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Structure and Function Relationship Study of Allium Organosulfur Compounds on Upregulating the Pi Class of Glutathione S-Transferase Expression

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ABSTRACT: Allium organosulfides are potential chemopreventive compounds due to their effectiveness on the induction of phase II detoxification enzyme expression. In this study, we examined the structure and function relationship among various alk(en)yl sulfides on the expression of the pi class of glutathione S-transferase (GSTP) in rat Clone 9 cells, and what mechanism is involved. Cells were treated with 300 μM dipropyl sulfide (DPS), dipropyl disulfide (DPDS), propyl methyl sulfide (PMS), and propyl methyl disulfide (PMDS) for 48 h. DPDS and PMDS displayed more potency on GSTP protein and mRNA induction than that of DPS and PMS. Next, we compared the effectiveness of DPDS, PMDS, and diallyl disulfide (DADS), which have the same number of sulfur atoms but differ in the side alk(en)yl groups. The maximum increases on protein expression, mRNA level, and enzyme activity were noted in cells treated with DADS, followed by DPDS and PMDS. A reporter assay showed that three disulfides increased GSTP enhancer I (GPE I) activity ($P < 0.05$) in the order DADS > DPDS $\geq$ PMDS. Electromobility gel shift assays showed that the DNA binding of GPE I to nuclear proteins reached a maximum at 1 to 3 h after alk(en)yl disulfide treatment. Supershift assay revealed that c-jun bound to GPE I. Silencing of extracellular signal-regulated kinase (ERK) 2 expression inhibited c-jun activation and GSTP induction. Results suggest that both the type of alk(en)yl groups and number of sulfur atoms are determining factors of allium organosulfides on inducing GSTP expression, and it is likely related to the ERK-c-Jun-GPE I pathway.

KEYWORDS: allium organosulfides, pi class of glutathione S-transferase, extracellular signal-regulated kinase, c-Jun, Clone 9 cells

■ INTRODUCTION

The genus *Allium* vegetables garlic and onion have garnered significant interest owing to their reported health benefits, which include antithrombotic, antiatherosclerotic, antidiabetic, and anticancer properties.[1−3] Epidemiologic evidence suggests that increased dietary consumption of garlic reduces the risk of colorectal, laryngeal, and endometrial cancer.[4,5] This anticarcinogenic activity has been attributed to the rich content of organosulfur compounds in garlic and onion. The type and content of different garlic and onion products differ dramatically, which is dependent on the means of plant tissue storage and processing.[6] For instance, by immersing fresh garlic into a vinegar or wine, S-acetylcysteine and S-acetylmercaptocysteine are two major organosulfur compounds in the aged garlic. By steam-distillation, garlic oil is composed of volatile alk(en)yl sulfides including diallyl disulfide (DADS), diallyl trisulfide (DATS), diallyl sulfide (DAS), and allyl methyl trisulfide, and a trace of propyl methyl disulfide (PMDS).[7] In onion oil, the organosulfur compounds include dipropyl disulfide (DPDS), dipropyl sulfide (DPS), propyl methyl sulfide (PMS), and PMDS.[8,9]

Cancer chemoprevention of garlic and onion organosulfur compounds has been proposed to be mainly due to their modulation on carcinogen metabolism, including the effects on both phase I and II detoxification enzymes[10,11] and cell cycle.[12] It has been demonstrated that G2/M arrest resulted by DADS is related to an increase of cyclin B1 protein levels in human gastric cancer BGC823 cells.[13] The antitumorigenic effect of DADS and DPDS can be attributed to the transcriptional upregulation of phase II detoxification enzymes, including glutathione S-transferases (GST),

UDP-glucuronyl transferases, and NAD(P)H-dependent quinone oxidoreductase, which accelerate carcinogen excretion.[10,11] Higher tissue levels of phase II detoxification enzymes lower susceptibility to chemical carcinogenesis.[14]

Among phase II detoxification enzymes, GST represents a major group that catalyzes the conjugation of glutathione with a variety of electrophilic xenobiotics and facilitates their excretion.[15] GST is divided into cytosolic, mitochondrial, and microsomal families. Seven distinct classes of cytosolic GST have been identified: alpha, mu, pi, sigma, theta, delta, and zeta.[16] Compared with other isozymes, the pi class of GST (GSTP) is more effective in the detoxification of the electrophilic α,β-unsaturated carbonyl compounds that are generated by radical reactions of lipids.[17] There has been considerable interest in the properties of GSTP, particularly in relation to its role in cell transformation and carcinogenesis.[18] GSTP activity has been used to evaluate the potency of chemoprevention agents in benzo[a]pyrene-induced cancer.[19] Moreover, in transgenic male Wistar rats, overexpression of GSTP inhibits the early phase of liver carcinogenesis.[20] The importance of GSTP in cancer prevention is further supported by the fact that benzo[a]pyrene-induced lung cancer is significantly elevated in GSTP-null mice.[21] Therefore, the expression of GSTP is regarded as an important determinant of protection against various chemical insults.

The inducibility of GSTP is generally attributed to the existence of a strong enhancer named GSTP enhancer I (GPE I), which has two 12-O- tetradecanoylphorbol 13-acetate (TPA) responsive element (TRE)-like sequences in the 5′ upstream region.[22] This enhancer on GSTP expression is regulated by multiple factors, mainly the activator protein-1 (AP-1), which is known to be a heterodimer or homodimer composed of c-Jun and c-Fos.[23] Several cellular stresses and cytotoxic chemicals engage the activation of mitogen-activated protein kinases, including c-Jun NH$_2$-terminal kinase, extracellular signal-regulated kinase (ERK), and p38 kinase, which in turn activate AP-1. Recently, we reported that DADS on GSTP expression is likely related to the activation of ERK and AP-1 in Clone 9 cells.[24] Activation of ERK signaling regulates the binding of c-Jun to the TRE in human lung cancer cells.[25] Therefore, ERK-c-Jun-GPE I signaling pathway may play an important role in GSTP expression by garlic and onion organosulfur compounds.

We previously reported that alkenyl sulfides DADS and DATS upregulate GSTP mRNA and protein expression.[24] However, much less is known about alkyl sulfides. In this study, we investigated the effect of DPS, DPDS, PMS, and PMDS on GSTP expression in rat liver Clone 9 cells and the induction potency was compared to that of DADS. In addition, the possible mechanism involved on GSTP transcription was examined.

■ MATERIALS AND METHODS

Materials. DPS, DPDS, PMS, PMDS, ethacrynic acid, HEPES, bovine serum albumin, deoxynucleotide triphosphate, poly(dI-dC), and β-mercaptoethanol were obtained from Sigma-Aldrich (St. Louis, MO). DADS was purchased from Tokyo Kasei Chemical Co. (Tokyo, Japan). RPMI-1640 medium and penicillin−streptomycin solution were obtained from Gibco Laboratory (Grand Island, NY). Trizol and lipofectamine were ordered from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Hyclone (Logan, UT). RNase inhibitor, oligo dT, and moloney murine leukemia virus reverse transcriptase were purchased from Promega Company (Madison, WI).

Cell Culture. Clone 9 cells, which were derived from normal rat livers, were obtained from Bioresources Collection and Research Center (BCRC, Taiwan). They were grown in RPMI-1640 medium supplemented with 10 mM HEPES, 1×10^5 units/L penicillin, 100 mg/L streptomycin, and 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO$_2$ 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, Lakes, NJ) at a density of 2.5×10^5 cells per dish and were allowed to grow for 24 h. Fresh culture medium containing 300 μM DPS, DPDS, PMS, PMDS, or DADS (Figure 1) was then added, and the cells were incubated for 48 h. Cells treated with 0.1% DMSO were used as controls.

Western Blot. Cells were washed twice with cold phosphate-buffered saline and were then lysed with potassium phosphate buffer (pH 7.0). The homogenates were then centrifuged at 10500g for 30 min at 4 °C. Protein concentrations were measured by using Coomassie Plus

CH$_3$-CH$_2$-CH$_2$-S-CH$_2$-CH$_2$-CH$_3$
dipropyl sulfide (DPS)

CH$_3$-CH$_2$-CH$_2$-S-S-CH$_3$
propyl methyl disulfide (PMDS)

CH$_3$-CH$_2$-CH$_2$-S-S-CH$_2$-CH$_2$-CH$_3$
dipropyl disulfide (DPDS)

CH$_2$=CH-CH$_2$-S-S-CH$_2$-CH=CH$_2$
diallyl disulfide (DADS)

CH$_3$-CH$_2$-CH$_2$-S-CH$_3$
propyl methyl sulfide (PMS)

Figure 1. Structures of allium organosulfur compounds.

Protein Assay Reagent Kit (Pierce Chemical Company, Rockford, IL). Five micrograms of cellular protein was separated by 10% SDS−polyacrylamide gel electrophoresis. Separated proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). Protein immunoblot analysis was carried out by use of the following: anti-GSTP (Transduction Laboratories, Lexington, KY), GSTA, and GSTM (Oxford Biomedical Research, Oxford, MI); β-actin (Sigma Chemical, St. Louis, MO); ERK1/2, c-Jun, phospho-ERK1/2, and phospho-c-Jun (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA) as primary antibody, and horseradish peroxidase−conjugated goat anti-rabbit IgG, goat anti-mouse IgG (all from Perkin-Elmer Life Sciences, Boston, MA), or rabbit anti-goat IgG (R&D Systems Inc., Minneapolis, MN) as secondary antibody. The blots were visualized by using an enhanced chemiluminescence kit (Perkin-Elmer Life Science, Boston, MA).

RT-PCR. Total RNA was extracted by using Trizol reagent. A total of 0.1 μg of RNA was used for the synthesis of first-stand cDNA. Reverse transcription was carried out in a programmable thermal cycler and was performed in 20 μL containing 25 mM Tris-HCl, 50 mM (NH$_4$)$_2$SO$_4$, 0.3% β-mercaptoethanol, 0.1 g/L bovine serum albumin, 5 mM MgCl$_2$, 1 mM of each deoxynucleotide triphosphate, 2.5 units of RNase inhibitor, and 0.5 mM oligo dT and moloney murine leukemia virus reverse transcriptase. 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. The sequences for the RT-PCR primers were as follows: for GSTP (forward, 5′-TTCAAGGCTCGCTCAAGTCCAC-3′; reverse, 5′-CTTGATCTT GGGGCGGGCACTG-3′); for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5′-GACGTGCCGCCTGGAGAAA-3′; reverse, 5′-GGGGGCCGAGTTGGGATAG-3′). The PCR reactions were performed as follows: 5 min at 94 °C; 35 cycles of 40 s at 94 °C, 40 s at 60 °C, and 120 s at 72 °C; and a final extension of 5 min at 68 °C. The PCR amplicons were then electrophoresed in 1%-agarose gels containing 1X TAE buffer (40 mM Tris, 20 mM glacial acetic acid, and 2 mM EDTA).

Transfection and Small Interfering RNA (siRNA). Cells were plated in 35 mm plastic tissue culture dishes at 70−80% confluence and then transfected with four synthesized ERK2 siRNAs (100 nM) or nontargeting control siRNA (si-control) by using DharmaFECT siRNA transfection reagent (all from Thermo Fisher Scientific, Lafayette, CO) for 24 h. The sense sequences of ERK2 siRNAs were as follows: (1) 5′-ACACUAAUCUCUCGUACAU-3′; (2) 5′-AAAAUAAGGUGCC-GUGGAA-3′; (3) 5′-UAUACCAAGUCCAUUGAUA-3′; and (4) 5′-UCGAGUUGCUAUCAAGAAA-3′. Cells were treated with allium organosulfides for indicated time and then lysed, and cell lysates were subjected to immunoblotting.

Enzyme Activity Assays. GST activity was measured by using ethacrynic acid as the substrate because of its better selectivity of the pi class isozyme.[26] Briefly, the reaction mixture in a final volume of 1 mL contained 100 mM potassium phosphate buffer (pH 6.5), 0.5 mM glutathione, 0.2 mM ethacrynic acid, and an appropriate amount of the total proteins. The ethacrynate−glutathione conjugate formed was measured at 270 nm.

Transient Transfection and Luciferase Activity Assay. The Luc-GPE reporter with −2713 to −2605 (GPE I) bp of the GSTP gene promoter region was constructed according to our previous study.[27] Clone 9 cells were plated at a density of 2×10^5 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 1 μg of the Luc-GPE vector by lipofectamine reagent and were then exposed to allium disulfides for an additional 20 h. Cells were then washed twice with cold phosphate-buffered saline and were lysed in 100 μL of lysis buffer. Luciferase activity was measured by using Luciferase Assay Reagent (Clontech, Palo Alto, CA) 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
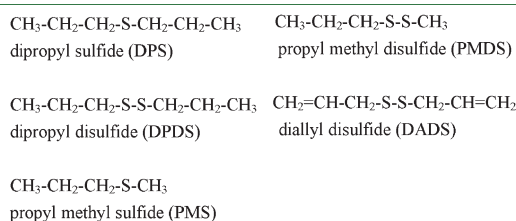
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O-nitrophenyl β-D-galactopyranoside as a substrate. The value for cells treated with DMSO vehicle alone was regarded as 1.

Electromobility Gel Shift Assay. Crude nuclear extracts were prepared according to the method described previously.[24] The Light-Shift Chemiluminescent electromobility gel shift assay (EMSA) Kit (Pierce Chemical Company, Rockford, IL) and synthetic biotin-labeled double-stranded GPE I consensus oligonucleotide (forward, 5′-AG-TAGTCAG TCACTATGATTCAGCAAC-3′; reverse, 5′-GTTGCTG AATCATAGTGACTGACTACT-3′) were used to measure whether allium sulfides changed GPE I binding activity with nuclear proteins. Unlabeled double-stranded GPE I (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 GPE I 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, Buckinghamshire, U.K.). The membrane was treated with streptavidin-horseradish peroxidase, and the nuclear protein−DNA bands were developed by using an enhanced chemiluminescence kit. In the supershift assay, nuclear protein was incubated with monoclonal anti-c-Jun antibody for 30 min after the binding reactions and was subjected to electrophoresis as described above.

Statistical Analysis. Statistical analysis was performed with commercially available software (SAS Institute Inc., Cary, NC). Data were analyzed by means of one-way ANOVA, and the significant difference among treatment means was assessed by use of Ducan's test. A value of $P < 0.05$ was considered to be significant.

■ RESULTS

Allium Alk(en)yl Sulfides on the Expression of GST Isozymes. To ensure that no cytotoxicity resulted from the 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 garlic organosulfides tested at the concentration up to 300 μM for 48 h resulted in cell viability greater than 85% (data not shown).

We first examine four alkyl sulfides DPS, DPDS, PMS, and PMDS on GSTP expression in the Clone 9 cells. The immunoblot assay showed that two alkyl disulfides, i.e. DPDS and PMDS, induced GSTP protein expression (Figure 2A). In addition, an increase of the other GST isozyme GSTM also resulted from DPDS and PMDS, although the extent of induction was less than that noted for GSTP. GSTA was not affected by both DPDS and PMDS. The levels of GSTP, GSTM, and GSTA, however, had a minor change resulting from sulfides with a single sulfur atom, i.e. DPS and PMS. RT-PCR revealed that changes of GSTP mRNA levels were consistent with those noted for protein expression (Figure 2B). Moreover, DPDS and PMDS dose-dependently increased GSTP protein levels in Clone 9 cells (Figure 2C). These results suggested that the induction of allium alkyl sulfides on GSTP expression was positively related to the number of sulfur atoms. This is similar to the findings reported in our previous work,[27] the sulfur atom numbers of three garlic diallyl sulfides are positively related to the induction efficiency on GSTP transcription.

Next, the differential induction on GSTP expression by alkyl disulfides (DPDS and PMDS) and alkenyl disulfide (DADS) was determined. As shown, all three disulfides increased GSTP protein (Figure 3A) and mRNA (Figure 3B) levels and DADS displayed the greatest induction compared with that of DPDS

and PMDS ($P < 0.05$). We additionally used ethacrynic acid as a substrate to measure GSTP enzyme activity. As noted, DPDS, PMDS, and DADS resulted in an increase of enzyme activity by 132%, 125%, and 298%, respectively, as compared with that of the control cells (Figure 3C).

GSTP Promoter Activity. To demonstrate the importance of GPE I in the GSTP expression in response to allium disulfides, we created a reporter construct (Luc-GPE) by ligating the genomic 109-bp GPE I segment (−2713 to −2605 bp) to the luciferase coding region. Results clearly indicated that the reporter activity was increased by DPDS, PMDS, and DADS. Luciferase activity in cells treated with DPDS, PMDS, and DADS was 109%, 78%, and 260%, respectively, higher than control cells ($P < 0.05$) (Figure 4). Again, the disulfides with allyl groups had the greatest increase among three compounds tested ($P < 0.05$).

Nuclear Protein Binding Activity to GPE I. EMSA indicated that, in the presence of DPDS, PMDS, and DADS, the binding of nuclear proteins to DNA reached a maximum at 1−3 h (Figure 5) in Clone 9 cells. The specificity of the DNA−protein interaction for GPE I 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). A similar increase in the DNA binding activity of GPE I was also noted by TPA, a GSTP inducer.
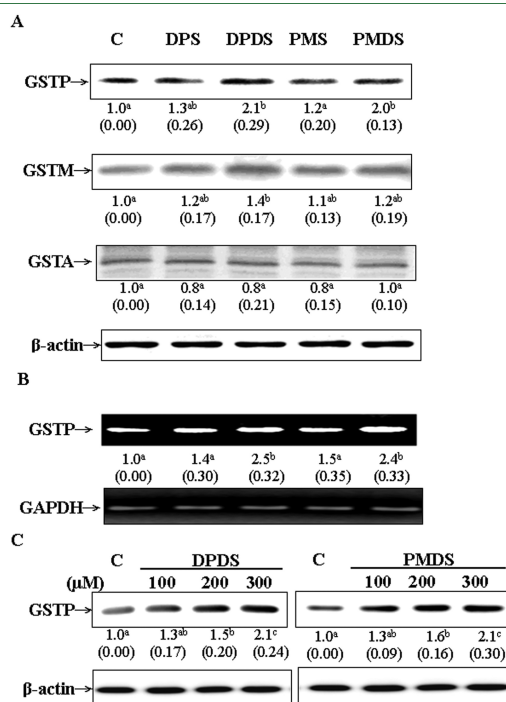

Figure 2. Changes of the pi class of glutathione *S*-transferase (GSTP) protein and mRNA levels by allium alkyl sulfides. Clone 9 liver cells were cultured with 0.1% DMSO alone (control, C) or with 100, 200, or 300 μM dipropyl sulfide (DPS), dipropyl disulfide (DPDS), propyl methyl sulfide (PMS), or propyl methyl disulfide (PMDS) for 48 h. (A) Expression of GSTP, GSTA, and GSTM protein was determined by immunoblotting. (B) Changes in GSTP mRNA levels induced by treatment with alkyl sulfides. (C) Changes in GSTP protein level induced by treatment with alkyl sulfides. The protein and mRNA levels were quantified by densitometry, and the level in the control cells was set at 1. Values are expressed as means (SD), n = 3. Means not sharing a common letter differ significantly, $P < 0.05$.
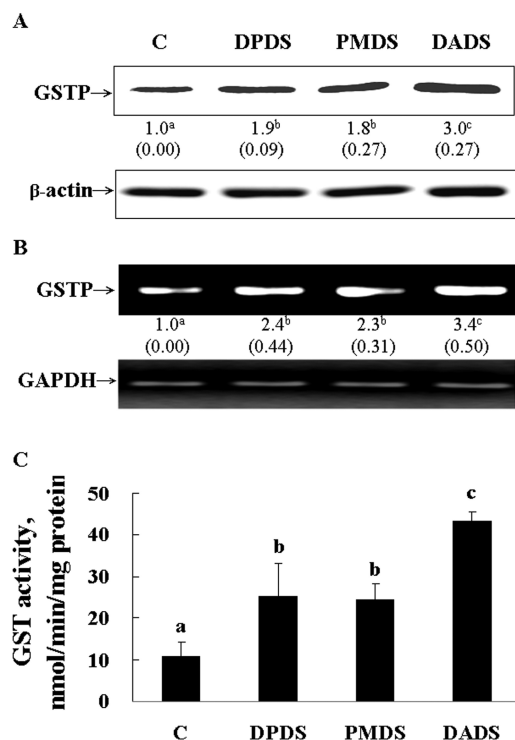
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A

	C	DPDS	PMDS	DADS
GSTP→	1.0ᵃ (0.00)	1.9ᵇ (0.09)	1.8ᵇ (0.27)	3.0ᶜ (0.27)

β-actin→

B

	C	DPDS	PMDS	DADS
GSTP→	1.0ᵃ (0.00)	2.4ᵇ (0.44)	2.3ᵇ (0.31)	3.4ᶜ (0.50)

GAPDH→

C

Figure 3. The expression of the pi class of glutathione *S*-transferase (GSTP) by various alk(en)yl disulfides in Clone 9 cells. Cells were treated with DMSO alone (control, C) or with 300 μM dipropyl disulfide (DPDS), propyl methyl disulfide (PMDS), or diallyl disulfide (DADS) for 48 h. GSTP protein (A), mRNA (B), and enzyme activity (C) were determined. The protein and mRNA levels were quantified by densitometry, and the level in the control cells was set at 1. Values are expressed as means (SD), $n = 3$. Means not sharing a common letter differ significantly, $P < 0.05$. Ethacrynic acid was used as a substrate for measuring GSTP activity because of its better specificity. Values are means $\pm$ SD, $n = 3–4$. Groups not sharing a common letter differ significantly, $P < 0.05$.
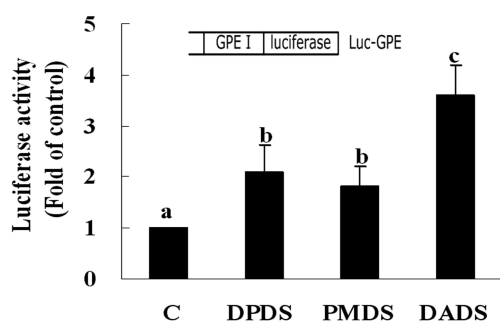

Figure 4. GSTP enhancer I (GPE I) is required for the upregulation of the pi class of glutathione *S*-transferase (GSTP) by dipropyl disulfide (DPDS), propyl methyl disulfide (PMDS), or diallyl disulfide (DADS). The GPE I-linked (-2713 to -2604 bp) Luc-reporter was transfected into the Clone 9 cells, and then the cells were treated with 300 μM DPDS, PMDS, or DADS for 20 h. Values are means $\pm$ SD, $n = 3$. Groups not sharing a common letter differ significantly, $P < 0.05$.

To further identify the transcription factor that is activated by DADS and bind to GPE I, a supershift assay with highly specific antibodies directed against c-Jun was performed (Figure 5). The increase of nuclear protein−DNA interaction by DADS
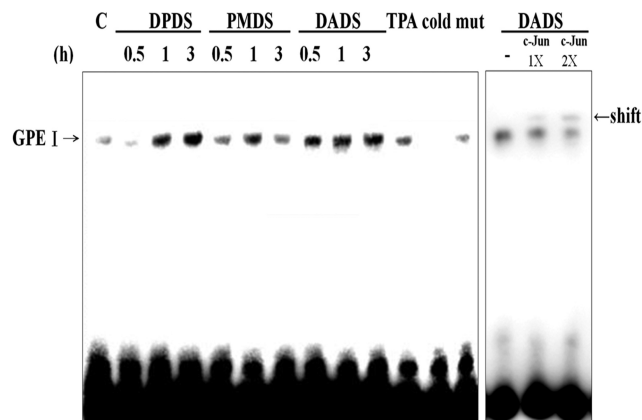

Figure 5. Activation of GPE I binding activity by various alk(en)yl disulfides in Clone 9 cells. Cells were treated with 300 μM dipropyl disulfide (DPDS), propyl methyl disulfide (PMDS), or diallyl disulfide (DADS) for the indicated times, and nuclear extracts were prepared to measure GPE I binding activity by electromobility gel shift assay (EMSA). Unlabeled double-stranded GPE I (200 ng) and a mutant double-stranded oligonucleotide were also used to confirm specific binding. For supershift assay, nuclear proteins isolated from the cells treated with DADS for 1 h were first reacted with GPE I oligonucleotides for 30 min and were then incubated with 1 μg (1×) or 2 μg (2×) of antibodies to c-Jun for an additional 30 min at room temperature. The subsequent supershift complexes were separated by 6% acrylamide gel electrophoresis. One representative immunoblot out of four independent experiments is shown.
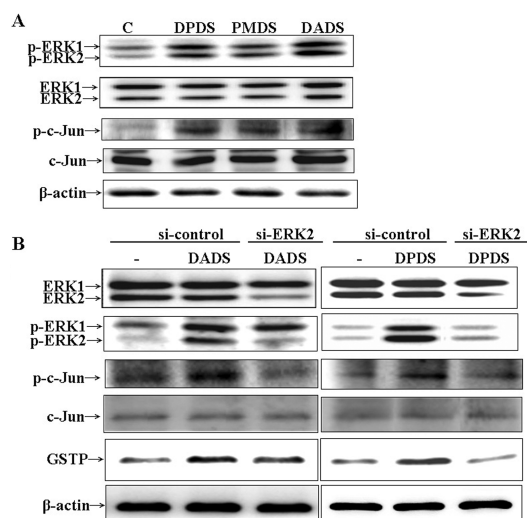

Figure 6. ERK2 knockdown suppressed alk(en)yl disulfide-induced GSTP protein expression. (A) Cells were treated with DMSO alone (C) or with 300 μM dipropyl disulfide (DPDS), propyl methyl disulfide (PMDS), and diallyl disulfides (DADS) for 0.5 h, and the phosphorylation of ERK and c-Jun was determined. (B) Cells were transfected with ERK2 siRNA (si-ERK2) or nontargeting control siRNA (si-control) for 24 h. The activation of c-Jun and ERK in the Clone 9 cells treated with DADS or DPDS for 0.5 h is shown. For GSTP protein determination, the transfected cells were treated with 300 μM DADS or DPDS for 48 h. One representative immunoblot out of three independent experiments is shown.

decreased in the presence of c-Jun antibody, and the supershift appeared in a dose-dependent manner.

ERK on GSTP Expression. The phosphorylation of ERK1/2 was increased by treating Clone 9 cells with DPDS, PMDS, or

DADS. With accompanying ERK activation, phosphorylation of c-Jun resulted (Figure 6A). It has been reported that ERK2 but not ERK1 plays a key role in rat hepatocyte. ERK2 is more important than ERK1 in replication of hepatocytes,[28] and ERK2 might regulate the expression of GSTP in this study. Therefore, knockdown of ERK2 by siRNA transfection was tested to determine the critical role of ERK2 in c-Jun activation and, thus, GSTP induction by allium disulfides. Immunoblots revealed that the activation of ERK1/2 and c-Jun was stimulated in the presence of DADS and DPDS (Figure 6B). In control groups, the activation of ERK2 and c-Jun and expression of GSTP protein were slightly inhibited in ERK2 siRNA-transfected cells. With ERK2 siRNA, cellular ERK2 level was dramatically decreased (vs si-control), which resulted in the phosphorylation of ERK2 by DADS and DPDS being alleviated. Activation of c-Jun and induction of GSTP expression by both disulfides was then suppressed.

■ DISCUSSION

Several lines of evidence have suggested that GSTP may play an important role in chemoprevention. GSTP is involved in the protection against alkylation caused by 4-nitroquinoline 1-oxide.[29] A point mutation in the GSTP gene that leads to a decrease in enzyme activity was also reported to be associated with increased cancer risk of the oral cavity, bladder, lung, testicles, larynx, and breast.[30] The importance of GSTP in cancer prevention is also supported by the fact that the 7,12-dimethyl-benz anthracene-induced skin cancer was significantly elevated in the GSTP null mice.[31] Therefore, higher GSTP activity allows cells to be better in protecting against chemical insult.

The Clone 9 cells, a permanently growing and nontransformed rat liver cell line, were derived from normal rat liver and retain an epithelial morphology. They have been used extensively as a model for hepatocyte functions, including mediating the expression of a number of phase II detoxification enzymes. In this study, DPDS, PMDS, and DADS are effective inducers of GSTP gene transcription, and DADS shows the greatest potency. In in vivo and in vitro studies, we reported that the induction of GSTP protein and mRNA levels in rat liver by DAS, DADS, and DATS was in the order of DATS ≥ DADS > DAS.[27,32] However, there was a study that reported that these compounds were not good inducers of GST in Hepa 1c1c7 cells.[33] It is not clear at present what causes such a differential structure−function relationship in modulating the GSTP, and this requires further study. Different experimental models and varied binding affinity of organosulfur compounds and their metabolic products may explain in part this discrepancy. In vivo study indicated that DPS in rat was metabolized into sulfone.[34] DADS was found to be reduced to allyl mercaptan in an isolated perfused rat liver and oxidized to diallyl thiosulfinate in rat liver microsomes.[35,36] DPDS is oxidized to dipropyl thiosulfinate in rat liver microsomes, whereas it is transformed to propylglutathione sulfide and propyl mercaptan by liver cytosol.[37]

In the present study, results clearly indicated that DPDS and PMDS displayed higher induction on GSTP mRNA and protein expression (Figure 2) than those of DPS and PMS, suggesting that the number of sulfur atoms plays a role in the upregulation of this phase II detoxification enzyme. Similar to this finding, the positive relationship between the number of sulfur atoms and GSTP expression has also been noted on DAS, DADS, and DATS in primary hepatocytes.[27] The reason that the compounds containing more sulfur atoms exhibit better inductive effect on

the GSTP expression is unclear. Bose et al. suggested that the disulfide chain might provide an appropriate spacing of the allyl groups in the GSTP-inducing activity of DADS.[38] In addition, it is also possible that the induction of phase II enzymes is often associated with oxidative stress,[24,39] and disulfides have been shown to cause oxidative damage though their ability to generate "active oxyen" species via redox cycling.[40]

In addition to the sulfur atom number, the levels in GSTP mRNA and protein were higher in cells treated with DADS than those exposed to DPDS and PMDS (Figure 3). It indicated that allium disulfides with allyl group exert stronger inducibility on GSTP expression than that with saturated propyl and methyl groups. The presence of allyl groups as well as the disulfide chain is required for maximum induction of GSTP in vivo by garlic organosulfur compounds.[38] Allium organosulfur compounds (such as DADS) that contain disulfur atoms and diallyl groups are more potent in inhibiting benzo[a]pyrene-induced forestomach cancer than are those containing monosulfur (such as DAS) and propyl groups (such as DPDS).[11,41] The chemopreventive efficacy of these organosulfur compounds correlated with their ability to increase the expression of GSTP.[42] Taken together, both the number of sulfur atoms and the type of alk(en)yl groups of allium organosulfur compounds are determining factors on upregulating GSTP expression. Among those sulfides examined, DADS, which is composed of two sulfur atoms and two allyl groups, showed the greatest induction, followed by DPDS and PMDS, and DPS and PMS had only minor effects. Structure−function relationship study has been widely used to examine the relative biological activity among phytochemicals sharing similar structure.[12,41] In the case of garlic organosulfur compounds, DATS revealed better growth inhibition of A375 skin cancer cells than did DADS and DAS.[12] In flavonoids, the order of potency at suppressing human liver HepG2 cancer cells is chalcones > flavones > isoflavones >flavanones.[43]

GPE I, which consists of two TRE-like sequences, acts as an enhancer and is required for the basal and inducible expression of GSTP in rat livers by a number of stimuli, such as lipoic acid and sulforaphane.[22,44] In this study, a 109 bp GPE I-Luc reporter was constructed and the change of luciferase activity was determined in the presence of alk(en)yl disulfides. Consistent with the changes of GST mRNA and protein levels, three disulfides tested significantly increased luciferase activity, and the greatest increase was noted in cells treated with DADS (Figure 4). It is interesting to explore how these allium organosulfur compounds work differentially on GSTP transcription. These results indicated that the differential induction potency among allium alk-(en)yl disulfides is likely to work through the modulation of GPE I activity, and the activation of intracellular signal transduction and transcription factors is the most likely explanation.

AP-1, which is composed mainly of c-Jun and c-Fos protein dimers, is the main transcription factor that binds to the TRE-like element in GPE I.[23] c-Jun is a member of a multiprotein family that has been implicated in several signal transduction pathways associated with cellular growth, neuronal regeneration, and cellular stress.[45−47] The results of our supershift assay in the present study clearly indicated that c-Jun was involved in the formation of the nuclear protein-GPE I complexes induced by DADS (Figure 5). Based on the fact that c-Jun is required for cellular defense against chemical agents,[48,49] it is likely that c-Jun functions as an important component that activates GPE I, followed by increasing GSTP expression, in the liver cells exposed to allium sulfides. In addition to c-Jun, the binding of other transcription factors to the TRE-like

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element in GPE I cannot be excluded. Nuclear factor erythroid-2 related factor 2 (Nrf2) is one of the candidates that has attracted a lot of attention, because of the sequence homology between the TRE-like sequences on GPE I (5′-AGTCAGTCACTAT**GATT**-**CAGCA**-3′) and the conserved sequences of the antioxidant response element (ARE, 5′-**GTGAC**NNN**GCA**-3′). The binding of Nrf2 to the ARE is well-known to upregulate the transcription of several antioxidant enzymes and phase II detoxification enzymes, including heme oxygenase 1, glutamate-cysteine ligase, GSTM, and NAD(P)H:quinone oxidoreductase.[50,51] Although it is not determined in this study, Nrf2 binding to GPE I has been reported to be responsible for upregulating GSTP transcription by DATS in primary rat hepatocytes[44] and also in the early carcinogenesis stage of rat H4IIE hepatoma cells.[52] Moreover, the induction of GSTP by 6-methylsulfinylhexyl isothiocyanate of wasabi and oltipraz is completely abrogated in Nrf2-deficient mice.[53]

Recently, in human pulmonary epithelial cells, ERK signaling, but not JNK1/2 and p38, was reported to be the main MAPK involved in activating the binding of c-Jun to the TRE by TPA.[25] The finding is supported in our result that allium sulfides increased the phosphorylation of ERK1/2, and then activation of c-Jun in Clone 9 cells (Figure 6A). Silibinin suppressed human osteosarcoma MG-63 cell invasion is attributed to its inhibition on the ERK-dependent c-Jun induction of matrix metalloproteinase-2.[54] In this study, ERK2 was chosen to be knockdown because ERK2 is more important than ERK1 in replication of hepatocytes.[28] As noted, when ERK2 expression was silenced by ERK2 siRNA, c-Jun activation by DADS and DPDS was suppressed (Figure 6B). In parallel, DADS and DPDS induction on GSTP expression disappeared. These findings strongly suggest that ERK2-c-Jun signaling is likely to play an important role in upregulating the transcription of this phase II detoxification enzyme. The result is consistent with our previous study that DATS on GSTP expression is dependent on the ERK-AP-1 signaling pathway. The activation of this pathway is likely related to transient changes in cellular redox states.[24] Moreover, Xu et al. indicated that the activation of the ERK signaling pathway is important for transcriptional activity of AP-1 and is involved in the regulation of cell death elicited by sulforaphane and phenethyl isothiocyanate in human prostate cancer PC-3 cells.[55] An understanding of the role of the ERK-c-Jun-mediated signal pathway in GSTP transcriptional regulation will help to clarify the possible molecular mechanism of allium organosulfur compounds in drug metabolism and cancer prevention.

In conclusion, both the number of sulfur atoms and the type of alk(en)yl groups are determining factors in the effectiveness of garlic and onion sulfides on upregulating GSTP expression. Moreover, differences in the potency among allium sulfides can be partly attributed to their differential activation of the ERK-c-Jun-GPE I signaling pathway.

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■ ABBREVIATIONS USED

AP-1, activator protein-1; ARE, antioxidant response element; DADS, diallyl disulfide; DAS, diallyl sulfide; DATS, diallyl trisulfide; DPDS, dipropyl disulfide; DPS, dipropyl sulfide; EMSA, electromobility gel shift assay; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; GSTP, pi class of GST; GPE I, GSTP enhancer I; Nrf2, nuclear factor erythroid-2 related factor 2; PMDS, propyl methyl disulfide; PMS, propyl methyl sulfide; siRNA, small interfering RNA; TPA, 12-O-tetradecanoylphorbol 13-acetate; TRE, TPA responsive element

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