

Blockade of Chronic High Glucose–Induced Endothelial Apoptosis by *Sasa borealis* Bamboo Extract

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Hyperglycemia is a causal factor in the development of diabetic vascular complications including impaired vascular smooth muscle contractility and increased cell proliferation. The present study was designed to investigate the effects of *Sasa borealis* water-extract (SBwE) on chronic hyperglycemia-induced oxidative stress and apoptosis in human umbilical endothelial cells (HUVEC). HUVEC were cultured in 5.5 mM low glucose, 5.5 mM glucose plus 27.5 mM mannitol as an osmotic control, or 33 mM high glucose for 5 days in the absence and presence of 1–30 µg/ml SBwE. Caspase-3 activation and Annexin V staining revealed chronic high glucose–induced endothelial apoptotic toxicity with a generation of oxidants detected by DCF-fluorescence, and these effects were reversed by SBwE at ≥ 1 µg/ml in a dose-dependent manner. Cytoprotective SBwE substantially reduced the sustained high glucose–induced expression of endothelial nitric oxide synthase and attenuated the formation of peroxynitrite radicals. The suppressive effects of SBwE were most likely mediated through blunting activation of PKC β 2 and NADPH oxidase promoted by high glucose. In addition, this bamboo extract modulated the high glucose–triggered mitogen-activated protein kinase–dependent upregulation of heat-shock proteins. Our results suggest that SBwE suppressed these detrimental effects caused by PKC-dependent peroxynitrite formation via activation of NADPH oxidase and induction of nitric oxide synthase and heat-shock protein family that may be essential

mechanisms responsible for increased apoptotic oxidative stress in diabetic vascular complications. Moreover, the blockade of high glucose–elicited heat-shock protein induction appeared to be responsible for SBwE-alleviated endothelial apoptosis. Therefore, SBwE may be a therapeutic agent for the prevention and treatment of diabetic endothelial dysfunction and related complications. Exp Biol Med 233:580–591, 2008

Key words: *Sasa borealis*; high glucose; NADPH oxidase; heat shock protein; endothelial apoptosis; peroxynitrite

Introduction

Uncontrolled hyperglycemia has been recognized as a major risk factor in the development of diabetic vascular complications (1, 2). The vascular lesions characteristic of experimental hyperglycemia and human diabetes imply that vascular endothelial cells are the primary cells targeted by the toxicity of high glucose (3). One diabetic vascular complication involves endothelial dysfunction characterized by impaired endothelium-dependent vasomotor responses. It has been shown that hyperglycemia induces endothelial dysfunction, possibly due to oxidative stress (2, 4). Elevated glucose has been shown to cause glucose oxidation by resulting in excess generation of reactive oxygen species (ROS) from vascular cells (5, 6). Accordingly, it is assumed that a reduction in cellular antioxidant reserves is responsible for triggering diabetic vascular complications (8, 9). However, the biochemical mechanisms responsible for the toxicity of high glucose in microcirculation remain elusive.

Various signaling pathways have been implicated in diabetes- and hyperglycemia-induced impaired vascular functions (6–10). Protein kinase C (PKC) is chronically activated in diabetic tissues, and its activation in normal blood vessels reduces endothelium-dependent relaxations, as in diabetes (10). The formation of ROS might lead to an activation of transcription factors for genes expressed in response to hyperglycemia (9). Thus, PKC activation and

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ROS production may explain impaired endothelium-dependent relaxations in diabetes. Endothelium-derived nitric oxide (NO) is an important component of vascular homeostasis through regulation of vascular tone, arterial pressure, platelet and leukocyte adhesion to the endothelial surface, and vascular smooth muscle cell proliferation. Nevertheless, little is known about the effects of hyperglycemia on the NO pathway. It has also been shown that mitogen-activated protein kinase (MAPK) pathways are involved in high glucose-induced cell damage (6, 9). High glucose-induced apoptosis in human umbilical vein endothelial cells (HUVEC) was mediated through NF- κ B-dependent c-Jun N-terminal kinase (JNK) activation that was prevented by the presence of vitamin C, and Akt survival signaling is observed in high glucose-triggered early phase (9). On the other hand, heat-shock proteins (Hsp) that perform functions in various intracellular processes as molecular chaperones for protein molecules under different kinds of environmental stress conditions (10, 12) can be triggered through MAPK pathways to comprise an acute adaptation to glycemic stress (13).

Sasa borealis bamboo has been used for making tea in the Far East, but little is known regarding its uses for traditional medicines. There is currently an intense attention in developing new antidiabetic agents from plants used for alternative medicines. It has been suggested that proanthocyanidins derived from cacao inhibits diabetes-induced cataract formation, possibly by virtue of its antioxidative activity (9). Recently, it has been shown that this bamboo extract exhibits antioxidant activity against the 1,1-diphenyl-2-picrylhydrazyl radical and cytoprotective effects against oxidative damage in HepG2 cells (14). The phenolic compounds isolated from this whole-plant extract showed inhibitory effects on the P-glycoprotein in adriamycin-resistant human breast cancer cells (15). Accordingly, *S. borealis* may be a novel plant for alternative medicinal uses.

Based on evidence in the literature that high glucose-induced oxidative stress may be a mechanism in the development of diabetic vascular complications (2, 4), this study attempted to examine antiapoptotic features of *S. borealis* bamboo water-extract (SBwE) in high glucose-exposed HUVEC and further explore the possible NADPH oxidase-dependent and MAPK-responsive mechanisms of SBwE-mediated downregulation of high glucose-induced apoptosis. Equimolar mannitol was used in parallel experiments to investigate whether high glucose-triggered endothelial dysfunction was independent of an increase in osmolarity generated by high glucose.

Materials and Methods

Chemicals and Materials. M199 medium chemicals, D-(+)-glucose, D-mannitol, human epidermal growth factor, and hydrocortisone were purchased from Sigma Chemical Co. (St. Louis, MO), as were all other reagents, unless specified otherwise. Collagenase was obtained from

Worthington Biochemicals (Lakewood, NJ). Fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were provided by Cambrex Corporation (East Rutherford, NJ). 3-(4,5-Dimethylthiazol-yl)-diphenyl tetrazolium bromide (MTT) was purchased from DUCHEFA Biochemie (Haarlem, Netherlands). Phycoerythrin-labeled Annexin V (Annexin V-PE) was obtained from BD Biosciences (Franklin Lakes, NJ) and 7-amino-actinomycin D (7-AAD) was from Invitrogen Co. (Carlsbad, CA). 4-Amino-5-methylamino-2',7'-difluorescein diacetate (DAF-FM) was obtained from Molecular Probes Inc. (Eugene, OR). Antibodies of human cleaved caspase-3, human endothelial nitric oxide synthase (eNOS), human phospho-apoptosis signal-regulating kinase-1 (ASK-1), human phospho-c-Jun N-terminal kinase (JNK), human phospho-p38 mitogen-activated protein kinase (p38 MAPK), human phospho-Akt, and human phospho-extracellular signal-regulated kinase 1/2 (ERK1/2) were provided by Cell Signaling Technology Inc. (Beverly, MA). Antibodies of human p22-phox and p47-phox were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of Crude Water Extracts of *S. borealis*. The fresh leaves of *S. borealis* were harvested from fields and mountains in the vicinity of Chuwol Mountain in Damyang (Chonnam, Korea). The collected leaves were washed thoroughly with tap water, and the leaves were then steamed at 100°C for 30 mins, dried at 30°C for 30 hrs, and pulverized using a mill. The leaf powder was extracted using a ton-extractor (Best Korea Co., Kwangju, Korea) with distilled water (1:20 wt/wt) at 95°C for 20 hrs. Finally, extracts were filtered through a cheese cloth, concentrated by a vacuum batch evaporator (Best Korea Co., Kwangju, Korea), and freeze-dried with a lyophilizer (SFDTS 10K, Samwon Freezing Engineering Co., Pusan, Korea).

The SBwE was solubilized in dimethylsulfoxide (DMSO) for culturing with cells; the final culture concentration of DMSO was <0.1%.

Culture of Human Endothelial Cells. HUVEC were isolated from umbilical cords using collagenase as described elsewhere (16). Cultures were maintained at 37°C in humidified atmospheres of 5% CO₂ in air. Cells were cultured in 25 mM HEPES-buffered M199 (with 5.5 mM glucose) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 g/ml streptomycin supplemented with 0.75 mg/ml human epidermal growth factor, and 0.075 mg/ml hydrocortisone. Endothelial cells were confirmed by their cobblestone morphology and uptake of acetylated low density lipoprotein (LDL) (17). For experiments, cells were used at confluence within 10 passages. To induce chronic endothelial hyperglycemia-induced toxicity, HUVEC (80%–90% confluence) were incubated in a 33 mM glucose-added M199 containing 2% FBS for 5 days in the absence or presence of SBwE. For osmotic control incubations, another set of endothelial cells was cultured

in 2% FBS containing M199 supplemented with 27.5 mM mannitol.

Cell Viability. After the 5-day incubation period under high-glucose conditions, the MTT assay was performed to measure cell viability (18). HUVEC were incubated in a fresh phenol red-free medium containing 1 mg/ml MTT for 3 hrs at 37°C. After unconverted MTT was washed out, the purple formazan product was dissolved in isopropanol with gentle shaking. Absorbance of formazan dye was measured at $\lambda = 570$ nm with background subtraction using $\lambda = 690$ nm.

Intracellular ROS Production. Superoxide oxidant generation of HUVEC was measured as a previously described method with a minor modification (19). This method was based on the conversion of 2',7'-dichlorodihydrofluorescein diacetate (DCHF) to 2',7'-dichlorofluorescein diacetate (DCF), a fluorescent dye. DCF fluorescence was measured to determine cellular ROS production. Cells challenged with 33 mM glucose were washed with phosphate-buffered saline (PBS) and loaded with 10 μ M DCHF freshly prepared in prewarmed M199 (+2% FBS) for 30 mins. All subsequent steps were conducted in the dark, and fluorescent images were taken using a fluorescence microscopy.

Western Blot Analysis. Western blot analysis was performed using whole-cell extracts from HUVEC as previously described (16, 20). The membrane was incubated for 3 hrs with primary polyclonal rabbit anti-human antibodies to detect cleaved caspase-3 (1:1000 dilution), eNOS (1:1000 dilution), protein kinase C β 2 (PKC β 2, 1:800 dilution; Sigma Co.), NADPH oxidase (p22phox and p47phox subunits, 1:200 dilution), phospho-ASK1 (Ser967, 1:1000 dilution), phospho-JNK (Thr183/Tyr185, 1:1000 dilution), phospho-p38 MAPK (Thr180/Tyr182, 1:1000 dilution), phospho-Akt (Thr308, 1:1000 dilution), phospho-ERK1/2 (Thr202/Tyr204, 1:1000 dilution), and Hsp (Hsp27, Hsp90 α/β). After washing with TBS-T buffer, the membrane was incubated for 1 hr with goat anti-rabbit IgG or goat anti-mouse IgG (1:10,000, Jackson ImmunoResearch Laboratories, West Grove, PA). Protein levels were determined by using Supersignal West Pico chemiluminescence detection reagents (Pierce Biotech. Inc., Rockford, IL) and Konica X-ray film (Konica Co., Tokyo, Japan). Incubation with monoclonal mouse β -actin antibody (1:1000) was also performed for the comparative control.

Flow Cytometry Evaluation of Early Apoptosis. After a 5-day culture challenge under high-glucose conditions, cells were washed with PBS and collected. Apoptosis in low glucose-, mannitol-, and high glucose-treated cells was assessed by staining with Annexin V-PE and 7-AAD. To prepare cell samples for the flow cytometric analysis, cells were resuspended in a binding buffer and stained with Annexin V-PE (1:40 dilution) and 1 μ g/ml 7-AAD for 15 mins. The cell samples were analyzed by Guava EasyCyte using CytoSoft software (Guava Technologies Inc., Hayward, CA).

Immunocytochemistry. After thorough washing with PBS, endothelial cells were incubated for 1 hr with 25% FBS in PBS to prevent any nonspecific binding. HUVEC were then fixed with ice-cold 4% formaldehyde for 30 mins. Fixed cells were washed with PBS containing 0.05% Tween 20, incubated overnight at 4°C with polyclonal rabbit anti-human eNOS antibody (1:100 dilution) or polyclonal rabbit anti-human Hsp27 antibody (1:100 dilution), and then incubated for 1 hr at 4°C with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:1000 dilution; Sigma) or with a cyanine 3-OSu-conjugated goat anti-rabbit IgG (1:1000 dilution; Rockland Co., Gilbertville, PA). Images were taken using an Olympus BX50 fluorescent microscope (Olympus Corp., Tokyo, Japan).

Measurement of Peroxynitrite Anion (ONOO⁻) Formation. The fluorescent dye DAF-FM is essentially nonfluorescent until it is nitrosylated by products of oxidation of nitric oxide. After a 5-day culture, all subsequent steps were conducted in the dark. After cells were washed twice with PBS, 10 μ M DAF-FM in prewarmed M199 (+2% FBS) was loaded for 3 hrs at 37°C. Fluorescent images were taken using an Olympus BX50 fluorescence microscope (Olympus Corp., Tokyo, Japan).

Data Analysis. The data are presented as mean $\pm$ SEM. Statistical analyses were conducted using Statistical Analysis Systems statistical software package (SAS Institute Inc., Cary, NC). Significance was determined by one-way ANOVA, followed by Duncan multiple range test for multiple comparisons. Differences were considered significant at $P < 0.05$.

Results

Inhibition of ROS-Triggered Apoptotic Cytotoxicity Under High-Glucose Conditions. This study examined endothelial oxidative stress caused by chronic exposure to high glucose levels. As expected, DCF staining did not reveal oxidant generation in cells incubated with 5.5 mM glucose in the absence or presence of 27.5 mM mannitol (Fig. 1A). Heavy nuclear staining in cells exposed to 33 mM glucose alone was observed, indicating a marked increase in the ROS oxidant generation. However, 1–10 μ g/ml SBwE concentration dependently suppressed DCF fluorescence (Fig. 1A).

Chronic exposure to high glucose caused $\geq 30\%$ decrease in the viability of endothelial cells, the predominant target of high ambient glucose in the vasculature (Fig. 1B). In contrast, a 5%–10% cell death was observed in mannitol osmotic controls. It was shown that proliferation did not decrease in HUVEC grown in the presence of 3–40 mM mannitol, indicating that the effect of high glucose on proliferation was not secondary to osmotic load (21). When cells were treated with SBwE at ≥ 1 μ g/ml, the rate of high glucose-induced cell death was significantly reduced. It should be noted that SBwE *per se* showed little cytotoxicity

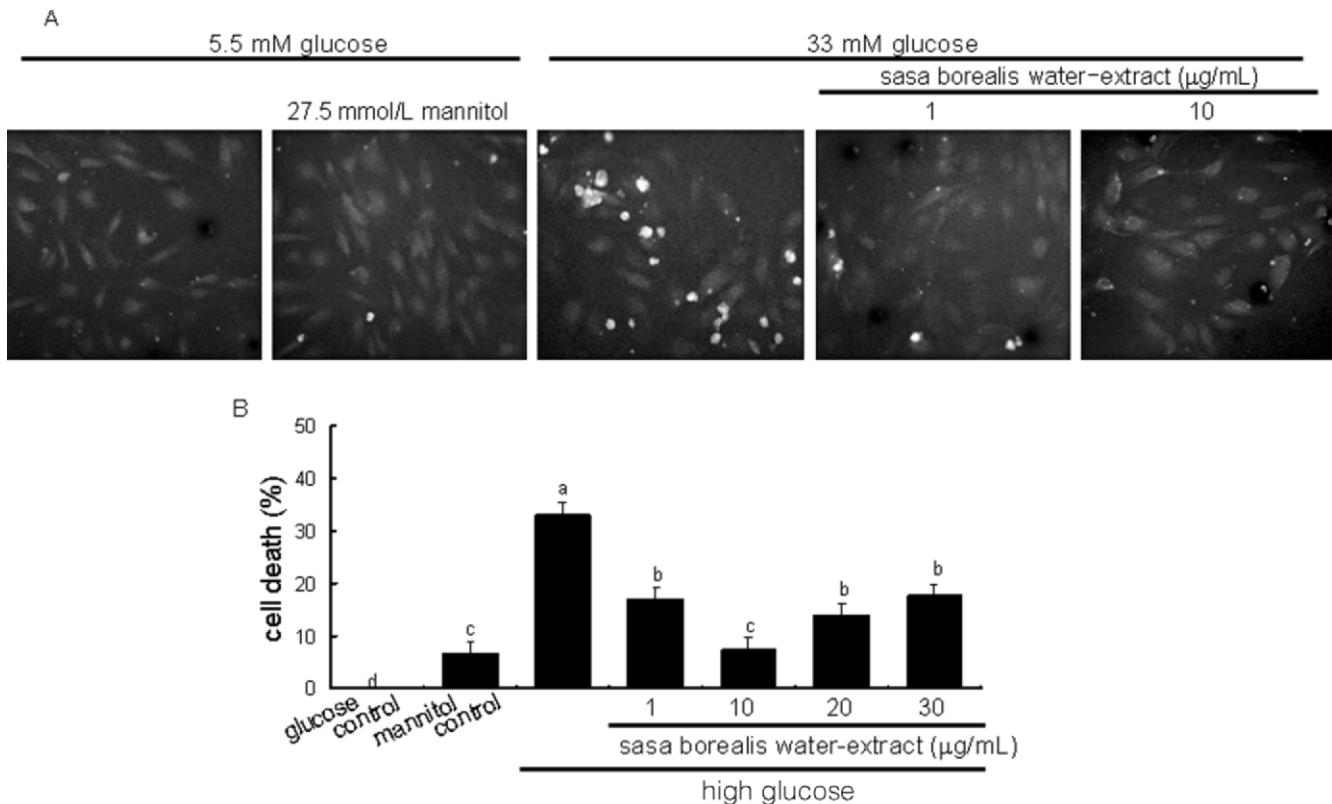


Fig. 1. Effects of SBwE on intracellular oxidant generation (A) and cell death (B) in HUVEC challenged with high doses of glucose. Endothelial cells were treated with 1–30 $\mu\text{g/ml}$ SBwE for 5 days in culture media consisting of 5.5 mM glucose (glucose controls), 5.5 mM plus 27.5 mM mannitol (mannitol controls), or 33 mM glucose. Oxidant generation was measured by DCHF fluorescence. Fluorescent images (three separate experiments) of representative controls and high glucose-treated cells were obtained using fluorescence microscopy. Magnification, $\times 200$. Cell death values are presented as mean $\pm$ SEM ($n = 6$) and expressed as percent cell death relative to glucose controls (cell death = 0%). Values not sharing a letter are different at $P < 0.05$.

at doses tested $\leq 30 \mu\text{g/ml}$. Treatment of endothelial cells with 10 $\mu\text{g/ml}$ SBwE completely prevented cytotoxicity triggered by high glucose (Fig. 1B). It is concluded that endothelial cell injury caused by high glucose may be at least in part attributed to an accumulation of intracellular oxidants (Fig. 1A).

Since oxidative stress appears to be a common apoptotic mediator (22), this study explored whether high glucose-triggered ROS formation might lead to cytotoxicity via apoptotic processes. In glucose controls, there was no sign of activation of caspase-3, a key enzyme involved in the apoptotic signaling cascades (Fig. 2A). However, high glucose resulted in a marked activation of caspase-3 relative to glucose controls or mannitol control cells, indicating that the treatment of HUVEC with high glucose instigated apoptotic cell death through eliciting cellular ROS (Fig. 1A). On the contrary, the caspase-3 activation in cells chronically exposed to high glucose was attenuated by a treatment with $\geq 1 \mu\text{g/ml}$ SBwE (Fig. 2A).

Further quantitation using double staining with Annexin V and 7-AAD confirmed a 2- to 3-fold increase in the number of Annexin V binding cells in early-stage apoptosis under high-glucose conditions (Fig. 2B). Early-apoptotic cells were defined as Annexin V-PE-positive and 7-AAD-

negative. In contrast, high glucose-elicited increase in the number of cells undergoing apoptosis was markedly diminished following SBwE addition; the inhibitory effects of 1 $\mu\text{g/ml}$ SBwE were statistically significant. It was also observed that the SBwE treatment resulted in the appearance of Annexin V-PE and 7-AAD double-positive late-apoptotic cells (Fig. 2B). Thus, SBwE attenuated high glucose-triggered apoptosis, leading to phosphatidylserine exposure at the endothelial cell surface.

Suppression of High Glucose-Induced eNOS Expression and Peroxynitrite Production. Recent evidence indicates that ONOO^- , a secondary oxidizing species, interacts with lipids, DNA, and proteins via direct oxidative reactions or via radical-mediated mechanisms, which in turn triggers cellular responses and commits cells to necrosis or apoptosis (23). This study investigated whether ONOO^- is implicated as a nitrosative stress in high glucose-induced endothelial apoptosis. There was a weak DAF staining in the glucose controls or osmotic control cells (Fig. 3A), indicating a lack of ONOO^- generation at the single-cell level. Cells exposed to high levels of glucose stained heavily, pointing to a marked increase in ONOO^- generation, whereas adding 1–10 $\mu\text{g/ml}$ SBwE to high glucose-exposed cells mitigated the

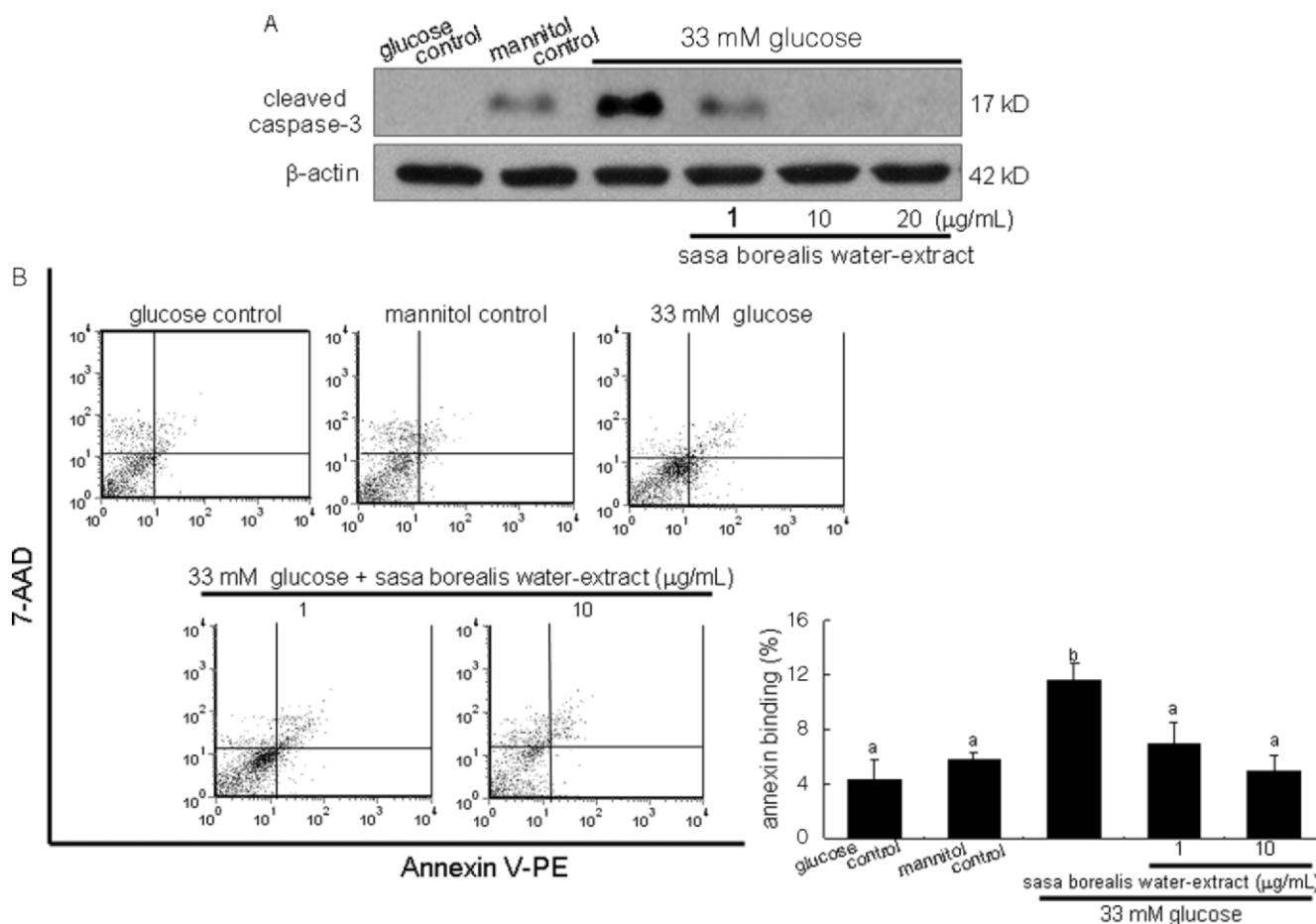


Fig. 2. Effect of SBwE on caspase-3 activation (A) and number of Annexin V-positive cells (B) under chronic high-glucose conditions. For the caspase-3 activation, cell lysates were electrophoresed on 10% SDS-PAGE gel, followed by Western blot analysis with a primary antibody against human cleaved caspase-3 (three independent experiments). β -Actin protein was used as an internal control. Representative flow cytometric dot plots (B). Apoptotic cell death was analyzed using two-color dot plots of Annexin V versus 7-AAD. Arithmetic values of the histograms are means $\pm$ SEM ($n=4$) of the Annexin V binding and consequent apoptosis percentage of endothelial cells incubated for 5 days in high glucose media with and without 1–10 μ g/ml SBwE. Values not sharing a letter indicate significant difference at $P < 0.05$.

DAF staining. This reactive nitrogen species appeared to be most likely responsible for endothelial apoptosis, with $\geq 30\%$ cell killing triggered by high glucose (Fig. 1B). Thus, it is deemed that the oxygen-dependent oxidants generated by high glucose would react with endothelial NO to form ONOO $^-$, which causes a loss of NO bioavailability.

When high glucose was applied to HUVEC for 5 days, the cellular level of eNOS protein was remarkably enhanced (Fig. 3B). The Western blot analysis revealed that ≥ 1 μ g/ml SBwE strikingly attenuated the expression of eNOS. Expression of eNOS was also elucidated by using immunocytochemical staining technique, in which the staining was apparent in high glucose-damaged cells (Fig. 3C). The staining of cells cotreated with 1 μ g/ml SBwE was indistinguishable from that of the glucose controls or mannitol-treated cells. Thus, the immunocytochemical staining results supported the Western blot data (Fig. 3B), showing a blockade of eNOS induction in 1 μ g/ml SBwE- and high glucose-treated HUVEC.

Attenuation of High Glucose-Triggered Up-regulation of Hsp and MAPK.

This study tested whether high glucose may potentiate endothelial expression of Hsp27 and Hsp90 α/β , which are important molecular chaperones essential for cellular integrity, and also assessed whether SBwE may inhibit increased Hsp expression. High glucose greatly induced Hsp27 expression after 24 hrs of exposure, and this induction was attenuated by adding ≥ 10 μ g/ml SBwE (Fig. 4A). In addition, the expression of Hsp90 α/β , enhanced in high glucose-treated HUVEC, was blunted with ≥ 10 μ g/ml SBwE. The induction of Hsp27 after high glucose exposure was also immunocytochemically determined using specific Hsp27 antibody (Fig. 4B). Staining in cells exposed to 24 hrs of high glucose levels was remarkable, indicating that Hsp27 was markedly induced. SBwE dampened fluorescent staining of Hsp27 in high glucose-exposed cells.

We examined whether SBwE inhibits Hsp27 expression and apoptosis of high glucose-exposed endothelial cells through downregulation of MAPK signaling cascades.

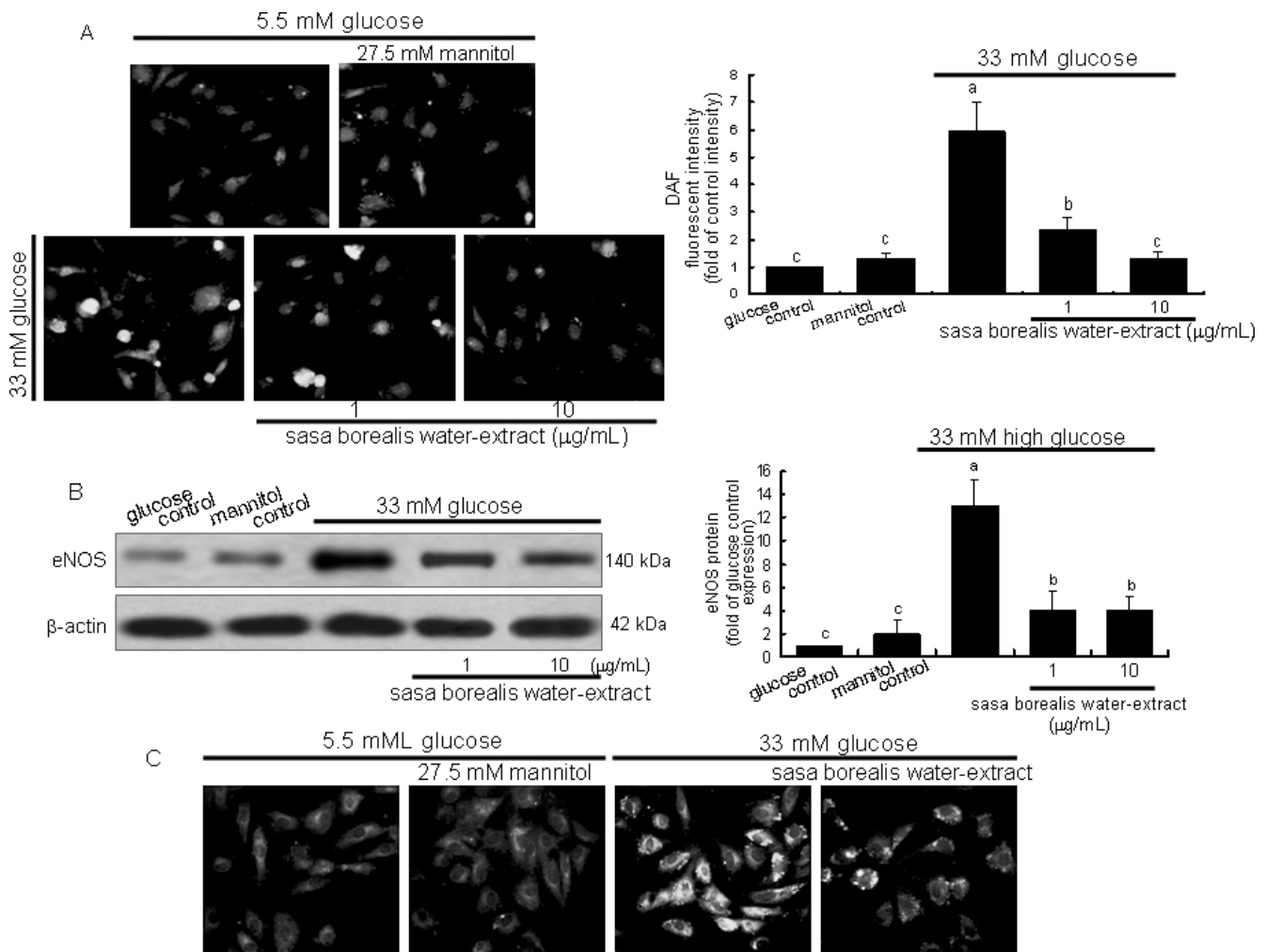


Fig. 3. Intracellular peroxynitrite accumulation (A) and Western blot analyses (B) of representative immunostaining microphotographs of eNOS protein expression (C) in HUVEC exposed to 5.5 mM or 33 mM glucose and treated with SBwE for 5 days (three separate experiments). Peroxynitrite formation was measured by loading with 10 μ M DAF-FM (A). After HUVEC culture protocols with SBwE, cell extracts were subjected to 10% SDS-PAGE and Western blot analysis with a primary antibody against eNOS (B). β -Actin protein was used as an internal control. In addition, immunocytochemical staining (C) was performed in the absence and presence of 10 μ M SBwE. Cells were fixed and subsequently incubated with rabbit polyclonal anti-human eNOS, and antibody localization was detected with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. Representative fluorescent images of controls and high glucose-treated cells were obtained using fluorescence microscopy. Magnification, $\times 200$. Bars represent the means $\pm$ SEM of three experiments. Values not sharing a letter indicate significant difference at $P < 0.05$. A color version of this figure is available in the online version of the article.

The effects of cytoprotective SBwE on the phosphorylation of ASK-1, JNK, p38 MAPK, Akt, and ERK1/2 were determined in HUVEC exposed to high glucose. The JNK phosphorylation was induced within 6 hrs after treatment with high glucose (data not shown), which was reversed by ≤ 1 μ g/ml SBwE in a dose-dependent manner, as in the phosphorylation of p38 MAPK (Fig. 5A). Likewise, the high glucose-triggered activation of ASK-1, a redox-sensitive MAPK kinase regulating JNK and p38 MAPK, was downregulated by a 24-hr cotreatment with SBwE. In addition, the activation of Akt and ERK1/2 triggered by high glucose was dose-dependently suppressed in SBwE-cotreated cells (Fig. 5B).

Blockade of NADPH Oxidase Subunits and PKC Induced by High Glucose. The expression of

p22phox and p47phox in HUVEC exposed to high glucose was greatly upregulated within 2 days (Fig. 6A). When cells were exposed to high glucose and cotreated with SBwE for 2 days, the expression of NADPH oxidase subunits was significantly reduced even at a concentration of 1 μ g/ml (Fig. 6B).

This study investigated the inhibitory effect of SBwE on PKC β 2 expression as the underlying mechanism down-regulating high glucose-induced expression of eNOS and NADPH oxidase. Adding SBwE to cells alleviated the glucose induction of PKC β 2 in a dose-dependent manner, with its near-complete inhibition at 10 μ g/ml (Fig. 6C). Thus, the PKC β 2 pathway appeared to be responsible for mediating the induction of eNOS and NADPH oxidase subunits. In addition, it is assumed that the blockade of

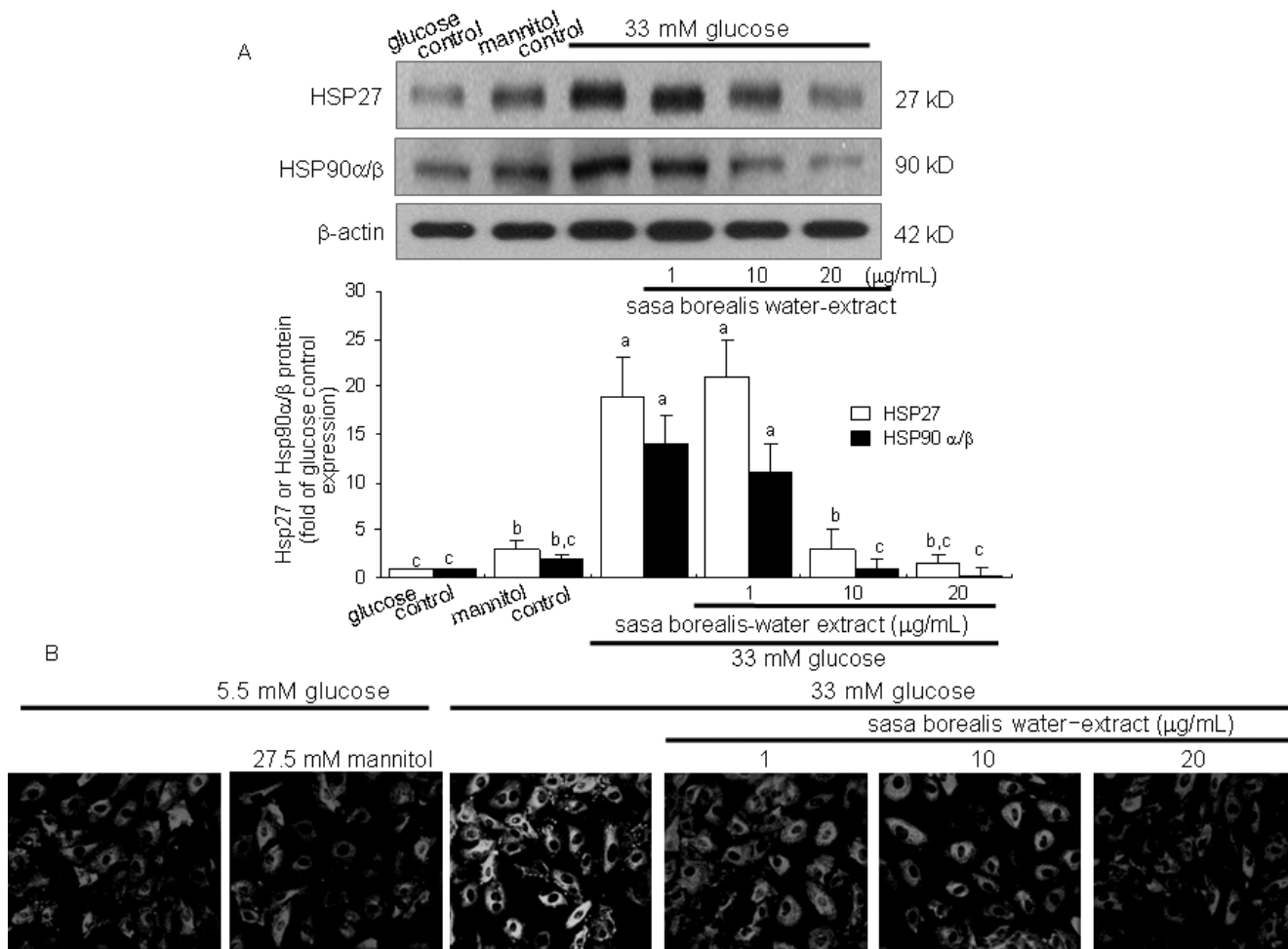


Fig. 4. Inhibition of high glucose-activated Hsp expression by SBwE. Human endothelial cells were treated with 1–20 $\mu\text{g/ml}$ SBwE under high glucose condition of 33 mM for 5 days. Total cell protein extracts were electrophoresed on 10% SDS-PAGE, followed by Western blot analysis with a primary antibody against human Hsp27 and Hsp90 α/β (A). β -Actin protein was used as an internal control. Bars represent the means $\pm$ SEM of three independent experiments. Values not sharing a letter indicate significant difference at $P < 0.05$. Immunocytochemical staining was also performed with rabbit polyclonal anti-human Hsp27. After cells were fixed and subsequently incubated with rabbit polyclonal anti-human Hsp27, antibody localization was detected with a cyanine 3-OSu-conjugated goat anti-rabbit IgG. Representative fluorescent images of controls and high glucose-treated cells were obtained using fluorescence microscopy. Magnification, $\times 200$.

PKC β 2 pathway by the SBwE addition blunted high glucose-triggered endothelial apoptosis, most likely by mitigating NO-dependent nitrosative stress.

Discussion

Six major observations resulted from this study. (i) SBwE at nontoxic doses of $<30 \mu\text{g/ml}$ dampened high glucose-induced endothelial apoptosis with a concomitant reduction of ROS generation. (ii) SBwE, when incubated at concentrations of $\geq 1 \mu\text{g/ml}$, markedly mitigated the expression of eNOS and the generation of peroxynitrite anion ONOO $^-$ enhanced by high glucose, indicating that high glucose-triggered eNOS upregulation appeared to be responsible for causing apoptotic cytotoxicity. (iii) SBwE blunted the expression of Hsp27 and Hsp90 α/β stimulated by high glucose, entailing possible association with eNOS for NO production. (iv) SBwE at doses $\geq 1 \mu\text{g/ml}$ inhibited the ASK-1 signaling-mediated activation of JNK and p38

MAPK enhanced by high glucose. (v) High glucose-induced expression of the NADPH oxidase subunits of p22phox and p44phox was attenuated in SBwE-treated HUVEC, proving that SBwE diminished consequent ROS production. (vi) Adding $\geq 1 \mu\text{g/ml}$ SBwE to HUVEC dulled high glucose-upregulated expression of PKC β 2. In addition, the activation of ERK1/2 and Akt was also substantially suppressed in SBwE-treated cells. These findings reveal that SBwE has the potential capability to hamper chronic high glucose-triggered endothelial apoptosis and resultant dysfunction (Fig. 7). The capability of SBwE to inhibit high glucose-induced ROS production is possibly mediated *via* a PKC β 2-responsive pathway and a redox-sensitive MAPK pathway entailing protein expression of NADPH oxidase, eNOS, and Hsp. However, it is quite possible that the inhibitory effects of SBwE on induction of ROS downstream proteins may be mediated *via* pathways in which ROS generation is not involved. Unfortunately, this

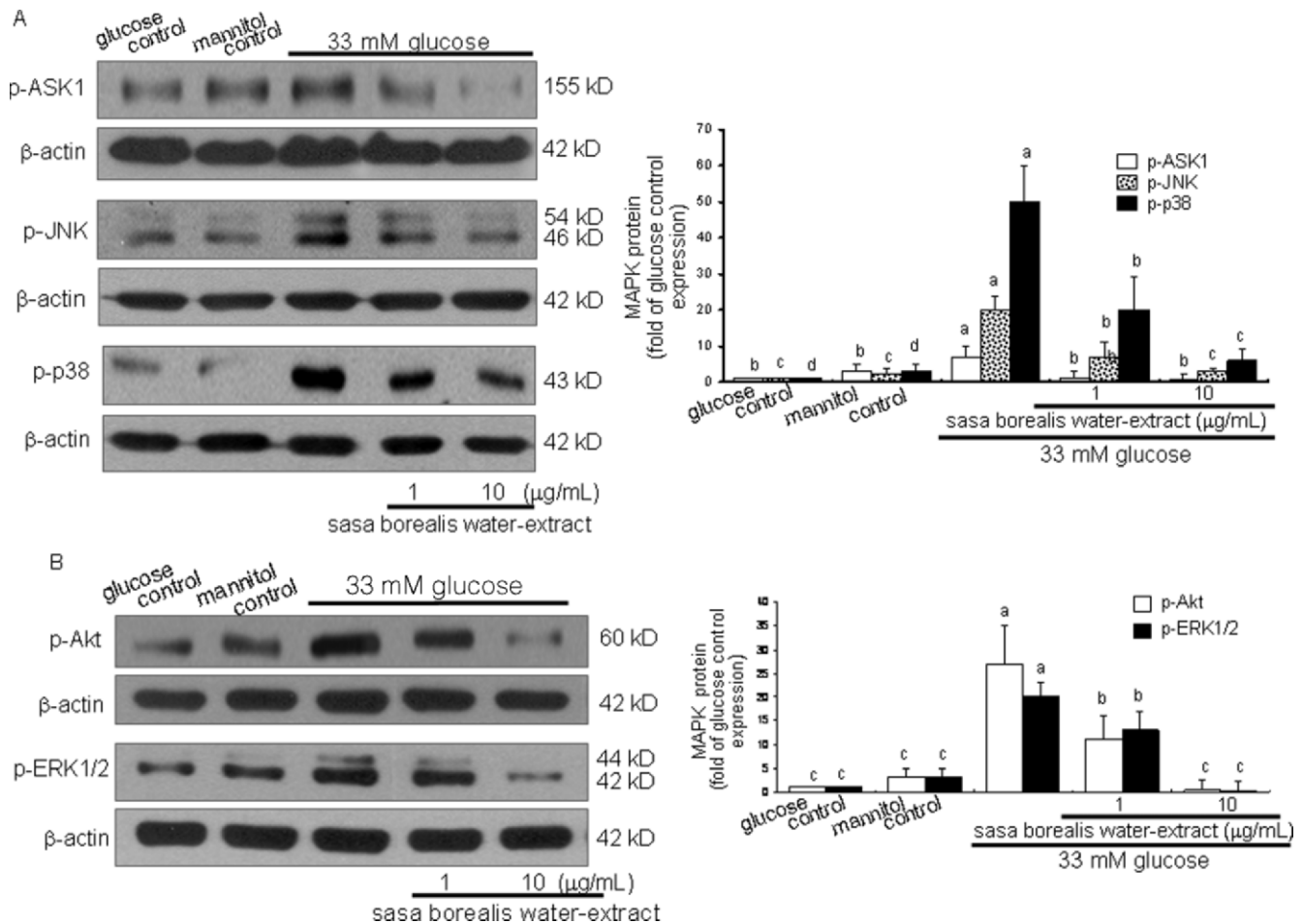


Fig. 5. Inhibition of activation of ASK1, JNK and p38 MAPK (A), and ERK1/2 and Akt (B) in 33 mM high glucose-exposed and/or 1–10 µg/ml SBwE-treated HUVEC. Total cell protein extracts were subjected to Western blot analysis using a primary antibody against human phospho-JNK, phospho-ASK1, phospho-p38 MAPK, phospho-Akt, phospho-ERK1/2, and β -actin. Representative blots shown are typical of four independent experiments. The letter p denotes a phosphorylated form of MAPK.

study did not investigate ROS-independent inhibition of SBwE on induction of these proteins. Different analogs of curcumin present in turmeric exhibited various anti-inflammatory and antiproliferative activities for NF- κ B or cell proliferation, which did not correlate with their ability to modulate the ROS status (24).

The endothelium is the prominent target of high ambient glucose in the pathological conditions of diabetes mellitus characterized by hyperglycemia and vascular complications (1). However, the mechanisms responsible for eliciting vascular complication triggered by hyperglycemia are not yet well established. Numerous studies have shown that hyperglycemia induces ROS, which hence triggers diabetic endothelial apoptosis and vascular dysfunction (2, 4, 6). Since oxidative stress contributes to cellular injury as a common apoptotic mediator, a reduction in the cellular antioxidant reserve might be responsible for endothelial dysfunction in diabetes (8, 9). It has been shown that agents blocking ROS production or improving cellular antioxidant defense prevented high glucose-induced cell damaging effects and apoptosis (25–27). The antioxidant *N*-

acetyl-L-cysteine, a glutathione precursor, attenuated hyperglycemia-induced rat myocyte death and compensatory hypertrophy through scavenging ROS and replenishing the intracellular glutathione pool (26). It should be noted that mannitol is known as a scavenger of hydroxyl radicals. However, this study employed mannitol as an osmotic control to examine any possible impact of osmotic load generated due to culturing with high glucose.

Sasa borealis has been used for making tea in the Far East. Nevertheless, there is little known about its use in traditional medicines. Accordingly, this study attempted to determine whether *S. borealis* could have novel alternative medicinal uses as an antidiabetic agent. Bioactivity-guided fractionation column chromatography demonstrated that flavone C-glucosides, isoorientin, and its derivative are present in *S. borealis* leaves throughout the year (14). These components display free radical scavenging activity and strong cytoprotective effects against tert-butylhydroperoxide-induced oxidative damage of HepG2 cells. The phenolic compounds isolated from *S. borealis* showed inhibitory effects on the P-glycoprotein in adriamycin-

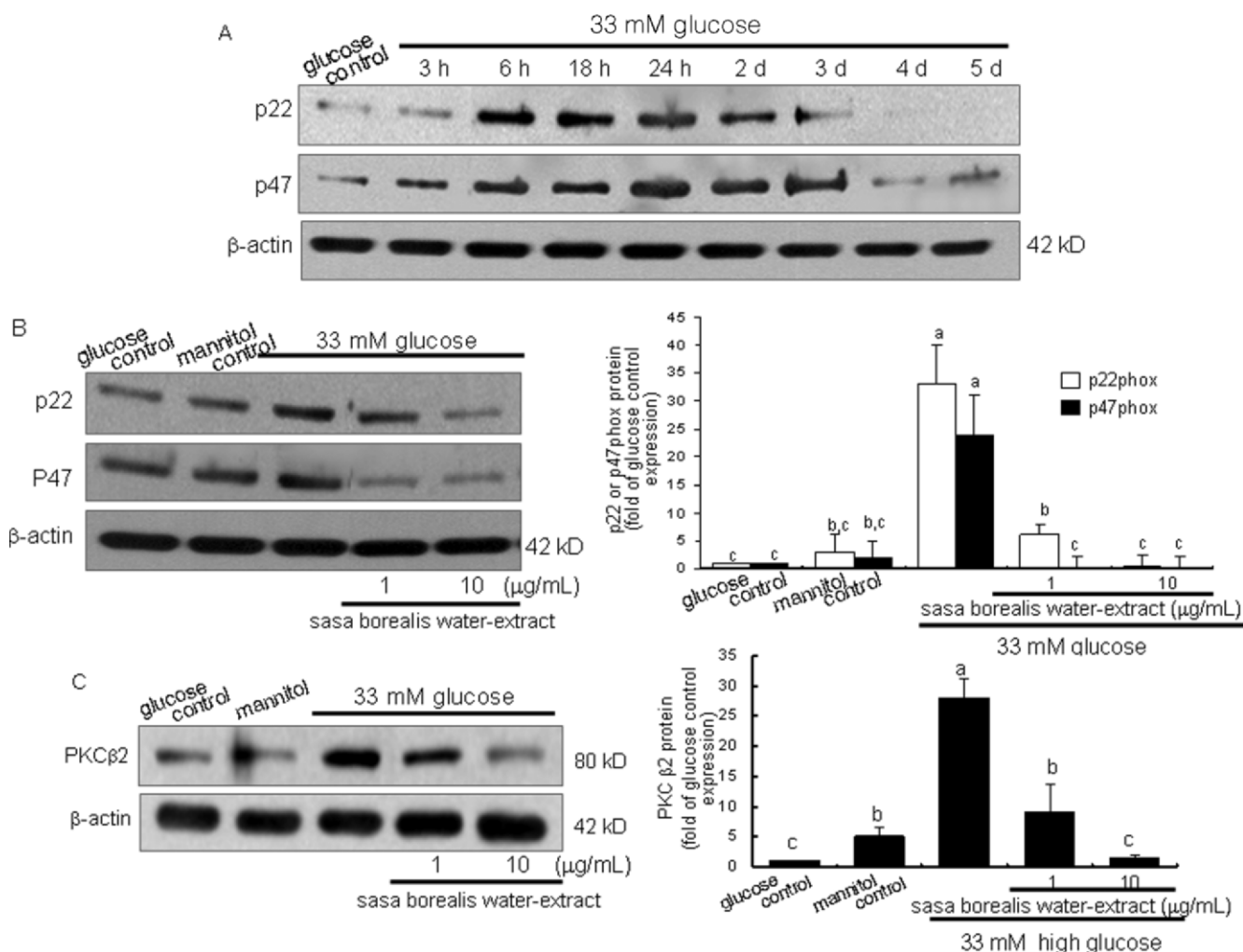


Fig. 6. Inhibition of activation of NADPH oxidase (A and B) and expression of PKCβ2 (C) in 33 mM glucose-exposed and SBwE-treated endothelial cells. Human vascular endothelial cells were exposed to control levels (5.5 mM) or high levels of glucose (33 mM) for 5 days and treated with 1–10 μg/ml SBwE. Total cell protein extracts were electrophoresed on 10% SDS-PAGE gel, followed by Western blot analysis with a primary antibody against human p22phox, p47phox and PKCβ2. β-Actin protein was used as an internal control. Bars represent the means ± SEM of three independent experiments. Values not sharing a letter indicate significant different at $P < 0.05$.

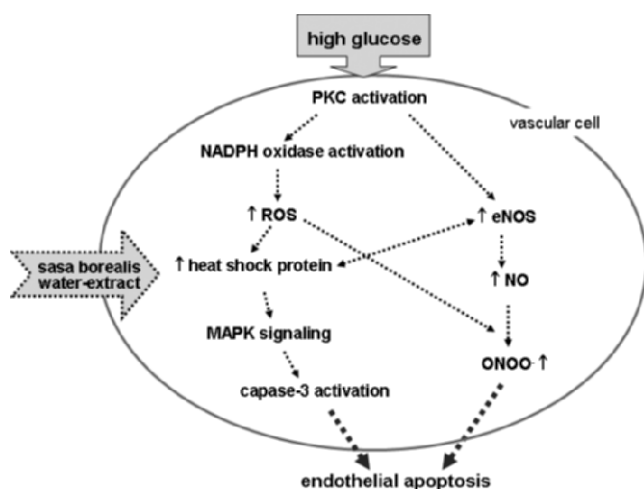


Fig. 7. Schematic diagram showing an inhibitory role of SBwE in chronic high glucose-induced endothelial dysfunction. The symbol indicates inhibition or blockade due to a SBwE treatment.

resistant human breast cancer cells (15). This study found that the treatment of high glucose-treated HUVEC with SBwE enhanced cytoprotective properties, most likely through the effects of antioxidant components present in the aqueous SBwE.

It was assumed that MAPK signaling pathways might be responsible for the protective effects of SBwE against the induction of apoptosis by high glucose. High levels of glucose stimulated hepatic stellate cell proliferation and upregulated the levels of activated ERK 1/2 with ROS production subsequent to proliferation and type I collagen production by hepatic stellate cells (28). Indeed, high glucose triggered ROS generation and caspase-3 activity in HUVEC through a PI3K/Akt-dependent pathway that facilitates HUVEC apoptosis (7). High glucose induced ERK-1 activity in mesangial cells through inhibition of NADPH oxidase, which was attenuated by pitavastatin, a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor (29). The

Akt survival signaling was observed in high glucose-triggered early phase, and excessive long-lasting ROS insult led to high glucose-induced HUVEC apoptosis due to sustained NF- κ B-dependent JNK activation with concomitant reduction of Akt survival signaling, which was prevented by the presence of vitamin C (30). PI3K inhibitors attenuated high glucose-stimulated eNOS expression, collectively indicating a contribution of PI3K/Akt/eNOS signaling in controlling the induction of apoptosis by high glucose. Consistent with these previous reports, this study found that exposure of HUVEC to high levels of glucose activated the MAPK signaling pathways and that inhibition of this activation was required for the endothelial cytoprotective effects of SBwE.

There is coregulation between Hsp and MAPK. The mycotoxin citrinin, a natural contaminant in foodstuffs and animal feeds, induced ROS and mitochondria-dependent apoptotic processes and inhibited Ras-mediated ERK survival signaling *via* inactivation of the Hsp90 α / β /multi-chaperone complex in embryonic stem cells (31). The inhibition of Hsp90 with geldanamycin increased the toxicity of cytochrome P450-mediated ROS in HepG2 cells through an early and sustained activation of the p38 MAPK pathway (32). This study found that SBwE markedly mitigated the induction of Hsp27 and Hsp90 α / β during oxidative stress due to exposure to high glucose and inhibited ROS-mediated stress-responsive MAPK death signaling *via* this Hsp blockade. Recently, it has been shown that fraxetin has significant neuroprotective effects against ROS-mediated apoptosis induced by mitochondrial complex I inhibitor rotenone by affecting the main protection system, including significant enhancement in Hsp70 expression at mRNA and protein levels (33). On the other hand, incubation of bovine aortic endothelial cells with antidiabetic metformin dramatically attenuated high glucose-induced reduction in the AMPK-dependent association of Hsp90 α / β with eNOS, resulting in increased NO bioactivity with a reduction in endothelial apoptosis caused by exposure to high levels of glucose (34). The soy isoflavone equol rapidly stimulated phosphorylation of ERK1/2 and PI3K/Akt and association of eNOS with Hsp90 α / β in human endothelial cells, leading to the activation of eNOS and increased NO production at resting cytosolic Ca²⁺ levels (35). Accordingly, it is inferred that HUVEC toxicity with high glucose might entail Hsp induction and its association with eNOS activated by oxidative stress during exposure to high glucose.

High glucose-induced endothelial dysfunction is known to involve increased NADPH oxidase-dependent ROS generation as ascertained in animal models of diabetes and in patients with diabetes (36, 37). Thus, possible therapeutic approaches to endothelial dysfunction and vascular complication caused by hyperglycemia comprise the use of antioxidants or direct pharmaceutical manipulation of NADPH oxidase. Intense interest is currently seen in naturally occurring compounds as antioxidants that

scavenge various types of radicals in aqueous and organic environments (38). Baicalein inhibited oxidative-stress-induced apoptosis *via* modulation of ERK activation and induction of hemoxygenase-1 gene expression in rat glioma cells C6 (39). These antioxidant compounds could prevent endothelial apoptotic toxicity triggered by high ambient glucose-elicited ROS generation. It was suggested that curcumin, a yellow curry pigment, might inhibit oxygen radical production caused by high glucose concentrations in a cell-free system, providing evidence that curcumin supplementation may prevent the cellular dysfunction associated with diabetes (40). Resveratrol present in red wine inhibited high glucose-induced oxidative stress and apoptosis of human leukemia cells by virtue of its antioxidant properties (41). The present results demonstrated that SBwE dampens glucose-induced endothelial apoptosis by downregulating the expression of p22phox and p47phox subunits and resultant ROS generation. Therefore, SBwE-targeted inhibition of NADPH oxidase could be a promising event to prevent oxidative stress implicated in diabetic endothelial dysfunction.

ROS production through NADPH oxidase upregulation is PKC-dependent and amplifies glucose signaling (42, 43). It has been shown that ROS is a downstream signal of PKC and NADPH oxidase in diabetic nephropathy (37) and that it amplified PKC signaling in high glucose-induced fibronectin expression of human peritoneal mesothelial cells (44). The PKC β isoform appears to be preferentially activated by high glucose levels and has been shown to be associated with diabetic vascular complications (45, 46). Thus, the PKC β signaling blockade has emerged as a therapeutic target mechanism against diabetic vascular complications, progressive β -cell dysfunction, and metabolic syndrome (46). Metformin, an antihyperglycemic drug, reduces high glucose-mediated intracellular ROS production in endothelial cells through the inhibition of PKC (47). Our data also show the involvement of PKC signaling in controlling high glucose-induced endothelial apoptosis entailing activation of NADPH oxidase and ROS production, which was markedly blocked by treatment with SBwE. Accordingly, the antioxidant activity of SBwE may afford an antihyperglycemic effect *in vivo* and improve diabetic endothelial dysfunction.

NO has emerged as a potent mediator of cellular damage and as a fundamental signaling machine regulating critical cellular function under pathophysiological conditions (23). However, it has been shown that most of the cytotoxicity attributed to NO is in fact due to ONOO⁻. High glucose caused the generation of ONOO⁻ in a proximal tubular epithelial cell line, leading to caspase-mediated apoptosis (36). Enhanced ROS generation within the glomerular microcirculation causes a loss of NO bioavailability most likely to yield ONOO⁻ and may contribute to renal vascular abnormalities observed in diabetic nephropathy (37). Accordingly, novel pharmacological strategies aimed at removing ONOO⁻ might represent powerful

therapeutic tools. Blockade of the signaling of PKC-dependent NADPH oxidase activation caused a reduction of ONOO⁻ formation, which appears to be a novel mechanism by which SBwE may reduce diabetic endothelial dysfunction. In addition, the ONOO⁻ scavenger ebselen significantly protected against high glucose-mediated apoptosis, implicating ONOO⁻ as a pro-apoptotic ROS in early diabetic nephropathy. Therefore, nitrosative stress triggered by ONOO⁻ in the presence of high glucose was abolished by treatment with SBwE. It should be noted that NO bioavailability was reduced due to the downregulation of eNOS expression despite SBwE supplementation. On the other hand, many studies have demonstrated that high glucose generates ONOO⁻ via increased inducible NOS activity, which is responsible for vascular-cell apoptosis (48, 49). Thus, a possible approach might be made to targeting beneficial actions of SBwE.

In summary, this hyperglycemia-mimetic HUVEC model demonstrated that SBwE has an anti-apoptotic activity protecting endothelial dysfunction possibly caused by high glucose-induced oxidative stress. This anti-apoptotic protection was most likely mediated through blunting PKC β 2-dependent and MAPK-responsive activation of NADPH oxidase- and Hsp/eNOS-mediated nitrosative stress triggered by ambient high glucose (Fig. 7). In addition, inhibition of Hsp27 induction followed by chronic oxidative stress during exposure to high levels of glucose was involved in blocking high glucose-induced endothelial apoptosis. *Sasa borealis* may be a medicinal plant for blunting high glucose-induced endothelial apoptosis leading to diabetes-associated vascular complications.

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