Effects of Glutathione on Antioxidant Response Element-Mediated Gene Expression and Apoptosis Elicited by Sulforaphane

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ABSTRACT

Sulforaphane (SFN) and its N-acetyl-l-cysteine (NAC) conjugate are effective inhibitors of tumorigenesis in animal models. These compounds induce the expression of the antioxidant response element (ARE)-related genes and cause apoptosis. We studied the role of reduced glutathione (GSH) in the activations of ARE-mediated gene expression, apoptosis, and the activation of c-Jun NH2-terminal kinase (JNK) in HepG2-C8 cells. The cellular level of GSH decreased transiently when cells were exposed to SFN and then increased from 4 h, reaching 2.2-fold over control at 24 h. In contrast, SFN-NAC did not change the GSH level substantially during the time of incubation. ARE expression increased in a dose-dependent manner up to 35 μM SFN and 75 μM SFN-NAC, respectively. The induction of ARE by SFN was 8.6-fold higher than that by SFN-NAC. Pretreatment with l-buthionine sulfoximine increased SFN-induced ARE expression significantly. The decrease in ARE expression at higher concentrations of SFN and SFN-NAC was correlated with accelerated apoptotic cell death, with a dose-dependent activation of caspase 3 activity by SFN. On addition of extracellular GSH within 6 h of treatment with SFN, the effect on ARE expression was blocked almost completely. SFN was able to activate JNK1/2, and that activation was blocked by treatment with exogenous GSH. Taken together, these results suggest that the biological effects of SFN and SFN-NAC on the induction of ARE-related gene expression and apoptosis could be different from each other; however, the different effects on ARE-related gene expression and apoptosis elicited by SFN can be blocked by the addition of GSH.

INTRODUCTION

Numerous epidemiological studies have shown that a diet containing vegetables reduces the risk of many types of cancers in humans. ITCs1 found in cruciferous vegetables such as broccoli, cauliflower, and Brussels sprouts have been proven to have chemopreventive effects in laboratory animals (1, 2). The main protective effects of ITCs against tumorigenesis are attributed to activities such as induction of phase 2 detoxifying enzymes (3), inhibition of cell growth by cell cycle arrest, and activation of apoptosis (4). The inhibitory effects on phase 1 drug-metabolizing enzymes, which are responsible for the bioactivation of carcinogens, are also a potential contributing factor for this chemoprotection (5, 6). SFN, an ITC first isolated from broccoli, has received intense attention for its cancer-chemopreventive effects in laboratory animals (1, 2). The main protective effects of ITCs against tumorigenesis are attributed to activities such as induction of phase 2 detoxifying enzymes (3), inhibition of cell growth by cell cycle arrest, and activation of apoptosis (4). The inhibitory effects on phase 1 drug-metabolizing enzymes, which are responsible for the bioactivation of carcinogens, are also a potential contributing factor for this chemoprotection (5, 6). SFN, 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 2 detoxifying enzymes among many natural compounds (1). The phase 2 detoxifying enzymes that can be induced by SFN contain the ARE sequence in the promoter region of their genes (7). Quinone reductase, GST Ya, and γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH biosynthesis, are the most common enzymes related with the ARE-mediated transcriptional pathway (8, 9). In addition, SFN was also capable of inducing the expression of phase 2 enzymes that lack the ARE, namely GST-a and microsomal GST (3). SFN is conjugated with GSH in rats and humans. γ-Glutamyl and glycy1 residues are released from SFN-GSH conjugate by two peptidases with the subsequent formation of the cysteine conjugate, which is ultimately metabolized to SFN-NAC by N-acetyltransferase. In vivo intake of SFN is mostly excreted in the urine as SFN-NAC (10, 11). On exposure of several cell lines to ITCs, the ITCs were accumulated in cells and might cause the induction of phase 2 enzymes (12). If the cells were treated with a GSH-depleting agent, the ITCs could not be accumulated in the cells, and the subsequent induction of phase 2 enzymes was blocked (13). Therefore, the conjugating potential of SFN with GSH in cells appears to be critically important for the induction of phase 2 enzymes.

Several laboratories have shown that the basic leucine zipper transcriptional factors, including Nrf1, Nrf2 (14), and small Maf (15), are implicated in the binding to and transcription activation of the ARE DNA sequences. However, the mechanism of signal transduction for the induction of phase 2 genes by SFN is not very clear, but it may be related to the activation of the mitogen-activated protein kinases such as ERK2 (16). Previously, we have shown that SFN induced the activation of ERK2 in HepG2 cells, whereas PEITC activated ERK2 and JNK. Transfection of ERK2 cDNA plasmid into HepG2 cells increased the expression of ARE reporter gene by SFN, whereas ERK inhibitor blocked the induction of ARE-related phase 2 enzymes in Hepa1c1c7 cells (16).

In this report, we study whether the cellular level of GSH would play a critical role in the induction of phase 2 gene, apoptosis, and signaling by SFN in HepG2-C8 cells. SFN-NAC was used to modulate the levels of GSH in cells because SFN-NAC does not conjugate with intracellular GSH. Therefore, SFN-NAC will have no effect on the transient decrease in GSH levels as observed in SFN-treated cells. We also used BSO, a widely used GSH-depleting agent, to investigate whether the cellular GSH level is playing a role in the SFN-induced ARE-mediated gene induction. The other objective of this study was to investigate whether the induction of phase 2 gene and apoptosis are driven simultaneously by SFN. We conclude that the transient decrease of intracellular GSH levels may be important in the induction of ARE-mediated phase 2 genes and that the activation of caspase 3 and JNK might be triggered by higher concentrations of SFN than the concentration of SFN required for the induction of ARE-mediated gene. Furthermore, exogenous treatment of GSH blocks the induction of ARE-mediated gene expression even several hours after the cells were treated with SFN.

MATERIALS AND METHODS

Cells and Chemicals. The HepG2-ARE-C8 cell line was established in our laboratory. Human hepatoma HepG2 cells (purchased from American Type Culture Collection) were transfected with pARE-TI-luciferase construct (provided by Dr. William Fahl, University of Wisconsin) using the FuGENE 6

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4 The abbreviations used are: ITC, isothiocyanate; SFN, sulforaphane; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun NH2-terminal kinase; GSH, reduced glutathione; NAC, N-acetyl-l-cysteine; BSO, l-buthionine sulfoximine; ARE, antioxidant response element; GST, glutathione-S-transferase; PEITC, phenethyl isothiocyanate; FBS, fetal bovine serum; GSSH, oxidized glutathione; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.
method (17). Twenty-four h after transfection, cells were cultured in fresh medium containing 0.8 mg/ml G418. Clonal cells were selected for growth by limiting dilution and confirmed by the inducible western blotting reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

MTS Assay for Cell Viability. Cells were cultured in 96-well plates at a density of 10^4 cells/well. Twenty-four h later, cells were treated with different doses of SFN and SFN-NAC in the presence and absence of GSH for 24 h. The MTS assay was performed with the CellTiter 96 Aqueous nonradioactive cell proliferation assay kit (Promega Corp., Madison, WI). The absorbance was read at 490 nm on an ELISA reader, and the percentage of cell survival was obtained.

Caspase Activity Assay. After treatments with several concentrations of SFN for the times indicated in Fig. 9, HepG2-C8 cells were washed twice with ice-cold PBS and lysed for 60 min at 4°C in a buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM β-glycerophosphate, 15 mM MgCl_2, 15 mM EDTA, 100 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 150 μM/ml digitonin as described previously (20). Cell lysates were homogenized by passing through a 23-gauge needle three times. Homogenates were kept on ice for 30 min and then centrifuged at 12,500 × g for 20 min at 4°C. The supernatants were transferred into new tubes, and protein concentrations were determined by the Bradford method (Bio-Rad). 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% 3-[3-cholamidopropyl(dimethylammonio)-l -propanesulfonic acid) as described previously (20). The fluorescence was measured with a FLX 800 microplate fluorescence reader (Winooski, VT), with excitation at 360 nm and emission at 460 nm.

RESULTS

Dose-Response of SFN and SFN-NAC on the Expression of ARE-TIF-Luciferase and Cell Viability. We investigated the expression of ARE-mediated gene expression via the activity of the luciferase reporter gene in the cytosol of the HepG2-C8 cells [HepG2 cells stably transfected with a PARE-TIF-luciferase reporter gene (18)]. SFN, which is known to be one of the most potent inducers for phase 2 detoxifying enzymes (7), was added to HepG2-C8 cells for the indicated times as described in Fig. 1A. The luciferase activity of HepG2-C8 cells was assayed as described in “Materials and Methods” and normalized by protein concentration of the cells. The fold induction of the luciferase activity was calculated by dividing treated samples over the control. As shown in Fig. 1A, the luciferase activity started to increase after 6 h of SFN treatment and then increased very rapidly at 12 h. It peaked at 18 h (at about 58-fold) and dropped a little bit at 24 h. HepG2-C8 cells were incubated with several doses of SFN and SFN-NAC conjugate for 24 h. The induction of luciferase activity was increased in a dose-dependent manner, and the maximum level was 116.6- and 13.5-fold of control at 35 μM SFN and 75 μM SFN-NAC, respectively (Fig. 1, B and C). Further increasing the concentrations of SFN and SFN-NAC dramatically reduced the activity of luciferase back to zero. Because SFN and its NAC conjugate did not inhibit in vitro activity of luciferase (data not shown), the
decrease of ARE-luciferase activity in cells treated with high concentrations of SFN and SFN-NAC appears to be driven by blockade of the expression of ARE-luciferase gene, cytotoxicity, and/or apoptosis induced by these compounds. The viability of HepG2-C8 cells was subsequently investigated using MTS assay. Cell viability was decreased with increasing concentrations of SFN and SFN-NAC and reached <20% of the viability of the control cells at 100 μM SFN and 300 μM SFN-NAC, respectively (Fig. 2, A and B). Interestingly, the slope of decreasing viabilities was very gradual in HepG2-C8 cells treated with SFN (20–35 μM) and SFN-NAC (<75 μM). However, as described above, in these concentration ranges of SFN and SFN-NAC, the inductions of ARE-luciferase activity were very potent, as shown in Fig. 1, B and C. These results suggested that when the expression of ARE-mediated gene was high, HepG2-C8 cells might become more resistant to the toxicity of SFN. To investigate whether GSH can protect the cytotoxic effects of SFN and SFN-NAC against the viabilities of the cells, HepG2-C8 cells were pretreated with exogenous GSH for 1 h, and then the cells were challenged with 100 μM SFN or 300 μM SFN-NAC. As shown in Fig. 2C, the cell viabilities were very low in HepG2-C8 cells when treated with these concentrations of SFN and SFN-NAC; however, the cell viabilities were improved dramatically back to the control levels with exogenous GSH pretreatment.

Effects of GSH on the Induction of ARE-Luciferase by SFN and SFN-NAC. As shown above in Fig. 1, B and C, the expression of the ARE-luciferase gene was induced by the treatments with SFN and SFN-NAC. This expression was further investigated in the presence of exogenous GSH. We chose two concentrations of these compounds, one (25 μM SFN and 50 μM SFN-NAC) was defined as “suboptimal” (lower concentration) for the ARE induction, and the other (50 μM SFN and 100 μM SFN-NAC) was an “overdose” in the induction of ARE (as shown in Fig. 2). As shown in Fig. 3A, in cells treated with suboptimal concentrations of SFN and SFN-NAC, addition of exogenous GSH decreased the expression of the ARE-luciferase gene. However, in contrast, for the completely diminished ARE expression with the higher “overdose” of SFN and SFN-NAC, the ARE induction was induced substantially by treatments of exogenous GSH with the maximum induction with 5.0 mM GSH. Next, we studied the effects of the time sequence of adding exogenous GSH to cells treated with SFN. As shown in Fig. 3B, HepG2-C8 cells were exposed to 25 μM SFN, and then 5 mM GSH was added at the time indicated, and finally, the cells were harvested after 24 h of SFN treatment. When cells were exposed to GSH and SFN simultaneously (0 h), ARE-luciferase was induced about 2–3-fold of the control cells. If GSH was added within 3 h after SFN treatment, SFN-induced ARE-luciferase was also minimal (about 2–3-fold of control). However, the inductive effect of SFN on ARE increased quite substantially when GSH was added after 9 h of SFN treatment, but this induction was still <50% of that of SFN treatment without addition of GSH.

Effects of SFN and SFN-NAC on the Cellular Level of GSH. To study the role of intracellular GSH, we measured the level of intracellular GSH after exposure of the cells to SFN and SFN-NAC. As shown in Fig. 4, the concentration of total GSH in HepG2-C8 cells at time 0 (control untreated cells) was about 51.6 nmol/mg protein. This concentration was slightly higher than those reported in other cell types (13). The concentration of GSSG was about 7.1 nmol/mg protein, and the level of GSH was around 44.5 nmol/mg protein. These values did not change significantly during the incubation with the DMSO vehicle. When HepG2-C8 cells were treated with 25 μM SFN for up to 24 h, the GSH level decreased in a time-dependent manner and reached a nadir of 25.4 nmol/mg protein at 4 h of SFN treatment (Fig. 4). Then, the level increased gradually after 4 h, reaching the control level at 12 h and 2.2-fold of the control at 24 h. Liquid chromatography-mass spectrometry analysis showed that the conjugation of SFN with intracellular GSH in HepG2-C8 cells reached a peak at 0.5 h after treatment with SFN, and then the level of SFN-GSH decreased, with a detectable level at 24 h after treatment.
with SFN (data not shown). Interestingly, however, the intracellular GSH level was not affected dramatically by the treatment of cells with 50 μM SFN-NAC (despite induction of ARE-luciferase activity as seen in Fig. 1C). Additionally, SFN-NAC did not change the GSH level in the early stage of incubation with HepG2-C8 cells, with only a slightly induced level at 24 h. To further ascertain whether the depletion of cellular GSH is playing a role in SFN-induced ARE activation, we used BSO, a GSH-depleting agent, to look at the effect of GSH on the ARE expression. As shown in Fig. 5, HepG2-C8 cells were preincubated with BSO at the indicated concentrations for 24 h, and the media containing BSO were removed and replaced with fresh media. The cells were then treated with DMSO (control), 25 μM SFN, and 35 μM SFN for another 24 h, and 250 μM BSO had the highest induction of ARE activity on the SFN-treated cells (about 2-fold on the 25 μM SFN-treated cells and 3-fold on the 35 μM SFN-treated cells, respectively), whereas various concentrations of BSO had no effect on the control cells (no SFN treatment).

**Effect of SFN on Caspase 3 Activation.** Previously, SFN has been shown to induce apoptosis in different cell types (4, 20–22); however, the molecular mechanism is still unclear. One of the key events leading to apoptosis is the activation of a cascade of intracellular ICE/Ced-3 proteases known as caspases (23). To date, at least 13 caspases have been identified in mammalian cells. Caspase 3 has been shown to play the final and critical role in the terminal, execution phase of apoptosis (24, 25). This enzyme cleaves a variety of substrates so that cells undergo the typical morphological and biochemical changes observed in apoptosis. The activity of caspase 3 was measured with Ac-DEVD-AMC in the lysate of HepG2 C8 cells treated with SFN. As shown in Fig. 6, caspase 3 activity was activated gradually during the incubation with SFN up to 24 h; furthermore, the activity of caspase 3 was increased in a dose-dependent manner. The caspase 3 activity was stimulated strongly with 50 μM SFN compared with 25 μM SFN. The activation of caspase 3 by 50 μM SFN was almost completely blocked by pretreatment with GSH at concentrations as low as 250 μM (Fig. 6, bottom panel). This result suggested that exogenous GSH could block one of the apoptotic processes induced by SFN via the caspase 3 pathway.

**Effect of SFN on JNK Activation.** To investigate the early signaling event modulated by SFN, HepG2-C8 cells were exposed to SFN for 1 h, and the activation of JNK1/2 was analyzed by the use of anti-phospho-JNK antibody. As shown in Fig. 7 (top panel), JNK1/2 were activated in a dose-dependent manner and reached a maximum activation with 50 μM SFN in these HepG2-C8 cells. Interestingly, the activation of JNK1/2 by 50 μM SFN was blocked almost completely by pretreatment with GSH at concentrations as low as 250 μM (Fig. 7, bottom panel).

**DISCUSSION**

The ARE was first identified in the 5'-flanking region of the rat GST Ya subunit gene (26). The expressions of ARE-mediated genes
including GSTs, quinone reductase, and γ-glutamylcysteine synthetase were induced by ITCs such as SFN in different cell types (1, 13). HepG2-C8 cells were stably transfected with pARE-TI-luciferase gene in HepG2 cells and established in our laboratory (18). This cell line has been proven to be a convenient tool for monitoring the induction of ARE-mediated phase 2 genes. In the present study, we investigated the role of GSH on the biological responses of SFN and SFN-NAC in HepG2-C8 cells. SFN and PEITC have been shown to decrease the cellular GSH level in several cell lines by direct conjugation with GSH within 30 min of addition (12). There are also reports showing that the GSH level was increased in human ARPE-19 cells and mouse papilloma cells after exposure to SFN for 24 h (13, 27). However, 48-h treatments with 15 μM SFN increased the GSH level only 18% of control in LNCaP cells (3), and the level was decreased significantly in HL60 cells by 24 h with 5 μM PEITC (28). As shown in the present study (Fig. 7), SFN significantly decreased the cellular GSH level at 4 h and then reached 2.2-fold of the control at 24 h. However, SFN-NAC conjugate had little effect on the GSH level, although SFN-NAC can induce ARE reporter gene (Fig. 2B). The difference in the changes of the cellular GSH level by SFN and SFN-NAC may be related to the limited conjugation status of SFN-NAC. Although SFN-NAC can be dissociated to SFN slowly under physiological pH (29), this may not provide sufficient time and amount for the dissociated SFN to alter the GSH level. BSO was proved to decrease the cellular GSH level efficiently without changing cell viability in the HepG2 cell line (30). The cellular GSH level was decreased 70% when treated with 100 and 500 μM BSO (31). The expression of ARE-luciferase by SFN was increased in HepG2-C8 cells that were preincubated with BSO for 24 h. The increase was BSO dose dependent up to 250 μM. This showed that decreasing GSH level is essential for ARE induction. The effect of SFN on the induction of ARE-luciferase was blocked if the GSH level of SFN-treated HepG2-C8 cells was increased by exogenous GSH or NAC. These data suggested that SFN or SFN-GSH (or SFN-NAC) by itself and/or coupled with the simultaneous decrease of cellular GSH level could be essential for the induction of ARE-mediated phase 2 genes. The cellular SFN concentration of HepG2-C8 cells treated with 50 μM SFN was higher than that of cells treated with 25 μM SFN. Therefore, the cellular GSH level would have been decreased in the early period of SFN treatment. If decreasing cellular GSH level per se was sufficient for ARE induction, then exposure of HepG2-C8 cells to 50 μM SFN should have induced the ARE-mediated genes more so to compensate for the stronger stress challenge as compared with that seen with 25 μM SFN. Half of the cells were alive with 50 μM SFN treatment, but there was completely no induction of ARE-luciferase at 24 h of treatment (Figs. 2A and 3A). Additionally, the activation of caspase 3 by 50 μM SFN was much higher than that by 25 μM SFN. As shown in Fig. 3, the viability of HepG2-C8 cells started to decrease rapidly when SFN concentration reached 50 μM. Hence, the decreased viability might be due to the induction of apoptosis as seen in the activation of caspase 3. The addition of exogenous GSH to HepG2-C8 cells, which were exposed to 50 or 100 μM SFN, showed an increase in the induction of ARE-luciferase, protected the cells against cell death, and blocked the activation of caspase 3 activity. These results implied that the treatments of exogenous GSH blocked SFN-induced apoptosis via possibly increasing the cellular level of GSH. However, the increased GSH level can also block the transiently decreased GSH level in cells exposed to suboptimal concentrations of SFN, where GSH might be important to trigger the expression of ARE-related genes (Fig. 5). It was reported previously that cellular accumulations of SFN and SFN-GSH were almost completely blocked in the presence of 5 μM GSH in the medium (12). However, as shown in Fig. 6, SFN-induced ARE-luciferase was blocked completely by adding GSH within 3 h after SFN treatment (although cellular SFN concentration reached plateau within 1 h) (13), similar to that seen when GSH was added at the same time as SFN. Even when GSH was added 9 h after SFN treatment, it could still block the induction of ARE-luciferase by >50%. Because SFN can penetrate into cells rapidly (12, 13, 32), 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 it could be related to the multidrug resistance-associated protein transporters as reported recently (33), which would export SFN-GSH out of the cells.

JNK is primarily activated by cellular stresses including heat, osmotic shock, DNA-damaging agents, chemotherapeutic agents, and hypoxia/reoxygenation agents (34). These activations resulted in cell growth arrest and cell death (8). In this regard, the activation of JNK by ITCs has been proposed to serve as a switch for apoptosis (25, 35, 36). We have shown previously that JNK1 was activated by PEITC in HeLa cells and HepG2 cells, whereas SFN slightly activated JNK1 and p38 but strongly activated ERK2 and ERK2 activation could be involved in ARE-mediated gene expression (16, 17). SFN has been reported to induce apoptosis in different mammalian cells from different laboratories (4, 21, 22). As shown in Fig. 7, SFN activated JNK1/2 in a dose-dependent manner, especially at concentrations greater than 25 μM, and 50 μM SFN showed the maximum activation. As described above, at 50 μM, SFN blocked the expression of ARE-luciferase, activated caspase 3, and induced about 50% cell death. These results suggested that JNK1/2 could play a role in SFN-induced apoptotic signaling, and future studies would yield further insights.

In conclusion, the inductive effect of ARE-luciferase by SFN is dependent on the reduction of the cellular level of GSH driven by SFN. Strong induction of phase 2 genes by SFN can potentially protect cells from its cytotoxic effect. However, when the cells are exposed to high concentrations of SFN, the expression of ARE-mediated genes would shut down completely, and the cells would start to die. The cell death effect is accelerated with >50 μM SFN. At these concentrations, SFN can activate caspase 3 as well as JNK1/2, which can be blocked by exogenous GSH. Interestingly, exogenously added GSH will also partially block ARE induction after 9 h of SFN exposure, implicating the overall important roles of GSH in SFN-mediated signaling, gene expression, and apoptosis. Future studies by integrating other kinds of cellular mediator(s), which can sense the extent of SFN-induced cellular stress and then decide the direction of signaling between survival and death, as exemplified with the role of p53 in DNA damage cellular response (37), will further elucidate the biological mechanisms of SFN.

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References


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