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RESEARCH ARTICLE

Diallyl disulfide and diallyl trisulfide protect endothelial nitric oxide synthase against damage by oxidized low-density lipoprotein

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Garlic is viewed as an effective health food against atherosclerosis. In this study, we examined whether diallyl disulfide (DADS) and diallyl trisulfide (DATS) protect endothelial nitric oxide synthase (eNOS) activation against oxidized LDL (ox-LDL) insult and through what mechanism. We found that DADS and DATS reversed the suppression of eNOS Ser1177 phosphorylation by ox-LDL, and wortmannin abolished the reversal by DADS and DATS. Similarly, the inhibition of cellular cGMP and nitric oxide production by ox-LDL was reversed by DADS and DATS ($p<0.05$). This increase in nitric oxide bioavailability by the allyl sulfides was attenuated by wortmannin. Immunoprecipitation assay revealed that DADS and DATS preserved the interaction of eNOS with caveolin-1 in the membrane. In addition, DADS and DATS suppressed the reduction of the cellular eNOS protein content by ox-LDL. When cycloheximide was added to block protein synthesis, DADS and DATS suppressed eNOS protein degradation similarly to that noted by MG132. Ox-LDL increased chymotrypsin-like proteasome activity, and this increase was inhibited by the allyl sulfides and MG132 ($p<0.05$). These results suggest that DADS and DATS protect eNOS activity against ox-LDL insult. This protection can be attributed partly to their mediation of phosphatidylinositol 3-kinase/protein kinase B signaling and prevention of eNOS degradation.

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1 Introduction

Atherosclerosis is a common vascular disease worldwide that leads to alterations and lesions in the inner walls of the blood vessels [1]. Although its etiology is multifactorial, endothelial dysfunctions, especially those elicited by oxidized low-density lipoprotein (ox-LDL), play a critical role in the pathogenesis of atherosclerosis [2, 3]. Ox-LDL promotes vascular dysfunction by exerting direct cytotoxicity on endothelial cells [4] and also by enhancing the production of inflammatory mediators, including pro-inflammatory cytokines, reactive oxygen species and arachidonic acid metabolites [5, 6]. Moreover, ox-LDL inhibits endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production, leading to interruption of NO-mediated responses in endothelial cells [7, 8]. Recently, it was reported that ox-LDL damage to NO production can also result partly

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Abbreviations: AMC, aminomethyl coumarin; **DAF-2 DA,** 4,5-diaminofluorescein diacetate; **DAS,** diallyl sulfide; **DADS,** diallyl disulfide; **DATS,** diallyl trisulfide; **Hsp90,** heat shock protein 90; **HUVEC,** human umbilical vein endothelial cells; **eNOS,** endothelial nitric oxide synthase; **NO,** nitric oxide; **ox-LDL,** oxidized LDL; **PI3K,** phosphatidylinositol 3-kinase; **PKB,** protein kinase B

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by the downregulation of cellular eNOS *via* the ubiquitin-proteasome pathway [9, 10]. Dysregulation of NO production has been demonstrated to be involved not only in the development of atherosclerosis, but also in the pathogenesis of several cardiovascular disorders, including essential hypertension, reperfusion injury and the myocardial depression associated with (septic) shock [11].

eNOS is a peripheral membrane protein in endothelial cells because of its co-translational *N*-myristoylation and post-translational cysteine palmitoylation [12]. Membrane-associated eNOS is localized in a specialized membrane structure called caveolae and is directly bound to caveolin [13]. NO plays an important role in maintaining vessel functions, including vascular tone, platelet aggregation, smooth muscular proliferation and leukocyte adhesion to endothelial cells [14–16]. For well-controlled normal NO production, eNOS activity is highly regulated by post-translational modifications, such as phosphorylation/dephosphorylation and protein–protein interactions [17]. Phosphorylation at Ser 1177 by protein kinase B (PKB) activates eNOS [18]. Binding to caveolin-1 and heat shock protein 90 (Hsp90) ensures recruitment of activated PKB to the eNOS-Hsp90-caveolin-1 activation complex and, thus, leads to the phosphorylation of eNOS [19, 20]. Disruption of the caveolin–eNOS activation complex and its association with PKB by ox-LDL deactivates eNOS [21].

Caveolae are flask-shaped vesicular invaginations in the plasma membrane that are considered to be a subset of lipid rafts [22]. They are enriched in cholesterol, sphingomyelin, glycolipids, caveolins and signal-transducing molecules such as eNOS and phosphatidylinositol 3-kinase (PI3K) and lipoprotein receptors such as SR-BP and CD36 [23–27]. Caveolin-1, -2 and -3 are caveolae-coat-associated proteins with a molecular weight of ~22 kDa. Caveolin-1 is ubiquitously expressed and abundant in endothelial cells and is the first caveolin recognized to be important in trafficking cholesterol between the endoplasmic reticulum and the plasma membrane [28]. Later, caveolin-1 was shown to be a negative regulator of eNOS enzymatic activity by reversibly keeping the enzyme in an inactive state [29]. Agonists that increase the intracellular Ca^{2+} concentration, such as bradykinin, promote eNOS dissociation from caveolin-1 and, thus, result in eNOS activation and translocation to sites within the cytosol [30]. The cycle is reversed when the intracellular Ca^{2+} level returns to baseline. These findings support the idea that the interaction between eNOS and caveolin-1 in membrane caveolae is the crucial step for activation of the enzyme.

Garlic (*Allium sativum* L.) has been used as a flavoring and medicinal agent for hundreds of years in many countries. It possesses diverse biological activities, including hypolipidemic, antitumorigenesis, antidiabetes, antioxidation, hepatic protection, antithrombosis and immune modulation effects [31, 32]. Evidence indicates that such health-related functions of garlic can be attributed to its rich content of various organosulfur compounds, including allicin, diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), and *S*-allyl cysteine [33]. In addition to its well-documented hypolipidemic and antithrombotic effects, garlic modulation of eNOS activity accounts for, at least in part, its anti-atherogenic effect. In an *in vivo* study, aged garlic extract rapidly increased NO production about 30–40% in mice; NO production then returned to the basal value at 2 h after administration [34]. *S*-allyl cysteine, the most abundant water-soluble organosulfur compound identified in aged garlic, increased NO bioavailability in human umbilical vein endothelial cells (HUVEC) as judged by measuring cGMP production [35]. However, the actual working mechanism of the effect of garlic and its organosulfur compounds on eNOS activation remain to be elucidated.

In this study, we hypothesized that garlic protection against ox-LDL-induced endothelium damage is possibly working through the eNOS activation associated with caveolin-1 and the PI3K/PKB dependent pathway. To test this hypothesis, we tested the effect of three garlic allyl sulfides, DAS, DADS and DATS, on eNOS and PKB activation and on the association of eNOS and caveolin-1 in ox-LDL-treated HUVEC. Moreover, we examined whether allyl sulfides attenuated the eNOS protein degradation induced by ox-LDL.

2 Materials and methods

2.1 Chemicals

DAS and DADS were purchased from Fluka Chemical (Buchs, Switzerland). DATS was purchased from LKT Laboratories (St. Paul, MN, USA). Medium 199 was from Gibco-BRL (Grand Island, NY). Polyclonal antibodies to PKB, phospho-PKB (Ser473), eNOS, and phospho-eNOS (Ser1177) were obtained from Cell Signaling Technology (Beverly, MA, USA) and Upstate Cell Signaling Solutions (New York, NY, USA). Polyclonal antibody to caveolin-1 was purchased from Chemicon International (Temecula, CA, USA). Monoclonal antibody to Hsp90 was purchased from StressGen Biotechnologies. (BC, Canada). HEPES, heparin, $CuSO_4$, actinomycin D, cycloheximide, wortmannin, and actin antibody were obtained from Sigma (St. Louis, MO, USA). Z-Leu-Leu-Leu-CHO (MG132) was purchased from Boston Biochem (Cambridge, MA, USA). Fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) was purchased from Calbiochem (San Diego, CA, USA).

2.2 LDL isolation and ox-LDL preparation

Blood was collected from healthy volunteers to isolate LDL. Written informed consent as approved by the Review Board for Human Research of the Chung-Shan Medical University

was signed by all participants. Plasma in the presence of EDTA was used to isolate LDL by sequential ultracentrifugation ($1.019 < d < 1.063$ kg/L) [36]. Afterward, native LDL was dialyzed at 4°C for 48 h against 500 volumes of PBS to remove EDTA. To initiate oxidation, LDL in an amount of 0.5 g/L protein was exposed to 5 μM $CuSO_4$ for 18 h. The generation of thiobarbituric acid-reactive substances was monitored by the fluorometric method as described by Fraga *et al.* [37], and the values of malondialdehyde equivalents increased from 0.76 ± 0.21 nmol/mg protein of native LDL to 24.3 ± 2.6 nmol/mg protein of $CuSO_4$-treated LDL. The freshly prepared ox-LDL was dialyzed at 4°C for 48 h against 500 volumes of PBS to remove Cu^{2+} and was sterilized by passage through a 0.2-μm filter. The protein contents of native LDL and the ox-LDL preparations were measured by the Lowry assay [38].

2.3 Cell treatments

HUVEC (Clonetics, San Diego, CA, USA) with passages between 7 and 9 were used in this study. Cells were grown in 10 mL of medium 199 supplemented with 20 mM HEPES (pH 7.4), 30 mg/L endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY, USA), 100 mg/L heparin, 20% fetal bovine serum (Biological Industries, Canada), 100 000 U/L penicillin, and 100 mg/L streptomycin at 37°C under 5% CO_2. For each experiment, HUVEC at 80% confluence were incubated with DADS or DATS at the indicated concentrations for 16 h and were then stimulated with 40 mg/L of ox-LDL for an additional 24 h. For the eNOS protein stability assay, cells were pre-incubated with DADS or DATS for 8 h followed by loading with 3.5 μM cycloheximide for an additional 8 h. Cells were then treated with ox-LDL for 12 h. For the eNOS mRNA stability assay, HUVEC were incubated with various concentrations of garlic allyl sulfides in the absence or presence of 1 mg/L actinomycin D for 4 h and were then stimulated with ox-LDL for an additional 20 h.

2.4 Western blot analysis

Cells were lyzed in a lysis buffer (10 mM Tris-HCl, pH 8, 0.1% Triton X-100, 320 mM sucrose, 5 mM EDTA, 1 mM PMSF, 1 mg/L aprotinin, 1 mg/L leupeptin, 1 mM dithiothreitol). The cell lysates were then sonicated at 20 W for 15 s in ice bath. All of the cell extracts were centrifuged at $20\,000 \times g$ at 4°C for 15 min. The supernatants were recovered and the total protein was analyzed by use of the Commassie Plus protein assay reagent kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of cellular proteins were then electrophoresed in an SDS-polyacrylamide gel, and proteins were then transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ, USA). Nonspecific binding sites on the membranes were blocked with 5% nonfat milk at 4°C overnight. Membranes were probed with rabbit anti-PKB, phospho-PKB, eNOS, phospho-eNOS, and β-actin antibody or mouse anti-Hsp90 antibody. The membranes were then probed with their respective secondary antibody labeled with horseradish peroxidase. The bands were visualized by using an enhanced chemiluminescence kit (Perkin Elmer Life Science, Boston, MA, USA) and were quantitated with an AlphaImager 2000 (Alpha Innotech, San Leandro, CA, USA).

2.5 Immunoprecipitation

The membrane fractions were prepared by use of a compartmental protein extraction kit (Chemicon International). The membrane proteins (600 μg) were incubated with 5 μg of anti caveolin-1 antibody overnight at 4°C. Then, 20 μL protein-A Sepharose was added and allowed to react by vortexing for 2 h at 4°C. Immunoprecipitates were collected by centrifugation, washed three times with immunoprecipitation buffer (40 mM Tris-HCl, pH 7.5, containing 1% NP-40, 150 mM NaCl, 5 mM EGTA, 1 mM DTT, 1 mM PMSF, 20 mM NaF, 1 mg/L aprotinin, 1 mg/L leupeptin, and 1 mM sodium vanadate), and subjected to SDS-PAGE.

2.6 RT-PCR

Total RNA of HUVEC was extracted by using Trizol reagent (Life Technologies, Rockville, MD, USA). Four micrograms of total RNA was used for the synthesis of first-strand cDNA by using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) in a 20 μL final volume containing 250 ng oligo-dT and 40 units RNase inhibitor. PCR was carried out in a thermocycler in a 50 μL reaction volume containing 20 μL of cDNA, BioTaq PCR buffer, 50 μM of each dNTP, 1.25 mM $MgCl_2$, and one unit of BioTaq DNA polymerase (BioLine, Randolph, MA, USA). Oligonucleotide primers of eNOS (forward: 5′-CAAGGGCACCGGCATCACCA-3′, reverse: 5′-CGCCG-CCAAGAGGACACCAGT-3′), caveolin-1 (forward: 5′-CCT-CCTCACAGTTTTCATCC-3′, reverse: 5′-CAATCACATCTT-CAAAGTCAATC-3′), and GAPDH (forward: 5′-GAGT-CAACGGATTTGGTCGT-3′, reverse: 5′-TTGATTTTGGA-GGGATCTCG-3′) were designed on the basis of published sequences [30]. Amplification was performed under standard conditions: denaturation at 94°C for 1 min, 32 cycles of amplification with annealing at 50°C for 1 min, and extension at 72°C for 1 min. PCR products were resolved in a 1%-agarose gel and were scanned by using a Digital Image Analyzer (AlphaImager 2000, Alpha Innotech).

2.7 Measurement of proteasome activity

The chymotrypsin-like activity of the proteasome was assessed in cell lysates by using the synthetic peptide substrate Suc-Leu-Leu-Val-Tyr linked to the fluorometric

reporter aminomethyl coumarin (AMC) as described previously [39]. Cell lysates prepared as described for Western blot analysis were incubated for 20 min in a reaction mixture containing an ATP regenerating system (50 mM Tris-HCl, pH 8.2, 18 mM KCl, 3 mM $Mg(CH_3COO)_2$, 3 mM $MgCl_2$, 1.1 mM DTT, 6 mM ATP, 5 mM phosphocreatine, 0.2 U phosphocreatinkinase) and 0.2 mM Suc-Leu-Leu-Val-Tyr AMC. AMC hydrolysis was quantified in an F-2000 fluorescence spectrofluorometer (Hitachi, Tokyo, Japan) with 355 nm excitation and 460 nm emission wavelengths. Enzymatic activity was normalized to protein concentration and was expressed as percent activity of control lysates.

2.8 NO measurement by confocal microscopy

Cellular NO production was assessed by using the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2 DA; Sigma) [40]. Once membrane-permeable DAF-2 DA is loaded into cells, intracellular esterase hydrolyzes the ester bond, which releases the fluorescent product DAF-2. This method is useful for real-time bioimaging of NO production in living cells with fine temporal and spatial resolution. HUVEC were seeded on gelatin-coated two-well chamber slides (Lab-Tek™ II Chamber Slide™ System; NUNC, Denmark). After treatment, cells were loaded with 3 μM DAF-2 DA for 30 min at 37°C. Then, the cells were fixed in 2% paraformaldehyde for 5 min at 4°C. To visualize DAF-2 DA fluorescence, the fixed cells were examined under a Zeiss Axiovert 510 Meta inverted confocal microscope (Carl Zeiss, Germany) with a ×10 objective lens, using an FL bulb and fluorescein isothiocyanate filters with a peak excitation wavelength of 494 nm and a peak emission wavelength of 517 nm.

2.9 Intracellular cGMP production

HUVEC were pretreated with each of the garlic allyl sulfides for 16 h and were then exposed to ox-LDL for an additional 24 h as stated above. At the end of treatment, HUVEC were placed on an ice bath and incubated for 60 min with 0.1 N HCl. Samples were prepared according to the manufacturer's instructions, and intracellular cGMP concentrations were measured by using the cGMP EIA kit obtained from Cayman Chemical (Ann Arbor, MI, USA).

2.10 Statistical analysis

Values are expressed as means ± SD ($n = 3$–5). Statistical analysis was performed with commercially available software (SAS Institute, Cary, NC, USA). Data were analyzed by means of one-way ANOVA, and the significant difference among treatment means was assessed by use of Tukey's test. A value of $p < 0.05$ was considered to be significant.

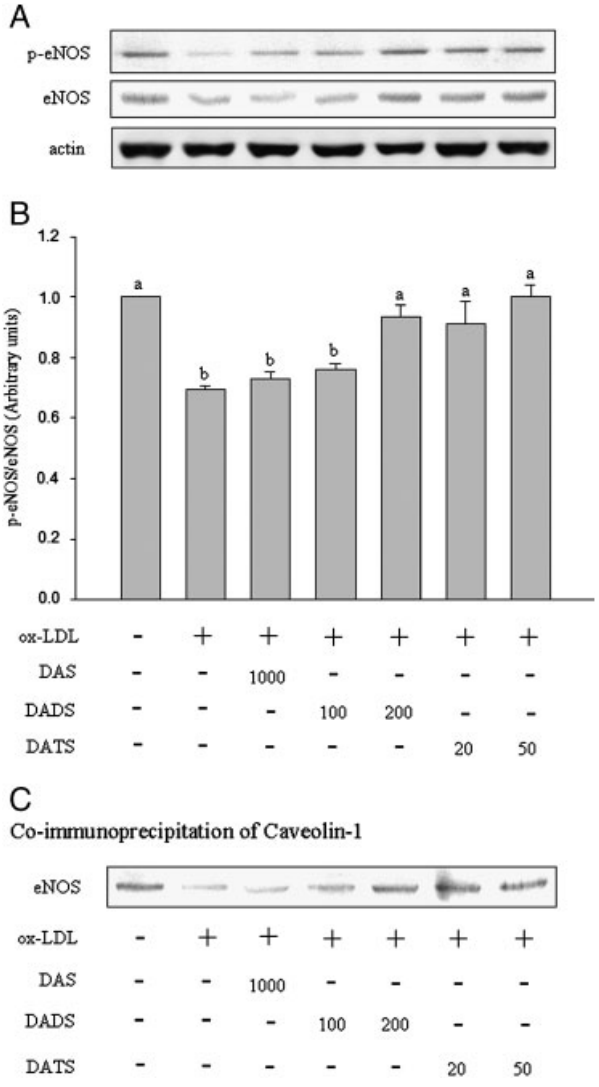

Figure 1. DADS and DATS increase eNOS phosphorylation and eNOS-caveolin-1 interaction in HUVEC exposed to ox-LDL. Cells were treated with DAS (1000 μM), DADS (100, 200 μM) or DATS (20, 50 μM) for 16 h and were then incubated with 40 mg/L ox-LDL for an additional 24 h. (A) Equal amounts of proteins (30 μg) were separated by 7.5% SDS-PAGE and were transferred to PVDF membranes for determination of total and phosphorylated eNOS by immunoblotting. (B) Protein bands of total and phosphorylated eNOS in each sample were quantified by densitometry and are expressed as the *p*-eNOS/eNOS ratio. The level in control cells was set as 1. Values are means ± SD ($n = 3$). ^{ab}Means without a common letter differ significantly, $p < 0.05$. (C) eNOS associated with caveolin-1 in the cellular membrane was examined by immunoprecipitation with an antibody to caveolin-1 followed by immunoblotting for eNOS.

3 Results

3.1 eNOS phosphorylation and interaction with caveolin-1

We used an immunoblot assay to determine whether garlic allyl sulfides protect eNOS activation against ox-LDL insult. With ox-LDL alone, both eNOS phosphorylation and total eNOS content were decreased compared with that in the control cells (Fig. 1A). With DADS and DATS pretreatment, the decrease in eNOS phosphorylation and total eNOS was reversed, which suggested that the two allyl sulfides may act not only on enzyme activation but also on protein preservation. DAS even showed minor protection at a concentration of 1000 μM. When we further expressed the changes in eNOS phosphorylation as the ratio of *p*-eNOS/total eNOS, the results revealed that ox-LDL resulted in a 30% decrease in phosphorylation (Fig. 1B). This decrease was dose-dependently reversed by pretreatment with DADS and DATS. The potency of protection among the three allyl sulfides examined was such that allyl sulfides with more sulfur atom numbers displayed greater protection, *i.e.* in the order of DATS > DADS > DAS.

eNOS activation is known to be dependent on the interaction of eNOS with caveolin-1 in membrane caveolae. To examine whether caveolin-1/eNOS colocalization was affected by DADS and DATS, we performed an immunoprecipitation assay. As shown in Fig. 1C, the amount of eNOS associated with membrane caveolin-1 apparently decreased with ox-LDL treatment. In the presence of garlic allyl sulfides, however, caveolin-1-associated eNOS was preserved. DAS showed no effect on eNOS interaction with caveolin-1.

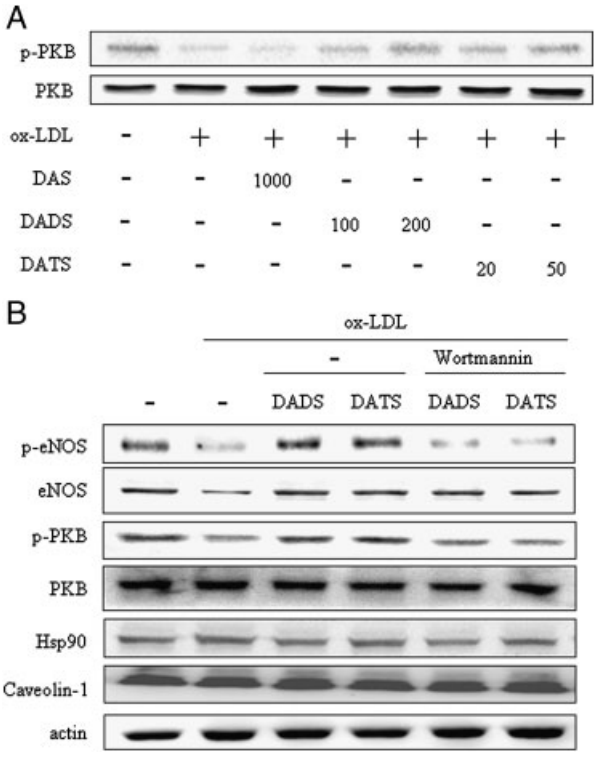

Figure 2. PI3K/PKB pathway in eNOS phosphorylation. Cells were preincubated with various concentrations of DAS, DADS or DATS for 16 h and were then treated with 40 mg/L ox-LDL for an additional 24 h. Total and phosphorylated PKB was measured by immunoblotting (A). For inhibitor treatments, wortmannin (100 nM) was added 1 h before the addition of 200 μM DADS or 50 μM DATS. Again, eNOS, PKB, caveolin-1 and Hsp90 were determined by immunoblotting (B).

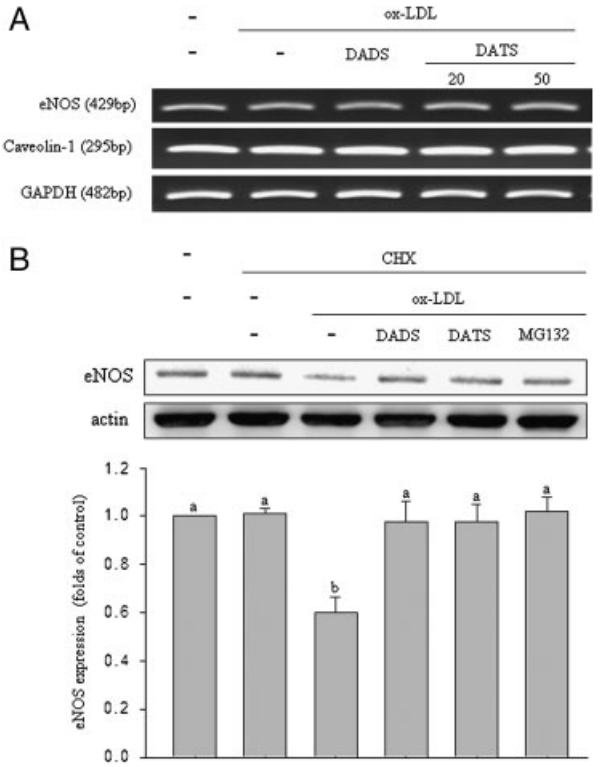

Figure 3. Effect of DADS and DATS on eNOS mRNA stability and protein degradation. (A) HUVEC were incubated with 1 mg/L actinomycin D in the absence or in the presence of 200 μM DADS or 20 or 50 μM DATS for 4 h and were then stimulated with ox-LDL for an additional 20 h. The expression of eNOS, caveolin-1 and GAPDH mRNA was analyzed by RT-PCR. (B) For the eNOS protein degradation assay, endothelial cells were incubated in the absence or presence of 200 μM DADS or 50 μM DATS for 8 h before loading with 3.5 μM cycloheximide (CHX) for an additional 8 h. Cells were then treated with ox-LDL for 12 h. Proteasome inhibitor MG132 was added 1 h before ox-LDL treatment. Equal amounts of proteins (30 μg) were separated by electrophoresis and were immunostained as described in Section 2. Relative changes in eNOS protein expression were measured from three independent experiments. The level in control cells was regarded as 1. [ab]Means without a common letter differ significantly, $p < 0.05$.

3.2 PKB and eNOS phosphorylation

With ox-LDL alone, PKB phosphorylation was suppressed (Fig. 2A). When the cells were pretreated with DADS and DATS, the suppression of PKB phosphorylation by ox-LDL was dose-dependently reversed. DAS did not change the phosphorylation/dephosphorylation state of PKB.

To verify whether the PI3K/PKB signaling pathway was involved in the protection of eNOS phosphorylation at Ser 1177, we tested the PI3K inhibitor wortmannin. In the presence of wortmannin, the DADS and DATS recovery of PKB phosphorylation disappeared (Fig. 2B). In parallel, the DADS and DATS protection of eNOS phosphorylation at Ser 1177 was blocked by wortmannin. The expression of Hsp90 and caveolin-1 in HUVEC was not changed by either of the allyl sulfides or of the PI3K inhibitor.

3.3 eNOS mRNA stability and protein degradation

As stated above, in addition to modulating eNOS activation, DADS and DATS could effectively preserve cellular eNOS contents in the presence of ox-LDL, suggesting that garlic allyl sulfides may also act on the stage of eNOS transcription and protein turnover in endothelial cells. To determine whether mRNA stability was affected, we assayed eNOS mRNA in the presence of actinomycin D. RT-PCR revealed that eNOS mRNA levels were not changed by ox-LDL whether in the absence or in the presence of allyl sulfides (Fig. 3A). A similar finding with no changes in eNOS

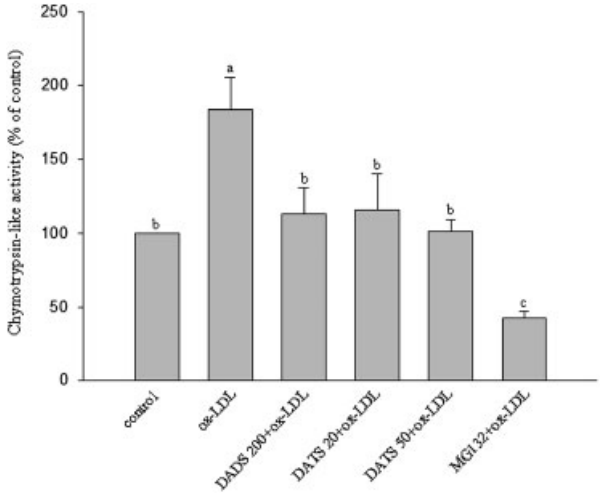

Figure 4. Proteasome activity in HUVEC. Cells were pretreated with 200 μM DADS or 20 or 50 μM DATS for 16 h and were then incubated with 40 mg/L ox-LDL for an additional 24 h. MG132 was added 1 h before ox-LDL treatment. Chymotrypsin-like proteasome activity was measured by using the synthetic peptide substrate Suc-Leu-Leu-Val-Tyr AMC. Values represent means ± SD ($n = 5$). [abc]Values without a common letter differ significantly, $p < 0.05$.

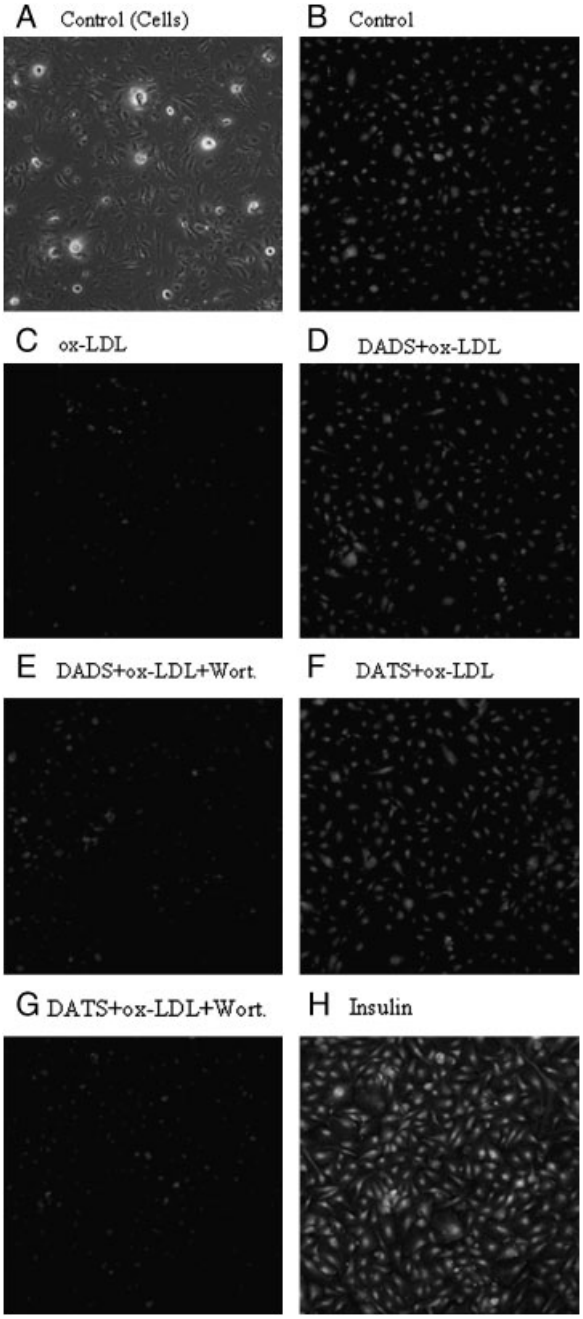

Figure 5. DADS and DATS protect NO production in ox-LDL-treated endothelial cells. HUVEC were preincubated with 200 μM DADS or 50 μM DATS for 16 h and were then stimulated by ox-LDL for an additional 24 h. Wortmannin (Wort., 100 nM) was added 1 h before allyl sulfide treatment. After treatment, cells were loaded with 3 μM DAF-2 DA for 30 min. Then, cells were fixed and the production of cellular NO was examined by using a Zeiss Axiovert 517 Meta inverted confocal microscope. Emission of green fluorescence (517 nm) from cells excited at 494 nm is indicative of NO production (B–G). The cell morphology of control cells was also photographed (A). Cells treated with insulin (100 nM) were regarded as a NO positive control (H).

mRNA among treatments was also noted in the absence of actinomycin D (data not shown). These results suggest that the differences in eNOS protein induced by ox-LDL and DADS and DATS were not likely to work at the level of gene transcription or transcript stability.

Next, cycloheximide was applied to HUVEC, and changes in eNOS protein level were determined. Cycloheximide alone did not change the total eNOS level in HUVEC (Fig. 3B). In the presence of ox-LDL, eNOS protein decreased by 41% ($p<0.05$). With DADS and DATS pretreatment, the decrease in eNOS content by ox-LDL was attenuated. The protection of the eNOS protein level by the two allyl sulfides against ox-LDL damage was similar to that noted in cells treated with the proteasome inhibitor MG132.

3.4 Chymotrypsin-like activity of proteasomes

Compared with that in the control cells, the chymotrypsin-like proteasome activity was significantly increased by ox-LDL ($p<0.05$) (Fig. 4). DADS at 200 μM and DATS at 20 and 50 μM significantly blocked this induction in chymotrypsin-like proteasome activity and reversed the enzyme activity to a level similar to that noted in control cells. In cells treated with MG132, chymotrypsin-like activity was only 23% of that in cells treated with ox-LDL alone.

3.5 NO production in HUVEC

Cellular NO production was measured by DAF-2 DA fluorescence with confocal microscopy. Higher fluorescence intensity indicates more NO production in cells. The results clearly showed that the cellular NO level in ox-LDL-treated endothelial cells was lower than that in the controls (Fig. 5B and C). With DADS and DATS pretreatment, NO production was reversed to a level similar to that in control cells (Figs. 5D and F). In the presence of wortmannin, this recovery of NO production by the garlic allyl sulfides was attenuated (Figs. 5E and G). These data strongly support the conclusion that DADS and DATS help to maintain eNOS activity in endothelial cells exposed to ox-LDL, leading to normal NO production.

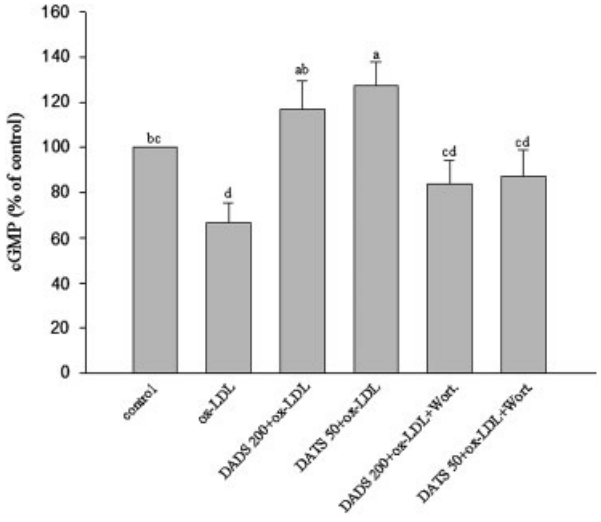

Figure 6. Recovery of cGMP production by garlic allyl sulfides in HUVEC exposed to ox-LDL. Cells were treated with garlic allyl sulfides, ox-LDL, and wortmannin (Wort.) as stated in Fig. 5. Cellular cGMP was determined by ELISA. Values represent means ± SD ($n=3$). [abcd]Values without a common letter differ significantly, $p<0.05$.

3.6 Cellular cGMP levels

Finally, we determined NO bioavailability by measuring cGMP production. Compared with that in control cells, ox-LDL caused a significant decrease in cGMP production in HUVEC by 33.5% ($p<0.05$) (Fig. 6). With 200 μM DADS or 50 μM DATS pretreatment, however, the cGMP content increased. In the presence of wortmannin, this recovery of cGMP production by DADS and DATS disappeared.

4 Discussion

Garlic consumed fresh or in preparations as aged garlic extract, garlic powder or steam-distilled garlic oil has been reported to display numerous biological activities, although the different preparations posses different active organosulfur constituents [41]. Alliin and *S*-allyl cysteine are the two most well studied representatives of garlic powder and aged garlic extract, respectively. More than a dozen volatile allyl sulfides have been identified in garlic oil, of which DADS and DATS are two of the most abundant. DADS and DATS are estimated to account for approximately 65–70% of the total sulfur-containing compounds in garlic oil [42, 43]. Although the protection provided by garlic in clinical trials remains controversial, the results of epidemiologic and *in vitro* studies suggest that, as a result of its hypolipidemic, anti-thrombosis, antioxidative and anti-inflammatory properties, garlic and its organosulfur compounds, including alliin, alliin-derived compounds and *S*-allyl cysteine, reduce the progression of cardiovascular disease [44, 45]. In a previous study, we reported that the protection of the vascular endothelium against ox-LDL insult by garlic allyl sulfides can be explained by the effectiveness of the allyl sulfides in down-regulating the expression of E-selectin and vascular cell adhesion molecule 1, leading to the suppression of leukocyte adhesion to endothelial cells [46]. Although garlic's protection of vessel function has also been attributed to its enhancement of eNOS activity and NO production [35, 47, 48], the actual working mechanism by which garlic and its organosulfur compounds affect endothelium NOS activation remains to be elucidated. In this study, we showed that DADS and DATS can effectively restore NO production

in HUVEC exposed to ox-LDL by enhancing eNOS activation and preventing eNOS degradation. This protection can be attributed in part to mediation of PI3K/PKB signaling and also the proteasome-dependent pathway. Our findings provide insight into the molecular action of DADS and DATS in protecting blood vessels against ox-LDL insult.

Because of the critical role of NO in maintaining vascular tone and the free radical characteristic of NO, abnormalities in production of NO in the endothelium resulting in either a decrease in its bioavailability or an excess production result in vascular dysfunction and accelerate atherosclerosis [49, 50]. For optimal NO production in response to diverse stimuli, endothelial eNOS is precisely regulated by multiple post-translational modifications, including phosphorylation, acylation and protein–protein interactions that act throughout the various stages of the enzyme's lifespan [17]. Inhibition of the PI3K/PKB pathway or mutation of the PKB site on the eNOS protein (at serine 1177) attenuates serine phosphorylation and prevents the activation of eNOS, demonstrating the critical role of PI3K/PKB signaling in modulating eNOS activity [51]. With respect to ox-LDL, it dephosphorylates PKB at Ser473 and in turn suppresses the activation of eNOS [21]. In this study, PKB and eNOS phosphorylation were decreased by ox-LDL (Figs. 1 and 2A) as reported previously. Cellular NO production was, thus, lower than that of control cells (Fig. 5). With DADS and DATS pretreatment, however, the suppression of PKB and eNOS phosphorylation by ox-LDL was abolished, and NO (Fig. 5) and cGMP (Fig. 6) production in endothelial cells was restored. In the presence of the PI3K inhibitor wortmannin; however, the protection of eNOS activation and also the recovery of NO production by DADS and DATS were attenuated (Fig. 2B). Taken together, these results provide strong evidence that the protection of NO production against ox-LDL insult by DADS and DATS is likely through the PI3K/PKB-dependent eNOS pathway.

It is clear that different extracellular stimuli modulate distinct kinase signaling pathways leading to eNOS phosphorylation and dephosphorylation in multiple serine and threonine residues [17]. In addition to PKB, cyclic-AMP-dependent kinase, AMP-dependent protein kinase, and calcium/calmodulin-dependent protein kinase II [17] catalyze Ser 1177 phosphorylation of eNOS. cAMP-mediated signaling pathway has been shown to be inhibited by ox-LDL in isolated arteries and thus interferes with the vessel dilation [52]. Among those kinases mentioned above, cyclic-AMP-dependent kinase and AMP-dependent protein kinase could be activated by garlic aqueous extract and allyl sulfides such as allylsulfinates in platelets and hepatocytes [53, 54]. Taken together, the signaling pathways other than the PI3K/PKB to phosphorylate Ser 1177 of eNOS by DADS and DATS cannot be excluded.

In response to diverse stimuli, the association of eNOS with caveolin-1 and Hsp90 in caveolae is required for PKB-dependent eNOS activation [55]. The impact of ox-LDL on eNOS activation can, thus, be caused by disrupting eNOS interaction with other membrane-associated proteins in caveolae, which leads to eNOS re-localization throughout the cell interior and not at the cell surface near the receptors and kinases that mediate eNOS activation [7, 56]. In an immunoprecipitation assay with caveolin-1, our data clearly showed that ox-LDL decreased eNOS association with membrane caveolin-1 (Fig. 1C). In the presence of garlic allyl sulfides, the reduction in eNOS–caveolin-1 complex formation was attenuated in a dose-dependent manner. This finding suggests that DADS and DATS likely act on the subcellular localization of eNOS in vascular endothelial cells and, thus, protect normal NO production against ox-LDL insult.

In eukaryotic cells, the ubiquitin-proteasome pathway represents the major route for intracellular protein degradation [57, 58]. This process is important for cells to prevent the accumulation of nonfunctional and potentially toxic proteins, such as those modified under oxidative stress. To avoid ox-LDL-induced structural modifications of cell proteins, because altered proteins may impair cell viability, the rate of protein ubiquitination and proteasome activity is increased [10]. In this study, the decrease in eNOS content by ox-LDL was attenuated by the proteasome inhibitor MG132 (Fig. 3B), which supports that ox-LDL activates proteasome activity, leading to enhancement of eNOS degradation, and then impairs NO production. Similar to MG132, DADS and DATS helped to preserve cellular eNOS content in the presence of ox-LDL (Fig. 1A). This finding raised the question of whether the garlic allyl sulfides mediate eNOS gene transcription or eNOS mRNA stability or act on eNOS degradation *via* the ubiquitin-proteasome pathway. The lack of effect of ox-LDL and allyl sulfides on eNOS mRNA levels in the absence (data not shown) or in the presence of actinomycin D (Fig. 3A) excludes the possibility that the changes in eNOS protein content are working at the level of eNOS transcripts. Thus, to further clarify the role of ubiquitin-proteasome, we studied the changes in cellular eNOS content in the presence of cycloheximide. As shown in Fig. 3B, DADS and DATS effectively blocked the decrease in eNOS protein expression by ox-LDL in endothelial cells. Consistent with the changes of eNOS content, an increase in the chymotrypsin-like activity of proteasome by ox-LDL was reversed by DADS and DATS (Fig. 4). These results suggest that suppression of proteasome-mediated eNOS protein degradation is likely to be one of the working mechanisms by which DADS and DATS preserve NO production in endothelium exposed to ox-LDL. Recently, protection of (−)-epicatechin against ox-LDL-elicited eNOS downregulation was attributed to the inhibition of proteasome degradation [9]. To our knowledge, ours is the first report to suggest that the protection of endothelial eNOS by DADS and DATS is related to the ubiquitin-proteasome pathway.

The three volatile allyl sulfides examined in this study differed in their number of sulfur atoms, and our results indicated that their potency in protecting eNOS activation

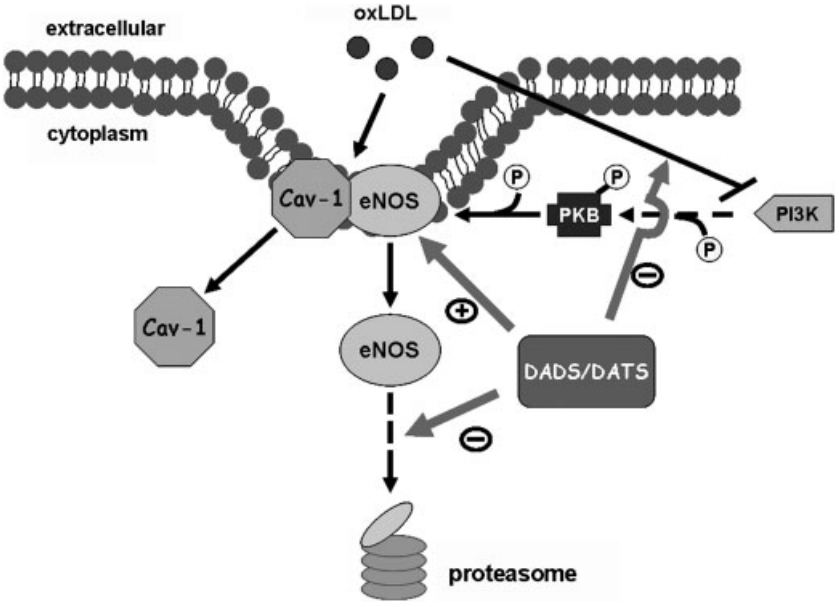

Figure 7. Schematic model of DADS and DATS protection on ox-LDL-induced eNOS damage. In vascular endothelial cells, ox-LDL results in eNOS disassociating from membrane caveolin-1 and increase of proteasome-dependent eNOS degradation. eNOS phosphorylation by PI3K/PKB is also inhibited by ox-LDL. In the presence of DADS and DATS, eNOS–caveolin complex and PI3K/PKB-dependent activation are restored and ox-LDL-induced proteasome-dependent eNOS degradation is inhibited.

was positively related to their number of sulfur atoms. DATS showed the best activity of the three compounds, followed by DADS, and DAS showed only minor protection. Such a structure–function relationship among garlic allyl sulfides was also reported in the upregulation of the π form of glutathione *S*-transferase expression, a phase II detoxification enzyme, in hepatocytes and in the suppression of E-selectin and vascular cell adhesion molecule-1 expression in vascular endothelial cells [46, 59].

Why allyl sulfides with more sulfur atoms had higher activity remains unclear, but their differential reactivity to the protein sulfhydryls is likely an explanation. It has been known for decades that garlic sulfur-containing compounds react to the sulfhydryl group of cysteine [60]. Recently, in HCT-15 and DLD-1 human colon cancer cell lines, DATS at 10 μM was reported to react with Cys-12β and Cys-354β of β-tubulin to form *S*-allylmercaptocysteine, which leads to microtubule network disruption and inhibits cell growth and proliferation [61]. In contrast, such a tubulin modification was not noted by DADS or DAS, even at the higher concentration of 100 μM. In addition to garlic, other organosulfur phytochemicals identified in cruciferous vegetables, *i.e.* sulforaphan and phenethyl isothiocyanate, have also been shown to be able to react to tubulin and other proteins [62, 63]. Although no evidence supports that garlic allyl sulfides directly change protein function by reacting with the cysteine residues of eNOS and PI3K/PKB and also other protein kinases, this possibility cannot be excluded.

From the viewpoint of nutriceutical, it is important to figure out whether the effective concentration of garlic allyl sulfides to maintain eNOS activity and NO production found in this study is achievable *via* the dietary supplement of garlic oil. Given that DATS accounts for 35% of garlic oil and the high absorption of garlic organosulfur compounds [43, 64], we estimated that dietary garlic oil supplementation at 0.8 mg/kg body weight could result in circulating DATS concentration of 20 μM. This means that a 70-kg man requires 56 mg garlic oil/day, corresponding to 19–22 g raw garlic cloves (1 kg raw garlic yields 2.5–3.0 g garlic oil by steam distillation). In a clinical study, Bordia showed 0.25 mg/kg bw garlic oil supplement for 10 months reduced circulating cholesterol and triglycerides in patients with coronary heart disease [65].

In summary, DADS and DATS can effectively attenuate ox-LDL insult on NO production by restoring eNOS–caveolin complex formation and PKB-dependent eNOS activation (Fig. 7). Furthermore, the two allyl sulfides may also exert protection by inhibiting ox-LDL-induced proteasome-dependent eNOS degradation. Our study presents new evidence that elucidates the molecular basis for garlic's protection of eNOS activation and NO production in vascular endothelial cells, and adds to our understanding of how garlic helps to prevent cardiovascular disease.

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