Sulforaphane and \(\alpha\)-Lipoic Acid Upregulate the Expression of the \(\pi\) Class of Glutathione S-Transferase through c-Jun and Nrf2 Activation\(^1,2\)

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Abstract

The anticarcinogenic effect of dietary organosulfur compounds has been partly attributed to their modulation of the activity and expression of phase II detoxification enzymes. Our previous studies indicated that garlic allyl sulfides upregulate the expression of the \(\pi\) class of glutathione S-transferase (GSTP) through the activator protein-1 pathway. Here, we examined the modulatory effect of sulforaphane (SFN) and \(\alpha\)-lipoic acid (LA) or dihydrolipoic acid (DHLA) on GSTP expression in rat Clone 9 liver cells. Cells were treated with LA or DHLA (50–600 \(\mu\)mol/L) or SFN (0.2–5 \(\mu\)mol/L) for 24 h. Immunoblots and real-time PCR showed that SFN, LA, and DHLA dose dependently induced GSTP protein and mRNA expression. Compared with the induction by the garlic organosulfur compound diallyl trisulfide (DATS), the effectiveness was in the order of SFN > DATS > LA = DHLA. The increase in GSTP enzyme activity in cells treated with 5 \(\mu\)mol/L SFN, 50 \(\mu\)mol/L DATS, and 600 \(\mu\)mol/L LA and DHLA was 172, 75, 122, and 117\%, respectively (\(P < 0.05\)). A reporter assay showed that the GSTP enhancer I (GPEI) was required for GSTP induction by the organosulfur compounds. Electromobility gel shift assays showed that the DNA binding of GPEI to nuclear proteins reached a maximum at 0.5–1 h after SFN, LA, and DHLA treatment. Super-shift assay revealed that the transcription factors c-jun and nuclear factor erythroid-2 related factor 2 (Nrf2) were bound to GPEI. These results suggest that SFN and LA in either its oxidized or reduced form upregulate the transcription of the GSTP gene by activating c-jun and Nrf2 binding to the enhancer element GPEI. J. Nutr. 140: 885–892, 2010.

Introduction

Epidemiologic studies have found that persons who consume a high proportion of vegetables and fruits in their diet may decrease their cancer risk (1,2). This can be partly attributed to the rich content of numerous phytochemicals in vegetables and fruits, including polyphenolic compounds, carotenoids, and organosulfur compounds (2–5). The accumulated evidence supports that garlic alliin-derived allyl sulfides and cruciferous isothiocyanates protect animals against a variety of chemical carcinogens (1,6). This chemoprevention can be partly explained by the potency of these phytochemicals in modulating the activity and gene expression of phase II detoxification enzymes (7–9).

Glutathione S-transferase (GST)\(^5\) is a phase II enzyme that catalyzes the conjugation of glutathione with a variety of electrophilic xenobiotics and facilitates their excretion. In mammals, 8 GST isozymes, including \(\alpha\) (\(\alpha\)), \(\mu\) (\(\mu\)), \(\omega\) (\(\omega\)), \(\pi\) (\(\pi\)), \(\sigma\) (\(\sigma\)), \(\theta\) (\(\theta\)), \(\zeta\) (\(\zeta\)), and \(\kappa\) (\(\kappa\)), have been identified (10). Recently, interest has grown in the physiologic properties of the \(\pi\) class of GST (GSTP), not only because of its function in drug detoxification but also because of its possible roles in cell transformation and carcinogenesis (11,12). GSTP activity has been used to evaluate the potency of chemoprevention agents in

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\(^{5}\) Abbreviations used: AP-1, activator protein-1; ARE, antioxidant response element; DATS, diallyl trisulfide; DHLA, dihydrolipoic acid; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EMSA, electromobility gel shift assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPEI, \(\pi\) class of glutathione S-transferase enhancer I; GST, glutathione S-transferase; GSTA, \(\alpha\) class of glutathione S-transferase; GSTM, \(\mu\) class of glutathione S-transferase; GSTP, \(\pi\) class of glutathione S-transferase; LA, \(\alpha\)-lipoic acid; NQO1, NAD(P)H-dependent quinone oxidoreductase 1; Nrf2, nuclear factor erythroid-2 related factor 2; SFN, sulforaphane; TRE, 12-0-tetradecanoylphorbol-13-acetate responsive element.
benz[a]pyrene-induced cancer (13). The importance of GSTP in cancer prevention is further supported by the fact that 7,12-dimethylbenzanthracene-induced skin cancer is significantly elevated in GSTP-null mice (14). Two enhancing elements were identified in the 5′ upstream region of the GSTP gene and were named GSTP enhancer I (GPEI, −2.5 kb) and II (GPEII, −2.2 kb) (15). GPEI has 2 phorbol-12-O-tetradecanoyl-13-acetate responsive element (TRE)-like elements that are considered to be required for basal and inducible expression of GSTP (16). Enhancers of GSTP expression are regulated by multiple factors, including activator protein-1 (AP-1), which is known to be a heterodimer or homodimer composed of the products of c-Jun and c-fos (17). Because the TRE-like elements in GPEI share sequences similar to those of the antioxidant response element (ARE), nuclear factor erythroid-2 related factor 2 (Nrf2) is also regarded as a possible transcriptional factor that binds to GPEI (18).

Sulforaphane (SFN), an isothiocyanate compound rich in cruciferous vegetables, has been demonstrated to be highly effective in affording protection against chemically induced cancer in animal models (6,9). This cytoprotection by SFN can be attributed to its activation of apoptosis and also its effective induction of the expression of phase II detoxification and antioxidant enzymes, including the α class of GST (GSTA), μ class of GST (GSTM), NAD(P)H-dependent quinone oxidoreductases 1 (NQO1), and γ-glutamylcysteine synthase (9,19,20). Recently, the upregulation of the gene expression of cytoprotective genes by SFN was shown to be dependent on Nrf2-ARE (21,22).

α-Lipoic acid (LA) is a thiol antioxidant distributed in vegetables, including broccoli, spinach, and tomatoes (23). LA and its reduced form, dihydrolipoic acid (DHLA), not only act as potent free radical scavengers and metal chelators (24) but also participate in the recycling of other cellular antioxidants, including vitamin C, vitamin E, and glutathione (25). Recently, the expression of several phase II enzymes was reported to be modulated by LA and DHLA. In human leukemia HL-60 cells and neuroblastoma SH-SY5Y cells, LA is effective at upregulating NQO1 gene transcription (26,27). LA induction of GSTA2 expression is likely associated with the phosophatidylinositol 3-kinase pathway (8). Regarding GSTP, however, it is not clear whether LA and DHLA induce the expression of this detoxification enzyme.

Recently, we reported that garlic oil and 2 of its major organosulfur components, diallyl disulfide and diallyl trisulfide (DATS), can effectively upregulate GSTP mRNA and protein expression. Moreover, GPEI is required for the induction of this phase II enzyme (28–30). In addition to the garlic allyl sulfides, we were also interested in examining whether organosulfur compounds not derived from garlic are also effective at upregulating GSTP expression and the possible transcription factors involved. Therefore, in the present study, we examined the modulatory effect of SFN, LA, and DHLA on GSTP expression in rat liver Clone 9 cells. Moreover, we compared the relative induction potency on GSTP of DATS, LA, DHLA, and SFN.

Materials and Methods

Materials. All other chemicals were purchased from Sigma-Aldrich unless specified otherwise. SFN and DATS were obtained from LKT Laboratories. RPMI-1640 medium and penicillin-streptomycin solution were obtained from Gibco Laboratory. RNase inhibitor, oligo dT, and Moloney murine leukemia virus RT were purchased from Promega. GSTP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer were obtained from Applied Biosystems. Fetal bovine serum was purchased from HyClone. Trizol and lipofectamine were ordered from Invitrogen.

Cell culture. Clone 9 cells, which were derived from normal rat livers, were obtained from Bioresources Collection and Research Center. They were grown in RPMI-1640 medium supplemented with 10 mmol/L HEPES, 100 KU/L penicillin, 100 mg/L streptomycin, and 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2 and 95% air. For all studies, cells between passages 4 and 10 were used. The cells were plated on 35-mm plastic tissue culture dishes (Falcon) at a density of 2.5 × 104 cells/dish and were allowed to grow for 24 h. Fresh culture medium containing various concentrations of DATS, LA, DHLA, or SFN was then added and the cells were incubated for the indicated times. Cells treated with 0.1% dimethylsulfoxide (DMSO) alone were used as controls.

SDS-PAGE and Western blot. Cells were washed twice with cold PBS and were then harvested in 300 μL of 20 mmol/L potassium phosphate buffer (pH 7.0). Supernatants were centrifuged at 10,000 × g for 30 min at 4°C. Protein concentrations were determined with the Coomassie Plus Protein Assay Reagent kit (Pierce Chemical). Four micrograms of cellular proteins from each sample was applied to 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene fluoride membranes (Millipore). The membranes were blocked at 4°C overnight with 50 g/L nonfat dry milk solution and were then incubated with primary antibodies against GSTP (Transduction Laboratories), GSTA, GSTM (all from Oxford Biomedical Research), NQO1, c-Jun, phospho-c-Jun, Nrf2 (all from Santa Cruz Biotechnology), or β-actin for 70 min at room temperature and were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG (all from Perkin Elmer Life Sciences), or rabbit anti-goat IgG (R&D Systems) secondary antibody. The bands were visualized by using an enhanced chemiluminescence kit (Perkin-Elmer Life Science).

Real-time PCR. Total RNA was extracted by using Trizol reagent. A total of 0.8 μg RNA was used for the synthesis of first-stand cDNA. RT was carried out in a programmable thermal cycler and was performed in 20 μL containing 25 mmol/L Tris-HCl (pH 8.3), 50 mmol/L (NH4)2SO4, 0.3% β-mercaptoethanol, 0.1 g/L bovine serum albumin, 5 mmol/L MgCl2, 1 mmol/L of each deoxynucleotide triphosphate, 2.5 U RNase inhibitor, and 2.5 mmol/L oligo dT and Moloney murine leukemia virus RT. The reaction mixture was incubated for 1 cycle at 42°C for 15 min, 99°C for 5 min, and 4°C for 10 min. Real-time PCR was carried out in an ABI Prism 7000 sequence detector (Applied Biosystems) by adding 5 μL cDNA, 10 μL Master Mixture, 5 μL ddH2O, and 1 μL GSTP (Rn02770492_gh) and GAPDH primer (Mm99999915_gl) to each microwell. The reaction was run with the following program: 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The comparative Ct (threshold cycle) method was used to determine the relative amount of GSTP mRNA (31). The ΔCt method was used for quantification of amplified gene targets according to the manufacturer’s protocol (Applied Biosystems). Briefly, the number of cycles required to reach a threshold level of log-based fluorescence (Ct value) was normalized to the Ct value of GAPDH gene in each sample. The relative expression value for GSTP gene was calculated as 2−ΔΔCt, where ΔΔCt represents difference of Ct between the GSTP gene and the GAPDH gene (ΔCt = CtGSTP gene − CtGAPDH gene); ΔΔCt = ΔCtGSTP gene − ΔCtGAPDH gene.

Enzyme activity assays. GST activity was measured by using ethanocrylic acid as the substrate because of its better selectivity of the π class isozyme (32). Briefly, the reaction mixture in a final volume of 1 mL contained 100 mmol/L potassium phosphate buffer (pH 6.5), 0.5 mmol/L glutathione, 0.2 mmol/L ethanocrylic acid, and an appropriate amount of the total proteins. The ethanocrylate-glutathione conjugate formed was measured at 270 nm. The GST activity was measured with 1-chloro-2,4-dinitrobenzene, whereas NQO-1 activity was determined using 2,6-dichloroindophenol as the substrate (33).
Expression and reporter constructs. The pTA-GSTP Luc reporter with GSTP gene promoter region was constructed as described previously (28). A 2.7-kb fragment of the gene for GSTP was inserted into the MluI and NheI site of pTA-SEAP/Luc vector (Clontech). In addition to the full-length construct (Luc-2713), 2 constructs with deletions from -2713 to -2605 bp (Luc-2604) and from -2713 to -2376 bp (Luc-2375) were generated. A reporter with the GPEI fragment was constructed by ligating the -2713 to -2605 bp segment into pTA-SEAP/Luc vector and was designated as Luc-GPE.

Transient transfection and luciferase activity assay. Clone 9 cells were plated at a density of 2.5 × 10^4 cells on 35-mm plastic tissue culture dishes and the dishes were incubated until 70% confluence was reached. Cells were transiently transfected for 5 h with 0.1 μg of the pTA-GSTP Luc vectors by lipofectamine reagent and were then exposed to each of the organosulfur compounds for an additional 15 h. Cells were then washed twice with PBS and were lysed in 100 μL of lysis buffer. Luciferase activity was measured by using Luciferase Assay reagent (Clontech) according to the manufacturer’s instructions. The luciferase activity of each sample was corrected on the basis of β-galactosidase activity, which was measured at 420 nm with O-nitrophenyl β-D-galactopyranoside as a substrate. The value for cells treated with DMSO vehicle alone was regarded as 1.

Electromobility gel shift assay. Electrophoretic mobility shift assay (EMSA) was performed according to our previous study (29). Cells were washed twice with cold PBS followed by scraping from the dishes with PBS. Cell homogenates were centrifuged at 2000 × g for 5 min. The cell pellet was allowed to swell on ice for 15 min after the addition of 200 μL of hypotonic buffer containing 10 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L EDTA, 0.5 mmol/L dithiothreitol (DTT), 0.5% Nonidet P-40, 4 mg/L leupeptin, 20 mg/L aprotinin, and 0.2 mmol/L phenylmethylsulfonyl fluoride. After centrifugation at 6000 × g for 15 min, pellets containing crude nuclei were resuspended in 50 μL of hypertonic buffer containing 10 mmol/L HEPES, 400 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L EDTA, 0.5 mmol/L dithiothreitol (DTT), 10% glycerol, 4 mg/L leupeptin, 20 mg/L aprotinin, and 0.2 mmol/L phenylmethylsulfonyl fluoride and were incubated for an additional 30 min on ice. The nuclear extracts were then centrifuged by centrifugation at 10,000 × g for 15 min and were frozen at -80°C until the EMSA was performed.

The LightShift Chemiluminescent kit (Pierce Chemical) and synthetic biotin-labeled double-stranded GPEI consensus oligonucleotide (forward: 5′-AGTAGTACATGCACTATGGTGCAAAC-3′; reverse: 5′-TGGTGCAGATCATGAGCTGACTACT-3′) were used to measure the effect of organosulfur compounds on GPEI nuclear protein-DNA binding activity. Unlabeled double-stranded GPEI (200 ng) and a mutant double-stranded oligonucleotide were also used to confirm specific binding. Two micrograms of nuclear protein, poly(dI-dC), and biotin-labeled double-stranded GPEI oligonucleotide were mixed with the binding buffer to a final volume of 20 μL and were incubated at room temperature for 30 min. The nuclear protein-DNA complex was separated by electrophoresis on a 6% Tris-boric acid-EDTA-polyacrylamide gel and was then electrotransferred to a Hybond-N+ nylon membrane (GE Healthcare). The membrane was treated with streptavidin-horseradish peroxidase and the nuclear protein-DNA bands were developed by using an enhanced chemiluminescence kit. In the super-shift assay, nuclear protein was incubated with 1 μg of monoclonal anti-c-Jun antibody for 30 min after the binding reactions and was subjected to electrophoresis as described above.

Immunoprecipitation. A total of 15 μg of nuclear proteins was first incubated with 1 μg anti-Nrf2 antibody overnight at 4°C. The cells were mixed with 0.1 g/L Protein A-Sepharose beads for 1 h at 4°C. Immunoprecipitated complexes were pelleted by centrifugation at 16000 × g for 2 min at 4°C. The pellet was washed 5 times with 1 mL IP buffer (40 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mmol/L NaCl, 5 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 4 mg/L aprotinin, 1 mg/L leupeptin, 20 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate) and was then subjected to electrophoresis followed by Western blot.

Results

GSTP protein expression. In this study, Clone 9 cells were incubated with 50 μmol/L DATS, 50–600 μmol/L LA or DHLA, or 0.2–5 μmol/L SFN for 24 h. To ensure that no cytotoxicity resulted by treatment with these organosulfur compounds, we first performed a cell viability assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method showed that each of the organosulfur compounds tested at the concentrations stated above resulted in cell viability >95% (data not shown).

Immunoblotting showed that LA, DHLA, and SFN dose dependently induced GSTP protein expression in Clone 9 cells (Fig. 1). LA and DHLA at 600 μmol/L caused a 5.4- and 4.8-fold increase, respectively, in the GSTP level compared with the control cells (P < 0.05). This induction was similar to that noted in cells treated with 50 μmol/L of DATS. It was interesting to note that SFN showed the greatest potency in upregulating GSTP expression among all the organosulfur compounds tested. An 8.1-fold induction in GSTP expression was reached when cells were exposed to 5 μmol/L SFN.

Organosulfur compounds affect GSTP mRNA level and activity. By real-time PCR, the increases in GSTP mRNA levels were consistent with the changes noted in protein expression. DATS caused a 1.1-fold increase in the GSTP mRNA level compared with the control cells (P < 0.05). There was a dose-dependent induction of GSTP mRNA in cells treated with LA, DHLA, and SFN. The increase in expression caused by SFN was higher than that caused by LA or DHLA (Fig. 2A). Again, enzyme activity toward ethacrynic acid was dose dependently increased by LA, DHLA, and SFN (Fig. 2B).

![FIGURE 1](https://academic.oup.com/jn/article-abstract/140/5/885/4689029/49505088468028) Protein levels of GSTP induced by organosulfur compounds. Cells were cultured with 0.1% DMSO alone (–) or with various concentrations of DATS, LA, DHLA, or SFN for 24 h. GSTP protein was determined by immunoblot assay. A total of 4 μg protein for each sample was applied for electrophoresis. Changes in GSTP protein expression were measured by densitometry. Data were normalized to β-actin expression. The level in control cells was set at 1. Each value represents the means ± SD, n = 4. Means without a common letter differ, P < 0.05.

Statistical analysis. Statistical analysis was performed with commercially available software (SAS Institute). Data were analyzed using 1-way ANOVA, and the significant difference among treatment means was assessed using Tukey’s test. Different from the 2 deletion constructs in the same treatment was analyzed by a separate ANOVA. A value of P < 0.05 was considered significant.

Organosulfur compounds and glutathione S-transferase expression 887
GSTP promoter activity. The different length constructs were transiently transfected into Clone 9 cells to examine whether the promoter activity of the GSTP gene was modulated by the organosulfur compounds and to locate the possible responsive sites. With the Luc-2713 reporter, 600 μmol/L of LA or DHLA and 5 μmol/L of SFN resulted in 2.0-, 1.5-, and 3.7-fold higher luciferase activity, respectively, than that in the control cells (P < 0.05) (Fig. 3A). A 1.7-fold increase in reporter activity was noted in cells treated with 50 μmol/L DATS. When the 22713-to-2605-bp region (GPEI) of the GSTP promoter was deleted (Luc-2604), however, this increase in reporter activity was completely abolished, and the activity was similar to that noted in cells transfected with Luc-2375.

To further demonstrate the importance of GPEI in GSTP expression in response to organosulfur compounds, a reporter construct (Luc-GPE) was created by ligating the genomic 109-bp GPEI segment (22713 to 2605 bp) to the luciferase coding region. The results clearly indicated that DATS, LA, DHLA, and SFN increased the reporter activity by 243, 189, 143, and 352%, respectively, compared with that in the control cells (P < 0.05) (Fig. 3B). These data establish that the GPEI bears the organosulfur compound-responsive element and that this element is essential for this stimulation of the promoter activity.

Protein binding activity on GPEI by EMSA. An EMSA was used to identify the transcription factors that were bound to GPEI. In the presence of the organosulfur compounds, the DNA binding activity reached a maximum at 0.5–1 h (Fig. 4A). Specificity of the DNA-protein interaction for GPEI was demonstrated by a competitive assay with 100-fold excess of unlabeled double-stranded oligonucleotide (cold) and also with a mutant double-stranded oligonucleotide (mut). Next, a supershift assay with highly specific antibodies directed against c-Jun and Nrf2 was performed. The GPEI nuclear protein band was abrogated and super-shift occurred in the presence of anti-c-Jun antibody (Fig. 4B). In addition, an immunoprecipitation with anti-Nrf2 antibody was performed before EMSA. As noted, anti-Nrf2 antibody diminished the binding of nuclear proteins to GPEI oligonucleotides (Fig. 4B). These data establish that the GPEI bears the organosulfur compound-responsive element and that this element is essential for this stimulation of the promoter activity.

Expression of other phase II enzymes. We also assessed the expression of other detoxification enzymes that are known to be upregulated by a Nrf2-dependent mechanism, including GSTA, GATM, and NQO-1, by immunoblots. As indicated, various concentrations of LA, DHLA, and SFN dose dependently
stimulated GSTA, GSTM, and NQO-1 protein contents as well as that noted for GSTP (Fig. 5A). In addition, enzyme activity toward 1-chloro-2,4-dinitrobenzene (Fig. 5B) and 2,6-dichloroindophenol (Fig. 5C) was increased by DATS, LA, DHLA, and SFN.

**Discussion**

The importance of GSTP in cancer prevention is supported by the finding that mice lacking this detoxification enzyme have a significantly increased incidence of 7,12-dimethylbenzanthracene-induced skin cancer (14). A point mutation in the GSTP gene that leads to a decrease in enzyme activity has also been reported to be associated with increased cancer risk of the oral cavity, bladder, lung, testicles, larynx, and breast (34). Moreover, because GSTP can be induced by numerous dietary factors, it is accepted that enhancement of GSTP expression and activity through a dietary regimen is a practical means of cancer chemoprevention. In fact, studies have shown that the suppression of benzo[a]pyrene-induced forestomach neoplastic formation in mice by garlic is positively related to the potency on modulating the expression of the GSTP enzyme (13,35). Garlic oil and garlic allyl sulfides, including diallyl disulfide and DATS, which are regarded as potent chemopreventive agents, are
effective GSTP inducers in the small intestine, liver, and lung (36). In this study, our results showed that organosulfur compounds from vegetables other than garlic also act as GSTP inducers with a differential potency. Moreover, we further showed that such upregulation of GSTP gene transcription by these organosulfur compounds is likely to be AP-1 and Nrf2 dependent.

In this study, LA, DHLA, and SFN dose dependently increased GSTP protein in Clone 9 cells (Fig. 1). Of the organosulfur compounds tested, SFN showed the greatest potency in upregulating GSTP expression, followed by DATS, whereas LA and DHLA were least effective. Such a discrepancy among organosulfur compounds is consistent with their differential increase in GSTP mRNA and enzyme activity (Fig. 2). Moreover, the LA, DHLA, and SFN treatments produced relatively greater induction over the controls in the GSTP protein than in the GSTP mRNA or enzyme activity. This might be related to unique regulation of GSTP mRNA stability and/or posttranslational mechanisms involving proteasomal degradation of newly synthesized GSTP proteins by these compounds (37). It is of interest to understand how these organosulfur compounds differentially regulate GSTP gene expression. Although no explanation for this finding is currently available, the differential pharmacologic properties of these organosulfur compounds in liver cells may be a possible explanation (38). Our findings suggest that the upstream signaling activating AP-1 and Nrf2 is likely to play a key role in the differential GSTP gene transcription.

To demonstrate the working mechanism by which the organosulfur compounds upregulate GSTP transcription, we constructed Luc-reporters with serial deletion of the 5’-flanking region of the GSTP gene promoter. These results clearly showed that the section from −2713 to −2605 bp is required for LA, DHLA, and SFN induction of GSTP expression in Clone 9 cells (Fig. 3). However, the second enhancer GPEII (−2604 to −2376 bp), which is adjacent to the GPEI, had no influence on the induction of the GSTP gene. This finding is consistent with the work of Okada et al. (16), who reported that GPEI is the main regulatory element responsible for GSTP induction. The published evidence suggests that AP-1 is the main transcription factor that binds to the TRE-like element in GPEI (17). AP-1 is mainly composed of c-Jun and c-Fos protein dimers. The results of our super-shift assay in the present study clearly indicated that c-Jun was involved in the formation of the nuclear protein-GPEI complexes induced by LA, DHLA, and SFN (Fig. 48).

In addition to AP-1, several other transcription factors have been reported to participate in the upregulation of GSTP expression. In undifferentiated F9 embryonic stem cells, which possess very low AP-1 activity, the GPEI element is active in an AP-1-independent fashion (39). Nrf2 is one of the transcription factors that attracts a lot of attention because of the sequence homology between the TRE-like sequences on GPEI (5’-TCGAGTCAGTCACTA-3’) and the conserved sequences of the ARE (5’-GTACNNNGCA-3’). Binding of Nrf2/MafK to the GPEI and upregulation of rat GSTP expression were shown during hepatocarcinogenesis (18). However, by treating RL34 liver epithelial cells with 15-deoxy-prostaglandin j2 (12,14), Nrf2 was thought to not be an important component responsible for transactivation of GPEI (40). Although the role of Nrf2 in modulating GSTP expression in rats is not well established, the importance of Nrf2 in regulating human and mouse GSTP gene transcription has been well documented (41,42). For instance, GSTP induction by 6-methylsulfonylhexyl isothiocyanate of wasabi, an analogue of SFN, was shown to be completely abrogated in Nrf2-deficient mice (43). To verify whether Nrf2 binds to the GPEI, we performed an assay combining immunoprecipitation and EMSA. Our results clearly showed that, in addition to AP-1, Nrf2 is likely to bind to the GPEI. c-Jun has been shown to be a binding factor in the activation of ARE-dependent transcription. Nrf2 in association with Jun proteins regulates ARE-mediated expression and coordinated induction of genes encoding detoxifying enzymes (44). The findings of a recent work by Levy et al. (45) support that c-Jun seems to be a partner of Nrf2 in the upregulation of ARE expression in human bronchial epithelial cells exposed to 4-hydroxy-2-nonenal, although the response varies with genes and cell types determined. In this study, the immunoprecipitation result showed that Nrf2 may not bind directly with c-Jun. Taken together, the EMSA results revealed that the upregulation of this phase II detoxification enzyme by LA and SFN is likely via multiple protein factors, at least c-Jun and Nrf2, that may act in a complex manner.

In response to numerous prooxidants and electrophilics, Nrf2 dissociates from Keap protein and quickly translocates from the cytosol into the nucleus, where it forms a heterodimer with small Maf and binds to the ARE. This binding of Nrf2 to the ARE upregulates the transcription of many cytoprotection enzymes. These include glutamate cysteine ligase, heme oxygenase 1, NQO1, and GST (21,27,43,46). In many types of cells, SFN has been regarded as a potent Nrf2 activator that leads to upregulation of NQO1 and GST isozymes including GSTA and GSTM (43,47,48). This increase in the levels of those detoxification enzymes accounts for, at least in part, the protection by SFN against chemical carcinogens such as benzo[a]pyrene-induced stomach and colon tumor formation (20,49). In the present study, an increase in GSTA, GSTM, and NQO1 was also noted in cells treated with SFN, which suggests that the Nrf2-ARE pathway was activated by treating Clone 9 cells with SFN (Fig. 5).

LA, in addition to its well-recognized role in acting as a coenzyme, is a natural antioxidant (24). LA is promptly taken up by cells, where it can be reduced to DHLA by enzymes such as dihydrolipoamide dehydrogenase, glutathione reductase, or thioredoxin reductase. DHLA produced inside the cell is a powerful reducing agent that can even reduce protein disulfides to protein sulfhydryls and also reduce cystine to cysteine, which is the limiting substrate for glutathione synthesis (50). Several in vivo studies have further provided evidence that LA supplementation decreases oxidative stress and restores reduced levels of other antioxidants under various physiologic and pathophysiologic conditions in brain and heart tissues and in RBC (51,52). In addition to acting as a coenzyme and antioxidant, recent work indicates that LA may also act as an inducer of several phase II detoxification enzymes, including GSTA and NQO1 through a CCAAT/enhancer binding protein and Nrf2-dependent pathway (8). In this study, we further showed that LA and DHLA activate AP-1 and Nrf2 translocation into the nucleus, where they bind to GPEI and upregulate GSTP transcription.

In summary, SFN, LA, DHLA, and DATS are effective inducers of GSTP gene transcription, and SFN shows the greatest potency. Moreover, AP-1 and Nrf2 binding to the enhancer element GPEI is essential for the induction of this phase II detoxification enzyme.

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890 Li et al.
organosulfur compounds and glutathione S-transferase expression 891


as potential inducers of phase II detoxification enzymes. 15-deoxy-


