Original Research

Buddleja officinalis suppresses high glucose-induced vascular smooth muscle cell proliferation: role of mitogen-activated protein kinases, nuclear factor-κB and matrix metalloproteinases

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Abstract

Diabetes mellitus is a well-established risk factor for vascular diseases caused by atherosclerosis. In the development of diabetic atherogenesis, vascular smooth muscle cell proliferation is recognized as a key event. Thus, we aimed to investigate whether an ethanol extract of *Buddleja officinalis* (EBO) suppresses high glucose-induced proliferation in primary cultured human aortic smooth muscle cells (HASMC). [^{3}H]-thymidine incorporation revealed that incubation of HASMC with a high concentration of glucose (25 mmol/L) increased cell proliferation. The expression levels of cell cycle protein were also increased by treatment with high glucose concentration. Pretreatment of HASMC with EBO significantly attenuated the increase of high glucose-induced cell proliferation as well as p38 mitogen-activated protein kinases (MAPK) and JNK phosphorylation. EBO suppressed high glucose-induced matrix metalloproteinase (MMP)-9 activity in a dose-dependent manner. In addition, EBO suppressed nuclear factor-κB (NF-κB) nuclear translocation and transcriptional activity in high glucose conditions. Taken together, the present data suggest that EBO could suppress high glucose-induced atherosclerotic processes through inhibition of p38, JNK, NF-κB and MMP signal pathways in HASMC.

Keywords: *Buddleja officinalis*, aortic smooth muscle cell, proliferation, MMP, NF-κB, MAPK

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Introduction

The fact that diabetic patients have an increased risk of atherosclerotic vascular disease has been well documented. However, the reason for this risk is less well understood.[1,2] The proliferation and migration of vascular smooth muscle cells are important steps in the formation and development of atherosclerotic lesions, and the degradation and remodeling of vascular basement membrane play a crucial role in the process. The effect of glucose on smooth muscle cell migration and proliferation has been studied, both of which are contributing factors to the development of atherosclerosis.[3] In many cells, transit through the G1-phase of the cell cycle and entry into the S-phase require the binding and activation of cyclin/CDK complexes, predominantly cyclin D1/CDK4 and cyclin E/CDK2.[4,5] The kinase activities of the cyclin/CDK complexes are negatively regulated by CDK inhibitors, such as $p21^{waf1/cip1}$ and $p27^{kip1}$.[6] However, there is little knowledge as to the mechanism including cell cycle protein by which elevated glucose may alter smooth muscle cell proliferation.

Increased proteolytic activity in the vessel wall mediates degradation of the extracellular matrix surrounding smooth muscle cells in response to injury. Gelatinase, matrix metalloproteinase (MMP)-2 (72 kDa) and MMP-9 (92 kDa) have been implicated as mediators of lesion development caused by atherosclerosis. Oxidative injury, due to the high glucose concentration, plays a central role in the development of diabetic complications.[7,8] Exposure to high glucose caused reactive oxygen species (ROS) production, leading to an increase in the state of cellular oxidative stress. Several of the growth factors that have been shown to increase intracellular ROS production as part of their intracellular signaling pathways include mitogen-activated protein kinases (MAPKs) and MMPs, important regulators in smooth muscle cell proliferation and differentiation.[9]

The flower buds of *Buddleja officinalis* Maximowicz (Loganiaceae) have been used traditionally as a folk remedy in Korea and China to treat vascular diseases, diabetes, conjunctival congestion and clustered nebulae.[10,11] Previously we reported that *B. officinalis* exhibited an anti-inflammatory

effect in endothelial cells.[12,13] In addition, it has been reported to contain terpenoids, flavonoids, phenylethanoids and saponins.[14] Although some constituents of *B. officinalis* have been identified, there are few studies verifying its pharmacological activity by a crude extract. Therefore, we examined the effects of an ethanol extract of *B. officinalis* (EBO) on high glucose-induced increase of MMP-2/-9 activities in primary cultured human aortic smooth muscle cells (HASMC). Thus, the aim of this study was to compare HASMC proliferation in a medium containing a physiologically normal glucose level and high glucose level, and to investigate the effect of EBO in high glucose-induced changes of HASMC proliferation.

Materials and methods

Materials

D-glucose, L-glucose, D-mannitol, gelatin and catalase were purchased from Sigma Chemical Company (St Louis, MO, USA). Lipofectamine LTX and CM-H_2DCFDA were purchased from Invitrogen Inc (Carlsbad, CA, USA). SB203580 and SP600125 were obtained from Biomol (Plymouth Meeting, PA, USA). Mouse monoclonal antibodies to cyclins, p27, β-actin and MMPs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies to CDKs, p21, NF-κB, phospho IκB-α and IκB-α were purchased from Santa Cruz Biotechnology. Goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Jackson Immunoresearch (West Grove, PA, USA). All other reagents were of the highest purity commercially available.

Preparation of an ethanol extract from *B. officinalis*

The flower buds of *B. officinalis* were purchased from Herbal Medicine Co-operative of Muju, Chonbuk, South Korea. A voucher specimen (No. HBE051) has been deposited in the Herbarium of the Professional Graduate School of Oriental Medicine, Wonkwang University, Korea. The dried flower buds of *B. officinalis* (600 g) were chopped into small pieces and soaked for 10 days in ethanol (99.0%, 1.5 L). The extract was filtered through Whatman No. 2 and then No. 5 filter paper and subsequently concentrated using a rotary evaporator (EBO, 14.44 g), and then used in the present study.

Cell cultures

Primary cultured HASMC (#C-007-5C), medium 231 and smooth muscle growth supplement were purchased from Invitrogen, which contains fetal bovine serum (5% v/v final concentration), recombinant human basic fibroblast growth factor, recombinant human epidermal growth factor, insulin, etc.[15] HASMC, plated at 4×10^4/well and used between passages 3 and 8, were maintained in EGM-2 medium in a humidified chamber containing 5% CO_2 at 37°C.

Measurement of cell proliferation

[^{3}H]-thymidine incorporation was measured to determine the effect on HASMC cell proliferation.[16] Quiescent cells were treated with 25 mmol/L glucose and EBO, respectively, and 1 μCi of [^{3}H]-thymidine was added (methyl-[^{3}H] thymidine 50 Ci/mmol; Amersham, Oakville, Ontario, Canada). After incubation for 24 h, cells were washed once with 2 mL of ice-cold phosphate-buffered saline (PBS) for 10 min, extracted three times with 2 mL of cold 10% trichloroacetic acid for five minutes each time and solubilized for at least 30 min at room temperature in 0.2 mL 0.3 N NaOH, 1% sodium dodecyl sulfate (SDS). After neutralizing with 0.2 mL 0.3 N HCl, [^{3}H]-thymidine activity was measured in a liquid scintillation counter (Beckman LS 7500, Fullerton, CA, USA). Each experiment was performed in triplicate or quadruplicate.

Cell viability test

To determine cell viability, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (0.5 mg) was added to 1 mL of cell suspension for four hours. After three washes of cells with PBS, the insoluble formazan product was dissolved in dimethyl sulfoxide. The optical density of each culture well was measured using a microplate reader at 590 nm (Multiskan, Thermo labsystems Inc, Franklin, MA, USA). The optical density in control cells was taken as 100% of viability.

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 H_2O, blocked with 5% skimmed milk powder in TBST (10 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 0.05% Tween-20) for one hour and incubated with the appropriate primary antibody at dilutions recommended by the supplier. Then the membrane was washed; primary antibodies were detected with goat anti-rabbit-IgG conjugated to horseradish peroxidase, and the bands were visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using the Chemi-doc image analyzer (Bio-Rad, Hercules, CA, USA).

RNA isolation and realtime quantitative reverse transcriptase-polymerase chain reaction

A kit from Qiagene (RNeasy Plus mini kit) was used for RNA isolation from cell cultures, and RNA quality was tested by measuring the ratio 260/280 nm in an ultraviolet spectrophotometer. Realtime quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was carried out in a 48-well plate using the Opticon MJ Research instrument (Bio-Rad Inc) and optimized standard SYBR Green 2-step qRT-PCR kit protocol (DyNAmo, Finnzymes, Finland). Specific sense and antisense primers used were as follows: MMP-2, 231 bp, sense: 5′ CAAAAA CAAGAAGACATACAT 3′; anti-sense: 5′ GCTTCCAAAC TTCACGCTC 3′; MMP-9, 223 bp, sense: 5′ TGGGGGGC AACTCGGC 3′; anti-sense: 5′ GGAATGATCTAAGCCC AG 3′. PCR was started at 95°C for 15 min (hot start) to activate the AmpliTaq polymerase, followed by a 45-cycle

amplification (denaturation at 94°C for 20 s, annealing at 60°C for 30 s, extension at 72°C for 60 s and plate reading at 60°C for 10 s). The temperature of PCR products was elevated from 65°C to 95°C at a rate of 0.2°C/1 s, and the resulting data were analyzed by using the software provided by the manufacturer.

Gelatin zymography

MMP-2 and MMP-9 enzymatic activities were assayed by gelatin zymography.[17] Samples were electrophoresed on 1 mg/mL gelatin containing 10% SDS-polyacrylamide gel. After electrophoresis, the gel was washed twice with washing buffer (50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 2.5% Triton X-100), followed by a brief rinsing in washing buffer without Triton X-100. The gel was incubated with incubation buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 10 mmol/L $CaCl_2$, 0.02% NaN_3, 1 μmol/L $ZnCl_2$) at 37°C. After incubation, the gel was stained with Coomassie brilliant blue R-250 and destained. A clear zone of gelatin digestion was represented with the MMP activity.

Intracellular ROS production assay

The fluorescent probe CM-H_2DCFDA was used to determine the intracellular generation of ROS by high glucose, as described elsewhere.[18] Briefly, the confluent HASMC in the 24-well culture plates were pretreated with EBO for one hour. After removing the EBO from the wells, HASMC were incubated with 20 μmol/L CM-H_2DCFDA for one hour. The HASMC were then stimulated with tumor necrosis factor (TNF)-α, and the fluorescence intensity (relative fluorescence units) was measured at an excitation and emission wavelength of 485 and 530 nm, respectively, using a spectrofluorometer.

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 Dschietzig *et al.*[19] Cells were harvested and washed with 1 mL buffer A (10 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.9, 1.5 mmol/L $MgCl_2$, 19 mmol/L KCl) for five minutes at 600 *g*. The cells were then resuspended in buffer A and 0.1% NP-40, left for 10 min on ice

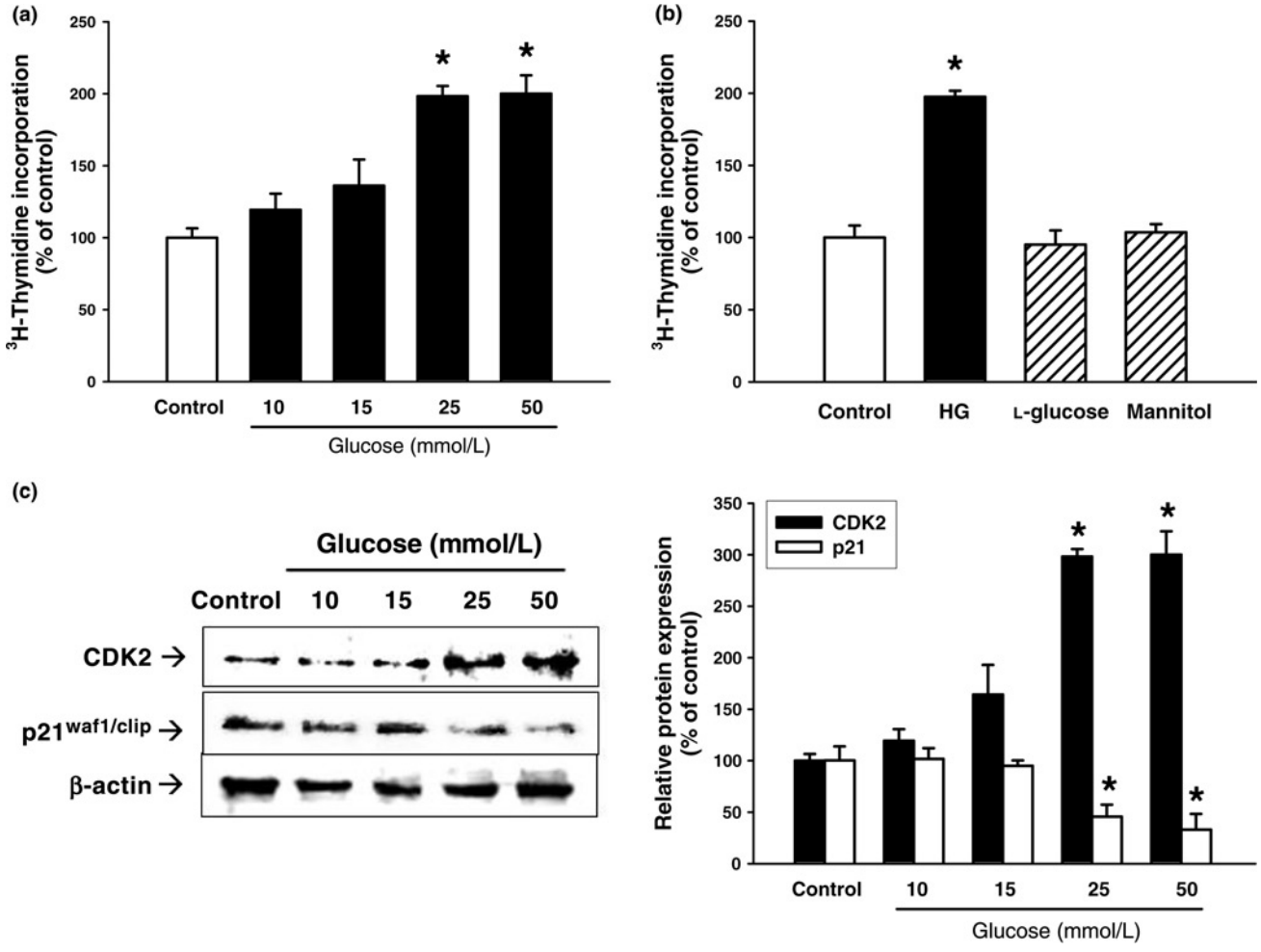

Figure 1 Effect of high glucose on human aortic smooth muscle cells (HASMC) proliferation. HASMC were treated with different dosages of D-glucose (0–50 mmol/L) for 24 h and pulsed with 1 μCi of [^{3}H]-thymidine for 24 h (a). Equal concentrations of 25 mmol/L D-glucose (HG), L-glucose and D-mannitol were added to HASMC (b). Effect of high glucose on the expression of CDK2 and p21$^{waf1/cip1}$ (c). Each electrophoretogram is representative of the results from five independent experiments. The right panel depicts quantitative data expressed as CDK2 (■) and p21$^{waf1/cip1}$ (□) to β-actin, and the results are expressed as the percentage of the control. Values are means ± SE of six independent experiments with triplicate dishes. $^*P < 0.01$ versus control

to lyse the cells and then centrifuged at 600 *g* for three minutes. The supernatant was saved as cytosolic extract. The nuclear pellet was then washed in 1 mL buffer A at 4200 *g* for three minutes, resuspended in 30 μL buffer C (20 mmol/L HEPES, pH 7.9, 25% glycerol, 0.42 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA), rotated for 30 min at 4°C, and then centrifuged at 14,300 *g* for 20 min. The supernatant was used as nucleus extract. The nucleus and cytosolic extracts were then analyzed for protein content using Bradford assay.

Transient transfection and luciferase reporter assay

The smooth muscle cells were grown to 60–80% confluence, and the cells were transiently co-transfected with the plasmids using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. Briefly, the transfection mixture containing 0.5 μg of 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 h, the cells were treated with EBO for 30 min and stimulated with high glucose for 24 h and then lysed. The luciferase and β-galactosidase activities were determined as described elsewhere using the Luciferase assay kit (Promega, Madison, WI, USA).[20] Luciferase activity was normalized with respect to β-galactosidase activity and expressed as a percentage of the activity of the high glucose group.

Statistical analysis

All the experiments were repeated at least three times. The results were expressed as a mean $\pm$ SE, and the data were analyzed using one-way analysis of variance followed by a Dunnett's test or a Student's *t*-test to determine any significant differences. $P < 0.05$ was considered as statistically significant.

Results

Effect of high glucose on HASMC proliferation

In the experiment to determine the effect of high glucose (25 mmol/L) on HASMC proliferation, we examined cell proliferation using [^{3}H]-thymidine incorporation. As shown in Figure 1a, exposure of HASMC to D-glucose (25 and 50 mmol/L) for 24 h induced cell proliferation up to 100 $\pm$ 2.5% and 102 $\pm$ 5.3% of control ($P < 0.05$), respectively. High glucose concentration can result in altered medium osmolarity. To rule out the involvement of hyperosmolarity in 25 mmol/L glucose-induced increase of cell proliferation, L-glucose and D-mannitol with an equal concentration of glucose were added to HASMC. Twenty-five millimolar mannitol and L-glucose did not affect [^{3}H]-thymidine incorporation (Figure 1b). In order to determine the effect of high glucose on cell cycle proteins, different doses of D-glucose (10–50 mmol/L) were added to HASMC and were examined by Western blotting. As shown in Figure 1c, high glucose (>25 mmol/L) significantly increased the expression level of CDK-2 in a dose-dependent manner. In contrast, high glucose decreased the level of $p21^{waf1/cip1}$, CDK inhibitory protein, in a dose-dependent manner.

Effect of EBO on high glucose-induced HASMC proliferation

To investigate the effect of EBO on high glucose-induced HASMC proliferation, [^{3}H]-thymidine incorporation was performed. [^{3}H]-thymidine incorporation revealed that [^{3}H]-thymidine incorporation increased by high glucose (25 mmol/L) was significantly decreased by pretreatment with 5–20 μg/mL EBO (Figure 2a). The high glucose-induced increase in the CDK2 or CDK4, cyclin D1 and cyclin E expression levels and the decrease in the $p21^{waf1/cip1}$ and $p27^{kip1}$ expression levels were attenuated by EBO. A dose-dependent manner was shown in concentrations of 5–10 μg/mL EBO (Figure 3). MTT assay demonstrated that EBO did not adversely affect the cell viability up to 50 μg/mL (Figure 2b). Therefore, it is confirmed that concentration of EBO did not show cytotoxicity in the present study.

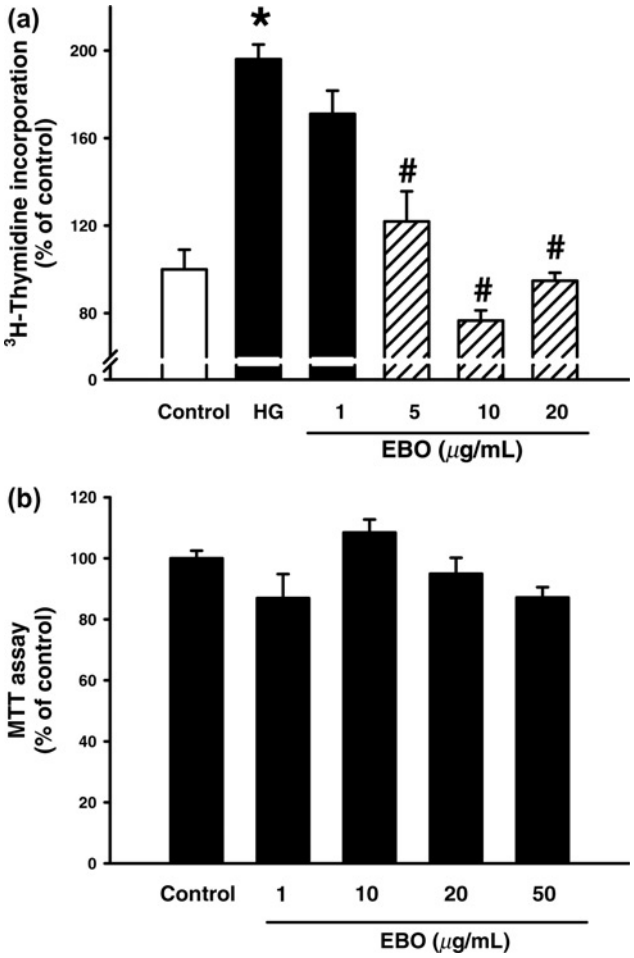

Figure 2 Effect of extract of *Buddleja officinalis* (EBO) on high glucose-induced human aortic smooth muscle cells (HASMC) proliferation. HASMC were pretreated with EBO (0–20 μg/mL) for 30 min prior to the treatment of 25 mmol/L glucose for 24 h. [^{3}H]-thymidine analysis was performed as described in the 'Materials and methods section' (a). Effect of EBO on cytotoxicity (b). HASMC were treated with various concentrations (1–50 μg/mL) of EBO for 48 h. Data are expressed as a percentage of basal value and are the means $\pm$ SE of five independent experiments with four dishes. $^*P < 0.01$ versus control, $^\#P < 0.05$ versus high glucose alone

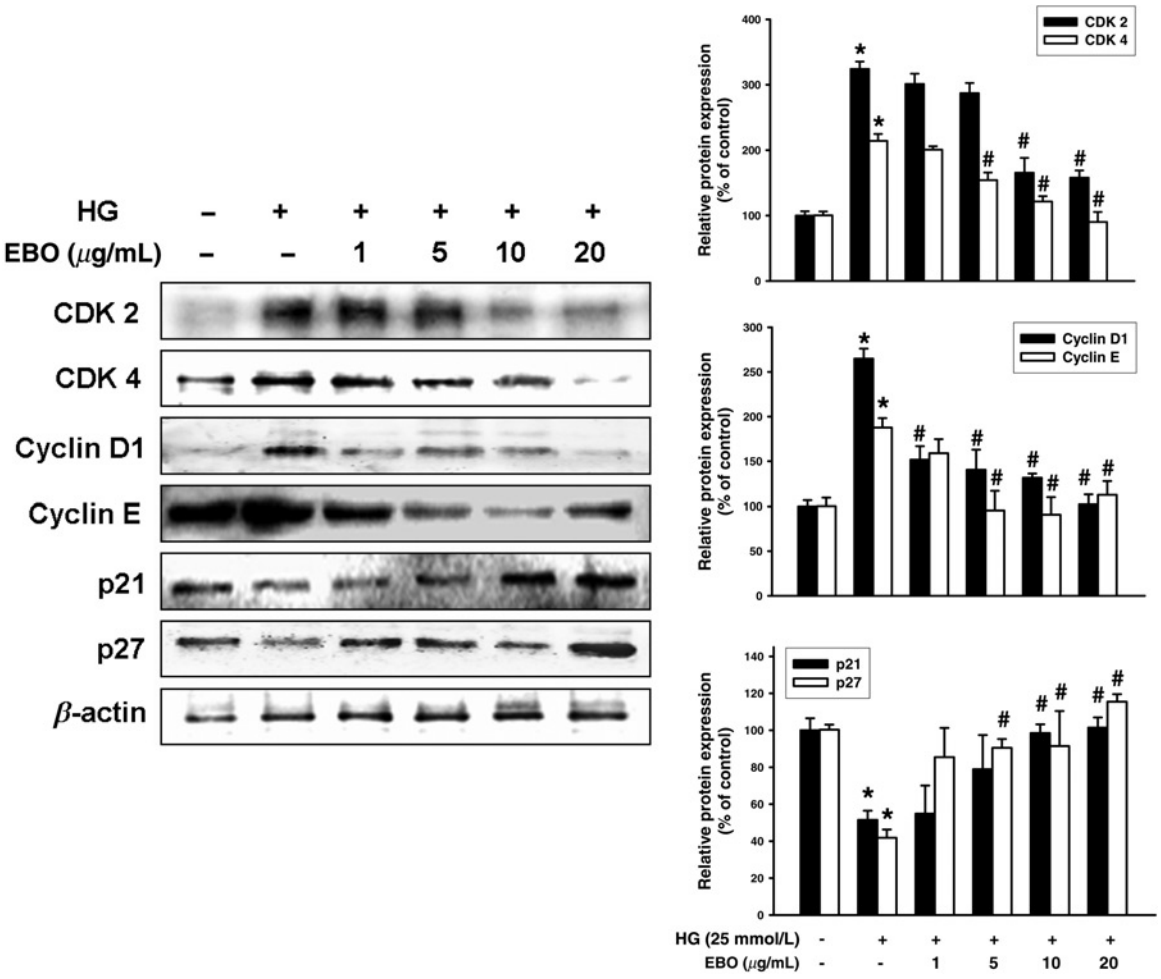

Figure 3 Effect of extract of *Buddleja officinalis* (EBO) on high glucose-induced cell cycle protein expression. Human aortic smooth muscle cells (HASMC) were pretreated with EBO (0–20 μg/mL) for 30 min prior to the treatment of 25 mmol/L glucose for 24 h. Western blotting analysis was performed as described in the 'Materials and methods' section. Each electrophoretogram is representative of the results from five independent experiments. Data are expressed as a percentage of basal value and are the means $\pm$ SE of five independent experiments with four dishes. $^{*}P < 0.01$ versus control, $^{\#}P < 0.05$ versus high glucose alone

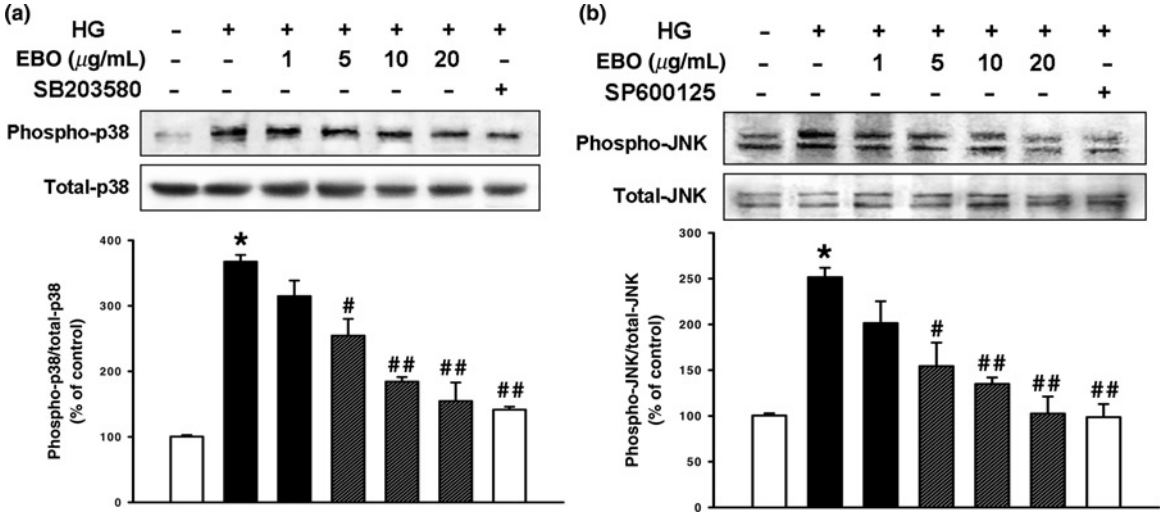

Figure 4 Effect of extract of *Buddleja officinalis* (EBO) on p38 mitogen-activated protein kinases (MAPK) (a) and JNK (b) phosphorylation in human aortic smooth muscle cells (HASMC). HASMC were pretreated with EBO (0–20 μg/mL) for 30 min prior to the treatment of 25 mmol/L glucose. SB203580 (1 μmol/L) and SP600125 (1 μmol/L) were used as positive control for p38 MAPK and JNK, respectively. Each electrophoretogram is representative of five different experiments. The lower panel depicts quantitative data expressed as phospho-p38 MAPK and phospho-JNK normalized to each total form, and the results are expressed as the percentage of the control. $^{*}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus high glucose alone

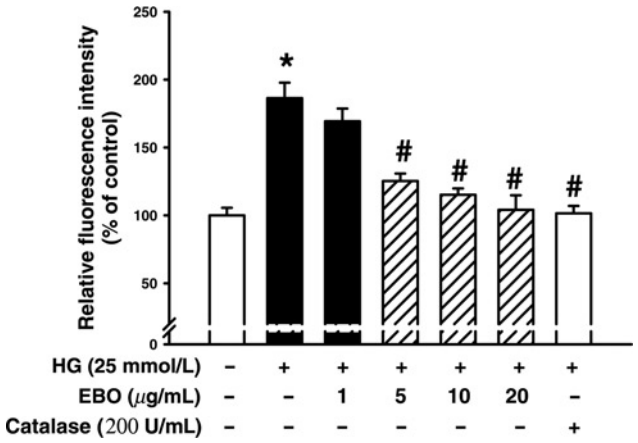

Figure 5 Effect of extract of *Buddleja officinalis* (EBO) on high glucose-induced intracellular reactive oxygen species production. Human aortic smooth muscle cells (HASMC) were pretreated with EBO (0–20 μg/mL) incubated with 20 μmol/L CM-H_2DCFDA for one hour. This was followed by stimulation with high glucose (25 mmol/L). The fluorescence intensity of HASMC was measured using a fluorescence microplate. The results are expressed as a mean percentage of the fluorescence intensity $\pm$ SE of five individual experiments with triplicate dishes. $^{*}P < 0.01$ versus control, $^{\#}P < 0.05$ versus high glucose alone

Involvement of MAPKs or ROS in the inhibitory effect of EBO on high glucose-induced HASMC proliferation

The involvement of MAPKs in the effect of high glucose on HASMC proliferation was examined. Figure 4 showed that high glucose-induced p38 MAPK and JNK phosphorylation were attenuated by pretreatment with EBO. In addition, ROS has been shown to activate various transcription factors as a common second messenger in various pathways leading to NF-κB activation.[21] Therefore, the level of intracellular ROS production was assessed by monitoring the fluorescence in order to determine whether EBO can reduce the level of high glucose-induced oxidative stress in HASMC. Figure 5 showed that pretreatment with EBO (5–20 μg/mL) significantly suppressed high glucose-induced increase of ROS production to 60.9%, 71.2% and 82.2% of high glucose-treated cells, respectively ($P < 0.05$).

Involvement of NF-κB in the inhibitory effect of EBO on high glucose-induced HASMC proliferation

In Western blotting analysis, p65 NF-κB expression, the active subunit of the NF-κB complex, was increased in

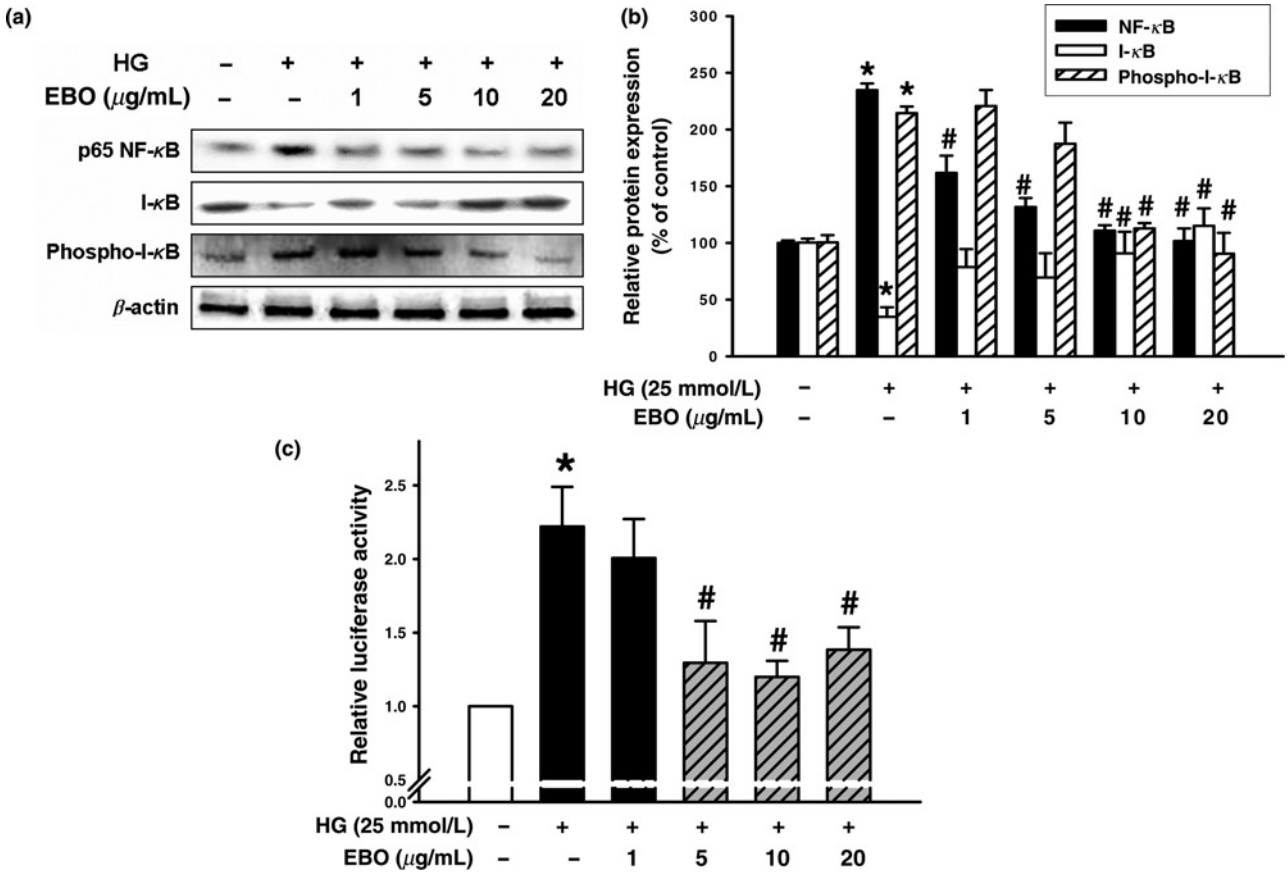

Figure 6 Effect of extract of *Buddleja officinalis* (EBO) on high glucose-induced nuclear factor-κB (NF-κB) activation. High glucose-induced nuclear translocation of NF-κB p65 was detected by Western blot analysis (a, b). Human aortic smooth muscle cells (HASMC) were pretreated with EBO and then stimulated with high glucose. The nuclear or total cellular protein extracts were prepared and blotted with the antibodies specific for NF-κB p65 (■), IκB-α (□) and p-IκB-α (▨), respectively. Effect of EBO on high glucose-induced NF-κB p65 promoter activity (c). HASMC were transiently transfected with pGL3-4κB-Luc. After 24 h, HASMC were pretreated with EBO for 30 min and then stimulated with high glucose for 24 h. This was followed by harvesting and their luciferase and]β-galactosidase activities were determined. The luciferase activities are expressed relative to high glucose. The results are expressed as a mean $\pm$ SE of five individual experiments with triplicate dishes. $^{*}P < 0.01$, compared with control, $^{\#}P < 0.05$, compared with high glucose alone

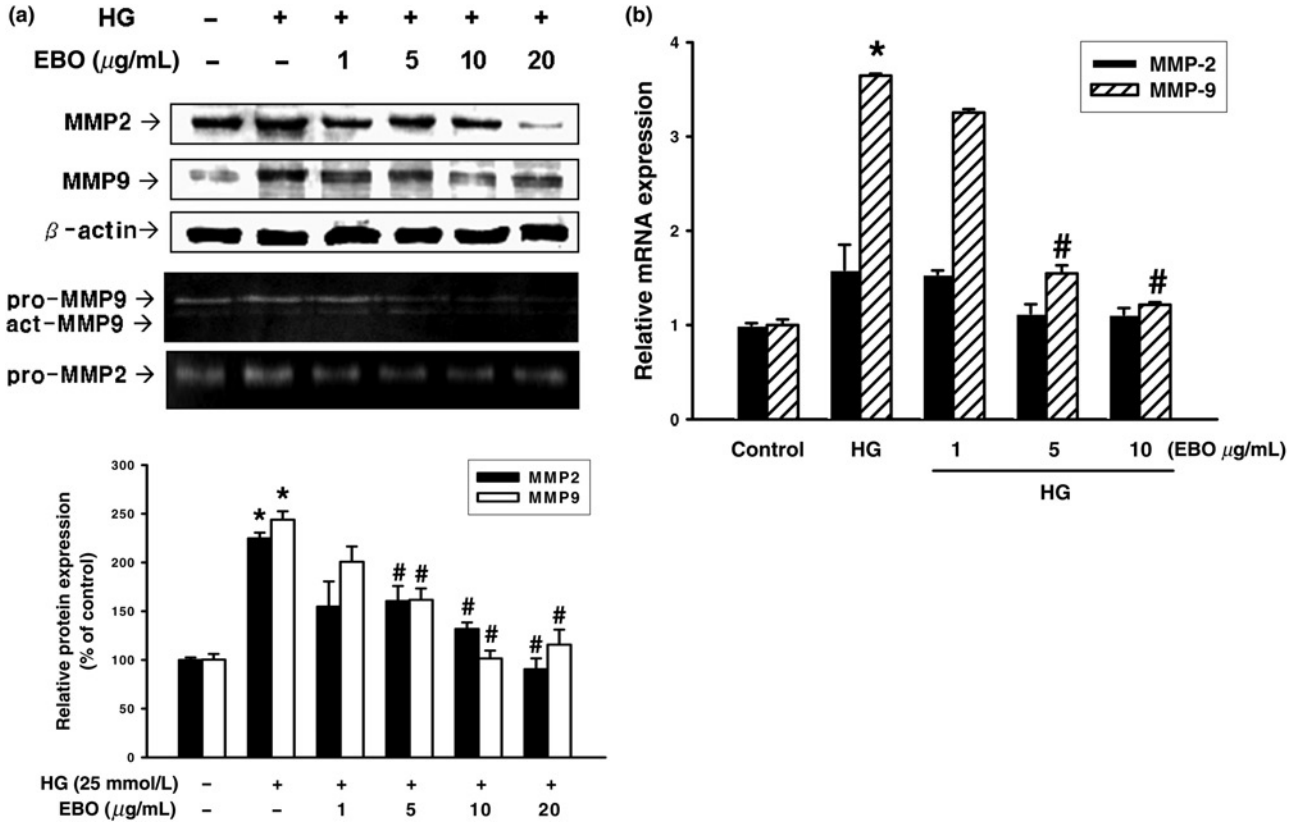

Figure 7 Effect of extract of *Buddleja officinalis* (EBO) on high glucose-induced matrix metalloproteinase (MMP)-2 and MMP-9 activity. (a) Human aortic smooth muscle cells (HASMC) were pretreated with EBO (0–10 μg/mL) for 30 min and then stimulated with high glucose for 24 h. The cell lysates were examined for the expression of MMP-2/-9 by Western blotting (top panel). The levels of MMP-2/-9 in conditioned medium were analyzed by gelatin zymography (bottom panel). The results are expressed as the percentage of the control and each electrophoretogram is representative of the results from five independent experiments. (b) Realtime quantitative reverse transcriptase-polymerase chain reaction analysis was performed using specific primers for MMP-2, MMP-9 and glyceraldehyde 3-phosphate dehydrogenase, respectively. Data are expressed as a fold of basal value and are the means $\pm$ SE of five independent experiments with four dishes. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus high glucose alone

the nuclear extracts and IκB-α phophorylation was also increased in the cytoplasmic extracts by high glucose (Figure 6a). Pretreatment with EBO decreased TNF-α-induced p65 NF-κB and phospho-IκB-α expression levels. In contrast, the IκB-α expression level was decreased by high glucose, and recovered by pretreatment with EBO in a dose-dependent manner. Transient transfection was performed using the NF-κB-dependent luciferase reporter plasmid in order to further examine the effects of EBO on NF-κB transcription activity. As shown in Figure 6b, high glucose increased NF-κB transcription activity and pretreatment with EBO significantly inhibited high glucose-induced NF-κB transcriptional activity over 5 μg/mL concentration.

Effect of EBO on high glucose-induced increase of MMP-2 and MMP-9

Since both MMP-2 and MMP-9 have been closely correlated with smooth muscle cell proliferation of vascular lesions and are activated by oxidative stress, the high glucose-induced MMP-2 and MMP-9 expressions and activities were examined.[22] The results of gelatin zymography revealed that culture medium by high glucose increased MMP-9 activity but not MMP-2. Western blotting also showed that high glucose increased MMP-9 expression. To determine the effect of EBO in preventing the increase of MMP-2 and MMP-9 activities by high glucose, HUVEC were cultured in serum-free media in the absence or presence of various concentrations of EBO. Pretreatment with EBO decreased MMP-9 expression and activity (Figure 7a). In addition, realtime qRT-PCR revealed that high glucose-induced increases of MMP-9 mRNA expression level were decreased by pretreatment with EBO (Figure 7b).

Discussion

Patients with diabetes are at higher risk for atherosclerotic disease than non-diabetic individuals with other comparable risk factors.[23–25] In the past several years, a number of studies have demonstrated that the proliferation and migration of vascular smooth muscle cells play a major role in the pathogenesis of atherosclerosis. *B. officinalis* inhibited high glucose-induced cell adhesion molecule and gelatinase expressions in vascular endothelial cells.[12,13] In the present study, the hypothesis of the inhibitory effect of *B. officinalis* on high glucose-induced HASMC proliferation was supported by our findings that pretreatment of EBO blocked high glucose-induced [^{3}H]-thymidine

incorporation and cell cycle protein expressions. The result of [^{3}H]-thymidine incorporation as an index of DNA synthesis indicated that EBO led to G1 cell cycle arrest in HASMC. Our Western blotting results also indicated that pretreatment with EBO of HASMC was found to result in significant down-modulation of all these regulatory molecules in the G1-phase of the cell cycle, specifically, cyclin D1, cyclin E, CDK2 and CDK4. $p21^{waf1/cip1}$ and $p27^{kip1}$ are regarded as universal inhibitors of cyclin/CDK complexes.[26] Pretreatment with EBO attenuated high glucose-induced increases of $p21^{waf1/cip1}$ and $p27^{kip1}$ expressions, suggesting that EBO-induced reduction of $p21^{waf1/cip1}$ and $p27^{kip1}$ could also be responsible for G1-phase arrest in HASMC.

Gelatinase, MMP-2 and MMP-9 play pivotal roles in the proliferation of HASMC, and the inhibitory effect on their expression is important in the search for therapeutic natural herbs.[27–29] The results of gelatin zymography and Western blotting revealed that pretreatment with EBO decreased high glucose-induced MMP-9 activity and expression. MMP-9 mRNA expression also increased by high glucose and pretreatment with EBO inhibited it in a dose-dependent manner (Figue 6b). This result suggested that MMP-9 is more sensitive to the inhibitory effect of EBO on high glucose-induced gelatinases in HASMC. A recent report concluded that MMP-9 is critical for the development of arterial lesions by regulating vascular smooth muscle proliferation.[30] MMP-9 gene expression can be activated by a number of signaling pathways, including those involving ERK1/2, p38 MAPK, JNK and PI3K/Akt, which are the upstream modulators of AP-1 or NF-κB.[31] High glucose-induced p38 MAPK and JNK phosphorylation were attenuated by pretreatment with EBO. Thus, our results demonstrated that EBO inhibited high glucose-induced HASMC proliferation through suppression of MMP-9 activity and p38 MAPK and JNK phosphorylation.

Excess accumulation of ROS has been reported in human and rodent diabetic vessels.[32,33] It is well known that ROS is associated with excess vascular smooth muscle cell proliferation through activation of MAPKs and transcription factors.[34,35] In the present experiment, the production of superoxide in HASMC was quantified by measuring the amount of CM-H_2DCFDA accumulation. Pretreatment with EBO significantly suppressed the high glucose-induced increase of ROS production, suggesting a potential antioxidant property of *B. officinalis*.

It has been also shown that activation of transcription factor NF-κB by high glucose is required for the transcriptional activation of MAPKs and MMPs.[36] Stimuli of high glucose lead to IκB-α phosphorylation.[37] Phosphorylated IκB-α resulted in the translocation of NF-κB to the nucleus, followed by the activation of specific target genes such as cyclin/CDK, MMP or MAPK.[38] The present study showed that pretreatment with EBO inhibited high glucose-stimulated translocation of NF-κB p65 to the nucleus and high glucose-induced activation of IκB-α phosphorylation. In addition, luciferase assay results demonstrated that EBO has some inhibitory effect on the NF-κB promoter specific to high glucose-induced HASMC proliferation. These findings indicate that EBO could contribute to the improvement of diabetic atherosclerosis associated with regulation of HASMC proliferation through inhibition of NF-κB activation.

To our knowledge, this study is the first to produce evidence for the antiproliferative effect of EBO in primary cultured HASMC. However, the therapeutic effect of EBO and the subsequent influence on HASMC proliferation for atherosclerosis are currently unknown. Thus, we further demonstrate here that *B. officinalis* suppresses high glucose-induced HASMC proliferation through inhibition of MMP-9 activity with its detailed molecular mechanisms including p38 MAPK, JNK, ROS and NF-κB, supporting the previous reports of its therapeutic potential in diabetic atherosclerosis.

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