



CTB glycoprotein (75 kDa) inhibits IgE releasing, TNF- α and IL-6 expressed by bisphenol A *in vivo* and *in vitro*

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ABSTRACT

Cudrania tricuspidata Bureau (CTB) has been used to treat allergies and inflammatory disease as folk medicine in Korea. The objective of this study is to determine whether a glycoprotein isolated from CTB (75 kDa) has a preventive potential of allergic inflammation caused by bisphenol A (BPA) in BALB/c mice and RBL-2H3 cells. Production of immunoglobulin (Ig) E and releasing of β -hexosaminidase and histamine at treatment of CTB glycoprotein (5–10 mg/kg, BW) were evaluated in mice serum. Activation of extracellular signal-regulated kinases (ERK) and p38 mitogen-activated protein kinase (MAPK), activator protein (AP)-1, expressions of pro-inflammatory cytokines, nitric oxide (NO) production and cyclooxygenase (COX)-2 were assessed in RBL-2H3 cells. In the results, CTB glycoprotein (10 mg/kg, BW) inhibited the production of IgE and releasing of β -hexosaminidase and histamine. Also, the CTB glycoprotein (100 μ g/ml) blocked phosphorylation of ERK1/2 and p38 MAPK, and the activation of AP-1, while it inhibited the NO production, activities of COX-2, tumor necrosis factor (TNF)- α , and interleukin (IL)-6, but not IL-1 β . Taken together, the results of this study indicated that the CTB glycoprotein modulates the expression of allergic inflammation-related factors via the suppression of MAPK/AP-1 activation.

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1. Introduction

Bisphenol A (BPA) is an estrogenic endocrine disrupting chemical (EDC) which has been utilized extensively in dentistry, food packaging, and lacquers (for coating food cans and water pipes) (Colborn et al., 1993). BPA has been implicated, in laboratory animals, as a cause of reproductive disorders and developmental abnormalities including reduced sperm production, increased cell proliferation in breast cancer cells, and stimulation of prolactin due to the interfering action of endogenous estrogens (Takao et al., 1999; Stoker et al., 1996; Hess-Wilson et al., 2006). More recently, it has been reported that BPA may interfere with and disrupt the immune system via a variety of cytokine signals (Segura et al., 1999; Yamashita et al., 2005) and may also promote the production of cytokines and immunoglobulins in macrophages and B cells *in vitro* (Hong et al., 2004; Yurino et al., 2004).

Allergic inflammation is a specific phenomenon occurring in tissues and cells, arising as the response of living systems to

confrontations with foreign organisms, pathogens, or mechanical disruption. Mast cells not only play a major role in the immediate hypersensitivity reaction by releasing chemical mediators such as histamine, serotonin and arachidonate metabolites, but can also express pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , IL-6, and interleukin (IL)-1 β during the early stages of inflammation (Drazen et al., 1996). It has been reported previously that the cytokines and chemokines secreted from mast cells are involved in the later phases of the inflammatory process. Additionally, several signal transduction-related intermediates, such as the protein kinases, are known to be involved in the inflammatory pathway (Cobb and Goldsmith, 2000). In particular, the phosphorylation of mitogen-activated protein kinases (MAPK) such as JNK, ERK, and p38 can result, either directly or indirectly, in subsequent activations of transcriptional factors. In particular, activator protein (AP)-1 is a redox-sensitive transcription factor that can regulate pro-inflammatory genes, including TNF- α , IL-6, IL-1 β , and COX-2 (Lee et al., 2004).

RBL-2H3 cells are an immortalized rat basophilic leukemia cell line which widely used *in vitro* tool for studying degranulation and signaling.

BPA plays an important role in IgE-dependent inflammatory allergic diseases (asthma, contact dermatitis, and anaphylaxis) and autoimmune diseases (rheumatoid arthritis and colitis) (Lee et al.,

Abbreviations: CTB, *Cudrania tricuspidata* Bureau; BPA, bisphenol A; IgE, immunoglobulin E; ERK, extracellular signal-regulated kinases; AP-1, activator protein-1; COX-2, cyclooxygenase-2; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; IL-1 β , interleukin-1 β .

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2003; Yurino et al., 2004). Tammes, eicosanoids, and pro-inflammatory cytokines are released in immediate and sustained inflammatory responses, and are then further amplified by the recruitment of other inflammatory cells (Gilfillan and Tkaczyk, 2006).

The deactivation of inflammatory mediators by treatment with certain compounds such as the phytolectoproteins isolated from natural products may prove to be an effective strategy for the treatment of certain inflammatory diseases. Recently, we isolated and characterized the phytolectoprotein from the edible fruit, *Cudrania tricuspidata* Bureau (CTB), which is used in traditional Korean folk medicine as a treatment for inflammation (Joo and Lim, 2009). The root bark of this species has also been employed in China for the treatment of lumbago, hemorrhages, and contusions (Jiangsu New Medical College, 1977). A broad variety of glycoprotein have been isolated from herbal plants and resources, and have proven useful as a source of therapeutic compounds for immunomodulation and anti-tumor activity; these qualities are generally attributed to the compounds' marked structural variability, which relies on the molecular mass, the degree of branching, and the conformation of sugar residues of different glycoproteins (Neville and Glossmann, 1974; Ooi and Liu, 2000). Thus, we surmise that CTB may also harbor a bio-functional substance with anti-inflammatory potential. In our preliminary study, we figured out that CTB glycoprotein has a molecular weight of 75 kDa with a carbohydrate content of 72.5% and a protein content of 27.5%, anti-oxidative properties, and certain other biological functions (Joo and Lim, 2009). This study basically differs from previously published article. Indeed, this study deals with anti-allergic inflammatory potential of CTB glycoprotein (75 kDa) in the pre-mast cells (RBL-2H3 cells) which is dominantly existed and circulated as basophilic cells in the blood, while the other was handled in the anti-inflammation effect by CTB glycoprotein (75 kDa) in the macrophages (Joo and Lim, 2009). To summarize, the goal of this study is to find remedy effect of CTB glycoprotein on allergy-related immune function caused by BPA. Namely, we studied here whether CTB glycoprotein can protect or remedy the allergy-related immune dysfunction caused by xenoestrogen (bisphenol A) exposures in experimental model.

Therefore, the principal objective of this study is to determine whether a glycoprotein isolated from CTB (CTB glycoprotein, 75 kDa) has a preventive potential of allergic inflammation caused by bisphenol A (BPA, 50 μ M) in BALB/c mice and RBL-2H3 cells. Production of immunoglobulin (Ig) E and degranulation of mast cells (releasing of β -hexosaminidase and histamine) at treatment with CTB glycoprotein (5–10 mg/kg, BW) were evaluated in mice serum using enzyme-linked immunosorbent assay (ELISA). Activation of mitogen-activated protein kinase (MAPK), activator protein (AP)-1, expressions of pro-inflammatory cytokines, and cyclooxygenase (COX)-2 were assessed by Western blot or RT-PCR assays.

2. Materials and methods

2.1. Chemicals

Bisphenol A (B239658), penicillin G (H0474), streptomycin (H0447), and trypsin (T4549) were obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified essential medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). The c-Jun, c-Fos, COX-2 and α -tubulin antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). The phospho-ERK 1/2, total-ERK 1/2, phospho-p38 MAPK, and total-p38 MAPK antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals and reagents were of the highest available quality.

2.2. Preparation of CTB glycoprotein

The CTB glycoprotein was isolated and purified from *C. tricuspidata* Bureau (CTB) fruit, as previously described (Joo and Lim, 2009). In brief, CTB fruits were obtained in October 2007 from the Naju traditional market located in Chonnam province, South Korea. The fruits were definitively identified by Dr. H.T. Lim (Chonnam National University). The CTB (1 kg) was chopped into small portions

and soaked in distilled water (20 L, w/v) for five months at 4 °C in a dark basement. The water extract was passed through Whatman filter paper (No. 2) to remove debris, then concentrated with a rotary evaporator (B465; Buchi, Flawil, Switzerland). The concentrated solution was freeze-dried (SFDS06; Samwon, Seoul, Korea) and the dried powder (8 g) was again dissolved in distilled water. The solution was then subjected to concanavalin A-Sepharose 4B affinity chromatography (24–45 mm, Sigma, C9017) and eluted with 0.5 M methyl α -D-glucopyranoside containing 0.5 M NaCl at pH 7.4. The eluted solution was dialyzed using a dialysis membrane (Spectra/por, MWCO 8000–12,000, CAL, USA) against 20 mM Tris-Cl (pH 7.4) at 4 °C overnight and lyophilized. The final production of CTB glycoprotein yielded about 5.68 g (1.49%) from the raw material. The eluted solution was then employed for further experimentation. To confirm, we conducted SDS-PAGE of a sample of protein (10–25 mg/ml) containing 0.1% SDS, using a 15% polyacrylamide mini-gel and a Mini-PROTEIN II electrophoresis cell (Bio-Rad) at 110 V and 30 mA for 2.5 h. The CTB glycoprotein was confirmed via staining with Schiff's reagent. (Neville and Glossmann, 1974), which is a specific staining reagent for the redox-mediated detection of glycoproteins. The purity of the CTB glycoprotein was greater than 98 $\pm$ 2%. After verifying the purity of the glycoprotein, we applied the glycoprotein to cell cultures for further study.

2.3. Animals and treatments

Female BALB/c mice (25 g, aged 6 weeks) were purchased from Daehan Lab (Animal Research Center Co., Ltd., Daejeon, South Korea). Animals were maintained in a clean room at a temperature between 23 $\pm$ 2 °C with a 12 h light/dark cycle, lights (an illumination intensity of 150–300 lux) on 0700 h and lights off 1900 h. The relative humidity was 55 $\pm$ 15% with air ventilation frequencies of 15–20 times/h. Animals housed according to Guiding Principles in the Use of Animal in Toxicology, adopted by the Society of Toxicology in 1989 at the Experimental Animal Room of College of Veterinary Medicine, Chonnam National University (CNU). The protocol of the animal study was approved by the Animal Care and Use Committee of the College of Veterinary Medicine, CNU. All mice were fed a commercial diet and water *ad libitum*, and kept for at least 1 week prior to the experiments. The body weight and food intake of each group were recorded once weekly. Mice were divided into the following five groups:

- Group 1: Control (n = 6);
- Group 2: 10 mg/kg CTB glycoprotein (n = 6);
- Group 3: 5 mg/kg BPA (n = 6);
- Group 4: 5 mg/kg BPA + 5 mg/kg CTB glycoprotein (n = 6); and
- Group 5: 5 mg/kg BPA + 10 mg/kg CTB glycoprotein (n = 6).

Mice in group 1 were fed a commercial diet and water *ad libitum*. Mice in group 2 were orally administrated 10 mg/kg CTB glycoprotein alone daily for 5 weeks. Mice in group 3 were intraperitoneally injected 5 mg/kg BPA containing 0.1 ml olive oil seven times a week for 4 weeks. The mice in groups 4 and 5 were orally administrated with CTB glycoprotein (5 and 10 mg/kg) daily for 5 weeks and co-treated with 5 mg/kg BPA seven times a week for 4 weeks. On 5th week, the mice blood was collected by a cardiac puncture under anesthesia condition with diethyl ether. The collected blood was centrifuged at 10,000g for 5 min at 4 °C. The supernatant was taken out and stored at –70 °C to measure the levels of β -hexosaminidase, histamine, and immunoglobulin (Ig) E.

2.4. β -hexosaminidase secretion and histamine release assay

The level of β -hexosaminidase was measured according to the methods of Matsubara et al. (2004). Briefly, aliquot (20 μ l) of serum was incubated with an equal volume of 1 mM p-nitrophenyl-N-acetyl β -D-glucosaminide in 0.1 M sodium citrate buffer (pH 4.5) at 37 °C for 1 h. The reaction was terminated by adding 200 μ l of stopping buffer (0.1 M Na₂CO₃/NaHCO₃, pH 10.0). The absorbance was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm.

The amount of histamine was assayed using a fluorometric assay (Shore et al., 1959). The sera were centrifuged at 10,000 g for 10 min at 4 °C and the supernatants were harvested. Afterwards, 2 ml of serum was mixed with 0.75 g of NaCl and 0.5 ml of 1 N NaOH, 5 ml of mixtures 5 ml of mixtures (3 ml of n-butanol and 2 ml of 0.1 N HCl). After centrifugation at 300g for 5 min at 25 °C, 1 ml of the lower HCl layer was recovered, mixed with 0.15 ml of 1 N NaOH and the subsequent product was added to 0.1 ml of 0.2% o-phthalaldehyde dissolved in methanol for 5 min at 25 °C to yield a fluorescent product. The reaction was terminated by adding 0.14 ml of 0.5 N H₂SO₄ and fluorescence intensity was measured using a fluorescent microplate reader (Dual Scanning SPECTRAMax, Molecular Devices Corporation, Sunnyvale, CA, USA) with the excitation set at 360 nm and the emission set at 440 nm. Histamine content was quantified by comparison to a standard curve generated by coupling histamine dihydrochloride to o-phthalaldehyde.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The amount of IgE was determined by sandwich ELISA using monoclonal antibodies of IgE (BD Biosciences, San Diego, CA, USA). Firstly, ELISA plates were coated with rat anti-mouse IgE. After coating, the sera were added into the plates in the

different concentration incubating overnight at 4 °C. After washing, biotinylated anti-mouse IgE was added. The plates were washed again and then streptavidin-horseradish peroxidase was added. After additional washes, TMB solution was added and developed for 10 min. The OD was determined at 405 nm in a Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Murine recombinant IgE was used for quantification of IgE as a standard.

2.6. Cell culture

RBL-2H3 cells were incubated in DMEM containing 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin at 37 °C and a 5% CO₂ atmosphere. The medium was exchanged twice a week. The cells (1×10^6 cells/ml) were divided into 35-mm culture dishes or 96-well flat bottom plates. The RBL-2H3 cells were then co-treated for 30 min with BPA (50 µM) to detect ERK1/2 and p38 MAPK activity, for 6 h to detect TNF-α and IL-6 mRNA expression, and for 2 h to detect COX-2. Additionally, in all experiments, the CTB glycoprotein was pre-administered at indicated concentrations for 30 min prior to the treatment of BPA.

2.7. Cytotoxicity of the CTB glycoprotein

Cellular viability was evaluated according to the method described by Mosmann (1983). The cells were treated with various concentrations of the CTB glycoprotein (25–100 µg/ml) and for suitable incubation times (0–24 h). Then, 2 µl of the (4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) solution which is dissolved 5 mg/ml in PBS as stock solution was added to each well. The cells were then incubated for another 4 h at 37 °C. After completely removing the medium, 50 µl of acidic isopropanol was added to each well, and the plates were read at 560 nm, using a Spectra-Count™ ELISA reader (Packard Instruments, Downers, IL, USA).

2.8. Measurement of nitric oxide

The amount of nitrite, a stable oxidized product of NO, was measured in cell culture media using the Griess method (Green et al., 1982). In brief, after treatment for 2 h, cells were centrifuged at 1000g for 10 min and supernatants were collected for determination of NO production. One hundred microlitres of cell culture supernatant was combined with 100 µl of Griess reagent [mixture of equal volume of 1% sulfanilamide in 2.5% H₃PO₄ and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in dH₂O] in 96-well plates and the mixture was reacted for 10 min at room temperature. The absorbance was measured at 540 nm with a MicroReader (Hyperion, Inc., USA) and the nitrite concentration was assessed using a standard curve of NaNO₂.

2.9. Preparations of protein extracts (whole and nuclear)

For the immunoblot of inflammatory-related proteins, protein extracts were prepared as previously described (Oh et al., 2007). The cells were then treated with 50 µM BPA in the presence of the CTB glycoprotein (50–100 µg/ml). For the immunoblot of COX-2, the treated cells were rinsed twice in PBS after removing the medium and scraped in 300 µl 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 a protease inhibitor cocktail. After cell lysis, the lysate was centrifuged for 30 min at 14,000g, 4 °C. The supernatants were collected and employed as whole cell lysates. In order to separate the cytosolic and nucleic protein extracts, the cells were scraped in 500 µl of 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 MgCl₂, 0.5% NP-40), followed by 5 min of centrifugation at 3000g. The supernatant and pellet were designated separately as nucleic protein extracts. The isolated nucleic protein extracts for the immunoblot of AP-1 were then re-suspended in 200 µl of 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 MgCl₂, 0.5% NP-40) containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany), and centrifuged for 15 min at 12,000g, 4 °C. The quantities of proteins were measured via the Lowry method (Lowry et al., 1951). Protein samples were stored at –70 °C prior to use.

2.10. Immunoblot analysis

Intracellular protein extracts were analyzed via 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, USA), the membranes were 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 then incubated for an additional 2 h at room temperature with a 1:3000 dilution of rabbit polyclonal antibodies (AP-1, ERK1/2, phospho-ERK1/2, p38 MAPK, and phospho-p38 MAPK, Cell Signaling, Beverly, MA; COX-2, Santa Cruz, CA, USA) 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, USA) in TBS-T buffer. The protein bands were then visualized via

enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK). The results of the immunoblot assays were calculated in terms of relative intensity, using Scion imaging software (Scion Image Beta 4.02, Frederick, MD, USA).

2.11. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from RBL-2H3 for 6 h using TRIZOL Reagent in accordance with the manufacturer's protocols (Invitrogen Life Technologies, Carlsbad, CA, USA). RT-PCR was conducted using the AccessQuick™ RT-PCR System (Promega). In brief, the RT-PCR mixture (50 µl) containing 1 µg of total RNA, 10 µM gene-specific sense and antisense primers, 1 µl of avian myeloblastosis virus reverse transcriptase, and 25 µl of AccessQuick™ Master Mix was prepared on ice. The following primer sequences of TNF-α, IL-6, IL-1β and GAPDH were used (sense and antisense, respectively): TNF-α (231 bp), 5'-TCT CAT CAGTTC TAT GGC CC-3' and 5'-GGG AGT AGA CAA GGT ACA AC-3'; IL-6 (223 bp), 5'-GTT CTC TGG GAA ATC GTG GA-3' and 5'-TGT ACT CCA GGT AGC TAT GG-3'; IL-1β (223 bp), 5'-TTG ACG GAC CCC AAA AGA TG-3' and 5'-AGA AGG TGC TCA TGT CCT CA-3'; GAPDH (446 bp), 5'-CAA AAG GGT CAT CAT CTC TG-3' and 5'-CCT GCT TCA CCA CCT TCT TG-3'. The amplifications were conducted with 35 cycles for TNF-α, IL-6, IL-1β and GAPDH (15 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C) and the RT-PCR amplification products were mixed with 2 µl loading buffer and separated on 1% agarose gel. The gels were then stained with 5 µg/mL ethidium bromide and photographed.

2.12. Real-time polymerase chain reaction

Complementary DNA (cDNA) was obtained from total RNA using the SuperScript First synthesis system RT-PCR kit (Invitrogen Corp.). Quantitative real-time polymerase chain reaction (qPCR) amplifications were performed in triplicate using the SYBR Green I assay and were carried out using Stratagene M3000 Sequence Detection System (Stratagene, USA). Specific primers were used for TNF-α (sense: 5'-GAC AAG CCT GTA GCC CAT GTT GTA-3', antisense: 5'-CAG CCT TGG CCC TTG AAG A-3'); IL-6 (sense: 5'-AAG CCA GAG CTG TGC AGA TGA GTA-3', antisense: 5'-TGT CCT GCA GCC ACT GGT TC-3'); IL-1β (sense: 5'-ACA GCA GAG CAC ACA AGC TT-3', antisense: 5'-CTG GCA ACC CTA CAA CAG AC-3') and GAPDH (sense: 5'-TGT GGA TCT GAC ATG CCG CC-3', antisense: 5'-AAC CAC CCT GTT GCT GTA GC-3'). The reaction were carried out in a 96-well plate in 20 µl reactions containing 2× SYBR Green Master mix (Invitrogen, USA)-2 pmol each of sense and antisense primer, and a conditions were 95 °C for 10 min, followed by 95 °C for 10 s, 58 °C for 50 s and 72 °C for 20 s, for 45 cycles. In each assay a standard curve was determined concurrently with examined samples. For standard curve determination, a six dilution series of standard samples, dilution up to 1/5, 1/25, 1/125, 1/625, and 1/3125 of the selected standard cDNA for each gene was utilized. Finally, relative quantitative values of each sample were determined with 1/25 diluted cDNA and were normalized with those of the GAPDH genes. Gene expression was quantified using a medication of the 2-ΔΔCt method as previously described (Livak and Schmittgen, 2001).

2.13. Statistical analysis

All experiments were conducted in triplicate, and the data were expressed as the means ± S.E. One-way analysis of variance (ANOVA) and Duncan's tests were conducted to detect significant differences among multiple comparisons (SPSS program, ver 11.0).

3. Results

3.1. Inhibitory effect of CTB glycoprotein on IgE production in BPA-stimulated ICR mice

Levels of IgE production increased after treatment with BPA alone compared to the control. However, when the mice were orally pre-administered with the CTB glycoprotein in the presence of BPA, level of IgE production after treatment with BPA decreased significantly in the presence of CTB glycoprotein in a dose-dependent manner. For instance, the amount of IgE was 89.05 ng/ml after treatment with BPA alone, as shown in Fig. 1. However, when the mice were orally pre-administered with the CTB glycoprotein, amounts of IgE decreased by 31.08 ng/ml at CTB glycoprotein (10 mg/kg, BW) in the presence of BPA, compared to the BPA treatment alone.

3.2. Inhibitory effect of CTB glycoprotein on releasing of β-hexosaminidase and histamine in BPA-stimulated ICR mice

β-Hexosaminidase and histamine release in the mouse serum were increased after treatment with BPA alone compared to the

control. However, in the mice that were pre-administered with the CTB glycoprotein in the presence of BPA, β -hexosaminidase and histamine release decreased significantly after treatment with BPA in the presence of CTB glycoprotein (5–10 mg/kg, BW) in a dose-dependent manner. For instance, β -hexosaminidase and histamine releases decreased 0.29- and 0.18-fold after the treatment with BPA in the presence of CTB glycoprotein (10 mg/kg, BW), as shown in Fig. 2A and B.

3.3. Cytotoxicity of CTB glycoprotein

To assess cytotoxicity, the RBL-2H3 cells were incubated with BPA alone or with BPA in the presence of the CTB glycoprotein (Fig. 3). When the cells were treated with BPA, the number of cells was unchanged. However, the number of cells in the group pre-treated with CTB glycoprotein for 24 h was slightly reduced relative to the controls. Interestingly, these reductions in cell number did not differ significantly between treatment groups in the different concentrations of the CTB glycoprotein. For instance, the viability of the RBL-2H3 cells was 92%, 95%, and 92% at 25, 50, and 100 μ g/ml of the CTB glycoprotein, respectively. This result means that the CTB glycoprotein (up to 100 μ g/ml) has no cytotoxicity.

3.4. Inhibitory effects of the CTB glycoprotein on ERK1/2 and p38 MAPK phosphorylation in BPA-stimulated RBL-2H3 cells

As shown in Fig. 4, we also assessed the inhibitory effects of the CTB glycoprotein on ERK1/2 and p38 MAPK phosphorylation in BPA-stimulated RBL-2H3 cells (Fig. 4). ERK1/2 phosphorylation increased significantly (2.90-fold) 30 min after BPA treatment (50 μ M), as compared to the control (Fig. 4A, lane 2). However, in the cells pre-treated for 30 min with the CTB glycoprotein (100 μ g/ml), BPA treatment precipitated a significant reduction in the expression of phospho-ERK1/2, by 0.59-fold relative to that of the cells treated only with BPA; there was no change in the total expression of ERK 1/2. In the p38 MAPK assay, the expression of p38 MAPK was significantly elevated in the cells treated with BPA alone (50 μ M), by 3.45-fold relative to the controls (Fig. 4B, lane 2). In the cells exposed to BPA after 30 min of pre-treatment with the CTB glycoprotein (100 μ g/ml), p38 MAPK expression was significantly reduced, by 0.46-fold relative to the cells treated solely with BPA; there was no change in the total expression of p38 MAPK.

3.5. Inhibitory activity of the CTB glycoprotein on AP-1 activation in BPA-stimulated RBL-2H3 cells

As shown in Fig. 5, AP-1 activity was markedly increased in the cells treated solely with 50 μ M BPA. However, when the cells were treated with 50 μ M BPA after pretreatment with the CTB glycoprotein (50–100 μ g/ml), AP-1 (c-Jun and c-Fos) activity decreased significantly in a concentration-dependent fashion. For instance, the relative intensities of c-Jun and c-Fos were augmented significantly, by 1.74- and 7.26- fold at 50 μ M BPA, respectively, relative to the controls. However, in the cells treated with BPA after first receiving pre-treatment with the CTB glycoprotein (100 μ g/ml), this augmentation of c-Jun and c-Fos activity was significantly suppressed, by 0.67 and 0.55-fold, respectively, relative to the BPA treatment-alone group (Fig. 5).

3.6. Inhibitory activity of the CTB glycoprotein on expressions of TNF- α , IL-6, and IL-1 β in BPA-treated RBL-2H3 cells

As shown in Fig. 6, the expressions of TNF- α and IL-6 were markedly up-regulated after 6 h of BPA treatment, relative to the controls. Interestingly, IL-1 β expression levels were lower in the

cells treated solely with BPA than in the controls, whereas TNF- α and IL-6 expression levels were higher than in the controls. However, TNF- α and IL-6 expression were significantly inhibited by BPA treatment when administered to cells pre-treated with the CTB glycoprotein (100 μ g/ml), relative to the cells treated with BPA alone. In the CTB glycoprotein-pretreated cells, TNF- α expression was reduced by 0.40-fold, IL-6 expression by 0.66-fold, and IL-1 β expression was reduced by 0.02-fold as the result of BPA treatment.

3.7. Inhibitory effect of CTB glycoprotein on NO production and COX-2 expression in BPA-stimulated RBL-2H3 cells

As shown in Fig. 7A, after the RBL-2H3 cells were treated with BPA (50 μ M) for 2 h, the relative production of NO increased compared to the control. However, when the RBL-2H3 cells were treated with CTB glycoprotein (50 and 100 μ g/ml) in the presence of BPA, it significantly reduced the NO production in a dose-dependent manner, compared to after the BPA treatment alone. For example, the levels of NO production were reduced 0.19- and 0.42-fold, respectively, after treatment with 50 and 100 μ g/ml CTB glycoprotein in the presence of BPA (50 μ M).

We also attempted to ascertain whether or not the CTB glycoprotein modulates the expression of pro-inflammatory protein (COX-2) in BPA-stimulated RBL-2H3 cells (Fig. 7B). As expected, the relative level of COX-2 was increased significantly upon exposure to BPA (50 μ M) for 2 h, whereas treatment with BPA after pre-treatment with the CTB glycoprotein significantly inhibited COX-2 expression in a concentration-dependent manner relative to the BPA treatment-alone group. As shown in Fig. 7, the band intensity of COX-2 was reduced substantially – by 0.55- and 0.67-fold – by additions of CTB glycoprotein at 50 μ g/ml and 100 μ g/ml in the presence of BPA, relative to the cells treated solely with BPA.

4. Discussion

Allergic inflammation is the results of inappropriate immune responses, often to common antigens such as plant pollen, food, or animal dander (O'Mahony et al., 2011). The sources of allergic occurring can be easily found from fast foods and preservatives, and chemicals which are used for production of children's toys and interior house holders (Berin, 2012). BPA, one of the chemicals, has a characteristic of depending on non-genomic pathways in allergy-related diseases. Exposure to BPA can indirectly promote the modulation of immune effects and lead to the development of allergies (Kang et al., 2006; Narita et al., 2007).

The results obtained from this study showed that CTB glycoprotein inhibits the activity of IgE production in BPA-administrated BALB/c mice (Fig. 1). The mast cell-mediated allergy reaction is associated with an increase in production of IgE which binds to receptors on the surface of mast cells. It stimulates the release of various mediators that promote human immediate hypersensitivity disease, mucus secretion, and increased vascular permeability. As shown Fig. 2, CTB glycoprotein inhibited β -hexosaminidase and histamine release in mouse serum.

In the pro-inflammatory mediator expression, treatment of CTB glycoprotein or BPA in RBL-2H3 cells was no significant cytotoxicity (Fig. 3). In the MAPKs, ERK1/2 and p38 MAPK phosphorylation are associated closely with the expression of pro-inflammatory cytokines (TNF- α , IL-6, and IL-1 β) and COX-2 in inflammatory diseases (Scherle et al., 1998; Carter et al., 1999). Our findings showed that the CTB glycoprotein inhibited expression of ERK1/2 and p38 MAPK, and AP-1 in RBL-2H3 cells (Figs. 4 and 5). The transcriptional activation of AP-1 appears to be a requirement for the induction of gene expression of pro-inflammatory mediators such as TNF- α , IL-6, IL-1 β , and COX-2 (Lee et al., 2004). In allergic patients,

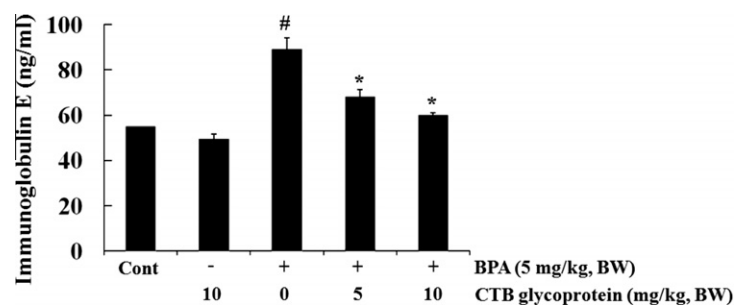


Fig. 1. Inhibitory effect of CTB glycoprotein on amount of IgE production in BPA-treated BALB/c mice. The amount of IgE was determined by sandwich ELISA using monoclonal antibody specific to IgE. All data are the means $\pm$ S.E. from triplicate experiments ($n = 6$), separately. [#] Represents a significant difference between treatments and control, $p < 0.05$. ^{*} Indicates a significant difference between BPA treatment alone and treatment of the CTB glycoprotein in the presence of BPA, $P < 0.05$. BW: body weight.

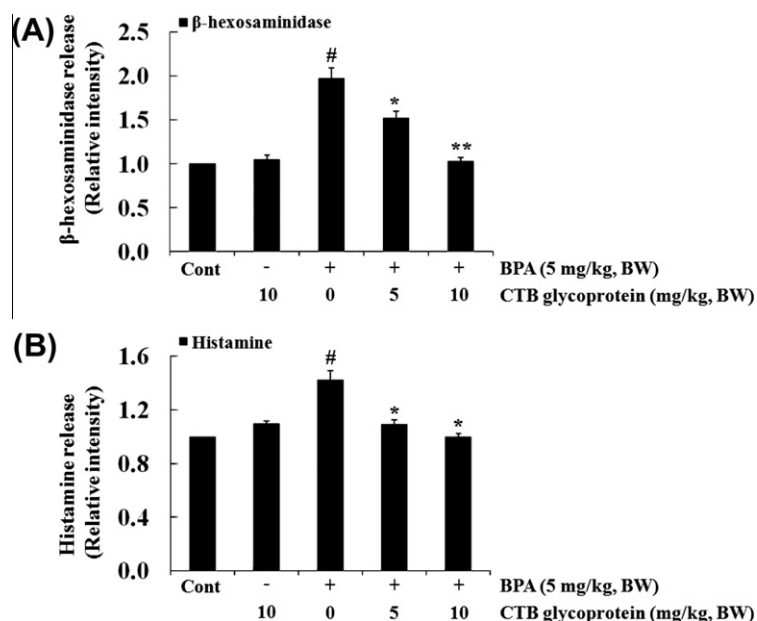


Fig. 2. Inhibitory effect of CTB glycoprotein on β -hexosaminidase and histamine releases in BPA-treated BALB/c mice. Secretion of β -hexosaminidase (A) and histamine release (B) were evaluated using a fluorescent microplate reader. All data are the means $\pm$ S.E. from triplicate experiments ($n = 6$), separately. [#] Represents a significant difference between treatments and control, $p < 0.05$. ^{*} and ^{**} Indicate significant differences between BPA treatment alone and the CTB glycoprotein treatment in the presence of BPA, $P < 0.05$ and $P < 0.01$, respectively. BW: body weight.

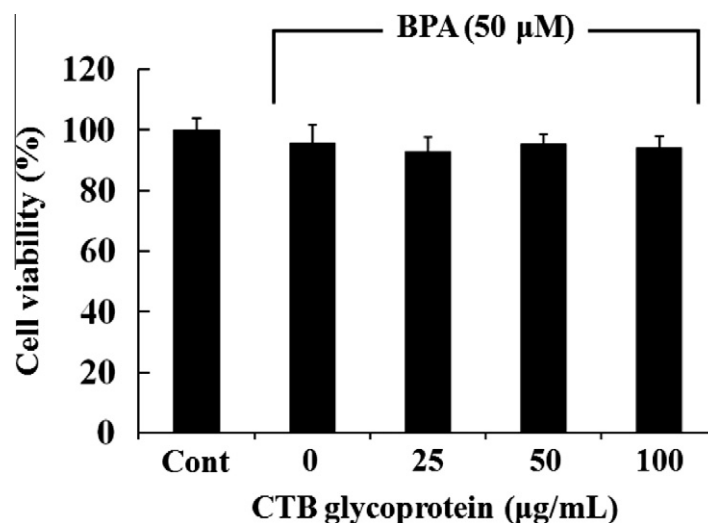


Fig. 3. Cytotoxicity of CTB glycoprotein in RBL-2H3 cells. The cells were treated with BPA alone and BPA in the presence of CTB glycoprotein (25–100 μ g/ml) for 24 h. Viability was assessed by MTT assay. The data are expressed as the means $\pm$ SE from triplicate experiments.

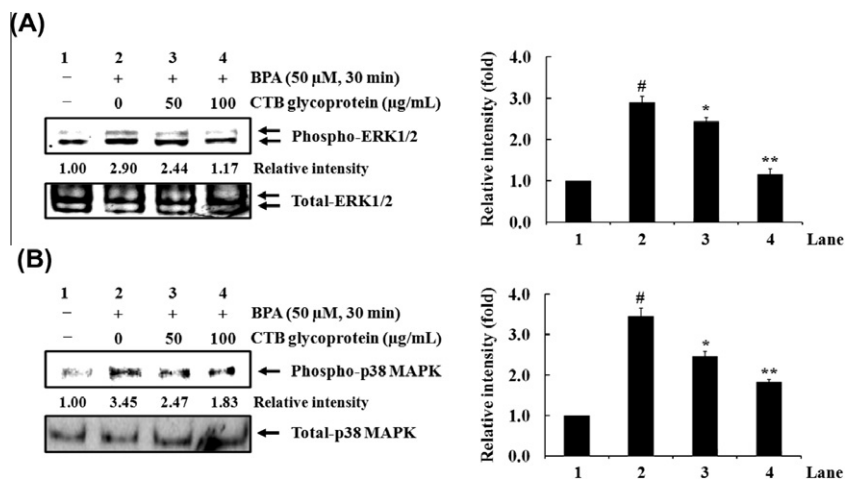


Fig. 4. Inhibitory effect of CTB glycoprotein on ERK1/2 and p38 MAPK activity in BPA-stimulated RBL-2H3 cells. To optimize the experimental conditions (time and concentration), RBL-2H3 cells were treated with BPA (50 μ M) and assessed with regard to ERK1/2 and p38 MAPK expression. The cells were exposed to BPA (50 μ M) for 30 min in the absence or presence of the CTB glycoprotein (50–100 μ g/ml). ERK1/2 and p38 MAPK were detected via Western blotting using anti-p38 MAPK, -ERK1/2, -phospho-p38 MAPK, and -phospho-ERK1/2 polyclonal antibodies. The relative intensities of either ERK1/2 (A) or p38 MAPK bands (B) were quantified using Scion Imaging Software (Scion Image Beta 4.02). # Represents a significant difference between BPA treatments and control, $p < 0.05$. * indicates a significant difference between BPA treatment and CTB glycoprotein pre-treatment, $P < 0.05$. ** indicates a significant difference between BPA treatment and CTB glycoprotein pre-treatment, $P < 0.01$.

increase of AP-1 expression is one of causes to produce the bronchial mucosa and nasal mucosa because it closely links to cytokines (TNF- α and IL-6) (Pawankar, 2005). Also, various studies have been reported that expression of AP-1 which can be activated by MAPKs (ERK, p38), because they are to be existing signals in the upstream pathway (Hsu et al., 2001; Palanki, 2002).

The results observed in these experiments revealed that the CTB glycoprotein significantly attenuates the expressions of TNF- α , IL-6, and COX-2 via an inhibitory effect on MAPK and AP-1—which are in the downstream signal pathway—but not on IL-1 β (Figs. 6 and 7). The activation of mast cells and their mediators, including cytokines, appears to contribute to the development of inflammation at inflammatory sites (Moller et al., 1998). In the pro-inflammatory cytokines, TNF- α , IL-6, and IL-1 β are known to play pivotal roles in a variety of cytokine-dependent inflammatory events. In the allergic response, they are secreted from mast cells and macrophages via an IgE-dependent signaling cascade. It has also been previously reported that TNF- α stimulates the survival of eosinophils, chemotaxis of neutrophils, and the expression of

adhesion molecules from endothelial cells, thereby contributing to chronic inflammation (Thomas, 2001).

Nitric oxide (NO) is an important messenger molecule that plays a role in the regulation of pro-inflammation properties in the early stages of inflammatory cell transmigration into sites of inflammation (Brown et al., 2011). COX-2 is upregulated in response to inflammation-associated tissue damage. COX-2 is also responsible for the pathogenesis of eosinophilic airway inflammation (Bochenek et al., 2004). In the results, the CTB glycoprotein inhibited the expressions of TNF- α , IL-6, IL-1 β , NO, and COX-2 in BPA-stimulated RBL-2H3 cells. Interestingly, IL-1 β expression was reduced compared to the control, while TNF- α and IL-6 expression levels were increased. Even though the reason for this will be required further study, IL-1 β expression can be probably established through another signal pathway of TNF- α and IL-6. Some recent studies have shown that protein kinase and transcription factor activations play a critical role in the expressions of pro-inflammatory cytokines including TNF- α , IL-6, and IL-1 β during the process of inflammation (Parronchi et al., 1992). Collectively, the results of

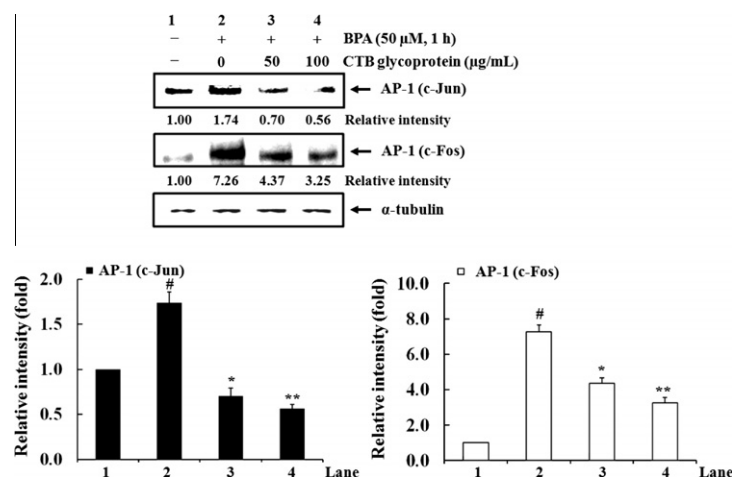


Fig. 5. Inhibitory effect of the CTB glycoprotein on AP-1 expression in BPA-stimulated RBL-2H3 cells. The cells were exposed to BPA (50 μ M) for 1 h in the absence or presence of the CTB glycoprotein (50–100 μ g/ml). The relative intensities of AP-1 (c-Jun) and c-Fos bands were quantified using Scion Imaging Software (Scion Image Beta 4.02). # Represents a significant difference between BPA treatments and control, $p < 0.05$. * indicates a significant difference between BPA treatment and CTB glycoprotein pre-treatment, $P < 0.05$. ** Indicates a significant difference between BPA treatment and CTB glycoprotein pre-treatment, $P < 0.01$.

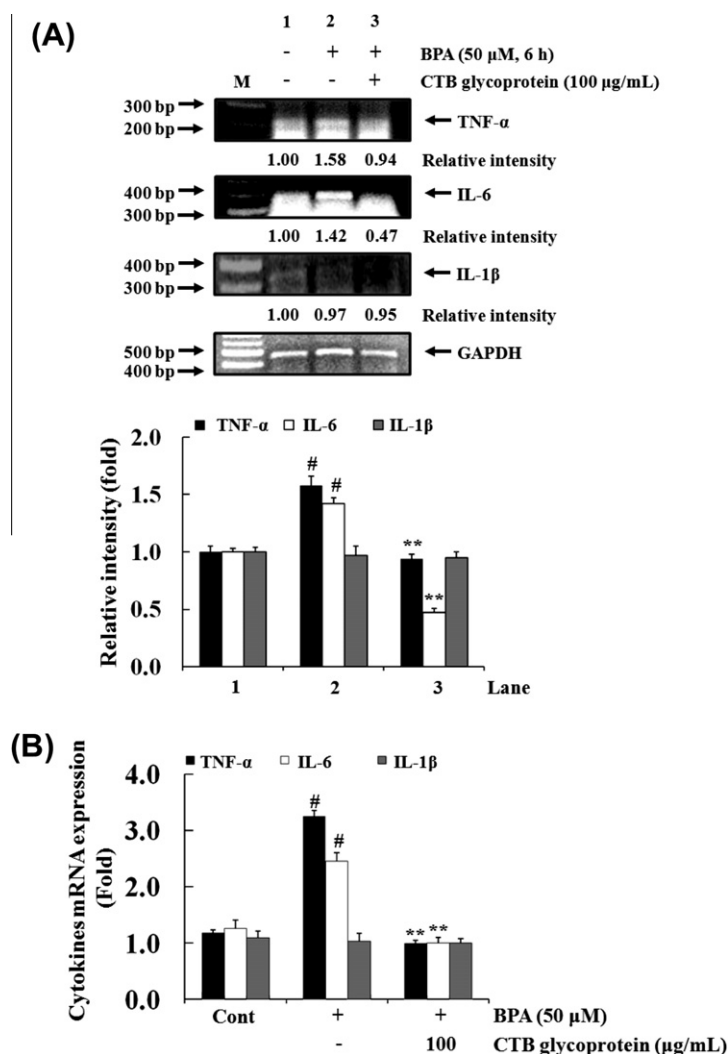


Fig. 6. Inhibitory effect of the CTB glycoprotein on TNF- α , IL-6, and IL-1 β expression in BPA-stimulated RBL-2H3 cells. The cells were exposed to BPA (50 μ M) for 6 h in the absence or presence of the CTB glycoprotein (100 μ g/ml). TNF- α , IL-6, and IL-1 β expression levels were determined via RT-PCR (A) qRT-PCR (B) using total RNA isolated from the BPA-treated RBL-2H3 cells. The relative intensities of TNF- α , IL-6, and IL-1 β were quantified with Scion Imaging Software (Scion Image Beta 4.02). GAPDH was used as an internal control. [#] Represents a significant difference between the BPA treatments and controls, $p < 0.05$. ^{**} Indicates a significant difference between BPA treatment and CTB glycoprotein pre-treatment, $P < 0.01$. M, marker.

this study demonstrate that the CTB-derived glycoprotein inhibited the production of pro-inflammatory mediators (TNF- α , IL-6, and IL-1 β , and COX-2), as well as the activations of MAPK and AP-1 expression, in BPA-stimulated RBL-2H3 cells.

From these stand points what CTB glycoprotein inhibits expression of TNF- α , IL-6, and IL-1 β , and COX-2, as well as the activations of MAPK and AP-1 expression, we can only speculate as to the possible mechanism underlying the inhibitory effect evidenced by the CTB glycoprotein in the presence of BPA. In this study, BPA (50 μ M) appeared to only weakly stimulate the activation of pro-inflammatory mediators, as their expressions were not apparent in the band intensity assessments. In the BPA (estrogen mimic environmental hormone) exertions, BPA binds to both estrogen receptor (ER) α and estrogen receptor (ER) β (Kuiper et al., 1998). Its binding affinity is $\approx 10,000$ -fold less than that of 17 β -estradiol (E2), and thus it should mimic or compete with estrogens only at the μ M range. At nanomolar concentrations, BPA exhibits estrogen-like activities similar to, or stronger than, E2. There may be several reasons for this interesting concentration-based property of BPA. First, differences in binding to serum proteins, transport, and metabolism may have accounted for the low-dose effect of BPA in animal studies (Welshons et al., 2006). Second, BPA is known to bind

differently, and also recruits a different set of co-regulators, within the ligand-binding domain of the ERs (Safe et al., 2002). Third, BPA elicits rapid responses via non-genomic mechanisms by activating membrane-anchored ERs (Watson et al., 2007), unidentified non-classical membrane estrogen receptors (ncmER, or the G-protein-coupled receptor 30 (GPR30) (Alonso-Magdalena et al., 2005; Filardo and Thomas, 2005). Fourth, BPA binds to estrogen related receptors (ERR), among which ERR γ evidences a relatively high binding affinity to BPA (Takayanagi et al., 2006). On the other hand, the CTB glycoprotein may directly deactivate BPA by forming a complex with BPA on the extracellular membrane, or may simply inhibit or disrupt the pro-inflammation-related upstream signals activated by BPA within the intracellular cytoplasm. The CTB glycoprotein itself has a fairly high molecular weight, and thus cannot penetrate the cell membrane. Hence, CTB glycoprotein interacts with BPA on the extracellular membrane or it simply transfers its signals into the cytoplasm disturbs the signals of BPA in the intracellular signal pathway. Subsequently, both signals interfere with one another, ultimately resulting in a blockage of pro-inflammatory signal expression.

In conclusion, the result obtained from this study showed that the CTB glycoprotein doesn't have any cytotoxicity and inhibits

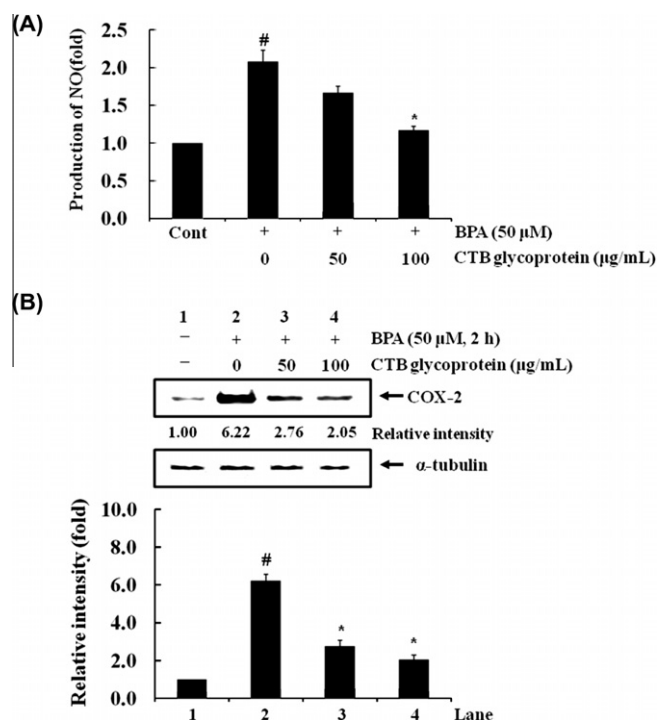


Fig. 7. Inhibitory effect of CTB glycoprotein on NO production and COX-2 expression in BPA-stimulated RBL-2H3 cells. The cells were exposed to BPA (50 μM) for 2 h in the absence or presence of the CTB glycoprotein (50–100 μg/ml). The supernatants were used for measurements of NO production (A). The relative intensity of the COX-2 band observed on Western blotting was calculated using Scion Imaging Software (B) (Scion Image Beta 4.02). [#] Represents a significant difference between BPA treatments and control, $p < 0.05$. ^{*} Indicates a significant difference between BPA treatment and CTB glycoprotein pre-treatment, $P < 0.05$.

the production of IgE and degranulation (releasing of β-hexosaminidase and histamine) at treatment with CTB glycoprotein (10 mg/kg, BW) in mice serum. Also, it showed that the CTB glycoprotein (100 μg/ml) inhibits the phosphorylation of ERK1/2 and p38 MAPK, and the activation of AP-1 (c-Jun and c-Fos). In the inflammation-related factors, their activities of COX-2, TNF-α, and IL-6 were reduced, but not IL-1β. Taken together, the results of this study indicated that the CTB glycoprotein modulates the expression of inflammation-related factors via the suppression of MAPK/AP-1 activation. Thus, we speculate that the CTB glycoprotein might be useful for prevention of BPA-induced allergic inflammation.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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