

Original Article

Ulmus davidiana ethanol extract inhibits monocyte adhesion to tumor necrosis factor-alpha-stimulated endothelial cellsKi Mo Lee^{a,1}, Hee Kyoung Joo^{a,1}, Yu Ran Lee^a, Myoung Soo Park^a,
Gun Kang^a, Sunga Choi^a, Kwon Ho Lee^b, Byeong Hwa Jeon^{a,*}^a Research Institute for Medical Sciences, Department of Physiology, School of Medicine, Chungnam National University, Daejeon, Korea^b Department of Physical Therapy, Joongbu University, Geumsan, Korea

ARTICLE INFO

Article history:

Received 13 January 2016

Received in revised form

26 February 2016

Accepted 25 March 2016

Available online 31 March 2016

Keywords:

endothelial cells

monocyte adhesion

Ulmus davidiana var. *japonica*

vascular cell adhesion molecule-1

ABSTRACT

Background: *Ulmus davidiana* var. *japonica* Rehder (UD) has long been used in traditional folk medicine in Asia. This study is designed to investigate the antiadhesive activity of the ethanol extract of UD (UDE) and its underlying mechanisms in cultured endothelial cells.

Methods: The dried root bark of UD was extracted with 80% (v/v) ethanol. The antiadhesive activity of the UDE was investigated in cultured human umbilical vein endothelial cells and human embryonic kidney epithelial 293T (HEK 293T) cells stably transfected with pGL3-vascular cell adhesion molecule (VCAM)-1-luc. Monocyte adhesion in endothelial cells was induced by tumor necrosis factor-alpha (TNF- α), and the protective effects of UDE on monocyte-endothelial cell adhesion, VCAM-1 expression, reactive oxygen species production, and nuclear factor- κ B activity were determined.

Results: Exposure to UDE at a concentration of 3–30 μ g/mL for 24 hours produced no detectable cytotoxicity in human umbilical vein endothelial cells, but it significantly inhibited TNF- α -induced monocyte adhesion and VCAM-1 expression. TNF- α treatment of HEK 293T/VCAM-1-luc cells resulted in increased luciferase activity of the VCAM-1 promoter, which was inhibited by treatment with UDE. Additionally, TNF- α -induced reactive oxygen species generation, nuclear translocation of nuclear factor- κ B, and I κ B α degradation in human umbilical vein endothelial cells were effectively reduced by treatment with 30 μ g/mL of UDE.

Conclusion: Our results indicated that UDE treatment inhibited TNF- α -induced monocyte adhesion in endothelial cells, suggesting that UD may reduce vascular endothelial inflammation.

© 2016 Korea Institute of Oriental Medicine. Published by Elsevier. This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

* Corresponding author. Department of Physiology, School of Medicine, Chungnam National University, 266 Munhwa-ro, Jung-gu, Daejeon 301-747, Korea.

E-mail address: bhjeon@cnu.ac.kr (B.H. Jeon).

¹ These authors contributed equally to this work.

<http://dx.doi.org/10.1016/j.imr.2016.03.006>

2213-4220/© 2016 Korea Institute of Oriental Medicine. Published by Elsevier. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

The vascular endothelium plays a central role in the regulation of vascular homeostasis. Alterations in endothelial function have been linked to a variety of inflammatory diseases, including atherosclerosis and diabetes mellitus.¹ Proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α), induce endothelial dysfunction by causing an increase in the expression of cell-surface adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1. These changes facilitate the adhesion of circulating blood cells, including neutrophils, leukocytes, and monocytes, to activated endothelial cells, ultimately leading to endothelial dysfunction.²

Monocyte rolling and adhesion to vascular endothelial cells are initial steps in the development of vascular inflammation.³ Among the adhesion molecules, VCAM-1 is a key molecule in the recruitment of leukocytes to endothelial cells. Upregulated VCAM-1 expression in endothelial cells is associated with several cardiovascular diseases that involve vascular inflammation. Nuclear factor (NF)- κ B signaling also plays a pivotal role in vascular inflammation.⁴

Ulmus davidiana var. *japonica* Rehder (Urticales: Ulmaceae) (UD) has traditionally been used for anticancer and anti-inflammatory therapy in Asia.^{5,6} Several compounds have been extracted from the stem and root barks of UD, and their functions have been investigated.^{7–9} In previous reports, an extract of UD (UDE) was found to attenuate ovalbumin-induced airway inflammation by increasing hemoxygenase-1 expression, and methanol extracts of UD showed an antiangiogenic effect.^{10,11} Recently, UDE was reported to induce vasorelaxation in an endothelium-dependent manner in rat aorta, suggesting that the vascular endothelium is the target tissue of UDE and that it has a protective effect against endothelial dysfunction.¹² However, the effect of UDE on monocyte adhesion to the endothelial cells remains unclear. This study aimed to examine the protective effect of UDE on TNF- α -induced monocyte adhesion and elucidate its underlying mechanism.

2. Materials and methods

2.1. Materials

The human monocyte cell line U937 and the human embryonic kidney cell line [human embryonic kidney epithelial 293T (HEK 293T)] were obtained from the American Type Culture Collection (Rockville, MD, USA), and human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Walkersville, MD, USA). Dulbecco's modified Eagle's medium, fetal bovine serum, and antibiotics were purchased from Gibco (Grand Island, NY, USA), and an endothelial growth medium (EGM-2) SingleQuot kit was purchased from Lonza. Human TNF- α was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dichlorofluorescein diacetate (H₂DCFDA) and 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester probes were purchased from Molecular Probes (Eugene, OR, USA). Antibodies against VCAM-1, NF- κ B p65, I κ B α , poly (ADP-ribose) polymerase (PARP), and glyceraldehyde

3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and β -actin antibody was purchased from Sigma-Aldrich.

2.2. Cell cultures and treatment with UD ethanol extracts

HUVECs were cultured in an endothelial growth medium (EGM-2), and the cells were used for up to six passages, according to the manufacturer's instructions. U937 and HEK 293T cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and antibiotics at the manufacturer's recommended concentration. Each cell line was incubated in an atmosphere of 95% air and 5% CO₂ at 37 °C. Ethanol extract of UD was prepared as described previously.¹² The freeze-dried UDE was thoroughly dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/mL prior to use in experiments. HUVECs at 80–90% confluence were treated with various concentrations of UDE (1–30 μ g/mL). As a vehicle-negative control, HUVECs were incubated in a culture medium containing DMSO (0.1%, v/v). Then HUVECs were stimulated with 15 ng/mL of TNF- α .

2.3. Cell viability measurement using bioluminescent assay

The effect of UDE on the viability of HUVECs was analyzed using a RealTime-Glo MT luminescent kit (Promega, Madison, WI, USA) in an opaque-walled assay plate, according to the manufacturer's instructions. The permeable viability prosubstrate in live cells is reduced to a luciferase substrate by metabolically active cells, while dead cells neither reduce the substrate nor produce a signal. The reduced substrate diffuses from cells into the surrounding culture medium and is then rapidly used by luciferase to produce a luminescent signal, which shows a correlation with viability. HUVECs in the assay-plate were treated with various concentrations of UDE (1–30 μ g/mL) for 24 hours. To continuously monitor the viability of the treated HUVECs in real time, the prosubstrate and luciferase were added at the same time as the UDE. Luminescence intensity at the desired time points was measured using a luminometer (Thermo Scientific, Rockford, IL, USA). Each sample was analyzed at least in triplicate.

2.4. Measurement of reactive oxygen species

The intracellular reactive oxygen species (ROS) level was measured using a membrane-permeant H₂DCFDA fluorogenic probe, as described previously.¹³ Briefly, 1×10^5 cells were seeded in a 12-well plate and allowed to attach overnight. The cells were pretreated with various concentrations of UDE (1–30 μ g/mL) for 1 hour and then stimulated with 15 ng/mL of TNF- α for 3 hours. Subsequently, the cells were stained with 5 μ M H₂DCFDA for 30 minutes at 37 °C. The cells were collected, and fluorescence was measured using a Fluoroskan Ascent microplate reader (Thermo Scientific) at wavelengths of 485 nm for excitation and 530 nm for emission.

2.5. Monocyte–endothelial cell adhesion assay

The monocyte–endothelial cell adhesion assay was performed as previously described.¹⁴ Briefly, U937 monocytes (1×10^7 cells/mL) were incubated with $1 \mu\text{M}$ 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester for 30 minutes at 37°C in Dulbecco's modified Eagle's medium. HUVECs (3×10^4 cells/well) were seeded in 96-well plates and grown until they reached confluent monolayers, and then pretreated with various concentrations of UDE (1 – $30 \mu\text{g/mL}$) for 1 hour, followed by stimulation with 15 ng/mL of TNF- α for 18 hours. The fluorescent-labeled U937 monocytes were then added to the TNF- α -stimulated HUVECs and incubated for further 2 hours. After washing out the unbound U937 three times, the amount of adherent monocytes was analyzed by measuring the fluorescent intensity at wavelengths of 485 nm for excitation and 530 nm for emission. Wells containing HUVECs alone were used as blanks.

2.6. Immunoblotting

Cells were harvested and the cell lysates were prepared as previously described.¹⁵ The total protein concentration was measured using the Bradford assay. For some experiments, nuclear and cytosolic fractions were prepared using a cell fractionation kit (Abcam, Cambridge, UK).¹³ The purity of the nuclear fraction was confirmed using an antibody against the nuclear marker PARP. The same concentration of protein was resolved by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane. Immunoblotting was performed using anti-VCAM-1, NF- κB p65, I $\kappa\text{B}\alpha$, PARP, GAPDH, and β -actin antibodies. The intensity of bands was quantified in an EpiChem 3 Darkroom using LabWorks software (UVP, Upland, CA, USA), followed by normalization of band densities to β -actin.

2.7. Immunocytochemistry

Immunocytochemical localization of the NF- κB p65 subunit was performed as previously described.¹⁶ HUVECs were cultured on glass coverslips in 12-well plates and treated with TNF- α after pretreatment with $10 \mu\text{g/mL}$ UDE. After washing with phosphate-buffered saline (PBS), cells were fixed with 4% (w/v) paraformaldehyde and permeabilized with 0.1% (v/v) Triton X-100. After blocking for 1 hour with PBS containing 2% (w/v) bovine serum albumin and 5% (v/v) horse serum, cells were incubated with NF- κB p65 antibody (1:500) overnight at 4°C and then labeled with Alexa Fluor 488-conjugated secondary antibody (1:500) for 1 hour in the dark at room temperature. Images were obtained by fluorescence microscopy.

2.8. Luciferase assay using in vitro imaging

For this experiment, HEK 293T cells were cotransfected with pGL3.1 VCAM-1 promoter firefly luciferase reporter plasmid and pcDNA4, and selected by 0.4 mg/mL Zeocin in culture for 4 weeks. To establish a clonal population stably expressing a homogenous level of luciferase, a couple of different clones were isolated to represent different intensities of luciferase activity. Following expansion, each clone was analyzed using

a Lumina XRMS in vitro imaging system (PerkinElmer Inc., Waltham, MA, USA) to ensure luciferase activity. The HEK 293T/VCAM-1-luc cells were plated in an opaque-walled 96-well plate (4×10^4 cells/well) and allowed to attach overnight. The cells were incubated with UDE in a concentration-dependent manner for 1 hour, followed by TNF- α stimulation and then further incubation for 6 hours. Subsequently, the cells were treated with luciferin for 30 minutes at 37°C , and the resulting biophotonic reaction was measured using the Lumina XRMS imaging system and analyzed using Living Image Software 4.4 (PerkinElmer Inc.).

2.9. Statistical analysis

The data are expressed as the mean \pm standard error of the mean (SEM). Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's multiple comparison tests using Prism 5 software (GraphPad, La Jolla, CA, USA), and $p < 0.05$ was considered significant.

3. Results

3.1. Effect of UDE on the viability of HUVECs

To investigate the potential cytotoxic effects of UDE, the effect of UDE treatment on the viability of HUVECs was examined using a bioluminescent assay. HUVEC viability was consistently high at the desired treatment concentrations of UDE (1 – $30 \mu\text{g/mL}$), but decreased viability was detected at $50 \mu\text{g/mL}$ of UDE (Fig. 1). HUVECs demonstrated more than 95% viability at $30 \mu\text{g/mL}$ UDE, indicating that it did not cause nonspecific

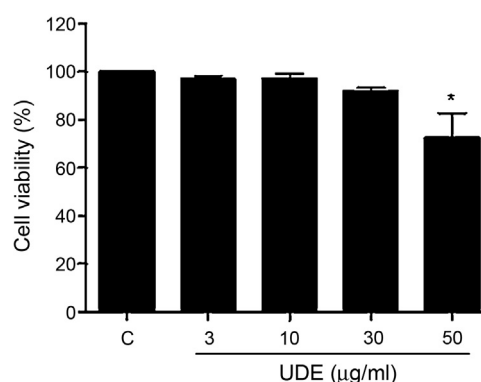


Fig. 1 – UDE treatment did not cause any detectable reduction of cell viability at 3–30 $\mu\text{g/mL}$ in HUVECs. The effect of UDE treatment for 24 hours on HUVEC viability at the indicated concentrations (0–50 $\mu\text{g/mL}$) was determined by a bioluminescent assay and then compared with that of DMSO-treated control cells. Data are presented as means \pm SEM ($n = 3$). Similar results were observed in replicate experiments.

* $p < 0.05$, compared with DMSO-treated control cells, as calculated by one-way ANOVA followed by Dunnett's test. ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; HUVEC, human umbilical vein endothelial cell; SEM, standard error of the mean; UDE, *U. davidiana* extract.

cytotoxicity at the highest concentration used for treatments (Fig. 1). The treated HUVECs were also observed under phase-contrast microscopy, and no effects on HUVEC morphology could be observed after UDE treatment even at 30 $\mu\text{g}/\text{mL}$ (data not shown). Collectively, it was determined that UDE treatment did not affect HUVEC viability adversely, and concentrations of < 30 $\mu\text{g}/\text{mL}$ can be considered safe.

3.2. Effect of UDE on monocyte adhesion in TNF- α -stimulated HUVECs

Recently, much evidence for the anti-inflammatory biological activity of natural agents has been reported in vascular inflammation models. The anti-inflammatory activity of these agents has generally been reported to be mediated by the inhibition of ROS generation, expression of adhesion molecules, and NF- κB activity, leading to reduced leukocyte adherence in the process of leukocyte trafficking. We determined the effect of UDE treatment on monocyte adhesion to endothelial cells. HUVECs were pretreated with the UDE (3–30 $\mu\text{g}/\text{mL}$) and then incubated with TNF- α for 18 hours. Adhesion of fluorescently labeled monocytes to stimulated

endothelial cells was observed under fluorescent microscopy (Fig. 2A). TNF- α -induced monocyte adhesion was inhibited by UDE treatment in a concentration-dependent manner. Exposure to 30 $\mu\text{g}/\text{mL}$ UDE resulted in a 21% decrease in monocyte adhesion compared with that in the DMSO-treated control cells (Fig. 2B). The decrease in fluorescence intensity induced by UDE treatment was found to occur in a concentration-dependent manner. These results indicated that UDE may inhibit monocyte adherence to the endothelial cell surface.

3.3. Effects of UDE on the adhesion molecule VCAM-1

During the process of monocyte trafficking, monocyte adherence is largely controlled by the regulation of surface-expressed, cell adhesion molecules in response to secreted chemokines. To examine whether UDE treatment modified monocyte adhesion by causing an altered expression of cell-surface adhesion molecules, we determined the expression level of the adhesion molecule VCAM-1 on the surface of TNF- α -stimulated HUVECs. Exposure of HUVECs to TNF- α induced a significant upregulation of VCAM-1 expression (Fig. 3A, 3B). Treatment with UDE caused a significant inhibition

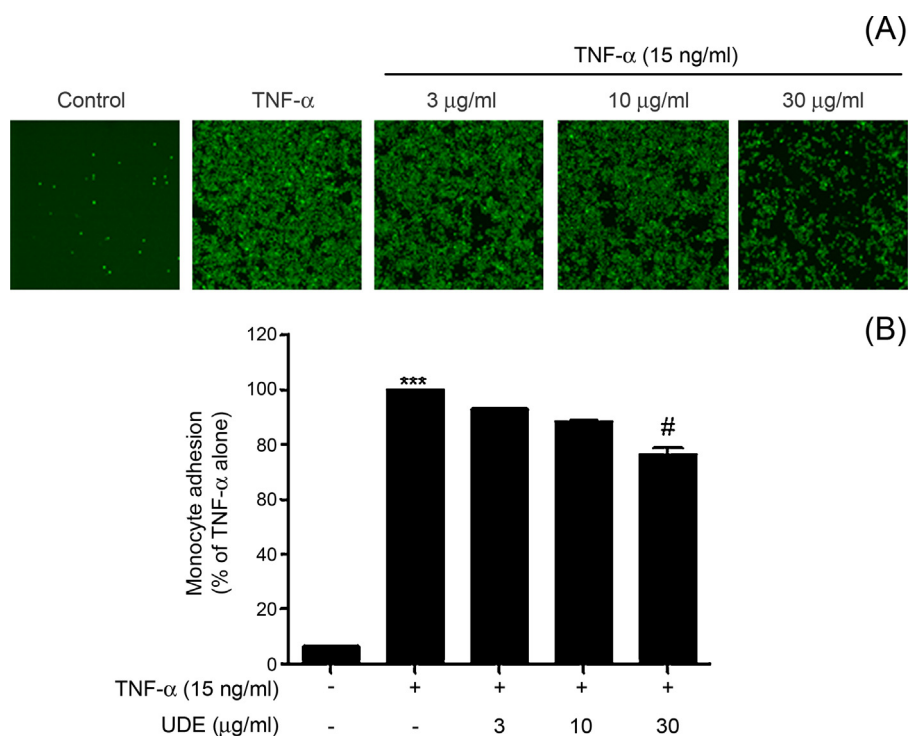


Fig. 2 – Quantitative adhesion assay of U937 monocytes to TNF- α -stimulated HUVECs. HUVECs were pretreated with various concentrations of UDE (0–30 $\mu\text{g}/\text{mL}$) for 1 hour followed by treatment with TNF- α for 18 hours. Fluorescent-labeled U937 monocytes were added onto TNF- α -stimulated HUVECs and then incubated for 90 minutes. (A) Fluorescent-labeled U937 cells adhering to the HUVECs were observed under a fluorescent microscopy at 100 \times magnification. (B) The number of U937 cells adhering to HUVECs was determined using a fluorometer. Data are representative of three independent experiments with similar results. Results are presented as means \pm SEM ($n = 3$).

* $p < 0.001$, compared with untreated control cells, as calculated by one-way ANOVA followed by Bonferroni's multiple comparison tests.

† $p < 0.01$, compared with control cells treated with TNF- α -only, as calculated by one-way ANOVA followed by Bonferroni's multiple comparison tests.

ANOVA, analysis of variance; HUVEC, human umbilical vein endothelial cell; SEM, standard error of the mean; TNF- α , tumor necrosis factor-alpha; UDE, *U. davidiana* extract.

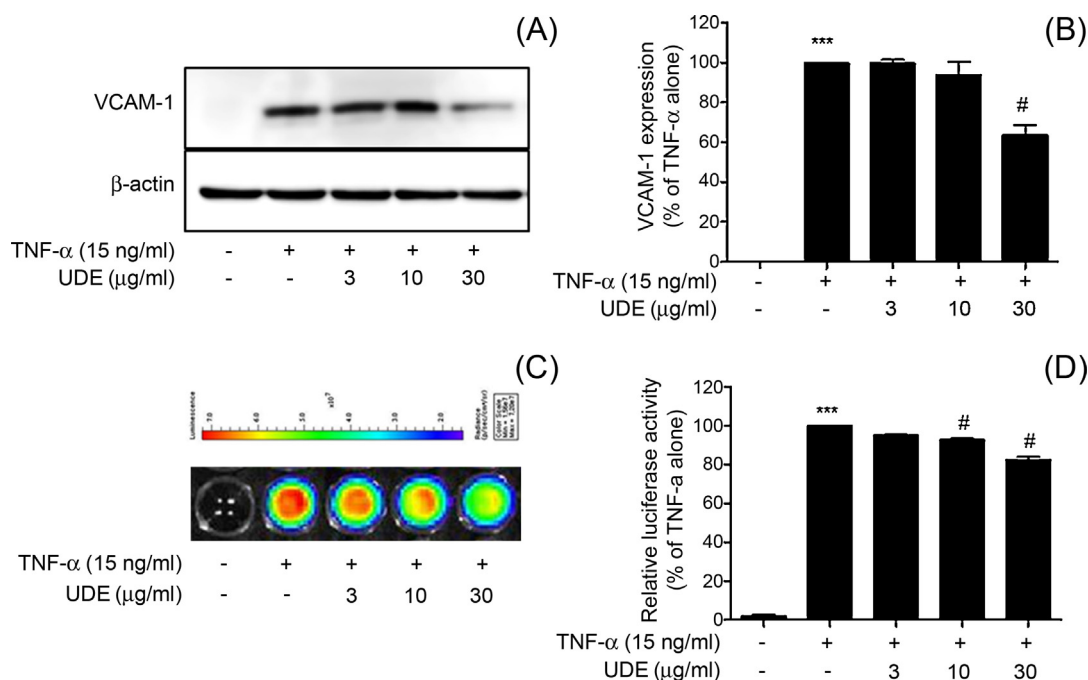


Fig. 3 – UDE treatment resulted in altered expression levels of the adhesion molecule VCAM-1 in TNF- α -stimulated HUVECs. **(A)** Immunoblotting for adhesion molecule VCAM-1 using lysates from TNF- α -stimulated HUVECs treated with DMSO or UDE at the indicated concentrations (0–30 μ g/mL). Blots were stripped and reprobbed with anti- β -actin antibody to correct for differences in protein loading. **(B)** The expression of VCAM-1 relative to that of β -actin was calculated for each treatment group based on the densitometric scanning data of each band. Similar results were observed in replicate experiments ($n=3$). **(C and D)** The transcriptional activity of VCAM-1 in TNF- α -stimulated HEK 293T cells was determined by a luciferase reporter gene assay. HEK 293T/VCAM-1-*luc* cells (4×10^4 cells/well) were plated in a black 96-well plate following treatment with various concentrations (0–30 μ g/mL) of UDE and then stimulated with TNF- α for 6 hours. **(C)** The bioluminescent images were taken following the addition of D-luciferin substrate (150 μ g/mL final concentration). **(D)** The luciferase activity, in units of total flux (photons/s) was quantified using an IVIS Lumina XRMS imaging system and analyzed by Living Image Software 4.4 (PerkinElmer Inc.). Values are expressed as the mean \pm SEM ($n=3$).

* $p < 0.001$, compared with untreated control cells, as calculated by ANOVA followed by Bonferroni's multiple comparison tests.

† $p < 0.01$ compared with control cells treated with TNF- α -only, as calculated by ANOVA followed by Bonferroni's multiple comparison tests.

ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; HEK, human epithelial kidney; HUVEC, human umbilical vein endothelial cell; SEM, standard error of the mean; TNF- α , tumor necrosis factor-alpha; UDE, *U. davidiana* extract; VCAM, vascular cell adhesion molecule.

of TNF- α -induced VCAM-1 expression in a concentration-dependent manner. At the maximum tested treatment concentration of UDE (30 μ g/mL), the TNF- α -induced increase in VCAM-1 expression was decreased by 36% (Fig. 3A, 3B).

Additionally, we analyzed the transcriptional activity of VCAM-1 using a luciferase reporter assay in HEK 293T/VCAM-1-*luc* cells, which were stably transfected with the human VCAM-1 promoter gene, and the results are shown in Fig. 3C and 3D. Indeed, an increase in the transcriptional activity of VCAM-1 was observed in TNF- α -stimulated HEK293T/VCAM-1-*luc* cells, which exhibited a bright biophotonic emission. Representative image and relative luciferase activities for VCAM-1 in TNF- α -stimulated HEK293T/VCAM-1-*luc* cells, after treatment with UDE, are shown in Fig. 3C and 3D. A significant decrease in VCAM-1 transcriptional activity was observed in UDE-treated TNF- α -stimulated HEK 293T/VCAM-1-*luc* cells. Similar to the results for VCAM-1 expression (Fig. 3A), UDE

treatment was also found to elicit a downregulation of VCAM-1 transcriptional activity. Owing to treatment with UDE at 30 μ g/mL, TNF- α -induced VCAM-1 transcriptional activity was inhibited by 17.4% (Fig. 3D).

3.4. Effect of UDE on ROS generation in TNF- α -stimulated HUVECs

Next, intracellular ROS generation in TNF- α -stimulated HUVECs after UDE treatment was measured using the H₂DCFDA fluorescent probe. As shown in Fig. 4, TNF- α stimulation induced increased intracellular ROS generation compared with that measured in DMSO-treated control cells. However, treatment with UDE led to a downregulation of ROS generation in a concentration-dependent manner, compared with that in HUVECs treated with TNF- α alone. In particular, treatment with 30 μ g/mL UDE almost completely abrogated

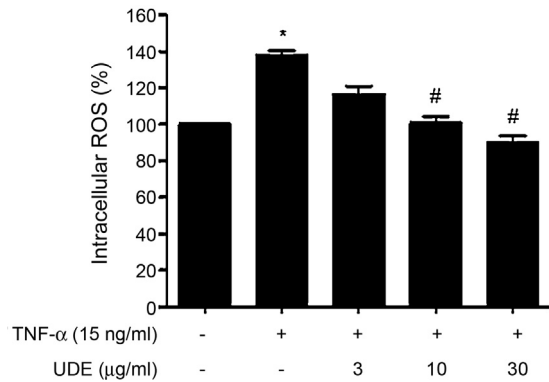


Fig. 4 – Treatment of UDE inhibited ROS generation in TNF- α stimulated HUVECs. HUVECs were pretreated with various concentrations (3–30 $\mu\text{g/mL}$) of UDE for 1 hour, followed by treatment with TNF- α for 3 hours. Then cells were labeled with the H_2O_2 -sensitive fluorescent probe, 5 mM of H_2DCFDA at 37 $^\circ\text{C}$ for 30 minutes. The fluorescence intensity in the cells was immediately assayed using a fluorometer. Data are presented as means \pm SEM ($n = 3$). * $p < 0.01$ compared with untreated control cells, as calculated by ANOVA followed by Bonferroni's multiple comparison tests.

† $p < 0.05$ compared with control cells treated with TNF- α only, as calculated by ANOVA followed by Bonferroni's multiple comparison tests.

ANOVA, analysis of variance; H_2DCFDA , dichlorofluorescein diacetate; HUVEC, human umbilical vein endothelial cell; ROS, reactive oxygen species; SEM, standard error of the mean; TNF- α , tumor necrosis factor-alpha; UDE, *U. davidiana* extract.

TNF- α -induced ROS generation, suggesting that UDE could effectively attenuate endothelial dysfunction by inhibiting TNF- α -induced ROS generation.

3.5. Effect of UDE on NF- κ B localization and signaling in TNF- α -treated HUVECs

The transcription factor NF- κ B, which acts as a central mediator of inflammatory responses, has been known to regulate the expression of adhesion proteins in TNF- α -induced vascular inflammation.^{17,18} The anti-inflammatory effect of many natural products results from the inhibition of NF- κ B activity in various cell types, which explains our results on UDE-mediated downregulation of the VCAM-1 proteins in TNF- α -stimulated HUVECs (Fig. 3A). To confirm the inhibition of NF- κ B in TNF- α -stimulated HUVECs under our experimental conditions, we determined the effect of UDE treatment on nuclear translocation of NF- κ B p65, which is necessary for its activation. Initially, we performed immunoblotting for NF- κ B p65 using nuclear and cytosol fractions prepared from TNF- α -stimulated HUVECs treated with different concentrations of UDE for 1 hour. As shown in Fig. 5A, UDE treatment caused a marked decrease in the nuclear level of NF- κ B p65 that was evident as low as 3 $\mu\text{g/mL}$ UDE after treatment. The UDE-mediated inhibition was also evident from a reciprocal increase of cytoplasmic localization of NF- κ B p65 at the

same concentration (Fig. 5A). The membranes were stripped and reprobed with anti-PARP and/or GAPDH antibodies to ensure equal protein loading, as well as to rule out cross-contamination of nuclear and cytoplasmic fractions (Fig. 5A). Additionally, I κ B degradation, which is a critical step for NF- κ B activation leading to nuclear translocation, was obviously prevented even with 3 $\mu\text{g/mL}$ UDE treatment (Fig. 5B).

Next, we performed immunocytochemistry to further examine the effect of UDE treatment on nuclear/cytoplasmic localization of NF- κ B p65. As shown in Fig. 5C, the NF- κ B p65 (green fluorescence) immunostaining was evident in both the nucleus (red fluorescence) and the cytoplasm of nonstimulated HUVECs compared with the mainly nuclear localization of NF- κ B p65 in TNF- α -stimulated cells. Similar to the result of Fig. 5A, UDE treatment significantly attenuated TNF- α -induced nuclear translocation of NF- κ B p65 and restricted it to the cytoplasm, indicating inhibition of its nuclear translocation and its activity (Fig. 5C).

4. Discussion

Here, we demonstrated that UDE treatment inhibits TNF- α -induced monocyte adhesion to endothelial cells, and that its antiadhesive activity might be due to the inhibition of VCAM-1 protein and/or transcriptional activity for VCAM-1.

In the present study, we showed the effect of UDE on endothelial cell viability. UDE at a concentration of less than 30 $\mu\text{g/mL}$ exerted antiadhesive activity without affecting endothelial cell viability. However, a high dose of UDE (more than 50 $\mu\text{g/mL}$) reduced cell viability, suggesting nonspecific cell toxicity at such high doses. UD is also used as a traditional medicine for its antitumor activities. In previous reports, UDE (more than 50 $\mu\text{g/mL}$) exerted antitumor activity.¹⁹ Therefore, pharmacological use of high doses of UDE may be possible, but requires further study and careful consideration of the benefits and risks. Our cell viability results could serve as fundamental data for the development of antitumor drugs lacking nonspecific cell toxicity.

Adhesion of circulating monocytes to the arterial endothelium is the initial event in the development of chronic inflammatory diseases such as atherosclerosis, and it is mainly regulated by a combination of cell-surface adhesion molecules. VCAM-1 is an adhesion protein that appears on the surface of endothelial cells after exposure to inflammatory cytokines. VCAM-1 is a ligand for the integrin receptor very late activation antigen (VLA)-4.²⁰ Binding of VCAM-1 to VLA-4 is a key regulator of monocyte adhesion in endothelial cells. The area of early atherosclerotic lesions in the aorta was reduced significantly in VCAM-1 knockout mice, indicating that VCAM-1 plays a dominant role in the initiation of atherosclerosis.²¹

In the present study, UDE treatment inhibited TNF- α -induced monocyte adhesion in endothelial cells. Additionally, western blot and promoter assays revealed that the antiadhesive activity of UDE is mainly due to the inhibition of TNF- α -induced VCAM-1 protein expression and its transcriptional activity. Especially, the effect of UDE on the transcriptional activity of VCAM-1 was evaluated in stable cell lines to avoid variable protein expressions among individual

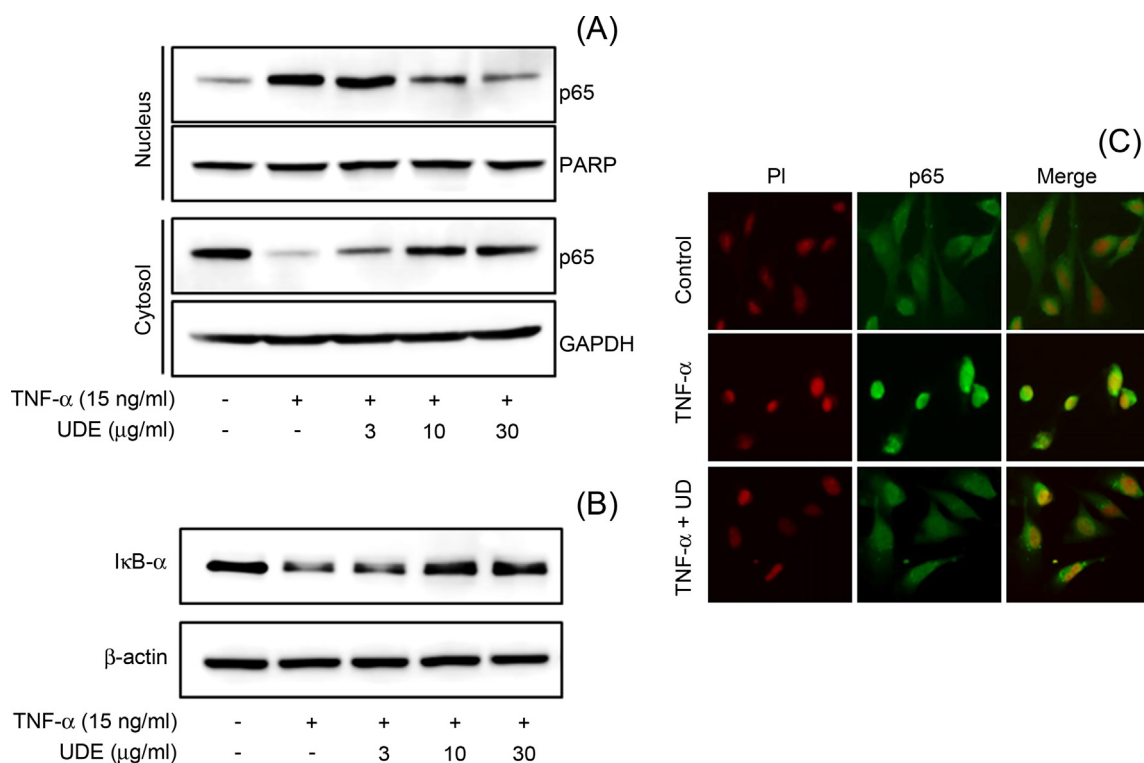


Fig. 5 – Treatment of HUVECs with UDE significantly inhibited TNF- α -induced NF- κ B activity. (A) A representative NF- κ B immunoblot using fractionated nuclear and cytosolic cell lysates from HUVECs stimulated with TNF- α following treatment with various concentrations (0–30 μ g/mL) of UDE for 1 hour. PARP and GAPDH were used as loading controls for nuclear and cytosolic proteins, respectively. **(B)** Immunoblot for I κ B α . HUVECs were pretreated with various concentrations (0–30 μ g/mL) of UDE for 1 hour and then stimulated with TNF- α for 30 minutes. The blot was stripped and reprobed with β -actin antibody to correct for differences in protein loading. Similar results were observed in replicate experiments ($n=3$). **(C)** Immunocytochemistry for the analysis of NF- κ B p65 localization following exposure to DMSO or 30 μ g/mL UDE in TNF- α -stimulated HUVECs for 30 minutes. Green and red fluorescence indicate staining for NF- κ B p65 and for the cell nucleus, respectively. Images were merged to detect the nuclear/cytosolic distribution of NF- κ B p65. Images are representative of three different experiments.

DMSO, dimethyl sulfoxide; HUVEC, human umbilical vein endothelial cell; NF- κ B, nuclear factor- κ B; PARP, poly (ADP-ribose) polymerase; TNF- α , tumor necrosis factor-alpha; UDE, *U. davidiana* extract.

cells that can occur with transient transfection. We generated stable HEK293T cell lines containing the region from -1716 to +119 of the human VCAM-1 promoter into pGL3.1 plasmids (kindly donated by Y.G. Kwon, Yonsei University).²² Then, this luciferase activity was analyzed using a high-sensitivity *in vitro* imaging system to visualize the luciferase signal. A significant decrease in VCAM-1 transcriptional activity was confirmed in UDE-treated TNF- α -stimulated HEK 293T/VCAM-1-luc cells.

ROS are created by a variety of cellular processes as part of cellular signaling events.²³ Proinflammatory cytokines, such as TNF- α , increase ROS generation in inflammatory diseases.^{24,25} In particular, Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mitochondrial ROS were shown to be involved in TNF- α -induced VCAM-1 expression and monocyte adhesion in endothelial cells.²⁶ In the present study, our data showed that treatment of cells with 30 μ g/mL UDE almost completely abrogated TNF- α -induced ROS generation, suggesting that UDE could effectively attenuate endothelial dysfunction by inhibiting TNF- α -induced ROS generation.

NF- κ B is regulated by the intracellular redox status.²³ It has been reported that oxidants that enhance ROS generation also induce NF- κ B activation. Indeed, excess generation of ROS is involved in the majority of signaling pathways that cause I κ B degradation and NF- κ B nuclear accumulation. Transcriptional activation of NF- κ B increases the expression of adhesion molecules, such as VCAM-1, subsequently leading to monocyte adhesion and transmigration across the endothelial barrier into the vessel wall.^{17,18} In this study, we clearly demonstrated that UDE attenuates TNF- α -induced NF- κ B p65 nuclear translocation and I κ B α degradation. Therefore, given these correlations between NF- κ B and endothelial dysfunction, we can speculate that UDE suppresses NF- κ B signaling.

Several kinds of candidate natural compounds can be involved in antiadhesive activity. Phytochemical components of UD that have been demonstrated to have cytoprotective effects in a purified form include epigallocatechin-3-gallate, catechin, triterpene ester, and sesquiterpene O-naphthoquinones.^{27–30} Based on our knowledge, UDE has been shown to have antiadhesive and vasorelaxant activities.

Therefore, UDE could be used as a part of a drug formula to inhibit chronic vascular inflammation, enhance blood flow, and/or facilitate blood circulation.

5. Conclusion

Taken together, we demonstrated that UDE inhibits TNF- α -induced monocyte adhesion in endothelial cells and that its antiadhesive activity was at least partly due to the inhibition of VCAM-1 expression, which is regulated by ROS and NF- κ B signaling. Our results provide new insight into the cellular mechanisms underlying the protective activity of UDE against endothelial dysfunction in chronic vascular inflammatory diseases.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2014R1A6A1029617). In addition, it was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through the Golden Seed Project, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (No. 213002043SBX20), and a NRF grant funded by the Korea government (2013R1A1A2060250).

REFERENCES

- Sena CM, Pereira AM, Seica R. Endothelial dysfunction—a major mediator of diabetic vascular disease. *Biochim Biophys Acta* 2013;1832:2216–31.
- Szmitko PE, Wang CH, Weisel RD, de Almeida JR, Anderson TJ, Verma S. New markers of inflammation and endothelial cell activation: Part I. *Circulation* 2003;108:1917–23.
- Mestas J, Ley K. Monocyte–endothelial cell interactions in the development of atherosclerosis. *Trends Cardiovasc Med* 2008;18:228–32.
- Kempe S, Kestler H, Lasar A, Wirth T. NF-kappaB controls the global pro-inflammatory response in endothelial cells: evidence for the regulation of a pro-atherogenic program. *Nucleic Acids Res* 2005;33:5308–19.
- Jun CD, Pae HO, Kim YC, Jeong SJ, Yoo JC, Lee EJ, et al. Inhibition of nitric oxide synthesis by butanol fraction of the methanol extract of *Ulmus davidiana* in murine macrophages. *J Ethnopharmacol* 1998;62:129–35.
- Kim KS, Lee SD, Kim KH, Kil SY, Chung KH, Kim CH. Suppressive effects of a water extract of *Ulmus davidiana* Planch (Ulmaceae) on collagen-induced arthritis in mice. *J Ethnopharmacol* 2005;97:65–71.
- Shin DY, Kim HS, Min KH, Hyun SS, Kim SA, Huh H, et al. Isolation of a potent anti-MRSA sesquiterpenoid quinone from *Ulmus davidiana* var. *japonica*. *Chem Pharm Bull (Tokyo)* 2000;48:1805–6.
- Kim YC, Lee MK, Sung SH, Kim SH. Sesquiterpenes from *Ulmus davidiana* var. *japonica* with the inhibitory effects on lipopolysaccharide-induced nitric oxide production. *Fitoterapia* 2007;78:196–9.
- Lee GY, Jang DS, Kim J, Kim CS, Kim YS, Kim JH, et al. Flavan-3-ols from *Ulmus davidiana* var. *japonica* with inhibitory activity on protein glycation. *Planta Med* 2008;74:1800–2.
- Lee MY, Seo CS, Ha H, Jung D, Lee H, Lee NH, et al. Protective effects of *Ulmus davidiana* var. *japonica* against OVA-induced murine asthma model via upregulation of heme oxygenase-1. *J Ethnopharmacol* 2010;130:61–9.
- Jung HJ, Jeon HJ, Lim EJ, Ahn EK, Song YS, Lee S, et al. Anti-angiogenic activity of the methanol extract and its fractions of *Ulmus davidiana* var. *japonica*. *J Ethnopharmacol* 2007;112:406–9.
- Cho EJ, Park MS, Kim SS, Kang G, Choi S, Lee YR, et al. Vasorelaxing activity of *Ulmus davidiana* ethanol extracts in rats: activation of endothelial nitric oxide synthase. *Korean J Physiol Pharmacol* 2011;15:339–44.
- Park MS, Kim CS, Joo HK, Lee YR, Kang G, Kim SJ, et al. Cytoplasmic localization and redox cysteine residue of APE1/Ref-1 are associated with its anti-inflammatory activity in cultured endothelial cells. *Mol Cells* 2013;36:439–45.
- Choi S, Park MS, Lee YR, Lee YC, Kim TW, Do SG, et al. A standardized bamboo leaf extract inhibits monocyte adhesion to endothelial cells by modulating vascular cell adhesion protein-1. *Nutr Res Pract* 2013;7:9–14.
- Kang G, Lee YR, Joo HK, Park MS, Kim CS, Choi S, et al. Trichostatin A modulates angiotensin II-induced vasoconstriction and blood pressure via inhibition of p66shc activation. *Korean J Physiol Pharmacol* 2015;19:467–72.
- Kim SN, Son SC, Lee SM, Kim CS, Yoo DG, Lee SK, et al. Midazolam inhibits proinflammatory mediators in the lipopolysaccharide-activated macrophage. *Anesthesiology* 2006;105:105–10.
- Chen CC, Rosenbloom CL, Anderson DC, Manning AM. Selective inhibition of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 expression by inhibitors of I kappa B-alpha phosphorylation. *J Immunol* 1995;155:3538–45.
- Iademarco MF, McQuillan JJ, Rosen GD, Dean DC. Characterization of the promoter for vascular cell adhesion molecule-1 (VCAM-1). *J Biol Chem* 1992;267:16323–9.
- Ahn J, Lee JS, Yang KM. Ultrafine particles of *Ulmus davidiana* var. *japonica* induce apoptosis of gastric cancer cells via activation of caspase and endoplasmic reticulum stress. *Arch Pharm Res* 2014;37:783–92.
- Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyj S, Hemler ME, et al. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell* 1990;60:577–84.
- Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, et al. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest* 2001;107:1255–62.
- Min JK, Kim YM, Kim SW, Kwon MC, Kong YY, Hwang IK, et al. TNF-related activation-induced cytokine enhances leukocyte adhesiveness: induction of ICAM-1 and VCAM-1 via TNF receptor-associated factor and protein kinase C-dependent NF-kappaB activation in endothelial cells. *J Immunol* 2005;175:531–40.
- Morgan MJ, Liu ZG. Crosstalk of reactive oxygen species and NF-kappaB signaling. *Cell Res* 2011;21:103–15.
- Kleinbongard P, Heusch G, Schulz R. TNFalpha in atherosclerosis, myocardial ischemia/reperfusion and heart failure. *Pharmacol Ther* 2010;127:295–314.
- Picchi A, Gao X, Belmadani S, Potter BJ, Focardi M, Chilian WM, et al. Tumor necrosis factor-alpha induces endothelial dysfunction in the prediabetic metabolic syndrome. *Circ Res* 2006;99:69–77.

26. Yu JHKC, Yoo DG, Song YJ, Joo HK, Kang G, Jo JY, et al. NADPH oxidase and mitochondrial ROS are involved in the TNF- α -induced vascular cell adhesion molecule-1 and monocyte adhesion in cultured endothelial cells. *Korean J Physiol Pharmacol* 2006;10:217–22.
27. Kim JP, Kim WG, Koshino H, Jung J, Yoo ID. Sesquiterpene O-naphthoquinones from the root bark of *Ulmus davidiana*. *Phytochemistry* 1996;43:425–30.
28. Lee MK, Sung SH, Lee HS, Cho JH, Kim YC. Lignan and neolignan glycosides from *Ulmus davidiana* var. *japonica*. *Arch Pharm Res* 2001;24:198–201.
29. Lorenz M, Wessler S, Follmann E, Michaelis W, Düsterhöft T, Baumann G, et al. A constituent of green tea, epigallocatechin-3-gallate, activates endothelial nitric oxide synthase by a phosphatidylinositol-3-OH-kinase-, cAMP-dependent protein kinase-, and Akt-dependent pathway and leads to endothelial-dependent vasorelaxation. *J Biol Chem* 2004;279:6190–5.
30. Jung MJ, Heo S, Wang M. HPLC analysis and antioxidant activity of *Ulmus davidiana* and some flavonoids. *Food Chem* 2010;120:313–8.