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Effect of *Polyopes lancifolia* Extract on Oxidative Stress in Human Umbilical Vein Endothelial Cells Induced by High Glucose

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ABSTRACT: The protective effect of *Polyopes lancifolia* extract on high glucose-induced oxidative stress was investigated using human umbilical vein endothelial cells (HUVECs). High concentration of glucose (30 mM) treatment induced HUVECs cell death, but *Polyopes lancifolia* extract, at concentrations of 25, 50, and 100 μg/mL, protected cells from high glucose-induced damage. Furthermore, thiobarbituric acid reactive substances, intracellular reactive oxygen species, and nitric oxide levels increased by high glucose treatment were effectively decreased by treatment with *Polyopes lancifolia* extract in a dose-dependent manner. Also, *Polyopes lancifolia* extract treatment reduced the overexpressions of inducible nitric oxide synthase, cyclooxygenase-2, and nuclear factor-kappa B proteins activation that was induced by high glucose in HUVECs. These results indicate that *Polyopes lancifolia* extract is a potential therapeutic material that will reduce the damage caused by high glucose-induced-oxidative stress associated with diabetes.

Keywords: *Polyopes lancifolia*, high glucose, oxidative stress, protective effects, HUVECs

INTRODUCTION

Diabetes mellitus is the most serious, chronic metabolic disorder and is characterized by high blood glucose levels (1,2). Currently, an estimated 347 million people worldwide have diabetes, and the World Health Organization (WHO) projects that diabetes deaths will increase by two-thirds between 2008 and 2030 (3). Diabetic complications are related to hyperglycemic levels, especially with the length of exposure to hyperglycemia. Long-term glycemic control is a significant predictor of both micro- and macro-vascular complications (4,5). Micro- and macro-vascular complications of diabetes have complexity of pathogenesis involving functional disorder of vascular endothelial cells, which are sensitive to stimulatory factors such as increased glucose concentration, glycation end products, reduced nerve growth factor, and oxidative stress (6-9).

Oxidative stress is a major contributor to the diabetic vascular complication, which was observed in diabetic patients (6,10,11). High glucose might contribute to increased oxidative stress and the release of reactive oxygen/nitrogen species (ROS/RNS) by several mechanisms (4). Reactive oxygen species (ROS) are generated by oxidative phosphorylation, nicotinamide adenine dinucleotide phosphate oxidase, xanthin oxidase, cytochrome P450 monooxygenase, and glucose autoxidation (12). Furthermore, hyperglycemia can reduce antioxidant enzyme defenses, thereby allowing ROS to accumulate, resulting in cellular and tissue damage (13,14). For this reason, decline of oxidative stress induced by hyperglycemia is an important issue to reduce the risk of diabetic complications (15).

Polyopes lancifolia (*Harvey*) *kawaguchi et wang* (PL), a type of seaweed usually found off the coast of Republic of Korea and Japan, holds medicinal value (16,17). In a previous study, Kim et al. (17) revealed that bromophenol purified from the PL may have the potential as a natural nutraceutical for α-glucosidase inhibitory activity. However, the specific effect of PL on the endothelial function related to diabetes has not been examined. Therefore, this study was designed to examine the effect of PL on oxidative damage in human umbilical vein endothelial cells induced by high glucose.

MATERIALS AND METHODS

Materials

The red alga, *Polyopes lancifolia* (*Harvey*) *kawaguchi et wang* (PL) was collected along the coast of Jeju Island, Korea. The samples were washed three times with tap water to

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remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water and freeze-dried. Dried sample was ground and sifted through a 50-mesh standard testing sieve. The sample was extracted with ten volumes of 80% methanol for 12 h three times at room temperature. The filtrate was then vacuum-evaporated to obtain the extract. After PL extract (PLE) was thoroughly dried for complete removal of solvent, the extract was then stored in a deep freezer (Nihon freezer Co., Tokyo, Japan) (−80°C).

Cell culture

Human umbilical vein endothelial cell (HUVECs) and 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., Rockville, MD, USA) at 37°C in a humidified atmosphere containing 5% CO_2, according to the supplier's recommendation and which were 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. Cells (4×10^4 cells/well), cultured in 24-well plates in a humidified atmosphere containing 5% CO_2 at 37°C, were pre-incubated with glucose (5.5 and 30 mM) for 48 h, followed by treatment with various concentrations (25, 50, and 100 μg/mL) of PLE 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 supernatant was measured spectrophotometrically at 540 nm.

Assay of lipid peroxidation

Lipid peroxidation, which was caused by influence of ROS generated with high glucose-induced oxidative damage in the cells, was measured by thiobarbituric acid reactive substances production. Cells (4×10^4 cells/well) were seeded in a 24-well plate 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 PLE and further incubated for 20 h. A 200 μL sample of each medium supernatant was mixed with 400 μL of TBARS solution 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).

Assay of intracellular ROS levels

Intracellular ROS levels were measured by the 2',7'-dichlorofluorescein diacetate (DCF-DA) assay. DCF-DA can be deacetylated in cells, where it can react 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 a 96-well plate 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 PLE and further incubated for 20 h. Thereafter, the medium was removed and the cells were washed twice with PBS (pH 7.4) and then incubated with 100 μM DCF-DA for 90 min at room temperature. Fluorescence was measured using a fluorescence plate reader (BMG LABTECH GmbH, Offenberg, Germany).

Assay of nitric oxide (NO) levels

The amount of nitrite accumulation, the end product of NO generation, was assessed by the Griess reaction. Cells (2×10^4 cells/well) were seeded in a 96-well plate 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 PLE 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 (Bio-Rad Laboratories Inc., Hercules, CA, USA), and a series of known concentrations of sodium nitrite was used as a standard.

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. 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 Lab.) with BSA as the standard.

Immunoblotting

iNOS, COX-2 expressions and NF-κB p65 DNA-binding activity were determined by western blot analysis. Total protein (20 μg) for iNOS, 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 (1:1,000; Abcam, Cambridge, UK) 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.

Statistical analysis

The data are represented as the mean±standard error (SE) 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 of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Neutral red cell viability

Fig. 1 shows the effect of *Polyopes lancifolia* extract (PLE) on cell viability in HUVECs treated with high glucose of 30 mM. When HUVECs were treated with 30 mM glucose for 48 h, the cell viability significantly decreased. Cell viability was decreased to 26.1% in 30 mM glucose-treated HUVECs, but PLE protected the cells from high glucose-induced damage, and reviving cell survival was significantly increased to 32.2%, 44.1%, and 73.4% at the concentrations of 25, 50, and 100 μg/mL, respectively.

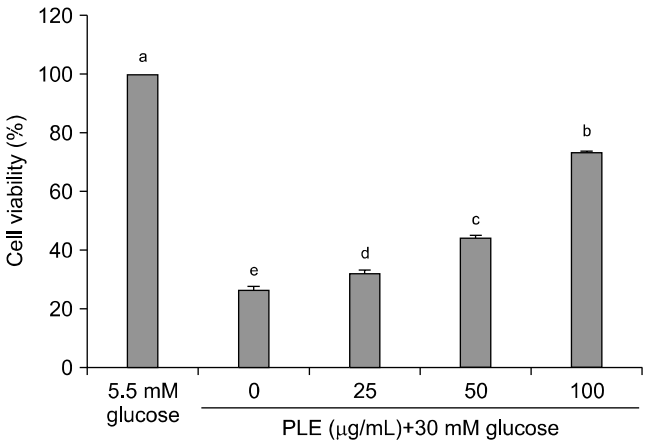

Fig. 1. Effect of PLE on cell viability in high-glucose-treated HUVECs. Cells in 24-well plates (4×10^4 cells/well) were pre-incubated with 5.5 or 30 mM glucose for 48 h, and then incubated in the absence of PLE or 25, 50, or 100 μg/mL of PLE for 20 h. The use of 5.5 mM glucose represents normal glucose and 30 mM represents high glucose. Each value is expressed as mean±SD (n=3). $^{a-e}$Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

Cell viability under oxidative stress induced by high glucose was measured by the neutral red uptake assay as reflected by lysosomal functions. Neutral red is uptaken into a cell owing to lysosomal activities (15). Our results evidenced that neutral red uptake was decreased owing to high glucose treatment, indicating that high glucose concentration might have a negative effect on the cell survival rate. However, the cell viability significantly increased with the addition of PLE in a dose-dependent manner. These findings suggest that PLE has the ability to protect the cell surface and lysosomal membranes of HUVECs from oxidative damage induced by high glucose treatment.

Lipid peroxidation

Inhibitory effect of PLE against lipid peroxidation induced by a high glucose was determined by measuring thiobarbituric acid reactive substance (TBARS). When HUVECs were incubated with 5.5 mM or 30 mM glucose for 48 h, TBARS level in the 30 mM glucose-treated HUVECs was significantly increased in comparison to the cells treated with 5.5 mM glucose. However, PLE protected lipid peroxidation of the cells in a dose-dependent manner. Importantly, TBARS was significantly decreased by 0.13 nmol MDA in the cells treated with 50 μg/mL of PLE together with high glucose.

Lipid peroxidation is one of the important interme-

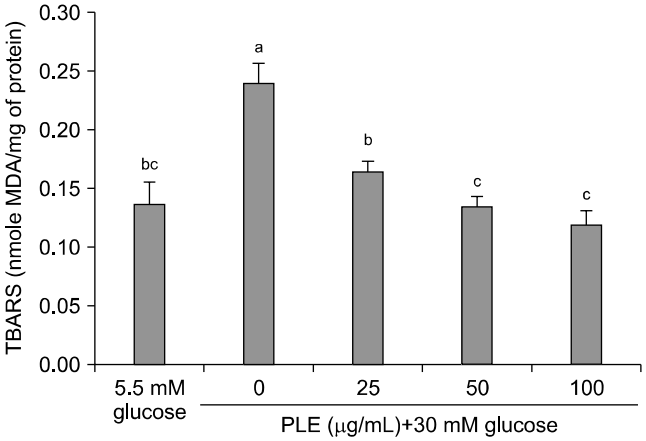

Fig. 2. Effect of PLE on TBARS generation in high-glucose-treated HUVECs. Cells in 24-well plates (4×10^4 cells/well) were preincubated with glucose and incubated in the absence or presence of PLE as described in the legend to Fig. 1. Each value is expressed as mean±SD (n=3). $^{a-c}$Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

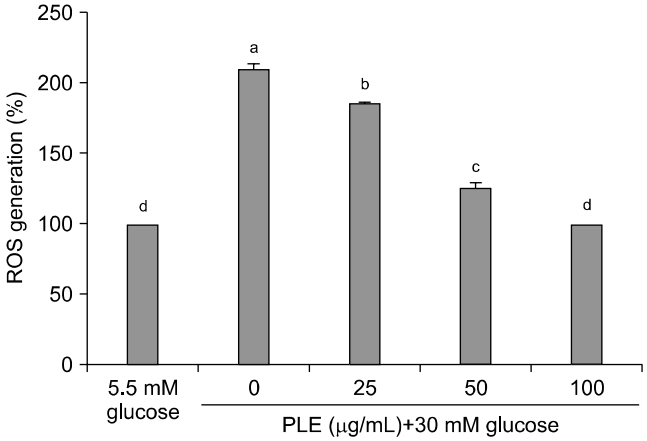

Fig. 3. Effect of PLE on intracellular ROS generation in high-glucose-treated HUVECs. Cells in 24-well plates (4×10^4 cells/well) were preincubated with glucose and incubated in the absence or presence of PLE as described in the legend to Fig. 1. Each value is expressed as mean±SD (n=3). $^{a-d}$Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

diary events in oxidative stress-induced cellular damage (18). In our study, high glucose induced lipid peroxidation in HUVECs and PLE inhibited TBARS formation effectively (Fig. 2). Lipid peroxidation is one of the consequences of oxidative stress and can be the cause of cell injury (19). In particular, the polyunsaturated fatty acid located in cellular membranes is highly prone to attack, which results in the generation of lipid peroxides (20). Lately, the levels of TBARS are increased in diabetic patients with hyperglycemia (21). 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.

Intracellular ROS generation

In an effort to express the effect of PLE on intracellular ROS induced by high glucose of 30 mM, we used DCF-DA as a probe for ROS measurement (Fig. 3). Production of ROS was substantially increased in the cells treated with the high glucose compared to the normal glucose treated cells. However, PLE inhibited the high glucose induced ROS production. Particularly, treatment with 100 μg/mL of PLE resulted in a significant decrease in intracellular ROS to 99.6%.

ROS are considered to be important mediators of several biologic responses, including cell proliferation, and extracellular matrix deposition. Recent observations indicated that hyperglycemia induces the generation of ROS and oxidative stress in various cell types (22). High ROS levels induce oxidative stress, which can result in a variety of biochemical and physiological lesions. Such cellular damage often injures metabolic function and leads to cell death (23). Our results showed that high glucose-treated cells increased the intracellular ROS level compared to normal glucose treatment while PLE significantly decreased the high glucose-induced ROS generation. These results suggest that PLE reduces oxidative stress via inhibiting ROS generation induced by high glucose treatment.

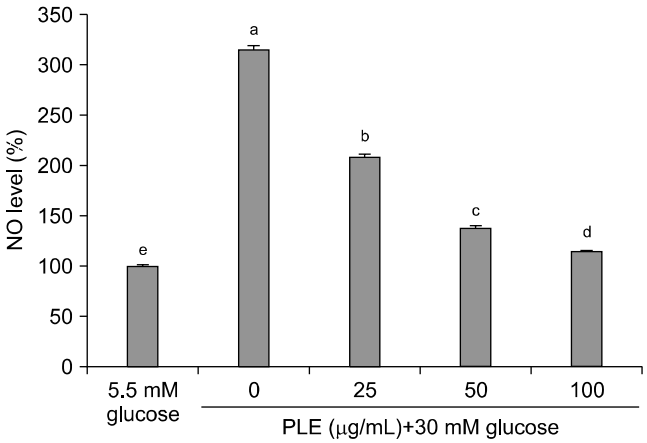

Fig. 4. Effect of PLE on NO level in high-glucose-treated HUVECs. Cells in 24 well plates (4×10^4 cells/well) were preincubated with glucose and incubated in the absence or presence of PLE as described in the legend to Fig. 1. Each value is expressed as mean±SD (n=3). $^{a-e}$Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

NO generation

As shown in Fig. 4, the level of NO in HUVECs was significantly increased by 30 mM glucose treatment (315.9%) compared to 5.5 mM glucose treatment. However, NO levels in PLE treated cells decreased significantly in a concentration-dependent manner, especially treatment with 100 μg/mL of PLE resulting in a significant decrease in intracellular NO to 114.2%.

The study have reported that high glucose treatment leads to overproduction of NO and O_2^-, which induces endothelial cell dysfunction (15). Hyperglycemia was related to an increase in NO production with a parallel enhancement of nitrotyrosine due to the attendant generation of superoxide radical and the consequent formation of peroxynitrite (24). NO reaction with superoxide anion (O_2^-) leads to the formation of peroxynitrite ($ONOO^-$), which can be decomposed, generating hydroxyl radicals ($^{\bullet}OH$) and nitrogen dioxide radicals ($^{\bullet}NO_2$). In addition, NO is one of the targets on intracellular antioxidative enzymes, resulting in the loss of their function (25,26). In this study, PLE effectively decreased NO production by high glucose induced oxidative stress. These results suggest that PLE might act effectively for NO levels and afford important protection against the oxidative stress induced by hyperglycemia.

iNOS and COX-2 expressions

After incubation for 48 h, iNOS and COX-2 protein levels were increased in 30 mM glucose treated HUVECs (Fig. 5). Densitometric analysis showed that iNOS and COX-2 protein levels in 30 mM glucose treated cells were 120% and 140% higher than those of 5 mM glucose, respectively. However, 50 μg/mL PLE treatment reduced these expression levels remarkably.

The proinflammatory enzymes, including iNOS and COXs, are known to be involved in the pathogenesis of many chronic diseases associated with oxidative stress (27). The level of NO released from iNOS under stimulation, such as high glucose or inflammatory conditions, is significantly elevated and forms the potent free radical $ONOO^-$ when combined with O_2^- (28). Therefore, an increase in iNOS production has been proposed to be responsible for multiple organ dysfunctions (29) and selective iNOS inhibition has been shown to attenuate or prevent this syndrome (30). COXs have been found in two forms, COX-1 and COX-2. COX-1, a housekeeping enzyme, is constitutively expressed in almost all mammalian tissues, but COX-2 is barely detectable under normal physiological conditions (31). As shown by our present study, high glucose induced the overexpression of COX-2 protein, and this was concentration-depend-

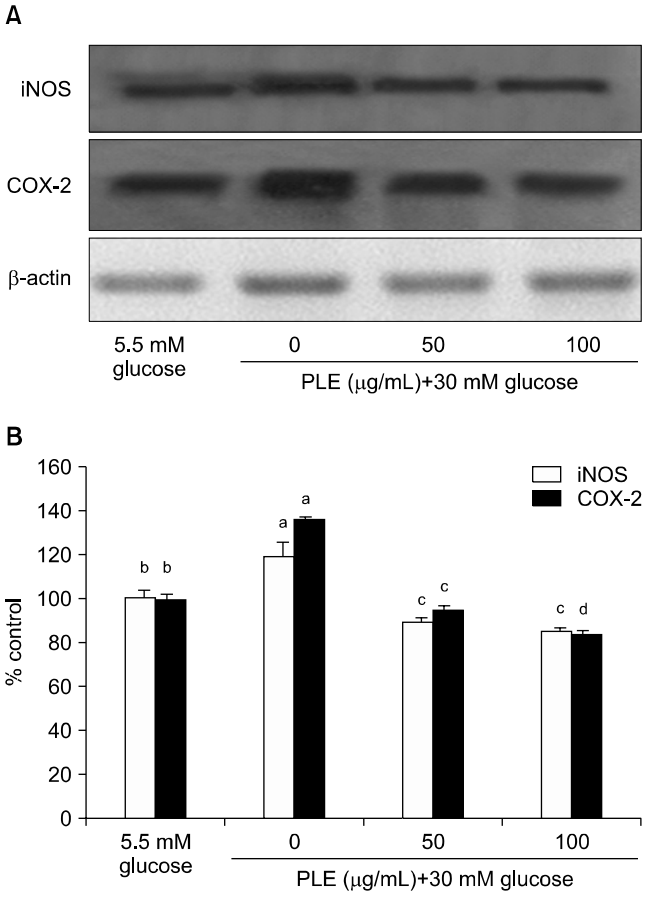

Fig. 5. Effect of PLE on high glucose-induced iNOS and COX-2 expressions in HUVECs. Equal amounts of cell lysates (30 μg) were subjected to electrophoresis and analyzed for iNOS and COX-2 expressions by western blot. Actin was used as an internal control. (A) iNOS and COX-2 protein expressions, (B) expression levels of iNOS and COX-2. Each value is expressed as the mean±SD (n=3). $^{a-d}$Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

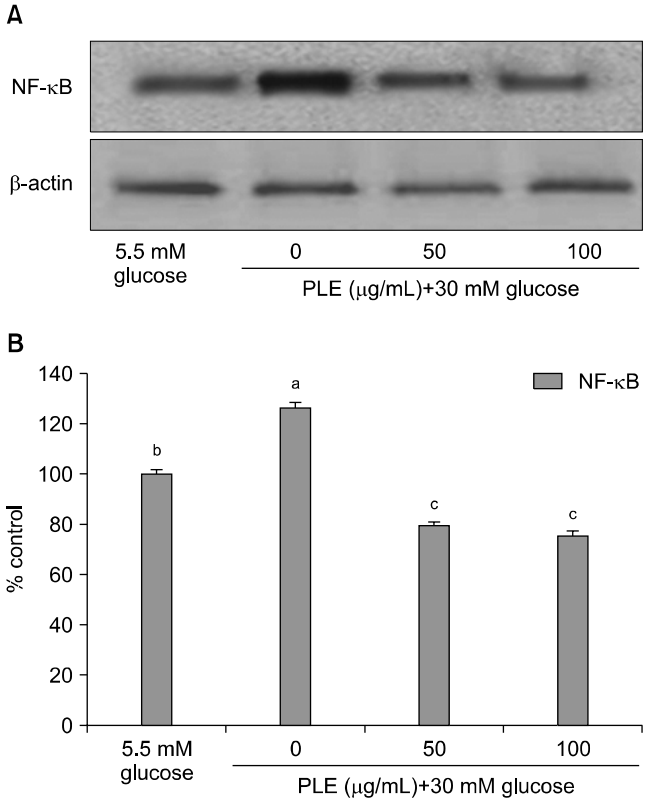

Fig. 6. Effect of PLE on high glucose-induced NF-κB p65 activation in HUVECs. Equal amounts of cell lysates (30 μg) were subjected to electrophoresis and analyzed for NF-κB activity by western blot. Actin was used as an internal control. (A) NF-κB p65 protein expression, (B) expression level of NF-κB p65. Each value is expressed as the mean±SD (n=3). $^{a-c}$Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

ently inhibited by treatment with PLE. Both iNOS and COX-2 are often present together and play fundamental roles in similar pathophysiological conditions, such as inflammation and chronic diseases associated with oxidative stress (32).

NF-κB activity

We determined the effect of PLE on NF-κB p65 activity because p65 is the major component of NF-κB in high glucose stimulated cells. The level of NF-κB p65 activity was obviously higher in 30 mM glucose-treated HUVECs compared to 5.5 mM glucose treatment (Fig. 6). However, when PLE concentration of 50 and 100 μg/mL were added to HUVECs, NF-κB p65 activities were decreased markedly; a significant inhibition of NF-κB activity was seen after 50 μg/mL of PLE treatment.

NF-κB p65, an oxidative stress responsive transcription factor, plays a significant role in the mechanism of cell injury and in the induction of iNOS and COX-2, which are both expressed as a result of NF-κB activation (33,34). In particular, NF-κB is activated in cells cultured under conditions of high glucose concentrations (35,36) and might play an important role in diabetes. There is evidence that exposure with endothelial cells under high glucose concentration induces the increase of NF-κB p65 activity and that specific inhibitors of the NF-κB factor significantly decreases high glucose-induced enhanced monocyte adhesion (37). In summary, this study demonstrated that PLE ameliorates an oxidative stress state induced by high glucose by its ability to suppress lipid peroxidation, ROS and NO production. Additionally, the data showed that PLE suppresses the oxidative stress process by modulating iNOS, COX-2, and NF-κB p65 activity.

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