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Differential effects of organosulfur compounds from garlic oil on nitric oxide and prostaglandin E_2 in stimulated macrophages

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Abstract

Objective: We investigated the inhibition of nitric oxide (NO) and prostaglandin E_2 (PGE_2) production by the garlic oil derivatives, diallyl sulfide (DAS), diallyl disulfide (DADS), and allyl methyl sulfide (AMS), in lipopolysaccharide (LPS)-activated RAW 264.7 cells.

Methods: Cells were treated with LPS (330 ng/mL) and various concentrations of DAS, DADS, and AMS. NO and PGE_2 released into the medium and expressions of inducible NO synthase and cyclooxygenase-2 protein were measured.

Results: All three compounds suppressed stimulated NO production, among which AMS exhibited the least inhibition. Western blot analysis showed that DAS and DADS, but not AMS, inhibited the corresponding inducible NO synthase expression. An in vitro study showed that all three compounds possess NO clearance activity, and that DADS and AMS were more effective than DAS. On the contrary, only DAS inhibited activated PGE_2 production and cyclooxygenase-2 protein expression.

Conclusions: The garlic derivatives, DAS, DADS, and AMS, differentially regulated the production of NO and PGE_2 in stimulated macrophages. DAS decreased stimulated NO and PGE_2 production by inhibiting inducible NO synthase and cyclooxygenase-2 expressions, and its enzyme inhibiting and NO clearance activity may also partly contribute to the suppression of NO. DADS inhibited activated NO production by decreasing inducible NO synthase expression and by directly clearing NO, whereas AMS suppressed NO mainly through its direct NO clearance activity. Further, neither DADS nor AMS showed any inhibitory effect on stimulated PGE_2 production.

Keywords: Garlic; Organosulfur compounds; Nitric oxide; Prostaglandin E_2; Inducible nitric oxide synthase; Cyclooxygenase

Introduction

Garlic (*Allium sativum* L.) has been widely used as a flavoring agent and as a traditional medicine to control diseases such as microbial infection, hyperlipidemia, and heart diseases [1,2]. Different studies have also demonstrated that garlic possesses anticarcinogenic and immunomodulatory effects [3–6], and organosulfur compounds isolated from different garlic preparations are suggested to be the bioactive components. For example, diallyl sulfide (DAS), diallyl disulfide (DADS), allyl methyl sulfide (AMS), and diallyl trisulfide (DAT) are principal compounds in garlic oil; S-allyl cysteine is the major compound in aged garlic extract [7,8]. Many of these organosulfur compounds have been identified as playing roles in anticarcinogenic processes [9].

Chronic infection and inflammation have been reported to be risk factors in carcinogenesis [10,11]. During infection or inflammation, various inflammatory mediators including nitric oxide (NO), prostaglandin E_2 (PGE_2), and many cytokines that are synthesized and released participate in host immune responses. NO is a small radical molecule that has many physiologic functions, being involved in vasorelaxation, neurotransmission, immunoregulation, and inflammation. NO is synthesized from L-arginine by nitric oxide synthase (NOS) in various cells and tissues [12]. Among isoforms identified, the inducible form of NOS (iNOS) is primarily expressed in macrophages and lymphocytes and is

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synthesized by infectious stimulants such as bacterial endotoxin lipopolysaccharide (LPS) and proinflammatory cytokines. Similarly, PGE_2 is also synthesized during infection by cyclooxygenase (COX). Two isoforms of COX have been identified, COX-1 and COX-2, and the latter is inducible after exposure to LPS and cytokines [11]. Therefore, NO and PGE_2 produced by iNOS and COX-2 are involved in antipathogenic processes, but the overproduction of NO and PGE_2 is also associated with oxidative stress and pathologic conditions such as chronic inflammation, degenerative neuronal diseases, and cancer [10,11,13]. Hence, high levels of NO or PGE_2 are potentially toxic, and agents that suppress the overproduction of NO and PGE_2, associated with chronic inflammation, may have possible roles in anticarcinogenic processes.

Garlic extract and its water-soluble organosulfur derivatives S-allyl cysteine, allicin, and ajoene have been reported to inhibit NO production in LPS- and/or interferon-γ–activated macrophages [14,15]. However, the effects of volatile organosulfur compounds from garlic oil on NO and PGE_2 have not been thoroughly examined. In this study, we used a murine macrophage RAW 264.7 cell line as a model to investigate the effects of DAS, DADS, and AMS, the principal components in garlic oil, on the LPS-stimulated production of NO and PGE_2. To understand the effects of these organosulfur compounds on the production of NO and PGE_2, nitrite and PGE_2 concentrations in stimulated cultured medium were monitored. To obtain further insights into the suppressive effects of these compounds, expressions of iNOS and COX-2 proteins were examined. The direct NO clearance activities of these compounds were also studied.

Materials and methods

Chemicals and biochemicals

DAS, DADS, LPS, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, sodium nitrite, and sodium nitroprusside were purchased from Sigma Chemical Co. (St. Louis, MO, USA). AMS was from ACROS (Fairlawn, NJ, USA), and absolute ethanol was purchased from Merck (Darmstadt, Germany). Dulbecco's Modified Eagle's Medium and fetal bovine serum were purchased from GIBCO (Grand Island, NY, USA). All other laboratory chemicals were of the highest quality available and were purchased from Sigma Chemical Co. and USB (Cleveland, OH, USA).

Cell culture

Murine monocyte-macrophage RAW 264.7 cells were obtained from the Culture Collection and Research Center (CRCC 60001; Hsinchu, Taiwan). Cells were grown as monolayers in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 95% air and 5% CO_2. Because dimethyl sulfoxide showed an effect on NO production, DAS, DADS, and AMS were dissolved in absolute ethanol, and the concentration of absolute ethanol added to the medium never exceeded 0.2% (v/v).

Assay for cytotoxicity

The viability of cells was examined with a CellTiter 96® Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA). Basically, cells were treated with LPS and various concentrations of DAS, DADS, and AMS for 24 h and then harvested to test for cytotoxicity. Live cells convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium to a formazan dye that can be detected at optical density of 492 nm by a microplate reader.

Assay for NO and PGE_2 production and release

The nitrite concentration in the culture medium was determined as an index of NO production. Nitrite was quantified spectrophotometrically after its reaction with a Griess reagent (1:1 mixture of 1% sulfanilamide/5% H_3PO_4 and 0.1% naphthyl ethylenediamine dihydrochloride) using sodium nitrite as a standard [16]. The production and release of PGE_2 in the culture medium was determined using a PGE_2 EIA commercial kit (Amersham Biosciences, Buckinghamshire, UK).

Sodium dodecylsulfate polyacrylamide gel electrophoresis and western blot analyses

To determine whether the production of NO and PGE_2 was associated with the expression of iNOS and COX-2 proteins, western blot analysis was performed. Cell lysate was isolated by treating cells with lysis solution (150 mM NaCl, 1% Triton X-100, 10 mM Tris-HCl buffer, pH 7.4, 5 mM ethylene diaminetetra-acetic acid, and 1 mM phenyl methylsulfonylfluoride), and cellular protein content was determined using a Bio-Rad protein assay kit (Hercules, CA, USA). After separation by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis, proteins were electroblotted onto a nitrocellulose membrane, and the blots were incubated with polyclonal antimouse iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blot was treated with peroxidase-conjugated immunoglobulin G (Novocastra Laboratories, Newcastle, UK), and specific bindings of the iNOS were detected with an enhanced chemiluminescent western detection system (Amersham Biosciences, Cleveland, OH, USA). Similarly, COX-2 protein was detected by the addition of monoclonal antimouse COX-2 antibody (BD Transduction Laboratories, Los Angeles, CA, USA) after the membrane was stripped by Restore TM western blot stripping buffer (Pierce, Rockford, IL, USA). The visu-

alized proteins were quantitated with Phoretix 1D Gel Analysis software (Phoretix International, Newcastle, UK).

In vitro NO clearance activity

To understand whether the lowered NO concentration in the culture medium was due to direct interaction between NO and garlic derivatives, sodium nitroprusside, an NO donor, was incubated with various concentrations of DAS, DADS, and AMS in a cell-free condition. The amounts of NO in the reaction mixture were then determined by the Griess reaction.

Statistical analysis

Values are expressed as mean ± standard deviation. One-way analysis of variance followed by Fisher's least significant difference test and Student's *t* test were used to determine statistical differences between groups by using SAS 6.12 (SAS Institute, Cary, NC, USA). Statistical significance of mean differences was based on $P < 0.05$.

Results

Effects of DAS, DADS, and AMS on NO and PGE_2 production

Treatment of RAW 264.7 cells with these compounds did not increase NO production (data not shown). Conversely, LPS treatment for 24 h significantly enhanced NO production, and cotreatment with DAS, DADS, and AMS suppressed this enhancement. As shown in Fig. 1, DAS, at concentrations ranging from 1 to 10 μM, inhibited 20% to 67% of the LPS-induced NO production in a dose-dependent manner. DADS (0.1~1 μM) and AMS (2~20 μM) showed similar inhibitory effects, but AMS exhibited the weakest inhibition. Except for 0.2 and 1 μM of DADS inhibiting 17% and 21% of the LPS-stimulated cell growth, respectively, no other concentration of DAS, DADS, or AMS showed any cytotoxicity to LPS-treated cells (data not shown), suggesting that the NO inhibitory effects of DAS, DADS, and AMS were not due to cell death. In contrast, these compounds showed different effects on stimulated PGE_2 production. Fig. 2 shows that DAS inhibited activated PGE_2 production to an extent similar to that of NO, but DADS and AMS showed no inhibitory effects, and 0.1 μM of DADS even slightly increased activated PGE_2 production. Therefore, DAS, DADS, and AMS differentially regulated the productions of NO and PGE_2 in stimulated RAW 264.7 cells.

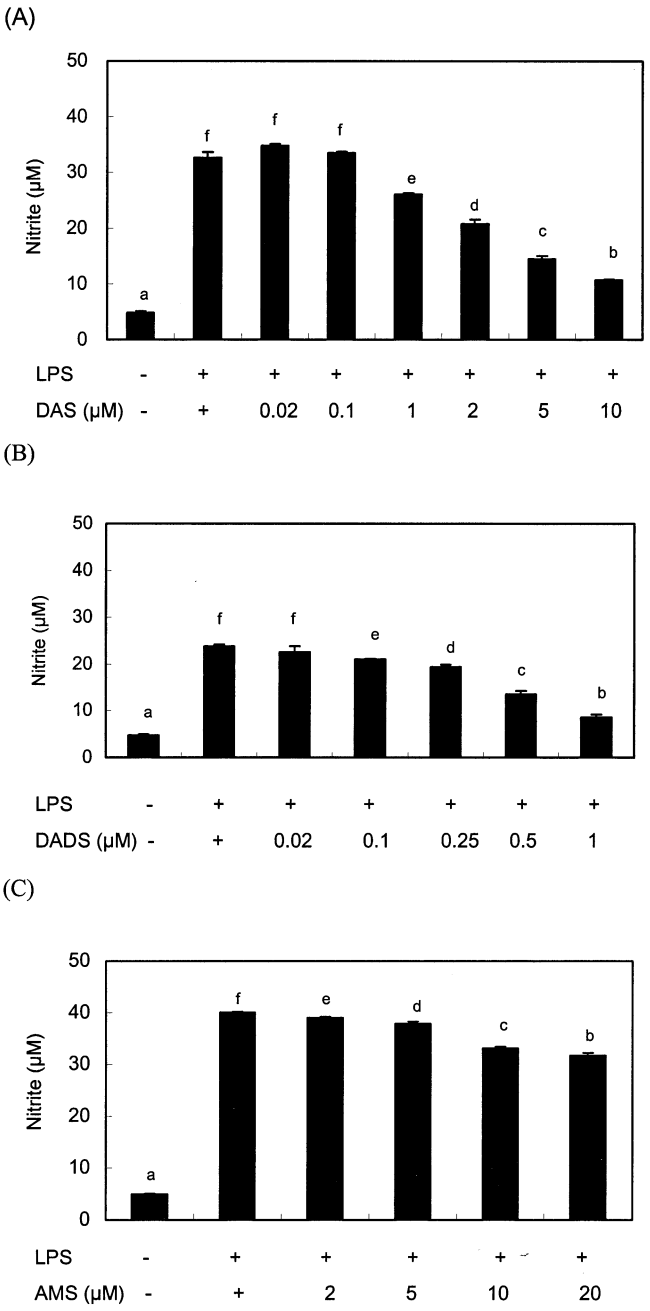

Fig. 1. Effects of DAS (A), DADS (B), and AMS (C) on LPS-activated nitric oxide released into the medium by RAW 264.7 cells. Cells were cotreated with LPS (330 ng/mL) and various concentrations of DAS, DADS, and AMS for 24 h. The nitrite content in the medium was then determined as described in MATERIALS AND METHODS. Values represent the mean ± standard deviation from four measurements. Data with different superscripts significantly differ ($P < 0.05$). AMS, allyl methyl sulfide; DADS, diallyl disulfide; DAS, diallyl sulfide; LPS, lipopolysaccharide.

Effects of DAS, DADS, and AMS on stimulated iNOS and COX-2

The effects of DAS, DADS, and AMS on the expression of activated iNOS and COX-2 proteins were examined by western blot analysis. Parallel to the results obtained from NO measurements, levels of stimulated iNOS protein decreased with DAS and DADS but not with AMS treatments (Fig. 3), and DAS showed a more prominent inhibition. Similarly, only DAS suppressed the stimulated COX-2 protein expression, but

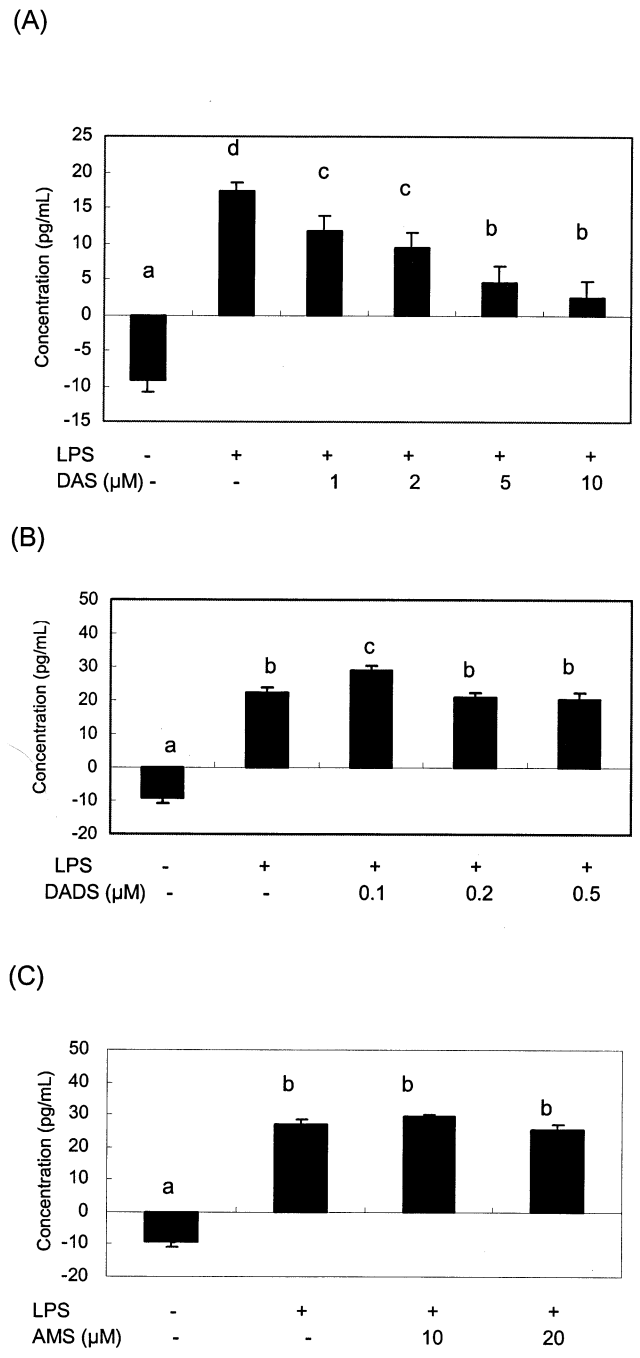

Fig. 2. Effects of DAS (A), DADS (B), and AMS (C) on LPS-activated PGE_2 released into the medium by RAW 264.7 cells. Cells were treated with LPS (330 ng/mL) and various concentrations of DAS, DADS, and AMS for 24 h. The PGE_2 concentration in the medium was then determined with a commercial enzyme-linked immunosorbent assay kit. Values represent the mean ± standard deviation from three measurements. Data with different superscripts significantly differ ($P < 0.05$). AMS, allyl methyl sulfide; DADS, diallyl disulfide; DAS, diallyl sulfide; LPS, lipopolysaccharide; PGE_2, prostaglandin E_2.

neither DADS nor AMS affected activated COX-2 expression. To obtain further insights into the inhibition, DAS was added to LPS-activated cells, and the levels of NO were then examined. The results showed that maximal NO inhibition was achieved in DAS and LPS cotreated cells, whereas less NO suppression was observed in LPS pretreated cells (Fig. 4A). Similar results were obtained for NO production after DADS treatment (Fig. 4B). These results suggest that the NO suppressive effect of DAS and DADS were regulated not only at the levels of translation and/or transcription but also at the level of post-translation. Interestingly, we observed that 6 h of LPS pretreatment gave the highest NO production.

Effects of DAS, DADS, and AMS on NO clearance

Because AMS suppression of NO production was not associated with decreased iNOS expression, and because garlic oil extract has been shown to possess antioxidative activity [8], direct NO clearance was examined. As shown in Fig. 5, all three compounds decreased the levels of NO generated from the NO donor, sodium nitroprusside, indicating that all three compounds interacted with NO and thus decreased its availability. Among these compounds, DAS was the least effective at NO clearance.

Discussion

In the present study, we demonstrate that DAS, DADS, and, to a lesser extent, AMS inhibit NO production by LPS-activated RAW 264.7 cells, and that the suppression induced by DAS and DADS was associated with decreased expression of iNOS protein, inhibited iNOS activity, and direct NO clearance, whereas AMS-induced inhibition was associated only with NO clearance. In addition, only DAS inhibited stimulated PGE_2 production by suppressing the expression of COX-2 protein, whereas DADS and AMS showed no such effect. Previously, Kim et al. [14] reported that garlic extract and its major component, S-allyl cysteine (20~80 μM), inhibit NO production in macrophages stimulated with LPS, interferon-γ, or LPS plus interferon-γ. Dirsch et al. [15] also indicated that allicin (10~100 μM) and ajoene (2.5~20 μM), active components in crushed garlic, can suppress LPS-stimulated NO production. Therefore, we first demonstrated that DAS, DADS, and AMS, major active components in garlic oil, suppress the inflammation- and cancer-associated mediators, NO and PGE_2, at lower concentration ranges (1~20 μM). Sparnins et al. [17] reported the importance of the allyl group in inhibiting tumor formation, and compounds with two allyl groups are more potent than those with one allyl group. Because all of these garlic derivatives that have NO-inhibitory activity consist of a common allyl group, and because DAS and DADS, compounds that contain two allyl groups, are more potent than AMS, which contains only one allyl group, in inhibiting stimulated NO production, the allyl structure of these compounds may contribute to the NO inhibiting effects. However, further investigation is required to identify this possibility.

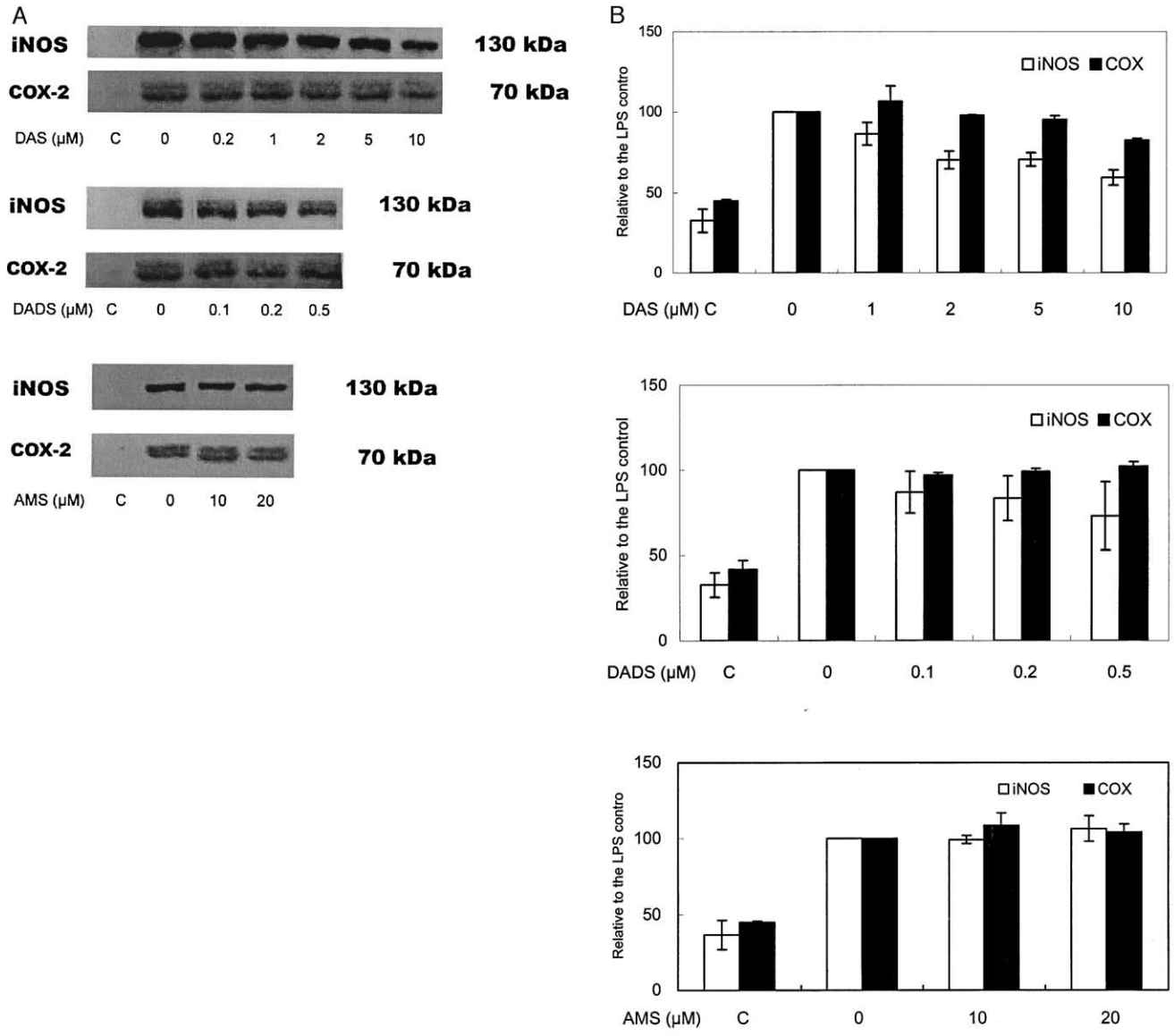

Fig. 3. Effects of DAS, DADS, and AMS on LPS-induced iNOS and COX-2 protein expressions in RAW 264.7 cells. (A) Cells were treated with LPS (330 ng/mL) and various concentrations of DAS, DADS, and AMS, and the cytosol was extracted after 24 h. Ten micrograms of cytosolic protein was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis, and the iNOS and COX-2 proteins were detected. These experiments were repeated two times, and similar results were obtained. (B) Densitometric quantification of the proteins. Values represent the mean ± standard deviation from two measurements. C, Control untreated cells; AMS, allyl methyl sulfide; COX-2, cyclooxygenase-2; DADS, diallyl disulfide; DAS, diallyl sulfide; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide.

Prolonged overproduction of NO and PGE_2 in response to bacterial endotoxin LPS or cytokines plays important roles in inflammation, which is a risk factor for certain cancers [10,11,13]. Large amounts of NO and increased formation of reactive oxygen species, including superoxide, by macrophages can lead to the generation of peroxynitrite ($ONOO^-$), which is a powerful oxidant and may readily decompose to the highly reactive hydroxyl radical (OH^-) and nitrogen dioxide (NO_2^-). These highly reactive and toxic compounds may react with macromolecules, such as proteins, DNA, and RNA, in cells and cause cellular or tissue damage [13]. Further, the increased formation of nitrosamines from the induction of iNOS contributes to the carcinogenic effect of NO [13]. In addition, iNOS and COX-2 are overexpressed in malignant tumors [11,18], and COX-2 inhibitors have been reported to possess antitumor activity [19]. Hence, compounds such as DAS, DADS, and AMS that suppress the overproduction of NO and PGE_2 may play protective roles in the development of inflammation and carcinogenesis.

DAS mainly inhibited stimulated NO and PGE_2 production through suppression of the expressions of iNOS and

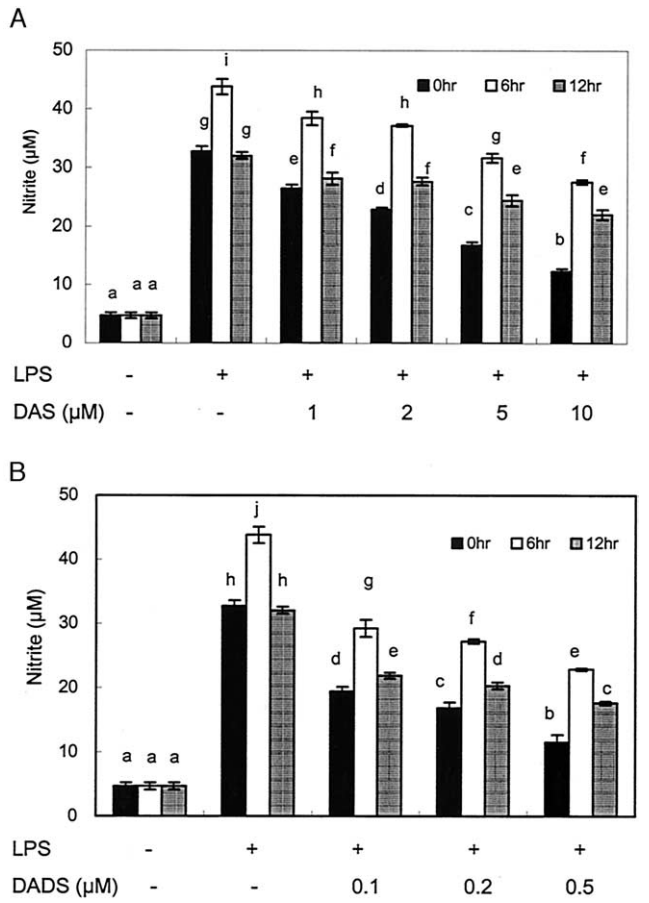

Fig. 4. Effects of DAS (A) and DADS (B) on nitric oxide production in LPS-pretreated RAW 264.7 cells. Cells were pretreated with LPS (330 ng/mL) for 0, 6, or 12 h, followed by treatment with various concentrations of DAS and DADS in the absence of LPS for up to 24 h. Nitrite content in the medium was then determined as described in MATERIALS AND METHODS. Values represent the mean ± standard deviation from four measurements. Data with different superscripts significantly differ ($P < 0.05$). DADS, diallyl disulfide; DAS, diallyl sulfide; LPS, lipopolysaccharide.

COX-2 proteins, inhibition of iNOS activity, and to a lesser extent through its NO clearance activity. Our results are consistent with other observations that demonstrate that iNOS and COX-2 proteins are coregulated because both proteins are induced by the activation of nuclear factor-κB (NF-κB) [20]. Various phytochemicals, including diterpenes [21], curcumin [22], resveratrol, and isothiocyanate [20,23] have been demonstrated to act through suppression of NF-κB activation to decrease iNOS and COX-2 expression. NF-κB is a transcription factor that controls transcription of a variety of inflammation-associated genes including *inos* and *cox-2*. In addition, NF-κB can be activated by oxidative stress and modulated by antioxidants [24], which may decrease intra- and extracellular oxidative stresses and thus inhibit nuclear NF-κB activation. Because garlic powder extract–treated human blood has been shown to decrease NF-κB activity in human embryonic kidney HEK 293 cells [25] and because DAS possesses antioxidant activity [26], DAS may act through suppression of NF-κB activation to decrease expressions of iNOS and COX-2. However, this possibility needs to be further investigated. In contrast, DADS and AMS inhibited LPS-activated NO production but showed no effect on stimulated PGE_2 production. Compared with DAS, DADS and AMS demonstrated higher efficiencies of NO clearance, so NO inhibition by DADS and AMS may be explained by their direct interaction with NO. This observation is consistent with several other studies that showed that DAS, DADS, and AMS possess antioxidant activities [26,27] because NO is a small radical molecule. In addition, DADS inhibited the expression of iNOS protein and acted similarly to DAS in the downregulation of NO production but showed no effect on COX-2 expression or PGE_2 production. Different cytokines are able to modulate the expressions of iNOS and COX-2, and it has been shown that DADS can modulate LPS-induced production of cytokines [25], so the equilibrium between different cytokines may explain the differential regulation of iNOS and COX-2 by DADS.

According to these results, garlic may have the potential to block the adverse effects evoked by excess NO production. One gram of crushed garlic would provide a dose of approximately 2 to 6 mg of allicin [28], the precursor of DADS, DAS, and AMS. Ideally, without considering digestion and absorption, this dose of allicin would produce approximately 1.8 to 5.4 μM of plasma DADS concentration in a 70-kg person based on a blood volume in the adult human that comprises 7% of body weight. This concentration of DADS would inhibit LPS-induced NO production. However, higher plasma concentration may be achieved by taking garlic supplements that contain larger amounts of these derivatives. Therefore, usual dietary consumption of garlic or its supplements would be beneficial in terms of its inhibitory effects on excess of NO and PGE_2 production.

In summary, we have demonstrated that DAS, DADS, and, to a lesser extent, AMS, derived from garlic oil, differentially regulate NO and PGE_2 production in mouse

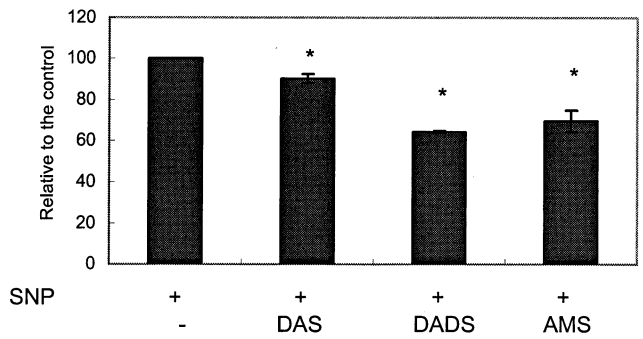

Fig. 5. Effects of DAS, DADS, and AMS on nitric oxide clearance. DAS (10 μM), DADS (0.2 μM), and AMS (20 μM) were incubated in a 10-mM sodium nitroprusside solution for 60 min, and the nitrite content in the reaction mixture was then determined using the Griess reagent. *Significantly different from the control group ($P < 0.05$). AMS, allyl methyl sulfide; DADS, diallyl disulfide; DAS, diallyl sulfide; SNP, sodium nitroprusside.

RAW 264.7 macrophages stimulated by LPS. DAS inhibits activated NO and PGE_2 production mainly by inhibiting the expressions of iNOS and COX-2 proteins and partly by iNOS enzyme inhibiting and NO clearance. DADS suppresses stimulated NO production by inhibiting iNOS expression and clearing NO, whereas AMS inhibits activated NO production mainly by NO scavenging. In addition, neither DADS nor AMS showed any effect on LPS-activated PGE_2 production. Various pathways have been suggested for the anticarcinogenic activity of garlic oil, including modulation of xenobiotic-metabolizing enzyme activities [17,29,30], inhibition of DNA adduct formation [31], induction of apoptosis [32], modulation of immune functions [5], and antioxidation [8]. Therefore, the results obtained from this study provide an alternative protective mechanism of garlic oil and provide information on the potential use of garlic or its supplements in chemoprevention or in pathologic conditions related to overproduction of NO and PGE_2.

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