# DATS Reduces LPS-Induced iNOS Expression, NO Production, Oxidative Stress, and NF-$\kappa$B Activation in RAW 264.7 Macrophages

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Diallyl trisulfide (DATS), diallyl sulfide (DAS), and diallyl disulfide (DADS) are the three major organosulfur compounds (OSCs) in garlic oil. In contrast to DADS and DATS, evidence of an anti-inflammatory effect of DATS is limited. In this study compares the efficacy of DATS with those of DAS and DADS on lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production in RAW 264.7 macrophages. The NO production in LPS-activated RAW 264.7 macrophages was suppressed by both DADS and DATS in a dose-dependent manner. At 100 $\mu$M, the nitrite levels of DADS- and DATS-treated cells were 57 and 34%, respectively, of cells treated with LPS alone. DAS, however, had no influence on NO production even at a concentration of 1 mM. Western blot and Northern blot assays showed that DADS and DATS but not DAS dose-dependently suppressed LPS-induced iNOS protein and mRNA expression in a pattern similar to that noted for NO production. LPS-induced cellular peroxide production was significantly inhibited by DADS and DATS ($P < 0.05$) but not by DAS. Electrophoresis mobility shift assays further indicated that DADS and DATS effectively inhibited the activation of NF-$\kappa$B induced by LPS. Taken together, these results indicate that the differential efficacy of three major OSCs of garlic oil on suppression of iNOS expression and NO production is related to the number of sulfur atoms and is in the order DATS > DADS > DAS. The inhibitory effect of DATS on LPS-induced iNOS expression is likely attributed to its antioxidant potential to inhibit NF-$\kappa$B activation.

**KEYWORDS:** Garlic oil; organosulfur compounds; inducible nitric oxide synthase; nuclear transcription factor-$\kappa$ B; macrophages

## INTRODUCTION

Nitric oxide (NO), the metabolic byproduct of the oxidation of L-arginine guanidino nitrogen, acts as a molecular messenger in various physiologic functions and in pathologic processes (*1*). In mammalian cells, at least three different isoforms of nitric oxide synthase (NOS) are responsible for NO production: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (*2*). In general, the small quantity of NO produced for neurotransmission (*3*) and vasodilation (*4*) is catalyzed by nNOS and eNOS, respectively, which are constitutively expressed and are activated through a $Ca^{2+}$-calmodulin-dependent pathway. On the other hand, the activation of iNOS is $Ca^{2+}$-calmodulin-independent, and the expression of iNOS is highly inducible by a variety of endogenous and exogenous stimulators in different cell types, such as macrophages, smooth muscle

cells, and hepatocytes (*5*, *6*). Although the large amount of NO produced by iNOS helps to kill pathogens (*7*), excessive NO production contributes to not only harmful cellular responses but also initiation and progression of inflammation, sepsis, atherosclerosis, and cancer (*8*−*11*).

Proteins of the Rel/nuclear transcription factor-$\kappa$ B (NF-$\kappa$B) family, including p65 (RelA), p50/p105 (NF-$\kappa$B1), p52/p100 (NF-$\kappa$B2), RelB, and c-Rel, appear as homodimers or heterodimers and perform as transcription factors (*12*). The expression of numerous genes is regulated by the activation of NF-$\kappa$B in diverse cell types. Binding of the activated NF-$\kappa$B to a unique DNA sequence in the iNOS promoter is crucial for the up-regulation of iNOS gene expression (*13*). In addition to NF-$\kappa$B, LPS-induced activation of activator protein-1 and cAMP response element binding protein is also involved in the iNOS expression (*14*, *15*). Activation of NF-$\kappa$B is necessary for LPS-induced iNOS expression, and using NF-$\kappa$B inhibitor blocks the iNOS expression and NO production in LPS-treated RAW 264.7 macrophages (*13*). In addition to iNOS, it is well

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recognized that NF-κB activates the downstream expression of several genes that are involved in the inflammatory response such as cyclooxygenase-2 (COX-2) (*16*). Accumulating evidence from studies evaluating the modulation of NF-κB activation and consequently the regulation of expression of inflammatory mediators in activated macrophages has shown that several herbal medicine and dietary compounds may be therapeutic for inflammatory diseases (*17*).

Because of its strong and unique taste and smell, garlic has been widely used as a flavoring agent for hundreds of years. Additionally, a large body of evidence from epidemiologic and laboratory investigations illustrates the health benefits of garlic consumption as a result of its antimicrobial, antioxidant, antithrombotic, antihypertensive, antiatherosclerotic, anti-inflammatory, hypolipidemic, hypoglycemic, hepatoprotective, immunomodulative, and chemopreventive properties (*18–22*). These health-related biological effects are attributed to the fact that garlic products are rich in special and diverse organosulfur compounds (OSCs) (*23*). Garlic extract and *S*-allylcysteine (SAC), the major water-soluble organosulfur component of aged garlic, as well as allicin and ajoene, compounds present in crushed garlic, showed an inhibitory effect on iNOS expression and NO production (*24–27*). Diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS), which differ in their number of sulfur atoms, are the three major OSCs of garlic oil (*28*). Although the influence of DAS and DADS on the production of cytokines and inflammatory mediators in lipopolysaccharide (LPS)-induced macrophages (*25*, *29*, *30*) has been evaluated, little is known about the effect of DATS on inflammatory responses or the mechanism involved in this inhibition. The objective of the present study is not only to compare the efficacy of DATS on modulating iNOS expression and NO production with those of DAS and DADS in LPS-stimulated RAW 264.7 macrophages but also to explore the possible molecular mechanism of these actions.

## MATERIALS AND METHODS

**Chemicals.** The mouse macrophage-like cell line RAW 264.7 was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan), and fetal bovine serum (FBS) was from Biowest LLC (Miami, FL). TRIzol reagent, RPMI 1640 medium, and medium supplements for cell culture were obtained from Invitrogen Corp. (Carlsbad, CA). LPS was obtained from Sigma Chemical Co. (St. Louis, MO). DAS of 100% purity was from Fluka Chemical Co. (Buchs, Switzerland), DATS of 100% purity was from LKT Laboratories Inc. (St. Paul, MN), and DADS of 98% purity was from Tokyo Kasei Chemical Industry (Tokyo, Japan). The specific antibody for iNOS was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). (α-$^{32}$P)-CTP was from Amersham International Company (Buckinghamshire, U.K.). The oligonucleotide primers for Northern blot, the biotin-labeled and unlabeled double-stranded NF-κB consensus oligonucleotide, and a mutant double-stranded NF-κB oligonucleotide for electrophoretic mobility shift assay (EMSA) were synthesized by MDBio Inc. (Taipei, Taiwan). All other chemicals were of the highest quality available.

**Cell Culture.** The RAW 264.7 macrophages (passage levels between 8 and 13) were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% (v/v) heat-inactivated FBS at 37 °C in a humidified atmosphere of 5% CO$_2$. Cells were plated at a density of $8 \times 10^5$ per 30-mm culture dish and were incubated until 90% confluence was reached. For the determination of cell viability, nitrite concentration, iNOS mRNA, and protein levels, cells were treated with various concentrations of DAS, DADS, or DATS in the presence of LPS for 24 h. For EMSA, cultures were preincubated with organosulfur components for 3 h and then treated with 1 μg/mL LPS for 90 min. All oil-soluble OSCs of garlic were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO added to medium was 0.1% (v/v).

**Cell Viability Assay.** The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used to measure cell respiration as an indicator of cell viability (*31*). After incubation with organosulfur compounds with or without LPS for 24 h, cells were incubated in RPMI medium containing 0.5 mg/mL MTT for 3 h. The medium was then removed, and 2-propanol was added to dissolve the formazan. After centrifugation at 5000*g* for 5 min, the supernatant fluid of each sample was transferred to 96-well plates and absorbance was read at 570 nm in a VersaMax tunable microplate reader (Molecular Devices Corp., Sunnyvale, CA). The absorbance in cultures treated with LPS alone was regarded as 100% cell viability.

**Nitrite Determination.** The nitrate in media was measured by use of the Griess assay (*32*) and was used as an indicator of NO synthesis in cells. Briefly, equal volumes of the culture supernatants and Griess solution [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% *N*-(naphthyl)-ethylenediamine dihydrochloride in 5% H$_3$PO$_4$] were added to 96-well plates at room temperature for 10 min. Absorbance at 550 nm was measured and calibrated by using a standard curve of sodium nitrite prepared in the culture medium.

**Western Blot Analysis.** Cells were washed twice with cold PBS and were then harvested in 150 μL of lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), and 20 μg/mL aprotinin, pH 7.4. Protein contents in each sample were quantified by use of the Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL). Equal amounts of proteins were denatured and separated on SDS–polyacrylamide gels and then transferred to poly(vinylidene difluoride) membranes (New Life Science Product, Inc., Boston, MA). Nonspecific binding sites on the membranes were blocked with 5% nonfat dry milk in a buffer containing 10 mM Tris-HCl and 100 mM NaCl, pH 7.5, at 4 °C overnight. The blots were then incubated sequentially with iNOS primary antibodies and horseradish peroxidase-conjugated anti-rabbit IgG. Immunoreactive protein bands were developed by using tetrahydrochloride 3,3′-diaminobenzedine and hydrogen peroxide as substrates and were quantified through densitometric analysis by Zero-Dscan (Scanalytics Inc., Fairfax, VA).

**Northern Blot Analysis.** Total RNA was isolated from cells by using TRIzol reagent as described by the manufacturer. The cDNA probe was prepared by reverse transcriptase Polymerase Chain Reaction as described previously (*33*). iNOS oligonucleotide primers (forward, 5′-CAGTTCTGC GCCTTTGCTCAT-3′; reverse, 5′-GGTGGTGCG-GCTGGACTTT-3′) were selected by using Primer Select (DNASTAR, Madison, WI). The PCR conditions were set as follows: denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min for 35 cycles followed by a 7-min extension at 72 °C. The band corresponding to the iNOS DNA fragment was labeled with [α-$^{32}$P]dCTP through use of an NEBlot kit (New England Biolabs Inc., Beverly, MA) and was used as a probe. For Northern blot analysis, 20 μg of each RNA sample was electrophoretically separated on a 1% agarose gel containing 6% formaldehyde and was then transferred to HyBond N+ membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ) as previously described (*34*). The membrane was prehybridized for 2 h at 42 °C in a solution containing 10× Denhardt's reagent (0.2% Ficoll, 0.2% polyvinylpyrolidone, and 0.2% bovine serum albumin), 5× SSPE (750 mM NaCl, 50 mM NaH$_2$PO$_4$, and 5 mM EDTA), 20 g/L SDS, 50% formamide, and 100 mg/L of single-stranded sheared salmon sperm DNA. The membrane was then hybridized in the same solution with an α-$^{32}$P-labeled iNOS cDNA probe at 42 °C overnight. After the wash, autoradiography was performed by exposing the membrane to SuperRx X-ray film (Kodak, Rochester, NY) at −80 °C with an intensifying screen. The bands on the X-ray film were measured with an AlphaImager 2000 (Alpha Innotech, San Leandro, CA).

**Peroxide Measurement.** The peroxide-scavenging activity of DAS, DADS, and DATS was assessed by using the probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes Inc., Eugene, OR) (*35*). After cells reached 90% confluence, RAW 264.7 macrophages were incubated in a phenol-red-free RPMI 1640 medium containing 10% (v/v) FBS and each of the OSCs for 3 h. After washing with PBS, cells were incubated in fresh medium with 50 μM DCFH-DA
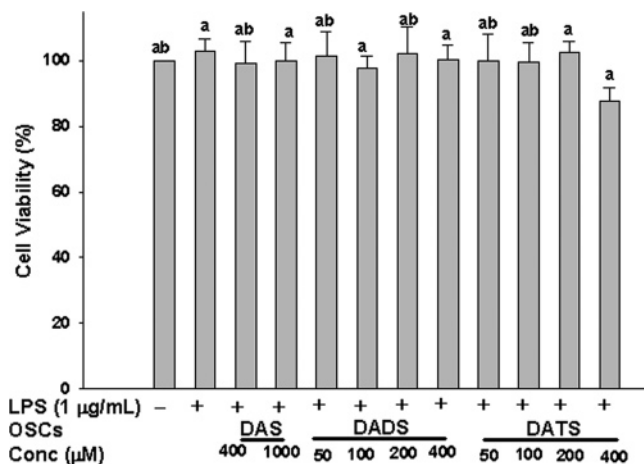
**Figure 1.** Effect of OSCs of garlic oil on cell viability. RAW 264.7 macrophages were treated with 1 $\mu$g/mL LPS alone or with different concentrations of DAS, DADS, and DATS for 24 h, and cell viability was measured using the MTT assay. Data are the mean ± SD of five separate experiments and are expressed as the percentage of the DMSO vehicle control. Values not sharing the same letter are significantly different ($P < 0.05$).



**Figure 2.** Suppression of LPS-induced NO production by OSCs of garlic oil. RAW 264.7 macrophages were treated with 1 $\mu$g/mL LPS alone or with different concentrations of DAS, DADS, and DATS for 24 h. The value for LPS-induced NO production, denoted as 100% response, was 41.39 ± 3.46 $\mu$M. Data are the mean ± SD of six separate experiments. Values not sharing the same letter are significantly different ($P < 0.05$).

for 45 min. DCFH-DA was removed, and the cells were washed twice with PBS and stimulated with 1 $\mu$g/mL LPS for an additional 90 min. As a control, an equal amount of DMSO was added to untreated cells. The fluorescent intensity of DCF was then analyzed in a FACScalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

**Nuclear Protein Preparation and EMSA.** Cells were preincubated with each of the allyl sulfides for 3 h before the addition of 1 $\mu$g/mL LPS for 90 min. Cells were then washed twice, scraped with cold PBS, and centrifuged. The pellets were resuspended in the hypotonic extraction buffer containing 10 mM HEPES, 10 mM KCl, 1 mM MgCl$_2$, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.2 mM PMSF, 4 $\mu$g/mL leupeptin, 20 $\mu$g/mL aprotinin, and 0.5% NP-40 for 15 min on ice and were then centrifuged at 6000$g$ for 15 min. Nuclear proteins were extracted by gentle mixing with 50 $\mu$L of hypertonic extraction buffer containing 10 mM HEPES, 0.4 mM KCl, 1 mM MgCl$_2$, 1 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 4 $\mu$g/mL leupeptin, 20 $\mu$g/mL aprotinin, and 10% glycerol at 4 °C for 30 min. The samples were then centrifuged at 10000$g$ for 15 min. The supernatant fluid containing the nuclear proteins was collected and stored at −70 °C until the EMSA was performed.

Three micrograms of nuclear extract, poly(dI-dC), and biotin-labeled double-stranded NF-$\kappa$B oligonucleotide (5′-AGTTGAGGGGACTTTC-CCAGGC-3′) was mixed with the binding buffer (LightShift Chemi-luminescent EMSA Kit; Pierce Chemical Co.) to a final volume of 20 $\mu$L and was incubated at room temperate for 30 min. An unlabeled and a mutant double-stranded NF-$\kappa$B oligonucleotide (5′-AGTTGAG-G$_C$GACTTTCCCAGGC-3′) were also used to confirm specific binding and protein binding specificity, respectively. The nuclear protein−DNA complex was separated by 6% Tris/Boric acid/EDTA−polyacrylamide gel electrophoresis and was then transferred to Hybond N+ membranes. The membranes were treated with streptavidin−horseradish peroxidase, and the nuclear protein−DNA bands were developed with the use of a SuperSignal West Pico kit (Pierce Chemical Co.).

**Statistical Analysis.** Data are expressed as the mean ± SD from at least four independent experiments. Differences among treatments were analyzed by ANOVA and Tukey's test by using Statistical Analysis System (Cary, NC). $P$ values of <0.05 were considered to be significant.

## RESULTS

**Cell Viability and NO Production.** We used the MTT assay to test whether the concentrations of the OSCs of garlic oil used caused cell damage (**Figure 1**). In the presence of LPS, there were no adverse effects on the growth of RAW 264.7 mac-rophages at concentrations up to 1.0 mM and 400 $\mu$M of DAS
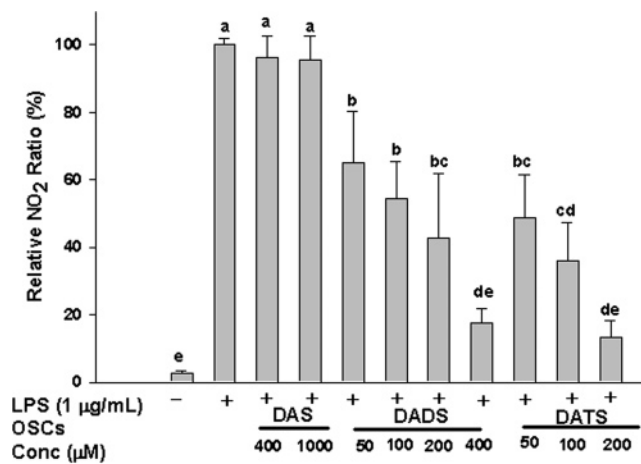
and DADS for 24 h, respectively. However, a 12.5% reduction in mitochondrial reductase activity was noted with 400 $\mu$mol/L DATS when compared with the DMSO vehicle control ($P <$ 0.05). On the basis of these results, the maximum concentrations of DAS, DADS, and DATS examined in the following assays were 1.0 mM, 400 $\mu$M, and 200 $\mu$M, respectively.

Nitrite production was substantially higher in the presence of LPS than with the DMSO vehicle control (**Figure 2**). With DADS and DATS treatments, LPS-induced nitrite production was significantly inhibited in a dose-dependent manner ($P <$ 0.05). At a concentration of 100 $\mu$M, 43 and 66% reductions in nitrite production were noted in cells treated with DADS and DATS, respectively. In contrast, even at a concentration of 1.0 mM, DAS had no significant effect on nitrite production. As shown by the cell viability assay, the decline in LPS-induced NO synthesis with DADS and DATS treatment was not due to cell damage.

**iNOS Protein and mRNA Expression.** The immunoblot assay showed that iNOS protein was undetectable in the resting RAW 264.7 macrophages and was highly induced in the presence of LPS (**Figure 3**). Consistent with the results for nitrite production, DAS had no significant effect on LPS-induced iNOS protein expression. Both DADS and DATS dose-dependently suppressed the iNOS protein level in the order DATS > DADS.

As noted for the changes in iNOS protein, the Northern blot assay further showed that LPS-induced iNOS mRNA expression was dose-dependently suppressed by DADS and DATS (**Figure 4**). Again, even at a concentration of 1 mM, DAS produced no significant change in LPS-induced iNOS mRNA expression. The iNOS mRNA levels of cells treated with 400 $\mu$M DADS or 200 $\mu$M DATS were 18 and 8%, respectively, of the levels of cells treated with LPS alone.

**Peroxide Scavenging Activity.** LPS significantly increased cellular peroxide production compared with that by unstimulated cells (**Figure 5**). Pretreatment with both DADS and DATS significantly inhibited the LPS-induced peroxide production as compared with that by cells treated with LPS only ($P < 0.05$). However, the peroxide level in DAS-pretreated cells was similar to that for cells treated with LPS alone.

**NF-$\kappa$B Activation.** An EMSA assay was performed to examine whether the suppression of iNOS expression by DADS and DATS was dependent on the inhibition of LPS-induced NF-
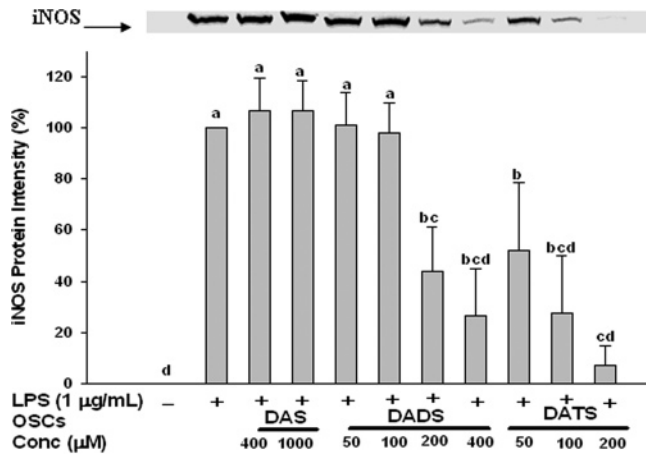
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**Figure 3.** Suppression of LPS-induced iNOS protein expression by OSCs of garlic oil. RAW 264.7 macrophages were treated with 1 $\mu$g/mL LPS alone or with DAS, DADS, and DATS at the concentration indicated for 24 h. The iNOS protein levels of each OSC-treated sample were quantified by densitometry and are expressed as the percentage of that observed with LPS alone. Data are the mean $\pm$ SD of at least four separate experiments. Values not sharing the same letter are significantly different ($P < 0.05$).
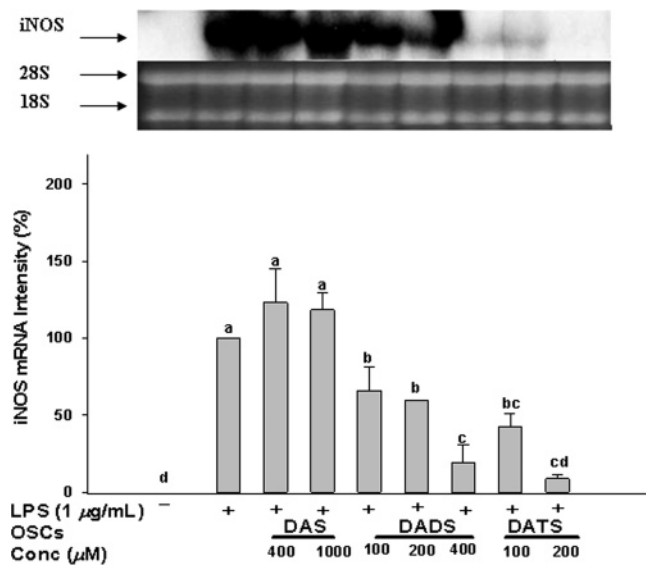


**Figure 4.** Suppression of LPS-induced iNOS mRNA expression by OSCs of garlic oil in RAW 264.7 macrophages. Total RNA was isolated by TRIzol reagent, and iNOS mRNA expression was analyzed by Northern blot. The iNOS mRNA was normalized by 28S mRNA expression, and the iNOS mRNA level of each OSC-treated sample is expressed as the percentage of maximal expression observed with LPS alone. Data are the mean $\pm$ SD of four separate experiments. Values not sharing the same letter are significantly different ($P < 0.05$).

$\kappa$B activation. As shown in **Figure 6**, the nuclear extract from LPS-stimulated macrophages showed a marked increase in NF-$\kappa$B DNA-binding activity compared with that in unstimulated macrophages. This increase in binding activity completely vanished with the addition of excess unlabeled, double-stranded NF-$\kappa$B consensus oligonucleotide, which illustrates the specificity of the NF-$\kappa$B nuclear protein–DNA interaction. Moreover, the addition of mutant double-stranded NF-$\kappa$B oligonucleotide caused only a slight change in the DNA binding of NF-$\kappa$B. Although DAS did not affect the NF-$\kappa$B activation stimulated by LPS, DADS and DATS pretreatment dose-dependently
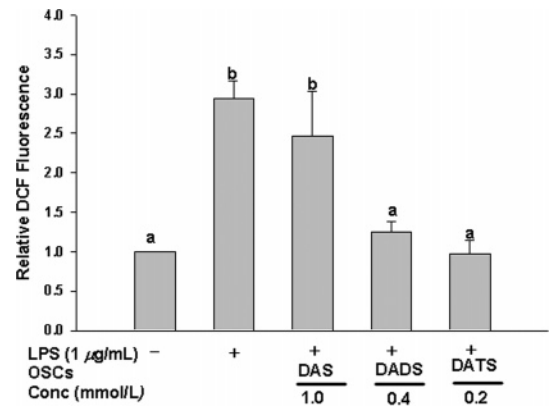


**Figure 5.** Suppression of peroxide production by OSCs of garlic oil. RAW 264.7 macrophages were preincubated with DAS, DADS, and DATS at concentrations indicated for 3 h followed by treatment with DCFH-DA for 45 min. After removal of DCFH-DA, cells were treated with 1 $\mu$g/mL LPS for an additional 90 min. The fluorescent intensity of DCF was detected in a FACScalibur flow cytometer and was expressed as the percentage of DMSO vehicle control in the absence of LPS. Data are the mean $\pm$ SD of three separate experiments. Values not sharing the same letter are significantly different ($P < 0.05$).
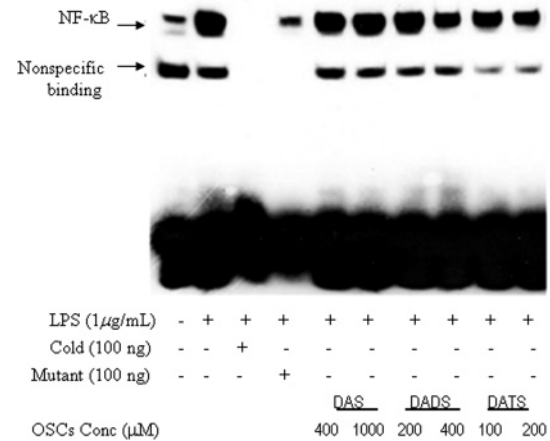


**Figure 6.** Suppression of LPS-induced NF-$\kappa$B nuclear protein DNA-binding activity by organosulfur compounds of garlic oil. RAW 264.7 macrophages were preincubated with various concentrations of DAS, DADS, and DATS for 3 h and were then treated with either the vehicle control or 1 $\mu$g/mL LPS for 90 min. Nuclear protein was prepared as described under Materials and Methods. NF-$\kappa$B nuclear protein DNA-binding activity was measured by EMSA. Bands for NF-$\kappa$B DNA-binding activity were detected by using streptavidin–horseradish peroxidase and were developed by using the SuperSignal West Pico kit (Pierce Chemical Co.).

suppressed the LPS-induced NF-$\kappa$B DNA-binding activity in the order DATS > DADS.

## DISCUSSION

In the past few years, garlic oil and its OSCs have gained much attention in the areas of chemoprevention (*36*), antimicrobial activity (*37*), antiatherosclerosis (*38*), antioxidation (*39*, *40*), and anti-inflammation (*25*, *29*, *30*). It has been shown that DAS and DADS reveal disparities in modulating NO, prostaglandin E$_2$, and cytokine production in activated macrophages, although the mechanism associated with this regulation is unknown (*29*, *30*). In contrast to DAS and DADS, considerably less is known about the efficacy of DATS on anti-inflammation. To our knowledge, this is the first paper to show that DATS is effective in the inhibition of LPS-induced iNOS expression and

NO production. Additionally, the effectiveness of inhibition of DAS, DADS, and DATS is related to the increased number of sulfur atoms. Our data further demonstrated that the influence of DATS and DADS on down-regulation of LPS-induced oxidative and NF-*κ*B activation is a possible mechanism responsible for their inhibitory effects on iNOS expression in stimulated macrophages.

In most unstimulated cells, NF-*κ*B covalently bound to inhibitor protein I*κ*B is sequestered in the cytoplasm (*41*). Exposure of the cells to external stimuli, such as inflammatory cytokines, oxidative stress, ultraviolet irradiation, or bacterial endotoxins, results in activation of NF-*κ*B through the stimulation of phosphorylation and degradation of I*κ*B*α* (*42*, *43*). The activated NF-*κ*B is then translocated to the nucleus, where it binds to the cis-acting *κ*B enhancer element of target genes and activates the expression of proinflammatory mediators, including iNOS (*13*, *44*). Our data showed that, with the exception of DAS, pretreatment with DADS and DATS can effectively suppress LPS-induced NF-*κ*B nuclear protein DNA-binding affinity and thus inhibit iNOS expression and NO production. On the basis of the results presented herein, oil-soluble OSCs of garlic show the similar molecular biological activity to SAC in modulating NF-*κ*B activation and then proinflammatory gene expression in stimulated macrophages (*24*).

Oxidant damage by reactive oxygen species (ROS) is known to contribute to cardiovascular (*45*) and neurodegenerative diseases (*46*), inflammation (*47*), and tumorigenesis (*48*). Awareness of the therapeutic action of aged garlic extract and SAC against those degenerative diseases is attributed to their antioxidant characteristics (*49*). Several lines of investigation suggest that suppression of LPS-induced ROS results in diminution of NF-*κ*B activity and subsequent inhibition of NF-*κ*B responded gene expression (*50*, *51*). In a recent study, we have reported that DAS, DADS, and DATS differentially modulate the rat liver tissue and red blood cell glutathione (GSH)-related antioxidation system, including GSH peroxidase, GSH reductase, and GSH-*S*-transferase activities and GSH level in the order DATS > DADS > DAS (*52*). Addition of the exogenous antioxidant 1 mM *N*-acetyl-L-cysteine reduced the hyperoxia-induced iNOS expression through restoring GSH level in rat liver (*53*). In this study, we further showed pretreatment of DADS and DATS but not DAS significantly decreases LPS-induced oxygen species by a fluorometric assay using DCFH-DA as a probe. It is likely that DATS up-regulated the cellular redox protection system in RAW 264.7 macrophages as in liver tissue. The parallel responses of DAS, DADS, and DATS in NF-*κ*B activation and peroxide scavenging activity suggest that the differential inhibition of LPS-induced iNOS expression by these three garlic allyl sulfides is likely attributed to, at least in part, their antioxidant activity and thus suppression of NF-*κ*B activation.

Of interest is that the number of sulfur atoms and allyl groups plays an important role in the biological feature of garlic OSCs (*54*). The correlation between the number of sulfur atoms of DAS, DADS, and DATS and their potency in modulating hepatic drug-metabolizing enzyme and antioxidant expression or activity was reported previously by ourselves and others (*40*, *52*, *55*). The number of sulfur atoms in these molecules is inversely correlated with the expression of phase I enzymes such as cytochrome P450 1A1, 2B1, and 3A1. In contrast, the up-regulation of phase II detoxification enzymes, that is, the placental form of glutathione *S*-transferase and quinone reductase, by DAS, DADS, and DATS is related to the increased number of sulfur atoms. In agreement with this structure–

function relationship, a similar inhibitory potency among DAS, DADS, and DATS has been noted in this study regarding LPS-induced events and in COX-2 expression in HEK 293 T cells (*56*). The reason DATS is the most potent inhibitor in NF-*κ*B responded proinflammatory gene expression remains to be clarified. The differences in their metabolites derived form the sulfides and the interaction between sulfides and protein thiols seem to be the possibilities to account for the structure–function relationship among these sulfides (*57*). Further studies are needed to explore how the structure–function relationship of these OSCs is involved in the regulation of chronic inflammation related diseases.

The NO produced by eNOS in normal arteries has an antiatherogenic effect (*6*). However, substantial evidence suggests that iNOS plays a pivotal role in modulating the inflammatory response, which is crucial in the pathology of many degenerative diseases (*58*). In atherosclerotic lesions, inflammatory cytokines induce iNOS expression in CD-68-positive macrophages, foam cells, and vascular smooth muscle cells and cause a burst of NO production. In addition to acting as a free radical, NO can react with superoxide anion and produce substantially reactive peroxynitrite, which causes nitrosation of cellular proteins and nucleic acids and also leads to peroxidation of membrane lipids (*59*). These findings indicate that attenuating iNOS induction in the arterial wall could become a new therapeutic strategy in atherosclerosis. In the present study, among the three major OSCs of garlic oil, DATS showed the most potent inhibition in LPS-induced iNOS expression. Further experiments should explore whether the inhibitory property of DATS in iNOS expression and also its modulatory property in inflammatory responses contribute to the antiatherogenic effect of garlic. Our findings about oil-soluble OSCs of garlic modulating iNOS expression and NO production, which was associated with the down-regulation of NF-*κ*B activity mediated by suppressed oxidative stress, provide a novel molecular approach of managing chronic inflammation-related diseases with garlic oil.

## ABBREVIATIONS USED

COX-2, cyclooxygenase-2; DADS, diallyl disulfide; DAS, diallyl sulfide; DATS, diallyl trisulfide; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; GSH, glutathione; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-*κ*B, transcription factor-kappa B; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; OSCs, organosulfur compounds; PMSF, phenylmethanesulfonyl fluoride; ROS, reactive oxygen species; SAC, *S*-allycysteine.

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