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Effect of Diallyl Sulfide on in Vitro and in Vivo Nrf2-Mediated Pulmonic Antioxidant Enzyme Expression via Activation ERK/p38 Signaling Pathway

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ABSTRACT: Increasing oxidative stress is intimately involved in the pathogenesis of lung failure. Nuclear factor-erythroid 2 related factor 2 (Nrf2) is a key element in redox homeostasis. Nrf2 regulates antioxidant-associated genes that are often the target of phytochemicals in chemoprevention. This study evaluated the effect of diallyl sulfide (DAS), which is present in garlic, on the expression of antioxidant enzymes in the rat lung and the Nrf2 modulation in MRC-5 lung cells. DAS increased the activities of glutathione *S*-transferase, glutathione reductase, and catalase as well as the GSH/GSSG ratio compared with the lung of untreated control rats ($p < 0.05$). The pulmonic superoxide dismutase, glutathione peroxidase, NAD(P)H:quinone oxidoreductase 1, and catalase mRNA levels were also significantly increased ($p < 0.05$) after DAS treatment. Following DAS treatment, DAS level was measured in the plasma after 7 days of oral administration, and the C_{max} value was 15 ± 4.2 μM. The total amount of pulmonic Nrf2 and the nuclear translocation of Nrf2 were elevated in DAS-treated rats, clarifying the effect of DAS on the modulation of antioxidant enzymes. Furthermore, DAS could induce nuclear translocation of Nrf2 via ERK/p38 signaling pathway in lung MRC-5 cells. This study demonstrates that DAS administration can significantly induce the activity of antioxidant enzymes in rat lungs and suggests a possible use for DAS as a dietary preventive agent against oxidative stress-induced lung injury.

KEYWORDS: *antioxidant enzymes, diallyl sulfide, MAPK, MRC-5 cells, Nrf2*

INTRODUCTION

The lung is constantly challenged with oxidative damage due to gas exchange and exposure to exogenous air pollutants. Reactive oxygen species (ROS), including superoxide anion, hydroxyl radicals, and hydrogen peroxide, have been reported to play a crucial role in airway inflammation and pathogenesis.[1] Oxidative stress is involved in the pathogenesis of many lung inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and pulmonary fibrosis, which is a major health problem in developing countries of the world.[2] Although the lung has a well-developed antioxidant system to protect itself against exposure to endogenous or exogenous oxidants, excessive levels of ROS can still cause inflammation in the lung.[3] Administration of some exogenous antioxidant compounds has been used in the prevention of or the therapeutic intervention in oxidative lung diseases in animal models.[4] Park et al.[5] have reported that quercetin can be helpful in preventing paraquat-induced lung injury by enhancing the activity of antioxidant enzymes in rats. Thus, induction of various detoxification and antioxidant genes can aid in the maintenance of cellular redox homeostasis and protect against oxidative damage.

Nuclear factor-erythroid 2 related factor 2 (Nrf2) is a redox-sensitive transcription factor, which binds to an antioxidant response element (ARE) in the promoter region of genes encoding several phase-II detoxifying/antioxidant enzymes and related stress-responsive proteins.[6] These enzymes and proteins include NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). The roles of these enzymes and proteins in oxidative tissue stress have been widely defined.[7,8] Under normal conditions, Nrf2 is inactive and is bound in the cytosol by Kelch-like ECH-associated protein 1 (Keap1). Nrf2 can be activated by diverse stimuli, including oxidants, pro-oxidants, antioxidants, and chemopreventive agents. Nrf2 can induce cellular rescue pathways against oxidative damage, abnormal inflammation, immune responses, apoptosis, and carcinogenesis.[9,10]

Diallyl sulfide (DAS), which is enriched in garlic, is one of the natural organosulfuric compounds that are beneficial to human health.[11,12] Recently, DAS has been reported to possess an anticancer effect in a colon cancer cell line.[13] Furthermore, DAS has been reported to modulate phase-II drug-metabolizing enzymes, including glutathione *S*-transferase (GST) and NQO1. Shaik et al.[14] showed that DAS regulates detoxification. Antioxidant enzymes and the prevention of oxidative stress in the liver may be important events in the protection against adverse effects related to oxidative injury. However, whether DAS can induce antioxidant enzymes and up-regulate their gene expression in rat lung tissue remains unclear. Thus, in this study, we used Sprague–Dawley (SD) rats to investigate the effect of the administration of DAS on the expression of pulmonic antioxidant enzymes. We also studied the mechanism of DAS-induced antioxidant enzymes using human embryonic MRC-5 lung cells (MRC-5).

MATERIALS AND METHODS

Chemicals. Minimum essential medium Eagle (MEM), fetal bovine serum (FBS), trypsin–EDTA (TE), L-glutamine, and penicillin–streptomycin (PS) antibiotic solution were obtained from Gibco BRL

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(Grand Island, NY). DAS was obtained from Alfa Aesar (Ward Hill, MA). Anti-HO-1 and anti-Nrf2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ERK, anti-phospho-ERK, anti-p38, anti-phospho-p38, anti-JNK, anti-phospho-JNK, antilamin B1, and anti-β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO). Molecular mass markers for proteins were obtained from Pharmacia Biotech (Saclay, France). PD98059, SB203580, and SP600125 were obtained from Biosource (Camarillo, CA). Trizol was obtained from Invitrogen (Carlsbad, CA). All fine chemicals were obtained from Showa Chemical (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO).

Cell Culture. Human embryonic lung cell line (MRC-5) cells (BCRC 60023) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). Cells were grown in MEM and supplemented with 10% FBS at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was renewed each day. Cells were subcultured weekly with 0.1% trypsin and 10 mM EDTA in PBS.

Animal Treatment. Male SD rats (200 ± 10 g) were used for the experiments. The rats were provided with food and water ad libitum and divided into five groups (six rats/group). To study the effect of DAS on the induction of pulmonic antioxidant enzymes in rats, DAS was given daily by gavage to the animals for 7 consecutive days. Previous studies have indicated that in rats treated orally with DAS at a dose of 500 mg/kg body weight, the hepatic phase-II detoxifying/antioxidant enzymes were induced.[15,16] Therefore, the dosage of 100 or 500 mg/kg body weight per day was chosen for DAS treatment in this study. The positive control group was treated with NAC at dosage of 500 mg/kg body weight. The vehicle group was treated with soybean oil, and the group treated with ddH_2O was the blank. At the end of the experiments, the rats were sacrificed by an overdose of carbon dioxide 2 h after the last treatment and subjected to the following analytical procedures: The lung was collected and rinsed extensively in cold phosphate-buffered saline (PBS). The rinsed lung tissues were cut into small pieces and rinsed twice with cold PBS, followed by homogenization in 20 parts (w/v) of ice-cold 50 mM phosphate buffer (pH 7.2) containing 2 mM EDTA using a tissue homogenizer with a Teflon pestle at 4 °C. The homogenate was centrifuged at 600*g* for 10 min to separate any cell debris, and the supernatant was further centrifuged at 10000*g* for 20 min to remove the mitochondria pellet. Finally, the supernatant was ultracentrifuged at 10000*g* for 60 min to obtain the supernatant containing the cytosol. The resulting cytosol aliquots were collected and preserved at −80 °C for enzymatic assay and Western blot (no longer than 1 week). All experimental procedures involving animals were conducted in accordance with National Institutes of Health guidelines. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University, Taichung, Taiwan (IACUC Approval 100-20).

Measurement of the GSH/GSSG Ratio. Total glutathione (GSH and GSSG) was measured according to a method modified from Yeh et al.[17] Lung tissue preparations were treated on ice with 6% (v/v) perchloric acid (PCA) containing freshly prepared 3 mM 1-methyl-2-vinylpyridinium trifluoromethanesulfonate and 1 mM EDTA to determine GSSG quantities. Lung tissue preparations were treated on ice with ice-cold 15% (w/v) TCA to determine GSH quantities. After centrifugation at 10000*g* for 10 min, 50 μL of the supernatant was added to GSH reductase (GRd; 1 U) and 75 μM DTNB. The reaction was initiated by the addition of 0.25 mM NADPH to a final volume of 200 μL. The change in absorbance was monitored at 410 nm for 5 min, and GSH and GSSG levels were calculated using pure GSH and GSSG as standards.

Assay of CAT Activity. CAT activity was measured using the method described by Aebi,[18] in which the disappearance of H_2O_2 is followed spectrophotometrically at 240 nm. The reaction medium consisted of 50 mM sodium phosphate buffer (pH 7.2) and 10 mM H_2O_2. Protein concentrations of cellular extracts were determined as the internal control by a commercial protein reagent kit (Bio-Rad, Hercules, CA). The results were expressed as nanomoles per milligram of protein.

Assay of GPx Activity. Lung tissue GPx activity was determined using the method of Flohe and Gunzler.[19] Briefly, 1 mM EDTA, 2 mM sodium azide, 100 μL of the sample, 100 μL of 10 mM GSH, 100 μL of GRd (2.4 U), and 100 μL of 1.5 mM NADPH were added to an assay cuvette containing 0.5 mL of 50 mM potassium phosphate (pH 7.0). The cuvette was incubated at 37 °C for 3 min. After the addition of 100 μL of 2 mM H_2O_2, the rate of NADPH consumption was monitored at 340 nm and 37 °C for 5 min. This reading was designated the total rate of NADPH consumption. The consumption of NADPH not dependent on enzymatic activity was also measured as above except that 100 μL of the sample was replaced by 100 μL of the sample buffer. The rate of enzyme-dependent NADPH consumption was obtained by subtracting the NADPH consumption rate not dependent on enzymatic activity from the total NADPH consumption rate. Protein concentrations of cellular extracts were determined as the internal control by a commercial protein reagent kit (Bio-Rad). GPx activity was calculated using the extinction coefficient of 6.22 mM/cm and expressed as nanomoles of NADPH consumed per minute per milligram of protein.

Assay of GRd Activity. GRd activity was determined using the method of Bellomo et al.[20] Lung homogenate solution (100 μL) was mixed with 900 μL of 100 mmol/L potassium phosphate buffer (pH 7.0) containing 1 mmol/L $MgCl_2 \cdot 6H_2O$, 50 mmol/L GSSG, and 0.1 mmol/L NADPH. This mixture was incubated for 3 min at room temperature. The absorbance change at 340 nm was recorded over the course of 3 min. Protein concentrations of cellular extracts were determined as the internal control by a commercial protein reagent kit (Bio-Rad). GRd activity was calculated using the extinction coefficient of 6.22 mM/cm and expressed as nanomoles of NADPH consumed per minute per milligram of protein.

Assay of GST Activity. The GSH *S*-transferase (GST) activity was determined using the method of Habig et al.[21] Lung homogenate solution (100 μL) was mixed well with 880 μL of 100 mmol/L potassium phosphate buffer (pH 6.5) containing 100 mmol/L GSH and 50 mmol/L 1-chloro-2,4-dinitrobenzene (CDNB). The absorbance change at 340 nm was recorded over the course of 3 min. Protein concentrations of cellular extracts were determined as the internal control by a commercial protein reagent kit (Bio-Rad). GST activity was calculated using the extinction coefficient of 9.6 mM/cm, and the result is expressed as nanomoles of CDNB−GSH conjugate formed per minute per milligram of protein.

Preparation of Plasma Samples for the Analysis of DAS by HPLC. Blood samples were collected into heparinized blood containers. The samples were centrifuged at 800*g* for 20 min. The harvested plasma was treated with 10% SSA and then centrifuged at 800*g* for 15 min. The supernatant was stored at −20 °C until it was analyzed. HPLC analysis was conducted after the supernatant was filtered with a 0.45 μm filter. The HPLC conditions used for the determination of DAS were as follows: an HPLC pump (Hitachi, Japan), an RP-18 GP column (250 × 4.6 mm, 5 μm), and a photodiode array detector (measured at 240 nm). Elution was carried out at room temperature. Elution utilized 70% (v/v) acetonitrile and 3% (v/v) tetrahydrofuran in water as solvent A and 7% (v/v) acetonitrile and 3% (v/v) tetrahydrofuran in water as solvent B. The elution gradient program was as follows: 100% A (20 min), 100% B (6.5 min), 100% A (23.5 min) at a flow rate of 1 mL/min. The injection volume for the standard and sample extracts was 20 μL. DAS was quantified using the external standard method. Quantification was based on peak area. Calibration curves of the standards were made by diluting stock standards in ethanol to yield 0−1000 mg/mL (DAS). Linear regression was fitted to the data to obtain regression coefficients >0.99 for DAS standard curves.

Cell Survival Assay. The cell toxicity of DAS was measured by MTT analysis. Briefly, MRC-5 cells (2×10^4 cells/mL) were seeded into 96-well microtiter plates. After co-incubation with DAS, the medium was removed and replaced with fresh medium containing 0.5 mg/mL MTT for 2 h at 37 °C. Violet crystal was converted from yellow MTT by living cells and was dissolved in DMSO. The optical

density was measured at 570 nm using a BMG Labtech FLUOstar fluorescence reader (Jena, Germany).

RNA Extraction and RT-PCR. Treated cells were washed three times with PBS, and Trizol (Invitrogen) was used to extract the intracellular RNA. Next, a RevertAid First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD) was used to translate RNA into cDNA, and Taq DNA polymerase (Fermemtas) was used to amplify the desired cDNA sequence. The primers used to amplify CAT, Cu/Zn SOD, GPx, HO-1, NQO1, and GAPDH were as follows: CAT, forward, CCCGATGTCCTGACCACCG, reverse, CTCTCCAGCGACTGTGGAG; Cu/Zn SOD, forward, GAAGGCCGTGTGCGTGCTG, reverse, GGACACATTGGCCACACCG; GPx, forward, TGCAACCAGTTCGGGAGGC, reverse, GAGATAGCACGGCAGGTCC; HO-1, forward, CCCACAGCTCGACAGCATG, reverse, GGGCCCATACCAGAAGGCC; NQO1, forward, CGCAGAGAGGACATCATTCA, reverse, CGCCAGAGATGACTCAACAG; GAPDH, forward, TCGGACGCCTGGTTACCAG, reverse, CCAGCCTTCTCCATGGTGG. Primer sequences were performed as described in Yeh et al.[17] The PCR products were electrophoresed on a 1.5% agarose gel, and the DNA was detected using SYBR Safe DNA gel stain (Invitrogen). The gel was then photographed using a BioDoc-It system (UVP, Cambridge, U.K.). Results were expressed as a ratio of the DNA signal relative to the corresponding GAPDH signal from each sample.

Preparation of Nuclear/Cytosol Extracts. To obtain nuclear/cytosol extracts lysates, the DAS-treated and untreated tissues or cells were centrifuged at 15*g* for 5 min at 4 °C and lysed using a Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA). The lysate was centrifuged at 13000*g* for 5 min, and nuclear extraction reagents were added, followed by vortexing for 15 s. This mixture was then centrifuged at 13000*g* for 10 min, and the protein content of the supernatant was determined with a bicinchoninic acid assay, using a commercial protein reagent kit (Bio-Rad).

Western Blotting. Treated cells were lysed in RIPA buffer (Millipore, Billerica, MA) and boiled at 100 °C for 10 min with 4× protein loading dye (8% SDS, 0.04% Coomassie Blue R-250, 40% glycerol, 200 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol). Samples were then subjected to SDS−polyacrylamide gel electrophoresis. Proteins were transferred onto cellulose nitrate membranes (Sartorius Stedim Biotech, Goettingen, Germany) and incubated with primary antibody (Nrf2, HO-1, phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38, lamin B1, or β-actin) overnight (1:1000 dilutions). Membranes were then incubated with a horseradish peroxide-conjugated secondary antibody (1:5000 dilutions) and analyzed using the Chemiluminescent ECL detection system (Millipore). Protein levels were normalized relative to lamin B1 or β-actin. Signal intensity was quantified using VisionWorks LS 6.3.3 (UVP).

Statistical Analysis. All data are expressed as the mean ± SD. An ANOVA was used to evaluate differences between multiple groups. Significant differences were subjected to Duncan's test to compare the means of two specific groups. A p value of <0.05 was considered to be significant.

RESULTS

Effect of DAS on the Relative Tissue Weight of SD Rats. There was no significant difference among the relative lung, heart, and liver tissue weight of rats with gavage of DAS (100 and 500 mg/kg of body weight) for 7 consecutive days when compared to the vehicle-only control group (data not shown). All rats remained in good health throughout the experimental period.

Effect of DAS on the Activity of Pulmonic Antioxidant Enzymes. The activities of pulmonic CAT, GST, GPx, and GRd in rats treated with DAS are shown in Figure 1A−D. Rats that received 100 or 500 mg/kg of DAS had significantly elevated pulmonic CAT, GST, GPx, and GRd activities as compared to the vehicle group ($p < 0.05$).

Effect of DAS on the GSH/GSSG Ratio. Oxidative stress of tissue generally involves the GSH system. The ratio of GSH/GSSG was measured, and the data are shown in Figure 1E. The levels of GSH in the rat lungs were significantly increased by DAS compared with the vehicle group. A significant decrease ($p < 0.05$) in GSSG levels was noted in the lungs of rats treated with DAS. Due to the changes in the GSH and GSSG levels, the GSH/GSSG ratio significantly increased ($p < 0.05$) in the lung of the rats treated with DAS in a dose-dependent manner.

Changes in mRNA Expression Levels of Antioxidant Enzymes in Rat Lungs following DAS Treatment. To further examine whether CuZnSOD, GPx, NQO1, HO-1, or CAT mRNA expression was modulated by DAS, total RNA was collected from treated cells. After reverse transcription, mRNA expression was analyzed by PCR. As shown in Figure 2, CuZnSOD, GPx, NQO1, and CAT mRNA expression levels in lung tissue were modulated by DAS in a dose-dependent manner. HO-1 mRNA levels also increased in DAS-treated rats.

Effect of DAS Treatment on Pulmonic HO-1 Protein Expression Levels. Several pieces of evidence exist to suggest that HO-1 plays a critical role in the adaptive response of lung tissues to a variety of stresses. To further evaluate the extent of HO-1 protein expression induced by DAS, rats were treated with 100 or 500 mg/kg body weight of DAS, and HO-1 protein expression in lung tissue was analyzed by Western blotting. HO-1 protein expression was quantified by densitometry and expressed as the induction fold of those in the corresponding control group. As shown in Figure 3, HO-1 protein expression was elevated by DAS and NAC treatments, and DAS elicited the strongest inductive effect in a dose-dependent manner.

DAS Stimulates Pulmonic Nrf2 Protein Expression. Because the gene transcription of antioxidant enzymes induced by DAS was dependent on mRNA expression (Figure 2), we further examined whether Nrf2 expression was modulated by DAS. The levels of Nrf2 protein expression in lung tissues were simultaneously modulated by DAS treatment in a dose-dependent manner (Figure 4A). Moreover, the translocation of Nrf2 protein to the nucleus was significantly increased in the DAS-treated rats in a dose-dependent manner (Figure 4B). These observations are consistent with the antioxidant gene expression results (Figures 2 and 4), suggesting that the Nrf2 protein accumulation might contribute to the induction of ARE-mediated antioxidant gene expression after DAS treatment.

Plasma Concentration of DAS in SD Rats after Treatment with DAS. To evaluate the plasma concentration of DAS in SD rats, the plasma was collected from DAS-treated rats. After preparation, the plasma concentration of DAS was analyzed by HPLC. A plasma concentration of 15 ± 4.2 μM DAS was detected after oral administration of 500 mg/kg body weight of DAS for 7 days in SD rats (Figure 5). However, plasma concentration of DAS after oral administration of 100 mg/kg body weight of DAS for 7 days in SD rats was not detected by HPLC. Because a mean maximum concentration of DAS of 15 ± 4.2 μM was detected in plasma by HPLC, all subsequent experiments involving DAS were performed using a concentration of 15 μM DAS.

Cytotoxicity of DAS to MRC-5 Cells. Enhancement of the antioxidant system by DAS treatment in vivo was observed; thus, the mechanism of action of DAS was further investigated in human embryonic MRC-5 lung cells (MRC-5). The cytotoxicity of DAS to MRC-5 cells was examined with a MTT assay. Twenty-four hours of incubation in medium containing 10−15 μM DAS did not decrease cell viability in MRC-5 cells; however, DAS treatment led to cytotoxicity at concentrations

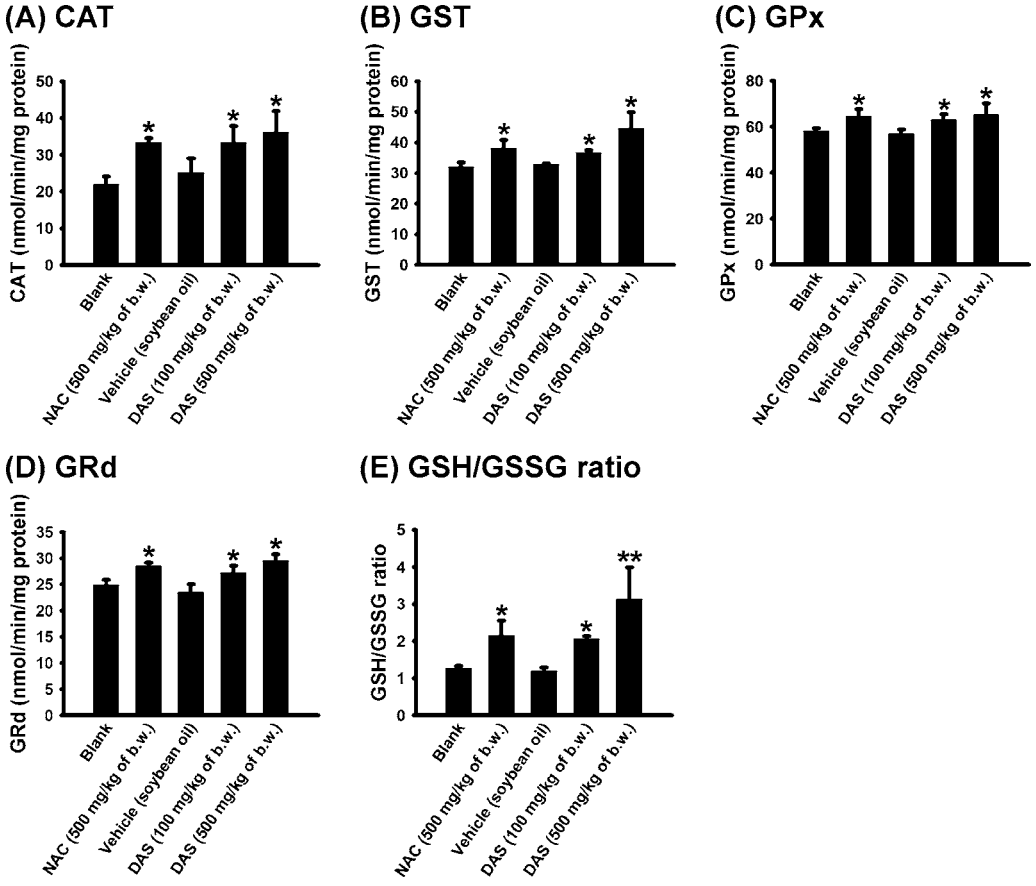

Figure 1. Effect of DAS on (A) CAT, (B) GST, (C) GPx, (D) GRd, and (E) GSH/GSSG ratio in lung tissue of SD rats. Either 500 mg/kg of body weight of *N*-acetylcysteine or diallyl sulfide or 100 mg/kg of body weight of diallyl sulfide was orally administered for 7 days. Values are expressed as the mean ± SD ($n = 6$). Values were significantly different (*, $p < 0.05$; **, $p < 0.01$) compared with the vehicle-only control group by Duncan's multiple-range test. Abbreviations: CAT, catalase; GST, glutathione *S*-transferase; GPx, glutathione peroxidase; GRd, glutathione reductase; GSH/GSSG, reduced/oxidized glutathione.

>25 μM (Figure 6). These results indicate that DAS does not induce cytotoxicity in MRC-5 cells at concentrations <15 μM.

Effect of DAS on Nrf2 Protein Expression in MRC-5 Cells. To investigate whether DAS could affect Nrf2 expression in MRC-5 cells, the cells were treated with 15 μM DAS for 2 h. Following treatment, the amount of Nrf2 protein was analyzed by Western blotting (Figure 7). After treatment with DAS, the amount of Nrf2 protein in the cytosol was markedly decreased ($p < 0.05$) at 1 and 2 h. Exposure of MRC-5 cells to DAS for 2 h resulted in elevating the amount of nuclear Nrf2, reaching a maximum at 1 h. The Nrf2 protein level of whole cell was constant following treatment with DAS. Due to the changes in the nuclear and cytosol levels of Nrf2 protein, the nuclear/cytosol ratio significantly increased ($p < 0.05$) in the DAS-treated MRC-5 cells (Figure 7B). These results suggest that induction of Nrf2 by DAS involves Nrf2-mediated ARE activation.

Involvement of the ERK/p38 Pathway in the Induction of Nrf2 Protein Expression by DAS in MRC-5 Cells. Many studies have demonstrated that several MAPKs, including ERK, JNK, and p38, are involved in regulating the phosphorylation of Nrf2 and the expression of ARE-mediated phase II and antioxidant genes. Thus, we examined whether the MAPK pathway is involved in the DAS-mediated nuclear translocation of Nrf2. As shown in Figure 8A, DAS increased the levels of phosphorylated ERK and p38. The same blots were probed with antibodies to total JNK, ERK, and p38 as protein loading controls. The DAS-mediated increase in Nrf2 protein expression was completely blocked by PD98059 (ERK inhibitor) and moderately blocked by SB203580 (p38 inhibitor). A similar concentration of SP600125 (JNK inhibitor) had no significant effect on Nrf2 protein expression (Figure 8B). These results indicate that a kinase in the ERK/p38 pathway might be involved in the regulation of DAS-mediated Nrf2 protein expression.

■ DISCUSSION

Free radicals can produce ROS in cells. ROS have been reported to contribute to cell and tissue damage, which is associated with several chronic lung inflammatory diseases.[22,23] Nadeem et al.[24] reported that the lung contains high levels of antioxidants, such as vitamin C, vitamin E, urate, GSH, SOD, CAT, and GPx. These antioxidants may prevent injuries induced by oxidation. In addition, natural agents may have physiological antioxidant properties, quenching ROS, thereby potentially modifying pathogenic mechanisms relevant to lung disease.[25] DAS is an organosulfuric component of garlic, which is well-known for its antioxidant property.[26,27] However, the effect of DAS on endogenous antioxidant enzymes in the rat lung has not been investigated. Thus, in this study, we examined the effect of DAS on the antioxidant defense system.

In the present study, the activities of pulmonic antioxidant enzymes (CAT, GST, GPx, and GRd) were significantly induced in the lung after administration of DAS or NAC

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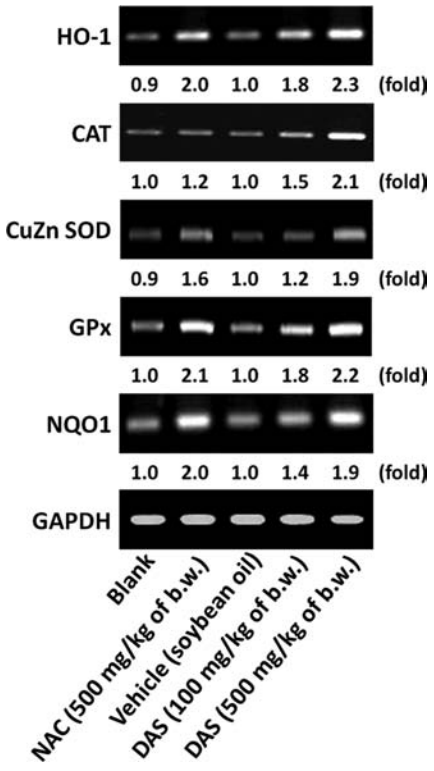

Figure 2. Effect of DAS on the mRNA levels of the genes of antioxidant enzymes in lung tissue of SD rats. Either 500 mg/kg of body weight of *N*-acetylcysteine or diallyl sulfide or 100 mg/kg of body weight of diallyl sulfide was orally administered for 7 days. Total RNA was reverse transcribed, and the cDNA products were amplified by PCR using gene-specific primers. Reactions corresponding to HO-1, CAT, CuZn SOD, GPx, and NQO1 were analyzed by agarose gel (1.0%) electrophoresis of the PCR products. Data shown are representative of three independent experiments.

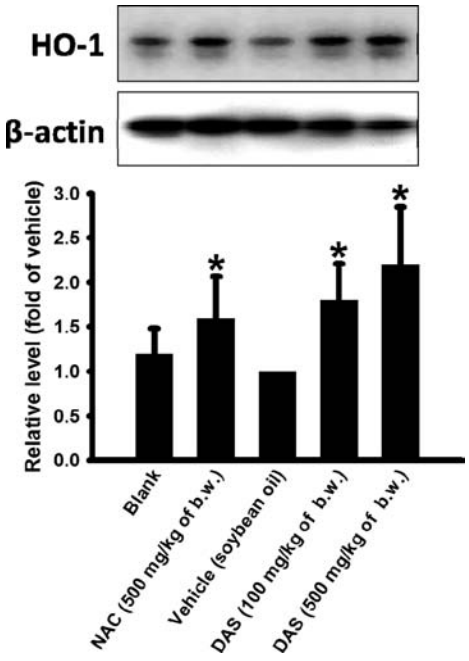

Figure 3. Effect of DAS on HO-1 protein expression in lung tissue of SD rats. Either 500 mg/kg of body weight of *N*-acetylcysteine or diallyl sulfide or 100 mg/kg of body weight of diallyl sulfide were orally administered for 7 days. Western blot analysis was performed using antibodies specific for HO-1 and β-actin. The intensity of the HO-1 protein band was detected by densitometric analysis and expressed in terms of fold change compared with the intensity of the control band. Data are expressed as the mean ± SD ($n = 6$). The ∗ indicates $p < 0.05$ when compared with the vehicle-only control group.

(Figure 1). We also found that the mRNA expression of HO-1, CAT, CuZnSOD, GPx, and NQO1 were higher in the lung tissue of the DAS treatment groups compared with the control group (Figure 2). The change in antioxidant enzyme activity was correlated with the change in the respective mRNA expression in the DAS-supplemented rats. A similar action of DAS was observed in the liver of rats treated orally with DAS at the dosage of 200 or 500 mg/kg of body weight for 4 days; the hepatic cytochrome P450 2B subfamily and glutathione *S*-transferase α subfamily were enhanced.[15] Hsu et al.[28] reported that after rats received rutin or *o*-coumaric acid for 8 weeks, the hepatic enzymatic activities of GPx, RGd, and GST as well as the GSH/GSSG ratio were markedly increased. This result suggests that the up-regulation of endogenous pulmonic phase II antioxidant enzymes could be achieved in vivo via the administration of phytochemical inducers. In addition, Yeh et al.[17] observed that the activities and mRNA levels of cardiac enzymes CAT and SOD of rats treated for 2 weeks with phenolic acids, such as gallic acid, ferulic acid, *p*-coumaric acid, or gentisic acid, were higher than the levels in untreated rats. Therefore, we assume that the mRNA expressions of CAT and GPx were altered by treatment with DAS, and these enzymes may be regulated on a transcriptional level.

GSH is a vital antioxidant that regulates the cellular redox status and protects airway epithelial cells from oxidant-induced lung injury and inflammation.[3] Kirkham and Rahman[29] reported that antioxidants might maintain cellular redox homeostasis (high GSH/GSSG) and reduce oxidative stress. Our results also showed that DAS treatment significantly decreased the level of GSSG in the lung, which can presumably stabilize GSH. Changes in the GSH and GSSG levels resulted in a significant increase in the GSH/GSSG ratio in the lung tissue treated with DAS, and the ratio was markedly ($p < 0.01$) increased with the high dose of DAS treatment (Figure 1E). Tripathi et al.[30] reported that mice treated intranasally with GSH show ameliorated responses to ovalbumin (OVA)-induced oxidative stress and decreased inflammation in the airway. Therefore, the significant increase in the GSH/GSSG ratio in the DAS-treated lung tissue may be indicative of reduced oxidative stress or an increased antioxidant capacity in the cells, thereby lowering the susceptibility to oxidative damage.

HO-1 is a stress-responsive enzyme, which is widely distributed in many mammalian tissues. It serves as a defense mechanism to metabolize cellular heme, which serves to increase biliverdin and carbon monoxide.[31] Recent evidence indicates that HO-1 plays a key role in the defense against oxidative stress. Yin et al.[32] reported that induction of HO-1 expression leads to a significant reduction in lipopolysaccharide-induced pulmonary edema, leucocyte influx, myeloperoxidase activity, and histopathologic insults in mice. Park et al.[5] also observed a significant increase in pulmonic HO-1 and antioxidant enzyme activity in mice after treatment with quercetin. In our study, we found that DAS markedly increased pulmonic HO-1 protein expression. Thus, we hypothesize that HO-1 can be induced by organosulfuric compounds.

There is accumulating evidence that Nrf2 is a key transcriptional factor that can activate an ARE, in turn regulating the expression of phase II detoxifying and antioxidant enzymes.

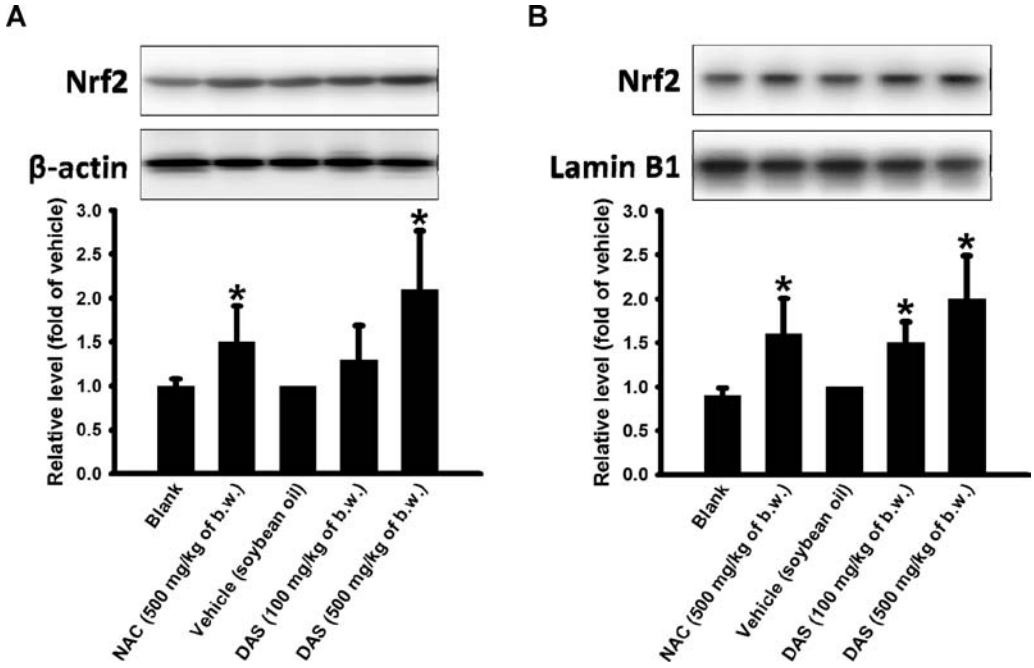

Figure 4. Effect of DAS on Nrf2 protein expression in lung tissue of SD rats: (A) whole cell lysates and (B) nuclear extracts detected by Western blot analysis with anti-Nrf2 and antilamin B1 antibodies. Either 500 mg/kg of body weight of *N*-acetylcysteine or diallyl sulfide or 100 mg/kg of body weight of diallyl sulfide was orally administered for 7 days. Data are expressed as the mean ± SD ($n = 6$). The ∗ indicates $p < 0.05$ when compared with the vehicle-only control group.

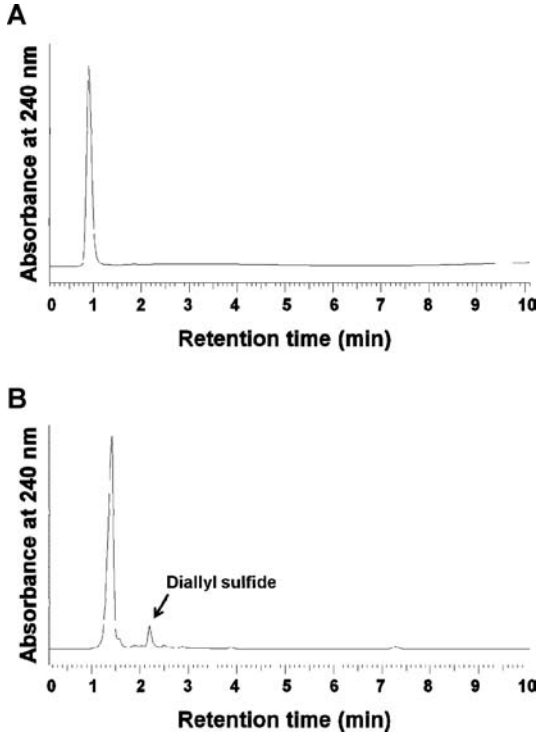

Figure 5. Typical HPLC chromatograms of rat plasma extracts of blood taken from rats (A) without or (B) with 7 days of 500 mg/kg of body weight of diallyl sulfide given by oral administration.

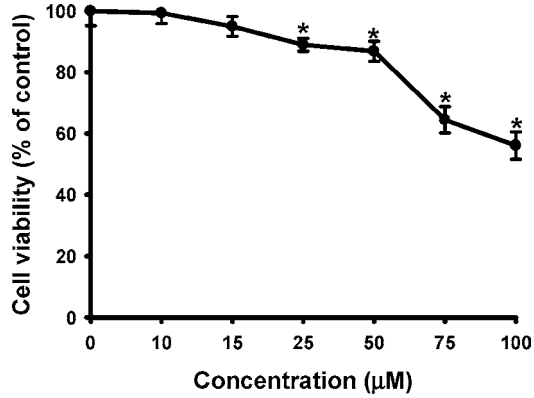

Figure 6. Effect of DAS on the viability of MRC-5 cells by MTT assay. Cells were treated with different doses of DAS (0−100 μM) for 24 h, and the viability of MRC-5 cells was detected by MTT assay. The ∗ indicates $p < 0.05$ when compared with the corresponding group with DAS treatment.

Hence, activation of Nrf2, which controls constitutive and inducible expression of phase II detoxifying and antioxidant genes, may be one of the protective mechanisms against xenobiotics.[33] Several chemopreventive compounds have been shown to induce antioxidant and detoxification genes.[34] Kalayarasan et al.[11] demonstrated that significant induction of Nrf2 was observed in the kidney tissue of animals following treatment with DAS. Farombi et al.[35] reported that curcumin-induced HO-1 expression via Nrf2 transcription factor activation was important for attenuating dimethylnitrosamine-induced liver injury in rats. Moreover, Na et al.[36] showed that (−)-epigallocatechin-3-gallate induced Nrf2-mediated antioxidant enzyme expression via phosphatidylinositol 3-kinase/Akt and ERK1/2 signaling in human mammary epithelial cells. These studies suggest that Nrf2 plays a key role in antioxidant-induced expression of several antioxidant enzymes and that Nrf2 can be induced by phytochemicals. The most significant finding in this study is that DAS can activate pulmonic Nrf2 in rats (Figure 4). The amount of pulmonic Nrf2 protein expression increased significantly in rats following DAS treatment. Fisher et al.[16] also observed that the hepatic NQO1 levels are increased through activation of Nrf2 by DAS oral administration at a

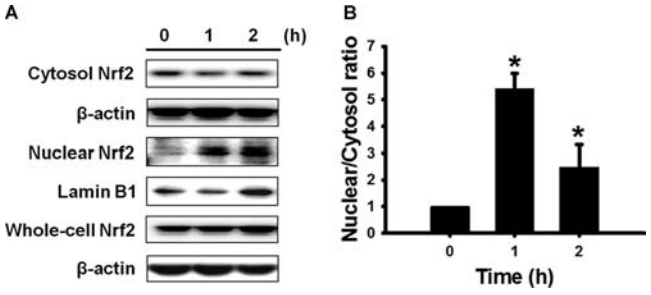

Figure 7. Effect of DAS on activated Nrf2 protein expression in MRC-5 cells. Cells were incubated with 15 μM DAS for 0, 1, or 2 h: (A) Cytosol, nuclear extracts, and whole cell lysates were subjected to Western blot analysis using antibodies specific to Nrf2, β-actin, and lamin B1. The result was quantified using a densitometer, and the Nrf2 value was plotted after normalization to values of β-actin or lamin B1. (B) The nuclear/cytosol ratio defines Nrf2 protein expression in nuclear/Nrf2 protein expression in cytosol. Data presented are the mean ± SD (n = 3). The ∗ indicates a significant difference from the control ($p < 0.05$).

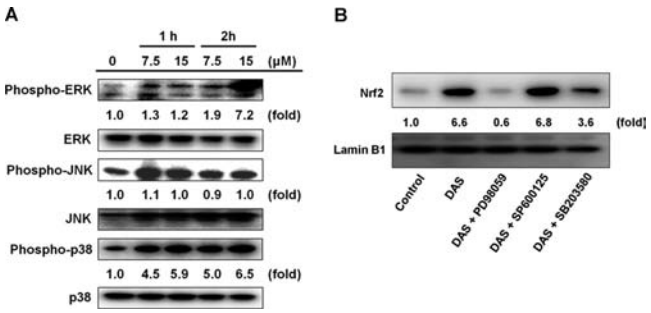

Figure 8. Effect of DAS-induced Nrf2 protein expression via ERK/p38 MAPK activation in MRC-5 cells: (A) Cells incubated in the absence or presence of DAS (7.5 or 15 μM) for the indicated time were subjected to Western blot analysis using phosphospecific antibodies to ERK, JNK, or p38. As a control, the same cell lysates were subjected to Western blot analysis using the corresponding antibodies to detect nonphosphorylated total ERK, JNK, or p38. (B) Cells were pretreated with SB203580 (10 μM), PD98059 (10 μM), or SP600125 (10 μM) for 1 h and were then exposed to 15 μM DAS for 1 h in the absence of inhibitor. Western blot analysis was performed using antibodies specific for Nrf2 and lamim B1. The intensity of the indicated Nrf2 proteins was detected by densitometric analysis and expressed in terms of fold change compared with the intensity of the control band. Data shown are representative of three independent experiments.

dosage of 500 mg/kg body weight in mice. In addition, Lubet et al.[37] showed that the hepatic genes with AREs are markedly induced in rats treated with 500 mg/kg body weight of DAS.

To clarify the modulation of Nrf2 by DAS, we used human embryonic MRC-5 lung cells to investigate the effect of DAS on Nrf2 translocation. We found that the plasma concentration of DAS in rat blood was 15 μM after 7 days of orally administered DAS. Therefore, experiments in cells were performed using 15 μM DAS. In the present study, an increased amount of Nrf2 nuclear translocation was observed in MRC-5 cells treated with DAS (Figure 7), suggesting that activation of Nrf2 may play a key role in the DAS-induced activation of antioxidant genes.

MAPK signaling cascades are stimulated by many extracellular stimuli, such as growth factors, cytokines, and various environment stresses. MAPK signaling cascades serve as a common signal transduction pathway for signals involved in proliferation, differentiation, functional activation, and stress responses. Many studies have demonstrated that MAPKs including ERK, JNK, and p38 are involved in regulating the phosphorylation of Nrf2 and ARE-mediated antioxidant gene expression.[22,38] Therefore, we investigated the MAPK pathway to further explore the upstream regulatory mechanisms involved in activation of Nrf2 induced by DAS. Results showed that ERK and p38 were phosphorylated in MRC-5 cells after DAS treatment (Figure 8A). Higher levels of phospho-ERK and phospho-p38 were also observed, whereas the levels of phospho-JNK did not change in DAS-treated cells when compared with the untreated MRC-5 cells. Inhibition of the phospho-ERK and phospho-p38 pathway by PD98059 and SB203580 inhibitors almost completely blocked DAS-induced Nrf2 protein expression, suggesting that ERK and p38 are key regulators of activation of Nrf2 induced by DAS (Figure 8B). Balogun et al.[39] reported that curcumin induced HO-1 expression by promoting the dissociation of the Nrf2−Keap1 complex in a p38-dependent manner. Chen et al.[40] also observed that resveratrol activated Nrf2-driven ARE activation and HO-1 expression via the ERK pathway. These studies suggest that MAPKs are important for Nrf2 activation and that MAPKs can be induced by phytochemicals.

In conclusion, our findings suggest that DAS effectively upregulates antioxidant genes and enzymes by activating Nrf2 in rat lung. Moreover, we demonstrate that Nrf2 activation induced by DAS may be triggered by the ERK/p38 pathways. The present study provides insight into the mechanism of DAS induction of antioxidant enzymes expression and suggests that DAS, a natural compound found in garlic, may be effectively utilized in the prevention of pulmonary injury.

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ABBREVIATIONS USED

ARE, antioxidant response element; CAT, catalase; DAS, diallyl sulfide; GPx, glutathione peroxidase; GRd, glutathione reductase; GST, glutathione *S*-transferase; HO-1, heme oxygenase-1; HPLC, high-performance liquid chromatography; Keap1, Kelch-like ECH-associated protein 1; NAC, *N*-acetyl-L-cysteine; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor-erythroid 2 related factor 2; SOD, superoxide dismutase.

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