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Plant Glycoprotein Modulates the Expression of Interleukin-1 β via Inhibition of MAP Kinase in HMC-1 Cells

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Dioscorea batatas Decne (DBD) is used to heal various disorders of the kidney and lungs as an herbal agent in Korea. The purpose of the present study was to determine whether the DBD glycoprotein regulates the inflammatory reaction stimulated by phorbol-12-myristate 13-acetate plus calcium ionophore A23187 (PMACI) in human mast cells (HMC-1). The results indicate that DBD glycoprotein decreased gene expression of interleukin (IL)-1 β and cyclooxygenase (COX)-2 in PMACI-stimulated HMC-1 cells through blocking of phosphorylation of p44/42 mitogen-activated protein kinase (MAPK) and p38 MAPK and DNA binding activities of nuclear factor (NF)- κ B and activator protein (AP)-1. The production of intracellular reactive oxygen species (ROS) and nitric oxide (NO) is gradually reduced by concentration-dependent DBD glycoprotein treatment in PMACI-stimulated HMC-1 cells. Hence, we propose the hypothesis that DBD glycoprotein can serve as a potent anti-inflammatory agent in the treatment of inflammatory allergic diseases through inhibition of inflammation-related signal transduction in mast cell activation.

Key words: *Dioscorea batatas* Decne (DBD) glycoprotein; interleukin-1 β ; intracellular reactive oxygen species; nitric oxide

Allergy is a hypersensitive response caused by immunoglobulin (Ig)E-dependent mast cell activation. In response to IgE receptor (Fc ϵ RI) cross-linking or other stimuli, mast cells initiate exocytosis of the contents of secretory granules. These include vasoactive amines, arachidonic acid metabolites, chemokines, and cytokines.^{1,2)} Of these, pro-inflammatory mediators such as nitric oxide (NO), cyclooxygenase (COX)-2, and

interleukin (IL)-1 β are responsible for activating mast cells in allergic inflammation and the hypersensitive response.^{3–5)}

Reactive oxygen species (ROS) are produced by enzymatic and non-enzymatic mechanisms in cells. Recent studies have reported that the level of intracellular ROS is related to cytokine production and degranulation by activating protein kinases and transcriptional factors in mast cells. Mitogen-activated protein kinases (MAPKs) and transcription factors, such as nuclear factor (NF)- κ B and activator protein (AP)-1 are well-known mediators in the expression of many pro-inflammatory genes.⁶⁾ MAPKs are serine and/or threonine kinases that transfer mitotic signals to the nucleus. They are activated by extracellular stimuli, including growth factor and various cytokines, and are implicated in the modulation of cytokine expression. The phosphorylation of MAPK eventually results in the direct or indirect activation of transcriptional factors. In particular, NF- κ B is redox-sensitive transcription factor translocated into the nucleus by phosphorylation, ubiquitination, and degradation of the I- κ B family under a cascade of stimuli.⁷⁾ AP-1, another redox-sensitive transcription factor, is also a key component in the regulation of pro-inflammatory genes, such as IL-1 β and COX-2.⁸⁾ Therefore, modulation of these factors from mast cells might provide a useful therapeutic strategy for allergic inflammatory diseases.

Dioscorea batatas Decne (DBD) is a dietary factor in health promotion, and it is used to treat various disorders of the lungs and kidneys in oriental medicine.^{9,10)} Recently, it has been discovered that dietary antioxidants occurring in natural materials have biological functions related to the immune system.¹¹⁾ In particular, many glycoproteins have been isolated from various

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Abbreviations: DBD, *Dioscorea batatas* Decne; PMA, phorbol-12-myristate 13-acetate; CI, calcium ionophore; IL-1 β , interleukin-1beta; COX-2, cyclooxygenase-2; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor-kappa B; AP-1, activator protein-1; ROS, reactive oxygen species; NO, nitric oxide

natural sources, such as mushrooms, fungi, algae, lichens, and plants, although the mechanisms of the immunobiological actions of glycoproteins are not yet fully understood. The biological activities of these glycoproteins have attracted attention in the biochemical and medical areas because of their immunomodulatory and anti-oxidative activities.^{12–15} In a previous study, we found that glycoprotein isolated from DBD (DBD glycoprotein) has an anti-apoptotic effect on radical-induced cell death due to its strong radical scavenging activity,^{16,17} but the role of DBD glycoprotein in mast-cell biology is unclear.

On this basis, we assumed that DBD glycoprotein can inhibit the expression of pro-inflammatory mediators in PMACI-stimulated mast cells. In the present study, to clarify the anti-inflammatory effect of DBD glycoprotein, we investigated the possible action of DBD glycoprotein on IL-1 β gene expression and COX-2 activation in PMACI-stimulated HMC-1 cells.

Materials and Methods

Chemicals. Phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, 2',7'-dichlorofluorescein diacetate, PD 98059, SKF 86002, SP 600125, pyrrolidine dithiocarbamate (PDTC), penicillin G, and streptomycin were obtained from Sigma (St. Louis, MO). Iscove's modified Dulbecco's medium (IMDM) and fetal bovine serum (FBS) were from Gibco BRL (Grand Island, NY). All other chemicals and reagents were of the highest analytical grade.

Preparation of DBD glycoprotein. DBD glycoprotein was isolated and purified from *Dioscorea batatas* Decne (DBD), as described previously.¹⁶ Briefly, the DBD (30 kg) was chopped into small portions and soaked in ddH₂O (20 liters, w/v) for several months in a dark basement. The water extracts were passed through Whatman filter paper (no. 2) in order to remove debris and concentrated with a rotary evaporator (B465; Buchi, Flawil, Switzerland). The concentrated solution was dried with a freeze-dryer (SFDS06; Sam Won, Seoul, Korea). The dried powder (8 g) was dissolved again in distilled water. The solution was adsorbed to concanavalin A-sepharose 4B affinity chromatography (24–45 mm, confirmed by Sigma, C9017) and eluted with 0.5 M methyl α -D-glucopyranoside containing 0.5 M NaCl at pH 7.4. The final dried sample was 1.6 mg (0.02% from the original sample). This was stored at –20 °C during the experimental period. To confirm the DBD glycoprotein, SDS–PAGE was performed with a sample of protein (50 mg/ml) containing 0.1% SDS on 15% polyacrylamide gel using Mini-Protein II (Bio-Rad, San Francisco, CA). The DBD glycoprotein was confirmed by staining with Schiff's reagent,¹⁸ a specific staining reagent for glycoprotein. The purity of the DBD glycoprotein was more than 95%. After verification of the high purity of the glycoprotein,

we determined that the DBD glycoprotein consisted of carbohydrate (83.75%) and protein (16.25%), as described previously.¹⁶

Cell culture and cytotoxicity. HMC-1 cells, a human mast cell line, were provided by H.-M. Kim (College of Oriental Medicine, Kyung Hee University, Seoul, Korea). The cells were grown in Iscove's media supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin at 37 °C in an atmosphere containing 5% CO₂. The medium was renewed twice a week. Cell viability was determined by MTT assay, as described previously.¹⁹ Briefly, the cells were treated with DBD glycoprotein (50–500 μ g/ml) for 24 h. After incubation, MTT stock solution (5 mg/ml) was added to each well, and the plates were incubated at 37 °C in a 5% CO₂ atmosphere for 4 h. Acidic isopropanol was then added to 96-well multi plates, and the plates were read at 560 nm using a SpectraCount™ (Packard Instrument, Meriden, CT).

Determination of intracellular ROS in HMC-1 cells. The amount of intracellular ROS was measured using non-fluorescent 2',7'-dichlorodihydrofluorescein (H₂DCF-DA). As a fluorogenic freely permeable tracer, H₂DCF-DA is specific for ROS assessment. It is deacetylated by intracellular esterases to non-fluorescent 2',7'-dichlorohydrofluorescein (DCFH), which is oxidized to the fluorescent compound 2',7'-dichlorofluorescein (DCF) by ROS. The cells were pre-incubated with 5 μ M H₂DCF-DA for 30 min at 37 °C, and then washed to remove excess H₂DCF-DA. Then they were treated with PMA (30 nM) and A23187 (1 μ M) (PMACI) for the indicated times, or co-treated with DBD glycoprotein (50–200 μ g/ml) in presence of PMACI for 30 min. Finally, fluorescence intensity was measured at an excitation of 485 nm and an emission of 530 nm using a fluorescence microplate reader (Dual Scanning SPECTRAmax, Molecular Devices Corporation, Sunnyvale, CA). The values were calculated as relative intensity of DCF fluorescence as compared with the control.

Preparation of cell extracts. Protein extracts were prepared as previously described.¹⁷ The cells were treated with PMA (30 nM) and A23187 (1 μ M) (PMACI) in the presence of DBD glycoprotein (50–200 μ g/ml), or 1 μ M PD 98059 (a MEK inhibitor) and 1 μ M SKF 86002 (a p38 inhibitor), 1 μ M SP 600125 (a JNK inhibitor), and 500 μ M PDTC (a NF- κ B inhibitor) for the indicated times. Then they were rinsed with PBS after the medium was removed, and lysed in 300 μ l of buffer A (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1.5 mM MgCl₂, 0.5% NP40, 25% glycerol) containing phosphatase inhibitor cocktail. For lysis, the cells were freeze-thawed, shaken for 30 min at 4 °C, and centrifuged at 14,000 $\times$ g for 30 min. The supernatant was designated as a whole extract.

To prepare nuclear extracts, the cells were resuspended in 400 μ l of hypotonic buffer B (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40), followed by centrifugation at $3,000 \times g$ for 5 min. The pelleted nuclear proteins were then resuspended in 200 μ l of NE buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40) containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany) and centrifuged at $12,000 \times g$ for 15 min. The amounts of protein were measured by the Lowry method.²⁰⁾ Samples of protein were stored at $-70^\circ C$.

Immunoblot analysis for MAPK and COX-2. Intracellular protein extracts were analyzed on 10% polyacrylamide mini-gel electrophoresis at 100 V for 2 h at room temperature using a Mini-PROTEIN II electrophoresis cell (Bio-Rad). After transfer onto nitrocellulose membranes (Millipore, Bedford, MA), the membrane was incubated for 1 h at room temperature in TBS-T solution (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% tween-20) containing 5% non-fat dry milk. The membranes were subsequently incubated for 2 h at room temperature with a 1:3,000 dilution of rabbit polyclonal antibodies (p44/42 MAPK, phospho-p44/42 MAPK, p38 MAPK, and phospho-p38 MAPK, Cell Signaling, Beverly, MA; and COX-2, Santa Cruz, CA) in TBS-T solution. After three washes with TBS-T, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Cell Signaling, Beverly, MA) in TBS-T. The protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK). The results obtained from immunoblot assay were calculated using Scion imaging software (Scion Image Beta 4.02, Frederick, MD) as the relative intensity.

Electrophoretic mobility shift assay (EMSA). To make the double stranded oligonucleotide, the strands of the oligonucleotides for NF- κ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3') and AP-1 (5'-TTC CGG CTG ACT CAT CAA GCG-3') were annealed in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) by heating them to $94^\circ C$ for 10 min. To probe NF- κ B and AP-1 double-stranded oligonucleotides, the oligonucleotides were labeled with [γ - ^{32}P] dATP (0.25 mCi, Amersham Pharmacia Biotech) by T4 polynucleotide kinase and purified with a QIAquick[®] Nucleotide Removal Kit according to the manufacturer's protocol (LRS Laboratory, Qiagen Distributor, Seoul, Korea). DNA-protein binding reaction was performed by incubation of the NF- κ B and AP-1 probes, and 10 μ g of nuclear protein extracts and 0.5 μ g/ml of poly dI/dC (Sigma Chemical) in binding buffer: 20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40 (buffer C), 20% ficoll 400, 100 mM HEPES, 300 mM KCl (buffer D), 0.2 M

DTT, and 20 mg/ml BSA at room temperature for 1 h. The DNA-protein complexes were resolved by applying 6% non-denaturing polyacrylamide gel in $0.5 \times TBE$ (45 mM Tris-borate, 1 mM EDTA). Electrophoresis was carried out at 200 V for 3 h in a cold room. Gels were then dried on 3 M blotting paper (Whatman, Maidstone, UK) and exposed to X-ray film at $-70^\circ C$ overnight.

Measurement of nitric oxide production. The cells were treated with PMA (30 nM) and A23187 (1 μ M) (PMACI) in the presence of DBD glycoprotein (50–200 μ g/ml) for 24 h in the 96-well multiple plate. Then they were centrifuged at $1,000 \times g$ for 10 min, and the supernatants were collected. NO production was measured as a function of nitrite (NO_2^-) concentration by the Green method.²¹⁾ Supernatants (50 μ l) were mixed with 100 μ l of 0.1% sulfanilamide and 100 μ l of 0.1% N-1-naphthylethylenediamine dihydrochloride in 2.5% polyphosphoric acid for 10 min. Absorbance was measured at 540 nm with a MicroReader (Hyperion, Boston, MA). Nitrite was quantified using sodium nitrate as a standard.

RNA isolation and RT-PCR. Total RNA was isolated from HMC-1 cells using TRIZOL[®] Reagent according to the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA). Reverse transcription polymerase chain reaction (RT-PCR) was performed by the SuperScript[™] One-Step RT-PCR System (Invitrogen, Carlsbad, CA). Briefly, an RT-PCR mixture (50 μ l) containing 1 μ g of total RNA, 10 μ M of gene-specific sense and antisense primers, 1 μ l of SuperScript[™] II RT/Platinum[®] Taq Mix, and 25 μ l of 2X Reaction Mix was assembled on ice. The following primer sequences of IL-1 β and GAPDH were used: IL-1 β (223 bp), sense (5'-TTG ACG GAC CCC AAA AGA TG-3'), and antisense (5'-AGA AGG TGC TCA TGT CCT CA-3'), and GAPDH (446 bp), sense (5'-CAA AAG GGT CAT CAT CTC TG-3') and anti-sense (5'-CCT GCT TCA CCA CCT TCT TG-3'). The amplifications were conducted with 35 cycles for IL-1 β and GAPDH (20 s at $95^\circ C$, 40 s at $60^\circ C$, and 30 s at $72^\circ C$), and the RT-PCR amplification products were mixed with 2 μ l of loading buffer and separated on 2% agarose gel. Then the gels were stained with 5 μ g/ml of ethidium bromide and photographed.

Statistical analysis. All experiments were done in triplicate, and data were expressed as means $\pm$ S.D. A one-way analysis of variance (ANOVA) and the Duncan test were used for significant differences of multiple comparisons (SPSS program, ver 11.0).

Results

Effect of DBD glycoprotein on intracellular ROS production in PMACI-stimulated HMC-1 cells

As shown in Fig. 1A, DBD glycoprotein did not show any cytotoxic effects up to 500 μ g/ml. On the other

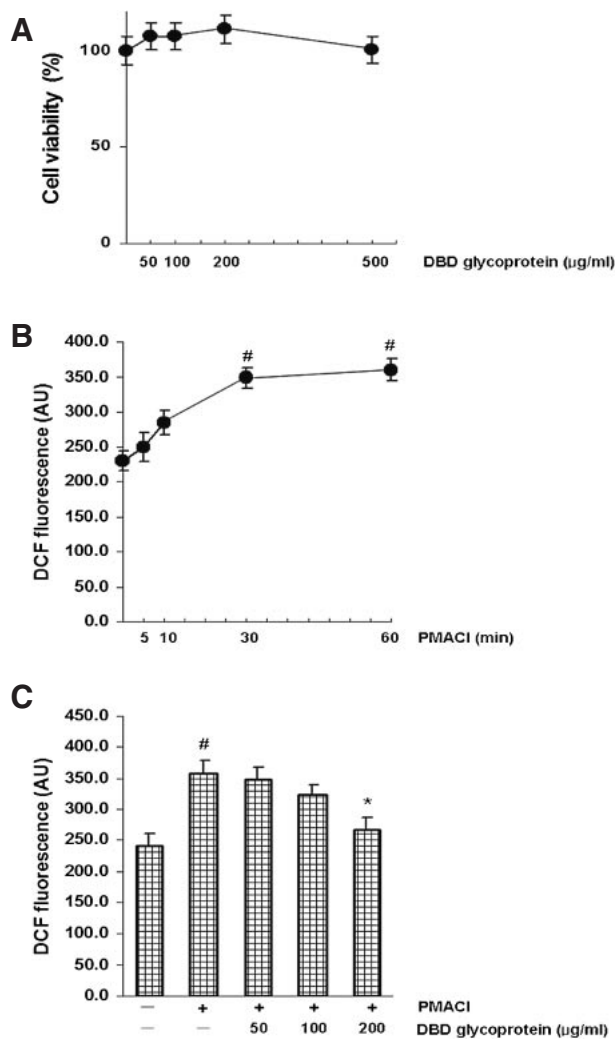


Fig. 1. Effects of DBD Glycoprotein on ROS Production in HMC-1 Cells.

Cells were treated with DBD glycoprotein (50–500 μg/ml) for 24 h (A). Cell survival was measured by MTT assay, as described in “Materials and Methods.” For measurement of intracellular ROS production, the cells were treated with PMACI for various times (B), or co-treated with DBD glycoprotein (50–200 μg/ml) in the presence of PMACI for 30 min (C). Results of ROS production showed no difference statistically between pre-treatment with DBD glycoprotein prior to 30 min and co-treatment with PMACI (data not shown). The values are presented in terms of relative intensity based on monitoring of dichlorodihydrofluorescein (DCF) fluorescence using a fluorescent microplate reader (Dual Scanning SPECTRA-max, Molecular Devices, Sunnyvale, CA). Data represent the means ± S.D. from triplicate experiments ($n = 3$). [#], significant difference between control and PMACI treatment, $P < 0.05$. ^{*}, significant difference with DBD glycoprotein treatment in the presence of PMACI and PMACI treatment alone, $P < 0.05$. AU, arbitrary unit.

hand, treatment with DBD glycoprotein resulted in inhibition of ROS production in PMACI-stimulated HMC-1 cells (Fig. 1B and C). When the cells were treated with PMACI for increasing times, the relative ROS amounts gradually increased, by 0.52 and 0.57 fold at 30 min and 1 h as compared with control. However, treatment with 100 and 200 μg/ml of DBD glycoprotein

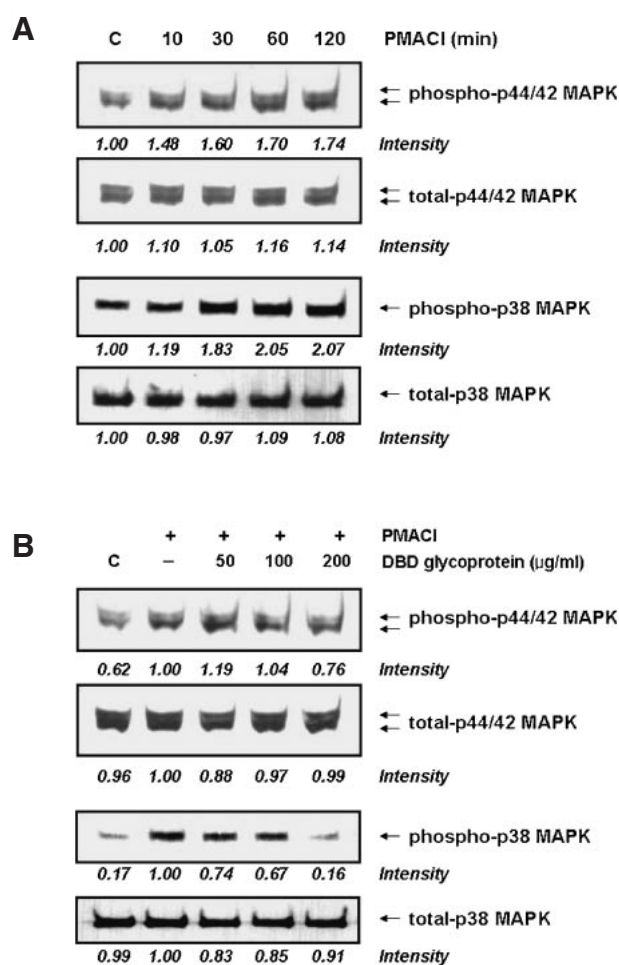


Fig. 2. Inhibitory Effects of DBD Glycoprotein on the Phosphorylation of p44/42 MAPK and p38 MAPK in PMACI-Stimulated HMC-1 Cells.

Cells were treated with PMACI for various times (A), or co-treated with DBD glycoprotein (50–200 μg/ml) in the presence of PMACI (B). The data represent triplicate experiments ($n = 3$). The relative intensities of the bands were calculated with Scion Imaging Software (Scion Image Beta 4.02, Frederick, MD). C, control.

in the presence of PMACI for 30 min diminished ROS amounts by 0.14 and 0.38 fold respectively as compared with the PMACI treatment.

Effect of DBD glycoprotein on phosphorylation of p44/42 MAPK and p38 MAPK in PMACI-stimulated HMC-1 cells

To determine whether stimulation by PMACI would trigger activation of MAPK cascades, activated MAPKs were determined by western blot analysis. The results were calculated using Scion Imaging Software, and were presented as relative intensities. As shown in Fig. 2A, phosphorylation of p44/42 MAPK, and p38 MAPK showed a maximum at 2 h, whereas there was no significant difference in the total expression of p44/42 MAPK, and p38 MAPK. For instance, the band intensities of p44/42 MAPK and p38 MAPK at the addition of PMACI increased by 0.74 and 1.07,

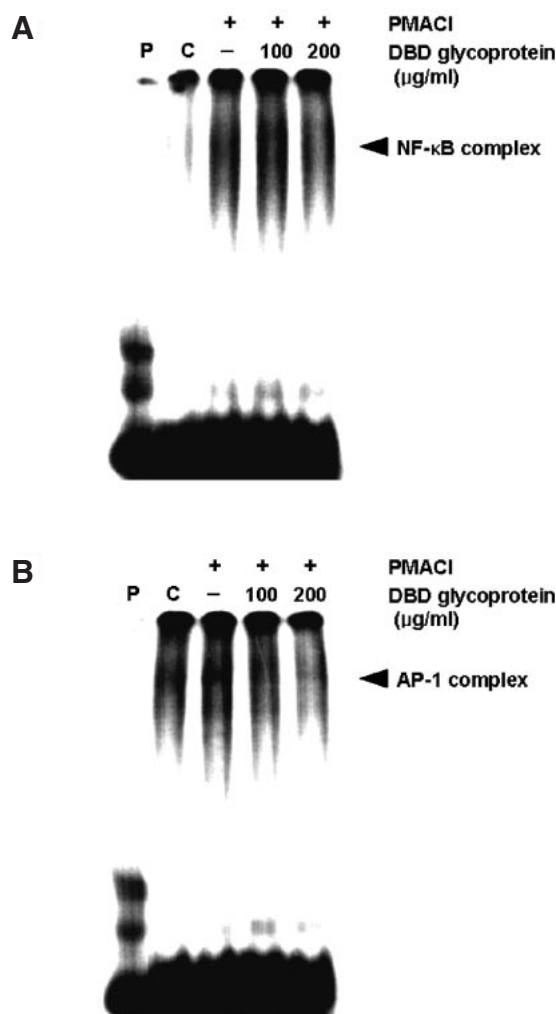


Fig. 3. Inhibitory Effects of DBD Glycoprotein on NF- κ B and AP-1 Binding Activities in PMACI-Stimulated HMC-1 Cells.

Cells were co-treated with DBD glycoprotein (100–200 μ g/ml) in the presence of PMACI for 2 h. The inhibitory effects of DBD glycoprotein on the DNA binding activities of NF- κ B (A) and AP-1 (B) were determined by EMSA. Lane P, probe alone; lane C, control; lane 3, PMACI; lane 4, DBD glycoprotein (100 μ g/ml) in the presence of PMACI; lane 5, DBD glycoprotein (200 μ g/ml) in the presence of PMACI.

respectively in the presence of PMACI. In contrast, treatment with DBD glycoprotein in the presence of PMACI resulted in an inhibition of p44/42 MAPK and p38 MAPK phosphorylation in HMC-1 cells. As shown in Fig. 2B, the values of band intensity for phosphorylated p44/42 MAPK and p38 MAPK decreased by 0.24 and 0.84 respectively at the addition of 200 μ g/ml of DBD glycoprotein in the presence of PMACI.

Effect of DBD glycoprotein on transcription factors in PMACI-stimulated HMC-1 cells

We examined the inhibitory effect of DBD glycoprotein on NF- κ B and AP-1 complex in PMACI-stimulated HMC-1 cells. As shown in Fig. 3A, nuclear extracts from PMACI-stimulated HMC-1 cells enhanced the DNA-binding activity of NF- κ B and AP-1, but treatment

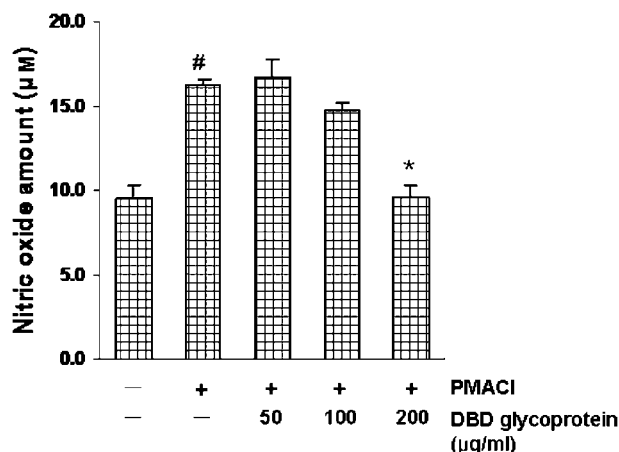


Fig. 4. Inhibitory Effect of DBD Glycoprotein on Nitric Oxide Production in PMACI-Stimulated HMC-1 Cells.

Cells were treated with PMACI for 24 h in the presence of DBD glycoprotein (50–200 μ g/ml). Production of NO was assessed indirectly by measuring nitrite levels as determined by a calorimetric method based on the Griess reaction. Data represent the means $\pm$ S.E. of triplicate experiments ($n = 3$). [#], significant difference between control and PMACI treatment, $P < 0.05$. ^{*}, significant difference with DBD glycoprotein treatment in the presence of PMACI and PMACI treatment alone, $P < 0.05$. C, control.

with DBD glycoprotein (200 μ g/ml) in presence of PMACI significantly inhibited the DNA-binding activity of NF- κ B and AP-1, in a concentration-dependent manner, as compared with PMACI treatment.

Effect of DBD glycoprotein on nitric oxide in PMACI-stimulated HMC-1 cells

When the cells were treated with PMACI, the amounts of nitric oxide significantly increased in the culture medium. For instance, the level of NO production increased significantly, by 6.7 μ M, in the presence of PMACI, but the addition of DBD glycoprotein (200 μ g/ml) in the presence of PMACI decreased the level of NO, as compared with PMACI alone (Fig. 4). For example, the amount of NO was diminished by 5.9 μ M at 200 μ g/ml of DBD glycoprotein, as compared with PMACI alone.

Effect of DBD glycoprotein on the expression of COX-2 and IL-1 β in PMACI-stimulated HMC-1 cells

To evaluate the effect of DBD glycoprotein on the expression of pro-inflammatory mediators, we analyzed the expression of COX-2 and IL-1 β in PMACI-stimulated HMC-1 cells by immunoblotting and RT-PCR respectively. As shown in Fig. 5, treatment with PMACI significantly enhanced the expression of COX-2 and IL-1 β as compared with unstimulated cells, but treatment with DBD glycoprotein in the presence of PMACI inhibited COX-2 and IL-1 β expression as compared with PMACI alone. For example, the value of the band intensity for COX-2 decreased by 0.37 with the addition of 200 μ g/ml of DBD glycoprotein as compared with PMACI treatment alone. In order to determine whether

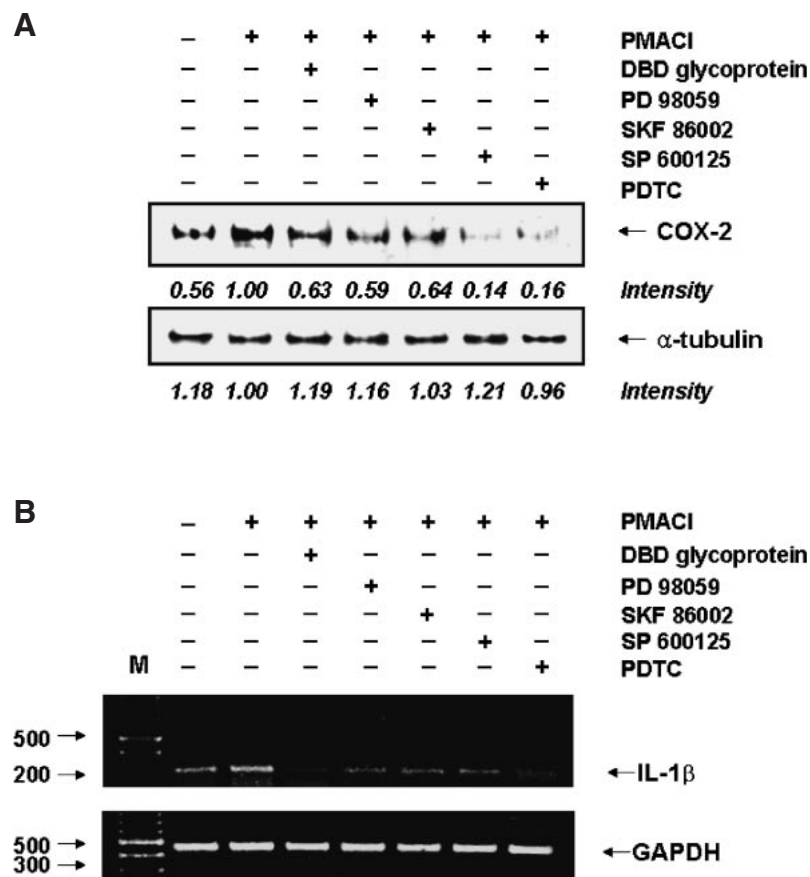


Fig. 5. Inhibitory Effect of DBD Glycoprotein on COX-2 and IL-1 β Expression in PMACI-Stimulated HMC-1 Cells.

Cells were treated with PMACI for 6 h in the presence of DBD glycoprotein (200 μ g/ml) and of specific inhibitors (PD 98059, 1 μ M, SKF 86002, 1 μ M, SP 600125, 1 μ M, and PDTC, 500 μ M). The inhibitory effects of DBD glycoprotein and various inhibitors on COX-2 activity were determined by immunoblotting analysis (A). The relative intensity of COX-2 was quantified using Scion Imaging Software as compared with the PMACI alone. α -tubulin was used as an internal control. The expression of IL-1 β mRNAs was determined by RT-PCR using total RNA isolated from HMC-1 cells (B). Electrophoresis was carried out on a 1% agarose gel. Lane 1, molecular weight maker; lane 2, control; lane 3, PMACI alone; lane 4, 200 μ g/ml of DBD glycoprotein in the presence of PMACI; lane 5, 1 μ M of PD 98059 in the presence of PMACI; lane 6, 1 μ M of SKF 86002 in the presence of PMACI; lane 7, 1 μ M of SP 600125 in the presence of PMACI; lane 8, 500 μ M of PDTC in the presence of PMACI. GAPDH was used as an internal control.

MAPK affects the expression of COX-2 and IL-1 β , HMC-1 cells were treated with PD 98059 (a MEK inhibitor), SKF 86002 (a p38 inhibitor), SP 600125 (a JNK inhibitor), and PDTC (a NF- κ B inhibitor) prior to PMACI treatment. Inhibition of MAPK with PD 98059 (p44/42 MAPK), SKF 86002 (p38 MAPK), and SP 600125 (JNK), and inhibition of NF- κ B with PDTC reduced the activities of COX-2 and IL-1 β as compared with PMACI treatment alone. For example, the values of the band intensity for COX-2 decreased by 0.41, 0.36, 0.86, and 0.84 with the addition of PD 98059, SKF 86002, SP 600125, and PDTC respectively as compared with PMACI treatment alone. α -tubulin and GAPDH were used as internal controls.

Discussion

Inhibition of mast cell activation has been proposed as a reasonable therapeutic target for allergic and inflammatory diseases.²²⁾ In the present study, we investigated

the anti-inflammatory properties of DBD glycoprotein in a PMACI-stimulated human mast cell line (HMC-1). It has been reported that HMC-1 cells are immature cells unable to bind specific IgE due to a lack of Fc ϵ RI receptors.²³⁾ Hence, we used PMACI as a substitute for the IgE/anti-IgE reaction in stimulating HMC-1 cells.

At local inflammatory sites, infiltrated mast cells and their mediators, including cytokines, contribute to the initiation and progression of the inflammatory process.^{24,25)} As an initiator of inflammation, IL-1 β cooperates with arachidonic acid metabolites to recruit inflammatory immune cells, leading to the development of the adaptive immune response.²⁶⁾ Consequently, it promotes inflammation, leukocyte infiltration, granuloma formation, and tissue fibrosis.²⁷⁾ Our results indicate that DBD glycoprotein inhibited the expression of IL-1 β and COX-2 and the production of nitric oxide in PMACI-stimulated HMC-1 cells, suggesting its anti-inflammatory properties. Recently, it has been found that both COX-2 and inducible NOS (iNOS) play important

roles in various inflammatory diseases.²⁸⁾ COX-2 is an inducible enzyme found at low concentrations in healthy tissues, but it is up-regulated in response to tissue damage during inflammation. Several studies have found that PGD₂, the major COX-2 metabolite released from activated mast cells, is responsible for the pathogenesis of eosinophilic airway inflammation.²⁹⁾ In this study, we found that COX-2 activation and NO production induced by PMACI were inhibited by treatment with DBD glycoprotein in HMC-1 cells. The results indicate that DBD glycoprotein has an anti-inflammatory effect, which might explain its beneficial effect in mast cell-mediated inflammatory diseases.

We further investigated to determine whether DBD glycoprotein has an inhibitory effect on signal transduction of mast cell degranulation. Especially, the MAP kinase signaling cascade plays an essential role in the initiation of inflammatory responses. Several recent studies have reported that suppression of MAPKs in mast cells can be a useful tool in reducing mast cell numbers in the inflammatory response.³⁰⁾ To investigate the inhibitory effect of DBD glycoprotein on the intracellular signaling network in pro-inflammatory cytokine expression, we evaluated the phosphorylation of MAP kinases. In the present study, DBD glycoprotein inhibited PMACI-induced activation of MAP kinases in HMC-1 cells, suggesting that it plays a leading part in controlling the inflammatory process. Among the MAP kinase subfamily, DBD glycoprotein inhibited the phosphorylation of p38 MAPK induced by PMACI more than other p44/42 MAPKs. In the phosphorylation of SAPK/JNK, DBD glycoprotein did not show any considerable reduction (data not shown). The MAP kinase pathway and its downstream transcription factors (NF- κ B and AP-1) are required for the expression of inflammatory mediator genes, including COX-2, iNOS, and IL-1 β . NF- κ B is a ubiquitous transcription factor that induces the transcription of cytokines and adhesion molecules that are important in inflammatory processes.³¹⁾ AP-1, another transcription factor known to be activated in response to IgE-mediated stimulation, also appears to be necessary for the production of particular cytokines.⁸⁾ Our results on EMSA revealed concentration-dependent inhibition of NF- κ B and AP-1 binding activity by DBD glycoprotein treatment in PMACI-stimulated HMC-1 cells. Based on the results, we speculate that DBD glycoprotein attenuates the activation of NF- κ B and AP-1 *via* inhibition of MAPK, and thus down-regulates IL-1 β and COX-2 expression.

On the other hand, the ROS produced by mast cells is a key element in the innate immunity and acute inflammatory reactions. Moreover, several reports have found that ROS can stimulate the production of cytokines as a second messenger *via* the MAP kinase cascade in activated mast cells.^{12,32)} To determine the inhibitory effect of DBD glycoprotein on PMACI-induced ROS production, we measured the amounts of

intracellular ROS using a fluorogenic tracer in PMACI-stimulated HMC-1 cells. As described in "Results," PMACI generated intracellular ROS in 30 min, but DBD glycoprotein suppressed PMACI-induced ROS production *via* inhibition of DCFH oxidation in the mast cells. The results for ROS production in the preliminary experiment statistically showed no difference between pre-treatment with DBD glycoprotein prior to 30 min and co-treatment with PMACI. Consequently, it can modulate the pro-inflammatory signals caused by PMACI in the downstream signal pathway. Based on our findings, it might be postulated that DBD glycoprotein has anti-oxidative and anti-inflammatory properties through modulation of the intracellular signaling network in HMC-1 cells. The results for DBD glycoprotein was similarly showed biological activity (anti-inflammation), like UDN glycoprotein, which was isolated from *Ulmus davidiana* Nakai, because both glycoproteins have an anti-oxidative, hygroscopic, high-polar character,³³⁾ although they show differences in molecular weight, and carbohydrate and protein moiety in the whole molecule, and are of plant origin.

In conclusion, our results indicate that DBD glycoprotein has anti-inflammatory potential due to activated mast cells through blocking of pro-inflammatory cytokine IL-1 β expression, COX-2 activation, and NO production. Hence, we speculate that it is applicable in the prevention and treatment of inflammatory diseases. Further study remains to modulate the expression of TNF- α and IL-6 by DBD glycoprotein at the molecular level.

Acknowledgments

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References

- 1) Marshall, J. S., Mast cell responses to pathogens. *Nat. Rev. Immunol.*, **4**, 787–799 (2004).
- 2) Rivera, J., and Gilfillan, A. M., Molecular regulation of mast cell activation. *J. Allergy Clin. Immunol.*, **117**, 1214–1225 (2006).
- 3) Kawata, R., Reddy, S. T., Wolner, B., and Herschman, H. R., Prostaglandin synthase 1 and prostaglandin synthase 2 both participate in activation-induced prostaglandin D2 production in mast cells. *J. Immunol.*, **155**, 818–825 (1995).
- 4) Moon, T. C., Murakami, M., Ashraf, M. D., Kudo, I., and Chang, H. W., Regulation of cyclooxygenase-2 and endogenous cytokine expression by bacterial lipopolysaccharide that acts in synergy with c-kit ligand and Fc epsilon receptor I crosslinking in cultured mast cells. *Cell Immunol.*, **185**, 146–152 (1998).
- 5) Swindle, E. J., and Metcalfe, D. D., The role of reactive oxygen species and nitric oxide in mast cell-dependent inflammatory processes. *Immunol. Rev.*, **217**, 186–205 (2007).

- 6) Cobb, M. H., and Goldsmith, E. J., Dimerization in MAP-kinase signaling. *Trends Biochem. Sci.*, **25**, 7–9 (2000).
- 7) Azzolina, A., Bongiovanni, A., and Lampiasi, N., Substance P induces TNF α and IL-6 production through NF kappa B in peritoneal mast cells. *Biochim. Biophys. Acta*, **1643**, 75–83 (2003).
- 8) Lee, Y. N., Tuckerman, J., Nechushtan, H., Schutz, G., Razin, E., and Angel, P., c-Fos as a regulator of degranulation and cytokine production in Fc ϵ RI-activated mast cells. *J. Immunol.*, **173**, 2571–2577 (2004).
- 9) Choi, E. M., Koo, S. J., and Hwang, J. K., Immune cell stimulating activity of mucopolysaccharide isolated from yam (*Dioscorea batatas*). *J. Ethnopharmacol.*, **91**, 1–6 (2004).
- 10) Akoruda, M. O., Genetic improvement of vegetable crops, yam (*Dioscorea* spp.). In “Genetic Improvement of Vegetable Crops,” ed. Kaslo, M., Pergamon Press, Oxford, pp. 717–733 (1984).
- 11) Scalbert, A., Manach, C., Morand, C., Remesy, C., and Jimenez, L., Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.*, **45**, 287–306 (2005).
- 12) Ooi, V. E., and Liu, F., Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. *Curr. Med. Chem.*, **7**, 715–729 (2000).
- 13) Oh, P. S., Lee, S. J., and Lim, K. T., Hypolipidemic and antioxidative effects of the plant glycoprotein (36 kDa) from *Rhus verniciflua* Stokes fruit in Triton WR-1339-induced hyperlipidemic mice. *Biosci. Biotechnol. Biochem.*, **70**, 447–456 (2006).
- 14) Oh, P. S., Lee, S. J., and Lim, K. T., Glycoprotein isolated from *Rhus verniciflua* Stokes inhibits inflammation-related protein and nitric oxide production in LPS-stimulated RAW 264.7 cells. *Biol. Pharm. Bull.*, **30**, 111–116 (2007).
- 15) Lee, S. J., and Lim, K. T., Inhibitory effect of phytoglycoprotein on tumor necrosis factor- α and interleukin-6 at initiation stage of colon cancer in 1,2-dimethylhydrazine-treated ICR mice. *Toxicol. Appl. Pharmacol.*, **225**, 198–205 (2007).
- 16) Oh, P. S., and Lim, K. T., Antioxidant activity of *Dioscorea batatas* Decne glycoprotein. *Eur. Food Res. Technol.*, **226**, 507–515 (2007).
- 17) Oh, P. S., and Lim, K. T., Protective activity of 30 kDa phytoglycoprotein from glucose/glucose oxidase-induced cell death in primary cultured mouse thymocytes. *Environ. Toxicol. Pharmacol.*, **25**, 114–120 (2008).
- 18) Neville, D. M. Jr., and Glossmann, H., Molecular weight determination of membrane protein and glycoprotein subunits by discontinuous gel electrophoresis in dodecyl sulfate. *Methods Enzymol.*, **32**, 92–102 (1974).
- 19) Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**, 55–63 (1983).
- 20) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275 (1951).
- 21) Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R., Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. *Anal. Biochem.*, **126**, 131–138 (1982).
- 22) Foreman, J. C., “Immunopharmacology of Mast Cells and Basophils,” ed. Foreman, J. C., Academic Press, New York, pp. 1–3 (1997).
- 23) Butterfield, J. H., Weiler, D., Dewald, G., and Gleich, G. J., Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leukemia Res.*, **12**, 345–355 (1988).
- 24) Marone, G., Mast cells in rheumatic disorders: master-minded or workhorse? *Clin. Exp. Rheumatol.*, **16**, 245–249 (1998).
- 25) Moller, A., Henz, B. M., Grutzkau, A., Lippert, U., Aragane, Y., Schwarz, T., and Kruger-Krasagakes, S., Comparative cytokine gene expression: regulation and release by human mast cells. *Immunology*, **93**, 289–295 (1998).
- 26) Hines, C., The diverse effects of mast cell mediators. *Clin. Rev. Allergy Immunol.*, **22**, 149–160 (2002).
- 27) Serlin, D. M., Kuang, P. P., Subramanian, M., O’Regan, A., Li, X., Berman, J. S., and Goldstein, R. H., Interleukin-1 β induces osteopontin expression in pulmonary fibroblasts. *J. Cell. Biochem.*, **97**, 519–529 (2006).
- 28) Kong, G., Kim, E. K., Kim, W. S., Lee, K. T., Lee, Y. W., Lee, J. K., Paik, S. W., and Rhee, J. C., Role of cyclooxygenase-2 and inducible nitric oxide synthase in pancreatic cancer. *J. Gastroenterol. Hepatol.*, **17**, 914–921 (2002).
- 29) Bochenek, G., Nizankowska, E., Gielicz, A., Swierczynska, M., and Szczeklik, A., Plasma 9 α , 11 β -PGF $_2$, a PGD $_2$ metabolite, as a sensitive marker of mast cell activation by allergen in bronchial asthma. *Thorax*, **59**, 459–464 (2004).
- 30) Koranteng, R. D., Swindle, E. J., Davis, B. J., Dearman, R. J., Kimber, I., Flanagan, B. F., and Coleman, J. W., Differential regulation of mast cell cytokines by both dexamethasone and the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580. *Clin. Exp. Immunol.*, **137**, 81–87 (2004).
- 31) Marquardt, D. L., and Walker, L. L., Dependence of mast cell IgE-mediated cytokine production on nuclear factor- κ B activity. *J. Allergy Clin. Immunol.*, **105**, 500–505 (2000).
- 32) Torres, M., and Forman, H. J., Redox signaling and the MAP kinase pathways. *Biofactors*, **17**, 287–296 (2003).
- 33) Lee, S. J., and Lim, K. T., Glycoprotein isolated from *Ulmus davidiana* Nakai regulates expression of iNOS and COX-2 *in vivo* and *in vitro*. *Food Chem. Toxicol.*, **45**, 990–1000 (2007).