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
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% CO2 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 (4×10⁴ cells/well) were cultured in 24-well plates and pre-incubated with glucose (5.5 and 30 mM) in humidified atmosphere containing 5% CO₂ 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 μL of culture supernatant. TBARS values were then extrapolated from the 1,1,3,3-tetraethoxypropane serial dilution standard curve. TBARS values were 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 (2×10⁶ cells/well) were seeded in 96-well plates and pre-incubated with glucose (5.5 and 30 mM) in humidified atmosphere containing 5% CO₂ 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 (2×10⁶ cells/well) were seeded in 96-well plates and pre-incubated with glucose (5.5 and 30 mM) in humidified atmosphere containing 5% CO₂ 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 sample of each medium supernatant was mixed with an equal volume of Griess reagent containing 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 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 MgCl₂, 15 mM CaCl₂, 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 MgCl₂, 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.

**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...
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. *Values with different alphabets are significantly different at p<0.05 as analyzed by Duncan’s multiple range test.

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 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...
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.

**ACKNOWLEDGMENTS**

This research was supported by Basic science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology.

**REFERENCES**

1. Ames B. 1998. Micronutrients prevent cancer and delay aging. *Toxicol Lett* 102: 5-18.
2. Baynes JW, Thorpe SR. 1999. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes* 48: 1-9.
3. Kukidume D, Nishikawa T, Sonoda K, Imoto K, Fujisawa K, Yano M, Motoshima H, Taguchi T, Matsumura T, Araki E. 2006. Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. *Diabetes* 55: 120-127.
4. Suształ K, Raif AC, Schiffer M, Bottinger EP. 2006. Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes* 55: 225-233.
5. Osakabe N, Yamagishi M, Natsume M, Yasuda A, Osawa T. 2004. Ingestion of proanthocyanidins derived from cacao inhibits diabetes-induced cataract formation in rats. *Exp Biol Med* 229: 33-39.
6. Johansen JS, Harris AK, Rychly DJ, Ergul A. 2005. Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. *Cardiovasc Diabetol* 4: 5-15.
7. Yolozawa T, Kim YA, Kim HY, Lee YA, Nonaka G. 2007. Protective effect of persimmon peel polyphenol against high glucose-induced oxidative stress in LLC-pK1 cells. *Food Chem Toxicol* 45: 1979-1987.
8. Chandini SK, Ganesan P, Blaskar N. 2008. *In vitro* antioxidant activities of three selected brown seaweeds of India. *Food Chem* 107: 707-713.
9. Kang JY, Khan MNA, Park NH, Cho JY, Lee MC, Fujii H, Hong YK. 2008. Antipyretic, analgesic, and anti-inflammatory activities of the seaweed *Sargassum fulvellum* and *Sargassum thunbergii* in mice. *J Ethnopharmacol* 116: 187-190.
10. Fukuyama Y, Kodama M, Miura I, Kinzyo Z, Kido M, Mori H, Nakayama Y, Takahashi M. 1989. Structure of an anti-plasmin inhibitor, eckol, isolated from the brown alga *Ecklonia kurome* Okamura and inhibitory activities of its derivatives on plasma plasmin inhibitors. *Chem Pharm Bull* 37: 549-553.
11. Lee SH, Min KH, Han JS, Lee DH, Park DB, Jung WK, Park PJ, Jeon BT, Kim SK, Jeon YJ. 2012. Effects of brown alga, *Ecklonia cava* on glucose and lipid metabolism in CS7BL/ KsJ-db/db mice, a model of type 2 diabetes mellitus. *Food Chem Toxicol* 50: 575-582.
12. Chung HY, Ma WC, Ang PO Jr, Kim JS, Chen F. 2003. Seasonal variations of bromophenols in brown algeae (Padina arborescens, Sargassum silicuasrum, and Lobophora variegata) collected in Hong Kong. *J Agric Food Chem* 51: 2619-2624.
13. Fautz R, Husen B, Hechenberger C. 1991. Application of the neutral red assay (NR assay) to monolayer cultures of primary hepatocytes: rapid colorimetric viability determination for the unscheduled DNA synthesis test (UDS). *Mutat Res* 253: 173-179.
14. Fraga CG, Leibovita RM, Roeder RG. 1988. Lipid peroxidation measured as thioarbituric-reactive substances in tissue slices: Characterization and comparison with homogenates and micsromoves. *Free Radic Biol Med* 4: 155-161.
15. Wang H, Joseph JA. 1999. Quantifying cellular oxidative stress by dichlorofluorescin assay using microplate reader. *Free Radic Biol Med* 27: 612-616.
16. Nath J, Powledge A. 1997. Modulation of human neutrophil inflammatory responses by nitric oxide: studies in unprimed and LPS-primed cells. *J Leukoc Biol* 62: 805-816.
17. Yamabe N, Kang KS, Goto E, Tanaka T, Yokozaawa T. 2007. Beneficial effect of corni fructus, a constituent of Hachimiji-gan, on advanced glycation end product mediated renal injury in streptozotocin-treated diabetic rats. *Biomed Pharmacol Bull* 30: 520-526.
18. Reusch JE. 2003. Diabetes, microvascular complications, and cardiovascular complications: what is about glucose? *J Clin Invest* 112: 986-988.
19. Sevanian A, Hochstein P. 1985. Mechanism and consequence of lipid peroxidation in biological systems. *Annu Rev Nutr* 5: 365-390.
20. Schmidtman S, Muller M, von Baehr R, Precht K. 1991. Changes of antioxidative homeostasis in patients on chronic
haemodialysis. *Neephrol Dial Transpl* 6: 71-74.

21. Kukidome D, Nishikawa T, Sonoda K, Imoto K, Fujisawa K, Yono M, Motoshima H, Taguchi T, Matsumura T, Araki E. 2006. Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. *Diabetes* 55: 120-127.

22. Li M, Absher PM, Liang P, Russell JC, Sobel BE, Fukagawa NK. 2001. High glucose concentrations induce oxidative damage to mitochondrial DNA in explanted vascular smooth muscle cells. *Exp Biol Med* 226: 450-457.

23. Du X, Stocklauser-Farber K, Rosen P. 1999. Generation of reactive oxygen intermediates, activation of NF-kappaB, and inducibin of apoptosis in human endothelial cells by glucose: role of nitric oxide synthase? *Free Radical Biol Med* 27: 752-763.

24. Suarez-Pinzon WL, Mahley JG, Strynadka K, Power RF, Szabo C, Rabinovitch A. 2001. An inhibitor of inducible nitric oxide synthase and scavenger of peroxynitrite prevents diabetes development in nod mice. *J Autoimmun* 16: 449-455.

25. Radi R, Beckman JS, Bush KM, Freeman BA. 1991. Peroxynitrite induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 288: 481-487.

26. Liu RH, Hotchkiss JH. 1995. Potential genotoxicity of chronically elevated nitric oxide; a review. *Mutat Res* 339: 73-89.

27. Kim JY, Park SJ, Yun KJ, Cho YW, Park HJ, Lee KT. 2008. Isoliquiritigenin isolated from the roots of *Glycyrrhiza uralensis* inhibits LPS-induced iNOS and COX-2 expression via the attenuation of NF-xB in RAW 264.7 macrophages. *Eur J Pharmacol* 584: 175-184.

28. Baeuerle PA, Baltimore D. 1996. NF-kappa B: ten years after. *Cell* 87: 13-20.

29. Han M, Wen JK, Zheng B, Zhang DQ. 2004. Acetylbritanniolateone suppresses NO and PGE2 synthesis in RAW 264.7 macrophages through the inhibition of iNOS and COX-2 gene expression. *Life Sci* 75: 675-684.

30. Hattori Y, Hattori S, Sato N, Kasai K. 2000. High-glucose-induced nuclear factor kappa B activation in vascular smooth muscle cells. *Cardiovasc Res* 46: 188-197.

31. Shishodia S, Majumdar S, Banerjee S, Aggarwal BB. 2003. Ursolic acid inhibits nuclear factor-kappa B activation induced by carcinogenic agents through suppression of I kappaBalpha kinase and p65 phosphorylation: correlation with down-regulation of cyclooxygenase 2, matrix metalloproteinase 9, and cyclin D1. *Cancer Res* 63: 4375-4383.