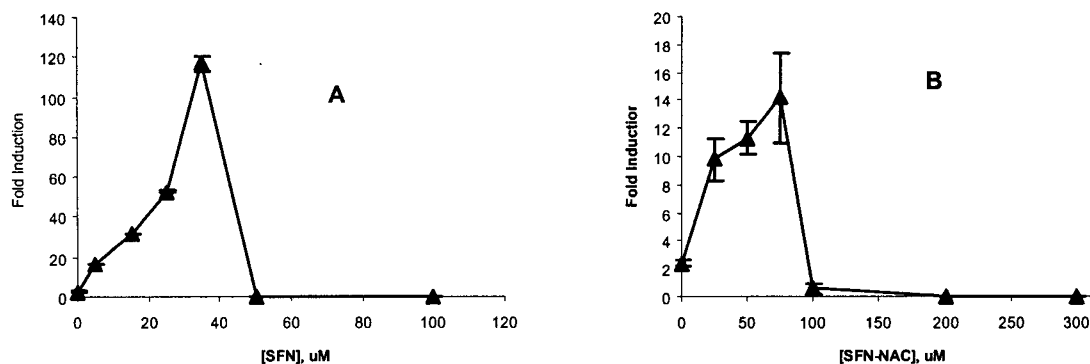

Fig. 4. Expression of ARE-LUC by SFN and SFN-NAC conjugate. HepG2 C8 cells were treated with several doses of SFN (A) and SFN-NAC conjugate (B) for 24 hrs and assayed the activity of luciferase. Bars, SE of triplicate data points.

Effects of SFN and SFN-NAC on the intracellular level of GSH

SFN and PEITC have been shown to decrease the intracellular GSH levels in murine hepatoma cells and human leukaemia cells by direct conjugation with reduced GSH (Xu and Thornalley, 2001) within 1 hr of incubation. However, SFN and PEITC induce the synthesis of GSH because the rate limiting enzyme for GSH synthesis, γ-GCS, has the ARE sequence in the promoter of its two subunit genes (Brooks et al., 2001; Xiao and Singh, 2002). There are some reports to show that intracellular GSH was increased in human ARPE-19 cells and mouse papilloma cells after exposure to SFN for 24 hr (Ye and Zhang, 2001; Gao et al., 2001). However, 48 hr treatments of SFN to 15 μM did not changed cellular GSH levels more than 18% in LNCaP cells (Brooks et al., 2001). The levels of GSH was decreased significantly in HL 60 cells by 24 hr culture with 5 μM PEITC. Therefore, it is meaningful to investigate whether SFN could increase the intracellular GSH levels in HepG2 C8 cells. The concentration of total GSH in HepG2 C8 cells at time zero was 61 nmol/mg protein. This concentration was little higher than in other cell types (Ye and Zhang, 2001). The concentration of GSSG was 9 nmol/mg protein, and the levels of reduced GSH was 52 nmol/mg protein. These values did not change significantly during incubation with vehicle. When HepG2 C8 cells were treated with several doses of SFN and SFN-NAC for 12 hr and 24 hr. The GSH levels after 24 hr treatment with 25 M SFN were increased gradually and reached to 1.8 fold but the intracellular levels of GSH after 12 hr treatment was not changed so much compared with the vehicle-treated control (Fig. 5). The treatment of SFN greater than 50 μM dropped the levels rapidly. The intracellular levels of

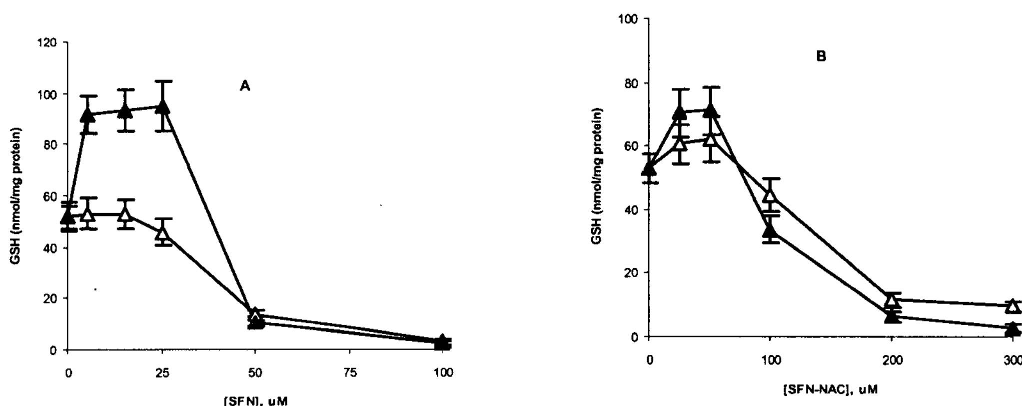

Fig. 5. Intracellular GSH levels in HepG2 C8 cells exposed to SFN or SFN-NAC conjugate. HepG2 C8 cells were incubated with SFN (A) and SFN-NAC (B) at the indicated concentrations for 12 (open) and 24 hr (solid).

GSH was about 1.3 fold at 25 μM SFN-NAC and was decreased gradually then reached near the levels of control cells at 100 μM in 12 and 24 hr treatment experiments. The patterns of SFN-NAC were quite different from the results of the cells treated with SFN and the differences seemed to be related with the conjugated status of SFN-NAC, even though, SFN-NAC can be dissociated to SFN slowly under physiological pH (Conaway et al., 2001).

Effects of GSH on the induction of ARE-luciferase by SFN

HepG2-C8 cells were exposed to 25 and 50 μM SFN and then 5 mM GSH was added at the time indicated, and finally the cells were harvested after 24 hr of SFN treatments. When cells were exposed to GSH and SFN simultaneously (0 hr), ARE-luciferase was induced about 2- to 3-fold of the control cells. If GSH was added within 3 hr after SFN treatment, SFN-induced ARE-luciferase was also minimal. However, the inductive effect of SFN on ARE increased quite substantially when GSH were added after 9 hr of SFN treatments. It was previously reported that cellular accumulations of SFN and SFN-GSH were almost completely blocked in the presence of 5 mM GSH in the medium (Zhang, 2000). However, as shown in Fig. 6, SFN-induced ARE-luciferase was blocked completely by adding GSH within 3 hr after SFN treatment (although cellular SFN concentration reached plateau within 1 hr, (unpublished observations, (Ye and Zhang, 2001)), similar to that when GSH was added at the same time as SFN. Even when GSH was added 9 hr after SFN treatment, it could still block the induction of ARE-luciferase by more than 50%. Since SFN can penetrate into cells rapidly (Zhang, 2000; Ye and Zhang, 2001; Zhang, 2001), these results indicated that exogenously added GSH could interfere with the sustenance of SFN-induced signaling and gene expression, however, the exact mechanism is currently unclear, but could be related to the multidrug resistance associated protein (MRP) transporters as reported recently (Zhang and Callaway, 2002) which would export SFN-GSH out of the cells.

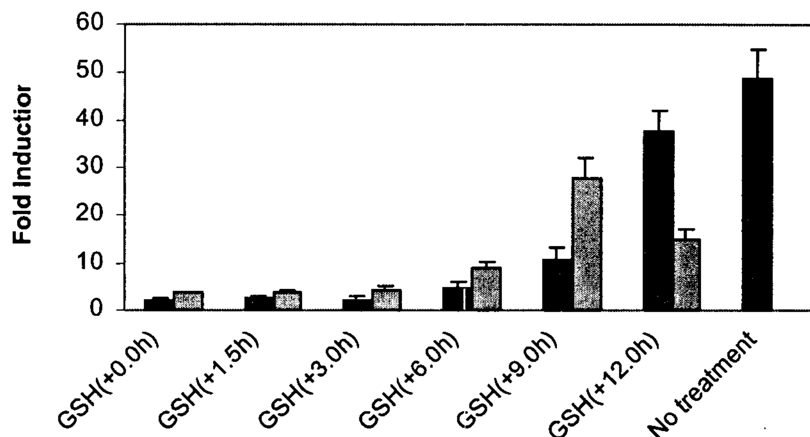

Fig. 6. Effect of GSH on the activation of SFN-induced expression of ARE-luciferase. HepG2-C8 cells were treated with 25 (solid) and 50 (open) μM SFN then 5 mM GSH was added at the time indicated. Cells were harvested at 24 hr. Bars, SE of triplicate data points.

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