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Modulatory Effects of Phytoglycoprotein (75 kDa) on Allergic Inflammatory Cytokines in Di(2-Ethylhexyl) Phthalate (DEHP)-Stimulated RBL-2H3 Cells

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ABSTRACT

This study investigated the inhibitory effect of a glycoprotein isolated from *Cudrania tricuspidata* Bureau (CTB glycoprotein) on di(2-ethylhexyl) phthalate (DEHP)-induced mast cell degranulation and related signaling cascade in RBL-2H3 cells. This experiment evaluated the intracellular Ca^{2+} level, and the activities of protein kinase C (PKC), mitogen-activated protein kinase (MAPK), transcription factor, and the cytokines in DEHP-treated RBL-2H3 cells. Our results revealed that the CTB glycoprotein in the presence of DEHP inhibits the release of histamine and expression of interleukin (IL)-4, IL-6, and TNF-α in RBL-2H3 cells. We also found that the CTB glycoprotein inhibits the intracellular Ca^{2+} level, translocation of PKC from cytosol to membrane and the phosphorylation of ERK1/2 in cells. Moreover, the CTB glycoprotein (100 μg/ml) has suppressive effects on transcriptional activation of nuclear factor (NF)-κB in DEHP-treated RBL-2H3 cells. The activation of NF-κB was collectively blocked by treatment with PKC inhibitor (staurosporine) as well as ERK1/2 inhibitor (PD98059), respectively. The results from these experiments indicated that the CTB glycoprotein inhibits release of histamine and expressions of IL-4, IL-6, and TNF-α via down regulations of PKC/MAPK and NF-κB on the stage of mast cell degranulation induced by DEHP. Moreover, oral administration of CTB glycoprotein (10–20 mg/kg) inhibited compound 48/80-mediated systemic reaction in mice. In conclusion, we speculated that the CTB glycoprotein might be one component for preparation of health supplements for prevention of allergic immune disorders. J. Cell. Biochem. 109: 124–131, 2010.

KEY WORDS: *CUDRANIA TRICUSPIDATA* BUREAU (CTB) GLYCOPROTEIN; DI(2-ETHYLHEXYL) PHTHALATE; HISTAMINE; PROINFLAMMATORY CYTOKINES

Recently, epidemiologic studies have shown that the prevalence of allergic disorders has been increased rapidly at a great rate among children and juveniles during the past several decades [Beasley et al., 2003] There are increasing evidences to support that environmental chemicals are contributed to the development of the allergic symptoms, which manifests as atopic dermatitis, allergic rhinitis and bronchial asthma [Bornehag et al., 2004; Jaakkola et al., 2004]. Many environmental chemicals can be absorbed through various routes such as ingestion, inhalation, and skin contact in human and animals. Consequently it can cause the allergic symptoms including itching, wheezing and airway hypersensitive response. Among the many environmental chemicals, di(2-ethylhexyl) phthalate (DEHP) has been widely used in PVC products, including vinyl flooring, wall coverings, food packaging, and children's toys [Schettler, 2006]. Recent study has revealed the positive association between allergic asthma in children and phthalate esters in house dust [Bornehag et al., 2004; Koike et al., 2009]. For example, exposure to DEHP can elevate immunoglobulin production in animals [Larsen et al., 2002, 2007].

Allergy is a hypersensitive reaction that is characterized by immunoglobulin-dependent mast cell activation. Through the antigen cross-linking to high affinity IgE receptors (FcεRI) on the cell surface, mast cells release immediately the preformed and de novo synthesized mediators such as histamine, arachidonic acid intermediates, and cytokines [Gilfillan and Tkaczyk, 2006; Galli et al., 2008]. Among the inflammatory substances released from

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mast cells, histamine is the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity [Petersen et al., 1996; Cook et al., 2002]. Also, proinflammatory cytokines including IL-4, -6, and TNF-α can be produced in the mast cell and potentiate inflammatory immune responses through the subsequent induction of other inflammatory mediators [Gilfillan and Tkaczyk, 2006; Theoharides and Kalogeromitros, 2006]. Activation of mast cells leads to phosphorylation of tyrosine kinase and mobilization of internal Ca^{2+}. This is followed by activation of protein kinase C, mitogen-activated protein kinases (MAPKs), and nuclear factor (NF)-κB, thereby producing various inflammatory mediators. Therefore, the modulation of degranulation-related mediators is considered to be a rational approach for regulating allergic responses.

Many researchers have investigated a possible therapeutic application of traditional medicines or health foods in allergic disease, because compounds of natural products do not have cytotoxicity and side effects onto body tissues [Li et al., 2000]. One of the Korean herbal plants, *Cudrania tricuspidata* Bureau (CTB) has been traditionally used for treating tumor, gastritis, liver damage, and inflammation in Korea [Park et al., 2006]. Recently, we have purified a glycoprotein from CTB (CTB glycoprotein) and found that it consisted of proteins (27.5%) and carbohydrates (72.5%). Our previous report indicated that the CTB glycoprotein has a strong antioxidative activity against radicals in a cell-free system, and suppressive effect on the Th2 response via the modulation of cytokine and related signal cascade in mouse lymphocytes [Joo and Lim, 2008; Oh and Lim, 2009]. However, there has been no study on the inhibitory ability of CTB glycoprotein on DEHP-stimulated mast cell biology.

It has been reported that RBL-2H3 cell line is an analog of rat mucosal mast cells [Seldin et al., 1985] and involves in the secretion of histamine and cytokines [Choi et al., 2004], because RBL-2H3 cells have similar functions as primary mast cells and normal basophils which can mediate allergic type I reaction [Barsumian et al., 1981; Teshima et al., 1994]. In this study, we investigated whether the CTB glycoprotein can inhibit the degranulation-related mediators induced by DEHP in RBL-2H3 cells. To confirm the effect of the CTB glycoprotein, we determined the possible action of the CTB glycoprotein on the activities of Ca^{2+}/PKC and MAPK.

MATERIALS AND METHODS

CHEMICALS

DEHP, penicillin G, and streptomycin were obtained from Sigma (St. Louis, MO). DMEM and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). Other chemicals and reagents were of the highest analytical grade.

PREPARATION OF CTB GLYCOPROTEIN

The CTB glycoprotein was isolated and purified from *C. tricuspidata* Bureau (CTB) fruit, as described previously [Joo and Lim, 2008]. Briefly, fruits of CTB were obtained in October 2007 from the Naju traditional market in the Chonnam province, South Korea. They were identified by Dr. H.T. Lim (Chonnam National University). CTB (1 kg) was chopped into small portions and soaked in distilled water (20 L, w/v) for several months in a dark basement. The water extract was passed through Whatman filter paper (No. 2) to remove debris and concentrated using a rotary evaporator (B465; Buchi, Flawil, Switzerland). The concentrated solution was freeze-dried (SFDS06; Samwon, Seoul, Korea). The dried powder (8 g) was dissolved again in distilled water. The solution was 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 with a dialysis membrane (Spectra/por, MWCO 8,000–12,000, CAL., USA) against 20 mM Tris–HCl (pH 7.4) at 4°C overnight and lyophilized. The eluted solution was used for further experimentation. To confirm, we performed 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 by staining with Schiff's reagent [Neville and Glossmann, 1974], which is a specific staining reagent for the glycoprotein detected through a redox reaction. The purity of the CTB glycoprotein is approximately more than 98%. After verification of the high purity of the glycoprotein, we treated cells with the glycoprotein for further studies.

ANIMALS AND CELL CULTURE

Female BALB/c mice (25 g), aged 6 weeks, were purchased from Daehan Lab (Animal Research Center Co., Ltd, DaeJeon, Co. Ltd., Daejeon, South Korea) and housed according to the "Guiding principles in the use of animals in toxicology," adopted by the Society of Toxicology in 1989 at the experimental animal room of the Veterinary College of Chonnam National University. Under optimal condition to minimize pain or discomfort, all mice were fed a commercial diet and water ad libitum, and kept at least 1 week prior to the injection of Compound 48/80. RBL-2H3 cells were provided by Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and atmosphere containing 5% CO_2. The medium was renewed twice a week. The cells (2.3×10^6 cells/ml) were dispensed into 96-well flat bottom plates or 6-well plates. Cell viability as a measure of the cytotoxicity of the CTB glycoprotein was determined by MTT method, as reported previously [Mosmann, 1983]. Briefly, the cells were treated with 100 μM DEHP, or cotreated with 100 μM DEHP and the CTB glycoprotein (25–100 μg/ml) for 24 h. After incubation, an MTT stock solution (5 mg/ml) was added into each well, and the plates were incubated at 37°C in 5% CO_2 atmosphere for 4 h. Acidic isopropanol was then added into 96-well multi plates, and the plates were read at 560 nm using a SpectraCount™ (Packard Instrument Co Downers, IL).

β-HEXOSAMINIDASE SECRETION ASSAY

Cells were treated with DEHP (100 μM) and cotreated with the CTB glycoprotein (25–200 μg/ml) for 15 min. At the end of incubation, aliquots (20 μl) of medium 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 μl of stopping buffer (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm [Matsubara et al., 2004].

HISTAMINE RELEASE ASSAY

Cells were treated with DEHP (100 μM) and cotreated with the CTB glycoprotein (25–200 μg/ml) for 15 min. Histamine content was measured by the o-phthaldialdehyde spectrofluorometric procedure [Shore et al., 1959]. The fluorescence intensity was measured using a spectrofluorometer (Molecular Devices) at an emission of 440 nm and an excitation of 360 nm. The data are expressed as relative fluorescence intensity.

MEASUREMENT OF INTRACELLULAR CA^{2+} CONCENTRATION

Changes in intracellular Ca^{2+} concentration were monitored with Fura-2/AM, as described previously [Kim and Shin, 2005]. RBL-2H3 cells in 35-mm culture dishes were rinsed twice with buffer A [140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, 5.5 mM HEPES (pH 7.4)]. They were then incubated in buffer A containing 0.5 μM Fura-2/AM with 5% CO_2 at 37°C for 45 min, rinsed twice with buffer A. After washing the dye from the cell surface, cells were pretreated with the CTB glycoprotein for 10 min before DEHP treatment. The fluorescent intensity was recorded using a fluorescence plate reader (Molecular Devices) at an emission of 500 nm and excitation of 340 and 380 nm. The fluorescence ratio (F_{340}/F_{380}) was calculated as an indicator of Ca^{2+}. The data are expressed as relative fluorescence intensity.

PREPARATION OF CELL EXTRACTS

Protein extracts were prepared as previously described [Oh and Lim, 2008]. The cells were treated with DEHP (100 μM), or cotreated with 100 μM DEHP and the CTB glycoprotein (50–100 μg/ml) for 2 h. The membrane and cytosolic extracts were prepared according to the method of Patton et al. [1989]. Briefly, RBL-2H3 cells were rinsed with PBS after removing the medium and lysed in 300 μl of buffer A (20 mM Tris–HCl pH 7.5, 0.25 M sucrose, 2 mM EDTA, and 2 mM EGTA) containing a protease inhibitor cocktail (Boehringer, Mannheim). The cells were briefly sonicated and centrifuged at 100,000*g* for 1 h to sediment all membranes and the insoluble cytoskeletal components. The supernatant was designated as a cytosolic fraction. The membrane proteins in the pellet were extracted with buffer B (20 mM Tris–HCl pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM EGTA, and 1 mM EDTA) containing a protease inhibitor cocktail (Boehringer, Mannheim) on ice for 30 min and centrifuged at 100,000*g* for 15 min at 4°C. The supernatant was saved as a detergent-soluble membrane fraction. On the other hands, either nucleic protein extract for immunoblotting of NF-κB or whole cellular protein extract for immunoblotting of ERK1/2 were isolated from RBL-2H3 cells as described previously [Oh and Lim, 2008]. The amount of protein was measured by the method of Lowry et al. [1951], and the cellular proteins were stored at −70°C prior to use.

IMMUNOBLOT ANALYSIS

Intracellular protein extracts were analyzed by 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 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% BSA. The membranes were subsequently incubated for 2 h at room temperature with rabbit polyclonal antibodies (1:3,000 dilution: PKC, ERK1/2, phospho-ERK1/2, NF-κB (p50 and p65), and α-tubulin) in TBS-T solution. After three time 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, MA) in TBS-T solution for 1 h at room temperature. The resulting protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK). The results of immunoblot assay were calculated as relative intensity using Scion imaging software (Scion Image Beta 4.02, MD).

RNA ISOLATION AND REVERSE TRANSCRIPT POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated from RBL-2H3 cells using TRIZOL® Reagent in accordance with the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA). Reverse transcription polymerase chain reaction (RT-PCR) was performed using the AccessQuick™ RT-PCR System (Promega). Briefly, the RT-PCR mixture (50 μl) containing 1 μg of total RNA, 10 μM gene-specific sense and antisense primers, 1 μl of AMV Reverse Transcriptase, and 25 μl of AccessQuick™ Master Mix was prepared on ice. The following primer sequences of IL-4, IL-10 and TNF-α were used (sense and antisense respectively): IL-4 (397 bp), 5′-ATG GGT CTC AAC CCC CAG CTA GT-3′ and 5′-GCT CTT TAG GCT TTC CAG GAA GTC-3; IL-6 (223 bp), 5′-GTT CTC TGG GAA ATC GTG GA-3′ and 5′-TGT ACT CCA GGT AGC TAT GG-3′; TNF-α (231 bp), 5′-TCT CAT CAG TTC TAT GGC CC-3′ and 5′-GGG AGT AGA CAA GGT ACA AC-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 IL-4, IL-10, IFN-γ and GAPDH (30 s at 94°C, 30 s at 60°C, 30 s at 72°C) and the RT-PCR amplification products were mixed with 2 μl of loading buffer and separated on a 1% agarose gel. The gels were then stained with 5 μg/ml ethidium bromide and photographed.

COMPOUND 48/80-INDUCED SYSTEMIC REACTION

Compound 48/80-induced systemic reaction was examined as previously described [Kim et al., 2005]. BALB/c mice were given an intraperitoneal injection of 0.008 g/kg body weight (BW) of the mast cell degranulator compound 48/80. CTB glycoprotein was dissolved in saline and administered orally at doses ranging from 5 to 10 mg/kg BW 1 h before the injection of compound 48/80 (n = 6/group). The responses in the Compound 48/80 experiments were represented in anaphylaxis and death rate, because it could not detect pain or discomfort which are appropriate indicators for measurement of physiological condition. Such responses (pain or discomfort) were appeared too quickly in short period time after injection of Compound 48/80. Therefore, we did not measure and represent the data of pain and discomfort responses in this study. Mortality was monitored for 1 h after induction of anaphylactic shock.

STATISTICAL ANALYSIS

All experiments were carried out in triplicate, and data were expressed as means ± SD. One-way analysis of variance (ANOVA)

and the Duncan test were carried out to determine significant differences of multiple comparisons (SPSS program, ver 11.0).

RESULTS

EFFECT OF CTB GLYCOPROTEIN ON DEGRANULATION AND HISTAMINE RELEASE IN DEHP-TREATED RBL-2H3 CELLS

As shown in Figure 1, activity of β-hexosaminidase increased 0.37-fold at addition of DEHP as compared with that in the control. However, treatment with the CTB glycoprotein gradually suppressed DEHP-induced increase of β-hexosaminidase activity in a concentration-dependent manner without any cytotoxic effects. That is, the values of β-hexosaminidase activity decreased by 0.62-fold in the case of addition of 100 μg/ml CTB glycoprotein in the presence of DEHP, compared with that for the DEHP treatment alone. In histamine release, treatment with the CTB glycoprotein also considerably decreased in a concentration-dependent manner. When the cells were treated with the 100 μg/ml CTB glycoprotein, the values of histamine release decreased 1.16-fold compared with the DEHP treatment alone.

EFFECT OF CTB GLYCOPROTEIN ON INTRACELLULAR CA^{2+} LEVEL IN DEHP-TREATED RBL-2H3 CELLS

As shown in Figure 2, intracellular Ca^{2+} level increased 0.18-fold at addition of DEHP as compared with that in the control. However, treatment with the CTB glycoprotein gradually suppressed DEHP-induced increase of intracellular Ca^{2+} level in a concentration-dependent manner. That is, the values of intracellular Ca^{2+} concentration decreased 1.44-fold after the addition of 100 μg/ml CTB glycoprotein in the presence of DEHP, compared with that for the DEHP treatment alone.

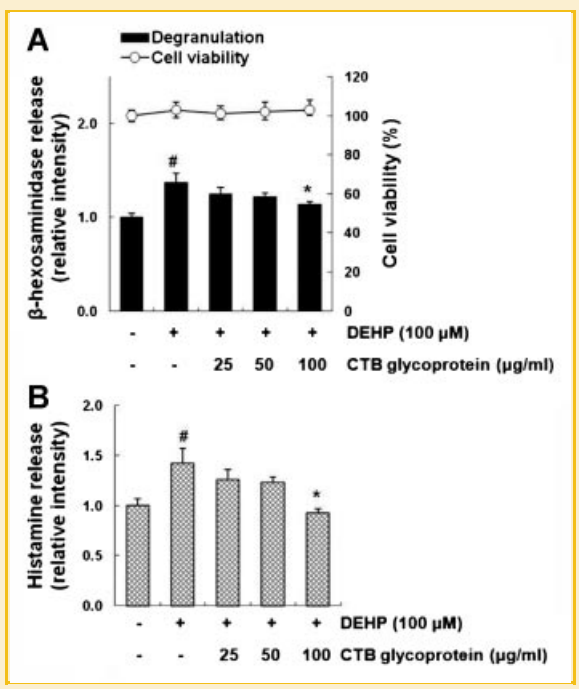

Fig. 1. Effect of the CTB glycoprotein on degranulation and histamine release in RBL-2H3 cells. Cells were treated with DEHP (100 μM) and cotreated with the CTB glycoprotein (25–100 μg/ml) for 15 min. Data are the means ± SE from triplicate experiments. # represents a significant difference between treatments and control, $P < 0.05$. * indicates a significant difference between DEHP treatment and the CTB glycoprotein treatment in the presence of DEHP, $P < 0.05$.

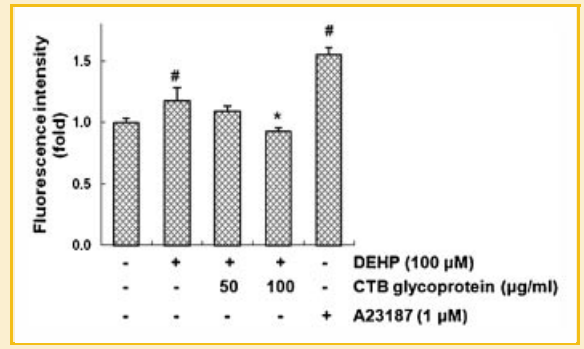

Fig. 2. Inhibitory effect of the CTB glycoprotein in the presence of DEHP on Ca^{2+} level in RBL-2H3 cells. Cells were treated with DEHP (100 μM) and cotreated with the CTB glycoprotein (50–100 μg/ml) for 1 min. Changes in intracellular Ca^{2+} concentration were monitored with Fura-2/AM, as described in Materials and Methods Section. Data are the means ± SE from triplicate experiments. # represents a significant difference between treatments and control, $P < 0.05$. * indicates a significant difference between DEHP treatment and the CTB glycoprotein treatment in the presence of DEHP, $P < 0.05$. A23187 (calcium ionophore) was used as an internal control.

EFFECT OF CTB GLYCOPROTEIN ON PKC TRANSLOCATION IN DEHP-TREATED RBL-2H3 CELLS

As shown in Figure 3, treatment with DEHP induced translocation of PKC from cytosol to membrane at 30 min. After exposure of 100 μM DEHP, the band intensity of PKC decreased 0.76-fold in cytosolic extracts, whereas it increased 1.60-fold in membrane extracts as compared with that in the control. However, treatment with the CTB glycoprotein resulted in a concentration-dependent inhibition of DEHP-induced PKC translocation in RBL-2H3 cells. That is, the band intensities of PKC in membrane fraction effectively decreased 0.15- and 0.4-fold after the addition of 50 and 100 μg/ml CTB glycoprotein, respectively, compared with that for the DEHP treatment alone.

EFFECT OF CTB GLYCOPROTEIN ON ERK1/2 PHOSPHORYLATION IN DEHP-TREATED RBL-2H3 CELLS

As shown in Figure 4A, treatment with DEHP induced phosphorylation of ERK1/2 at 1 h, whereas there is no significant difference in the total expression level of ERK1/2. That is, the band intensity of ERK1/2 significantly increased by 0.36-fold in the presence of DEHP as compared with that in the control. However, treatment with the CTB glycoprotein resulted in a concentration-dependent inhibition of DEHP-induced ERK1/2 phosphorylation in RBL-2H3 cells. That is, the band intensities of phosphorylated ERK1/2 considerably decreased 0.69-fold after the addition of 100 μg/ml CTB glycoprotein, compared with band intensities after DEHP treatment alone. When cells were pretreated with 1 μM staurosporine (PKC inhibitor)

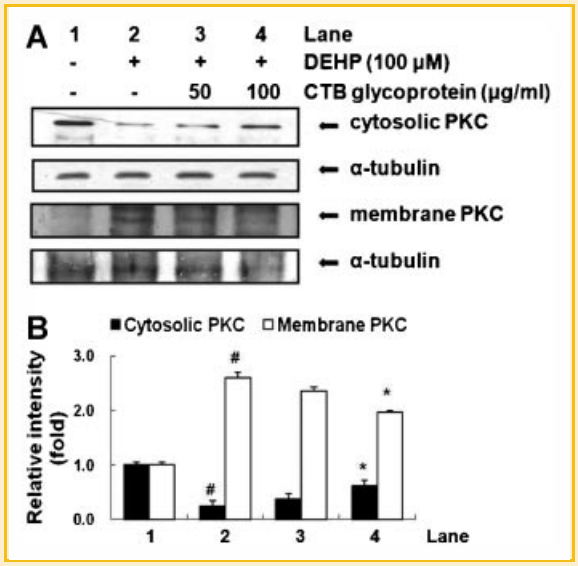

Fig. 3. Inhibitory effect of the CTB glycoprotein in the presence of DEHP on PKC translocation in RBL-2H3 cells. Cells were treated with DEHP (100 μM) or cotreated with the CTB glycoprotein (50–100 μg/ml) and DEHP for 1 h (A). The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02, MD) (B). All data are the means ± SE from triplicate experiments. # represents a significant difference between treatments and control, $P < 0.05$. * indicates a significant difference between DEHP treatment and CTB glycoprotein treatment in the presence of DEHP, $P < 0.05$. Lane 1, control; lane 2, DEHP alone; lane 3, 50 μg/ml CTB glycoprotein in the presence of DEHP; lane 4, 100 μg/ml CTB glycoprotein in the presence of DEHP. α-tubulin was used as an internal control.

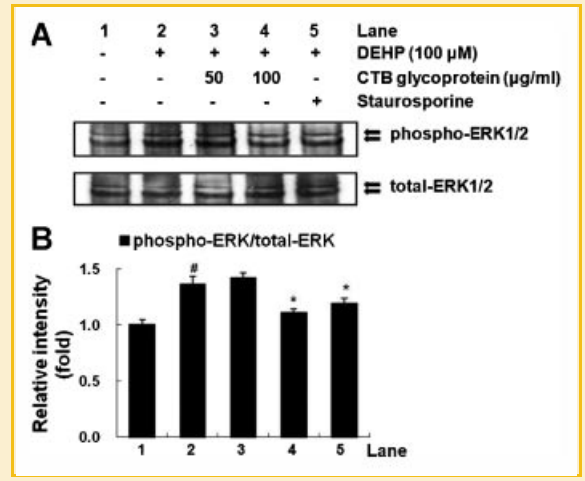

Fig. 4. Inhibitory effect of the CTB glycoprotein in the presence of DEHP on phosphorylation of ERK1/2 in RBL-2H3 cells. Cells were treated with DEHP (100 μM) or cotreated with the CTB glycoprotein (50–100 μg/ml) and DEHP for 1 h (A). The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02, MD) (B). All data are the means ± SE from triplicate experiments. # represents a significant difference between treatments and control, $P < 0.05$. * indicates a significant difference between DEHP treatment and CTB glycoprotein treatment in the presence of DEHP, $P < 0.05$. Lane 1, control; lane 2, DEHP alone; lane 3, 50 μg/ml CTB glycoprotein in the presence of DEHP; lane 4, 100 μg/ml CTB glycoprotein in the presence of DEHP; lane 5, staurosporine in the presence of DEHP.

prior to DEHP treatment, the band intensity of phospho-ERK1/2 decreased by 0.47-fold as compared to band intensity after DEHP treatment.

EFFECT OF CTB GLYCOPROTEIN IN THE PRESENCE OF DEHP ON TRANSCRIPTION FACTOR IN DEHP-TREATED RBL-2H3 CELLS

As shown in Figure 5A, treatment with DEHP increased the expression level of NF-κB after 2 h. That is, the relative intensities of NF-κB (p50) and NF-κB (p65) increased 0.3- and 0.63-fold in the presence of DEHP, respectively, compared with that in the control. However, treatment with the CTB glycoprotein in the presence of DEHP inhibited the expression of NF-κB (p50) and NF-κB (p65) in a concentration-dependent manner, compared with the level of expression after DEHP treatment. As shown in Figure 5B, the band intensities of NF-κB (p50) and NF-κB (p65) decreased considerably; 1.36- and 0.87-fold after the addition of 100 μg/ml CTB glycoprotein in the presence of DEHP, respectively, compared with the band intensities after DEHP treatment alone. Moreover, the band intensities of NF-κB (p50) decreased by 1.46- and 1.6-fold at pretreatment with staurosporine and PD98059 (ERK1/2 inhibitor) prior to DEHP treatment in RBL-2H3 cells, compared with that in DEHP treatment alone. In the case of NF-κB (p65), the values of band intensities also decreased by 0.74- and 0.73-fold at pretreatment with staurosporine and PD98059 prior to DEHP treatment in RBL-2H3 cells, compared with the band intensities after DEHP treatment alone.

EFFECT OF CTB GLYCOPROTEIN ON GENE EXPRESSION OF CYTOKINES IN DEHP-STIMULATED RBL-2H3 CELLS

As shown in Figure 6, treatment with DEHP increased expression levels of IL-4, IL-6, and TNF-α at 4 h as compared to the control. However, treatment with the CTB glycoprotein in the presence of DEHP inhibited the expression level of IL-4, IL-6, and TNF-α as compared to the DEHP treatment alone. GAPDH was used as an internal control.

EFFECT OF CTB GLYCOPROTEIN ON COMPOUND 48/80-INDUCED SYSTEMIC REACTION

After the intraperitoneal injection of compound 48/80 (0.008 g/kg), the mice were monitored for 1 h, and then the mortality rate was determined. As shown in Table I, injection of compound 48/80 into mice induced fatal shock in 100% of animals. However, when the CTB glycoprotein was administered orally at concentrations ranging from 5 to 20 mg/kg BW for 1 h, the mortality with compound 48/80 was dose dependently reduced.

DISCUSSION

It has been commonly recognized that traditional medicines isolated from natural products have positive effects on the prevention and healing of various immune disorders. Recently, the biological activities of various phytoglycoproteins were evaluated in the biochemical areas such as tumorigenesis and immune system [Ooi and Liu, 2000; Oh and Lim, 2008], because they have hygroscopic

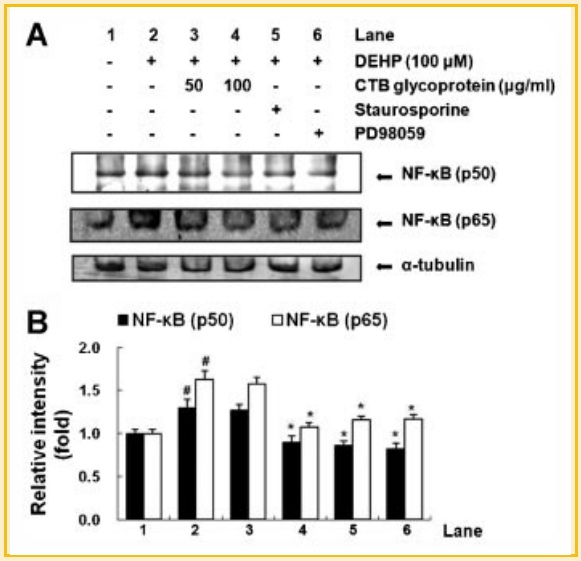

Fig. 5. Inhibitory effect of the CTB glycoprotein in the presence of DEHP on NF-κB activity in RBL-2H3 cells. Cells were treated with DEHP (100 μM) or cotreated with the CTB glycoprotein (50–100 μg/ml) and inhibitors (staurosporine and PD98059) for 2 h (A). The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02, MD) (B). All data are the means ± SE from triplicate experiments. # represents a significant difference between treatments and control, $P < 0.05$. * indicates a significant difference between DEHP treatment alone and treatment with the CTB glycoprotein in the presence of DEHP, $P < 0.05$. Lane 1, control; lane 2, DEHP alone; lane 3, 50 μg/ml CTB glycoprotein in the presence of DEHP; lane 4, 100 μg/ml CTB glycoprotein in the presence of DEHP; lane 5, staurosporine (PKC inhibitor) in the presence of DEHP; line 6, PD98059 (ERK1/2 inhibitor) in the presence of DEHP. α-tubulin was used as an internal control.

characters and high polarity. The present study demonstrated that the CTB glycoprotein inhibits the DEHP-induced degranulation of RBL-2H3 cells by decreasing the expression of cytokines and release of histamine via modulation of Ca^{2+}/PKC and ERK1/2 activity in cells.

DEHP displays an allergic inflammatory activity in cultured neutrophils of both human and animals [Gourlay et al., 2003; Lee et al., 2004]. Also, it is able to increase serum IgG1 and lung inflammatory cell levels at high concentration in animal experiment [Larsen et al., 2007]. These observations point out that DEHP has potential to interact with the immune system, thereby enhancing the development of allergic inflammatory disorders. Our results showed that the CTB glycoprotein inhibits the expressions of IL-4, IL-10, and TNF-α, and production of histamine in DEHP-treated RBL-2H3 cells. In addition, their inhibitory effects are related to the modulation of intracellular Ca^{2+} level, PKC translocation, ERK1/2 phosphorylation, and NF-κB transcriptional activation. These results suggest that the CTB glycoprotein may attenuate allergic reaction via inhibition of degranulation caused by DEHP in RBL-2H3 cells.

To investigate the inhibitory effect of the CTB glycoprotein on the degranulation of mast cells induced by DEHP, we initially monitored following release of two different mediators, β-hexosaminidase and histamine in RBL-2H3 cells. Among the various inflammatory mediators, β-hexosaminidase and histamine are released by exocytosis when mast cells are immunologically activated [Petersen

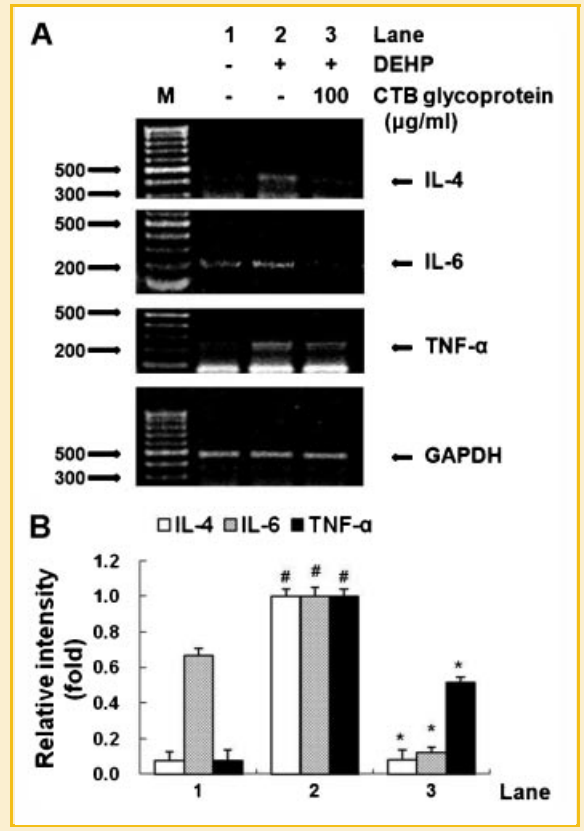

Fig. 6. Inhibitory effect of the CTB glycoprotein in the presence of DEHP on expressions of cytokine in RBL-2H3 cells. Cells were treated with DEHP (100 μM) or cotreated with the CTB glycoprotein (50–100 μg/ml) and DEHP for 4 h. The expressions of IL-4, IL-6 and IL-10 mRNAs were determined by RT-PCR using total RNA isolated from DEHP-treated RBL-2H3 cells. The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02, MD). All data are the means ± SE from triplicate experiments. # represents a significant difference between treatments and control, $P < 0.05$. * indicates a significant difference between DEHP treatment alone and treatment with the CTB glycoprotein in the presence of DEHP, $P < 0.05$. Lane 1: molecular weight maker; lane 2, control; lane 3, DEHP alone; lane 4, 100 μg/ml CTB glycoprotein in the presence of DEHP. GAPDH was used as an internal control.

et al., 1996; Gilfillan and Tkaczyk, 2006]. Thus inhibitions of β-hexosaminidase and histamine releases from mast cells are closely related to the reliable parameter to predict possible antiallergic activity of natural compounds. Our results showed that treatment with DEHP stimulates the degranulation of mast cells through releases of β-hexosaminidase and histamine. However, the releases of β-hexosaminidase and histamine were significantly inhibited by treatment with the CTB glycoprotein in DEHP-treated RBL-2H3 cells, indicating its antiallergic potential.

We next investigated whether the CTB glycoprotein inhibits the proinflammatory mediators including cytokines in RBL-2H3 cells. Among proinflammatory cytokines, IL-4, IL-6, and TNF-α are thought to play critical roles in allergic inflammation [Theoharides and Kalogeromitros, 2006]. In particular, IL-4 induces endothelial cells to produce eosinophil chemotactic factor and eotaxin [Rothenberg et al., 1995], and stimulates expression of adhesion molecules such as vascular cell adhesion molecule-1 on endothelial

TABLE I. Effect of the CTB Glycoprotein on Compound 48/80-Induced Systemic Reaction in Mice

CTB glycoprotein treatment (mg/kg BW)	Compound 48/80 (8 mg/kg BW)	Mortality (%)
None (saline)	−	0
None (saline)	+	100
5	+	83.3
10	+	44.4
20	+	11.1

Groups of mice (n = 6/group) were orally administered with 100 μl saline or CTB glycoprotein. CTB glycoprotein was given at various doses 1 h before the compound 48/80 injection. The compound 48/80 solution was given intraperitoneally to the group of mice. Mortality (%) within 1 h following compound 48/80 injection was represented as the number of dead mice × 100/total number of experimental mice in triplicate experiments.

cellular surface [Schleimer et al., 1992]. Moreover, IL-4 has an important role in the regulation of Th2 cells and B cells through stimulation of the switch of the antibody isotype to IgE [Herrick et al., 2000; Murphy and Reiner, 2002]. Like IL-4, IL-6, and TNF-α are also potent inflammatory mediators that induce response of delay type hypersensitivity (DTH). TNF-α is involved in cutaneous neutrophil recruitment, gastric inflammation, and bacterial immunity [Malaviya et al., 1996; Furuta et al., 1997]. Our results showed that the CTB glycoprotein significantly inhibited the DEHP-stimulated expressions of IL-4, IL-6, and TNF-α in a dose-dependent manner. This result suggests that the antiallergic effect of the CTB glycoprotein is a result of its reduction of IL-4, IL-6, and TNF-α expression in RBL-2H3 cells.

We also examined that the CTB has an inhibitory effect on the signal transduction of degranulation in RBL-2H3 cells. Recent studies have demonstrated that increase of intracellular Ca^{2+} triggers a concerted series of early signaling events such as histamine release and expressions of proinflammatory cytokines including IL-4, -6, and TNF-α in mast cell activation [Beaven et al., 1984]. In addition, the expression of proinflammatory mediators is regulated by the activation of PKC, MAPKs, and NF-κB, and these events can lead to pathogenesis of allergic inflammation in asthma and other allergic disorders [Marquardt and Walker, 2000; Abdel-Raheem et al., 2005]. Our results showed that treatment with DEHP stimulates the elevation of intracellular Ca^{2+} level, the translocation of PKC from cytosol to membrane, and the phosphorylation of ERK1/2 in RBL-2H3 cells. However, the activities of PKC and ERK1/2, and intracellular Ca^{2+} level were inhibited effectively by treatment with the CTB glycoprotein in DEHP-treated RBL-2H3 cells, suggesting that antiallergic activity of the CTB glycoprotein is related the modulation of PKC and ERK1/2 activation in cells. Indeed, the DEHP-induced phosphorylation of ERK1/2 was inhibited by pretreatment with staurosporine (PKC inhibitor), indicating that the phosphorylation of ERK1/2 is affected by PKC translocation in DEHP-treated RBL-2H3 cells. In addition, transcriptional activation of NF-κB is responsible for mast cell degranulation by inducing the expression of proinflammatory cytokines gene because consensus binding sites for the transcription factor are present in the TNF-α and IL-6 gene [Marquardt and Walker, 2000]. We found that treatment with DEHP induces the activation of NF-κB (p50) and NF-κB (p65), where this activation was effectively inhibited by treatment with the CTB glycoprotein in RBL-2H3 cells. Interestingly, the DEHP-induced transcriptional activation of NF-κB was effectively inhibited by pretreatment with staurosporine and PD98059 (ERK1/2 inhibitor), suggesting that the transcriptional activity of NF-κB is influenced by translocation of PKC and phosphorylation of ERK1/2 in DEHP-treated cells. On the basis of our findings, we postulated that the CTB glycoprotein has an antiallergic potential for the expressions of proinflammatory cytokines via the regulation of PKC/MAPK and NF-κB in DEHP-stimulated RBL-2H3 cells.

We further examined the antiallergic effect of CTB glycoprotein on compound 48/80-induced systemic reaction in mice. Stimulation with compound 48/80, which has been used as a direct and convenient reagent to study the mechanism of anaphylaxis, increases the permeability of the lipid bilayer membrane by causing a perturbation in the membrane, thereby inducing histamine release from mast cells [Ennis et al., 1980; Mousli et al., 1990]. Our results showed that the CTB glycoprotein has antiallergic property by inhibiting compound 48/80-induced systemic allergic reaction. This result indicates that the CTB glycoprotein inhibits the mast cell-mediated immediate-type allergic reaction by having a membrane-stabilizing action in mice.

In conclusion, our results showed that the CTB glycoprotein inhibited the degranulation of mast cells via modulation of histamine release and cytokine expression. In addition, it regulated the mobilization of the intracellular Ca^{2+}, the phosphorylation of PKC/MAPK, and activity of NF-κB. Further study is necessary to elucidate the precise mechanism underlying antiallergic effect of CTB glycoprotein using animal model system.

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