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Effects of Diarylheptanoids on the Tumor Necrosis Factor-α-Induced Expression of Adhesion Molecules in Human Umbilical Vein Endothelial Cells

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Atherosclerosis is a chronic inflammatory disease that is characterized by infiltration of mononuclear lymphocytes into the intima through the expression of adhesion molecules on the arterial wall. In the present study, we report the inhibitory effects of two diarylheptanoids, 5-*O*-methylhirsutanonol (**1**) and oregonin (**2**), isolated from the methanolic extracts of *Alnus japonica* leaves, on the expression of adhesion molecules in human umbilical vein endothelial cells (HUVECs). Compounds **1** and **2** inhibited tumor necrosis factor (TNF)-α-induced up-regulation of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which also prevented adhesion of monocytes to HUVECs, and slightly suppressed the mRNA expression of the inflammation-associated gene interleukin-1β (IL-1β). A further study demonstrated the inhibitory effect of compound **1** on DNA-binding of nuclear factor κB (NF-κB) and on the phosphorylation and degradation of inhibitory factor κBα (IκBα) in TNF-α-stimulated HUVECs. These results indicate that compounds **1** and **2** may be useful in the prevention and treatment of atherosclerosis through attenuation of adhesion molecule expression by inhibition of NF-κB activation.

KEYWORDS: Diarylheptanoids; 5-*O*-methylhirsutanonol; oregonin; HUVECs; VCAM-1; ICAM-1; inflammation; atherosclerosis

INTRODUCTION

The recruitment of circulating monocytes/leukocytes to inflamed sites in the arterial wall is one of the earliest detectable events in atherogenesis. Monocytes/macrophages play important roles in the development of atherosclerosis (*1, 2*). Endothelial cells recruit leukocytes by selectively expressing major adhesion molecules on the surface: for example, vascular cell adhesion molecules (VCAM-1), intercellular adhesion molecules (ICAM-1), and endothelial cell selectin (E-selectin) (*3, 4*). These adhesion molecules and pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), which are commonly found in atherosclerotic lesions, have been known to contribute to the inflammatory process (*5, 6*) Previous studies have shown that cytokines such as TNF-α change the shape and motility of endothelial cells, which could contribute to vascular leakage at the site of inflammation (*7*), and that such cytokines stimulate an increase in the expression of cell adhesion molecules and genes involved in regulating the vessel tone and thrombosis (*8*).

The mechanism of VCAM-1 induction on endothelial cells early after feeding an atherogenic diet probably depends on the inflammation instigated by modified lipoprotein particles accumulating in the arterial intima in response to the hyperlipidemia (*9*). Some constituents of modified lipoprotein particles, such as oxidized phospholipids and short-chain aldehydes arising from lipoprotein oxidation, can induce transcriptional activation of the VCAM-1 gene, which is mediated, in part, by nuclear factor κB (NF-κB) (*10*). Many studies have shown that NF-κB activation is required for the up-regulation of adhesion molecules such as ICAM-1 and VCAM-1, which are responsible for monocyte adhesion and increased vascular inflammation (*11, 12*). Reactive oxygen species (ROS) may up-regulate pro-inflammatory gene expression by activating NF-κB, a process that is itself sensitive to the cellular redox state (*13*). Therefore, there has been increasing interest in developing the potential therapeutic value of ROS-antioxidants as inhibitors of NF-κB from various plants (*14*).

Alnus japonica Steud is a Betulaceae tree found in damp areas of mountain valleys and has been used in traditional Asian medicine (*15*). A series of diarylheptanoids classified into linear

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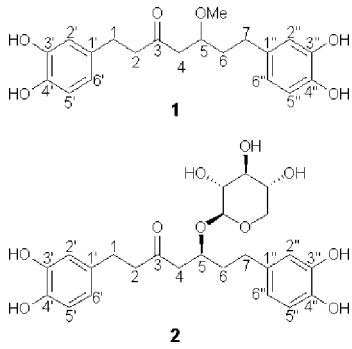

Figure 1. Structures of two diarylheptanoids, 5-*O*-methylhirsutanonol (**1**) and oregonin (**2**), isolated from *A. japonica*.

and cyclic types are isolated from *A. japonica* Steud. The diarylheptanoids are known to have a variety of biological activities, such as anticancer, antifungal, antihepatotoxic, and anti-inflammatory activities (*16–19*), and to have antiatherosclerotic effects via suppression of the adhesion of leukocytes to endothelial cells (*20*). We have previously shown that two diarylheptanoids, 5-*O*-methylhirsutanonol (**1**) and oregonin (**2**), isolated from *A. japonica* Steud, exhibited potent antioxidant activity against low-density lipoprotein (LDL) oxidation (*21, 22*). Antioxidants may play an important role in the prevention of atherosclerosis accompanied by expression of cell adhesion molecules (*8, 23, 24*).

In the present study, we investigated the effects of compounds **1** and **2** (**Figure 1**) on the adhesion of monocytes to human umbilical vein endothelial cells (HUVECs) and on the expression of adhesion molecules in HUVECs and the underlying mechanisms.

MATERIALS AND METHODS

General Experimental Materials. The reagents, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), and 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). CAM monoclonal antibodies and recombinant human TNF-α were provided by R&D Systems Inc. (Minneapolis, MN, USA). The endothelial cell basal medium-2 (EBM-2) Bullet kit was purchased from Clonetics (San Diego, CA, USA), and all other tissue culture reagents were obtained from GIBCO-BRL (Gaithersburg, MD, USA).

Isolation of Compounds 1 and 2. Dried leaves of *A. japonica* (1.0 kg) were extracted with 100% MeOH (4 L) at room temperature for 2 weeks. After filtration, the methanol was evaporated, the remaining extracts were suspended in water and then further partitioned with *n*-hexane, then $CHCl_3$, and finally EtOAc. Filtration and evaporation gave three fractions, *n*-hexane-extracted (35.2 g), $CHCl_3$-extracted (25.4 g), and EtOAc-extracted (30.0 g) residues. Compounds **1** and **2** were isolated from the EtOAc-extracted residues using silica gel column chromatography and preparative reverse-phase HPLC (*21, 22*).

Cell Culture. HUVECs were obtained from Clonetics, grown on gelatin-coated culture dishes or 6-well plates in EBM-2, and used for the experiments within the first 3 or 4 passages. The growth medium was changed every other day until cells reached confluence. The cells were grown in monolayers at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, and were used for experiments at >80% confluence. THP-1 monocytes were obtained from the American-Type Culture Collection (Rockville, MD, USA) and were cultured in RPMI-1640 (Invitrogene, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco) under the conditions used for HUVECs.

Cell Adhesion Assay. The monocytoid cell line THP-1 was used essentially as described (*25*) but with minor modifications. Briefly, regularly passaged THP-1 cells were labeled with the fluorescent dye BCECF-AM, at a final concentration of 10 μM in RPMI-1640 culture medium at 37 °C for 1 h. The labeled cells were harvested by centrifugation, washed three times with phosphate-buffered saline (PBS) before suspension in the medium, and added to HUVECs growing in 96-well culture plates. The coincubation was done at 37 °C for 1 h, and unbound THP-1 cells were removed by stringent washing four times with medium (RPMI-1640, 1% FBS), and twice with PBS. THP-1 cells bound to HUVECs were lyzed with 50 mM Tris-HCl (pH 8.0), 0.1% (w/v) SDS, and the fluorescence was measured on a spectrofluorometer (Wallac 1420, Perkin-Elmer, Turku, Finland) at 485 nm excitation and 535 nm emission wavelength.

Measurement of Monocyte Migration. Migration of THP-1 cells was assayed in 96-well plates using the ChemoTx kit (Neuro Probe Inc., Gaithersburg, MD, USA) with a collagen-coated polycarbonate filter with 5.0-μm pores. The lower chambers were filled with 30 μL of medium obtained from cultured THP-1 cells that were treated with 10 μM of compounds **1** and **2** for 2 h and were coincubated with 100 nM of phorbol 12-myristate 13-acetate (PMA) as differentiating agent and 100 ng/mL of LPS as stimulant for 18 h. Then, 25 μL of 1.25×10^4 THP-1 cells was loaded onto the filter membrane. After incubation at 37 °C for 6 h, cells that had migrated into the lower chamber were counted by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA, USA).

Flow Cytometry for Cell Adhesion Molecule Expression. The flow cytometry was performed as described (*26*). Briefly, HUVECs were plated in 6-well plates, grown to 70% confluence, pretreated with compounds **1** or **2** (5 ∼ 50 μM) for 2 h, and followed by stimulation with TNF-α (10 ng/mL) and induction of VCAM-1 for 6 h or ICAM-1 for 12 h. Cells were labeled with fluorescein isothiocyanate (FITC)-conjugated primary antibody (R&D Systems, Inc., Minneapolis, MN, USA) for 1 h in the dark. The expression of cell-surface adhesion molecules was measured as fluorescence intensity by a FACS Calibur instrument (Becton Dickinson, San Jose, CA, USA).

Measurement of Cell-Surface Expression of Adhesion Molecules. The cell-surface expressions of VCAM-1 and ICAM-1 on HUVECs were performed essentially as described (*26*) but with some modifications using cell ELISA. The adhesion molecule expressed cells were washed with PBS and fixed with 4% (v/v) paraformaldehyde for 30 min at 4 °C. FBS (1% (v/v) in PBS) was used as a blocking reagent. After washing with PBS, cells were incubated with FITC-conjugated anti-VCAM-1 or anti-ICAM-1 monoclonal antibody (R&D Systems, Inc., Minneapolis, MN, USA) for 1 h at 4 °C. The cell-surface expressions of VCAM-1 and ICAM-1 were measured with a spectrofluorometer (Wallac 1420, Perkin-Elmer, Turku, Finland) at 485 nm excitation wavelength and 535 nm emission wavelength, and microphotographs were also obtained using a fluorescence microscopy with a fluorescein blue filter.

Total RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). HUVECs were pretreated with 20 and 50 μM of compounds **1** or **2** for 2 h before being incubated with 10 ng/mL of TNF-α for 12 h. The cells were harvested, and total RNA was isolated by RNeasy mini columns (Qiagen, Santa Clarita, CA, USA) according to the manufacturer's instructions. A sample (1 μg) of total RNA was used for the synthesis of the first strand cDNA using the Omniscript (Qiagen, Santa Clarita, CA, USA) according to the manufacturer's instructions. For amplification of VCAM-1, ICAM-1, IL-1β, and β-actin, the following primers were used: for VCAM-1, 5′-ACT GTT TAT TAC AGC CCC GC-3′ (sense), 5′-ACT TCA ACG ATG GGG ACT TG-3′ (antisense); for ICAM-1, 5′-GTC GAA GGT GGT TCT TCT GAG C-3′ (sense), 5′-TCC GTC TGC AGG TCA TCT TAG G-3′ (antisense); for IL-1β, 5′-CTG AGC ACC TTC TTT CCC TTC ATC T-3′ (sense), 5′-GCG TGC AGT TCA GTG ATC GT-3′ (antisense); for β-actin, 5′-AAC ACC CCA GCC ATG TAC G-3′ (sense), 5′-ATG TCA CGC ACG ATT TCC C-3′ (antisense). For PCR amplification, the following conditions were used: 95 °C for 2 min for one cycle; 95 °C for 1 min; 55 °C for 30 s; and 72 °C for 1 min for 27–35 cycles. The amplified PCR products were separated on 1.2% agarose gel and then stained with ethidium bromide. The density of bands was scanned and semiquantified using a calibrated Densitometer GS-800 (BioRad, Hercules, CA) with Quantity One software (version 4.4.0). The relative band intensity was considered as reflecting the expression level of β-actin protein.

Western Blot Analysis. Total cell extracts (for ICAM-1 and VCAM-1) and cytoplasmic extracts (for IκBα and phosphor-IκBα) were

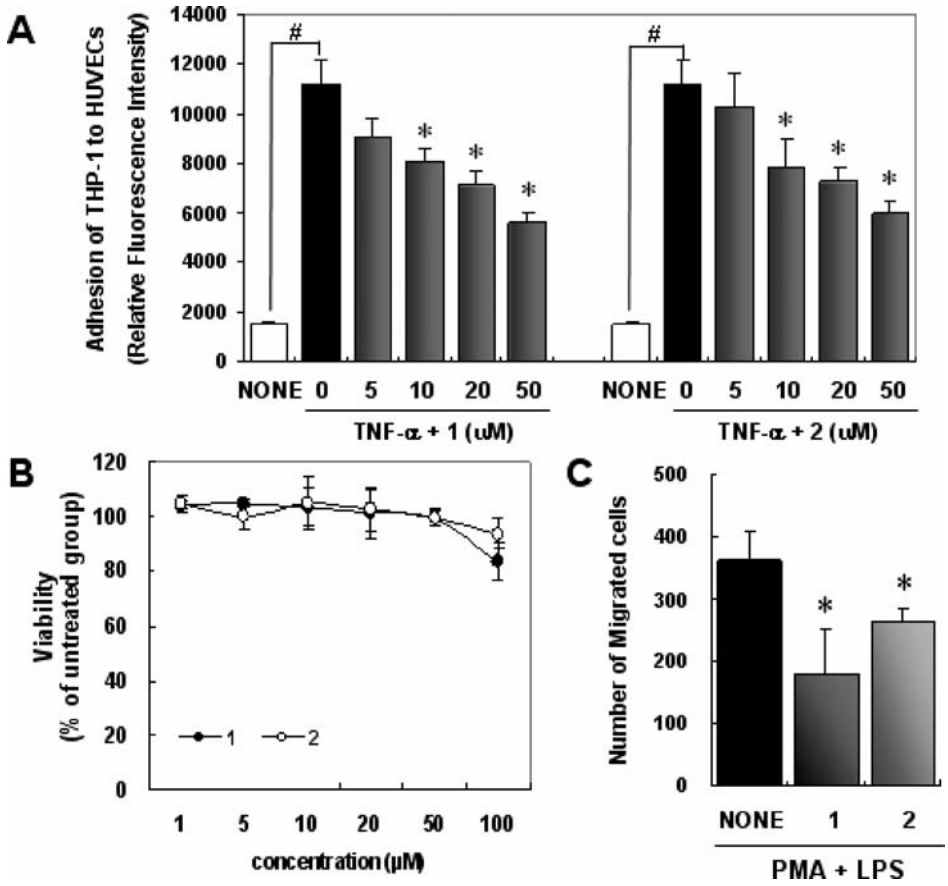

Figure 2. Effects of compounds **1** and **2** on adhesion and migration of THP-1 cells to HUVECs, stimulated with TNF-α. (**A**) Cells were pretreated with compounds **1** and **2** for 2 h and then treated with 10 ng/mL TNF-α for 8 h. THP-1 monocytes previously labeled with BCBCF-AM were added (2×10^5 cells/well) to washed endothelial cell monolayers ($n = 4$ replicates per sample). The cell adhesion assay was performed as described under the Materials and Methods section. Data shown are the mean ± SD ($n = 4$). $^{\#}P < 0.01$ vs media alone-treated group. $^{*}P < 0.05$ vs TNF-α alone-treated group. (**B**) Cell viability of compounds **1** and **2** in HUVECs by MTT assay. Cells were pretreated with compounds **1** and **2** for 72 h. (**C**) Measurement of the relative migration of THP-1 monocytes. THP-1 cells were stimulated with LPS and PMA for 18 h. Then, conditioned medium was added to the lower chamber of ChemoTx plates, and 1.25×10^4 THP-1 cells were loaded onto the 5 μm pore size filter. After incubation for 6 h, the migrated cells were counted by flow cytometry. $*P < 0.05$ vs PMA and LPS alone-treated group.

fractionated by electrophoresis on 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by blocking solution [10 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.1% Tween 20, and 5% skim milk]. The membranes were incubated with anti-VCAM-1, anti-ICAM-1, anti-IκBα, antiphospho-IκBα, and anti-actin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, the blots were washed three times with washing buffer (20 mM Tris, 160 mM NaCl, 0.1% Tween 20) followed by 1 h of incubation with the appropriate horse radish peroxidase-conjugated secondary antibody. The peroxidase bound to the blot was detected using the ECL Western blot kit (Elipis biotech, Korea). This immunoblot analysis was performed three times independently.

Nuclear Extracts and Gel Electromobility Shift Assay (EMSA). Cells pretreated with compound **1** were stimulated for 2 h with TNF-α (10 ng/mL); then, preparation of nuclear extracts was done as described by Lee et al. (*27*). Cell nuclei were extracted and lysed with hypotonic buffer, and nuclei were pelleted by centrifugation at 3000*g* for 5 min. Nuclear lysis was performed using a hypertonic buffer [50 mM Hepes (pH 7.9), 400 mM KCl, 0.1 mM EDTA, 2 μg/mL leupeptin, 10% (v/v) glycerol]. Following lysis, the samples were centrifuged at 14,000 *g* for 15 min, and the supernatant was retained for use in the DNA binding assay. Nuclear proteins were quantified via the Bradford method at 595 nm. EMSA (Promega) were conducted with ^{32}P-labeled double-strand oligonucleotides having consensus recognition sequences for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′), or Oct-1 (5′-TGTCGAATGCAAATCACTAGAA-3′). The DNA–protein complexes were then resolved on a 5% nondenaturating polyacrylamide gel in 0.5 × TBE running buffer [44.5 mM Tris-HCl, pH 8.0, 44.5 mM boric acid, 1 mM EDTA]. The gels were dried and exposed to X-ray film.

Statistical Analysis. The results are expressed as mean ± standard deviation (SD). Statistical analysis was done using Student's *t*-test. A value of $P < 0.05$ was accepted as statistically significant.

RESULTS AND DISCUSSION

So far, the studies of diarylheptanoids have been focused on their anti-inflammatory activity via inhibition of 5-lipoxygenase, COX-2, and MMP-9 (*18*, *28*, *29*). Among them, oregonin (compound **2**) has been known to have in vitro and macrophage-mediated antioxidant activities (*21*, *30*) and anti-inflammatory activities on the LPS-induced iNOS and COX-2 protein in macrophages and microglial cells (*19*, *31*). Recently, we have reported that oregonin and hirsutanone are potent antioxidants against LDL oxidation as well as the macrophage-mediated LDL oxidation (*21*). We also isolated 5-HM (compound **1**), another diarylheptanoid, from the metanolic extracts of *A. japonica* leaves.

Because the adhesion of leukocytes or monocytes to the vascular endothelium is an important step in the reaction to inflammation and development of atherosclerotic lesions, the effects of several diarylheptanoids have been investigated for

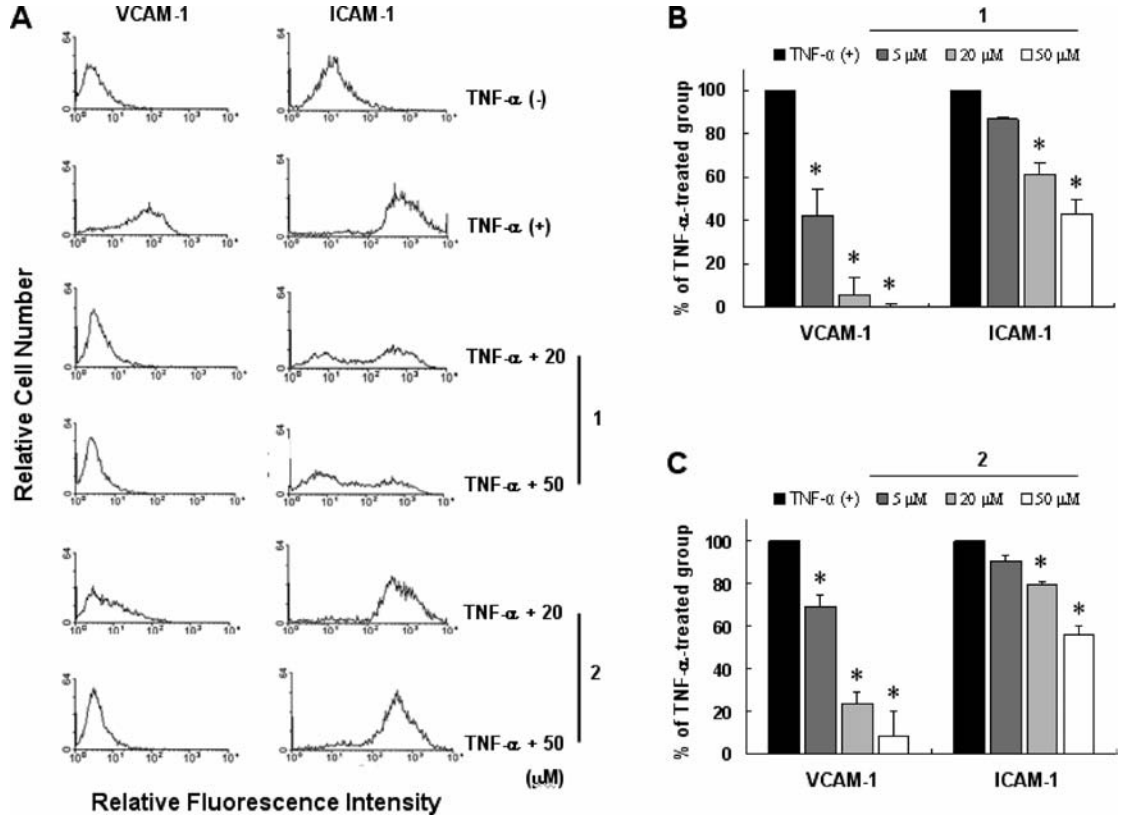

Figure 3. Effects of compounds **1** and **2** on TNF-α-induced expression of adhesion molecules on HUVECs. The expression of adhesion molecules on the surface of HUVECs was analyzed by flow cytometry, as described in the Materials and Methods section. (**A**) The effects of compounds **1** and **2** on the TNF-α-induced expression of VCAM-1 and ICAM-1, respectively. The data represented one of three separate replicate experiments. (**B**) The inhibitory effect of compound **1** on the expression of VCAM-1 and ICAM-1 in a dose-dependent manner. (**C**) The inhibitory effect of compound **2** on the expression of VCAM-1 and ICAM-1 in a dose-dependent manner. The results represent the mean ± SD ($n = 3$). $*P < 0.05$ vs TNF-α alone-treated group.

their anti-inflammatory effects by way of suppression of the adhesion of leukocytes to endothelial cells (*20*). Yamazaki et al. (*20*) reported that three phenolic diarylheptanoids reduced the adhesion of U937 and EoL-1 to TNF-α-treated HUVECs and were effective in suppressing both IL-1β and TNF-α-induced expression of adhesion molecules on the surface of the endothelial cells. The adhesion of monocytes to the vascular endothelial cells and their subsequent recruitment into the artery wall are key features in the early stage of atherosclerosis (*5*). Adhesion molecules such as VCAM-1 and ICAM-1 are expressed in human coronary atherosclerotic plaques and play a role in this disease (*32, 33*). In this study, we first investigated the effects of compounds **1** and **2** on the adhesion of monocytes to HUVECs and on the expression of adhesion molecules by moderating NF-κB-dependent inflammatory signals in HUVECs. To evaluate suppression of the adhesion of monocytes to endothelial cells, we examined adhesion of THP-1 cells to HUVECs stimulated with TNF-α (10 ng/mL) for 8 h. The time of incubation and the concentrations (0–50 μM) of compounds **1** and **2** used in these experiments had no effect on the viability as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and morphology of endothelial cells (**Figure 2B**). Compounds **1** and **2** (5 ∼ 50 μM) dramatically inhibited the adhesion of THP-1 cells to TNF-α-stimulated HUVECs in a dose-dependent manner (**Figure 2A**). This result suggests that compounds **1** and **2** might inhibit the expression of adhesion molecules (VCAM-1 and ICAM-1) on TNF-α-stimulated HUVECs. Also, to investigate whether compounds **1** and **2** affect cell migration, we performed a chemotaxis assay. Leukocyte migration after adhesion to endothelium, as well as expression of adhesion molecules and cell accumulation, plays a crucial role in the perpetuation of a chronic inflammatory reaction, lipid deposition, and formation of atheromas (*2, 5*). The LPS induced transcriptional response in THP-1 cells (*34*). Therefore, we showed that PMA- and LPS-treated THP-1 monocytes could induce various chemokines, and then, THP-1 monocytes migration increased significantly. However, the preincubation with 10 μM of compounds **1** and **2** showed significant decreases of THP-1 monocyte migration, by 50% and 27%, respectively (**Figure 2C**). These beneficial properties of two diarylheptanoids on circulating monocytes may prevent excessive accumulation of monocytes on the arterial wall during the process of atherosclerosis.

The effect of other antioxidants on the expression of adhesion molecules is relatively well known. Above all, *N*-acetylcysteine, verapamil, and flavonoids are selective inhibitors of the cytokine-stimulated synthesis of VCAM-1 (*8, 35, 36*). Wolle et al. (*36*) demonstrated that a flavonoid, 2-(3-amino-phenyl)-8-methoxy-chromene-4-one (PD 098063), has been shown to selectively block the TNF-α-stimulated NF-κB activation and transcription and cell-surface expression of VCAM-1 in endothelial cells, but they had no effect on ICAM-1 expression. In our results, compounds **1** and **2**, having antioxidant activity (*21, 22*), decreased the expression of VCAM-1 and ICAM-1 in TNF-α-stimulated HUVECs (**Figure 3**). When pretreated with 20 μM compound **1**, the expression of TNF-α-induced VCAM-1 and ICAM-1 was inhibited by 93.8 ± 8.1% and a 38.5 ± 5.3%, respectively, as compared to that of the TNF-α-treated group (**Figure 3**, panels **A** and **B**). Compound **2** at 20 μM also showed 76.6 ± 5.6% and 20.8 ± 1.8% inhibition of the expression of TNF-α-induced VCAM-1 and ICAM-1, respectively (**Figure 3**, panels **A** and **C**).

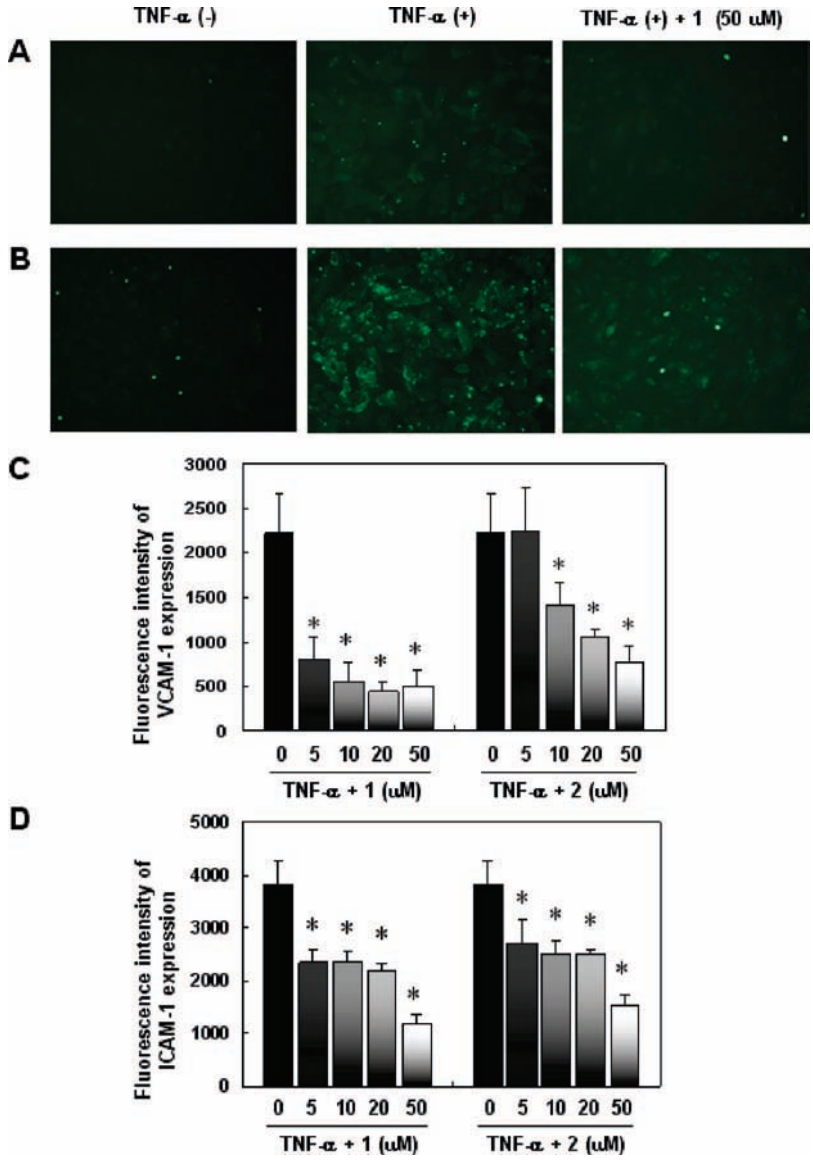

Figure 4. Effects of compounds **1** and **2** on TNF-α-induced cell surface expression of adhesion molecules. Cells were pretreated with compounds **1** and **2** for 2 h and then treated with 10 ng/ml TNF-α for 8 h. Cells were incubated with a FITC-conjugated VCAM-1 (panels **A** and **C**) and ICAM-1 (panels **B** and **D**) antibody diluted 1:50 at 4 °C for 1 h. After washing, fluorescence microphotographs were obtained using fluorescence microscopy with a fluorescein blue filter (panels **A** and **B**). Original magnification: x200. The bar graphs (panels **C** and **D**) represent quantitative results obtained using a spectrofluorometer at 485 nm excitation wavelength and 535 nm emission wavelength. The data represent the mean ± SD ($n = 4$). $*P < 0.01$ vs TNF-α alone-treated group.

To confirm this result, we quantified with fluorescence microscopy and spectrofluorometry for the cell-surface expression of these adhesion molecules on HUVECs. As shown in **Figure 4**, panels **A** and **B**, expression of VCAM-1 and ICAM-1 on TNF-α untreated endothelial cells was hardly detectable, but the expression of these molecules was increased significantly by TNF-α. In the case of compound **1**, pretreatment with only a small dose (5 μM) markedly suppressed the expression of TNF-α-induced VCAM-1 (**Figure 4C**). Compound **2** also inhibited the expression of TNF-α-induced VCAM-1 in a dose-dependent manner, but the inhibitory effect of compound **2** was relatively less than that of compound **1**. In the case of ICAM-1, compounds **1** and **2** at 5–20 μM exhibited similar but significant inhibitory effects, and compounds **1** and **2** at 50 μM showed much stronger inhibitory activity (**Figure 4D**).

To assess the effect of compounds **1** and **2** on the transcriptional levels of VCAM-1, ICAM-1, and IL-1β genes, the mRNA levels were determined by RT-PCR. When treated with 50 μM compounds **1** and **2**, the mRNA expression of VCAM-1 was significantly reduced, 79.4 ± 0.8% and 74.4 ± 4.8%, respectively (**Figure 5**, panels **A** and **B**). Compound **2** also inhibited the mRNA expression of VCAM-1, although less than that of compound **1**. Compounds **1** and **2** at 50 μM concentrations showed 46.8 ± 6.6% and 32.1 ± 11.2%, respectively, inhibition in mRNA expression of ICAM-1. Compound **1** is more potent than compound **2** in the suppression of VCAM-1 and ICAM-1 gene expression. Pro-inflammatory cytokines, such as TNF-α and IL-1β, stimulate the expression of cell adhesion molecules in endothelial cells (*6*, *8*). Treatment with compounds **1** and **2** slightly reduced the mRNA expression of IL-1β. Therefore, this result shows that compounds **1** and **2** possess potential to moderate, due in part to both paracrine and autocrine, mechanisms by cytokines, IL-1β or TNF-α in endothelial cells (*8*). The inhibitory effects of compounds **1** and **2** on VCAM-1 and ICAM-1 mRNA expressions were comparable with their inhibi-

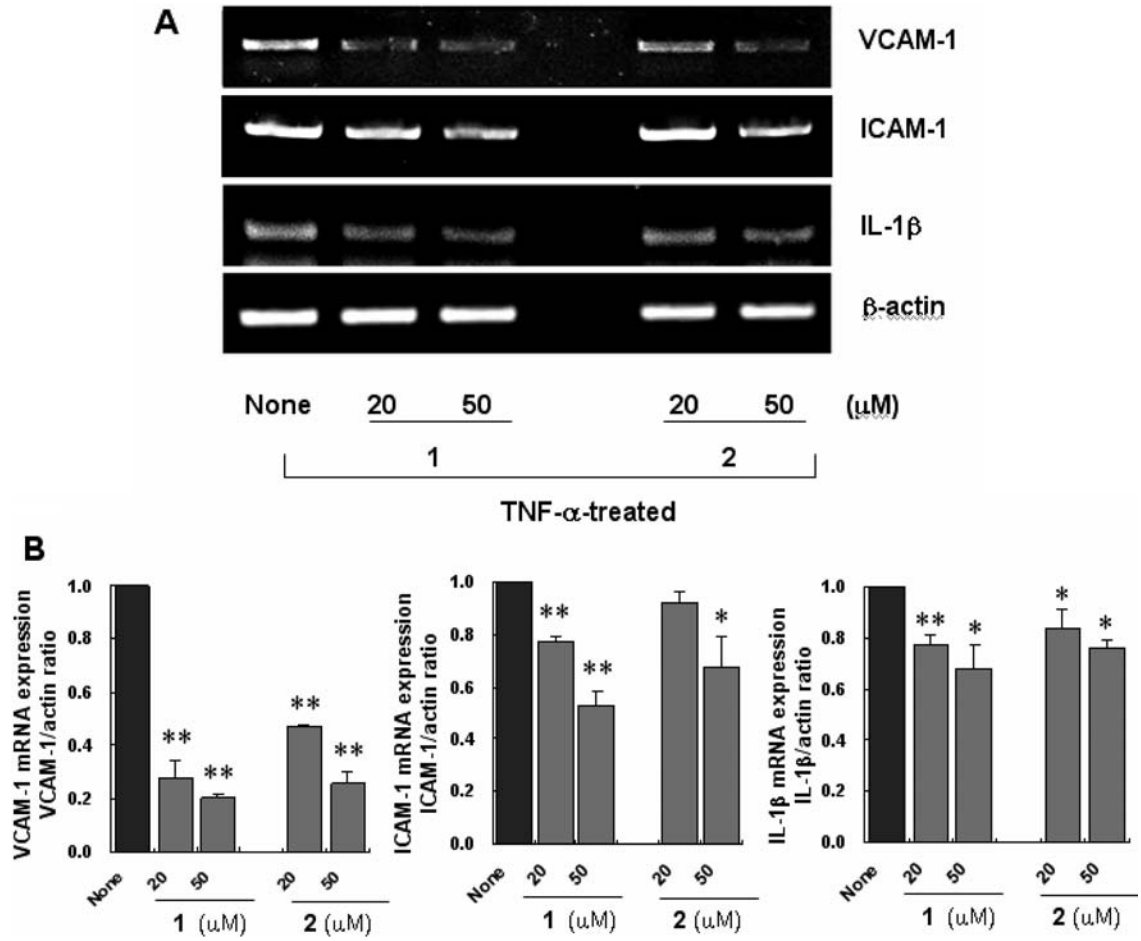

Figure 5. Effects of compounds **1** and **2** on the mRNA expression of VCAM-1, ICAM-1, and IL-1β. (**A**) The mRNA expression subjected to RT-PCR analysis using specific primers for human VCAM-1, ICAM-1, IL-1β, and β-actin as described in the Materials and Methods section. (**B**) Semiquantification of relative band intensities from two independent experimental results was determined by densitometry. $*P < 0.05$, $**P < 0.01$ vs TNF-α alone-treated group.

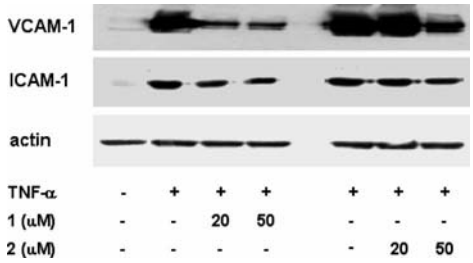

Figure 6. Effects of compounds **1** and **2** on the expression levels of cell adhesion molecules. Cells were pretreated with compounds **1** and **2** for 2 h and then treated with 10 ng/ml TNF-α for 8 h. The cell extracts were subjected to 10% SDS-PAGE and Western blot analysis with the respective primary antibody against VCAM-1 and ICAM-1. β-Actin protein was used as an internal control.

tory effects on TNF-α-induced cell surface protein expression of VCAM-1 and ICAM-1, as shown in **Figure 3**.

In light of the results achieved so far, it seems that the antiatherosclerotic ability of diarylheptanoid derivatives is no less than known antioxidants with antiatherosclerotic ability (*8*, *23*, *24*, *36*) Western blot analysis was used to address whether or not compounds **1** and **2** block the TNF-α-induced expression of VCAM-1 and ICAM-1. Expression of these adhesion molecules was markedly increased by TNF-α; however, the pretreatment with 50 μM compounds **1** and **2** demonstrated conspicuous inhibition of the expression of VCAM-1 (**Figure 6**). At 20 μM, cells treated with compound **1** exhibited a substantial inhibition of the expression of VCAM-1 protein, whereas compound **2** did not inhibit their expression. However, in the case of ICAM-1 protein, 50 μM compounds **1** and **2** exhibited mild inhibitory activity similar to that of **Figure 5**.

In general, cytokine-induced transcription of genes for adhesion molecules in HUVECs is regulated by an antioxidant-sensitive mechanism, involving NF-κB activation (*8*, *10*). TNF-α, IL-1β, or LPS-induced NF-κB activation plays a key role in the production of chemotactic cytokines and adhesion molecules (*37*). So, inhibition of NF-κB activation interacts with suppression of VCAM-1 expression on HUVECs (*38*). To further investigate the molecular mechanism responsible for the inhibitory effects of the two diarylheptanoids on adhesion molecule expression (**Figures 5** and **6**), the effect of compound **1** was examined on TNF-α-stimulated NF-κB activation in HUVECs by performing an EMSA. As shown in **Figure 7A**, TNF-α (10 ng/mL) significantly increased the DNA-binding activity of NF-κB within 2 h. However, in the presence of compound **1** (10, 20, and 50 μM), NF-κB binding activity was suppressed remarkably (**Figure 7**, panels **A** and **B**). Together, to determine whether or not the inhibitory effect of compound **1** on TNF-α-stimulated NF-κB activation was because of inhibition of IκBα degradation and phosphorylation, the cytosolic IκBα levels following treatment of TNF-α in the presence or absence of compound **1** were detected. TNF-α induced the degradation of IκBα protein after stimulation for 5 min. However, the treatment of compound **1** sustained the IκBα protein, namely, nearly the control level (**Figure 7C**). These results suggest that compound

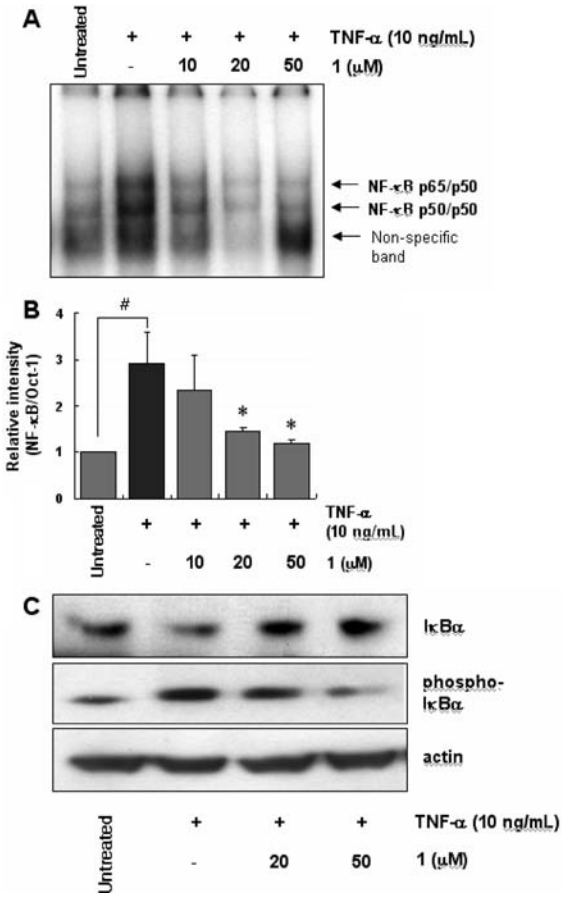

Figure 7. Effect of compound **1** on TNF-α-induced NF-κB activation through phosphorylation and degradation of IκBα. (**A**) EMSA analysis of the nuclear extracts was conducted using a ^{32}P-labeled NF-κB oligonucleotide probe. (**B**) Quantification of relative NF-κB activity against Oct-1 from three independent experimental results was determined by densitometry. $*P < 0.05$ vs TNF-α alone-treated group. (**C**) Western blot analysis for phosphorylation and degradation of IκBα. HUVECs were pretreated with vehicle (DMSO) or 20 and 50 μM compound **1** for 2 h before being incubated with TNF-α for 5 min.

1 has antiatherosclerotic activity, perhaps because of its ability to decrease intracellular NF-κB signaling, which leads to down-regulation of adhesion molecule expression and monocyte accumulation.

In summary, we demonstrated here that two diarylheptanoids, compounds **1** and **2**, inhibit THP-1 cell adhesion to TNF-α-stimulated HUVECs by suppressing the gene expression of adhesion molecules, VCAM-1 and ICAM-1. Moreover, compounds **1** and **2** slightly suppressed the expression of inflammation-associated gene IL-1β. Our results also showed that the inhibitory effect of compound **1** on adhesion molecule expression is mediated by blocking NF-κB. The less lipophilic compound **1** showed stronger inhibitory effects than compound **2** on the expression of VCAM-1 and ICAM-1. The results suggest that compounds **1** and **2** may be useful in the prevention and treatment of atherosclerosis through attenuation of adhesion molecule expression by inhibition of NF-κB activation.

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