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Protective Effect of *Padina arborescens* Extract against High Glucose-induced Oxidative Damage in Human Umbilical Vein Endothelial Cells

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ABSTRACT: Dysfunction of endothelial cells is considered a major cause of vascular complications in diabetes. In the present study, we investigated the protective effect of *Padina arborescens* extract against high glucose-induced oxidative damage in human umbilical vein endothelial cells (HUVECs). High-concentration of glucose (30 mM) treatment induced cytotoxicity whereas *Padina arborescens* extract protected the cells from high glucose-induced damage and significantly restored cell viability. In addition, lipid peroxidation, intracellular reactive oxygen species (ROS), and nitric oxide (NO) levels induced by high glucose treatment were effectively inhibited by treatment of *Padina arborescens* extract in a dose-dependent manner. High glucose treatment also induced the overexpressions of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and NF-κB proteins in HUVECs, but *Padina arborescens* extract treatment reduced the overexpressions of these proteins. These findings indicate the potential benefits of *Padina arborescens* extract as a valuable source in reducing the oxidative damage induced by high glucose.

Keywords: *Padina arborescens*, diabetes, oxidative damage, high glucose, HUVECs

INTRODUCTION

Diabetes is a common metabolic disease characterized by abnormally high plasma glucose levels, leading to major complications, such as diabetic neuropathy, retinopathy, and cardiovascular diseases (1). Several recent studies have demonstrated that hyperglycemia can cause glucose to undergo autooxidation to generate intermediates that lead to the formation of ROS, nitric oxide (NO), peroxynitrite ($ONOO^-$), and advanced glycation end products (AGE), which cause various complications of diabetes (2). Prolonged hyperglycemia is the major factor in the etiology of atherogenic pathogenesis in diabetes, which causes 80% of total mortality in diabetic patients. One diabetic vascular complication involves endothelial dysfunction characterized by impaired endothelium-dependent vasomotor responses. Previous studies have shown that hyperglycemia induces endothelial dysfunction, possibly due to oxidative stress (3,4). Accordingly, a reduction in cellular antioxidant reserves is responsible for triggering diabetic vascular complications (5,6). Therefore, antioxidants can prevent pathological damage caused by hyperglycemia-induced oxidative stress associated with diabetes (7).

Marine algae have demonstrated free radical scavenging activities, and thus may help slow aging and prevent some chronic diseases. In particular, brown algae display a variety of biological activities, including antioxidant (8), anti-inflammatory (9), anti-coagulant (10) and anti-hyperlipidemic properties (11). *Padina arborescens*, a type of brown alga popular in Korea and Japan as a food ingredient and marine herb, contains biologically active compounds such as bromophenols (12). However, in high glucose, the effect of *Padina arborescens* on oxidative damage of HUVECs is unclear. In the present study, we investigated the protective effects of *Padina arborescens* extract (PAE) against high glucose-induced oxidative damage using human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Materials

The brown alga, *Padina arborescens* (Phylum Ochrophyta, Class Phaeophyceae, Order Dictyotales, Family Dictyota-

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ceae) was collected along the coast of Jeju Island, Korea. The sample was washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water and maintained in a medical refrigerator at −20°C. Thereafter, the frozen samples were lyophilized and homogenized with a grinder prior to extraction. *Padina arborescens* was extracted with ten volumes of 80% methanol for 12 h three times at room temperature. The filtrate was then evaporated at 40°C to obtain the methanol extract. The *Padina arborescens* extract (PAE) was thoroughly dried for complete removal of solvent and stored in a deep freezer (Nihon Freezer Co., Tokyo, Japan) (−80°C).

Cell culture

Human umbilical vein endothelial cells (HUVECs) and the endothelial cell basal medium-2 (EBM-2) bullet kit were purchased from Clonetics Inc. (San Diego, CA, USA). Cells were cultured in EGM-2 containing 2% fetal bovine serum (FBS; GIBCO Inc., Grand Island, NY, USA), at 37°C in a humidified atmosphere containing 5% CO_2 according to the supplier's recommendations, and used between passages 3 and 6.

Assay of neutral red cell viability

Cell viability was assessed by measuring the uptake of the supravital dye neutral red (13). Cells (4×10^4 cells/well) cultured in 24-well plates were pre-incubated with glucose (5.5 and 30 mM) in humidified atmosphere containing 5% CO_2 at 37°C for 48 h. After 48 h of incubation, the cells were treated with various concentrations (25, 50, and 100 μg/mL) of PAE and further incubated for 20 h. Thereafter, the medium was carefully removed from each well, and replaced with 0.5 mL of fresh medium containing 1.14 mmol/L neutral red. After 3 h of incubation, the medium was removed and the cells were washed twice with phosphate buffered saline (PBS, pH 7.4). The incorporated neutral red was released from the cells by incubation in the presence of 1 mL of cell lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L dithiothreitol (DTT), and Triton X-100 (1%, v/v)] containing acetic acid (1%, v/v) and ethanol (50%, v/v) at room temperature for 15 min. To measure the dye taken up, the cell lysis products were centrifuged and absorbance of the supernatant was measured spectrophotometrically at 540 nm (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Assay of lipid peroxidation

Lipid peroxidation, which was caused by influence of ROS generated with a high glucose-induced oxidative damage in the cells, was measured by thiobarbituric acid reactive substances (TBARS) production. Cells (4×10^4 cells/well) were seeded in 24-well plates and pre-incubated with glucose (5.5 and 30 mM) in humidified atmosphere containing 5% CO_2 at 37°C for 48 h. After 48 h of incubation, the cells were treated with various concentrations (25, 50, and 100 μg/mL) of PAE and further incubated for 20 h. A 200 μL sample of each medium supernatant was mixed with 400 μL of TBARS solution and then boiled at 95°C for 20 min. The absorbance at 532 nm was measured and TBARS concentrations were extrapolated from the 1,1,3,3-tetraethoxypropane serial dilution standard curve. TBARS values were then expressed as equivalent nmoles of malondialdehyde (MDA) (14).

Assay of intracellular ROS levels

Intracellular ROS levels were measured by the 2',7'-dichlorofluorescein diacetate (DCF-DA) assay (15). DCF-DA can be deacetylated in cells by reacting quantitatively with intracellular radicals to convert into its fluorescent product, DCF, which is retained within the cells. Therefore, DCF-DA was used to evaluate the generation of ROS in oxidative damage. Cells (2×10^4 cells/well) were seeded in 96-well plates and pre-incubated with glucose (5.5 and 30 mM) in humidified atmosphere containing 5% CO_2 at 37°C for 48 h. After 48 h of incubation, the cells were treated with various concentrations (25, 50, and 100 μg/mL) of PAE and further incubated for 20 h. Thereafter, the medium was removed and the cells were washed twice with phosphate buffered saline (PBS, pH 7.4) and incubated with 100 μM DCF-DA for 90 min at room temperature. Fluorescence was measured using a fluorescence plate reader (BMG LABTECH GmbH, Offenburg, Germany).

Assay of nitric oxide (NO) levels

The amount of nitrite accumulation, the end product of NO generation, was assessed by the Griess reaction (16). Cells (2×10^4 cells/well) were seeded in 96-well plates and pre-incubated with glucose (5.5 and 30 mM) in humidified atmosphere containing 5% CO_2 at 37°C for 48 h. After 48 h of incubation, the cells were treated with various concentrations (25, 50, and 100 μg/mL) of PAE and further incubated for 20 h. Thereafter, each 50 μL of culture supernatant was mixed with an equal volume of Griess reagent [0.1% N-(1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5% phosphoric acid] and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in a microplate absorbance reader and a series of known concentrations of sodium nitrite was used as the standard curve.

Total and nuclear protein extracts

Cells were homogenized with ice-cold lysis buffer containing 250 mM NaCl, 25 mM Tris-HCl (pH 7.5), 1% v/v NP-40, 1 mM DTT, 1 mM PMSF, and protein inhibitor

cocktail (10 μg/mL aprotinin, 1 μg/mL leupeptin). The cells were then centrifuged at 20,000×*g* for 15 min at 4°C. The supernatants were used as total protein extracts (17). For nuclear protein extracts, cells were homogenized with ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 15 mM $CaCl_2$, 1.5 M sucrose, 1 mM DTT, and protease inhibitor cocktail (10 μg/mL aprotinin, 1 μg/mL leupeptin). Then, the cells were centrifuged at 11,000×*g* for 20 min at 4°C. The supernatants were resuspended with extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 10 mM DTT, and protease inhibitor cocktail (10 μg/mL aprotinin, 1 μg/mL leupeptin). The samples were shaken gently for 30 min and centrifuged at 21,000×*g* for 5 min at 4°C. The pellets were used as nuclear protein extracts. The total and nuclear protein contents were determined by the Bio-Rad protein kit (Bio-Rad Laboratories Inc.) with BSA as the standard.

Immunoblotting

iNOS and COX-2 expressions and NF-κB p65 DNA-binding activity were determined by western blot analysis (17). Total protein (20 μg) for iNOS and COX-2 protein levels and nuclear protein (20 μg) for NF-κB were electrophoresed through 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Separated proteins were transferred electrophoretically to a pure nitrocellulose membrane, blocked with 5% skim milk solution for 1 h, and then incubated with primary antibodies (Abcam, Cambridge, UK; 1:1,000) overnight at 4°C. After washing, the blots were incubated with goat anti-rabbit or goat anti-mouse IgG HRP-conjugated secondary antibody for 1 h at room temperature. Each antigen-antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-1000 plus (FUJIFILM, Tokyo, Japan). Band densities were determined by an image analyzer (Multi Gauge V3.1, FUJIFILM Corp. Valhalla, NY, USA) and normalized to β-actin for total protein and nuclear protein.

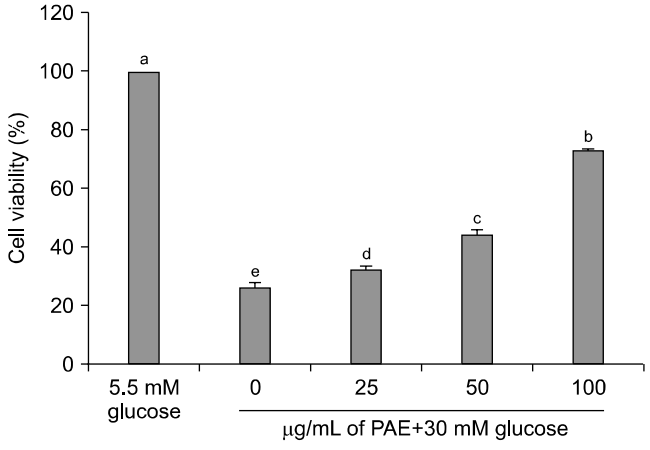

Fig. 1. Effect of PAE on high glucose-induced-oxidative damage of HUVECs. HUVECs were preincubated with normal glucose (5.5 mM) and high glucose (30 mM) for 48 h. Thereafter, HUVECs were treated with various concentrations (25, 50, and 100 μg/mL) of PAE and further incubated for 20 h. After an incubation of 20 h, cell viability was determined by neutral red assay. Each value is expressed as mean±SD in triplicate experiments. [a-e]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

Statistical analysis

The data are represented as the mean±standard deviation (SD) of triplicate experiments. The statistical analysis was performed using SAS 9.0 software (SAS Institute Inc., Cary, NC, USA). The values were evaluated by one-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range tests, and p-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Effect of PAE on high glucose-induced cell viability

In an effort to express whether *Padina arborescens* extract (PAE) protect the cells from cellular damage induced by high glucose (30 mM), cell viability was examined by neutral red assay. As shown in Fig. 1, a high glucose treatment without PAE decreased cell viability to 26.1%, while PAE protected the cells against the cellular damage induced by 30 mM glucose in a dose-dependent manner; especially, treatment with 100 μg/mL of PAE significantly increased cell viability to 77.2%.

The exposure of endothelial cells to high glucose increases the production of reactive oxygen species at the mitochondrial level, leading to increased cellular apoptosis (18). Exposure of HUVECs to high glucose resulted in a significant decrease of cell viability; however, PAE treatment inhibited cell death. Taken together, these results suggest that PAE protect HUVECs from high glucose-induced oxidative damage.

Effect of PAE on high glucose-induced lipid peroxidation

The effect of PAE on lipid peroxidation in high glucose treated HUVECs was determined by measuring TBARS, a lipid peroxidation product (Fig. 2). TBARS level of the normal glucose (5.5 mM glucose) treated cells was recorded as 0.14 nmol MDA, whereas that of the high glucose-treated cells was recorded as 0.24 nmol MDA. However, treatment with PAE together with high glucose significantly inhibited TBARS formation, indicating protection against lipid peroxidation. When the cells were treated with 100 μg/mL of PAE, TBARS was significantly decreased by 0.15 nmol MDA.

Lipid peroxidation may be a form of cell damage that is mediated by free radicals (19). Lipid peroxidation also

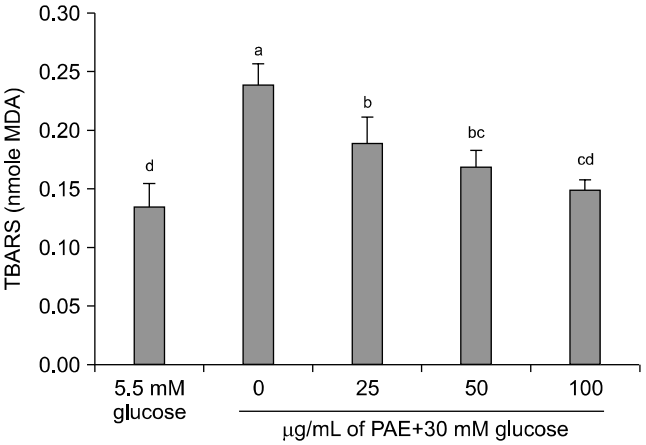

Fig. 2. Effect of PAE on high glucose-induced lipid peroxidation of HUVECs. HUVECs were treated with glucose and PAE in the same method as described in Fig. 1 legend. Lipid peroxidation was assayed by measuring the amount of TBARS formation. Each value is expressed as mean±SD in triplicate experiments. [a-d]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

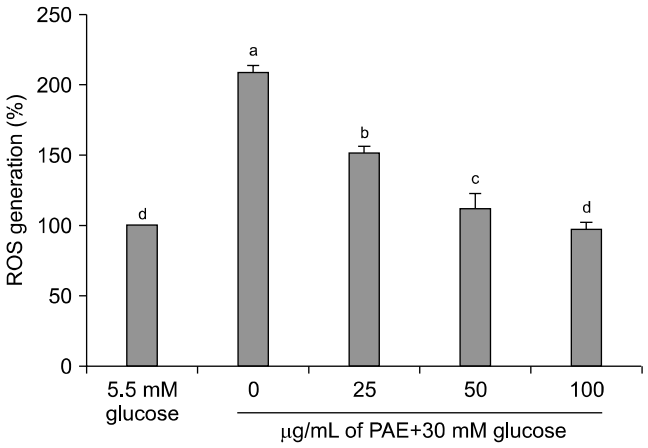

Fig. 3. Effect of PAE on high glucose-induced intracelular ROS of HUVECs. HUVECs were treated with glucose and PAE in the same method as described in Fig. 1 legend. After an additional 20 h, the intracellular ROS generated was detected by spectrofluorometry after the DCFH-DA treatment. Each value is expressed as mean±SD in triplicate experiments. [a-d]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

is one of the consequences of oxidative damage and has been suggested as a general mechanism for cell injury and death. Recently, the levels of TBARS are increased in diabetic patients (20). Therefore, the inhibition of lipid peroxidation is considered to be an important index of antioxidant activity as well as anti-diabetic activity induced by oxidative stress. In this study, high glucose induced lipid peroxidation in HUVECs and PAE inhibited TBARS formation effectively. The presently demonstrated protective action of PAE on TBARS formation can be attributed to its antioxidative effect.

Effect of PAE on high glucose-induced intracellular ROS

As shown in Fig. 3, the generation of intracellular ROS in HUVECs increased significantly after treatment with 30 mM high glucose compared with 5.5 mM normal glucose. When HUVECs were cultured with 30 mM glucose, intracellular ROS level increased significantly to 208.7%. However, treatment of PAE decreased dose-dependently the ROS level in the cells induced with 30 mM glucose; especially, treatment with 100 μg/mL of PAE resulted in a significant decrease in intracellular ROS to 98.82%. Therefore, PAE significantly decreased the elevated ROS level induced by high glucose.

Numerous studies have shown that hyperglycemia induces ROS, which hence triggers diabetic endothelial apoptosis and vascular dysfunction (21,22). Furthermore, high ROS levels induce oxidative stress, which can result in a variety of biochemical and physiological lesions. Our result showed that treatment of HUVECs with 30 mM glucose significantly increased the intracellular ROS level. However, PAE inhibited the high glucose-induced ROS generation. The present results indicate that PAE

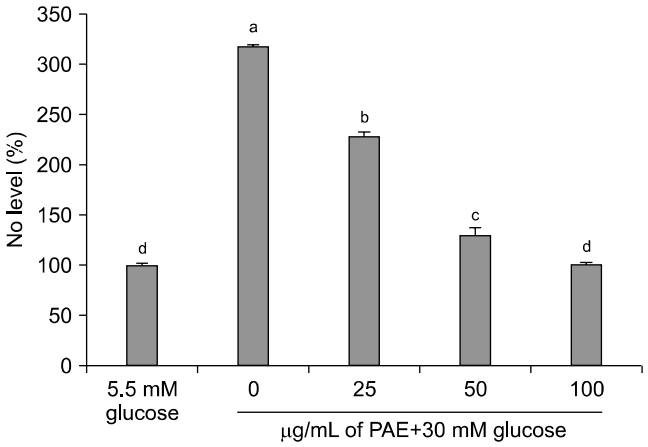

Fig. 4. Effect of PAE on high glucose-induced NO production of HUVECs. HUVECs were treated with glucose and PAE in the same method as described in Fig. 1 legend. After an additional 20 h, produced NO levels was assessed by the Griess reaction. Each value is expressed as mean±SD in triplicate experiments. [a-d]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

may play a role of protection on endothelial cell injury induced by ROS.

Effect of PAE on high glucose-induced NO

As shown in Fig. 4, the level of NO in HUVECs was significantly elevated by 30 mM glucose treatment compared with 5.5 mM glucose treatment. However, NO levels in PAE treated cells were significantly decreased. The level of NO in HUVECs treated with 30 mM high glucose is 315.9%, but treatment with 100 μg/mL of PAE together with high glucose exposure resulted in a

significant decrease in intracellular NO to 101.1%. PAE scavenged NO produced by high glucose-induced oxidative stress.

The impairment of NO bioavailability may also be responsible for vascular complications in diabetes. High glucose treatment leads to overproduction of nitric oxide (NO) and the superoxide anion (O_2^-) (23,24). NO and O_2^- separately cause ischemic renal injury; however, the toxicity and damage is multiplied as NO and O_2^- combine to produce reactive peroxynitrite ($ONOO^-$), which leads to serious toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modifications. In addition, NO and O_2^- induce highly reactive oxidative damage associated with diabetes (25). In the present study, we found that PAE scavenged NO produced by high glucose-induced oxidative stress. These findings suggest that PAE might confer important protection against the oxidative stress induced by hyperglycemia.

Effect of PAE on high glucose-induced iNOS and COX-2 expressions

To determine whether PAE inhibits high glucose-induced overexpressions of iNOS and COX-2 proteins, PAE concentrations of 50 and 100 μg/mL were added to HUVECs. As shown in Fig. 5, the levels of iNOS and COX-2 expressions were clearly higher in 30 mM glucose-treated HUVECs than with 5.5 mM glucose treatment. However, these expression levels by treatment with PAE were reduced markedly. The 100 μg/mL of PAE showed maximum inhibitory effects on both iNOS and COX-2 expressions. Actin was used as a housekeeping control gene.

iNOS is one of three key enzymes generating NO from arginine. Basically, NO plays a pivotal role in many body functions, although, its over production can lead to cytotoxicity, inflammation, and autoimmune disorders (26). Therefore, iNOS inhibitors are essential for prevention of inflammatory diseases associated with oxidative stress. COX-2 is another enzyme that plays a pivotal role in the mediation of inflammation, and catalyzed the rate-limiting step in prostaglandin biosynthesis (27). Thus, inhibition of COX-2 can provide an effective strategy for

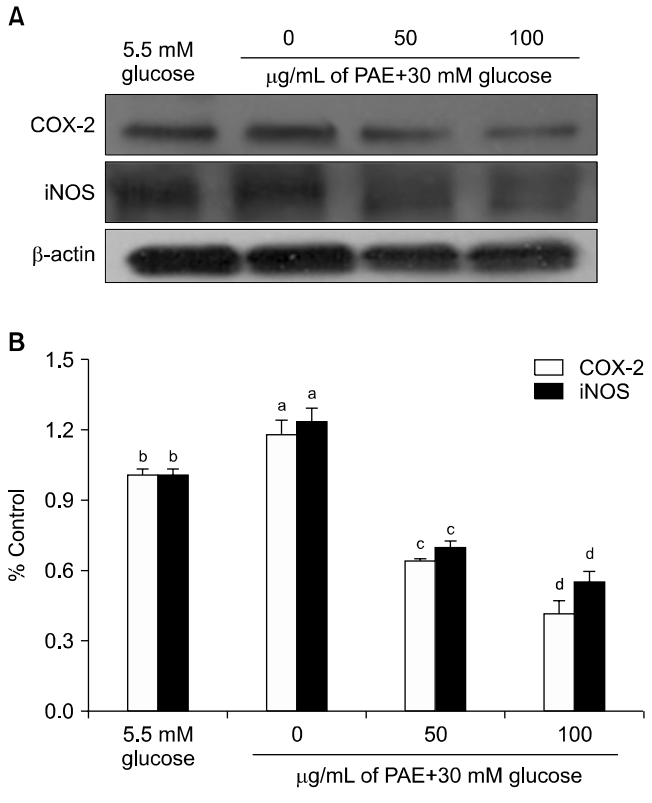

Fig. 5. Effect of PAE on high glucose-induced iNOS and COX-2 expression of HUVECs. HUVECs were treated with glucose and PAE in the same method as described in Fig. 1 legend. After an additional 20 h, cells were homogenized with ice-cold lysis buffer and then centrifuged to extract total protein extract. Cell lysates were electrophoresed, and the expression levels of iNOS and COX-2 were detected with specific antibodies in western blot analysis. (A) iNOS and COX-2 protein expression, (B) expression levels of iNOS and COX-2. Each value is expressed as mean±SD in triplicate experiments. [a-d]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

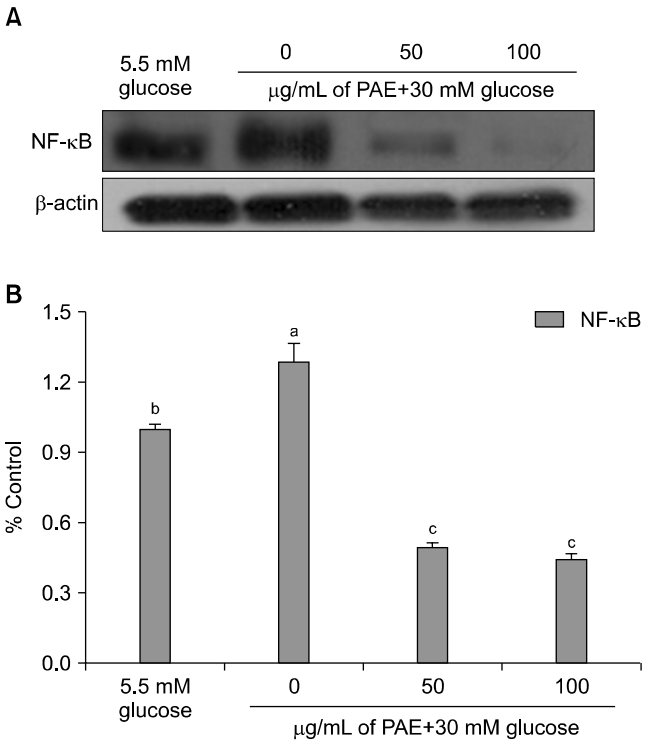

Fig. 6. Effect of PAE on high glucose-induced NF-κB activation of HUVECs. HUVECs were treated with glucose and PAE in the same method as described in Fig. 1 legend. After an additional 20 h, cells were homogenized with ice-cold lysis buffer and then centrifuged to extract total protein extract. Cell lysates were electrophoresed, and the expression NF-κB was detected with specific antibodies in a western blot analysis. Each value is expressed as mean±SD in triplicate experiments. [a-c]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

inhibiting the oxidative damage. In the present study, high glucose induced the over expression of iNOS and COX-2 proteins, and this was concentration-dependently inhibited by treatment with PAE. These findings indicate that PAE can alleviate oxidative damage by inhibiting expressions of iNOS and COX-2 enzymes.

Effect of PAE on high glucose-induced NF-κB activation

The effect of PAE on NF-κB activation was carried out using nuclear extracts obtained from HUVECs stimulated with high glucose of 30 mM in the presence or absence of PAE (Fig. 6) Treatment with high glucose was found to increase NF-κB activation as compared to that exhibited by normal glucose treatment. However, treatment of PAE in the cells exposed to high glucose decreased NF-κB activation in a concentration-dependent manner (Fig. 6). 100 μg/mL of PAE showed a maximum inhibitory effect of NF-κB activity. Actin was used as a house-keeping control gene.

NF-κB, a transcription factor that is responsive to oxidative stress, plays an important role in the mechanism of cell injury and in the induction of iNOS and COX-2, which are expressed as a result of NF-κB activation (28,29). In particular, NF-κB is activated in cells cultured under conditions of high glucose concentrations (30). NF-κB activation is suppressed by anti-inflammatory agents and antioxidant inhibitors (31). We presently observed that PAE inhibited high glucose-induced NF-κB activation in HUVECs (Fig. 6), indicating that NF-κB reduction by PAE may contribute to the attenuation of intracellular oxidative damage.

In conclusion, we demonstrated that PAE is a potential therapeutic agent that will reduce the damage caused by hyperglycemia-induced oxidative damage associated with diabetes.

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