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Diallyl Sulfides (DAS) and Diallyl Disulfides (DADS) Exhibit a Suppressive Effect on the Proliferation and Migration of Vascular Smooth Muscle

− Research Note −

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Abstract

Previous studies report that organo-sulfur compounds derived from garlic inhibited smooth muscle cell (SMC) proliferation and induced apoptosis of cancer cells. Recently, lipid-soluble compounds such as diallyl sulfides (DAS) and diallyl disulfides (DADS) have been reported to more effectively suppress tumor cell proliferation. However, there were few studies on the suppressive effects of lipid-soluble garlic sulfur compounds on the proliferation and migration of vascular smooth muscle cells (VSMC). Therefore, this study investigated the effect of DAS and DADS on VSMC proliferation/migration induced by oleic acid (OA), a principal fatty acid in circulating triglyceride of blood stream. Assays performed include a tetrazole (MTT) assay, a wound healing assay and a Western blots. VSMC proliferations were enhanced by OA in a dose-dependent manner at concentrations of 10~50 μM and inhibited by DAS and DADS compared to non-treated control. OA-induced proliferations were also attenuated by DAS and DADS. OA-induced cell migrations were 2.5 times higher than non-treated control, and they were significantly attenuated by DAS (32% at 150 μM and 50% at 200 μM) and DADS (40% at 150 μM and 46% at 200 μM). OA-induced cell migration was also attenuated by PD98059 (ERK inhibitor), SB203580 (P38 inhibitor) and particularly by LY204002 (PI3K inhibitor) and SP600125 (JNK2 inhibitor). Additionally, Western blot assays showed that OA-induced JNK1/2-phosphorylation was down-regulated after treatment with DAS and DADS. In conclusion, the findings of our study support the idea that DAS and DADS may have a suppressive effect on the proliferation and migration of OA-induced VSMC and that this effect may be partly associated with PI3K and JNK2 pathways.

Key words: diallyl sulfides, diallyl disulfides, vascular smooth muscle cell, proliferation, migration

INTRODUCTION

Enhanced proliferation and migration of vascular smooth muscle cells (VSMC), and their accumulation into the intimal layer of vasculature are one of major process in the pathogenesis of vascular disorders such as atherosclerosis and post-angioplasty restenosis (1-3).

Previous studies reported that garlic-derived organo-sulfur compounds inhibited SMC proliferations and induced apoptosis of cancer cells by blocking the cell cycle in G_0/G_1 phases or G_2/M phases. Garlic organo-sulfur compounds, particulary allicin and ajoene have been mostly investigated for their apoptotic effects on cancer cells (4-6). According to Miron et al. (4), allicin induced growth inhibition and elicited apoptotic events such as blebbing, mitochondrial membrane depolarization, cytochrome c release into the cytosol, activation of caspase 9 and caspase 3 and DNA fragmentation. Ajoene inhibits protein prenylation and arterial SMC proliferation (7). Ajoene also leads to covalent modification of the cysteine SH group of the CAAX motif of protein prenyl group acceptors, which may contributes to the ability of ajoene to inhibit SMC proliferation (7).

Recently, lipid-soluble compounds such as diallyl sulfides (DAS) and diallyl disulfides (DADS) have been reported to more effectively suppress the tumor cell proliferation (8-10). Nigam and Shukla (11) showed the antimutagenic properties of DAS on 7,12-dimethylbenz [a] anthracene (DMBA)-a carcinogenic polycyclic aromatic hydrocarbon, which breaks DNA strand in mouse skin. In addition, Thejass and Kuttan (12) reported a suppressive effect of DADS on angiogenesis in *in vitro* models using

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human umbilical vein endothelial cell (HUVECs). However, there were few studies on the suppressive effects of lipid-soluble garlic sulfur compounds (DAS or DADS) on the proliferation and migration of VSMC. Therefore, we aimed to investigate the suppressive effect of DAS and DADS on VSMC proliferation and migration.

MATERIALS AND METHODS

Materials

Diallyl sulfide (DAS), diallyl disulfide (DADS), oleic acid bound to bovine serum albumin (OA bound to BSA), β-actin and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, fetal bovine serum (FBS), Dulbecco's phosphate buffered saline and antibiotics (10,000 U penicillin, 10,000 μg streptomycin) were purchased from GIBCO (Grand Island, NY). Cell lysis buffer (10×) and anti-phospho-SAPK/JNK (Thr183/Tyr185), LY294002 (PI3K inhibitor) were obtained from Cell Signaling Technology (Beverly, MA). PD98059 (ERK inhibitor), SB203580 (p38 kinase inhibitor), the horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit HRP conjugated antibody) and Western blotting luminal reagent for enhanced chemiluminescence detection of Western blots were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SP600125 (JNK2 inhibitor) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) were obtained from InvivoGen (San Diego, CA) and Amresco Inc. (Solon, OH), respectively. The vascular smooth muscle cell line (A10) derived from rat thoracic aorta was obtained from BioBud Inc. (Seoul, Korea).

Cell culture conditions

Cells were cultured in 75 cm^2 tissue culture flasks and grown at 37°C under a humidified 5% CO_2 atmosphere in a high glucose (10 mM)-containing DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were grown to >70% confluence and those between passages 9 and 15 were used for experiments.

Cell proliferation

A10 cells were seeded on 48 well plates at a density of 1×10^5 cells/well in 200 μL of DMEM containing 10% FBS. After cell growth (>70% confluence) and further 24 hr-incubation in serum-free medium, cells were incubated for 24 hr after being treated with OA bound to BSA, DAS, DADS (0, 10, 25, 50, 100, 150 or 200 μM) separately or in combination. After addition of 200 μL MTT and a 2 hr-incubation, the media was removed and 200 μL of DMSO was added to each well to dissolve any formazan crystals that formed, and the optical density was measured with ELISA microplate reader (VERSA max, Molecular Devices Corp, Sunnyvale) at 570 nm.

Cell migration

Cells (1×10^5 cells/well) were seeded in a collagen-coated well plate (Nunc Multidishes, Rochester, NY, USA) with DMEM containing 10% FBS. After cell growth (>70% confluence) and further 24 hr-incubation in serum-free medium, cells were incubated with BSA-bound OA (50 μM) in the presence or absence of DAS and DADS (150 μM, 200 μM). Cells were also tested with 10 μM of PD98059 (ERK inhibitor), B203580 (p38K inhibitor), LY294002 (PI3K inhibitor) and SP600125 (JNK2 inhibitor), respectively. A clear area was made in the monolayer with a narrow tip and further incubated for 24 hr in serum-free media. The distance of the cells moving to the cleared area was measured and the fold change was calculated in comparison with the moving distance of control (non-modulated cells).

Western blot analysis

Cells were harvested in a lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, 1 μg/mL leupeptin. Samples containing equal amounts of cellular proteins were loaded on 8～15% SDS polyacrylamide gels. Following electrophoresis, the proteins were transferred to nitrocellulose membranes (Schleicher and Schuell BioScience GmbH, Germany). After blocking in 5% skim milk, the membranes were immunoblotted with anti-phospho-JNK1/2 and with IgG HRP-conjugated antibody. Visualization of the blot was performed with the ECL Western blotting system (Amersham Biosciences Corp.).

Statistical analysis

Statistical analysis was performed with SPSS version 12.0 for Windows (Statistical Package for the Social Science, SPSS Inc, Chicago, Ill). Differences between control and treated groups were analyzed by Mann-Whitney test (nonparametric independent *t*-test) and repeated measure ANOVA. Data are presented as mean ±standard error (SEM) and a two-tailed value of $p<0.05$ was considered statistically significant.

RESULTS

Effects of oleic acid, DAS and DADS on VSMC proliferation

Compared to non-treated control cells, cell growth was significantly increased in a dose-dependent manner after

treatment with OA at concentrations of 10, 25 and 50 μM. The significant growth was still observed at concentrations 100 and 150 μM of OA, but the levels were relatively lower than those at 50 μM of OA (data not shown). When VSMCs were treated with DAS and DADS at concentrations ranging from 10 to 200 μM, cell growth was significantly inhibited at the 150 μM and 200 μM levels for both DAS and DADS, compared with control. OA-induced cell proliferation was also suppressed by DAS (about 14% at 150 μM and 20% at 200 μM) and DADS (about 16% at 150 μM and 22% at 200 μM) (Fig. 1).

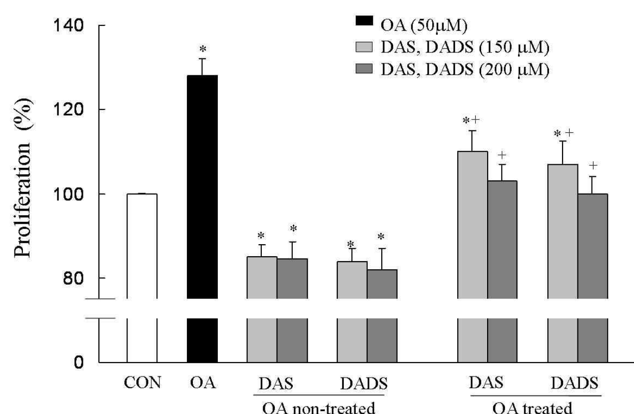


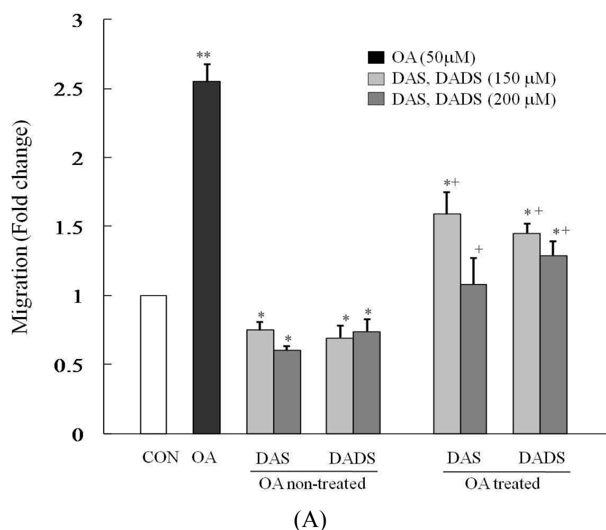
Fig. 1. The effect of DAS and DADS on OA-induced VSMC proliferation. The cell proliferation (%) migration compared with control group. Data are presented as mean±SEM from six duplicated, independent experiments. $^{*}p<0.05$ compared with control (non-treated), $^{+}p<0.05$ compared with OA-treated. VSMC: vascular smooth muscle cells, BSA-bound OA: oleic acid bound to albumin from bovine serum, CON: control (non-treated cells), DAS: diallyl sulfides, DADS: diallyl disulfides.

Inhibitory effect of DAS and DADS on OA-induced VSMC migration

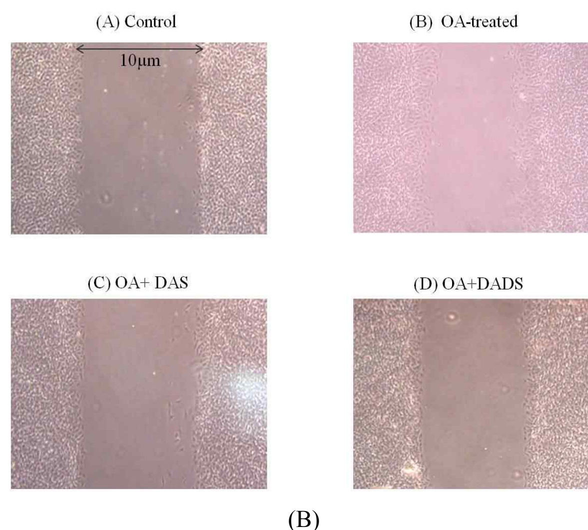
Fig. 2A and Fig. 2B show the effect of OA, DAS and DADS on the motility of VSMC. The concentrations of OA (50 μM), DAS (150 μM and 200 μM) and DADS (150 μM and 200 μM) for the migration test were chosen based on the results for proliferation. VSMC migrated into the clear area when stimulated with OA and the distance of migration was 2.5 times longer than that of non- modulated cells. On the other hand, cells treated with DAS or DADS migrated less than the control cells. OA-induced migrations were significantly suppressed by DAS (32% at 150 μM and 50% at 200 μM) and DADS (40% at 150 μM and 46% at 200 μM). Particularly, the attenuation by 200 μM of DAS was close to the control level.

Signaling pathways related to OA-induced VSMC migration

In order to examine which signaling pathways are linked to OA-induced VSMC proliferation and migration, we incubated the cells with PD98059 (ERK inhibitor), SB203580 (p38K inhibitor), LY204002 (PI3K inhibitor) and SP600125 (JNK2 inhibitor), inhibitors for major signaling pathways and then compared the distances of the cells' movements. Cells migrated significantly less than controls when incubated with LY204002 and SP600125, but not with PD98059 and SB203580.



(A)



(B)

Fig. 2. The effect of DAS and DADS on OA-induced VSMC migration. Fig. 2A presents the fold changes of cell migration compared with control group. Data are presented as mean±SEM from six duplicated, independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$ compared with control (non-treated), $^{+}p<0.05$ compared with OA-treated. Fig. 2B presents the images for VSMC migrations (A) control (non-treated cells) (B) OA only treated cells, (C) DAS (200 μM) on OA treated cells, (D) DADS (200 μM) on OA treated cells. VSMC: vascular smooth muscle cells, BSA-bound OA: oleic acid bound to albumin from bovine serum, CON: control (non-treated cells), DAS: diallyl sulfides, DADS: diallyl disulfides.

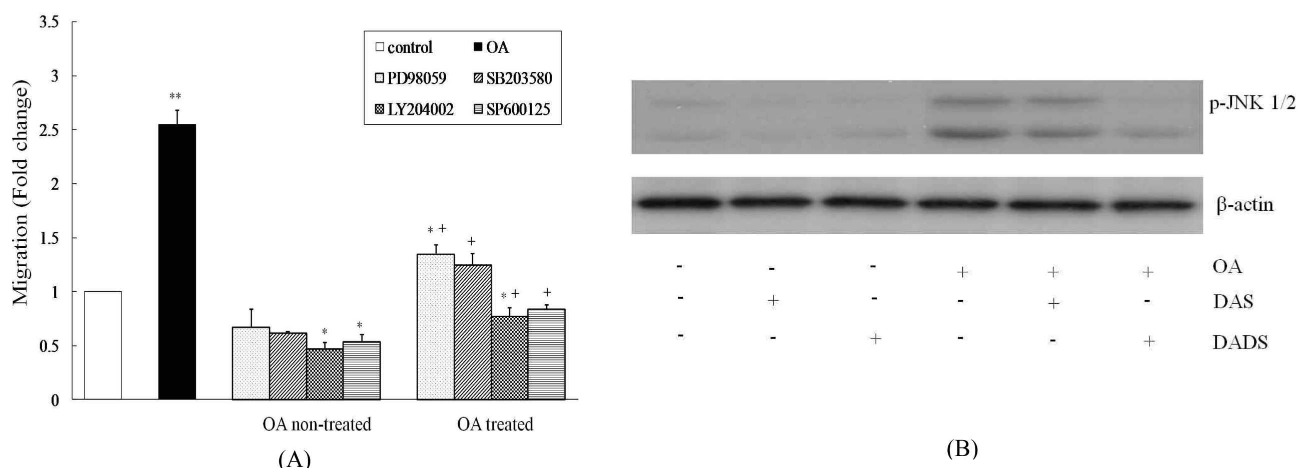


Fig. 3. Signaling pathways related to the inhibition of OA-induced VSMC migration. Fig. 3A presents the fold changes of cell migration compared with control group in the presence or absence of PD98059 (ERK inhibitor), B203580 (p38K inhibitor), LY294002 (PI3K inhibitor) and SP600125 (JNK2 inhibitor), respectively. Data are presented as mean±SEM from six duplicated, independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$ compared with control (non-treated), $^{+}p<0.05$ compared with OA-treated. Fig. 3B. Western blot analysis was performed to examine whether DAS or DADS can inhibit JNK1/2-phosphorylation. Upper lane is representative immunoblots for anti-phospho-JNK1/2 antibody, and the second lane is for β-actin for correction. The first column of each lane indicates control (non-modulated cells). VSMC: vascular smooth muscle cells, BSA-bound OA: oleic acid bound to albumin from bovine serum, CON: control (non-treated cells), DAS: diallyl sulfides, DADS: diallyl disulfides, ERK: signal-regulated kinase, PI3K: phosphatidylinositol 3-kinase, JNK: Jun N-terminal kinase, p-JNK1/2: phosphorylated JNK1/2.

OA-induced migrations were significantly attenuated by all of these molecules. In particular, the attenuation by LY204002 and SP600125 went down almost to control level (Fig. 3A).

In addition, Western blot assays show that OA, DAS and DADS are involved in the phosphorylation of JNK1/2. The increased p-JNK1/2 was detectable following treatment of OA and the increased levels were significantly attenuated by DAS and DADS (Fig. 3B).

DISCUSSION

This study aimed to investigate the effect of DAS and DADS, the garlic sulfur compounds, on the proliferation and migration of VSMC induced by OA, a major fatty acid of blood stream triglycerides. Our findings supported the idea that DAS and DADS may effectively suppress the proliferation and migration of VSMC induced by OA. In addition, our data suggested the suppression might be related to the signaling pathways of JNK1/2 and PI3K.

Proliferation and migration of VSMC are thought to be the important processes involved in the pathogenesis of vascular disorders such as atherosclerosis and post-angiorestenosis, and also closely associated with hypertriglyceridemia (2,8). OA is a principal fatty acid in triglycerides and has its highest concentration in plasma. Several reports demonstrate that OA may induce proliferation and migration of SMC via sequential signaling pathways, including activation of PKC, ERK and PI3K, and generation of reactive oxygen species (13-15). In our present study, VSMC proliferation was induced by the stimulation of OA at concentrations of 10 to 150 μM. Interestingly, a dose-dependent increase of cell proliferation was observed at OA concentrations ≤50 μM (28% increase). This result may be in accordance with the report of Yun et al. (2). Some reports suggest that OA promoted cell proliferation, but that the high concentration might be toxic to cell growth (16). The average concentration of OA measured in human plasma is 2∼3 times higher than 50 μM (17). Thus, the less-induced proliferation by OA at 150 μM could not be explained only by toxicity.

Allyl sulfur compounds derived from garlic are known to inhibit cell proliferation and induce apoptosis of cancer cells and endothelial cells (8,9,18). In our study, DAS and DADS significantly suppressed not only the VSMC proliferation and migration itself but also the OA-induced cell growth at concentration of 150 and 200 μM. This suppressive effect may be explained by the previous reports that DAS and DADS specifically blocked the cell cycle in G_0/G_1 phase or G_2/M phase (9), thus attenuating the reduction of cells in G_0/G_1 phases or the increase of cells in S and G_2/M phases induced by OA (2,19). Additionally, our results suggested that OA-induced cell proliferation/migration may be partly associated with the PI3K and JNK1/2 signaling pathway. In our study, OA-induced VSMC proliferation and migration were sig-

nificantly attenuated when pre-incubated with inhibitors of ERK, p38K, PI3K and JNK2. Interestingly, the attenuation was much larger when inhibiting PI3K and JNK2. The key role of JNK is as a mediator in several steps of angiogenesis: cell migration and proliferation, matrix invasion and network formation (20). In addition, PI3K has been known to activate the Akt signaling pathway that is located upstream of JNK (21). Kavurma et al. (21) also suggested that SMC proliferation/ migration are differentially regulated by ERK, JNK and p38K and are more dependent on the JNK pathway than others. In our study, we performed a Western Blot assay to see if DAS and DADS are involved in the JNK2 signaling pathway. The up-regulated JNK1/2 phosphorylation in OA-induced cells showed remarkably reduced expression with the co-treatment with DAS, and particularly with DADS.

To identify the precise step in the down/up regulation of cell proliferation/migration, we need to do further confirmation. According to Ciapaite et al. (22), the function of HUVEC for the activation of apoptotic and inflammatory pathways may be influenced by the type of fatty acid. According to Artwohl et al. (23), fatty acids induce apoptosis in human smooth muscle cells depending on their chain length, saturation and duration of exposure. For example, OA could stimulate more cell proliferation than palmitate (22). Therefore, it was worth testing cell proliferation and migration with different kinds of fatty acids, including palmitate. In addition, Western Blot assays looking at other signaling pathways were run; for example, PI3Ks, whose inhibitor significantly blocked OA-induced migration, is shown in our results. We also considered the new marker peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α). Zhu et al. (24) showed an interesting result with PGC-1α, suggesting that this transcriptional coactivator of peroxisome proliferator-activated receptor-γ, as well as a regulator of expression of many genes coding for mitochondrial proteins, may be a key modulator of high glucose-induced proliferation and migration in VSMCs. He also suggested that elevation of PGC-1α in VSMC could be a useful strategy in preventing the development of diabetic atherosclerosis. Zhang et al. (25) investigated PGC-1α inhibition of OA-induced proliferation and migration of rat VSMC. They reported that OA stimulates VSMC proliferation and migration via suppression of PGC-1α expression while palmitic acid reverses the effects of OA by inducing PGC- 1α expression (25). Further investigation into the role of PGC-1α on the influence of different kinds of fatty acids on VSMC, as well as the association of the role of PGC-1α with DAS or DADS, is necessary. However, our findings suggest that DAS and DADS may effectively suppress the OA induced proliferation and migration of VSMC.

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NOMENCLATURE

CON: control (non-treated cells)
DAS: diallyl sulfides
DADS: diallyl disulfides
ERK: extracellular signal-regulated kinase
JNK: Jun N-terminal kinase
OA: oleic acid bound to albumin from bovine serum
p-JNK1/2: phosphorylated JNK1/2
PI3K: phosphatidylinositol 3-kinase
VSMC: vascular smooth muscle cells, BSA-bound

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