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Effects of polysaccharides isolated from *Inonotus obliquus* against hydrogen peroxide-induced oxidative damage in RINm5F pancreatic β-cells

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Abstract. The purpose of the present study was to elucidate the cytoprotective effects of polysaccharides isolated from *Inonotus obliquus*. The polysaccharides were extracted from the fruiting body of *I. obliquus* (PFIO) and the liquid culture broth of *I. obliquus* (PLIO). The effects of PFIO and PLIO on hydrogen peroxide (H_2O_2)-induced oxidative damage of RINm5F pancreatic β-cells were comparatively investigated using an MTT assay, immunofluorescent staining, flow cytometry, and western blot analyses *in vitro*. The results of the present study demonstrated that treatment with PFIO and PLIO decreased DNA fragmentation and the rate of apoptosis. In addition, pretreatment of cells with PFIO and PLIO prior to H_2O_2 exposure resulted in increased insulin secretion and scavenging activity for intracellular reactive oxygen species, as compared with treatment with H_2O_2 alone. The results of the present study suggested that PFIO and PLIO may exert protective effects against H_2O_2-induced oxidative stress via the regulation of mitogen-activated protein kinases, nuclear factor-κB and apoptotic proteins. Therefore, PFIO and PLIO may have potential merit as a medicinal food for the prevention of diabetes.

Introduction

Reactive oxygen species (ROS), including superoxide anions, hydroxyl radicals and hydrogen peroxide (H_2O_2), are generated in cells by environmental elements, primarily via mitochondrial respiratory cellular metabolism (1). Under normal conditions, ROS are efficiently neutralized by cellular antioxidant mechanisms. However, when the generation of ROS increases, the resulting imbalance can cause various cellular dysfunctions. This type of cellular damage, particularly in the pancreas, may lead to deleterious effects, and could potentially cause diabetes (2,3). Diabetes mellitus is a common metabolic disease that is associated with chronic inflammation, hyperglycemia, obesity, hyperlipidemia, hyperinsulinemia and insulin resistance (4). Individuals with diabetes have high blood sugar caused by β-cell dysfunction (5). H_2O_2 can inflict damage on vulnerable cell types, including RINm5F pancreatic β-cells, which may lead to apoptosis due to intracellular ROS generation (6,7).

Mushrooms have been used as an effective medicinal food and traditional therapy for centuries; they contain several compounds, including polyphenols and polysaccharides (particularly beta-glucan), which provide health benefits due to their antioxidative effects (8-12). Among them, the mushroom *Inonotus obliquus* has been used as a traditional natural medicine with notable efficacy (13-15). Several studies have reported that *I. obliquus* does not induce any adverse side effects when used in drugs and food for the prevention and treatment of diabetes. In a previous study, a culture broth of *I. obliquus* had significant effects on alloxan-induced diabetic mice (16,17). However, it has been previously noted that while its effects on diabetes have been studied extensively *in vivo*, the number of *in vitro* studies is insufficient. Therefore, the present study aimed to confirm that the antioxidant potential of *I. obliquus* protects against β-cell death and may therefore prevent diabetes. The present study examined the preventive effects of polysaccharides isolated from *I. obliquus* on H_2O_2-induced oxidative damage in RINm5F pancreatic β-cells. In addition, polysaccharides from the fruiting body of *I. obliquus* (PFIO) and polysaccharides from a liquid culture broth of *I. obliquus* (PLIO) were compared, and the results of the study confirmed that they inhibit the destruction of pancreatic β-cells in H_2O_2-induced oxidative stress via the modulation of cellular signaling pathways.

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Materials and methods

Materials. RPMI-1640 media, fetal bovine serum (FBS), penicillin/streptomycin and trypsin-ethylenediaminetetraacetic acid (EDTA) were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Dichlorodihydrofluorescein diacetate (H_2DCF-DA) and an apoptotic assay kit were obtained from Molecular Probes (Thermo Fisher Scientific, Inc.). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), isopropyl alcohol, H_2O_2, Hoechst 33342, mitochondria isolation kit and the rat/mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (cat. no. EZRMI-13K) were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Antibodies against c-Jun N-terminal kinase (JNK; dilution, 1:1,000; cat. no. 9252), phosphorylated (p)-JNK (dilution, 1:1,000; cat. no. 9255S), extracellular signal-regulated kinase (ERK; dilution, 1:1,000; cat. no. 4695), p-ERK (dilution, 1:1,000; cat. no. 9101S), p38 (dilution, 1:1,000; cat. no. 9212), p-p38 (dilution, 1:1,000; cat. no. 4631S), cleaved caspase-3 (dilution, 1:1,000; cat. no. 9664S), nuclear factor (NF)-κB p65 (dilution, 1:1,000; cat. no. 3034), and horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Ig)G (dilution, 1:2,000; cat. no. 7074) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against β-actin (dilution, 1:1,000; cat. no. sc-47778), B-cell lymphoma 2 (Bcl-2; dilution, 1:1,000; cat. no. sc-7382), Bcl-2-associated X protein (Bax; dilution, 1:1,000; cat. no. sc-493), caspase-3 (dilution, 1:1,000; cat. no. sc-7272), apoptosis-inducing factor (dilution, 1:200; AIF; cat. no. sc-13116), cytochrome *c* (dilution, 1:200; cat. no. sc-7159), and HRP-conjugated goat anti-mouse IgG (dilution, 1:2,000; cat. no. sc-2005) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The RINm5F (CRL-11605; American Type Culture Collection, Manassas, VA, USA) cell line was a clone derived from the RIN-m rat islet cell line. The cells were kindly provided by Professor S. Y. Choi (Hallym University, Chuncheon, South Korea). All other chemicals were analytical grade.

Preparation of samples. Dried fruiting bodies of *I. obliquus* (IO) were purchased from ChagaIn (Seoul, South Korea) and were pulverized in a blender. Ground mushroom (20 g) was subsequently extracted with distilled water (60 ml) at 121˚C for 2 h. Extracts were centrifuged at 600 x *g* for 25 min at 4˚C and were filtered through 0.45 μm Whatman filter paper (Whatman 4) to remove insoluble matter prior to freeze-drying. The entire procedure was repeated three times. Polysaccharides were precipitated from the resuspended extracts using 95% ethanol, and were collected by filtration through 0.45 μm Whatman filter paper. The supernatant precipitant was dialyzed using a dialysis tube (molecular weight cut-off, 12,400; Sigma-Aldrich; Merck Millipore) for 5 days to remove low-molecular-weight compounds. The extracted PFIO was then used for further experiments. The liquid culture broth of *I. obliquus* was filtered and centrifuged (600 x *g*, 25 min, 4˚C) to remove fragments of debris. The supernatant was extracted in the same manner as PFIO. The extracted PLIO were then used for further experiments.

Cell culture. RINm5F cells were maintained in RPMI-1640 medium supplemented with 10% inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. The cells were cultured to ~80% confluence and were harvested with 0.25% trypsin-EDTA. The resulting cells were diluted appropriately for reseeding in culture petri dishes or in test plates.

Cell viability assay. To determine the effects of PFIO and PLIO on cell viability the cells were treated with H_2O_2. Briefly, RINm5F cells were seeded in 12-well plates (2.5x10^4 cells/well in 1 ml medium) and were incubated for 72 h. Subsequently, 300 μM H_2O_2 was added to the cells (for 2 h) that had been pretreated with or without PFIO or PLIO (1-100 μg/ml for 24 h). Cell viability was evaluated using the MTT assay. MTT solution (0.5 ml) was added to each well, which was then incubated for 2 h at 37˚C. The formazan crystals in each well were then dissolved in isopropyl alcohol, and the absorbance was measured at 595 nm using an ELISA microplate reader (model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Intracellular ROS scavenging activity and image analysis. To determine the effects of PFIO and PLIO on oxidative stress-induced ROS generation, the cells were treated with or without PFIO or PLIO (1-100 μg/ml) for 20 h, and were then treated with 0.3 mM H_2O_2 for 2 h. After 2 h, 5 μM H_2DCF-DA solution in phosphate-buffered saline (PBS) was added to each well of the plate, which was incubated for 2 h at 37˚C and the fluorescence was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using a microplate spectrofluorometer. Image analysis of intracellular ROS production was performed by seeding RINm5F β-cells in coverslip-loaded 12-well plates and treating in the aforementioned manner. After washing twice with PBS, the cells were mounted under glass coverslips using Vectashield (Brunschwig Chemie, Amsterdam, Netherlands) and the cells were observed. Images of the stained cells were captured using a fluorescence microscope (Nikon Corporation, Tokyo, Japan).

Annexin V/propidium iodide (PI) staining. Cells undergoing apoptosis were identified using a fluorescein isothiocyanate (FITC)-labeled Annexin V/PI apoptosis detection kit (Molecular Probes; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. PI can be used to differentiate necrotic, apoptotic and normal cells, since this agent cannot penetrate the membrane and is generally excluded from viable cells. Cells were pretreated with or without various concentrations of PFIO or PLIO (50 or 100 μg/ml) for 20 h, and/or were then treated with 0.3 mM H2O2 for 2 h. Briefly, the cells were harvested with trypsin-EDTA, washed with PBS, and were centrifuged at 600 x *g* for 5 min to collect the cell pellet. The number of cells was adjusted to 1x10^6 cells/ml. The cells were then resuspended in binding buffer [10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$ (pH 7.4)] and were stained with FITC-labeled Annexin V/PI at room temperature for 15 min in the dark. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The percentage of apoptotic cells was calculated using Cell Quest software (version 4.0.4; BD Biosciences). Cells in the early phase of apoptosis were Annexin V-positive and PI-negative; however, cells in the late stages of apoptosis were Annexin V-positive and PI-positive. The apoptotic index (%)

was calculated as the sum of cells in the early and late phases of apoptosis divided by the total number of events.

Hoechst 33342 staining. In order to examine the degree of nuclear condensation, the nuclear morphology of cells was evaluated using the cell-permeable, DNA-specific fluorescent dye Hoechst 33342. RINm5F cells were seeded in 24-well plates and incubated for 24 h. Cells were pretreated with or without various concentrations of PFIO or PLIO (50 or 100 μg/ml) for 20 h, and/or were then treated with 0.3 mM H_2O_2 for 2 h. Cells were incubated for 30 min with 5 μg Hoechst 33342 (stock solution, 10 mg/ml), and were fixed for 20 min at room temperature in 4% formaldehyde. Images of the stained cells were collected using a Nikon fluorescence microscope in order to examine the degree of nuclear condensation. Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis.

Measurement of caspase-3 activities. Caspase activity was determined with a fluorimetric assay using the enzyme substrate Z-DEVDAMC for caspase-3 (Molecular Probes; Thermo Fisher Scientific, Inc.), which is specifically cleaved by the enzyme at the Asp residue to release the fluorescent group, 7-amino-4-methyl coumarin. Cells were pretreated with or without various concentrations of PFIO or PLIO (50 or 100 μg/ml) for 20 h, and/or were then treated with 0.3 mM H_2O_2 for 2 h. Cells were harvested and processed according to the manufacturer's protocol. Fluorescence was measured continuously for a period of 60 min at multiple time points at 350 and 450 nm excitation and emission, respectively.

Measurement of insulin secretion. To determine the amount of insulin secreted, cells were pretreated with or without various concentrations of PFIO or PLIO (50 or 100 μg/ml) for 20 h, and/or were then treated with 0.3 mM H_2O_2 for 2 h. After incubation, 1 ml of Krebs-Ringer's bicarbonate buffer [115 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM $NaHCO_3$, 10 mM HEPES (pH 7.4), and 0.2% bovine serum albumin] was added for 30 min at 37˚C, after which the cells were incubated in Krebs-Ringer's bicarbonate buffer containing 5 or 20 mM glucose for 2 h at 37˚C. The cell culture medium was collected from the treated cells, and the level of insulin released into the medium was measured using a rat/mouse insulin ELISA kit according to the manufacturer's protocol.

Preparation of subcellular fractions. After various treatments, the mitochondrial fraction was prepared using a mitochondria isolation kit (Sigma-Aldrich; Merck Millipore) according to the manufacturer's protocol. Briefly, after various treatments, cells were harvested and resuspended in 0.65-2 ml lysis buffer. The homogenate was incubated on ice for 5 min, two volumes of 1X extraction buffer were added, and the solution was centrifuged at 600 x *g* for 10 min at 4˚C. Following centrifugation, the supernatant was transferred to fresh 1.5 ml tubes and centrifuged at 11,000 x *g* for 10 min at 4˚C. The supernatant was removed, and the pellet was suspended in a CelLytic M cell lysis reagent with protease inhibitor cocktail (1:100; v/v). Nuclear extracts were prepared by lysing nuclei in a high salt buffer supplemented with protease and phosphatase inhibitors using a nuclear extraction kit (Affymetrix, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol.

Western blot analysis. The treated cells were washed in 1X PBS and were lysed in lysis buffer (10 mM Tris-HCl, pH 7.5; 10 mM NaH_2PO_4/$NaHPO_4$, pH 7.5; 130 mM NaCl; 1% Triton X-100, 10 mM NaPPi; 1 mM phenylmethylsulphonyl fluoride; 2 μg/ml pepstatin A) for 30 min on ice. The lysates were centrifuged at 12,000 x *g* for 30 min at 4˚C. The supernatant was collected, and protein content in the supernatant was measured using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc.) prior to western blot analysis. The total or fractionated protein samples (50 μg per lane) were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 1.5% skim milk in 1X Tris-buffered saline containing 0.1% Tween 20 for 30 min, prior to incubation with the appropriate primary antibodies at 4˚C overnight. Subsequently, the samples were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. An enhanced chemiluminescence kit (EMD Millipore, Billerica, MA, USA) was used to develop the luminescent signal.

Statistical analysis. Experimental results are presented as the mean ± standard error of the mean, and were from at least three independent experiments. Statistical analysis was performed to evaluate significant differences using Student's t-test, or one-way analysis of variance and Duncan's multiple range tests (SAS version 9.1; SAS Institute, Inc., Cary, NC, USA) for comparing multiple groups. $P<0.05$ was considered to indicate a statistically significant different.

Results

Protective effects of PFIO and PLIO on H_2O_2-treated RINm5F cells. To determine the cytotoxic effects of PFIO and PLIO, cell viability was determined using the MTT assay. PFIO and PLIO did not cause any cytotoxicity at a 100 μg/ml concentration (Fig. 1A). Therefore, the maximum concentration of PFIO and PLIO used for follow-up studies was 100 μg/ml. In the present study, H_2O_2 was used to induce oxidative stress in RINm5F cells. To confirm the cytotoxicity of H_2O_2, it was added in various concentrations (10-700 μg/ml). H_2O_2 was able to induce oxidative stress in RINm5F cells, and decreased viability in a dose-dependent manner. Compared with the control group, the viability of RINm5F cells treated with 300 μg/ml H_2O_2 for 2 h was reduced by ~60% (Fig. 1B). Therefore, 2 h duration was selected for the exposure to 300 μg/ml H_2O_2. To evaluate whether PFIO and PLIO exerted protective effects on H_2O_2-treated cells, cells were pretreated with PFIO or PLIO. The viability of PFIO- and PLIO-treated cells increased; however, the protective effect of PFIO was greater than the effect of PLIO (Fig. 1C and D).

Inhibitory effects of PFIO and PLIO on ROS generation in H_2O_2-treated RINm5F cells. The inhibitory effects of PFIO and PLIO on H_2O_2-induced ROS generation in RINm5F β-cells was determined using the ROS-sensitive fluorescent

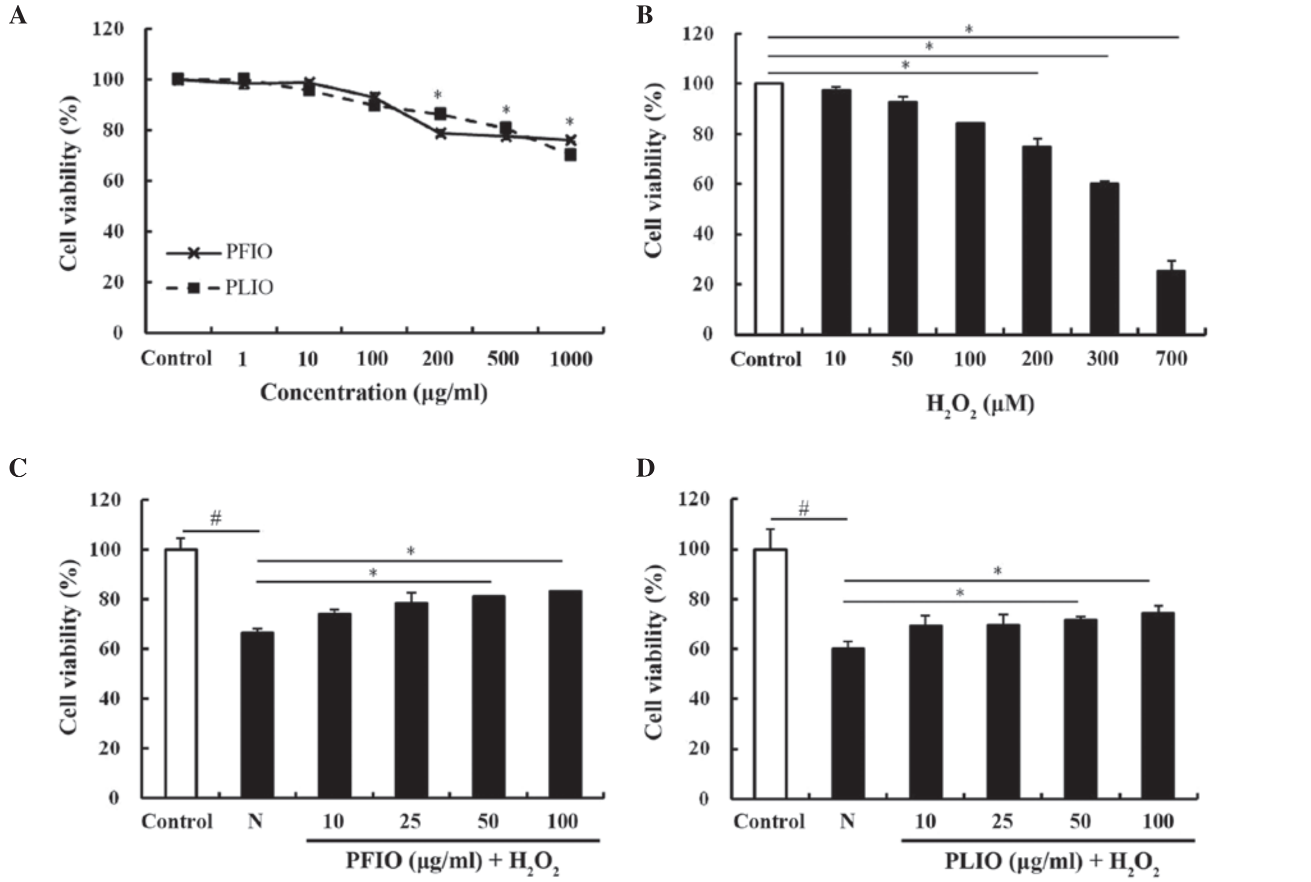

Figure 1. Cell viability of RINm5F pancreatic β-cells. (A) To determine the non-toxic concentrations of PFIO and PLIO, cells were exposed to various concentrations of PFIO and PLIO (1-1,000 μg/ml) for 24 h. $^*P<0.05$ vs. the control group. (B) Cytotoxicity was examined following exposure to various concentrations of H_2O_2 for 2 h. $^*P<0.05$ vs. the control group. Protective effects of (C) PFIO and (D) PLIO in oxidative stress-damaged RINm5F pancreatic β-cells. Cell viability was determined by MTT assay. $^\#P<0.05$ vs. the control group; $^*P<0.05$ vs. the H_2O_2-only treatment group. Data are presented as the mean ± standard error of the mean. C, control; N, H_2O_2 treatment alone; H_2O_2, hydrogen peroxide; PFIO, polysaccharides derived from *Inonotus obliquus* fruiting body; PLIO, polysaccharides derived from *I. obliquus* liquid culture broth.

probe, H_2DCF-DA. H_2DCF-DA is a cell-permeable dye that is diverted by intracellular esterase into its non-fluorescent form, DCFH. DCFH is not cell permeable and is oxidized by H_2O_2 to DCF. PFIO (100 μg/ml) exerted an inhibitory effect on H_2O_2-treated cells, as demonstrated by a decrease in intracellular ROS levels, which was similar to untreated controls (Fig. 2A). The inhibitory effects of PLIO on H_2O_2-treated cells were weaker compared with PFIO (Fig. 2B). Furthermore, the fluorescence intensity of H_2DCF-DA was enhanced in the microscopic images of H_2O_2-treated RINm5F cells; however, the fluorescence intensity of cells pretreated with PFIO and PLIO was decreased (Fig. 2C). These data suggest that PFIO and PLIO may prevent H_2O_2-induced oxidative stress through the scavenging of intracellular ROS.

Effects of PFIO and PLIO on H_2O_2-induced apoptosis of RINm5F cells. To evaluate whether the inhibitory effects of H_2O_2 on RINm5F β-cells were associated with apoptosis, double staining using FITC-labeled Annexin V and PI was performed by flow cytometry. The apoptotic rate was significantly increased to 35.6% in RINm5F β-cells following treatment with H_2O_2 for 2 h; however, pretreatment with 100 μg/ml PFIO markedly inhibited H_2O_2-induced apoptosis in RINm5F β-cells, and the inhibitory effects of PLIO were also confirmed (Fig. 3A and B). To investigate DNA condensation and/or fragmentation in H_2O_2-induced apoptosis, chromatin in RINm5F β-cells was stained using Hoechst 33342. Only in RINm5F β-cells treated with H_2O_2 was microscopic DNA fragmentation detected. Pretreatment with 100 μg/ml PFIO or PLIO decreased H_2O_2-induced chromatin condensation, suggesting that PFIO and PLIO may exert protective effects on oxidative stress-induced apoptotic cell death in RINm5F β-cells by inhibiting DNA fragmentation (Fig. 3C).

Effects of PFIO and PLIO treatment on the expression of mitogen-activated protein kinases (MAPKs) and apoptosis-associated proteins. A previous study demonstrated that the phosphorylation of MAPK proteins is associated with the regulation of mitochondrial permeability-mediated activation of apoptotic proteins, including the Bcl-2 protein family and cytochrome *c* (18). To further confirm the effects of PFIO and PLIO on the H_2O_2-induced apoptosis of RINm5F β-cells, the present study detected the expression of phosphorylated proteins from the MAPK signaling pathway (ERK, JNK and p38) in RINm5F cells using western blot analysis. In cultured cells exposed to H_2O_2, the N group exhibited increased levels of p-MAPKs compared with the control. Conversely, pretreatment with 100 μg/ml PFIO or PLIO inhibited the H_2O_2-dependent

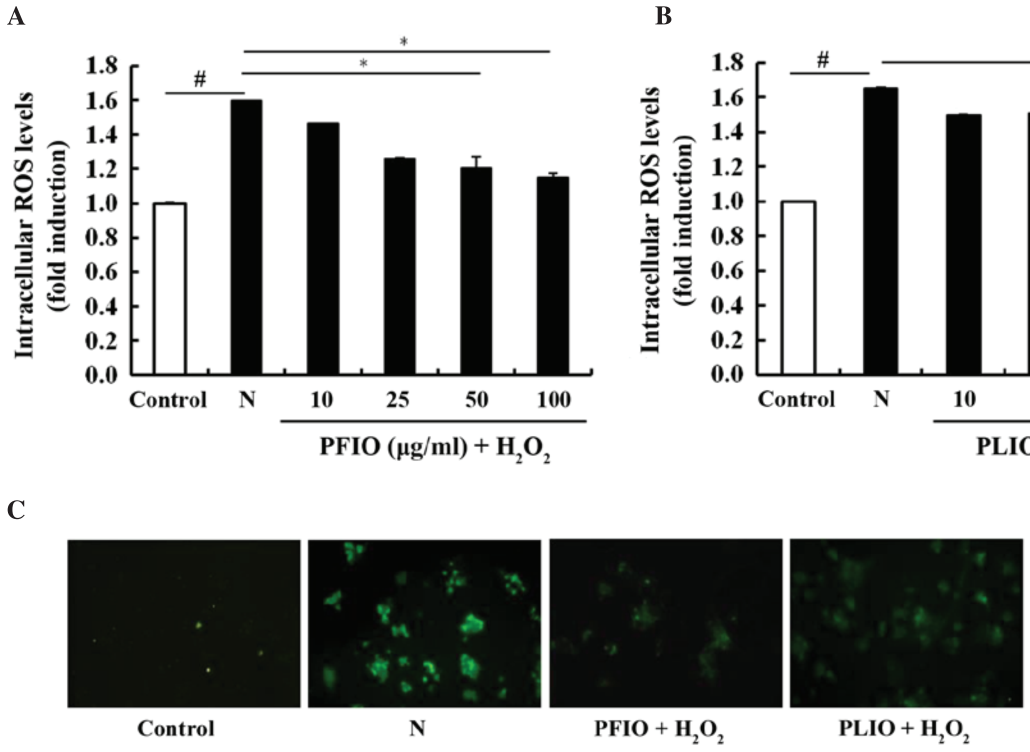

Figure 2. Inhibitory effects of PFIO and PLIO on H_2O_2-induced generation of ROS in RINm5F cells. Intracellular ROS scavenging activity of (A) PFIO and (B) PLIO. Data are presented as the mean ± standard error of the mean. $^{\#}$P<0.05 vs. the control group; *P<0.05 vs. the H_2O_2-only treatment group. (C) Intracellular ROS scavenging activity was investigated using the dichlorodihydrofluorescein diacetate method. ROS levels in RINm5F cells were determined by fluorescence microscopy. Magnification, x400. C, control; N, H_2O_2 treatment alone; H_2O_2, hydrogen peroxide; PFIO, polysaccharides derived from *Inonotus obliquus* fruiting body; PLIO, polysaccharides derived from *I. obliquus* liquid culture broth; ROS, reactive oxygen species.

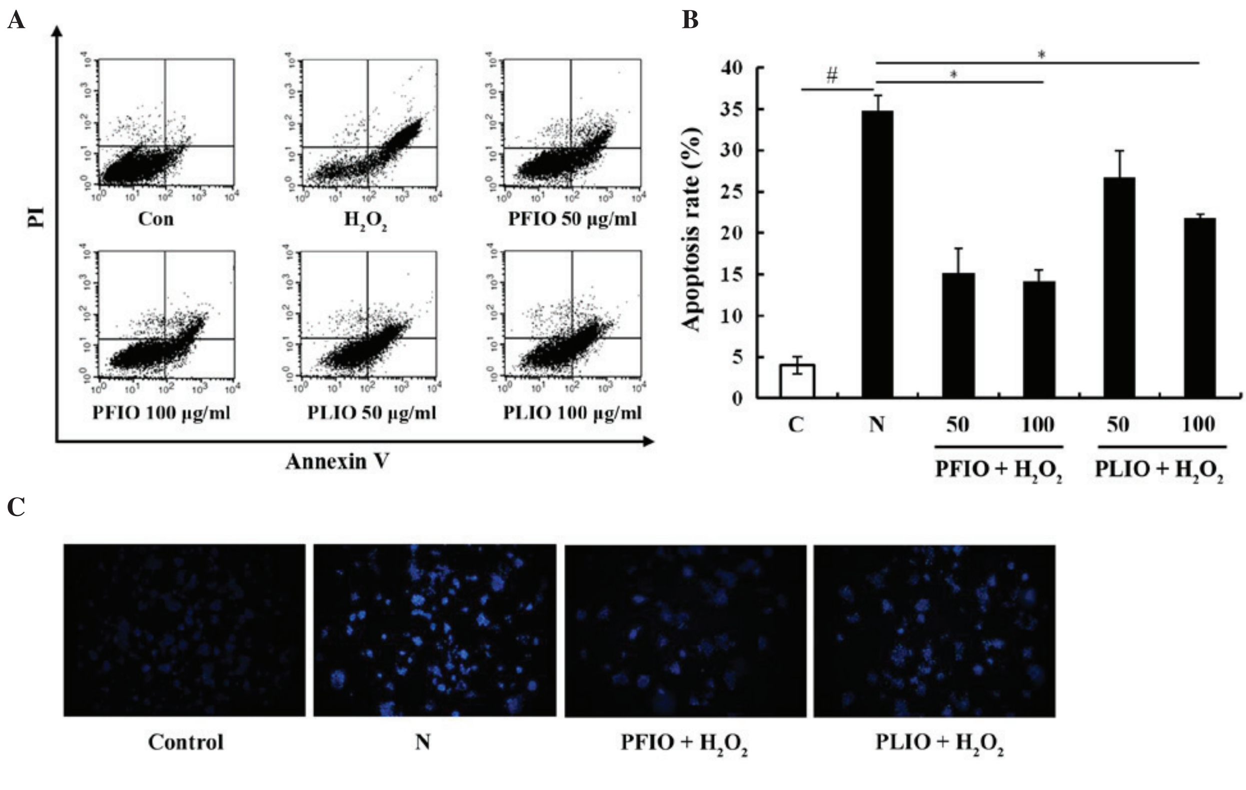

Figure 3. Effects of PFIO and PLIO on H_2O_2-induced apoptosis and inhibition of DNA fragmentation in RINm5F pancreatic cells. (A) Apoptotic cells were detected using Annexin V/PI double staining methods and were analyzed by flow cytometry. (B) Graph represents the percentage of apoptotic cells, quantified from the upper right plus lower right quadrants of (A). Data are presented as the mean ± standard error of the mean. $^{\#}$P<0.05 vs. the control group; *P<0.05 vs. the H_2O_2-only treatment group. (C) DNA fragmentation in RINm5F cells was determined by staining with fluorescent Hoechst 33342 dye. Magnification, x200. C, control; N, H_2O_2 treatment alone; H_2O_2, hydrogen peroxide; PFIO, polysaccharides derived from *Inonotus obliquus* fruiting body; PLIO, polysaccharides derived from *I. obliquus* liquid culture broth; PI, propidium iodide.

phosphorylation of ERK. However, PFIO and PLIO did not alter the phosphorylation of JNK (Fig. 4A). Apoptosis is well known to occur via two pathways, the intrinsic and extrinsic pathways (18). The intrinsic pathway is associated with activation of the Bcl-2 family of proteins and the release of cytochrome *c*, whereas the extrinsic pathway is characterized by the activation of AIF, caspase-8 and caspase-10. The present study confirmed that pretreatment with 100 μg/ml PFIO or PLIO did not alter the activation of AIF compared with in the H_2O_2-only RINm5F cells (data not shown); however, treatment with H_2O_2 alone decreased the expression of Bcl-2 compared with the control, whereas treatment with H_2O_2 alone increased the expression of Bax compared to the control. Treatment with H_2O_2 alone increased mitochondrial release of cytochrome c into the cytosol compared with the control. However, treatment with 100 μg/ml PFIO or PLIO inhibited mitochondrial release of cytochrome c into the cytosol compared with the N group. Furthermore, pretreatment with PFIO increased the expression of Bcl-2 compared with the N group, whereas PFIO treatment decreased the expression of Bax compared to the N group (Fig. 4B).

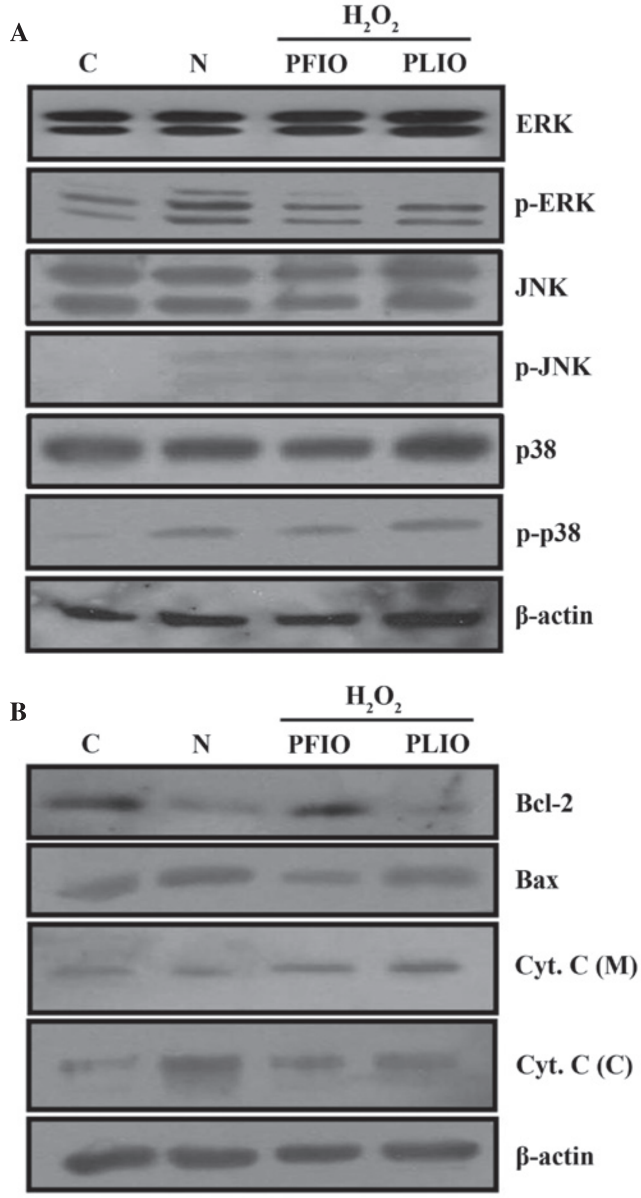

Figure 4. Effects of PFIO and PLIO on H_2O_2-induced apoptosis-associated protein expression in RINm5F pancreatic cells. Treated cells were harvested and lysates were prepared. (A) Expression levels of ERK, p-ERK, JNK, p-JNK, p38 and p-p38 were assessed by western blot analysis. (B) Expression levels of the Bcl-2 family of proteins and fractional Cyt. C were determined by western blot analysis. Equal loading of total proteins in each sample was confirmed by β-actin expression. C, control; N, H_2O_2 treatment alone; H_2O_2, hydrogen peroxide; PFIO, polysaccharides derived from *Inonotus obliquus* fruiting body; PLIO, polysaccharides derived from *I. obliquus* liquid culture broth; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p-, phosphorylated; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; Cyt. C, cytochrome *c*; (C), cytosolic fraction; (M), mitochondrial fraction.

Effects of PFIO and PLIO treatment on NF-κB translocation and caspase-3 activation. Caspase-3 is an important protein in the procession of apoptosis; therefore, the present study examined the activation of caspase-3 and its expression using a caspase-3 assay kit and western blot analysis. Initially, the effects of PFIO or PLIO on cleaved caspase-3 expression in RINm5F cells were determined. The expression level of cleaved caspase-3 in H_2O_2-treated cells was increased compared with the control. However, there were no marked differences in cleaved caspases-3 expression between the PFIO or PLIO groups and the N group (Fig. 5A). Caspase activity was also measured; treatment with 100 μg/ml PFIO or PLIO significantly inhibited H_2O_2-induced caspase-3 activity compared with the N group (Fig. 5B). NF-κB is involved in oxidative stress-induced cell death in several cell types (19). The present study examined the translocation of NF-κB from the cytosol to the nucleus, and demonstrated that H_2O_2 treatment of RINm5F cells increased the NF-κB p65 translocation from the cytosol into the nucleus. However, pretreatment with PFIO or PLIO in RINm5F cells prior to H_2O_2 treatment did not induce any change in the translocation of NF-κB p65 compared with the N group (Fig. 5C).

Effects of PFIO and PLIO on insulin secretion of H_2O_2-treated RINm5F cells. To investigate whether PFIO and PLIO have potential for the prevention of H_2O_2-induced β-cell dysfunction in RINm5F rat insulinoma cells, the efficacy of insulin release was examined following pretreatment with PFIO or PLIO. Insulin secretion was measured using a rat/mouse insulin ELISA kit. Compared with H_2O_2-treated RINm5F cells, pretreatment with PFIO increased insulin secretion; however, pretreatment with PLIO did not increase insulin secretion (Fig. 6).

Discussion

Increased exposure to H_2O_2 generates ROS, which induces exogenous stress in RINm5F cells. Excess ROS generation can be inhibited by natural antioxidants, as well as synthetic antioxidants, including butylated hydroxyanisole and butylated hydroxytoluene (20,21). However, synthetic antioxidants possess adverse side effects and toxicity compared with natural antioxidants (22). Therefore, there has been a gradual increase in demand for a safe substitute, such as antioxidants extracted from natural foods (23,24). Among natural foods, polysaccharides isolated from mushrooms have previously been reported to be bioavailable and non-toxic compounds that possess antioxidant activity (25-28). Therefore, the present study evaluated the anti-diabetic efficacy of a natural antioxi-

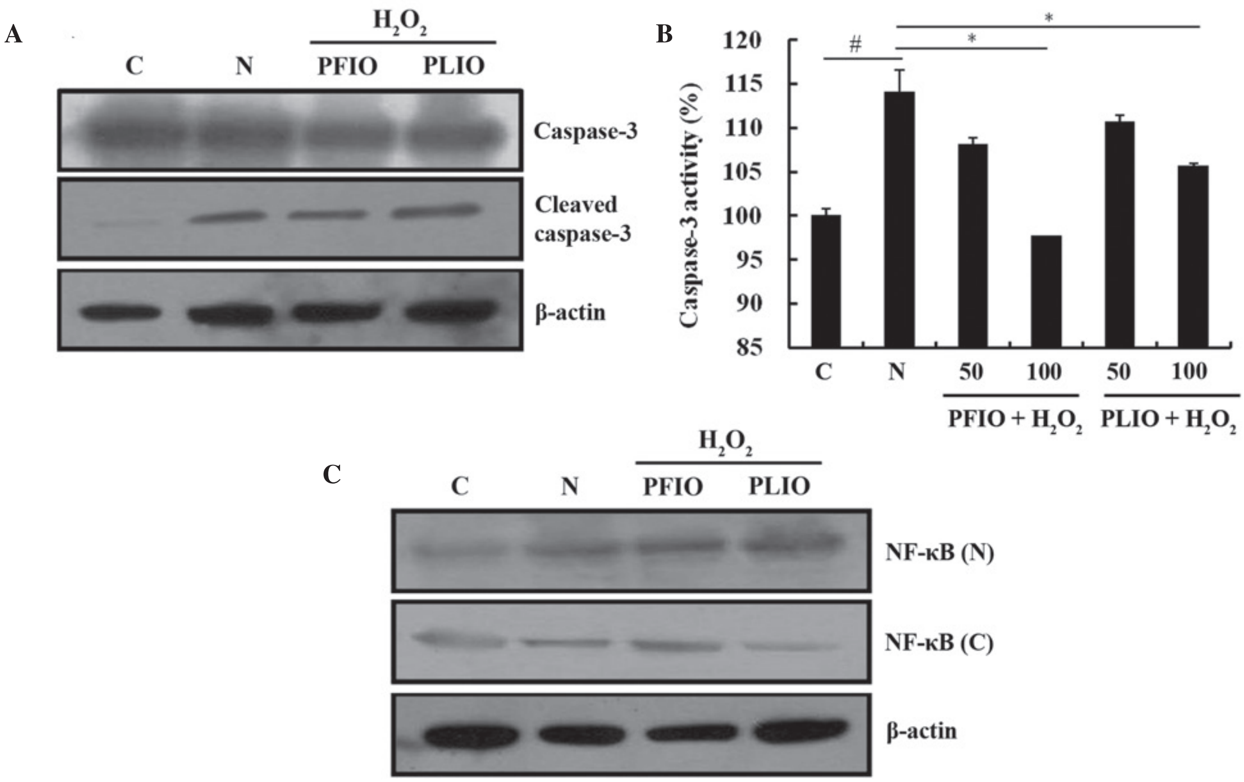

Figure 5. Effects of PFIO and PLIO on the translocation of NF-κB and activation of caspase-3 in H_2O_2-treated RINm5F cells. Cells were harvested and the lysates were prepared. (A) Protein expression levels of caspase-3 and cleaved caspase-3 were determined by western blotting. Equal loading of total proteins in each sample was confirmed by β-actin expression. (B) To determine the effects of PFIO and PLIO on caspase-3 activity in RINm5F β-cells, preparation of cell lysates and measurement of caspase-3-like activity was performed using a caspase assay kit. Data are presented as the mean ± standard error of the mean. $^{\#}P<0.05$ vs. the control group; $^{*}P<0.05$ vs. the H_2O_2-only treatment group. (C) Protein expression levels of fractional NF-κB were determined by western blotting. Equal loading of total proteins in each sample was confirmed by β-actin expression. C, control; N, H_2O_2 treatment alone; H_2O_2, hydrogen peroxide; PFIO, polysaccharides derived from *Inonotus obliquus* fruiting body; PLIO, polysaccharides derived from I. *obliquus* liquid culture broth; NF-κB, nuclear factor-κB; (C), cytosolic fraction; (N), nuclear fraction.

dant isolated from *I. obliquus* on H_2O_2-induced generation of ROS in pancreatic β-cells.

Treatment with H_2O_2 significantly decreased cell viability, which was restored by PFIO and PLIO pretreatment (Fig. 1C and D). Excessive ROS generation is associated with apoptosis, resulting in mitochondrial translocation of Bax, and the release of cytochrome *c* from the mitochondrial fraction to the cytosol. Subsequently, cytochrome *c* in the cytosol activates caspase-3, which has a crucial role in the apoptotic pathway. In the present study, H_2O_2 treatment of RINm5F cells increased the protein expression levels of the pro-apoptotic protein Bax and the release of cytochrome c from the mitochondria to the cytosol compared with the control, whereas H_2O_2 treatment in RINm5F cells decreased the expression levels of the anti-apoptotic protein Bcl-2 compared with the control, suggesting H_2O_2 treatment induced apoptosis of RIN5mF pancreatic cells (Fig. 4B). Among the various signaling pathways that respond to stress, MAPK family members are crucial for the maintenance of cells. It has been previously demonstrated that ERK is important for cell survival, whereas JNK and p38 are considered to be stress responsive and, thus, involved in apoptosis (29). The present study demonstrated that H_2O_2-induced apoptosis is prevented by pretreatment with PFIO and PLIO. According to the results of the present study, the H_2O_2-only group exhibited upregulation of MAPK phosphorylation and other distinct characteristics of apoptosis. However, pretreatment of cells with

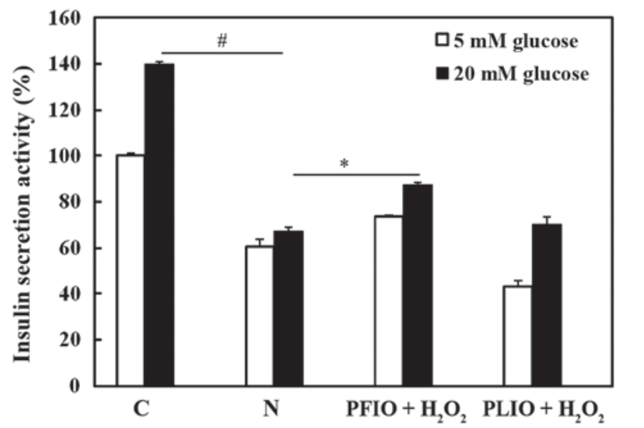

Figure 6. Effects of PFIO and PLIO on insulin secretion in H_2O_2-induced RINm5F β-cells. After treatment, the supernatants were collected, and insulin release was measured using a rat/mouse insulin enzyme-linked immunosorbent assay kit. Data are presented as the mean ± standard error of the mean. $^{\#}P<0.05$ vs. the control group; $^{*}P<0.05$ vs. the H_2O_2-only treatment group. C, control; N, H_2O_2 treatment alone; H_2O_2, hydrogen peroxide; PFIO, polysaccharides derived from *Inonotus obliquus* fruiting body; PLIO, polysaccharides derived from *I. obliquus* liquid culture broth.

PFIO and PLIO reduced phosphorylation of MAPKs (Fig. 4A). Although the effects of the PLIO-treated group may seem insignificant, it did have efficacy. The translocation of NF-κB from the cytosol to the nucleus is associated with the phosphorylation of MAPK proteins. However, in the present study, H_2O_2-treated cells with or without PFIO and PLIO treatment did not induce marked changes in the levels of NF-κB nuclear translocation. The treatment of RINm5F cells with PFIO or PLIO would explain the effects on oxidative stress-induced cell damages independent of NF-κB activation (Fig. 5C). Finally, insulin secretion was significantly inhibited in RINm5F cells exposed to H_2O (30,31). PFIO-treated cells exhibited a marked increase in insulin secretion compared with cells treated with PLIO (Fig. 6).

In conclusion, the results of the present study demonstrated that PFIO and PLIO not only scavenged intracellular ROS but also downregulated the phosphorylation of ERK, which may lead to inhibition of cleaved caspase-3 in RINm5F pancreatic β-cells after H_2O_2-treatment. These effects may result in a decreased apoptotic cell rate. Therefore, these results indicated that since ROS scavenging in cells is important for cellular therapy, *I. obliquus* may be considered a potential therapeutic agent for the prevention of diabetes.

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