第一部份

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Sulforaphane and α-Lipoic Acid Upregulate the Expression of the π 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 π class of glutathione *S*-transferase (GSTP) through the activator protein-1 pathway. Here, we examined the modulatory effect of sulforaphane (SFN) and α-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 μmol/L) or SFN (0.2–5 μmol/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 μmol/L SFN, 50 μmol/L DATS, and 600 μmol/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. doi: 10.3945/jn.110.121418.

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 A (α), M (μ), O (ω), P (π), S (σ), T (θ), Z (ζ), and K (κ), have been identified (10). Recently, interest has grown in the physiologic properties of the π 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, π class of glutathione *S*-transferase enhancer I; GST, glutathione *S*-transferase; GSTA, α class of glutathione *S*-transferase; GSTM, μ class of glutathione *S*-transferase; GSTP, π class of glutathione *S*-transferase; LA, α-lipoic acid; NQO1, NAD(P)H-dependent quinone oxidoreductases 1; Nrf2, nuclear factor erythroid-2 related factor 2; SFN, sulforaphane; TRE, 12-*O*-tetradecanoylphorbol-13-acetate responsive element.

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benzo[*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*-tetradecanoate-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 phosphatidylinositol 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% 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) at a density of 2.5×10^5 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 $10{,}000 \times g$ 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 antibody 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 Perkinelmer 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 $(NH_4)_2SO_4$, 0.3% β-mercaptoethanol, 0.1 g/L bovine serum albumin, 5 mmol/L $MgCl_2$, 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 ddH_2O, 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 $2^{-\Delta\Delta Ct}$, where $\Delta\Delta$Ct represents difference of Ct between the GSTP gene and the GAPDH gene ($\Delta Ct = Ct_{GSTP\ gene} - Ct_{GAPDH\ gene}$; $\Delta\Delta Ct = \Delta Ct_{GSTP\ gene} - \Delta Ct_{calibrator}$).

Enzyme activity assays. GST activity was measured by using ethacrynic 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 ethacrynic acid, and an appropriate amount of the total proteins. The ethacrynate-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 *Mlu*I and *Nhe*I 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 2.5×10^5 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 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 $MgCl_2$, 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 $MgCl_2$, 1 mmol/L EDTA, 0.5 mmol/L 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 obtained by centrifugation at 10,000 × *g* for 15 min and were frozen at −80°C until the EMSA was performed.

The LightShift Chemiluminescent EMSA kit (Pierce Chemical) and synthetic biotin-labeled double-stranded GPEI consensus oligonucleotide (forward: 5′-AGTAGTCAGTCACTATGATTCAGCAAC-3′; reverse: 5′-GTTGCTGAATCATAGTGACTGACTACT-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 supershift 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, 1 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.

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.

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-2yl)-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. 2***A*). Again, enzyme activity toward ethacrynic acid was dose dependently increased by LA, DHLA, and SFN (Fig. 2*B*).

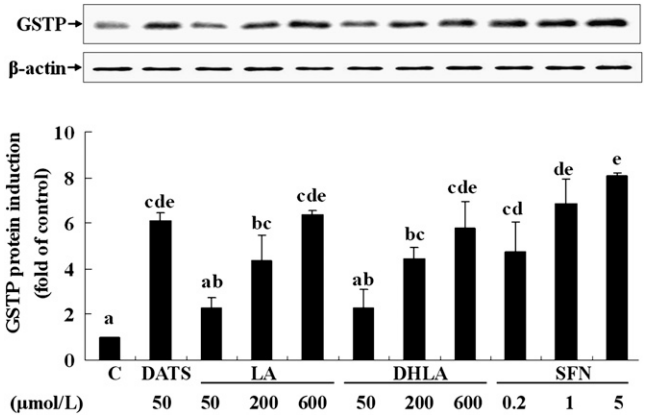

FIGURE 1 Protein levels of GSTP induced by organosulfur compounds. Cells were cultured with 0.1% DMSO alone (−) or with various concentrations of DATS, LA, DHLA, or SFN for 24 h. GSTP protein was determined by immunoblot assay. A total of 4 μg protein for each sample was applied for electrophoresis. 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, $P < 0.05$.

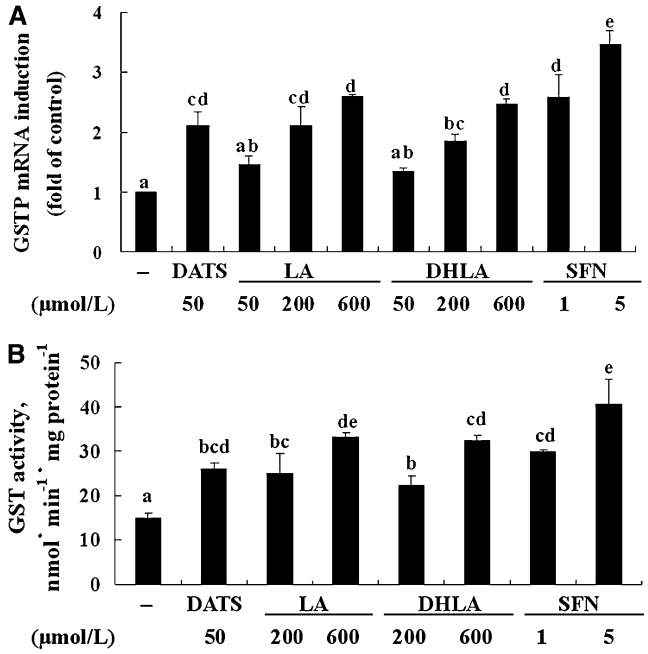

FIGURE 2 mRNA expression and enzyme activity of GSTP induced by organosulfur compounds. Cells were treated with DMSO alone (−) or with various concentrations of DATS, LA, DHLA, or SFN for 24 h. (*A*) Real-time PCR of GSTP mRNA expression. The GSTP mRNA level in the control cells was regarded as 1. (*B*) GST activity determined by using ethacrynic acid as a substrate. Values are means ± SD, $n = 3$–4. Groups without a common letter differ, $P < 0.05$.

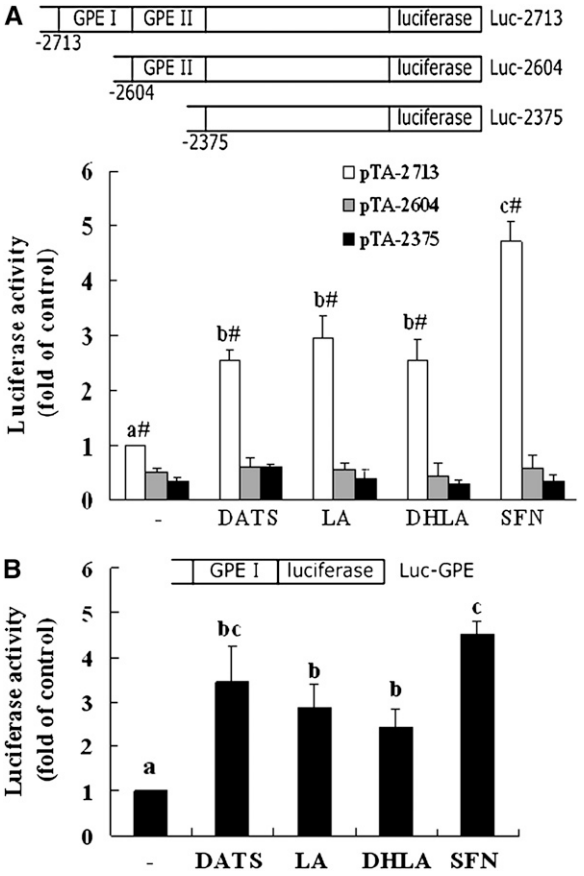

FIGURE 3 GPEI is required for the upregulation of GSTP by phyto-derived organosulfur compounds. (*A*) Cells were transfected with different constructs and were then treated with 50 μmol/L DATS, 600 μmol/L LA, 600 μmol/L DHLA, or 5 μmol/L of SFN for 15 h. The luciferase activity of cells transfected with pTA-2713 and treated with DMSO alone (−) was regarded as 1. (*B*) The GPEI-linked construct was transfected into cells and the cells were treated with DATS, LA, DHLA, or SFN for 15 h. Values are means ± SD, $n = 3$–4. Groups in the same construct without a common letter differ, $P < 0.05$. #Different from the 2 deletion constructs in the same treatment, $P < 0.05$.

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. 3***A*). A 1.7-fold increase in reporter activity was noted in cells treated with 50 μmol/L DATS. When the −2713- 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 (−2713 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. 3*B*). 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. 4***A*). 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 super-shift 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. 4*B*). 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. 4*B*). Accompanied by the decrease in Nrf2-GPEI binding, Nrf2 in the nuclear immunoprecipitates was increased in cells treated with organosulfur compounds (Fig. 4*C*). The activation of c-Jun after immunoprecipitation with anti-c-Jun antibody was consistent with the result of organosulfur compound-induced nuclear Nrf2 accumulation. Following the immunoprecipitation, whether c-Jun interacts to Nrf2 by serving as a partner was determined. No Nrf2 was detected in the nuclear immunoprecipitates with c-Jun antibody (Fig. 4*C*). Similarly, in the Nrf2 immunoprecipitates, there was no p-c-Jun detected.

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

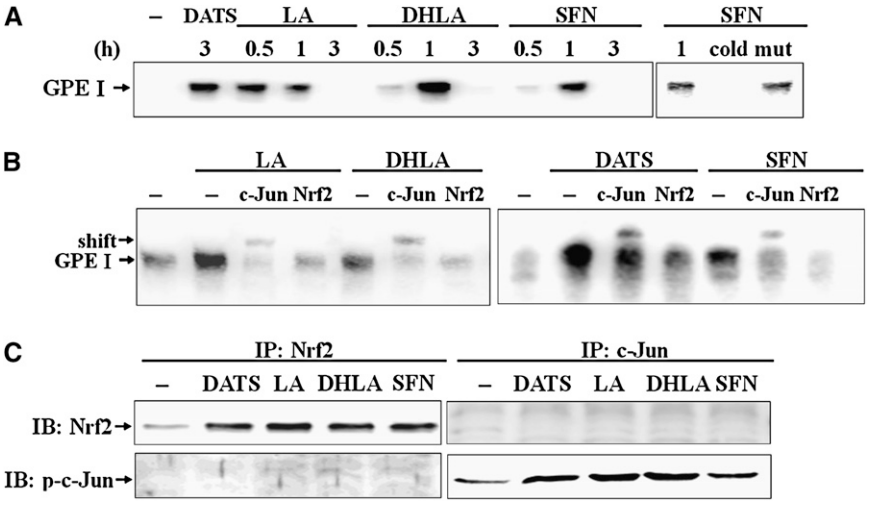

FIGURE 4 Activation of GPEI binding activity by phytoderived organosulfur compounds. (*A*) Cells were treated with 200 μmol/L DATS, 600 μmol/L LA, 600 μmol/L DHLA, or 5 μmol/L SFN for the indicated times and nuclear extracts were prepared to measure GPEI binding activity by EMSA. Free probe at the bottom is not shown. (*B*) Nuclear proteins isolated from the cells treated with DATS for 3 h and LA, DHLA, SFN for 1 h were first added with GPEI oligonucleotides into each reaction for 30 min and were then incubated with antibodies to c-Jun for an additional 30 min at room temperature. The subsequent super-shift complexes were separated by 6% acrylamide gel electrophoresis. Aliquots of the supernatant after immunoprecipitation with anti-Nrf2 antibody were used for EMSA. (*C*) Nuclear extracts isolated from the cells treated with DATS for 3 h and LA, DHLA, and SFN for 1 h were subjected to immunoprecipitation (IP) with anti-Nrf2 or anti-c-Jun antibody. Aliquots of pellet after IP (15 μg) were used for immunoblotting (IB) with anti-Nrf2 or anti-phospho-c-Jun antibody. The results shown are representative of 4 experiments.

stimulated GSTA, GSTM, and NQO-1 protein contents as well as that noted for GSTP (**Fig. 5***A*). In addition, enzyme activity toward 1-chloro-2,4-dinitrobenzene (Fig. 5*B*) and 2,6-dichloroindophenol (Fig. 5*C*) 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

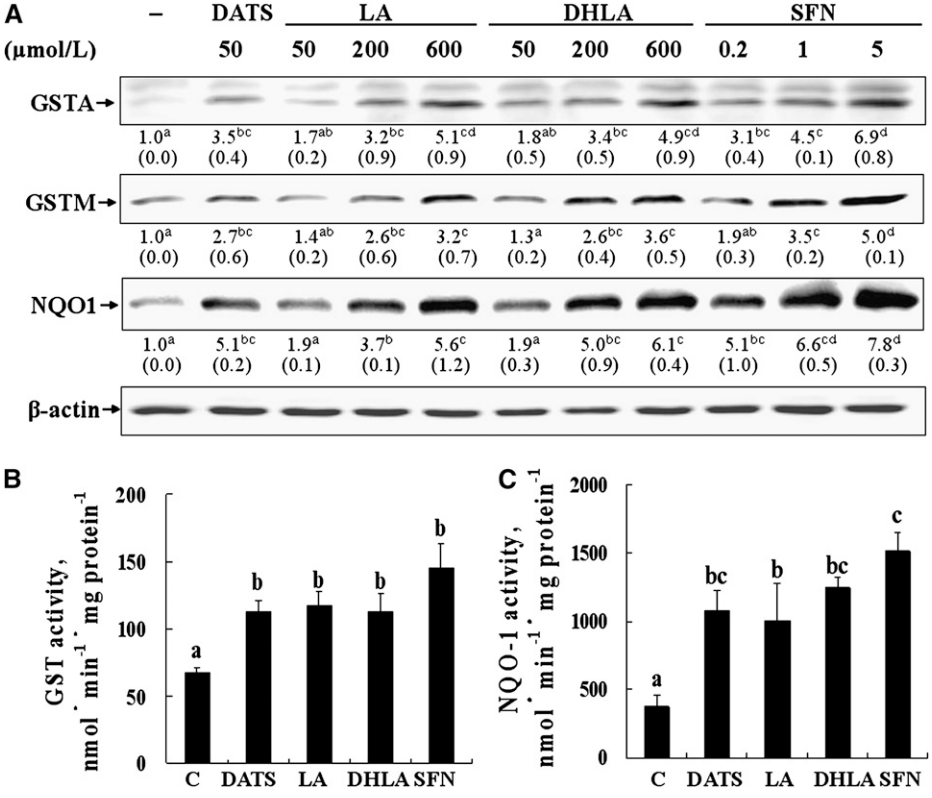

FIGURE 5 Protein expression and enzyme activity of the phase II detoxification enzymes induced by organosulfur compounds. Cells were cultured with 0.1% DMSO alone (−) or with various concentrations of DATS, LA, DHLA, or SFN for 24 h. (*A*) GSTA, GSTM, and NQO1 protein were determined by immunoblot assay. A total of 8 μg protein for each sample was applied for electrophoresis. The protein was quantified by densitometry and the level in control cells was set 1. Values are means (SD), $n = 3$. Means without a common letter differ, $P < 0.05$. Cells were cultured with 50 μmol/L DATS, 600 μmol/L LA, 600 μmol/L DHLA, or 5 μmol/L SFN for 24 h to determine GST (*B*) and NQO1 (*C*) activity. Values are means ± SD, $n = 3$–4. Groups without a common letter differ, $P < 0.05$.

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 Okuda 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. 4*B*).

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′-AGTCAGTCACTATGATTCAGCA-3′) and the conserved sequences of the ARE (5′-GTGACNNNGCA-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-Δ-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-methylsulfinylhexyl 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 regulate 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.

Acknowledgments

C.K.L., H.W.C., and C.W.T. designed research; C.W.T., K.L.L., and C.K.L. conducted research; Y.P.C., and A.H.L. analyzed data; C.K.L. and C.W.T. wrote the paper. C.W.T. had primary

responsibility for final content. All authors read and approved the final manuscript.

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第二部份

緣由與目的

現代人重視生活品質，希望藉由攝取自然的食材來達到保護身體健康和預防疾病發生，已有許多實驗證實蔬菜和水果中含有不少種類的植物化學因子(phytochemicals)，具調節人體代謝的功能，並有助於抗氧化、抗發炎、抗過敏及抗癌的作用，包括 phenolic compounds、flavonoids、carotenoids、indoles 和含硫化合物 (sulfur compounds)等。含硫化合物廣泛存在各式各樣食物內，比如存於蔥蒜的有機硫成份 (organosulfur compounds, OSCs)中的二烯丙基二硫化物(dially disulfide, DADS)和二烯丙基三硫化物 (dially trisulfide, DATS)、硫辛酸(α-lipoic acid, LA)及其還原態二氫硫辛酸 (dihydrolipoic acid, DHLA)和蘿蔔硫素(sulforaphane, SFN)等。由本計畫第一部份的研究發現，DATS、LA、DHLA 和 SFN 顯著誘發大鼠 Clone 9 細胞株中肝臟解毒酵素 phase II 系統 GSTP 的酵素活性、mRNA 和蛋白質表現，也可以增加 GSTP 的 luciferase 活性，除此之外，其他 phase II 酵素如 GSTA、GSTM 和 NQO-1 的蛋白質和酵素活性也顯著增加，接著自行建構 GSTP 報導基因 (reporter gene)，轉殖入 Clone 9 肝細胞株，結果顯示：GSTP 基因啟動中的 GST enhancing element I (GPE I)是 DATS、LA、DHLA 和 SFN 增加轉錄作用所必須。第二部分將研究其後續的可能分子機制，由計畫主持人在 2007 年發表的 paper 指出，DADS 和 DATS 經由 mitogen-activated protein kinase (MAPK)中 ERK 和 JNK 訊息路徑誘發大鼠 Clone 9 肝細胞的 GSTP 表現。因此本實驗將探討其他非屬蔥蒜的含硫化合物：LA、DHLA 和 SFN 是否也走相同的訊息傳導路徑，此外，近年有研究發現 PI3K (phosphatidylinositol 3-kinase)/ Akt 路徑參與調控大鼠初代肝細胞的 GSTP 表現，也將一併討論。

材料與方法

[實驗材料]

1. Clone 9肝細胞株

Clone 9 細胞為大鼠正常肝細胞，購自台灣生物資源保存與研究中心(Bioresources Collection and Research Center, BCRC)。

2. 含硫成分試藥

(1) Diallyl trisulfide (DATS)購自 LKT Laboratories Inc. (St. Paul, MN)，純度 99%。

(2) Lipoic acid (LA)購自Sigma Chemical Co. (St. Louis, MO)，純度 99%。

(3) Dihydrolipoic acid (DHLA)購自Sigma Chemical Co. (St. Louis, MO)，純度 99%。

(4) Sulforaphane購自Sigma Chemical Co. (St. Louis, MO)，純度 98%。

[實驗方法]

1. 細胞處理

將 Clone 9 細胞株培養於含 10%胎牛血清、1×10^5 unit/L penicillin及 100 mg/L streptomycin 之 RPMI-1640 培養液中，培養條件設為 37℃與 5% CO_2。接種 1×10^6 個細胞於 3 cm之培養盤中，生長至 80% confluence後，分別加入不同濃度之含硫化合物，分別於指定的時間點收取細胞，4℃下以超高速離心 (10,500 g) 30 分鐘，所得上清液即為蛋白質液，分別以Western blot 測定GSTP蛋白質表現。在探討轉錄因子的上游活化路徑(MAPK或PI3K/Akt)部分，細胞先預處理 PD98059 (MAPK/ERK 抑制劑, 20 μM)或 wortmannin (PI3K抑制劑, 1 μM) 1 小時，再處理含硫化合物。

2. 西方墨點法 (Western blotting)

細胞蛋白質濃度測得後，將細胞質原液與 5 X sample buffer 混合，並將每一樣品的最終蛋白質濃度調整為 0.4 μg/μl，於 95℃下乾浴 5 分鐘使蛋白質變性，取適量體積之樣品注入 SDS-PAGE 的樣品槽中，以 Tank buffer (含 25 mM Tris- HCl、192 mM glycine 及 0.1% SDS，pH 8.3)為電泳液在 130 伏特電壓進行電泳。

電泳完成後，取下膠體，切除 stacking gel，將 separating gel 浸泡於 transfer buffer (含 80% 25 mM Tris-HCl、192 mM glycine 及 20% methanol)中，同時截取一片與 separating gel 大小相同之 PVDF 膜，先將此膜浸泡於 99.5%酒精 5

分鐘，再與膠片、濾紙及海綿一起浸泡於 transfer buffer 約 5 分鐘，依序將海綿、濾紙、膠片、PVDF 膜、濾紙、海綿放置於三明治式塑膠板中，固定後，放入轉漬槽中，在冰浴下以 100 伏特進行轉漬 90 分鐘。

完成轉漬的 PVDF 膜，先以冰冷的 buffer A (含 25 mM Tris-HCl、150 mM NaCl 及 0.3% Tween-20，pH 7.4) 清洗一次，加入 Ponceau S solution 染色並在確定蛋白質分離之位置後，剪裁 PVDF 膜，以冰冷的 buffer A 清洗三次，每次 5 分鐘，隨後放置於含 5% skim milk 的 buffer B (含 25 mM Tris-HCl 及 150 mM NaCl，pH 7.4)中，4℃下隔夜或室溫下 2 小時，進行 blocking 反應。取出 PVDF 膜以冰冷的 buffer A 清洗三次，每次 5 分鐘，接著依一級抗體種類不同，於室溫反應 70 分鐘或 4℃下反應 overnight。取出後，PVDF 膜以冰冷的 buffer A 清洗三次，每次 5 分鐘，隨後加入二級抗體，依種類不同，室溫下反應 40 或 50 分鐘，再以冰冷的 buffer A 清洗三次，每次 5 分鐘。最後以 Enhanced Chemiluminescence Reagent (ECL kit) 呈色，並以冷光螢光數位分析儀 (LAS-4000, FUJIFILM, Japan)照相分析蛋白質表現。

結果與討論

圖一、不同含硫化合物對 Clone 9 細胞 ERK、JNK、p38 和 Akt 蛋白質磷酸化之影響

為探討不同含硫化合物 (DATS、LA、DHLA 和 SFN)誘發大鼠 Clone 9 細胞 GSTP 蛋白質表現是否經由 MAPK 或 PI3K/Akt pathway，以西方墨點分析法分析 ERK、JNK、p38 和 Akt 蛋白質磷酸化的情形。結果如圖一所示，Clone 9 細胞 ERK 蛋白質磷酸化在 DATS (50 μM)、LA (600 μM)、DHLA (600 μM)和 SFN (5 μM)處理之下會顯著增加 (p<0.05)。DATS、LA、DHLA 和 SFN 的 p-ERK 誘發倍數與控制組相比，分別為 5.3 倍、9.6 倍、5.1 倍、6.2 倍和 5.2 倍，而 Clone 9 細胞 JNK 和 p38 的蛋白質磷酸化情形與控制組相比沒有顯著差異 (p<0.05)。此

外，Clone 9 細胞 Akt 蛋白質磷酸化在 DATS、LA、DHLA 和 SFN 處理之下會增加 (p<0.05)，p-Akt 誘發倍數與控制組相比，分別為 3.1 倍、3.5 倍、2.9 倍、3 倍和 3.1 倍 (p<0.05)。

圖二、MAPK/ERK 抑制劑 PD98059 對不同含硫化合物誘發 Clone 9 細胞 ERK 蛋白質磷酸化之影響

大鼠 Clone 9 細胞先以 PD98059 (20 μM)預處理 1 小時後，再分別加入 DATS (50 μM)、LA (600 μM)、DHLA (600 μM)或 SFN (5 μM)培養 5 分鐘，比較細胞 ERK 蛋白質磷酸化的情形。由圖二結果顯示，PD98059 可顯著抑制不同含硫化合物 (DATS、LA、DHLA 和 SFN)誘發 Clone 9 細胞 ERK 蛋白質磷酸化 (p<0.05)。

圖三、PI3K/Akt 抑制劑 wotmannin 對不同含硫化合物誘發 Clone 9 細胞 Akt 蛋白質磷酸化之影響

大鼠 Clone 9 細胞先以 wortmannin (1 μM)預處理 1 小時後，再分別加入 DATS (50 μM)、LA (600 μM)、DHLA (600 μM)或 SFN (5 μM)培養 5 分鐘，比較細胞 Akt 蛋白質磷酸化之抑制情形。由圖三結果顯示，wortmannin 可顯著抑制不同含硫化合物 (DATS、LA、DHLA 和 SFN)誘發 Clone 9 細胞 Akt 蛋白質磷酸化 (p<0.05)。

由以上結果可以得知，DATS (50 μM)、LA (600 μM)、DHLA (600 μM)或 SFN (5 μM)誘發大鼠 Clone 9 細胞 GSTP 蛋白質表現可能經由 MAPK/ERK 和 PI3K/Akt 路徑，接著探討 MAPK/ERK 抑制劑 PD98059 和 PI3K 抑制劑 wortmannin 是否能持續其抑制效果，進而影響下游 GSTP 的蛋白質表現。

圖四、MAPK/ERK 抑制劑 PD98059 對不同含硫化合物誘發 Clone 9 細胞 GSTP 蛋白質表現之影響

大鼠 Clone 9 細胞先以 PD98059 (20 μM)預處理 1 小時後，再分別加入 DATS (50 μM)、LA (600 μM)、DHLA (600 μM)或 SFN (5 μM)培養 24 小時，比較細胞 GSTP 蛋白質表現情形。結果由圖四顯示，PD98059 可顯著抑制不同含硫化合物 (DATS、LA、DHLA 和 SFN)誘發 Clone 9 細胞 GSTP 蛋白質表現 ($p<0.05$)。

圖五、 PI3K/Akt 抑制劑 wotmannin 對不同含硫化合物誘發 Clone 9 細胞 GST 蛋白質表現之影響

大鼠 Clone 9 細胞先以 wortmannin (1 μM)預處理 1 小時後，再分別加入 DATS (50 μM)、LA (600 μM)、DHLA (600 μM)或 SFN (5 μM)培養 24 小時，比較細胞 GSTP 蛋白質之抑制情形。由圖五結果顯示，wortmannin 對不同含硫化合物 (DATS、LA、DHLA 和 SFN) 誘發 GSTP 蛋白質表現沒有顯著影響 ($p<0.05$)。

由以上結果可以得知，DATS (50 μM)、LA (600 μM)、DHLA (600 μM) 或 SFN (5 μM) 可能主要透過 MAPK/ERK 路徑誘發 Clone 9 細胞 GSTP 蛋白質表現，而 PI3K/Akt pathway 則不影響。在計畫主持人過去的研究指出，DADS 和 DATS 可能透過 MAPK pathway 來活化 Clone 9 細胞的轉錄因子 AP-1，同時 AP-1 也為誘發 GSTP 的關鍵角色，則有待後續的研究，將釐清不同含硫化合物 (DATS、LA、DHLA 和 SFN)、MAPK/ERK pathway 和 AP-1 三者之間的關係。

結論

綜合上述結果，大蒜有機化合物 DATS 與非屬大蒜的含硫化合物 (LA、DHLA 和 SFN)顯著誘發大鼠 Clone 9 細胞 GSTP 的酵素活性、蛋白質和 mRNA 表現，而這可能是透過 MAPK 訊息傳遞路徑中 ERK 蛋白質的磷酸化，上調 GSTP 的表現。

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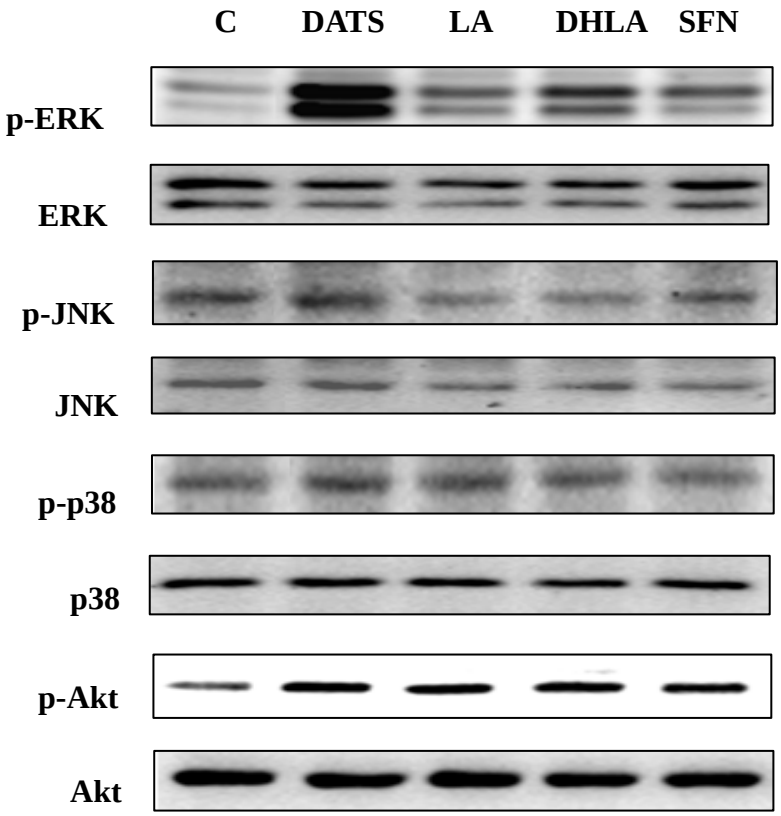
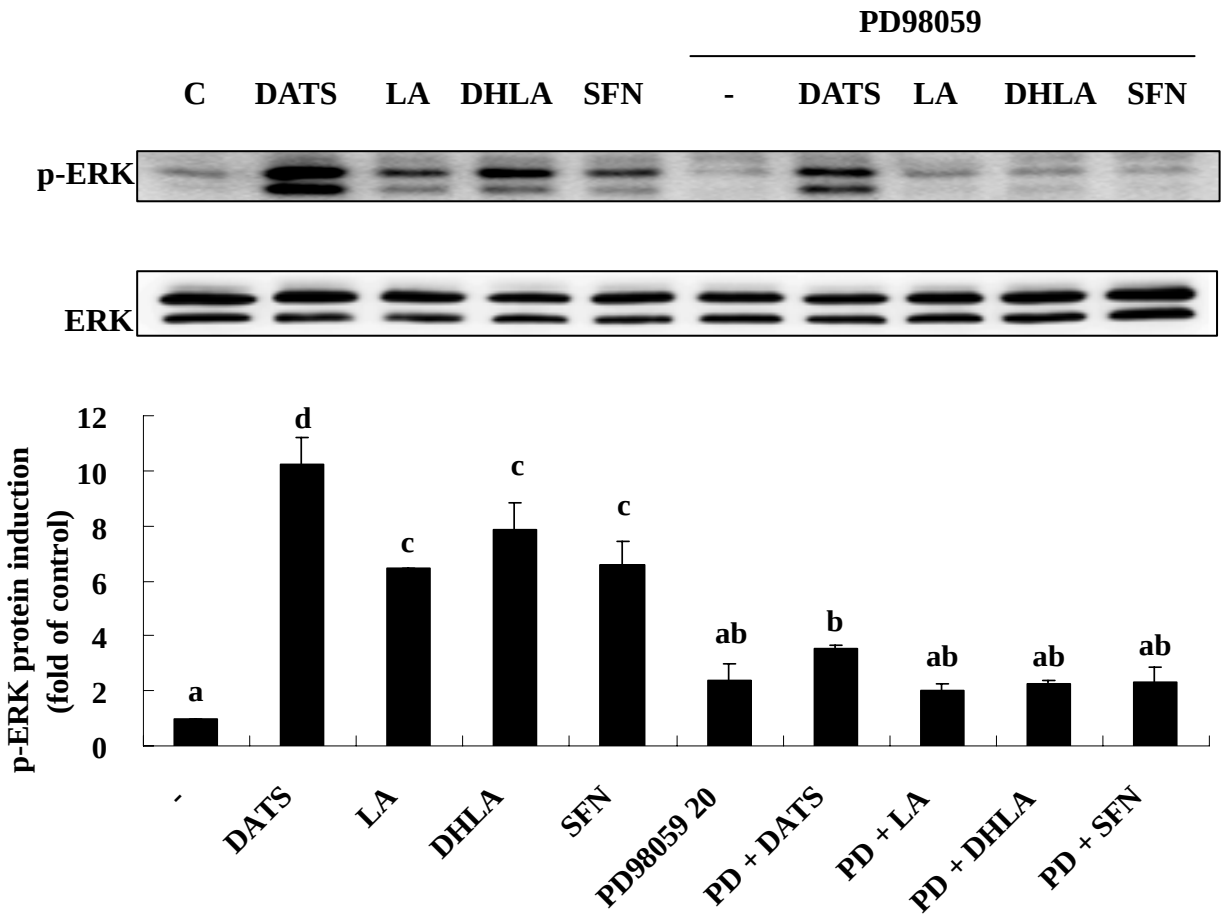


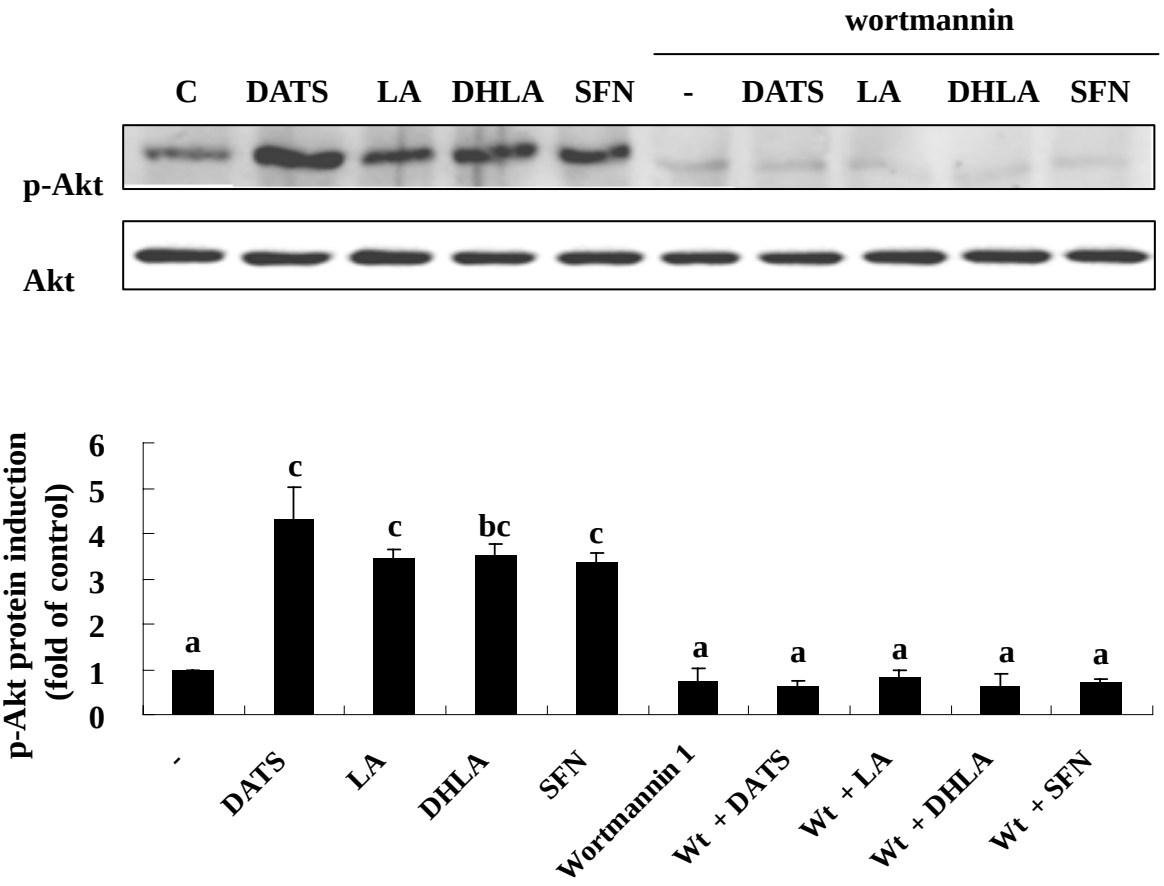
圖一、不同含硫化合物對 Clone 9 細胞 ERK、JNK、p38 和 Akt 蛋白質磷酸化之影響

大鼠 Clone 9 細胞分別處理 DATS (50 μM)、LA (600 μM)、DHLA (600 μM) 或 SFN (5 μM)，培養 5 分鐘後收取細胞質液，控制組細胞 (control, C)則僅給予 0.1 % DMSO 處理。



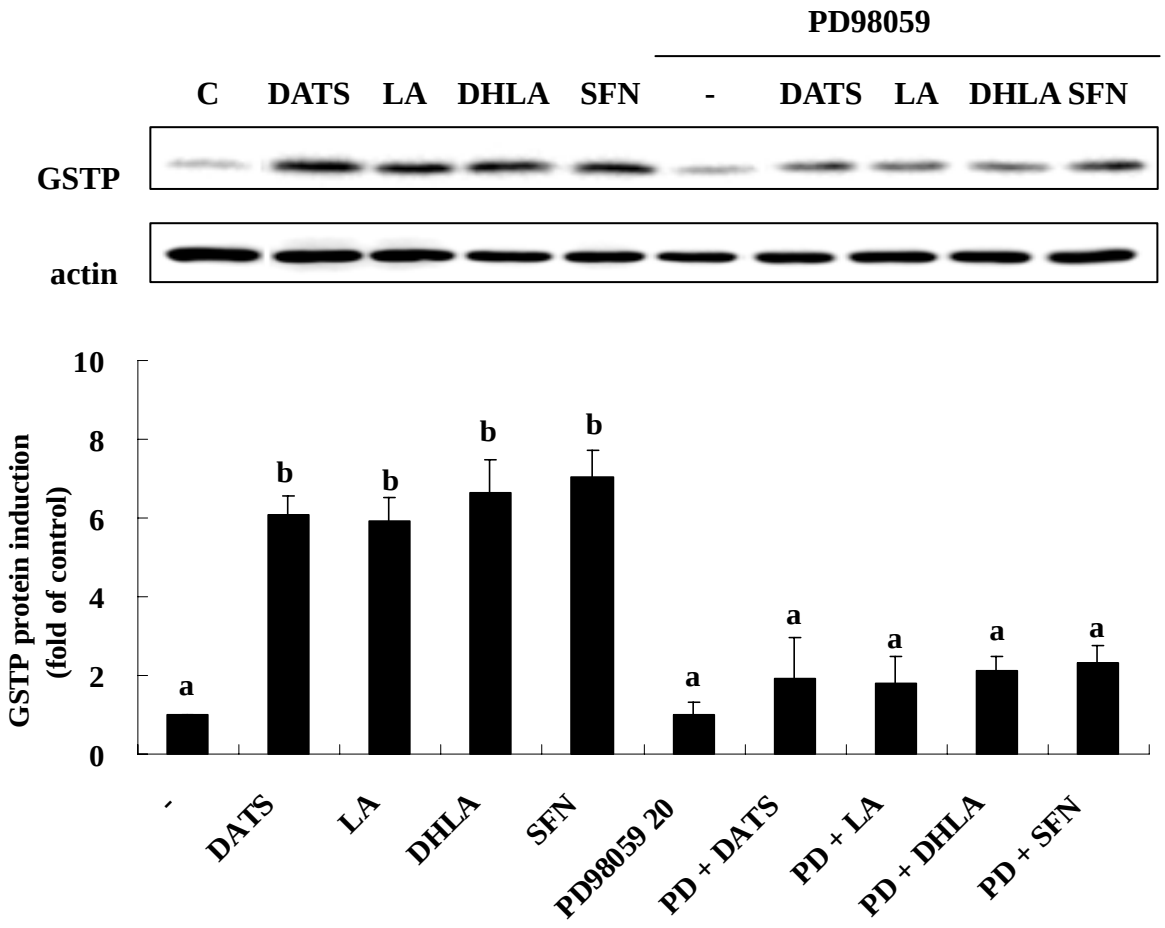
圖二、MAPK/ERK 抑制劑 PD98059 對不同含硫化合物誘發 Clone 9 細胞 ERK 蛋白質磷酸化之影響

大鼠 Clone 9 細胞分別處理 DATS (50 μM)、LA (600 μM)、DHLA (600 μM) 或 SFN (5 μM)，培養 5 分鐘後收取細胞質液，控制組細胞 (control, C)則僅給予 0.1 % DMSO 處理。



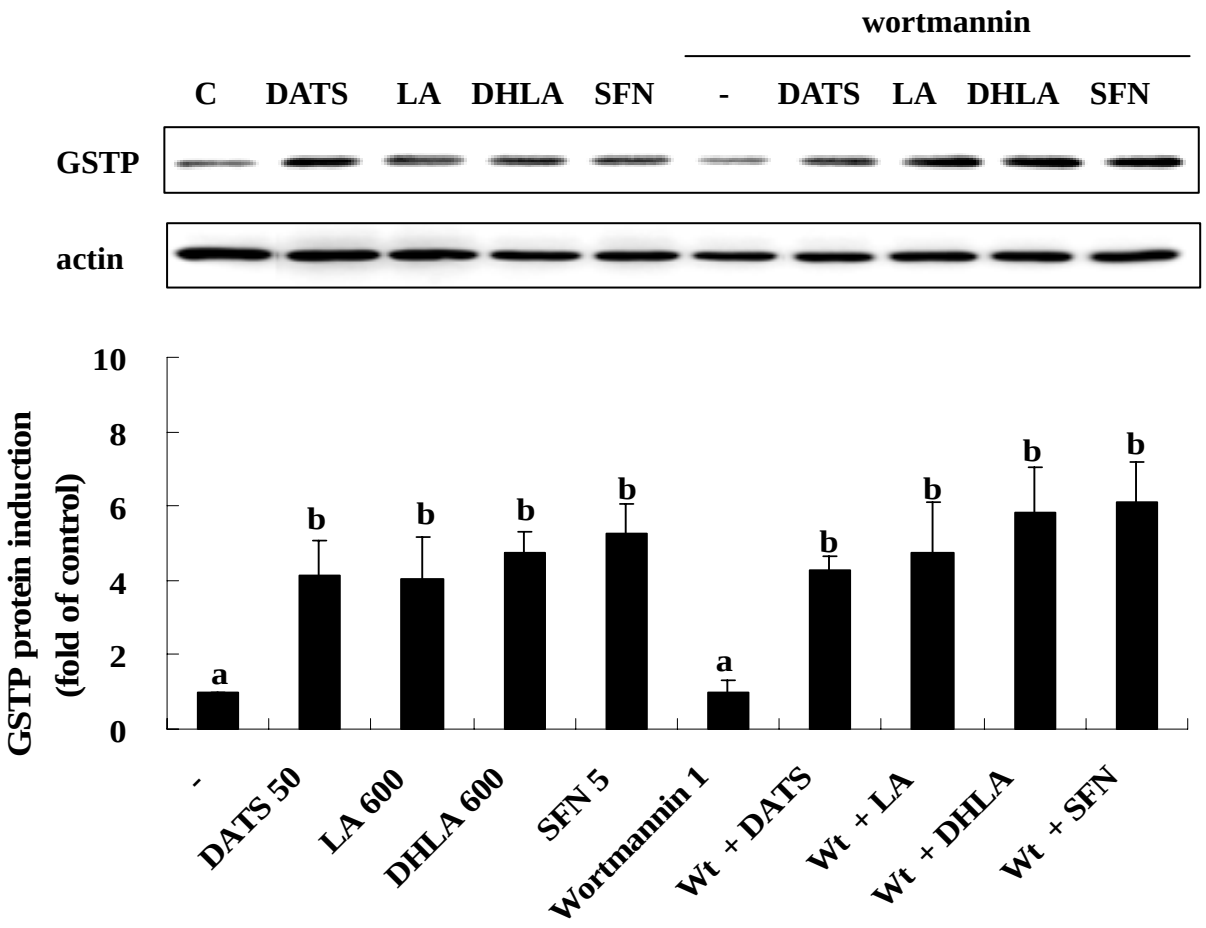
圖三、PI3K 抑制劑 wortmannin 對不同含硫化合物誘發 Clone 9 細胞 Akt 蛋白質磷酸化之影響

大鼠 Clone 9 細胞先以 wortmannin (1 μM)預處理 1 小時後，再分別加入 DATS (50 μM)、LA (600 μM)、DHLA (600 μM)或 SFN (5 μM)，培養 5 分鐘後收取細胞質液，控制組細胞 (control, C)則僅給予 0.2 % DMSO 處理。



圖四、MAPK/ERK 抑制劑 PD98059 對不同含硫化合物誘發 Clone 9 細胞 GSTP 蛋白質表現之影響

大鼠 Clone 9 細胞先以 PD98059 (20 μM)預處理 1 小時後，再分別加入 DATS (50 μM)、LA (600 μM)、DHLA (600 μM)或 SFN (5 μM)，培養 24 小時後收取細胞質液，控制組細胞 (control, C)則僅給予 0.2 % DMSO 處理。



圖五、PI3K 抑制劑 wortmannin 對不同含硫化合物誘發 Clone 9 細胞 GSTP 蛋白質表現之影響

大鼠 Clone 9 細胞先以 wortmannin (1 μM)預處理 1 小時後，再分別加入 DATS (50 μM)、LA (600 μM)、DHLA (600 μM)或 SFN (5 μM)，培養 24 小時後收取細胞質液，控制組細胞 (control, C)則僅給予 0.2 % DMSO 處理。