Cynandione A inhibits lipopolysaccharide-induced cell adhesion via suppression of the protein expression of VCAM-1 in human endothelial cells

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Abstract. Cynandione A (CA) is one of the most active compounds in the roots of *Cynanchum wilfordii*, the extracts of which have been used extensively in East Asia to treat various diseases including anti-ischemic stroke. In the present study, the anti-adherent activity of CA in lipopolysaccharide (LPS)-stimulated human umbilical vascular endothelial cells (HUVECs) was investigated. CA markedly reduced the expression of vascular adhesion molecule-1 (VCAM-1) by LPS in HUVECs. The results also demonstrated that CA significantly reduced the expression of pro-inflammatory and chemoattractant cytokines, including interleukin (IL)-1β, IL-6, IL-8, monocyte chemoattractant protein-1 and tumor necrosis factor-α, in LPS-activated human endothelial cells. CA inhibited the phosphorylation of mitogen-activated protein kinases, including the extracellular signal-regulated kinase 1/2 and p38 kinases. It was found that CA decreased the IKK/IκB-α phosphorylation of inhibitor of nuclear factor (NF)-κB kinase/inhibitor of NF-κB-α, suppressed translocation of the NF-κB p65 subunit into the nucleus and inhibited the transcriptional activity of NF-κB. CA also decreased human monocyte cell adhesion to endothelial cells in LPS-stimulated conditions. These results demonstrated that CA inhibited the protein expression of VCAM-1 and pro-inflammatory cytokines by suppressing the transcriptional activity of NF-κB. The results also suggested that CA may be important in the development of anti-inflammatory drugs by inhibiting the expression of cell adhesion molecules.

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Introduction

During vascular inflammation, numerous cytokines and adhesion molecules are overexpressed, which leads to the homing of various immune cells into tissues (1-3). Major events in the homing of immune cells include the migration of leukocytes from the blood, their firm adhesion to the vascular endothelium and transmigration across the vascular endothelium (4). Several types of adhesion molecule are important in the endothelium cell adhesion process. Among these cell adhesion molecules, vascular adhesion molecule-1 (VCAM-1/CD106) is known to be increased in human endothelial cells by pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and various inflammatory stimuli, including reactive oxygen species (ROS) and lipopolysaccharide (LPS) (5,6). In addition, the phosphorylation of mitogen-activated protein kinases (MAPKs), phosphoinositide-3 kinase and the nuclear factor (NF)-κB pathways regulates the activation of the gene expression of VCAM-1 (3,7,8). According to previous reports, the NF-κB signaling pathway in human endothelial cells is important in regulating cell-to-cell adhesion in the inflammatory response by inflammatory mediators associated with increased expression of VCAM-1. NF-κB protein is known to be a pivotal transcription factor for the expression of inflammatory cytokine-mediated VCAM-1 (9,10). The progressive accumulation of leukocyte adhesion to the endothelium may cause vascular inflammation.

LPS is a lipoglycan and a known endotoxin present in the outer membrane of Gram-negative bacteria, which affects inflammation and immune cell activation in animals (11). The binding of LPS to toll-like receptor 4, present in immune cells, affects various signal transduction pathways, particularly the activation of inflammatory signaling pathways involving MAPK/inhibitor of NF-κB (IκB) kinase (IKK)/IκB/NF-κB (12). In non-stimulated conditions, the p65 subunit of the NF-κB protein is concealed by the IκB-α protein complex in the cytoplasm. However, when activated by external stimuli, including inflammatory molecules, the IKK-α/β subunit undergoes a successive phosphorylation cascade (13). Subsequently, the activated and released p65 subunit of NF-κB is translocated into the nucleus and binds to DNA promoter regions, increasing the transcription of

inflammatory-mediated genes, including cell adhesion molecules (14,15).

In ancient oriental medicine, active ingredients isolated from natural products and herbal extracts have been used to treat various diseases. Previous studies have shown that *Cynanchum wilfordii* root extracts have been used in traditional medicine for the therapeutic treatment and prevention of diseases, including vascular disease, arteriosclerosis and cancer (16-18). The active ingredient of *C. wilfordii* roots, cynandione A (CA; Fig. 1A), has been isolated in a number of studies (19,20). In addition, several studies investigating the biological and pharmaceutical activity of CA have revealed it exhibits protective activity against toxicity by various stimulant agents in rat hepatocytes and cortical neurons (21-23). Among investigations on the inhibitory effects of CA on anti-inflammatory activity, few have reported on the molecular mechanism underlying the anti-adhesion effects of CA in vascular inflammation. In the present study, it was demonstrated that CA inhibited the mRNA and protein expression of VCAM-1 in LPS-induced human umbilical vascular endothelial cells (HUVECs). The results confirmed that CA inhibited the expression of pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1) and TNF-α, in LPS-activated HUVECs. It was also shown that CA inhibited the phosphorylation of IKK and the transcriptional activity of NF-κB via downregulating the phosphorylation of MAPKs. Finally, the anti-adhesion activity of CA was confirmed using FITC-labeled immune cells. These findings may be beneficial for the development of a useful therapeutic agent for vascular inflammatory diseases, including atherosclerosis.

Materials and methods

Materials and cell culture. Human endothelial cells were cultured in medium 199 containing fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin) prepared by Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anti-human VCAM-1 (1:200; cat no. SC-13160) and anti-b-actin (1:200; cat no. SC-47778) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The antibodies targeting phosphorylated (p-)c-Jun N-terminal kinase (JNK; 46 kDa, 1:1,000; cat no. MAB1205), p-extracellular signal-regulated kinase (ERK)1/2 (44,42 kDa, 1:1,000; cat no. MAB18251), p-p38 (38 kDa, 1:500; cat no. MAB8691), JNK, ERK and p38 antibodies were from R&D Systems, Inc. (Minneapolis, MN, USA). Antibodies against p-IKK α/β (87, 85 kDa, 1:1,000; cat no. #2697) p-IκB-α (40 kDa, 1:2,000; cat no. #9246), anti-p65 (65 kDa, 1:1,000; cat no. #8242) and all non-phosphorylated antibodies were prepared and acquired from Cell Signaling Technology, Inc. (Beverly, MA, USA). Signal inhibitors AG490 (a Janus kinase 2 inhibitor), U0126 (an MAPK kinase/ERK inhibitor) and SB203580 (a p38 inhibitor) were purchased from Cell Signaling Technology, Inc., and PDTC (an NF-κB inhibitor) was from EMD Millipore (Billerica, MA, USA). The primers used for polymerase chain reaction (PCR) analysis were prepared from Bioneer Corporation (Daejeon, Korea). Other chemicals were purchased commercially from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany).

The HUVECs (BMS, Seoul, Korea) were grown over three to six passages in cell culture medium at 37˚C under a humidified 95 and 5% (v/v) mixture of air and CO_2 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 3 ng/ml bFGF and 5 U/ml heparin. The U937 human monocyte-like cell line (American Type Culture Collection, Manassas, VA, USA; CRL-1593.2™) was also cultured in complete RPMI-1640 cell culture medium under the same conditions as the HUVECs.

Cell viability assay. The HUVECs ($1x10^4$ cells/well) were removed from the cell growth medium and incubated in serum-free medium for 18 h. These cells were then incubated with or without LPS (1 µg/ml) in the presence of each indicated CA concentration (0, 5, 10, 20 and 40 µM) for 48 h at 37˚C Cell viability was measured by the addition of 50 µg/ml 3-(4,5-dimetnythiazol-2-yl)-2,5-diphenyl-thetazolium bromide (MTT). Following incubation for 2 h, purple formazan had formed, the supernatants were removed from each well and 100 µl DMSO was added to completely dissolve the formazan crystals. Data was quantified by measuring the absorbance at 540 nm using a spectrophotometric multi-well microplate reader (Multiskan MS; Thermo Fisher Scientific, Inc.).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA from HUVECs was isolated according to the manufacturer's protocol using an RNA-Bee isolation kit (Tel-Test, Inc., Friendswood, TX, USA). The cDNA was prepared by reverse transcription using M-MuLV reverse transcriptase (Fermentas; Thermo Fisher Scientific, Inc.) and PCR amplification was performed according to the manufacturer's protocol using an AccuPower PCR PreMix (Bioneer Coporation; Daejeon, Korea). Using 250 ng of cDNA and 10 pmole/µl of forward and reverse primers, PCR was performed as follows: Intercellular adhesion molecule (ICAM)-1 (556 bp, annealing temp. 60˚C, 25 cycles), forward 5'-CAGTGACCA TCTACAGCTTTCCGG-3' and reverse 5'-GCTGCTACCACA GTGATGATGACAA-3'; VCAM-1 (742 bp, annealing temp. 62˚C, 32 cycles), forward 5'-AATTTATGTGTGTGAAGG AG-3' and reverse 5'-GCATGTCATATTCACAGAA-3'; IL-1β (264 bp, annealing temp. 62˚C, 33 cycles), forward 5'-GGA TATGGAGCAACAACTGG-3' and reverse 5'-ATGTACCAG TTGGGGAACTG-3'; IL-6 (497 bp, annealing temp. 54˚C, 30 cycles), forward 5'-TGACAAACAAATTCGGTACAT CC-3' and reverse 5'-ATCTGAGGTGCCCATGCTAC-3'; IL-8 (292 bp, annealing temp. 63˚C, 28 cycles), forward 5'-ATGACT TCCAAGCTGGCCGTGGCT-3' and reverse 5'-TCTCAG CCCTCTTCAAAAACTTCTC-3'; MCP-1 (261 bp, annealing temp. 62˚C, 30 cycles), forward 5'-TCTGTGCCTGCTGCT CATAG-3' and reverse 5'-TTTGCTTGTCCAGGTGGTCC-3'; and TNF-α (444 bp, annealing temp. 62˚C, 32 cycles), forward 5'-GACTGACAAGCCTGTAGCCCATGTTGTTGTAGCA-3' and reverse 5'-GCAATGATCCCAAAGTAGACCTGCCCA GAC-3'; β-actin (482 bp, annealing temp. 58˚C, 28 cycles), forward 5'-TGAGACCTTCAACACCCCAG-3' and reverse 5'-CACTGTGTTGGCGTACAGGT-3'. Each band intensity was detected for all bands and standardization relative β-actin and measured using ImageJ software (Ver.1.51e; National Institutes of Health, Bethesda, MD, USA).

Western blot analysis. The HUVECs were treated with cell lysis buffer to obtain cell extracts. Protein quantity was determined using a Bradford assay kit according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.) and equal quantities of protein (30 μg) were separated by performing electrophoresis on 6-15% SDS-PAGE gels. The proteins were then transferred onto a nitrocellulose membrane, which was gently agitated and washed with 3-5% non-fat dry milk in Tris-buffered saline-Tween-20 buffer, containing 0.5 mol/l Tris-HCl (pH 7.5), 0.15 mol/l NaCl and 1 g/l Tween-20. The membrane was incubated with each primary antibody overnight at 4˚C according to manufacturer's protocol followed by the secondary antibodies (1:2,000) 2 h at room temperature which was then visualized through enhanced chemiluminescence and exposure to X-ray films. The intensity of each band was measured as aforementioned using ImageJ software.

Luciferase reporter assay. VCAM-1 promoter region information was obtained by searching human VCAM-1 gene sequences (www.ncbi.nlm.nih.gov/), following which the pVCAM-1/Luc plasmid construct, containing the -1,350 to +45 bp promoter region in the pGL3-basic vector (Promega, Madison, WI, USA), was constructed. The HUVECs were transiently transfected with empty vector (pGL3-basic), constructed pVCAM-1/Luc or pNF-κB/Luc (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) using Lipofectamine™ 2000 reagent (Stratagene; Agilent Technologies, Inc.). Following 24 h of transfection, cell extracts were prepared and luciferase activity was detected using the *Renilla* luciferase assay kit according to the manufacturer's protocol (Promega). Data was quantified by measuring the fluorescence intensity at 480 nm (excitation) and 560 nm (emission) using a fluorescence multi-well microplate reader (Wallac 1420 Victor 2; PerkinElmer, Inc., Waltham, MA, USA).

Fluorescence microscopy analysis. Growth medium was removed from the HUVECs and serum-free medium containing CA was stimulated with or without LPS (1 μg/ml) for 2 h. The HUVECs were incubated overnight at 4˚C with the FITC-labeled NF-κB p65 antibody [(F-6) Alexa Fluor^{R}488; 65 kDa; 1:100; cat. no. SC-8008; Santa Cruz Biotechnology, Inc.], and then treated with 3.7% paraformaldehyde solution to preserve the cell state and then washed in PBS. The fluorescence intensities were analyzed for the distribution and translocation of p65 protein. The fixed cells were stained with mounting solution containing 4'-6-diamidino-2-phenylindole to indicate the location of nuclei in the cells.

Cell adhesion assay. The U937 cell line has the monocyte phenotype and can therefore be used in monocyte adhesion experiments, which were performed as described in a previous study (24). The HUVECs were cultured to confluence in 6-well plates and pre-treated with each signal inhibitor (10 μM) with CA at the indicated concentrations for 12 h. The U937 cells were cultured in 1% FBS and 5 μg/ml calcein-AM (Invitrogen; Thermo Fisher Scientific, Inc.) RPMI medium at 37˚C for 30 min for fluorescent labeling. The fluorescence-labeled U937 cells ($1x10^7$) were re-suspended in HUVEC culture medium and then incubated for 30 min with the pre-cultured HUVECs. The non-adherent fluorescent U937 cells were washed with PBS and the fluorescence intensity of attached cells was quantitated using a Wallace Victor2 1420 Multi-label counter (PerkinElmer, Inc.).

Statistical analysis. Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. The data are presented as the mean ± standard deviation and statistical comparisons between groups were performed using one-way analysis of variance followed by Student's t-tests using SigmaPlot software (version 11; Systat Software Inc., San Jose, CA, USA). $P<0.05$ was considered to indicate a statistically significant differences.

Results

CA inhibits the LPS-induced mRNA and protein expression of VCAM-1 in HUVECs. The present study examined cell viability using an MTT assay to determine the *in vitro* experimental concentration of CA. As shown in Fig. 1B and C, the cells were treated with CA for 24 h in the presence or absence of LPS. It was confirmed that treatment of the HUVECs with CA was not toxic up to a concentration of 40 μM CA. Based on this information, the mRNA expression levels of ICAM-1 and VCAM-1 were examined in the LPS-induced HUVECs at the indicated concentrations of CA. The results showed that CA did not affect the mRNA expression of ICAM-1, however, the mRNA expression of VCAM-1 was decreased in a concentration-dependent manner in the LPS-induced HUVECs (Fig. 1D). Subsequently, the results of western blot analysis showed that the protein expression levels of ICAM-1 and VCAM-1 were significantly increased by LPS stimulation in the HUVECs. Similar to the mRNA expression levels, CA markedly inhibited the protein expression of VCAM-1 and had no effect on the protein expression level of ICAM-1 in the LPS-stimulated HUVECs (Fig. 1E).

CA inhibits the pro-inflammatory cytokine expression in LPS-stimulated HUVECs. The present study subsequently examined the activity of CA on the mRNA expression levels of IL-1β, IL-6, IL-8, MCP-1 and TNF-α in HUVECs. In the LPS-stimulated HUVECs, the gene expression levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, MCP-1 and TNF-α increased ~6-9-fold, compared with those in the untreated control. In the CA-treated HUVECs, the mRNA level of each pro-inflammatory cytokine was significantly decreased in a CA concentration-dependent manner (Fig. 2).

Role of MAPKs in the inhibition of VCAM-1 by CA in LPS-induced HUVECs. To reveal the molecular mechanism for the inhibitory activity of CA on the expression of VCAM-1 and pro-inflammatory cytokines, the present study analyzed the phosphorylation of MAPKs in the LPS-induced HUVECs with or without CA exposure. The effects of CA and commercial signal inhibitors on the mRNA expression levels of VCAM-1 and pro-inflammatory cytokines were examined in the LPS-activated HUVECs. The HUVECs were treated with AG490 (a Janus kinase inhibitor), U0126 (an MEK/ERK inhibitor), SB203580 (a p38 inhibitor), PDTC (an NF-κB inhibitor) and at the indicated concentration of CA. After 1 h,

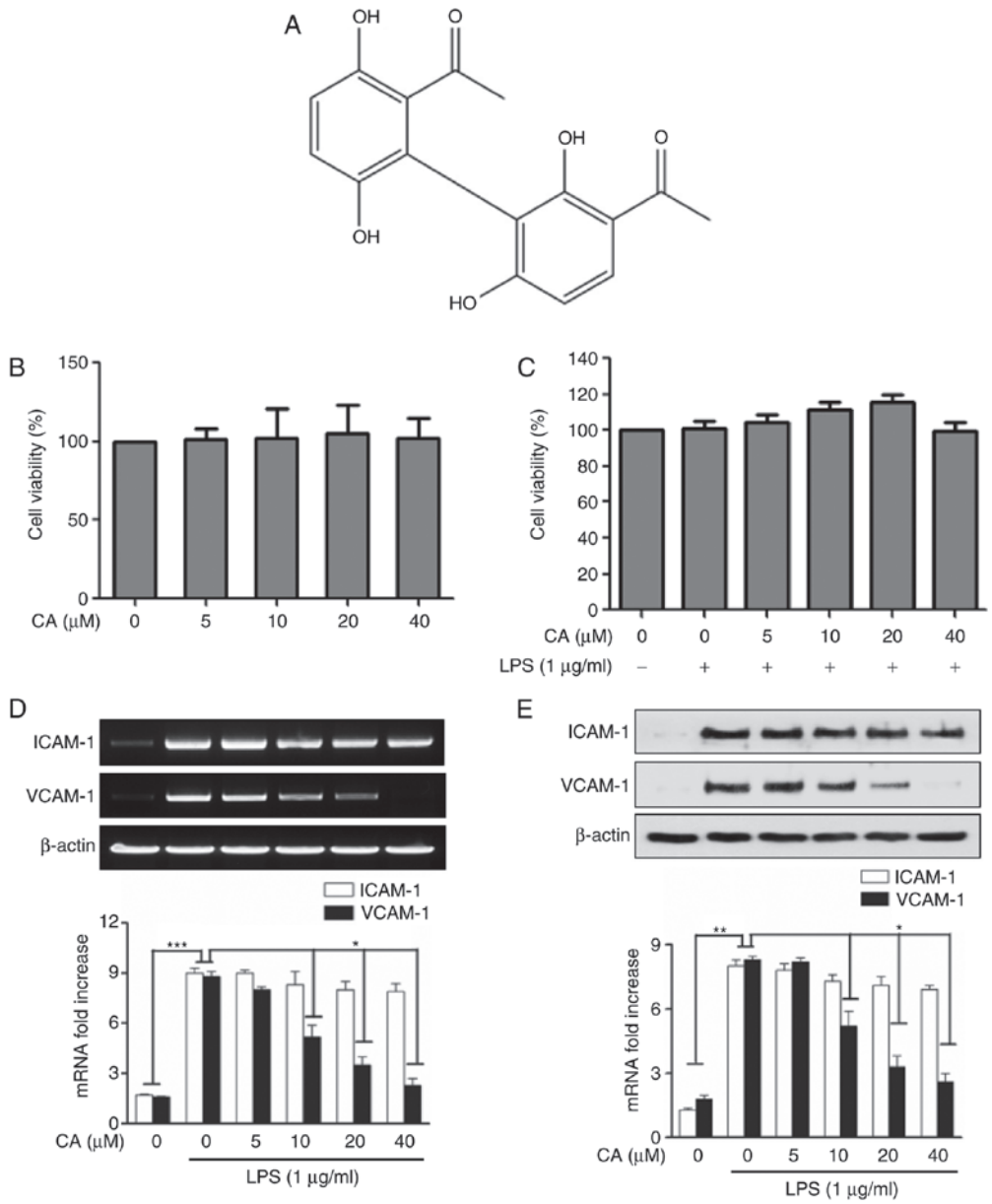

Figure 1. Effects of CA on mRNA and protein expression of cell adhesion molecules in LPS-stimulated human umbilical vascular endothelial cells. (A) Chemical structure of CA. Cell viability assessment of (B) CA-treated cells and (C) cells treated with CA+LPS was evaluated using a 3-(4,5-dimetnythiazol-2-yl)-2,5-diphenyl-thetazolium bromide assay. The cells were cultured for 24 h at each indicated CA concentration and the results were measured by optical density value. Results are shown as a percentage of the negative control, which was treated with medium alone. Each value is presented as the mean ± standard deviation. (D) Reverse transcription-quantitative polymerase chain reaction experiments were performed to compare mRNA levels of cell adhesion molecules. The values for intensity are presented as the mean ± standard deviation of three independent experiments. ***P<0.001, compared with treatment with medium alone; *P<0.05, compared with treatment with LPS alone. (E) Western blot analyses were performed to compare the expression levels of cell adhesion molecules. The intensity of each band was compared and data are presented as a graph. The values for intensity are presented the mean ± standard deviation from three independent experiments. **P<0.01, compared with treatment with medium alone; *P<0.05, compared with treatment with LPS alone. CA, cynandione A; LPS, lipopolysaccharide; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular adhesion molecule-1.

the incubated HUVECs in the commercial signal inhibitors and CA were stimulated with LPS. Our data showed that, with the exception of AG490, all the commercial signal inhibitors inhibited the mRNA and protein levels of VCAM-1 in LPS-induced HUVECs. CA inhibited the mRNA and protein expression levels of VCAM-1 to levels similar to those in the U0126, SB203580 and PDTC treatment groups (Fig. 3A). This result indicated that key signal inhibitors, including U0126, SB203580 and PDTC, inhibited the expression of VCAM-1 and the upstream signaling molecules of VCAM-1 in the LPS-stimulated HUVECs.

Subsequently, the present study examined the effect of CA on cellular signaling molecules activated by LPS-stimulation in HUVECs. The inhibitory activity of CA on the phosphorylation of the most well known MAPK pathways, including JNK, ERK1/2 and p38 proteins, were then confirmed in LPS-induced HUVECs. The results indicated that CA decreased the phosphorylation of ERK1/2 and p38 proteins in the LPS-stimulated HUVECs in a concentration-dependent manner, whereas CA had minimal effect on the phosphorylation of JNK (Fig. 3B). These results suggested that the inhibitory activity of CA on the expression of VCAM-1 may be associated with

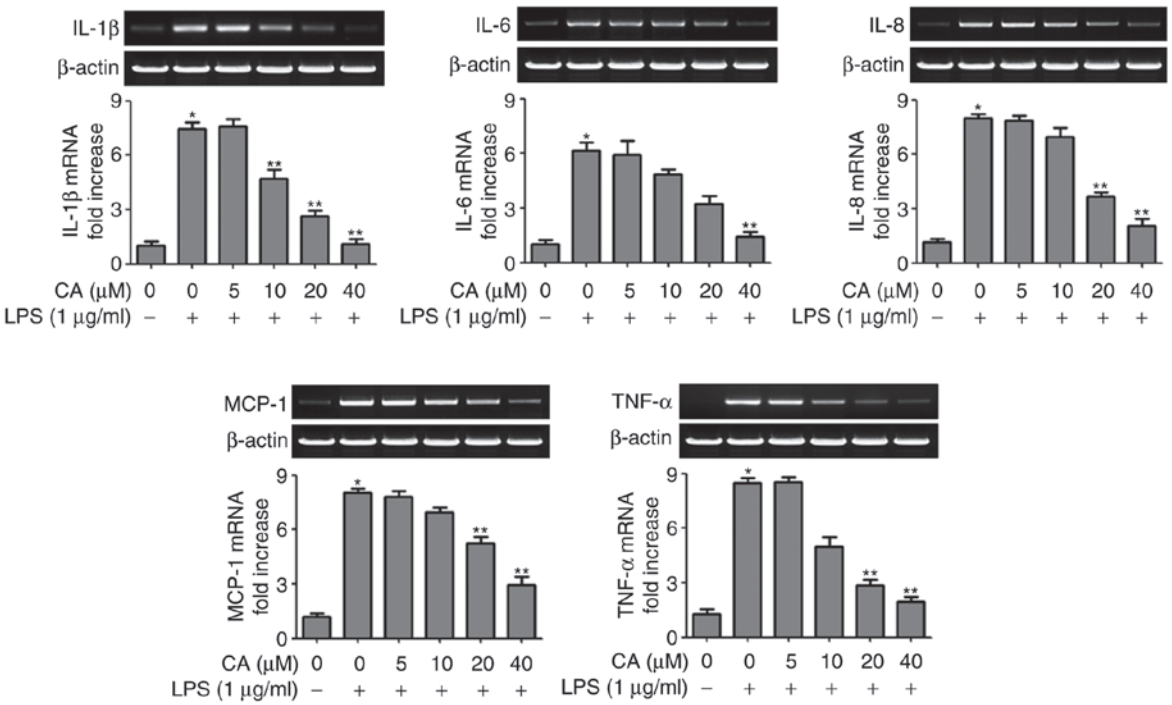

Figure 2. Inhibitory effect of CA on expression of LPS-induced pro-inflammatory cytokines and chemoattractant cytokines in HUVECs. Reverse transcription polymerase chain reaction analyses were performed to compare the mRNA expression levels of IL-1β, IL-6, IL-8, MCP-1 and TNF-α. HUVECs were pretreated with CA at the indicated concentration for 1 h and then treated with LPS. The values for intensity are presented as the mean ± standard deviation of three independent experiments. *P<0.01, compared with treatment with medium alone; **P<0.05, compared with treatment with LPS alone. HUVECs, human umbilical vascular endothelial cells; CA, cynandione A; LPS, lipopolysaccharide; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α.

inhibition of the phosphorylation of ERK1/2 and p38 kinase in LPS-stimulated HUVECs.

To analyze the effect of CA on MAPK downstream signaling pathways, the present study confirmed the activity of CA on the phosphorylation of IKK/IκB-α signal transduction. The NF-κB p65 subunit is an important transcription factor for the expression of VCAM-1 in LPS-stimulated HUVECs (25). The nuclear translocation of the p65 subunit is activated by the phosphorylation of IκB-α. Therefore, the effect of CA on the phosphorylation of IKK was examined. The results demonstrated that CA significantly inhibited the phosphorylation of IKK in LPS-stimulated HUVECs. In addition, it was found that CA inhibited the phosphorylation of IκB-α protein and suppressed the degradation of IκB-α protein in the cell cytosolic fraction (Fig. 3C). These results suggested that CA significantly inhibited IKK/IκB-α signal transduction. The protein levels of p65 in the nucleus of cells treated with CA in the LPS-stimulated HUVECs were examined. In the nuclei of resting state cells, a basic level of p65 protein was identified, and in the cells treated with LPS alone, the nuclear translocation of p65 subunit was significantly increased. In cells stimulated with LPS in the presence of CA, it was confirmed that the protein expression of p65 was decreased in a concentration-dependent manner. The reduced protein expression of p65 was similar to that in the non-stimulated cells (Fig. 3D). The inhibitory activity of CA on the expression of VCAM-1 and pro-inflammatory cytokines may be due to the decrease in nuclear p65 translocation through inhibition of the phosphorylation of MAPK/IKK/IκB-α and degradation of IκB-α.

CA inhibits LPS-induced transcription of the VCAM-1 promoter and activation of NF-κB. The present study investigated whether CA regulates the transcriptional activity of the VCAM-1 gene using VCAM-1 luciferase reporter gene constructs. The transfected HUVECs were treated with or without CA and then activated with LPS. Stimulation of the transfected cells with LPS promoted luciferase activity by ~5-6-fold over that in the mock transfected cells. CA significantly reduced the LPS-activated induction of VCAM-1 in the transfected HUVECs at the transcriptional level (Fig. 4A). The regulatory activity of CA on transcriptional activation was examined using the established pNF-κB/Luc reporter gene construct by LPS stimulation. As shown Fig. 4B, the results demonstrated that CA reduced transcriptional activity by LPS in a dose-dependent manner in the HUVECs transfected with the pNF-κB/Luc reporter gene construct. To analyze the activity of CA on the translocation of the p65 subunit into the nucleus, fluorescence microscopy was used to analyze the HUVECs treated with or without CA. It was confirmed that the fluorescence intensity in the nucleus was increased in cells treated with LPS alone, and a large quantity of p65-FITC antibody was present in the nucleus. By contrast, CA decreased fluorescence intensity in the nucleus by LPS stimulation, and a similar result was observed in the cells treated with PDTC, which is an NF-κB specific signaling inhibitor (Fig. 4C).

CA inhibits the LPS-induced adhesion of U937 cells to HUVECs. The overproduction of cell adhesion molecules, including VCAM-1, by LPS likely increases the adherence of monocytes to endothelial cells in the early stages of vascular

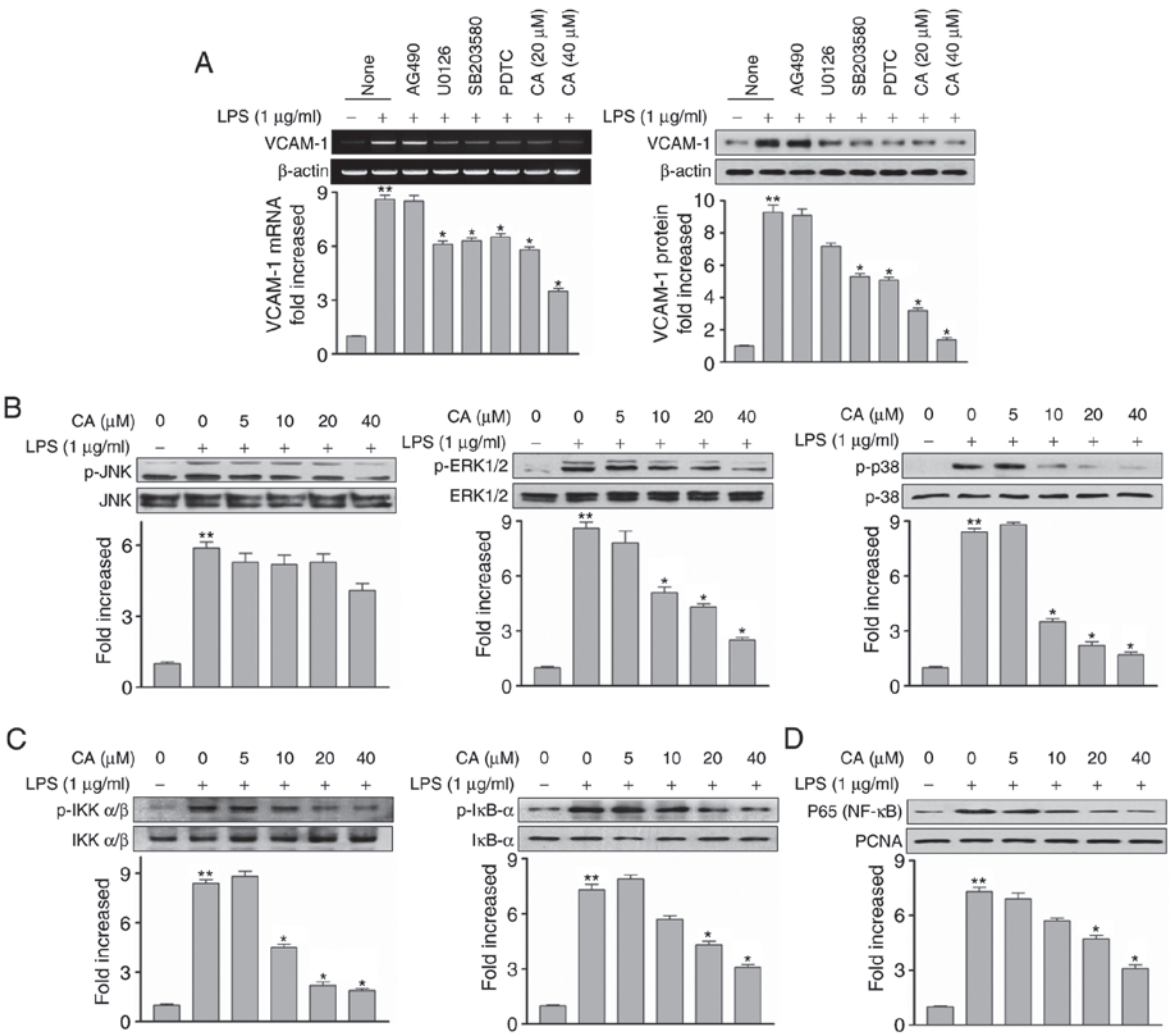

Figure 3. Effects of CA on the phosphorylation of mitogen-activated protein kinase/IKK/IκB and analysis of the translocation of p65 into the nucleus of LPS-activated HUVECs. (A) HUVECs were pretreated with the indicated signal inhibitor, AG490 (10 μM), U0126 (10 μM), SB203580 (10 μM) or PDTC (10 μM) for 1 h, and stimulated with LPS with or without CA for 24 h. mRNA and protein expression levels of VCAM-1 were detected using reverse transcription-quantitative polymerase chain reaction and western blot analyses, respectively. (B) HUVECs were starved for 6 h and then pre-treated with CA for 1 h, followed by activation by LPS for 30 min. Phosphorylation patterns were analyzed using western blot analysis. (C) HUVECs were starved for 6 h and then pre-treated with CA for 1 h, followed by activation by LPS for 30 min. The activation levels of p-IKK, p-IκB-α, IKK and IκB-α were detected using western blot analysis. (D) HUVECs were starved for 6 h and then pre-treated with CA for 1 h, followed by activation with LPS for 2 h. Proteins in the nuclear extract fraction were prepared and NF-κB p65 subunit translocation levels were analyzed using western blot analysis. **P<0.01, compared with the negative control; *P<0.05, compared with treatment with LPS alone. HUVECs, human umbilical vascular endothelial cells; CA, cynandione A; LPS, lipopolysaccharide; VCAM-1, vascular adhesion molecule-1; NF-κB, nuclear factor-κB; IκB, inhibitor of NF-κB; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; p-, phosphorylated.

inflammation. Therefore, in order to demonstrate the effect of CA on U937 cell adhesion to endothelial cells, the present study compared cell adhesion activity with various signal inhibitors in the presence of CA in the LPS-induced HUVECs. In the case of cells treated with LPS alone, the adhesion activity of the U937 cells was markedly increased compared with that in the control cells. The data showed that U0126, SB203580 and PDTC significantly inhibited U937 adhesion in the LPS-induced HUVECs, whereas AG490 had no effect. The LPS-activated HUVECs were treated with CA (20 or 40 μM), and it was shown that CA inhibited U937 adhesion to the HUVECs (Fig. 5). The data showed that CA inhibited the LPS-induced expression of VCAM-1 and suppressed U937 adhesion to HUVECs.

Discussion

In the present study, it was found that CA had anti-adhesive activity with regard to the adhesion of U937 monocytes to HUVECs via suppressing the expression of VCAM-1 and pro-inflammatory mediators in LPS-stimulated HUVECs. The inhibitory activity of CA on the expression of cell adhesion molecules was examined in LPS-induced HUVECs, and it was demonstrated that CA treatment inhibited the mRNA and protein expression levels of VCAM-1. The inhibition of VCAM-1 protein expression in LPS-induced HUVECs was due to the fact that CA inhibited the phosphorylation and degradation of IκB-α, the activation of NF-κB, and the translocation of NF-κB p65 subunit to the nucleus. Therefore, the

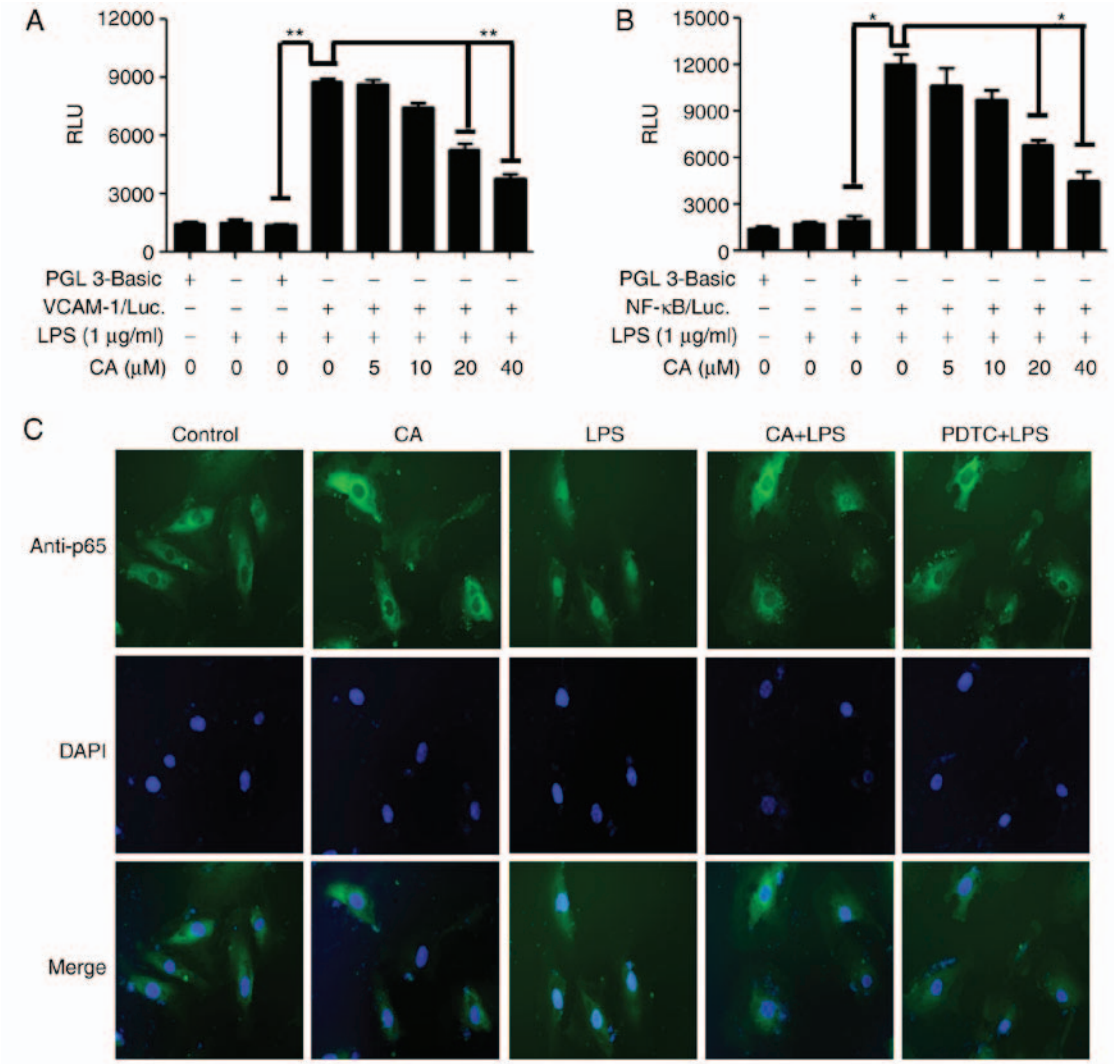

Figure 4. Effects of CA on the transcriptional activity of NF-κB and luciferase reporter genes in transiently transfected HUVECs. (A) pVCAM-1/Luc transfected HUVECs were cultured for 24 h, and treated with indicated concentrations of CA and 1 μg/ml LPS for 24 h. The values for relative luciferase intensity are shown as the mean ± standard deviation of three independent experiments (n=3). **P<0.01, compared with the mock transfectant; **P<0.01, compared with treatment with LPS alone. (B) pNF-κB/Luc transfected HUVECs were cultured for 24 h, and treated with indicated concentrations of CA and 1 μg/ml LPS for 24 h. The values for relative luciferase intensity are shown as the mean ± standard deviation from three independent experiments (n=3). *P<0.05, compared with the mock transfectant; *P<0.05, compared with treatment with LPS alone. (C) Nuclear translocation of NF-κB p65 subunit was analyzed following CA (40 μM) and PDTC (10 μM) treatment for 1 h. Cells were activated for 2 h with LPS and then observed via fluorescence microscopy (magnification, x100). HUVECs, human umbilical vascular endothelial cells; CA, cynandione A; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; VCAM-1, vascular adhesion molecule-1; Luc, luciferase.

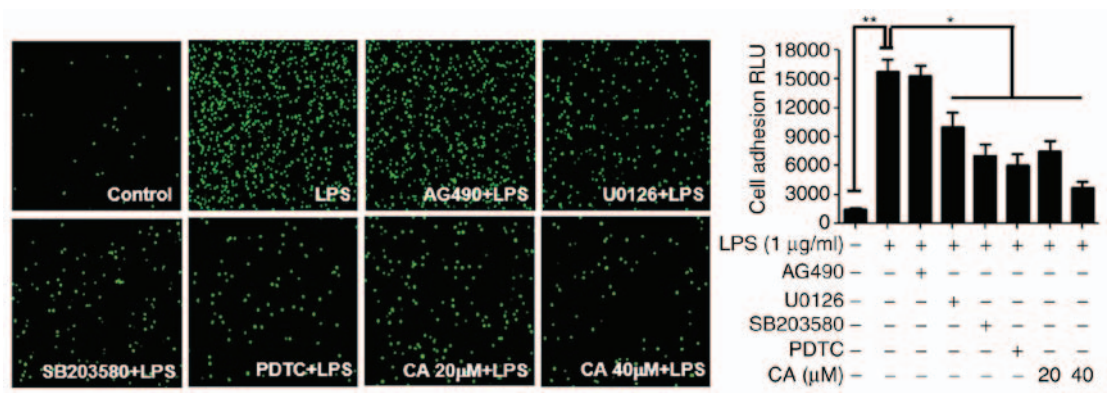

Figure 5. CA reduces U937 monocyte adhesion to endothelial cells. Cells were pretreated with or without each signal inhibitor (10 μM) and CA at the indicated concentration for 12 h. Adhesion of U937 cells to HUVECs was quantitatively measured by the fluorescence intensity of FITC-labeled U937 cells adherent to LPS-activated HUVECs and then observed via fluorescence microscopy (x40). The values for relative luciferase intensity are shown as the mean ± standard deviation of three independent experiments (n=3). **P<0.01, compared with the negative control; *P<0.05, compared with treatment with LPS alone. HUVECs, human umbilical vascular endothelial cells; CA, cynandione A; LPS, lipopolysaccharide.

anti-adhesion effect of U937 monocytes to vascular endothelial cells by CA was the result of the inhibition of vascular inflammation, which prevented activation of the NF-κB signaling pathway in HUVECs.

Chronic vascular inflammation is a main cause of various vascular inflammatory diseases. In vascular inflammatory sites, endothelial cells stimulated by inflammatory stimuli, including LPS, are known to interact with other leukocytes through a variety of adhesion molecules, including VCAM-1, inflammatory cytokines and chemokines (2,26). The firm adhesion of leukocytes to the endothelium is critical in early atherosclerotic plaque development and in vascular inflammation. LPS is a component of the Gram-negative bacterial cell membrane and is known to induce inflammation *in vivo*. According to previous reports, LPS is known to increase the expression and secretion of cytokines, including IL-1β, IL-6, IL-8, MCP-1 and TNF-α, in HUVECs (27-29). IL-8 and MCP-1 are chemoattractant cytokines, which are involved in the accumulation of monocytes in vascular inflammation plaque lesions (30). TNF-α and other inflammatory stimuli have been reported to accrete the production of VCAM-1 in endothelial cell membranes, which can promote leukocyte-to-endothelium adhesion in vascular plaques (31). Therefore, inhibiting the secretion of pro-inflammatory cytokines and the expression of cell adhesion molecules may be important for the development of therapeutic agents for atherosclerosis caused by vascular inflammation.

The expression of VCAM-1, pro-inflammatory cytokines and chemoattractant cytokines, including IL-8 and MCP-1, in LPS-induced cells is regulated by the MAPK/IKK/IκB signaling cascade and activation of NF-κB (9,10,32). In addition, interactions with transcription factor binding elements, including NF-κB, can affect the activation of cell adhesion molecules and inflammatory mediators by LPS stimulation (32). In inflammatory conditions, the present study confirmed that CA inhibited the expression of VCAM-1 and pro-inflammatory cytokines. The results following signal inhibitor treatment demonstrated that ERK1/2 and p38 kinase were more important than JAK2 in the LPS-induced expression of VCAM-1. To characterize the mechanism underlying the effect of CA in LPS-induced HUVECs, the present study analyzed the phosphorylation pattern of the MAPK/NF-κB signaling pathway. CA markedly inhibited the phosphorylation of ERK1/2 and p38, and weakly inhibited the phosphorylation of JNK. This result indicated that CA served as a selective phosphorylation inhibitor of ERK1/2 and p38 kinase. It was also shown that CA affected the inhibition of the phosphorylation of IKK/IκB-α, which is a downstream signaling pathway of MAPKs (14,15). These data demonstrated that CA acted as a specific phosphorylation inhibitor of upstream molecules, including IKK and MAPKs, in the NF-κB pathway.

In the resting state, the NF-κB protein has two subunits, p65 and p50, which exist as a heterotrimer with IκB protein in the cytoplasm. In the presence of external stimuli, the IκB protein is activated via phosphorylation, and the p65 subunit is released and translocated to the nucleus for target gene activation (33). The present study showed that CA inhibited nuclear translocation of the p65 subunit, and the results suggested that CA had an inhibitory effect on the transcriptional activity of NF-κB using a NF-κB/Luc reporter gene. The luciferase reporter assays demonstrated that CA suppressed the expression of the VCAM-1/Luc reporter gene in the LPS-stimulated HUVECs. The upregulation of VCAM-1 had functional effects, specifically the adhesion of monocytes to the endothelium in vascular inflammatory lesions. It was found that CA suppressed U937 monocyte adhesion to activated HUVECs by the inhibition of MAPK/IKK/IκB-α, which correlated with the decreased expression of VCAM-1. These results provided additional evidence for the involvement of the MAPK/IKK/IκB/NF-κB signaling pathway in LPS-induced monocyte adhesion.

According to previous reports, natural compounds, including flavonoids, phytosteroids and small phenolic molecules, have various pharmacological activities, including anti-inflammatory activity (34,35). CA is an acetophenone isolated from the roots of *Cynanchum* extracts, and is known to be a natural compound with effective anti-oxidant, anti-inflammatory and neuroprotective effects (19,20).

In this present study, it was demonstrated that CA markedly inhibited the expression of LPS-induced VCAM-1 in HUVECs. The result showed that the LPS increased the expression levels of ICAM-1 and VCAM-1 in the HUVECs. However, it was confirmed that CA only inhibited the expression of VCAM-1 (Fig. 1). The activation of ICAM-1 by endotoxins, including LPS, in HUVECs has been demonstrated in a number of studies (36). In particular, the inhibition of cell adhesion molecules using *C. wilfordii* root ethanol extract and its active ingredients have shown no effect on the expression of ICAM-1 (18). Taken together, the results of the present and previous studies suggest that CA likely has no significant effect on the expression of ICAM-1. The present study further examined the inhibition of the expression of VCAM-1 at the molecular levels and suggested a mode of action in the anti-adhesion activity of CA. CA decreased the levels of IL-1β, IL-6, TNF-α, IL-8 and MCP-1 induced by LPS. At the molecular level, CA acted to inhibit the activation of NF-κB by inhibiting the phosphorylation of IKK/IκB-α, and inhibiting p38 and ERK1/2 MAPKs. In addition, CA substantially inhibited the adhesion of U937 to HUVECs in a dose-dependent manner. These results suggested that CA may be used as a novel drug candidate for the treatment of vascular inflammation by the inhibition of monocyte infiltration.

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Competing interests

The authors declare that they have no competing interests.

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