

Diallyl-disulfide, an organosulfur compound of garlic, attenuates airway inflammation via activation of the Nrf-2/HO-1 pathway and NF-kappaB suppression



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ABSTRACT

Diallyl disulfide (DADS) is a major organosulfur compound found in garlic oil that is widely used as a flavoring agent. In this study, we evaluated the effects of DADS on airway inflammation using an ovalbumin-induced model of allergic asthma and RAW264.7 cells. DADS decreased nitric oxide production with a reduction in the levels of interleukins (IL)-1 β and IL-6 in RAW264.7 cells stimulated with LPS. DADS also reduced the expression of proinflammatory proteins including inducible nitric oxide synthase (iNOS), nuclear factor (NF)- κ B, and matrix metalloproteinase (MMP)-9, and it enhanced the expression of anti-oxidant proteins including Nrf-2 and hemeoxygenase (HO)-1. In *in vivo* experiments, DADS decreased the inflammatory cell count in the bronchoalveolar lavage fluid (BALF) with IL-4, IL-5, IL-13, and immunoglobulin (Ig) E. These results were consistent with the histological analysis. DADS attenuated the airway inflammation and mucus hypersecretion induced by OVA challenge. In addition, DADS induced the activation of Nrf-2 and the expression of HO-1. In contrast, DADS reduced the activation of NF- κ B, iNOS and MMP-9. In conclusion, DADS reduced the airway inflammation via regulation of Nrf-2/HO-1 and NF- κ B. These results suggest that DADS might represent a useful new oral therapy to treat allergic asthma.

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1. Introduction

Eosinophil-rich airway inflammation, goblet cell hyperplasia and hyperresponsiveness are important features of allergic asthma. Allergic asthma is a complex response that is associated with various factors. In allergic asthma, exposure to allergens cause an imbalance between the T helper type 1 (Th1) and Th2 responses and an overproduction of reactive oxygen species (ROS), resulting in asthmatic responses in the airway (Mehta et al., 2009; Won et al., 2010). In particular, airway inflammation is aggravated the asthmatic responses. Inflammatory cells, particularly eosinophils, can produce inflammatory cytokines, chemokines and various growth factors that may induce persistent inflammatory, mucus hypersecretion and airway hyperresponsiveness (Lee et al., 2011). Currently, asthma patients are treated with immunosuppressants, bronchodilators and anti-histamine. However, the use of these drugs is limited due to adverse effects. Therefore, many researchers

have sought to develop alternative medicines that are more effective for asthma and have less toxic properties. Indeed, various material for treating asthma have been reported based on *in vivo* and *in vitro* experiments (Kim and Yang, 2011; Kuo et al., 2012; Sun et al., 2013).

Diallyl disulfide (DADS) is a major organosulfur compound of garlic oil. Garlic has been widely used as a flavoring agent and as a traditional medicine to treat various diseases, such as microbial infections, hyperlipidemia, and heart disease (Chang et al., 2005). DADS also has a various pharmacological properties, such as anti-oxidant and anti-tumor activities. Furthermore, DADS inhibits thrombotic aggregation and enhances macrophage function and hypolipidemic activities (Yeh and Liu, 2001; Lin et al., 2008; Rai et al., 2009; Yang et al., 2009). In recent studies, DADS has been shown to exhibit a significant reduction in nitric oxide production and prostaglandin E2 levels as well as suppression of proinflammatory cytokines and chemokines, including interleukin-1 β (IL-1 β), tumor necrosis factor (TNF)- α , and monocyte chemoattractant protein-1 (MCP-1), via inhibition of the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in

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lipopolysaccharide-stimulated BV microglia (Park et al., 2012). However, there has been no study on the effects of DADS with regard to allergic asthma. Based on the properties of DADS, we investigated whether DADS effectively attenuates allergic asthma induced by an allergen challenge.

We therefore investigated the inhibitory effects of DADS on airway inflammation in asthma using a mouse model of ovalbumin-induced asthma and RAW264.7 cells. To investigate the possible mechanism of DADS, we evaluated the abundance of pro-inflammatory and antioxidant proteins in lung tissue and RAW264.7 cells.

2. Materials and methods

2.1. In vitro experiment

2.1.1. Cell culture and viability

The RAW264.7 murine macrophage cell line was purchased from the American Type Culture Collection (ATCC; MD, USA). The cells were incubated at 37 °C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Gibco Inc., NY, USA) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 5.5% fetal bovine serum (FBS; Gibco Inc., NY, USA). The cells were also treated with 0.05% DMSO as a vehicle control. The cells were seeded into 96-well plates at a density of 5×10^4 cells/well and incubated with serum-free medium in the presence of different concentrations of DADS. After incubation for 24 h, the cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) methods. All of the experiments were performed in triplicate.

2.1.2. Measurement of nitric oxide (NO) and ROS production

The cells (2.5×10^5 cells/mL) were seeded in 96-well plates in phenol red-free DMEM, treated with different concentrations (62.5, 125, 250, and 500 ng/mL) of DADS (Tokyo Chemical Ins., Tokyo, Japan) for 1 h, and then incubated in the presence of LPS (1 µg/mL) for 24 h. Nitrite accumulation in the culture medium was measured using Griess reagent (Promega Corporation, WI, USA). The absorbance at 535 nm was measured using a microplate reader (Bio-Rad Laboratories, CA, USA). The induction of oxidative stress was monitored using 2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR), which is converted into highly fluorescent DCF by cellular peroxides, including hydrogen peroxide. To evaluate ROS production, the culture medium was treated with 20 µM DCF-DA for 10 min at 37 °C. ROS activity was measured by measuring the fluorescence at 488 nm excitation and 525 nm emission on a fluorescence plate reader (Perkin-Elmer, MA, USA).

2.1.3. Measurement of proinflammatory cytokine production

The cells were treated as described above, and the concentrations of IL-1β and IL-6 in the culture medium were quantified using a sandwich ELISA kit (R&D Systems, MN, USA) according to the manufacturer's instructions. The absorbance at 450 nm was measured in a microplate reader (Bio-Rad Laboratories). The absolute concentrations were obtained by comparison with standard curves generated with the same ELISA plates.

2.1.4. Western blotting

The cells were treated as described above and then incubated in the presence of LPS (1 µg/mL) for 1 h or 24 h. The cells were collected by centrifugation, washed twice with phosphate-buffered saline (PBS), and suspended in an extraction lysis buffer (Sigma-Aldrich) containing protease inhibitors. The protein concentration was determined using a protein assay reagent (Bio-Rad Laboratories) according to the manufacturer's instructions. Equal amounts of total cellular protein (30 µg) were resolved by 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with blocking solution (5% skim milk), followed by an overnight incubation at 4 °C with the appropriate primary antibody. The following primary antibodies and dilutions were used: anti-β-actin (1:2000 dilution; Cell Signaling, MA, USA); anti-iNOS (1:200 dilution; Abcam, MA, USA); anti-HO-1 (1:250 dilution; Abcam). The blots were washed three times with Tris-buffered saline containing Tween 20 (TBST), and then incubated with a 1:10,000 dilution of a horseradish peroxidase (HRP)-conjugated secondary antibody

(Jackson Immuno Research, PA, USA) for 30 min at room temperature. The blots were again washed three times with TBST, and then developed using an enhanced chemiluminescence (ECL) kit (Thermo, CA, USA). In addition, the nuclear and cytosol extractions were conducted using commercial kit (Thermo, CA, USA) to evaluate the expression of Nrf2 and NF-κB induced by DADS.

2.1.5. Gelatin zymography

Cell supernatant was loaded for gelatin zymography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) zymography was performed to determine the gelatinase activity according to Heussen and Dowdel (1980). Briefly, the zymogram gels consisted of 10% SDS-PAGE containing 1% gelatin as an MMP substrate. The gels were washed in 2.5% Triton X-100 for 1 h to remove the SDS and then incubated at 37 °C for 16 h in developing buffer (1 M Tris-HCl, pH 7.5 with CaCl₂). Thereafter, the gels were stained with 25% methanol/8% acetic acid containing Coomassie brilliant blue. The gelatinase activity was visualized as white bands on a blue background, representing areas of proteolysis.

2.2. In vivo experiment

2.2.1. Experimental procedure

Specific pathogen-free female BALB/c mice (6 weeks old) were purchased from the Koatech Co., (Pyeongtaek, Korea) and used after 2 weeks of quarantine and acclimatization. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology.

The mice divided into four groups ($n = 7$). The mice were sensitized on days 0 and 14 via an intraperitoneal injection of 20 µg OVA (Sigma-Aldrich) emulsified with 2 mg aluminum hydroxide in 200 µL phosphate-buffered saline (PBS) (pH 7.4). On days 21, 22 and 23, the mice received an airway challenge with OVA (1% (w/v)) for 1 h using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan). DADS or montelukast (positive control) was orally administered to mice at a dose of 30 mg/kg body weight 1 h before the OVA challenge. In preliminary study, DADS was administered to mice at doses of 30 and 60 mg/kg body weights. The experimental procedure was the same with present study. BALF cell counts were observed the significant decreases in both 30 and 60 mg/kg treated mice. However, 30 mg/kg treated mice was more effective than 60 mg/kg treated mice. Based on these results, we determined 30 mg/kg, as an experimental dose. Montelukast, a positive drug was developed as a cysteinyl leukotriene (cys-LT)-1 receptor antagonist and was successfully introduced into the market (Lynch et al., 1999; Korenblat et al., 2001). Methacholine responsiveness was assessed 24 h after the final challenge by indirectly using single-chamber, whole body plethysmography (Allmedicus, Seoul, Korea). The experimental procedures were shown in Table 1. Bronchoalveolar lavage fluid (BALF) samples were obtained for analysis. The mice were sacrificed 48 h after the final challenge via an intraperitoneal injection of pentobarbital (50 mg/kg; Hanlim Pharm. Co., Seoul, Korea), and a tracheostomy was performed. To obtain the BALF, ice-cold PBS (0.5 mL) was infused into the lung three times and then withdrawn each time via tracheal cannulation (a total volume 1.5 mL). Total inflammatory cell numbers were determined by counting cells in at least five squares of a hemocytometer, after exclusion of dead cells by Trypan blue staining. Differential cell counts in the BALF were performed using Diff-Quik® staining reagent (B4132-1A; IMEB Inc., CA, USA), according to the manufacturer's instructions.

2.2.2. Measurement of ROS production in BALF

The induction of oxidative stress was monitored using DCF-DA (Molecular Probes), which is converted into highly fluorescent DCF by cellular peroxides, including hydrogen peroxide. Briefly, the cells from the BALF were washed with PBS, and the total cells (5×10^3) was counted. The BALF cells were treated with 20 µM DCF-DA for 10 min at 37 °C. Intracellular ROS activity was measured by measuring the fluorescence at 488 nm excitation and 525 nm emission on a fluorescence plate reader (Perkin-Elmer).

2.2.3. Measurement of the levels of cytokines in BALF and IgE in serum

The levels of IL-4 (sensitivity: 2 pg/mL), IL-5 (sensitivity: 7 pg/mL), IL-13 (sensitivity: 1.5 pg/mL), and IFN-γ (sensitivity: 2 pg/mL) in BALF were measured using sandwich ELISA kits (R&D System) according to the manufacturer's protocols. The levels of total IgE and OVA-specific IgE in the serum were also measured by ELISA. Microtiter plates were coated with anti-IgE antibodies (anti-mouse IgE; 10 µg/mL; Serotec, Oxford, UK) in PBS-Tween 20 and then incubated with samples of BALF

Table 1
Experimental procedure.

Groups	Sensitization (days 0 and 14)	Drug treatment (once daily from days 21 to 23)	Inhalation (days 21, 22, and 23)	Methacholine responsiveness	Sacrifice
NC	PBS (i.p.)	PBS (p.o.)	PBS	Day 24	Day 25
OVA	OVA/Alum (i.p.)	PBS (p.o.)	1% OVA	Day 24	Day 25
Mon	OVA/Alum (i.p.)	Montelukast (30 mg/kg, p.o.)	1% OVA	Day 24	Day 25
DADS	OVA/Alum (i.p.)	SCRT (30 mg/kg, p.o.)	1% OVA	Day 24	Day 25

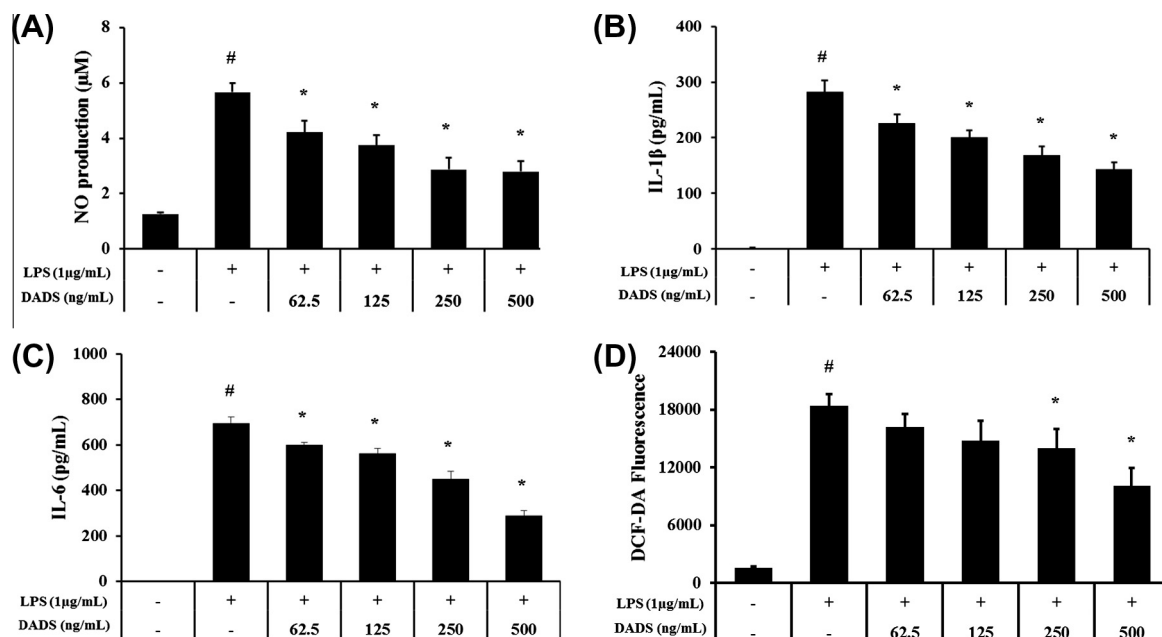


Fig. 1. DADS reduces the production of proinflammatory mediator in RAW264.7 cells stimulated with LPS. (A) NO production, (B) IL-1β, (C) IL-6, (D) ROS production. The cells were treated with different concentrations (62.5, 125, 250, and 500 ng/mL) of DADS for 1 h, and then incubated in the presence of LPS (1 μg/mL) for 24 h. The values were expressed as Mean ± SD. [#]Significantly different from control, $P < 0.05$; ^{*}Significantly different from cells treated with LPS alone, $P < 0.05$.

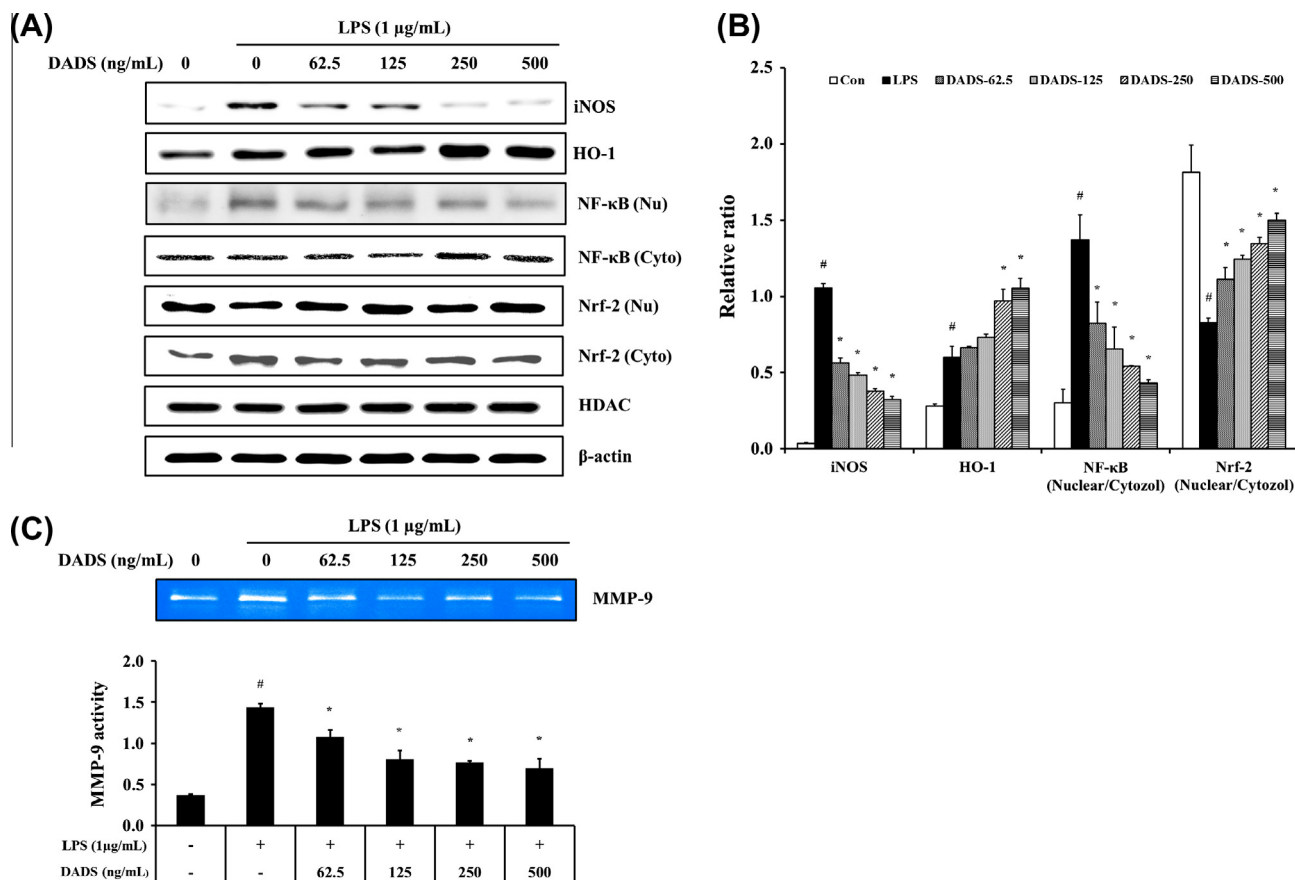


Fig. 2. DADS decreases proinflammatory proteins and increases antioxidant proteins expression in RAW264.7 cells stimulated with LPS. (A) Western blotting, (B) relative ratio, (C) gelatin zymography. The cells were pretreated with DADS for 1 h and then stimulated with LPS for 24 h. Equal amounts of protein in the cell lysates were electrophoresed to determine the levels of iNOS, MMP-9 and HO-1 protein expression. MMP-9 activity was assessed using gelatin zymography. To investigate the activation of Nrf-2 and NF-κB, the cells were pretreated with DADS for 1 h and then stimulated with LPS for 1 h. Equal amounts of protein in the nuclear and cytosolic fraction of the cell lysates were electrophoresed and the levels of Nrf-2 and NF-κB in both the nuclei and cytosol were determined using specific antibodies. The values were expressed as Mean ± SD. [#]Significantly different from control, $P < 0.05$; ^{*}Significantly different from cells treated with LPS alone, $P < 0.05$.

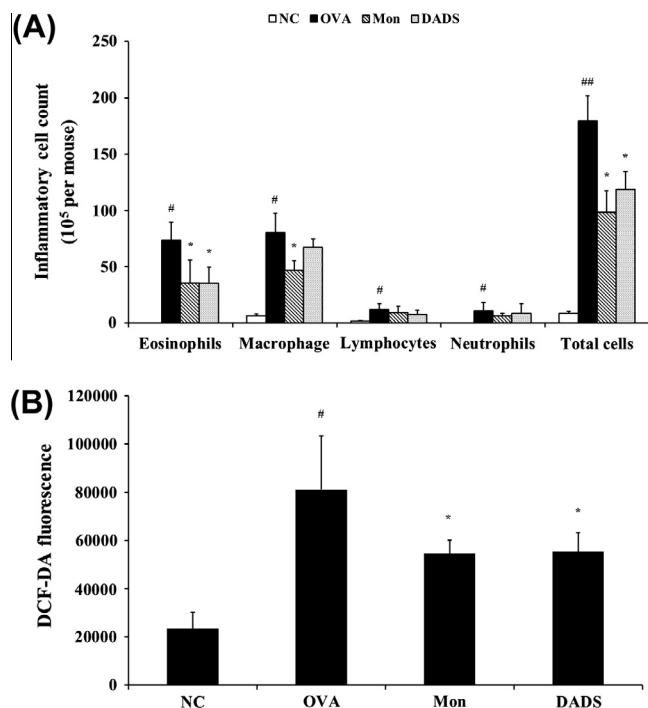


Fig. 3. DADS inhibits the recruitment of inflammatory cells and ROS production in the BALF of mice. The cells were isolated by centrifugation and stained with Diff-Quik[®] staining reagent. (A) Inflammatory cell count, (B) ROS production. The cell numbers were determined using a light microscope to count the cells in at least five squares of a hemocytometer after excluding the dead cells using Trypan blue. NC; normal control mice treated with PBS only; OVA; OVA-sensitized/challenged mice; Mon; montelukast (30 mg/kg) + OVA sensitized/challenged mice; DADS; DADS (30 mg/kg) + OVA-sensitized/challenged mice. The values were expressed as Mean $\pm$ SD ($n = 7$). [#]Significantly different from negative control, $P < 0.05$; ^{*}Significantly different from OVA challenged mice, $P < 0.05$.

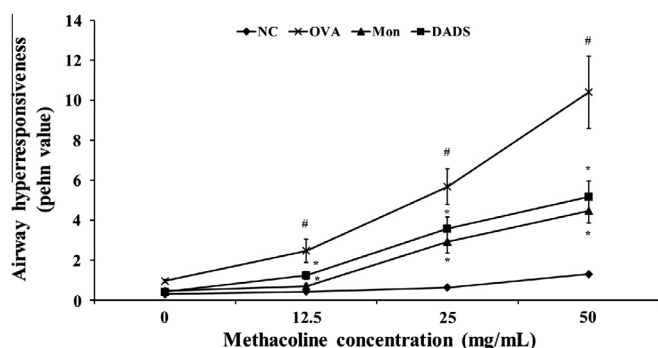


Fig. 4. DADS reduces airway hyperresponsiveness induced by OVA challenge. Airway responsiveness was assessed 24 h after the final challenge by indirectly using single-chamber, whole body plethysmography (Allmedicus, Seoul, Korea). The values were expressed as Mean $\pm$ SD ($n = 7$). [#]Significantly different from negative control, $P < 0.05$; ^{*}Significantly different from OVA challenged mice, $P < 0.05$.

or plasma. The plates were then washed four times, and 200 μ L of o-phenylenediamine dihydrochloride (Sigma–Aldrich) was added to each well. The plates were incubated for 10 min in the dark and the absorbance was measured at 450 nm.

2.2.4. Immunoblotting

Lung tissue was homogenized (1/10 w/v) using a homogenizer with a tissue lysis/extraction reagent (Sigma–Aldrich) containing a protease inhibitor cocktail (Sigma–Aldrich). In addition, to extract the nuclear protein, the lung tissue was homogenized using an extraction kit (Thermo). Each protein concentration was determined using Bradford reagent (Bio-Rad). Western blotting was performed as described above, and the levels of MMP-9, iNOS, Nrf2, HO-1 and NF- κ B were determined.

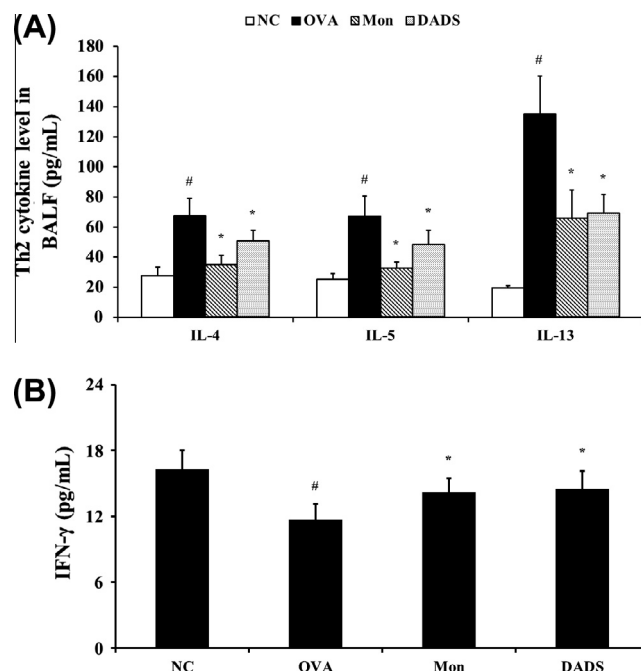


Fig. 5. DADS decreases the levels of IL-4, IL-5 and IL-13 and increases IFN- γ level in BALF. (A) Th2 cytokine levels, (B) IFN- γ level. The Th2 cytokines in the BALF were determined using ELISA. The values were expressed as Mean $\pm$ SD ($n = 7$). [#]Significantly different from negative control, $P < 0.05$; ^{*}Significantly different from OVA challenged mice, $P < 0.05$.

Table 2

The levels of total IgE and OVA-specific IgE in serum.

Groups	Total IgE (μ g/mL)	OVA-specific IgE (ng/mL)
NC	0.22 $\pm$ 0.16	–
OVA	2.36 $\pm$ 0.34 [#]	367.97 $\pm$ 90.63 [#]
Mon	1.75 $\pm$ 0.28 [*]	198.48 $\pm$ 82.95 [*]
DADS	1.78 $\pm$ 0.17 [*]	255.19 $\pm$ 73.42 [*]

NC; normal control mice treated with PBS only; OVA; OVA-sensitized/challenged mice; Mon; montelukast (30 mg/kg) + OVA-sensitized/challenged mice; DADS; DADS (30 mg/kg) + OVA-sensitized/challenged mice.

[#] Significantly different from NC, $P < 0.05$.

^{*} Significantly different from OVA, $P < 0.05$.

2.2.5. Histology

After the BALF samples were obtained, the lung tissue was fixed in 4% (v/v) paraformaldehyde. The tissues were paraffin-embedded, sectioned at a 4 μ m thickness and stained with a H&E solution (Sigma–Aldrich) and periodic acid–Schiff (PAS) (IMEB Inc., CA, USA) to estimate inflammation and mucus production, respectively. Quantitative analysis of inflammation and mucus production was determined using an image analysis (Molecular Devices, Inc., Sunnyvale, CA).

2.3. Statistical analysis

The data are expressed as the means $\pm$ standard error of the mean (SEM). Statistical significance was determined using analysis of variance (ANOVA) followed by a multiple comparison test with Dunnett's adjustment. P values < 0.05 were considered significant.

3. Results

3.1. In vitro experiment

3.1.1. Effects of DADS on NO, IL-1 β , IL-6, and ROS production in LPS-stimulated RAW264.7 cells

The production of NO was significantly increased in LPS-activated cells. In contrast, DADS-treated cells presented significantly inhibited NO production due to LPS treatment in a dose-dependent

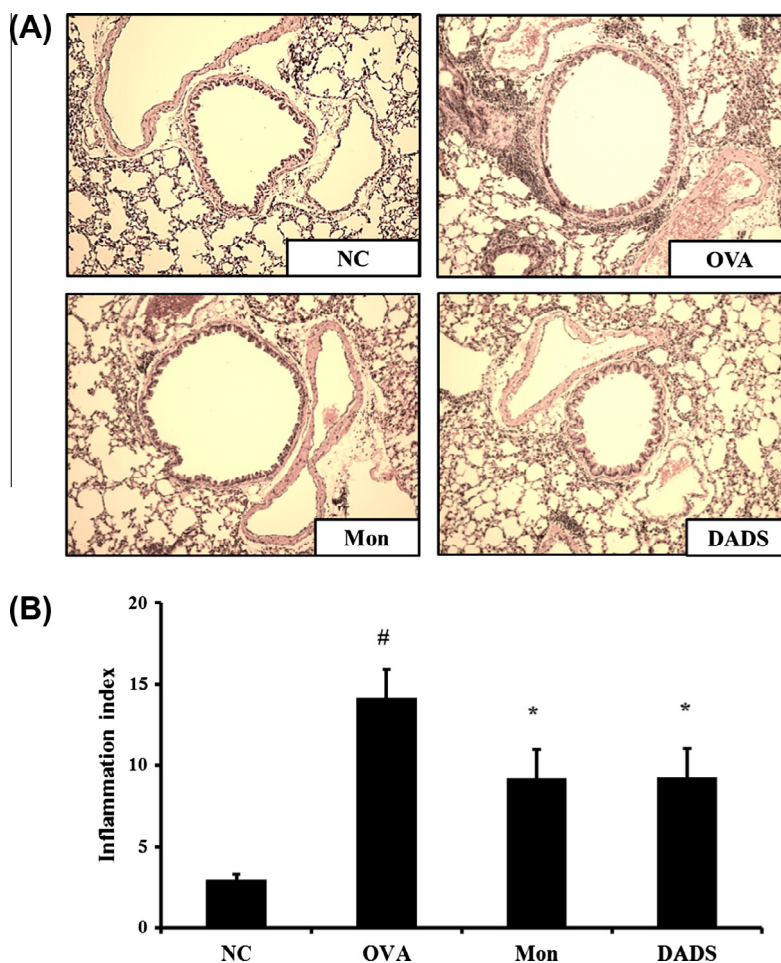


Fig. 6. DADS attenuates airway inflammation induced by OVA challenge. (A) Histological examination of airway inflammation in the lung tissue by H&E staining. (B) Quantitative analysis for inflammatory responses. The values were expressed as Mean $\pm$ SD ($n = 7$). #Significantly different from negative control, $P < 0.05$; *Significantly different from OVA challenged mice, $P < 0.05$.

manner (Fig. 1A). The results for IL-1 β and IL-6 were consistent with those for NO production. The LPS-stimulated cells markedly increased the levels of IL-1 β and IL-6 compared with the normal controls, whereas the DADS-treated cells significantly decreased the levels of IL-1 β and IL-6 in a dose-dependent manner compared with those of LPS-stimulated cells (Fig. 1B and C). The ROS production was significantly increased in LPS-activated cells. However, DADS-treated cells exhibited the significant reduction in the ROS production in concentration-dependent manner (Fig. 1D).

3.1.2. Effects of DADS on the expression of proinflammatory and antioxidant protein in LPS-activated RAW264.7 cells

LPS-stimulated cells showed increases in the expression of iNOS. In contrast, DADS-treated cells had significantly reduced the iNOS expression compared with the LPS-stimulated cells (Fig. 2A and B). HO-1 expression had increased in LPS-stimulated cells. DADS-treated cells exhibited the significant increase in HO-1 expression compared with LPS-treated cells. LPS-stimulated cells had increased Nrf-2 expression in cytosol and decreased in nuclear. However, DADS-treated cells had significantly Nrf-2 expression in nucleus (Fig. 2A and B). The ratio of nuclear to cytosolic Nrf-2 expression increased more in the DADS-treated cells than in the LPS-stimulated cells. In addition, NF- κ B activation was markedly increased in the LPS-stimulated cells. However, NF- κ B activation was effectively inhibited in the DADS-treated cells. As shown in Fig. 2C, DADS-treated cells showed reduced MMP-9 activity in comparison with LPS-stimulated cells.

3.2. In vivo experiment

3.2.1. Effects of DADS on the inflammatory cell count and ROS production in the BALF of OVA-challenged mice

OVA-challenged mice showed significant increases in the numbers of eosinophils and other inflammatory cells compared with the negative controls. In contrast, DADS-treated mice showed a significantly decreased influx of eosinophils into the airways compared with the OVA-challenged mice (Fig. 3A). OVA-challenged mice exhibited significantly increased ROS production in the BALF compared with the negative controls. In contrast, the DADS-treated mice showed significantly decreased ROS production in the BALF compared with the OVA-challenged mice (Fig. 3B).

3.2.2. Effects of DADS on methacholine responsiveness of OVA-challenged mice

The Penh value of OVA-challenged mice was significantly higher than that of the negative controls at any concentration between 12.5 and 50 mg/mL of methacholine (Fig. 4). In the DADS treated mice, the Penh value was significantly reduced compared with that of the OVA-challenged mice. These reductions were similar to those observed in montelukast-treated mice.

3.2.3. Effects of DADS on proinflammatory cytokines in BALF and IgE in serum of OVA-challenged mice

The levels of IL-4, IL-5, and IL-13 were significantly increased in the OVA-challenged mice compared with the negative controls (Fig. 5A). In contrast, the administration of DADS induced a

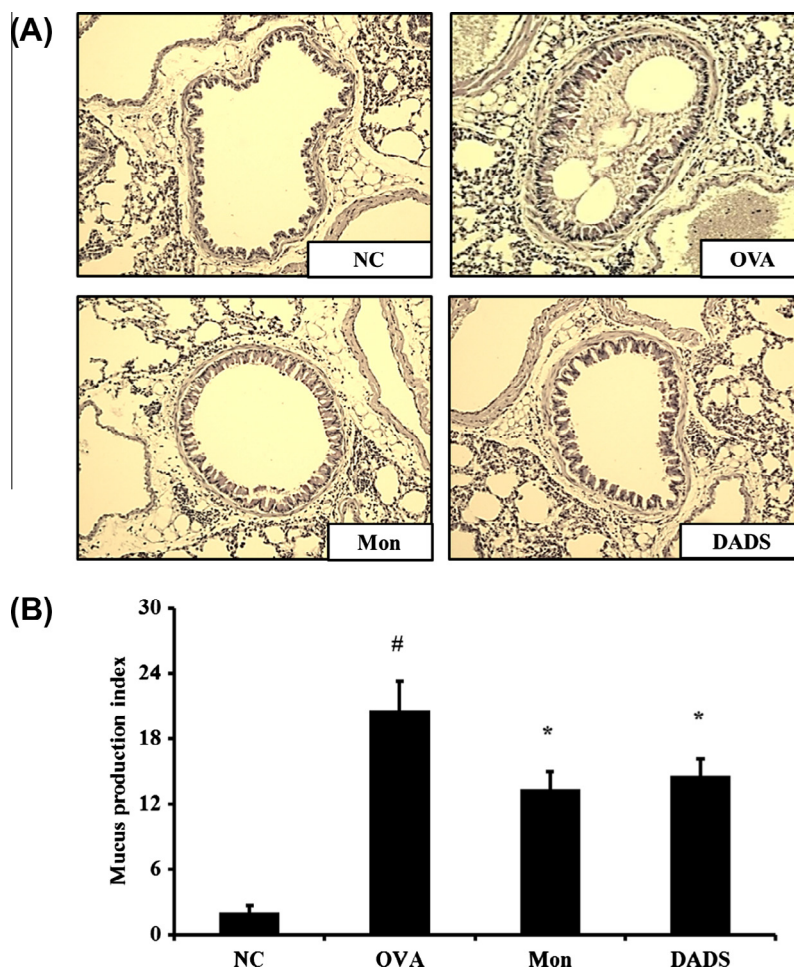


Fig. 7. DADS reduces mucus production induced OVA challenge. (A) Histological examination of mucus production in the lung tissue by PAS stain. (B) Quantitative analysis for mucus production. The values were expressed as Mean $\pm$ SD ($n = 7$). [#]Significantly different from negative control, $P < 0.05$; ^{*}Significantly different from OVA challenged mice, $P < 0.05$.

significant reduction in these increased levels of IL-4, IL-5, and IL-13 in BALF from the OVA-challenged mice. The IFN- γ level was significantly decreased in the OVA-challenged mice compared with the negative controls (Fig. 5B). However, the administration of DADS exhibited the significant increase in IFN- γ level compared with the OVA-challenged mice. The total IgE and OVA-specific IgE were also markedly increased in the OVA-challenged mice, whereas these levels were significantly decreased in DADS-treated mice (Table 2).

3.2.4. Effects of DADS on the inflammatory response and mucus production in lung tissues of OVA-challenged mice

OVA-challenged mice showed a marked infiltration of inflammatory cells into the peribronchiol and perivascular lesions in the lung tissue (Fig. 6A and B). However, DADS-treated mice decreased the inflammatory cell infiltration compared with the OVA-challenged mice. In lung sections stained with PAS, an overproduction of mucus was observed in the bronchial airway of OVA-challenged mice. In contrast, the DADS-treated mice showed a reduction in mucus production (Fig. 7A and B).

3.2.5. Effects of DADS on the expression of proinflammatory and antioxidant proteins in the lung tissue of OVA-challenged mice

The OVA-challenged mice showed a significant increase in the expression of iNOS in the lung tissue compared with the negative controls. However, in the DADS-treated mice, we observed a meaningful reduction in the expression of iNOS compared with

the OVA-challenged mice (Fig. 8A and B). NF- κ B activation was significantly reduced in the DADS-treated mice compared with the OVA-challenged mice. In contrast, Nrf-2 expression in the nuclei of the lung cells was decreased in the OVA-challenged mice, and it was markedly increased in the DADS-treated mice compared with the OVA-challenged mice. HO-1 was overexpressed in the DADS-treated mice compared with the OVA-challenged mice. In addition, The MMP-9 activity was increased in the OVA-challenged mice compared with the negative controls, whereas they were significantly decreased in the DADS-treated mice compared with the OVA-challenged mice (Fig. 8C).

4. Discussion

In this study, we found that DADS effectively suppressed airway inflammation in OVA-induced asthma. DADS suppressed increased NO production, proinflammatory cytokines and protein expression in RAW264.7 cells stimulated with LPS. DADS also significantly increased the expression of antioxidant proteins, including Nrf-2 and HO-1. In our *in vivo* studies, DADS markedly attenuated the asthmatic responses, such as airway inflammation, methacholine responsiveness, and mucus hypersecretion with reduction in Th2 cytokines and IgE, and elevation of IFN- γ . In addition, DADS treatment resulted in a significant reduction in proinflammatory proteins, including NF- κ B, MMP-9 and iNOS and marked increases in Nrf-2 and HO-1 levels.

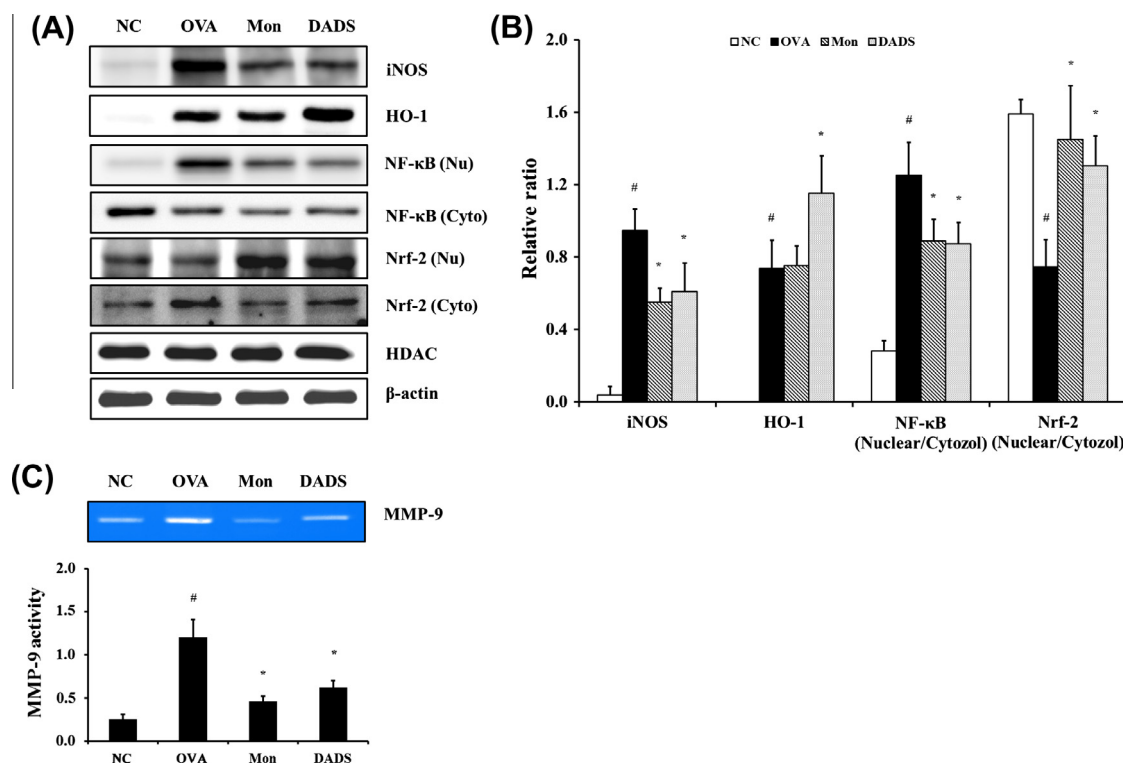


Fig. 8. DADS inhibits the expression of proinflammatory proteins and enhances the expression of antioxidant protein. (A) Western blot, (B) relative ratio, (C) gelatin zymography. The values were expressed as Mean $\pm$ SD ($n = 7$). #Significantly different from negative control, $P < 0.05$; *Significantly different from OVA challenged mice, $P < 0.05$.

Airway inflammation is an important feature of asthma and it is caused by various proinflammatory factors. NF- κ B regulates a number of the genes involved in the immune and inflammatory responses. NF- κ B activation is enhanced by proinflammatory stimuli, such as LPS, TNF- α , phorbol 12-myristate 13-acetate (PMA), which caused the phosphorylation of I κ B (Rhee et al., 2007). NF- κ B is translocated to the nucleus by the degradation of I κ B, and then it binds to NF- κ B binding sites in the promoter regions of proinflammatory genes and induces the transcription of proinflammatory mediators, including iNOS, COX2 and MMP-9 (Lee et al., 2012). The overexpression of iNOS and COX2 increase the NO and prostaglandins E₂ (PGE₂), which aggravate inflammatory responses. MMP-9 increases the inflammatory cell recruitment and releasing proinflammatory cytokines, such as IL-4, IL-5 and IL-13 (Klein et al., 2004). In addition, MMP-9 causes the degradation of the extracellular matrix, which induces airway remodeling in asthma (Rhee et al., 2007). In this study, we found that DADS treatment markedly reduced the expression of iNOS, COX2 and MMP-9, with decreases in NF- κ B activation in RAW264.7 cells stimulated by LPS. These results of our *in vitro* experiments were consistent with those of the *in vivo* experiments. OVA-induced asthmatic mice exhibited marked increases in the expression of iNOS and COX2 with elevation of NF- κ B activation. DADS effectively suppressed the MMP-9 activity and expression in the lung tissue with a reduction in the levels of IL-4, IL-5, IL-13 and IgE. In addition, OVA-induced asthmatic mice significantly decreased the IFN- γ level, whereas DADS-treated mice significantly increased in comparison to the OVA-induced asthmatic mice. These results were strongly supported by the histological analysis of the lung tissues. DADS attenuated the airway inflammation, mucus hypersecretion and methacholine responsiveness induced by OVA exposure. These results indicate that DADS suppresses the inflammatory responses via down-regulation of NF- κ B activation.

Oxidative stress is also closely related to inflammatory responses, such as asthma and chronic obstructive pulmonary diseases. HO-1 is an inducible enzyme that catalyzes the rate-limiting step in the oxidative degradation of cellular heme into carbon monoxide, biliverdin and free iron. It provides a host defense mechanism that protects against oxidative stress and contributes to the anti-inflammatory activity (Ryter et al., 2006; Park et al., 2010). Previous studies have demonstrated that HO-1 expression is regulated via the activation of transcription factors, including Nrf-2, cAMP response element (CRE)-binding protein (CREB) and activator protein-1 (Paine et al., 2010). The promoter region of the HO-1 gene has antioxidant response elements (AREs) to which Nrf-2 can directly bind. The activation of Nrf-2 causes dissociation of Nrf-2 from Kelch-like ECH-associated protein 1 (Keap 1), which is followed by translocation of Nrf-2 to the nucleus, where it modulates the expression of HO-1 (Srisook et al., 2005). In addition, many studies have reported that the Nrf-2 signaling pathways negatively regulate the NF- κ B mediated inflammatory responses (Han et al., 2013). In this study, DADS caused the overexpression of HO-1 with elevation of Nrf-2 translocation to the nucleus in LPS-stimulated RAW264.7 cell. These results were consistent with those of *in vivo* experiment. OVA-induced asthmatic mice showed the reduction in Nrf-2 translocation to the nucleus with elevated ROS production. In contrast, the administration of DADS induced a significant increase in Nrf-2 translocation to the nucleus with reduced ROS production compared with OVA-induced asthmatic mice. With increasing Nrf-2 translocation, DADS caused the overexpression of HO-1 in lung tissue. These results indicate that DADS attenuates inflammatory responses via enhancing the antioxidant status induced by Nrf-2 activation.

In summary, the present study demonstrated the anti-inflammatory effects of DADS in *in vitro* and *in vivo* experiments. DADS inhibited the expression of proinflammatory mediators in

LPS-stimulated RAW264.7 cells and OVA-induced asthmatic mice, and it reduced the activation of NF- κ B, which was accompanied by an induction of HO-1 via Nrf-2 activation. These results suggest that DADS may have therapeutic potential for effectively inhibiting the progression of airway inflammation in asthma.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

- Chang, H.P., Huang, S.Y., Chen, Y.H., 2005. Modulation of cytokine secretion by garlic oil derivatives is associated with suppressed nitric oxide production in stimulated macrophages. *Journal of Agricultural and Food Chemistry* 53, 2530–2534.
- Han, C.W., Kwun, M.J., Kim, K.H., Choi, J.Y., Oh, S.R., Ahn, K.S., Lee, J.H., Joo, M., 2013. Ethanol extract of *Alismatis Rhizoma* reduces acute lung inflammation by suppressing NF- κ B and activating Nrf2. *Journal of Ethnopharmacology* 146, 402–410.
- Heussen, C., Dowdel, E.B., 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrate. *Analytical Biochemistry* 102, 196–202.
- Kim, D.Y., Yang, W.M., 2011. Panax ginseng ameliorates airway inflammation in an ovalbumin-sensitized mouse allergic asthma model. *Journal of Ethnopharmacology* 136, 230–235.
- Klein, G., Vellenga, E., Fraaije, M.W., Kamps, W.A., de Bont, E.S., 2004. The possible role of matrix metalloproteinase (MMP)-2 and MMP-9 in cancer, e.g. acute leukemia. *Critical Reviews in Oncology/Hematology* 50, 87–100.
- Korenblat, P.E., Antileukotriene Working Group, 2001. The role of antileukotrienes in the treatment of asthma. *Annals of Allergy, Asthma and Immunology* 86, 31–39.
- Kuo, C.Y., Wang, T., Dai, T.Y., Wang, C.H., Chen, K.N., Chen, Y.P., Chen, M.J., 2012. Effect of the Velvet Antler of Formosan Sambar Deer (*Cervus unicolor swinhoei*) on the prevention of an allergic airway response in mice. *Evidence Based Complementary and Alternative Medicine*, 481318.
- Lee, M.Y., Seo, C.S., Lee, J.A., Lee, N.H., Kim, J.H., Ha, H., Zheng, M.S., Son, J.K., Shin, H.K., 2011. Anti-asthmatic effects of *Angelica dahurica* against ovalbumin-induced airway inflammation via upregulation of heme oxygenase-1. *Food and Chemical Toxicology* 49, 829–837.
- Lee, M.Y., Yuk, J.E., Kwon, O.K., Oh, S.R., Lee, H.K., Ahn, K.S., 2012. Zuonin B inhibits lipopolysaccharide-induced inflammation via downregulation of the ERK1/2 and JNK pathways in RAW264.7 macrophages. *Evidence Based Complementary and Alternative Medicine* 2012, 728196.
- Lin, Y.T., Yang, J.S., Lin, S.Y., Tan, T.W., Ho, C.C., Hsia, T.C., Chiu, T.H., Yu, C.S., Lu, H.F., Weng, Y.S., Chung, J.G., 2008. Diallyl disulfide (DADS) induces apoptosis in human cervical cancer Ca Ski cells via reactive oxygen species and Ca²⁺-dependent mitochondria-dependent pathway. *Anticancer Research* 28, 2791–2799.
- Lynch, K.R., O'Neill, G.P., Liu, Q., Im, D.S., Sawyer, N., Metters, K.M., Coulombe, N., Abramovitz, M., Figueroa, D.K., Zeng, Z., Connolly, B.M., Bai, C., Austin, C.P., Chateaufort, A., Stocco, R., Greig, G.M., Kargman, S., Hooks, S.B., Hosfield, E., Williams Jr., D.L., Ford-Hutchinson, A.W., Caskey, C.T., Evans, J.F., 1999. Characterization of the human cysteinyl leukotriene CysLT1 receptor. *Nature* 399, 789–793.
- Mehta, A.K., Arora, N., Gaur, S.N., Singh, B.P., 2009. Choline supplementation reduces oxidative stress in mouse model of allergic airway disease. *European Journal of Clinical Investigation* 39, 934–941.
- Paine, A., Eiz-Vesper, B., Blasczyk, R., Immenschuh, S., 2010. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. *Biochemical Pharmacology* 80, 1895–1903.
- Park, S.Y., Kim, Y.H., Kim, E.K., Ryu, E.Y., Lee, S.J., 2010. Heme oxygenase-1 signals are involved in preferential inhibition of pro-inflammatory cytokine release by surfactin in cells activated with porphyromonas gingivalis lipopolysaccharide. *Chemico-Biological Interactions* 188, 437–445.
- Park, H.Y., Kim, N.D., Kim, G.Y., Hwang, H.J., Kim, B.W., Kim, W.J., Choi, Y.H., 2012. Inhibitory effects of diallyl disulfide on the production of inflammatory mediators and cytokines in lipopolysaccharide-activated BV2 microglia. *Toxicology and Applied Pharmacology* 262, 177–184.
- Rai, S.K., Sharma, M., Tiwari, M., 2009. Inhibitory effect of novel diallyldisulfide analogs on HMG-CoA reductase expression in hypercholesterolemic rats: CREB as a potential upstream target. *Life Science* 85, 211–219.
- Rhee, J.W., Lee, K.W., Kim, D., Lee, Y., Jeon, O.H., Kwon, H.J., Kim, D.S., 2007. NF- κ B-dependent regulation of matrix metalloproteinase-9 gene expression by lipopolysaccharide in a macrophage cell line RAW264.7. *Journal of Biochemistry and Molecular Biology* 40, 88–94.
- Ryter, S.W., Alam, J., Choi, A.M., 2006. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiological Reviews* 86, 583–650.
- Srisook, K., Kim, C., Cha, Y.N., 2005. Molecular mechanisms involved in enhancing HO-1 expression: de-repression by heme and activation by Nrf2, the “one-two” punch. *Antioxidants and Redox Signaling* 7, 1674–1687.
- Sun, J., Li, L., Wu, J., Liu, B., Gong, W., Lv, Y., Luo, Q., Duan, X., Dong, J., 2013. Effects of baicalin on airway remodeling in asthmatic mice. *Planta Medica* 79, 199–206.
- Won, H.Y., Sohn, J.H., Min, H.J., Lee, K., Woo, H.A., Ho, Y.S., Park, J.W., Rhee, S.G., Hwang, E.S., 2010. Glutathione peroxidase 1 deficiency attenuates allergen-induced airway inflammation by suppressing Th2 and Th17 cell development. *Antioxidants and Redox Signaling* 13, 575–587.
- Yang, J.S., Chen, G.W., Hsia, T.C., Ho, C.C., Lin, M.W., Lin, S.S., Yeh, R.D., Ip, S.W., Lu, H.F., Chung, J.G., 2009. Diallyl disulfide induces apoptosis in human colon cancer cell line (COLO205) through the induction of reactive oxygen species endoplasmic reticulum stress, caspases cascade and mitochondrial-dependent pathways. *Food and Chemical Toxicology* 47, 171–179.
- Yeh, Y.Y., Liu, L., 2001. Cholesterol-lowering effect of garlic extracts and organosulfur compounds: human and animal studies. *Journal of Nutrition* 131, 989S–993S.