

Effect of *Buddleja officinalis* on High-Glucose-Induced Vascular Inflammation in Human Umbilical Vein Endothelial Cells

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In this study, we aimed to investigate whether an aqueous extract of *Buddleja officinalis* (ABO) suppresses high-glucose-induced vascular inflammatory processes in the primary cultured human umbilical vein endothelial cells (HUVEC). The high-glucose-induced increase in expression of cell adhesion molecules (CAMs) such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial-selectin (E-selectin) was significantly attenuated by pretreatment with ABO in a dose-dependent manner. Enhanced cell adhesion caused by high glucose in co-cultured U937 and HUVEC was also blocked by pretreatment with ABO. Pretreatment with ABO also blocked formation of high-glucose-induced reactive oxygen species (ROS). In addition, ABO suppressed the transcriptional activity of NF- κ B and I κ B phosphorylation under high-glucose conditions. Pretreatment with N(G)-nitro-L-arginine methyl ester (L-NAME), an endothelial nitric oxide (NO) synthase inhibitor, attenuated the protective action of ABO on high-glucose-induced CAM expression, suggesting a potential role of NO signaling. The present data suggest that ABO could suppress high-glucose-induced vascular inflammatory processes, and ABO may be closely related with the inhibition of ROS and NF- κ B activation in HUVEC. Exp Biol Med 233:694–700, 2008

Key words: *Buddleja officinalis*; adhesion molecules; ROS; NF- κ B; HUVEC

Introduction

Macrovascular complications including atherosclerosis are the leading cause of morbidity and mortality in patients with diabetes mellitus (1). Hyperglycemia is thought to be an important regulator of vascular lesion development. In particular, endothelial cells in human atherosclerotic lesions have increased cell adhesion molecules (CAMs) expression such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial-selectin (E-selectin) (2). Under these conditions, numerous leukocytes adhere to the vascular endothelium, transmigrate the endothelium, and aggravate endothelial dysfunction and tissue injury. Endothelial cells and monocytes cultured in media of high glucose concentration exhibit increased adhesiveness, suggesting that leukocyte binding through adhesion molecules increases under conditions of high glucose concentration (3).

Oxidative injury due to high glucose concentration plays a central role in the development of diabetic complications (4). Production of reactive oxygen species (ROS) such as superoxides and peroxynitrite together with inflammatory factors including chemokines, cytokines, and adhesion molecules have been shown to be increased in atherosclerotic lesions (5, 6).

NF- κ B is a transcription factor for the inflammatory process and a ubiquitously expressed multiunit transcription factor that is activated by diverse signals, possibly *via* phosphorylation of the I κ B subunit and its dissociation from the inactive cytoplasmic complex, followed by translocation of the active dimers p50 and p65 to the nucleus (7).

Several traditional herbal medicines are widely used for the treatment of diabetes and diabetic complications in the Far East including Korea. The flower bud of *Buddleja officinalis* Maximowicz (Loganiaceae) is one of the earliest and most important crude herbs used in traditional Oriental medicine for the treatment of diabetes and vascular diseases (8, 9). *Buddleja officinalis* Maximowicz contains terpenoids, flavonoids, phenylethanoids, and saponins, and has been

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reported to protect PC12 cells from apoptosis and the oxidative stress induced by the 1-methyl-4-phenylpyridinium ion, as well as to inhibit eicosanoid generation by leukocytes (6, 10). However, the anti-inflammatory action of *B. officinalis* Maximowicz under diabetic atherosclerotic conditions has not been well examined. Thus, we examined the anti-inflammatory effects of an aqueous extract of *B. officinalis* Maximowicz (ABO) on high-glucose-induced vascular inflammation in primary cultured human umbilical vein endothelial cells (HUVEC).

Materials and Methods

Extraction of Aqueous Fraction from *B. officinalis* Maximowicz. The flower buds of *B. officinalis* Maximowicz were purchased from an herbal medicine cooperative association in Jeonbuk Province, Korea. A voucher specimen (No. DH-12) has been deposited in the Herbarium of the Professional Graduate School of Oriental Medicine, Wonkwang University (Korea). Dried *B. officinalis* Maximowicz (600 g) was extracted with 2 liters of boiled distilled water at 100°C for 2 hrs. The yield of the aqueous extract of *B. officinalis* Maximowicz was approximately 15.6% of the plant powder. The extract was centrifuged at 1000 g for 20 mins at 4°C and filtered with Whatman No. 3 filter paper. The resulting supernatant was lyophilized to produce a powder, which was then kept at 4°C.

Cell Culture. Primary cultured HUVEC and endothelial cell growth medium (EGM-2) containing 2.5% fetal bovine serum (FBS) and growth supplements were purchased from Cambrex (East Rutherford, NJ) (11). HUVEC which were used between passages 3 and 8 were maintained in EGM-2 in a humidified chamber containing 5% CO₂ at 37°C.

Cell Enzyme Linked Immunosorbent Assay (ELISA). ELISA was used to determine the level of ICAM-1, VCAM-1, and E-selectin expression on the cell surface, as previously described with minor modifications (12). Briefly, HUVEC were fixed by 1% glutaraldehyde and exposed to mouse anti-human VCAM-1, ICAM-1, or E-selectin antibodies at 1:1000 dilution in the PBS containing 1% skim milk for 2 hrs at room temperature. The cells were washed and incubated with a horseradish-peroxidase-conjugated secondary antibody. The expression of VCAM-1, ICAM-1, or E-selectin was quantified by adding a peroxidase substrate solution (40 mg *o*-phenylenediamine and 10 µl 30% H₂O₂ in 100 ml 0.05 M citrate-phosphate buffer). After incubation for 30 mins at 37°C, the reaction was stopped by addition of 5 N H₂SO₄, and the absorbance of each well was measured at 490 nm by a Multiskan microplate reader (Thermo LabSystems Inc., Franklin, MA).

Monocyte-Endothelial Cell Adhesion Assay. The cell adhesion assay was modified as described (13). Briefly, regularly passaged U937 cells were labeled with 10 µg/ml 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM, Sigma Chemical

Co., St. Louis, MO) at 10 µM final concentration in RPMI-1640 medium containing 10% fetal FBS at 37°C for 30 mins. The labeled cells were harvested by centrifugation and washed three times with phosphate-buffered saline (PBS) before suspension in the medium, and added to HUVEC in six-well culture plates at 4 × 10⁵ cells/ml. The co-incubation was done at 37°C for 30 mins and nonadhering U937 cells (American Type Culture Collection, Manassas, VA) were removed by stringent washing two times with PBS. U937 cells bound to HUVEC were measured by fluorescence microscopy (Leica DMIRB, Leica, Germany) and were lysed with 50 mM Tris-HCl, pH 8.0, containing 0.1% sodium dodecyl sulfate (SDS). The fluorescent intensity was measured using a spectrofluorometer (F-2500, Hitachi, Tokyo, Japan) at an excitation and emission wavelength of 485 nm and 535 nm, respectively. The adhesion data are represented in terms of the fold change compared with the control values.

Intracellular ROS Production Assay. The fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) was used to determine the intracellular generation of ROS by high glucose described elsewhere (14). Briefly, the confluent HUVEC in the 24-well plates were pretreated with ABO for 30 mins. After removing the ABO from the wells, the cells were incubated with 5 µM dichlorodihydrofluorescein (DCF) for 30 mins. The cells were then stimulated with 25 mM glucose for 1 hr, and the fluorescence intensity (relative fluorescence units) was measured at an excitation and emission wavelength of 485 nm and 530 nm, respectively, using a spectrofluorometer (Hitachi).

Preparation of Cytoplasmic and Nucleus Extracts. The cells were rapidly harvested by sedimentation, and nuclear and cytoplasmic extracts were prepared on ice as previously described by the method of Mackman *et al.* (15). Cells were harvested and washed by centrifugation with 1 ml buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 19 mM KCl) for 5 mins at 600 g. The cells were then resuspended in buffer A and 0.1% NP-40, left for 10 mins on ice to lyse the cells, and then centrifuged at 600 g for 3 mins. The supernatant was saved as cytosolic extract. The nuclear pellet was then washed by centrifugation in 1 ml buffer A at 4200 g for 3 mins, resuspended in 30 µl buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA), rotated for 30 mins at 4°C, then centrifuged at 14,300 g for 20 mins. The supernatant was used as nucleus extract. The nucleus and cytosolic extracts were then analyzed for protein content using the Bradford assay.

Western Blot Analysis. Cell homogenates (40 µg of protein) were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. Blots were then washed with H₂O, blocked with 5% skimmed milk powder in Tris-Buffered Saline Tween-20 (TBST) (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.05% Tween-20) for 1 hr, and incubated with the appropriate primary antibody at

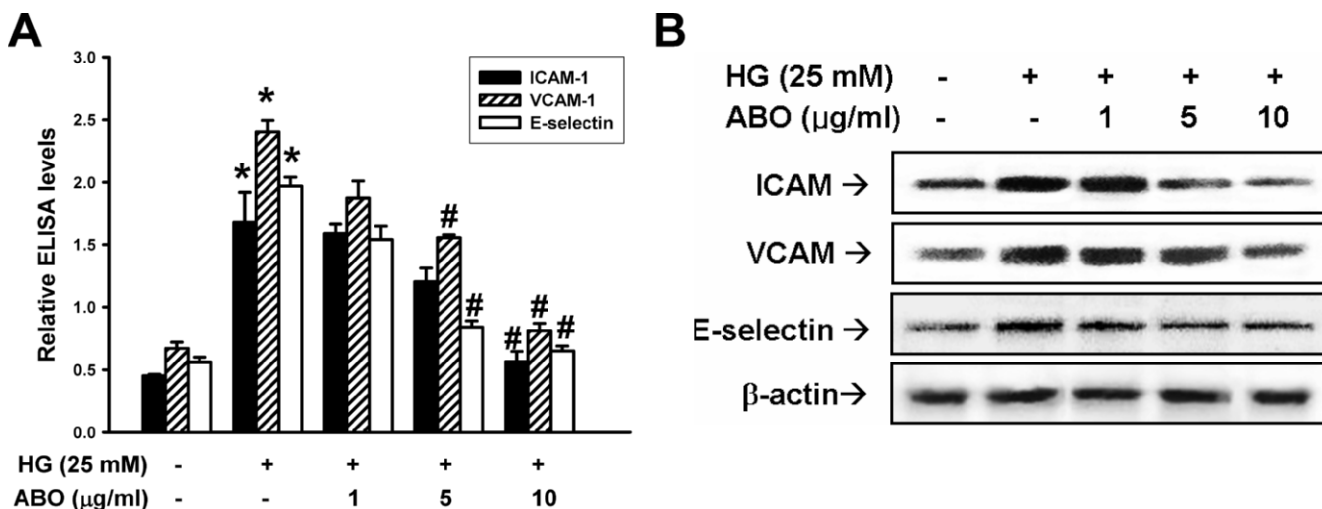


Figure 1. Effect of ABO on endothelial cell surface expression of ICAM-1, VCAM-1, and E-selectin. (A) HUVEC were preincubated with ABO (0–10 μg/ml) for 30 mins prior to the treatment of high glucose (25 mM) for 48 hrs, respectively. The cells were fixed with glutaraldehyde, and the CAMs expressions were analyzed by cell ELISA. Values are means $\pm$ SE of six independent experiments with triplicate dishes. (B) Western blots of ICAM-1, VCAM-1, and E-selectin were detected as described in Materials and Methods. Each electrophoretogram is representative of the results from five independent experiments. * $P < 0.05$ vs. control, # $P < 0.05$ vs. high glucose alone.

dilutions recommended by the supplier. Then the membrane was washed and primary antibodies were detected with goat anti-rabbit-IgG conjugated to horseradish peroxidase, and the bands were visualized with enhanced chemiluminescence (Amersham Bioscience, Buckinghamshire, UK). Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using the ChemiDoc image analyzer (Bio-Rad Laboratories, Hercules, CA).

Transient Transfection and Luciferase Reporter Assay. The endothelial cells were grown to 60%–80% confluence, and the cells were transiently cotransfected with the plasmids using Lipofectamine LTX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, the transfection mixture containing 0.5 μg of either the pGL3–4κB-Luc and 0.1 μg of pCMV-β-gal was mixed with the Lipofectamine LTX reagent and added to the cells. After 24 hrs, the cells were treated with ABO for 30 mins and stimulated with high glucose for 24 hrs and then lysed. The luciferase and β-galactosidase activities were determined as described elsewhere using a luciferase assay kit (Promega, Madison, WI) (16). The luciferase activity was normalized with respect to the β-galactosidase activity and is expressed as a percentage of the activity of the high glucose group.

Statistical Analysis. Data are expressed as a mean $\pm$ SE, and the data were analyzed using one-way ANOVA followed by a Dunnett's test or Student's *t* test to determine any significant differences. A *P* value < 0.05 was considered significant.

Results

Effect of ABO on High-Glucose-Induced Vascular Inflammation. In the experiment to determine whether ABO inhibits high-glucose-induced ICAM-1,

VCAM-1, and E-selectin expression, various ABO concentrations ranging from 1–10 μg/ml were added to HUVEC. As shown in Figure 1A, pretreatment with ABO decreased high-glucose (25 mM)-induced ICAM-1, VCAM-1, and E-selectin expression with the maximum inhibitory effect being observed at 10 μg/ml. Coincident with the cell ELISA, Western blot analysis showed that ABO decreased high-glucose-induced ICAM-1, VCAM-1, and E-selectin expression levels (Fig. 1B). In this study, MTT assay demonstrated that ABO (1–10 μg/ml) did not alter any cytotoxicity (data not shown).

To explore the effect of ABO on endothelial cell-leukocyte interaction, we examined the adhesion of U937 cells to high-glucose-activated HUVEC under static conditions. Control confluent HUVEC showed minimal binding to U937 cells, but adhesion was markedly increased when treated with high glucose. Pretreatment with ABO decreased the number of U937 cells adhering to high-glucose-induced HUVEC at concentrations over 5 μg/ml (Fig. 2).

Effect of ABO on High-Glucose-Induced ROS Formation. ROS has been implicated as a common second messenger in various pathways leading to NF-κB activation (4, 6). Thus, the level of intracellular ROS production was assessed by monitoring the fluorescence in order to determine whether ABO can reduce the level of high-glucose-induced oxidative stress in HUVEC. Figure 3 showed that the DCF fluorescence level showed a statistically significant increase after incubation with 25 mM glucose. However, pretreatment with 10 μg/ml ABO significantly inhibited high-glucose-induced DCF-sensitive cellular ROS levels. In addition, ABO itself did not induce oxidative stress.

Effect of ABO on High-Glucose-Induced NF-κB Activation. Various transcription factors including NF-κB have been activated by ROS in cultured endothelial cells

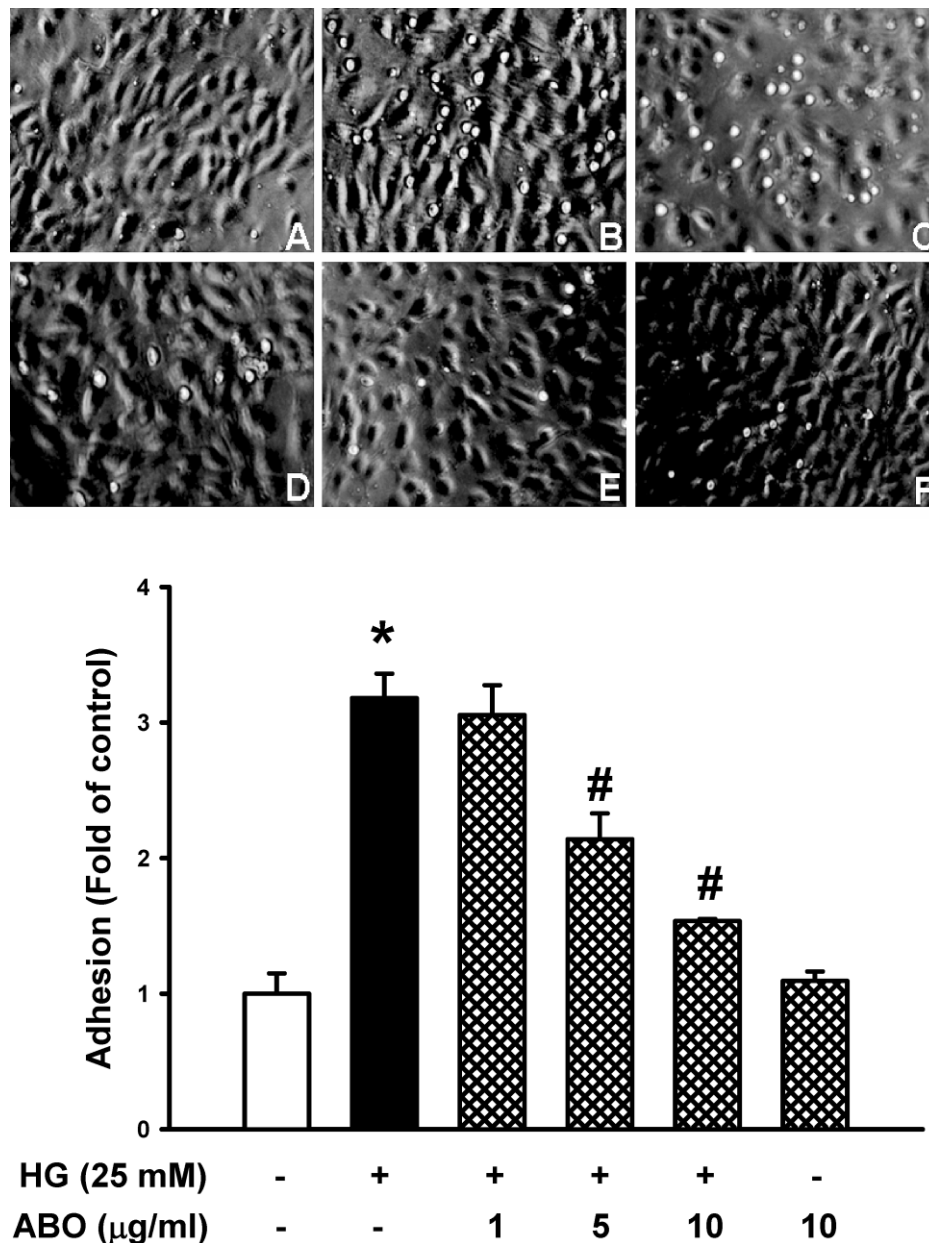


Figure 2. Effect of ABO on the adhesion of monocytes to high glucose-stimulated HUVEC. Cells were pretreated with ABO for 30 mins and then stimulated with high glucose for 48 hrs. Adhesion of fluorescence-labeled U937 cells to HUVEC was determined as described in Materials and Methods. (A) Control. (B) High glucose (25 mM). (C) Cotreated with high glucose and ABO (1 µg/ml). (D) Cotreated with high glucose and ABO (5 µg/ml). (E) Cotreated with high glucose and ABO (10 µg/ml). (F) ABO (10 µg/ml) alone. The amounts of adherent U937 cells were monitored by fluorescence microscopy. Low panel indicates ratio of fluorescence intensity. Values are means $\pm$ SE of six independent experiments with triplicate dishes. * $P < 0.05$ vs. control, # $P < 0.05$ vs. high glucose alone.

(17). Thus, we measured whether or not ABO could suppress the NF- κ B promoter in HUVEC. Transient transfections were performed using the NF- κ B-dependent luciferase reporter plasmid in order to further examine the effects of ABO on the NF- κ B transcription activity. As shown in Figure 4A, high glucose increased NF- κ B transcription activity and concentrations over 5 µg/ml ABO significantly inhibited high-glucose-induced NF- κ B transcriptional activity. In Western blot analysis, the active subunit of the NF- κ B complex, p65 NF- κ B expression, was

increased in the nuclear extracts; high glucose also increased phospho-I κ B expression in the cytoplasmic extracts (Fig. 4B). Pretreatment with ABO decreased high-glucose-induced p65 NF- κ B and phospho-I κ B expression levels.

Involvement of Endothelial Nitric Oxide Synthase (eNOS) in Protective Effect of ABO on High-Glucose-Induced Vascular Inflammation. In experiments designed to determine whether ABO-mediated nitric oxide signaling is involved in protective effect against the high-glucose-induced vascular inflammation, HUVEC were

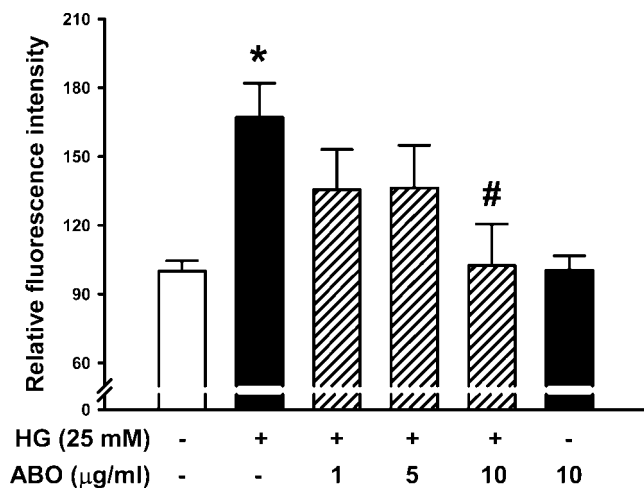


Figure 3. Effect of ABO on high-glucose-induced ROS formation. Cells were pretreated with ABO (0–10 $\mu\text{g/ml}$) for 30 mins and then stimulated with high glucose or ABO (10 $\mu\text{g/ml}$) alone for 1 hr. The intracellular level of ROS was measured by fluorescence microplate, and the relative ROS levels were quantified. The values are expressed as a mean percentage of the fluorescence intensity $\pm$ SE of five individual experiments performed in triplicate. * $P < 0.05$ vs. control, # $P < 0.05$ vs. high glucose alone.

pretreated with L-NAME (nitric oxide synthase inhibitor, 1–100 μM) before the ABO treatment. After exposure to high glucose, expression of CAMs was measured by Western blot. Figure 5 shows that ABO significantly prevented the high-glucose-induced ICAM-1, VCAM-1, and E-selectin expressions. Pretreatment with L-NAME attenuated the protective effect of ABO in high-glucose-induced ICAM-1, VCAM-1, and E-selectin expression in a dose-dependent manner. This result suggests a potential role of NO signaling in high-glucose-induced vascular inflammation.

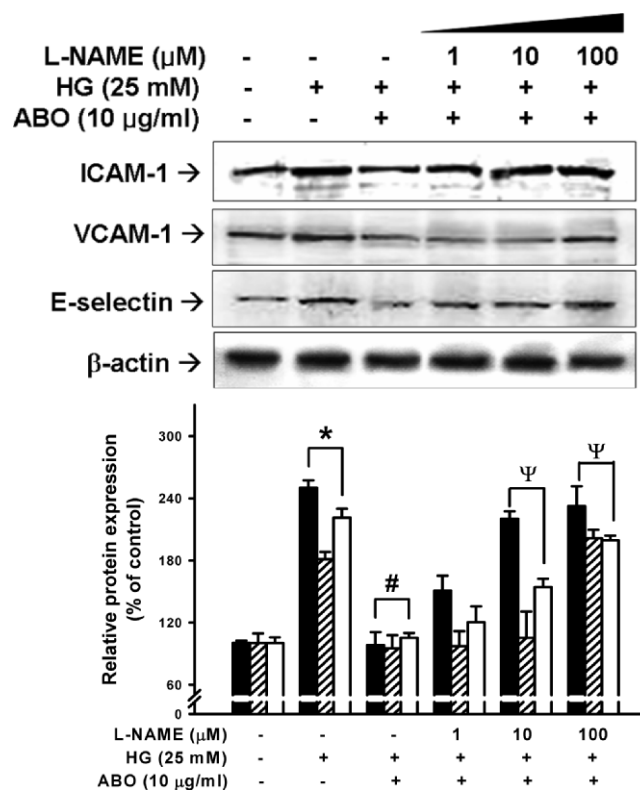
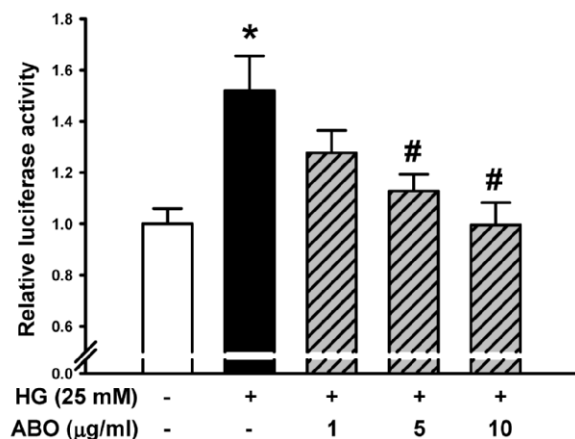


Figure 5. Effect of L-NAME on protective effect of ABO against high glucose-induced increase of ICAM-1, VCAM-1, and E-selectin expression. The HUVEC were preincubated with L-NAME (1–100 μM) for 30 mins prior to the treatment of ABO (10 $\mu\text{g/ml}$) for 30 mins, followed by high glucose treatment for 48 hrs. The lower panel depicts quantitative data expressed as ICAM-1 (■), VCAM-1 (▨), and E-selectin (□) normalized to β -actin, and the results are expressed as the % of the control. Each electrophoretogram is representative of the results from five independent experiments. * $P < 0.05$ vs. control, # $P < 0.05$ vs. high glucose alone, ψ $P < 0.05$ vs. ABO + high glucose.

A



B

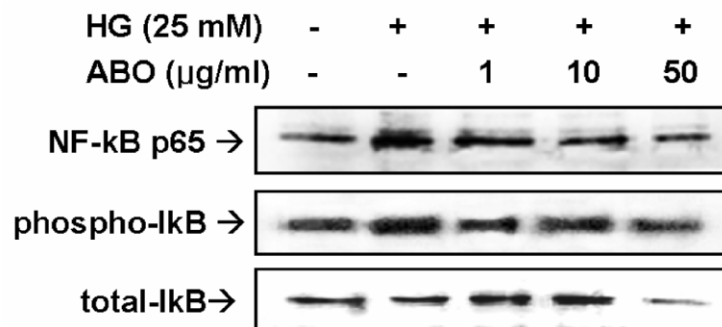


Figure 4. Effect of ABO on high-glucose-induced NF- κ B activation. (A) HUVEC were transiently transfected with pGL3-4 κ B-Luc and pCMV- β -gal. After 24 hrs, the cells were pretreated with ABO for 30 mins and then stimulated with high glucose. This was followed by harvesting and determining their luciferase, and β -galactosidase activities were determined. The luciferase activities are expressed relative to the basal value and are the means $\pm$ SE of five independent experiments with four dishes. (B) HUVEC were pretreated with ABO, respectively. Band represents 65 kDa of NF- κ B p65, 41 kDa of phospho I κ B- α , and I κ B- α specific antibody. Each electrophoretogram is representative of the results from five independent experiments. * $P < 0.05$ vs. control, # $P < 0.05$ vs. high glucose alone.

Discussion

This report presents evidence suggesting that ABO blocked expression of high-glucose-induced CAMs, ROS production, and NF- κ B activation in primary cultured HUVEC. In addition, NO was involved in the inhibitory effect of ABO on high-glucose-induced CAMs expression. One of the earliest events in the vascular inflammation process is the adhesion of monocytes to the endothelium, which is followed by their infiltration and differentiation into macrophages. This key step is mediated by an interaction between monocytes and the molecules expressed on the endothelial cell surface (18, 19). These cell adhesion molecules primarily mediate the adhesion of monocytes specifically found in atherosclerosis lesions to the vascular endothelium. In this study, high glucose (25 mM) increased ICAM-1, VCAM-1, and E-selectin expression when compared with nontreated control (5 mM), and pretreatment with ABO inhibited their expression in a dose-dependent manner. Moreover, the high-glucose-induced adhesion of monocytes to HUVEC was markedly inhibited by ABO. These results suggest that ABO may be useful in preventing the hyperglycemia-induced vascular inflammatory process in endothelial cells. It is supported by an *in vivo* report on the anti-inflammatory effect of *B. officinalis* Maximowicz extract in transient middle cerebral artery occlusion in rats (20).

Recent reports have demonstrated that natural extracts induce inhibition of cell adhesion molecules and monocyte adhesion to endothelial cells *via* the suppression of NF- κ B activation under pathophysiologic conditions including high glucose or cytokines (3, 21). In the present study, we found that ABO treatment blocked high-glucose-induced NF- κ B p65 translocation in HUVEC. Pretreatment with ABO inhibited high-glucose-induced NF- κ B p65 expression and activity in a dose-dependent manner. Our previous report supported the theory that medicinal plant extracts such as rhubarb suppress NF- κ B p65 expression in the vascular endothelial inflammation process (22). Our results suggest that high-glucose-induced NF- κ B activation is inhibited by ABO, indicating that ABO has some inhibitory effect on the NF- κ B promoter specific to the high-glucose-induced adhesion molecules in HUVEC.

Recent observations indicate that hyperglycemia triggers the generation of free radicals and oxidant stress in various cell types (23, 24). Reactive oxygen species are considered to be important mediators of several biologic responses, including cell proliferation and extracellular matrix deposition. Thus, agents that inhibit ROS production or enhance cellular antioxidant defenses system can prevent high-glucose-induced vascular inflammation. In addition, the formation of oxygen-derived radicals might lead to an activation of NF- κ B; ROS-mediated NF- κ B activation plays an important role in the pathogenesis of atherosclerosis. Involvement of ROS and NF- κ B in the induction of apoptosis by high glucose has been demonstrated in

cultured human endothelial cells (25). In our result, high glucose increased cellular ROS production. Pretreatment with ABO significantly inhibited high-glucose-induced ROS production, suggesting a role of antioxidant activity. However, it is known that H₂O₂ induces NF- κ B translocation, suggesting NF- κ B inhibition observed here may be secondary to ROS effect (26). Thus, for antioxidant activity, ABO appears to serve as a messenger directly or indirectly mediating the inhibition of NF- κ B activation.

Interestingly, we found that pretreatment with ABO could suppress high-glucose-induced adhesion molecules, while eNOS inhibitor L-NAME, which could decrease endogenous NO production, attenuated the protective effect of ABO on the high-glucose-induced inflammation process. It is suggested that eNOS/NO pathways may be involved for vascular protection in high-glucose-induced inflammation. In diabetes, elevated blood glucose, altered insulin signaling, increased ROS, and inflammation can act together or separately leading to a decrease in NO bioavailability (27). Recent study demonstrated that the endothelial NO/cGMP system serves as an endogenous defense mechanism against vascular inflammation and vasoconstriction (28, 29). Our result supported the finding that impaired release of NO from vascular beds results in increased leukocyte-endothelium interactions *via* increase of ICAM-1, VCAM-1, and E-selectin (30, 31). This finding provides an additional mechanism for ABO-induced NO-elicited protection of HUVEC. However, other groups have reported that *B. officinalis* Maximowicz inhibits LPS-induced nitric oxide production in mouse microglial cells (20). Many of the adverse effects of nitric oxide, including its ability to induce apoptosis, are linked to the availability of superoxide radicals because of their ability to form another potent oxidizer, peroxynitrite (32, 33). Thus, abolishing ABO's effect on L-NAME may also have implications for inhibiting ROS.

In the Far East including Korea, China, and Japan, the flower buds of *B. officinalis* Maximowicz have been used for the treatment of diabetes and vascular diseases. We suspect that these beneficial effects could be due to biologically active components of *B. officinalis* Maximowicz such as flavonoids and saponins, which are well known as potent antioxidants. Whether the antioxidant effects of ABO are due to the integrated effects of these components or due to a single pure compound requires further investigation. In conclusion, the present data suggest that *B. officinalis* Maximowicz could suppress high-glucose-induced vascular inflammatory processes *via* inhibition of oxidative stress and NF- κ B activation, which may be closely related with the activation of vascular eNOS/NO pathways in primary cultured HUVEC.

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