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Diallyl Disulfide and Diallyl Trisulfide Up-Regulate the Expression of the π Class of Glutathione *S*-Transferase via an AP-1-Dependent Pathway

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Garlic organosulfur compounds are recognized as potential chemopreventive compounds. This protection is related to the induction of phase II detoxification enzymes. We previously reported that diallyl disulfide (DADS) and diallyl trisulfide (DATS) up-regulate the gene expression of the π class of glutathione *S*-transferase (GSTP) and that an enhancer element named GPE I is required for this induction. In the present study, we further investigated the signal pathway involved in DADS and DATS up-regulation of this detoxification enzyme in Clone 9 cells. Cells were cultured with 25−200 μmol/L of DADS or DATS for 24 h. Western and Northern blots showed that both garlic allyl sulfides concentration dependently induced GSTP protein and mRNA expression, respectively. Changes in GST activity toward ethacrynic acid were consistent with the increase in GSTP expression ($P < 0.05$). Electromobility gel shift assay showed that the DNA binding activity of nuclear activator protein-1 (AP-1) is concentration-dependently increased in the presence of DADS and DATS as compared with that of the control cells. The phosphorylation of c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), but not of p38, was stimulated in the presence of both garlic allyl sulfides. Pretreatment with SP600125 and PD98059, which are JNK and ERK inhibitors, respectively, abolished the increase in AP-1-DNA binding activity and also the induction of GSTP protein by either allyl sulfide. Our results indicate that the effectiveness of DADS and DATS on GSTP expression is likely related to the JNK-AP-1 and ERK-AP-1 signaling pathways and, thus, that DADS and DATS enhance the binding of AP-1 to GPE I.

KEYWORDS: Garlic organosulfur compounds; π class of glutathione *S*-transferase; gene expression; mitogen-activated protein kinase; activator protein-1

INTRODUCTION

The biological activities of garlic include antithrombotic, antiatherosclerotic, antidiabetic, and antioxidant activities and immune modulation (*1*−*4*). Moreover, epidemiologic evidence suggests that increased dietary consumption of garlic (*Allium sativum* L.) reduces risk of stomach and colon cancers (*5*). These health-related functions are generally attributed to the rich content in fresh garlic of γ-glutamylcysteine and to the numerous derivatives, named organosulfur compounds, formed during garlic storage and processing (*6*).

Diallyl disulfide (DADS) and diallyl trisulfide (DATS) are two major volatile allyl sulfides that account for 70% of the total organosulfur compounds in garlic oil (*7*). During the past decades, both allyl sulfides have been reported to possess diverse biological activities (*8*, *9*). The chemopreventive effect of DADS and DATS has been proposed to be due to modulation of carcinogen metabolism, including both effects on phase I and II detoxification enzymes (*10*, *11*) and interference with the cell cycle (*12*, *13*). For instance, DADS and DATS can arrest the cell cycle in the G(2)-M phase in J5 human liver tumor cells and human A549 lung cancer cells, thus resulting in cell apoptosis (*12*). More recently, the arresting of human prostate cancer cells in the G(2)-M phase by DATS was shown to be caused by hyperphosphorylation of Cdc25C (*13*). In addition, the chemopreventive use of DADS and DATS can be partly attributed to their effectiveness in the transcriptional up-regulation of phase II detoxification enzymes, including glutathione *S*-transferase (GST), UDP-glucuronyl transferase, and NAD(P)H-dependent quinone oxidoreductase, which accelerate carcinogen excretion (*10*, *11*, *14*). Indeed, DATS suppression

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of benzo(*a*)pyrene-induced forestomach neoplastic formation in mice was attributed to its up-regulating the expression of the π class of GST (GSTP) (*11*). 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 (*15*).

Recently, interest has been growing in the physiologic properties of GSTP, not only because of its action in drug detoxification but also because of its possible roles in cell transformation (*16*, *17*). Compared with other isozymes, GSTP is more effective in the detoxification of electrophilic α,β-unsaturated carbonyl compounds that are generated by radical reactions of lipids (*18*). Because it is highly inducible during carcinogenesis, GSTP expression is regarded as an important determinant of cancer susceptibility and a reliable marker of tumorigenesis (*19*). The inducibility of GSTP is generally attributed to the existence of a strong enhancer (GSTP enhancer I, GPE I) in the 5′ upstream region (*20*). GPE I has two 12-*O*-tetradecanoylphorbol-13-acetate response-like elements (TREs), which are thought to be required for the basal and inducible expression of this phase II detoxification enzyme (*21*, *22*). Multiple transcriptional factors, mainly activator protein-1 (AP-1), bind to TREs and up-regulate GSTP expression (*23*). A number of cellular stresses and cytotoxic chemicals engage c-Jun NH_2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 kinase, which in turn activate AP-1. Upon activation, AP-1 regulates the expression of a wide variety of genes involved in various biological processes in cell proliferation, differentiation, transformation, apoptosis, inflammation, and immune responses (*24*).

In a recent work, we reported that GPE I is essential for the up-regulation of GSTP by DADS and DATS (*25*). However, whether both garlic allyl sulfides act on the mitogen-activated protein kinase (MAPK)-AP-1 signaling pathway and, thus, up-regulate GSTP transcription is not clearly elucidated. In the present study, we first investigated the role of AP-1 and its upstream signal factors. Second, we determined whether other enhancer or suppressor elements in addition to GPE I are responsible for the induction of GSTP expression by DADS and DATS.

MATERIALS AND METHODS

Materials. DADS (purity ~75%) and DATS (purity ~98%) were purchased from Tokyo Kasei Chemical Co. (Tokyo, Japan) and LKT Laboratories (St. Paul, MN), respectively. Ethacrynic acid and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI-1640 media, fetal bovine serum, and penicillin-streptomycin solution were obtained from Gibco Laboratory (Grand Island, NY). Trizol and lipofectamine were ordered from Invitrogen (Carisbad, CA). SP600125 (JNK inhibitor) and PD98059 (MAPK/ERK kinase (MEK) inhibitor) were purchased from TOCRIS (Ellisville, MO). Antibodies against JNK and phospho-JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against ERK, phospho-ERK (Thr202/Tyr204), p38, and phospho-p38 (Thr180/Tyr182) were purchased from Cell Signaling Technology (Beverly, MA).

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 mmol/L HEPES, 1×10^5 unit/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 (Nuck, Roskilde, Denmark) at a density of 1×10^6 cells per dish and were allowed to grow for 24 h. Fresh culture medium containing various concentrations of DADS or DATS was then added, and the cells were incubated for indicated time. Inhibition of kinase activity by SP600125 or PD98059 at a concentration of 20 μmol/L was performed 1 h before garlic allyl sulfide treatment. Cells treated with 0.1% dimethylsulfoxide (DMSO) alone were used as controls.

Northern Blot Analysis. The specific cDNA probe for the GSTP gene was amplified by RT-PCR and labeled with α-^{32}P-dCTP with the use of a NEBlot kit (New England and Biolabs Inc., Beverly, MA) as described previously (*26*). Total RNA was isolated from Clone 9 cells by using Trizol reagent. Briefly, the RNA sample was electrophoretically separated on an agarose gel and transferred to a HyBond N^+ membrane (Amersham, Little Chalfont, UK). The membrane was then prehybridized and hybridized as described (*26*). Autoradiography was performed by exposing the membrane to Kodak SuperRx X-ray film at −80 °C with an intensifying screen.

SDS-PAGE and Western Blot. Cells were washed twice with cold PBS and were harvested in 500 μL of 20 mmol/L potassium phosphate buffer (pH 7.0). Supernatants were centrifuged at 10000*g* for 30 min at 4 °C. Protein concentrations were determined by the Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Company, Rockford, IL). Equal amounts of proteins from each sample were applied to 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene fluoride membranes. To measure the expression of GSTP and actin, the membranes were blocked at 4 °C overnight with 50 g/L nonfat dry milk solution and then incubated with antibodies against GSTP (Transduction Laboratories, Lexington, KY) or actin (Sigma Chemical, St. Louis, MO). After incubation with the horseradish peroxidase−conjugated secondary antibody, color was developed by adding hydrogen peroxide and tetrahydrochloride diaminobenzidine as peroxidase substrates. For the detection of MAPK, the membranes were incubated overnight at 4 °C with anti-JNK1, anti-ERK1/2, and anti-p38 MAPK or anti-phospho-activated JNK1, ERK1/2, and p38 MAPK antibodies. The bands were detected by using an enhanced chemiluminescence kit (Perkin-Elmer Life Science, Boston, MA).

Preparation of Nuclear Extract. Clone 9 cells were treated with DMSO or allyl sulfides for 3 h and were then washed twice with cold PBS followed by scraping from the dishes with PBS. Cell homogenates were centrifuged at 2000*g* for 5 min. The supernatant was discarded, and 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 $MgCl_2$, 1 mmol/L EDTA, 0.5 mmol/L DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 0.5% Nonidet P-40, 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 $MgCl_2$, 1 mmol/L EDTA, 0.5 mmol/L DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 10% glycerol, and 0.2 mmol/L phenylmethylsulfonyl fluoride and incubated for an additional 30 min on ice. The nuclear extracts were then obtained by centrifugation at 10000*g* for 15 min and were frozen at −80 °C until the electromobility gel shift assay (EMSA) was performed.

Electromobility Gel Shift Assay. EMSA was performed according to our previous study (*27*). The LightShift Chemiluminescent EMSA Kit (Pierce Chemical Company, Rockford, IL) and synthetic biotin-labeled double-stranded AP-1 consensus oligonucleotides (5′-CGCTTGATGACTCAGCCGGAA-3′) were used to measure the effect of allyl sulfides on AP-1 nuclear protein−DNA binding activity. Two micrograms of nuclear protein, poly(dI-dC), and biotin-labeled double-stranded AP-1 oligonucleotides 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% TBE-polyacrylamide gel and was then electrotransferred to a Hybond-N^+ nylon membrane. The membrane was treated with streptavidin-horseradish peroxidase, and the nuclear protein−DNA bands were developed by using an enhanced chemiluminescence kit.

Peroxide Measurement. Detection of intracellular oxidative states was performed by using the probe 2,7-dichlorofluorescin diacetate (DCF-DA) (Molecular Probes Inc., Eugene, OR) (*28*). After cells reached 90% confluence, Clone 9 cells were incubated in RPMI 1640 medium containing 10% fetal bovine serum and each of the allyl sulfides for 0.5, 4, or 16 h. As a control, an equal amount of DMSO was added to untreated cells. After being washed with PBS, cells were

incubated in fresh medium with 50 μmol/L DCF-DA for 50 min. The DCF fluorescent was then performed in a FACScalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

Biochemical Assays. GST activity was measured according to the method of Habig et al. (*29*) by using ethacrynic acid as the substrate because of its better selectivity for the π class isozyme (*30*). Briefly, the reaction mixture in a final volume of 1 mL contained 100 mmol/L potassium phosphate buffer (pH 6.5), 0.25 mmol/L glutathione, 0.2 mmol/L ethacrynic acid, and an appropriate amount of the cytosolic proteins. The ethacrynate−glutathione conjugate formed was measured at 270 nm.

Expression and Reporter Constructs. The pTA-GSTP Luc reporter with 2.7 kb GSTP gene promoter region was constructed as described previously (*25*). Briefly, the rat GSTP promoter region was generated by PCR amplification with rat genomic DNA as a template. The oligonucleotide primer (forward: 5′-GCCTCAGCTGGTAAATG-GATAA-3′; reverse: 5′-AAAGGCCCCAGAGCCGCCAGCC-3′) was designed on the basis of the published sequence (*20*, *31*). The PCR amplicons were then identified in 1%-agarose gels containing 1X TAE buffer (40 mmol/L Tris, 20 mmol/L glacial acetic acid, and 2 mmol/L EDTA). The band corresponding to the designated length was excised, and the DNA was purified by using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). In addition to the full-length construct (Luc E), 4 constructs with deletions from −2713 to −2186 bp (Luc 1), −2185 to −1578 bp (Luc 2), −1577 to −1028 bp (Luc 3), and −1027 to −151 bp (Luc 4) were generated.

Transient Transfection and Luciferase Activity Assay. 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 4 h with 0.1 μg of the pTA-GSTP Luc vectors by lipofectamine reagent and were then changed to fresh culture media for 5 h before being exposed to DADS or DATS for an additional 12 h. Cells were then washed twice with PBS and were lysed in 100 μL of lysis buffer (Clontech, Palo Alto, CA). 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 *O*-nitrophenyl β-D-galactopyranoside as a substrate. The value for cells treated with DMSO vehicle alone was regarded as 1.

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 Tukey's test. A value of $P < 0.05$ was considered to be significant.

RESULTS

GSTP mRNA and Protein Expression and Enzyme Activity. The immunoblot assay showed that 5−200 μmol/L of DADS and DATS concentration dependently increased GSTP protein levels in Clone 9 cells, and the increase in expression caused by DATS was higher than that caused by DADS (**Figure 1A**). By Northern blot, the increases in GSTP mRNA levels were consistent with the changes noted in protein expression (**Figure 1B**). We also measured the enzyme activity of GSTP by using ethacrynic acid as a substrate. Compared with that in the control cells, enzyme activity increased in a concentration-dependent manner in cells treated with DADS and DATS ($P < 0.05$) (**Figure 1C**). Increases of 90% and 110% in GSTP enzyme activity were noted in cells treated with 200 μmol/L DADS and DATS, respectively, as compared with the control cells.

Allyl Sulfides and AP-1 Activation. To demonstrate that AP-1 is involved in the induction of GSTP expression by garlic allyl sulfides, we performed EMSAs. Upon treatment with DADS and DATS, the DNA binding activity of AP-1 nuclear protein increased concentration dependently compared with that of the control cells (**Figure 2A**). After pretreatment with the JNK and ERK inhibitors SP600125 (**Figure 2A**) and PD98059

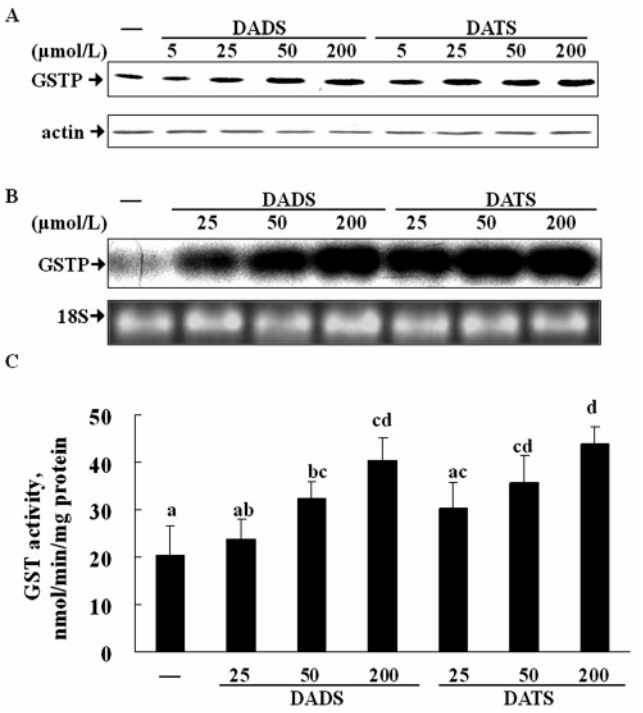

Figure 1. Diallyl disulfide (DADS)- and diallyl trisulfide (DATS)-induced expression of the π class of glutathione *S*-transferase (GSTP) in Clone 9 cells. Clone 9 cells were cultured with 0.1% dimethylsulfoxide alone (—) or with 5, 25, 50, or 200 μmol/L of DADS or DATS for 24 h. (**A**) GSTP protein was determined by immunoblot assay. A total of 5 μg of protein of each sample was applied for electrophoresis. (**B**) Changes in GSTP mRNA levels induced by DADS or DATS treatment. (**C**) The activity of GST was determined by using ethacrynic acid as a substrate. Values are means ± SD, $n = 3$∼5. Groups not sharing a common letter differ significantly, $P < 0.05$.

(**Figure 2B**) however, the increase in AP-1 binding to DNA induced by either allyl sulfide was inhibited. DNA binding activity also increased in cells treated with 50 μmol/L *t*-butyl hydroquinone (t-BHQ), an antioxidant and a well-known phase II detoxification enzyme inducer, although to a lesser extent than in cells treated with DATS (**Figure 2C**).

Allyl Sulfides and MAPK Kinases. MAPK signaling pathways, including JNK, ERK, and p38, are upstream mediators of AP-1. The activation of each MAPK kinase was determined 1 h after the addition of garlic allyl sulfides. Immunoblot analysis showed that DADS and DATS concentration-dependently increased JNK and ERK phosphorylation (**Figure 3A**,**B**), but not p38 phosphorylation (**Figure 3C**). Pretreatment of cells with SP600125 or PD98059 suppressed the induction of JNK and ERK activation by DADS and DATS. We finally determined whether the up-regulation of GST protein expression by allyl sulfides is suppressed in the presence of either JNK or ERK inhibitor. As noted, SP600125 and PD98059 pretreatment suppressed the expression of this phase II detoxification enzyme ($P < 0.05$) (**Figure 4**).

Allyl Sulfides and Cellular Peroxides. We investigated changes in cellular oxidative states as a potential indication of garlic allyl sulfide-induced MAPK-AP-1 activation in Clone 9 cells. In the presence of DADS and DATS, cells underwent a dramatic increase in reactive oxygen species flux as early as 0.5 h ($P < 0.05$), which then decreased to the basal level at 4 h (**Figure 5**). It is interesting to note that at 16 h the intensity of DCF fluorescence of those cells incubated with DADS and DATS was even lower than that in the control cells ($P < 0.05$).

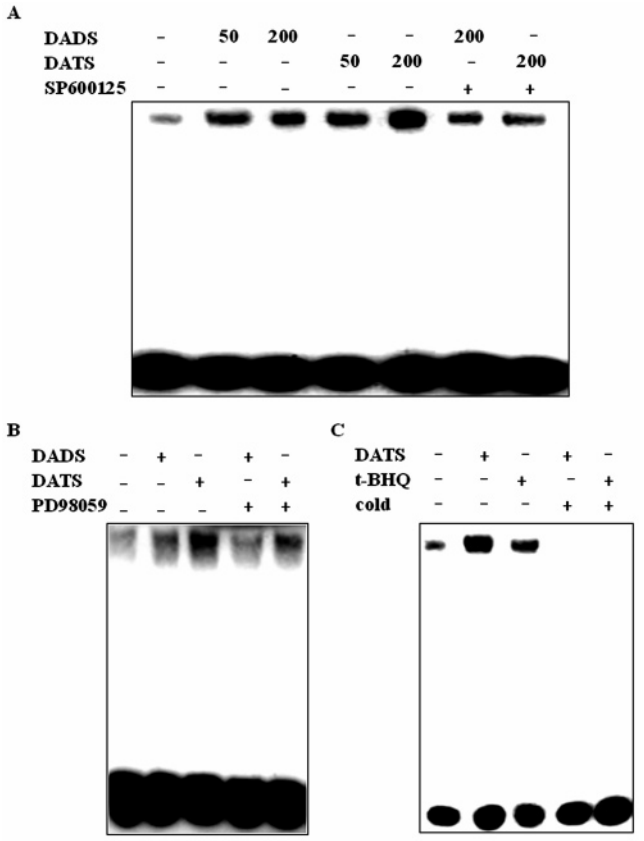

Figure 2. Activation of AP-1 nuclear protein DNA binding activity by garlic allyl sulfides in Clone 9 cells. (**A**) Cells were treated with 200 μmol/L diallyl disulfide (DADS) or diallyl trisulfide (DATS) for 3 h, and then nuclear extracts were prepared to measure AP-1 nuclear protein DNA binding activity by electromobility gel shift assay. For inhibitor treatment, SP600125 was added 1 h before allyl sulfide treatment. (**B**) Cells were pretreated with PD98059 for 1 h and were then exposed to 200 μmol/L DADS or DATS for 3 h in the presence or absence of the ERK inhibitor. (**C**) Cells were incubated with 200 μmol/L DATS or 50 μmol/L *tert*-butylhydroquinone (t-BHQ) for 3 h. Unlabeled double-stranded AP-1 oligonucleotide (100 ng) was added for the competition assay. The band for AP-1 nuclear protein-DNA binding was detected by using a streptavidin-horseradish peroxidase and was developed by using an Amersham ECL kit.

GSTP Promoter Activity. Luciferase-reporter constructs were transiently transfected into Clone 9 cells to determine whether the promoter activity of the GSTP gene was modulated by the garlic allyl sulfides and to locate the putative responsive sites. DADS and DATS significantly increased GSTP promoter activity by 3.4- and 3.9-fold, respectively, compared with that in the control (Luc E) cells (**Figure 6**). This induction of reporter activity was completely abolished when the −2713 to −2186 bp region of the GSTP promoter (Luc 1), which contains GPE I, was deleted. This result is consistent with our previous report that GPE I is required for DADS and DATS up-regulation of GSTP expression. Further deletion to −1578 bp (Luc 2) resulted in a partial but significant recovery of the allyl sulfide-induced luciferase activity ($P < 0.05$). A 1.7- and 2.5-fold increase in luciferase activity was noted with the Luc 2 reporter in the presence of DADS and DATS, respectively, compared with the Luc 1. After further deletion to −1028 bp (Luc 3) and −151 bp (Luc 4), the recovery of GSTP promoter activity disappeared. These results suggest that elements in addition to GPE I in the GSTP promoter region likely play a role in the modulation of transcription by DADS and DATS.

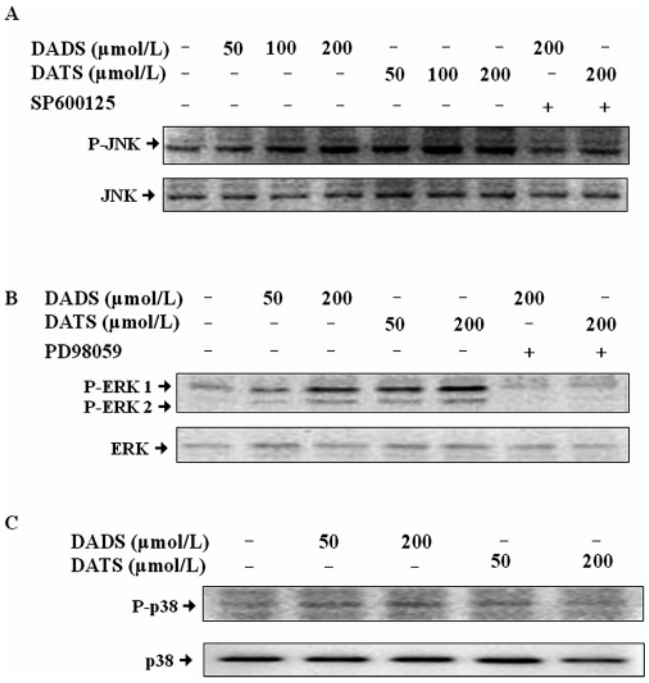

Figure 3. Activation of JNK (**A**), ERK (**B**), and p38 (**C**) phosphorylation in Clone 9 cells by garlic allyl sulfides. Cells were treated with various concentrations of diallyl disulfide (DADS) or diallyl trisulfide (DATS) for 1 h in the presence or absence of SP600125 or PD98059, which were added to cells 1 h before allyl sulfide treatment. Activation of ERK1/2, JNK, and p38 was assessed by immunoblot analysis of the phosphorylated forms (P-) of the mitogen-activated protein kinases in whole cell lysates. Results were confirmed by four separate experiments, and a representative blot is shown.

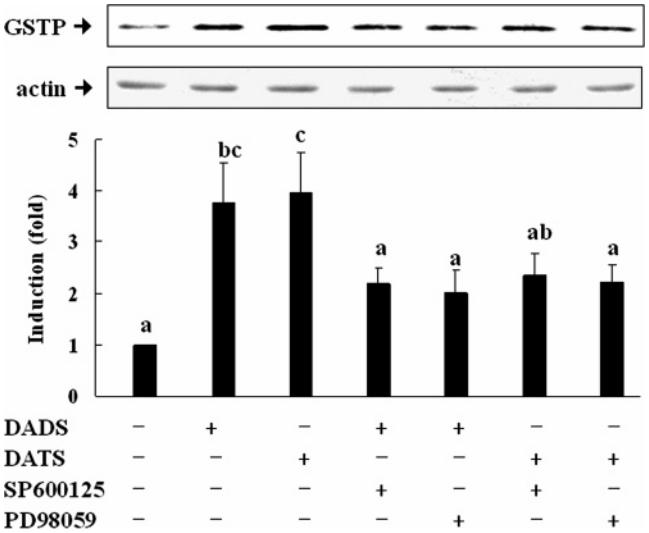

Figure 4. Mitogen-activated protein kinase inhibitors suppress garlic allyl sulfide-induced GSTP protein in Clone 9 cells. Cells were incubated with 200 μmol/L diallyl disulfide (DADS) or diallyl trisulfide (DATS) for 24 h in the presence or absence of SP600125 or PD98059. Cytosolic proteins were prepared, and GSTP levels were measured by immunoblot assay. Changes in GSTP protein expression were measured by densitometry. The level in control cells was regarded as 1. Each value represents the means ± SD of three independent experiments. Groups not sharing a common letter differ significantly, $P < 0.05$.

DISCUSSION

The expression of GSTP is highly inducible not only by a variety of exogenous xenobiotics but also by several dietary

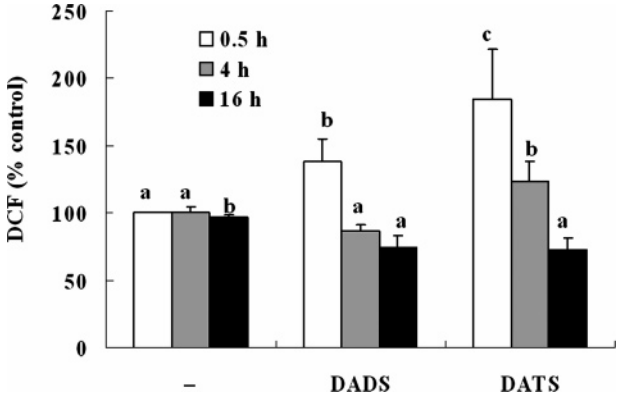

Figure 5. Changes in cellular peroxides by garlic allyl sulfides. Clone 9 cells were treated with 200 μmol/L of diallyl disulfide (DADS) or diallyl trisulfide (DATS) for 0.5, 4, and 16 h. The DCF fluorescent was performed by a FACScalibur flow cytometer. The fluorescence intensities in control cells are expressed as 100%. Values are means ± SD, $n = 3\sim6$. Groups not sharing a common letter differ significantly at the same time, $P < 0.05$.

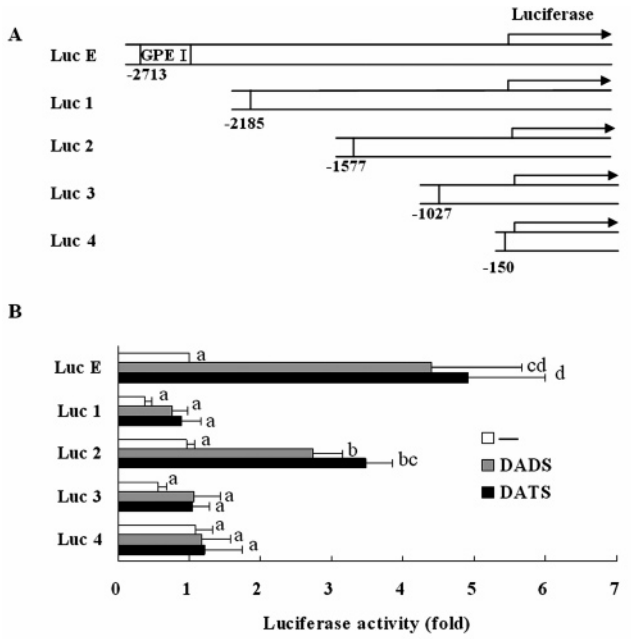

Figure 6. Changes in the GSTP promoter activity by garlic allyl sulfides. Serial deletions of the pTA-GSTP Luc DNA constructs (pTA-2713, pTA-2185, pTA-1577, pTA-1027, and pTA-150) were transfected into Clone 9 cells, which were then treated with 0.1% dimethylsulfoxide alone (—) or with 200 μmol/L of diallyl disulfide (DADS) or diallyl trisulfide (DATS) for 12 h. The luciferase activity of cells transfected with pTA-2713 and treated with dimethylsulfoxide alone (—) was regarded as 1. Values are means ± SD, $n = 5$. Groups not sharing a common letter differ significantly, $P < 0.05$.

factors, including nutrient and nonnutrient factors (*26*, *32*, *33*). In the past few years, we have shown that both the amount and type of fats and amino acids are two nutrients that effectively modulate the expression of this phase II detoxification enzyme (*26*, *32*). Increases in hepatic GSTP protein and mRNA levels are noted in rats fed a diet rich in corn oil and also a diet in which fish oil is substituted for corn oil (*26*). In an in vitro study, limiting the supply of methionine and cysteine enhanced GSTP expression in primary rat hepatocytes (*32*). Organosulfur compounds of garlic are also known to be effective inducers of GSTP (*33*). The differences in the chemical properties and physiologic roles of these dietary inducers suggest that the modulatory mechanism on the GSTP gene cannot be attributed to a single pathway. To our knowledge, limited evidence is available for the actual molecular mechanism of action of fish oil, sulfur amino acids, and garlic organosulfur compounds on this GST isozyme. In a previous study, we showed that an enhancer element named GPE I is essential for the up-regulation of GSTP gene transcription by DADS and DATS (*25*). In the present study, we further demonstrate that DADS and DATS up-regulate GSTP expression via the ERK and JNK signaling pathways, which activate the binding of AP-1 to GPE I. Furthermore, the luciferase-reporter activity assay identified a silencer that is likely involved in the modulation of the GSTP gene by DADS and DATS.

GPE I contains two TRE-like elements, and both elements are required for the basal and inducible expression of GSTP (*21*, *22*). Deletion of the TRE abolishes the induction of GSTP transcription by 3,4,5,3′,4′-pentachlorinated biphenyl in primary hepatocytes (*21*). Because the TRE-like sequence on GPE I (5′-AG*TCAGTCA*CTA*TGATTCA*GCA-3′) is similar to the core sequence of the AP-1 binding site (5′-TGACTCA-3′), AP-1 is thought to be the key transcription factor in the induction of GSTP. Our present results clearly show that the DNA binding activity of AP-1 is increased in the presence of DADS and DATS and that inhibiting AP-1 activation by pretreating the cells with SP600125 or PD98059 suppresses GSTP protein expression. An understanding of the role of the AP-1-mediated signal pathway in GSTP transcriptional regulation will help to clarify the possible molecular mechanism of action of garlic components in drug metabolism and cancer prevention.

MAPK kinase phosphorylates and activates MAPKs. MAPKs including ERK, JNK, and p38 are known to be a common signal mediating AP-1 activity (*24*). ERK and JNK are often responsive to different extracellular signals (*34*, *35*). However, they can also be activated by the same stimuli, such as mitogenic signals, growth factors, oncogenic *Ras*, stress signals, ultraviolet radiation, and oxidative stress (*36*−*39*). Activation of ERK and JNK generally contributes to cell survival or proliferation (*40*, *41*), and activation of p38 precedes the induction of apoptosis (*42*). During the past few years, evidence has indicated that activation of MAPK is closely associated with the biological effects of garlic. For instance, phosphorylation of ERK and JNK is responsible for the DATS-induced apoptosis of human PC-3 prostate cancer cells and hepatoma HepG2 cells (*43*). DADS-induced SH-SY5Y neuroblastoma cell apoptosis was highly dependent on the activation of the reactive oxygen species-dependent JNK/c-Jun pathway (*44*). Moreover, DADS arrest of HL-60 cells in the G(2)/M phase is associated with the activation of p38 (*45*). In the modulation of phase II detoxification enzymes, MAPK activation has also been shown to be responsible for NAD(P)H-dependent quinone oxidoreductase, UDP-glucuronosyl transferase, and quinone reductase induction by isothiocyanates, including sulforaphane, phenethyl isothiocyanate, and benzyl isothiocyanate (*46*−*48*).

In the present study, we showed that ERK 1/2 and JNK 1 activation occurs before the induction of GSTP by allyl sulfides and that this up-regulation and AP-1 binding activity to DNA are suppressed by PD98059 and SP600125. This evidence indicates that the ERK 1/2- and JNK 1-AP-1 signaling pathways are important in the up-regulation of GSTP by DADS and DATS. In contrast, the lack of change in p38 suggests that it has a minor role in the action of both allyl sulfides. To our knowledge, this is the first report showing that the induction of GSTP activity by DADS and DATS is dependent on the ERK-AP-1 and JNK-AP-1 signaling pathways.

As stated, the ERK- and JNK-AP-1 signaling pathways have been shown to play an important role in the up-regulation of GSTP gene transcription by DADS and DATS. The involvement of other transcription factors, however, cannot be excluded. Among those possible transcriptional factors, Nrf2 is the most likely candidate. The binding of Nrf2 to the ARE is well-known to up-regulate the transcription of several phase II detoxification enzymes, including NAD(P)H:quinone oxidoreductase and heme oxygenase (*49*). Although the GSTP gene promoter region lacks a typical ARE core sequence, evidence has shown that the Nrf2/Mafk heterodimer can bind to GPE I and up-regulate GSTP transcription in the early hepatocarcinogenesis stage of rat H4IIE hepatoma cells (*50*). Moreover, the induction of GSTP by 6-methylsulfinylhexyl isothiocyanate of wasabi and oltipraz was completely abrogated in Nrf2-deficient mice (*51*). Taken together, these data suggest that Nrf2 may play a role in the induction of GSTP expression by DADS and DATS. Further study is required to answer this question.

AP-1 activation is known to be closely associated with increases in reactive oxygen species production (*52*). DADS-initiated neuroblastoma cell death is dependent on reactive oxygen species activation of the JNK/c-Jun signaling cascade (*44*). However, reactive oxygen species generation is reduced in the JNK-/- fibroblast. Generation of reactive oxygen species by DATS is suggested as a causative for the JNK activation (*53*). In the present study, the change in cellular redox status by allyl sulfides was monitored by DCF-DA cytofluorometric assay. A transient increase in cellular oxidative stress by DADS and DATS was noted in Clone 9 cells. Peroxide production increased during the first 30 min and then decreased to the basal level at 4 h; at 16 h, peroxide levels were even lower than in the control cells (**Figure 5**). This suggests that the activation of MAPK-AP-1 signaling can be explained, at least in part, by a transient change in redox states caused by DADS and DATS. This explanation is supported by the finding that (−)-epigallocatechin-3-gallate activation of the MAPK-AP-1 signaling pathway and, thus, the induction of HT-29 human colon cancer cell apoptosis are prevented by glutathione and *N*-acetyl-L-cysteine (*54*, *55*).

An enhancer element located at −2.6 to −2.7 kb, i.e., GPE I, was shown to be essential for DADS and DATS up-regulation of GSTP transcription in our previous study. In the present study, we further constructed a series of pTA-GSTP Luc deletions to examine the existence of other regulatory elements that are involved in the garlic allyl sulfide induction of GSTP. In addition to GPE I, two candidates are worthy of further attention. The one located between −2.0 and −1.5 kb acts as a silencer, and the other located at −1.5 to −1.0 kb acts as an enhancer. To our knowledge, this is the first report of an enhancer different from GPE I and II in the GSTP gene promoter. The existence of a negative regulatory element in the GSTP gene promoter has also been reported by Sakai and colleagues (*20*). However, the actual site reported in their work was more proximate to the transcription start site, i.e., at approximately −0.4 kb. Recently, three factors termed silencer factor A, silencer factor B, and silencer factor C were shown to bind to this silencer region (*56*). Evidence suggests that the regulation of the transcription of this phase II detoxification enzyme is likely via multiple DNA elements and protein factors that act in a complex manner.

In conclusion, the up-regulation of GSTP transcription by DADS and DATS is dependent on the JNK-AP-1 and ERK-AP-1 signaling pathways, the activation of which is likely related to transient changes in cellular redox states. In addition to GPE I, other regulatory elements in the GSTP gene promoter likely play a role in the induction of this phase II detoxification enzyme.

ABBREVIATIONS

AP-1, activator protein-1; ARE, antioxidant response element; t-BHQ, *tert*-butylhydroquinone; DADS, diallyl disulfide; DATS, diallyl trisulfide; DCF-DA, dichlorofluorescin diacetate; DMSO, dimethylsulfoxide; EMSA, electromobility gel shift assay; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; GSTP, π class of GST; GPE I, GSTP enhancer I; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; TRE, 12-*O*-tetradecanoylphorbol-13-acetate response element.

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