

Inhibitory Effect of Glycoprotein Isolated from *Cudrania tricuspidata* Bureau on Expression of Inflammation-Related Cytokine in Bisphenol A-Treated HMC-1 Cells

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Abstract—*Cudrania tricuspidata* is one of the most omnipresent traditional herbal drugs for anti-inflammation and anti-tumor. The purpose of the present study was to determine whether the CTB glycoprotein regulates the inflammatory reaction stimulated by bisphenol A (BPA) in human mast cells (HMC-1). Thus, we investigated that CTB glycoprotein inhibits the degranulation of histamine, expression of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), as a mitogen activated protein (MAP) kinase, nuclear transcription factors involving nuclear factor (NF)- κ B and Activator protein (AP)-1, cyclooxygenase (COX)-2. The results indicated that CTB glycoprotein decreased gene expression of cytokines of IL-4, IFN- γ , interleukin (IL)-1 β and cyclooxygenase (COX)-2 in BPA-stimulated HMC-1 cells. Hence, we speculate that CTB glycoprotein can use as a potent anti-inflammatory agent for inflammatory allergic diseases.

KEY WORDS: CTB glycoprotein; IL-4; IFN- γ ; IL-1 β ; inflammation; HMC-1 cells; bisphenol A.

INTRODUCTION

Endocrine-disrupting chemicals (EDC) can interfere with natural hormone cycles in humans or animals, potentially affecting metabolism, development, reproduction, and growth. Humans and animals can be exposed to exogenous, anthropogenic EDCs through contaminated surface waters [1]. Concentrations of some EDCs in surface waters are detected in the parts per trillion or parts per billion range, but evidence is mounting that, even at these low levels, EDCs may adversely impact animals, especially humans [2, 3]. Bisphenol A (BPA), one of a endocrine disruptors, is one

of the most important monomer in polycarbonate production. Polycarbonates are used in numerous consumer products, including food and water containers, baby bottles, PVC gloves, linings of metal food and beverage cans, medical tubing, epoxy resins, and dental fillings. BPA has been reported to alter several metabolic functions as an allergen [4, 5].

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 such as histamine, leukotrienes, proteases, prostaglandins. One of the secretory granules, histamine is a key biogenic amine responsible for allergic inflammatory reactions mediated through 4 distinct receptors such as H1, H2, H3 and H4 receptors. Pro-inflammatory cytokines that are released by activated mast cells play an important role in allergic inflammation.

These include vasoactive amines, arachidonic acid metabolites, chemokines, and cytokines [6, 7]. Of these, pro-inflammatory mediators such as cyclooxygenase

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(COX)-2, and cytokines (IFN- γ , IL-4, and IL-1 β) are responsible for activating mast cells in allergic inflammation and the hypersensitive response [8–10]. 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 [11]. 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. MAPKs have three major sub-families, such as extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK) [12, 13]. ERK plays a major role in the proliferation, differentiation and survival of the cells induced by various growth factors [14, 15]. In contrast, JNK and p38 MAPK are activated by environmental stress such as H₂O₂, irradiation, heat shock, hyperosmotic shock and inflammatory cytokines [16], and play a critical role in the inflammatory cytokine expression [17].

AP-1, another redox-sensitive transcription factor, is also a key component in the regulation of pro-inflammatory genes, such as cytokines and COX-2 [18]. Activator protein 1 (AP-1) is involved in cellular proliferation and transformation, and is a possible molecular target of chemotherapy for cancer and inflammatory diseases [19].

It has reported that the expressions of cyclooxygenase (COX)-2 under inflammatory circumstance are elevated through the activation of transcriptional factors in response to pro-inflammatory cytokines and ROS [20]. COX-2, one of the major mediators of the inflammatory reaction, is also strongly induced in activated monocytes/macrophages. Several recent studies demonstrated that PGD₂, which is the COX-2 metabolite released from activated mast cells, is also essential for the pathogenesis of eosinophilic airway inflammation [21].

For the past three decades, many polysaccharide protein complexes (glycoproteins) have been isolated from mushrooms, fungi, yeasts, algae, lichens, and plants [22]. In recent years, we have reported that plant glycoprotein has inhibition effects of inflammation in different cells. Glycoproteins isolated from *Gardenia jasminoides* Ellis (GJE glycoprotein) and *Rhus verniciflua* Stokes (RVS glycoprotein) modulates inflammatory cytokines and inhibits Inflammation-Related Protein [23, 24]. In these concepts, plant-originated glycoproteins

have distinctive different bioactive characters. *Cudrania tricuspidata* Bureau (CTB) is a deciduous trees widely distributed in Korea, China, and Japan. The cortex and root bark of this species have been used as a traditional medicine for the treatment of neuritis and inflammation [25]. Moreover, the roots of *C. tricuspidata* Bur. have been used for the treatment of gonorrhea, rheumatism, jaundice, hepatitis, boils, scabies, bruising, and dysmenorrhea [26].

Therefore, we investigated whether or not CTB glycoprotein has inhibits intracellular ROS, MAPK pathway and transcription factors in bisphenol A (BPA)-stimulated Human mast cell (HMC-1) cells.

MATERIALS AND METHODS

Chemicals

All the plastic materials were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ). Ethylene diamine tetra acetate (EDTA, E5134), penicillin G (H0474), streptomycin (H0447), ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA, E4378), nonidet P-40 (NP-40, N0896), phenyl methane sulfonyl fluoride (PMSF, P7626), obtained from Sigma (St. Louis, MO, USA). Iscove's Modified Dulbecco's Medium (IMDM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Other chemicals and reagents were of the highest analytical grade available.

Preparation of CTB Glycoprotein

CTB glycoprotein was isolated and purified from *Cudrania tricuspidata* Bureau (CTB) fruits, as described previously [27]. Briefly, fruits of *Cudrania tricuspidata* Bureau (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, CA, USA) against 20 mM Tris–Cl (pH 7.4) at 4°C overnight and lyophilized to yield 5.68 g (1.49%). Samples used to confirm characterization of CTB glycoprotein. Also, 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 [28], 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. From electrophoretic analysis, we confirmed that the CTB glycoprotein has 75 kDa molecular weights.

Cell Culture

HMC-1 cells, a human mast cell line, were provided by H.M. Kim (College of Oriental Medicine, Kyung Hee University, Seoul, Korea). HMC-1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS) and 100 IU/mL penicillin/streptomycin (Life Technologies) at 37°C in an atmosphere containing 5% CO₂. HMC-1 cells were passaged every 3–4 days.

Histamine Measurement

Histamine content was measured by fluorometric assay [29]. To determine the amounts of total cellular histamine, HMC-1 cells (1.0×10^6 cells) were suspended and sonicated in Tyrode buffer. Then, the cell lysates were centrifuged at $10,000 \times g$ for 10 min and the supernatant was harvested. After mixing 2 mL of the supernatant with 0.75 g of NaCl and 0.5 mL of 1 N NaOH, 5 mL of the 3:2 (v/v) mixture of *n*-butanol and chloroform was added, and mixed for 5 min. The solution was centrifuged for 5 min at $300 \times g$ and 4 mL of the upper organic solvent layer was recovered. The recovered layer was mixed with 2 mL of *n*-heptane and 1.5 mL of 0.1 N HCl. After centrifugation at $300 \times g$ for 5 min, 1 mL of the lower HCl layer was recovered, mixed with 0.15 mL of 1 N NaOH and the subsequent product was added with 0.1 mL of 0.2% *o*-phthalaldehyde dissolved in methanol for 5 min at room temperature 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 quantitated by comparison to a standard curve generated by coupling histamine dihydrochloride to *o*-phthalaldehyde.

Western Blot

Nuclear protein extract isolated from HMC-1 cells were separated in a 10% polyacrylamide mini-gel at 100 V for 2 h at room temperature using a Mini-PROTEAN II electrophoresis cell (Bio-Rad). After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The transferred 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% (v/v) Tween-20] containing 5% (w/v) non-fat dry milk. The membranes were subsequently incubated for 2 h at room temperature with rabbit polyclonal antibody (Santa Cruz Biotechnology, CA, USA) in TBS-T solution containing 5% non-fat dry milk. 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 Technology, MA, USA) in TBS-T containing 5% non-fat dry milk. The protein bands were visualized by incubation with enhanced chemiluminescence (Amersham Pharmacia Biotech., England, UK).

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from HMC-1 cells using TRIZOL[®] Reagent according to the manufacture's protocol (Invitrogen life Technologies, Carlsbad, CA). Reverse transcription polymerase chain reaction (RT-PCR) was performed using the SuperScript[™] One-Step RT-PCR System (Invitrogen, Carlsbad, CA). Briefly, 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 IFN- γ , IL-4, and IL-1 β were used: IFN- γ (346 bp) sense (5'-GCC AGG GCA CAC TGA TTG AA-3') and antisense (5'-GCT GGT GAA TCA CTC TGA TG-3'), IL-4 (397 bp) sense (5'-ATG GGT CTC AAC CCC CAG CTA GT-3') and

antisense (5'-GCT CTT TAG GCT TTC CAG GAA GTC-3'), IL-1 β (332 bp) sense (5'-TTG ACG GAC CCC AAA AGA TG-3') and anti-sense (5'-AGA AGG TGC TCA TGT CCT CA-3'). The amplifications were conducted with 30 cycles for IL-1 β (30 s at 95°C, 30 s at 60°C, 60 s at 72°C) and the RT-PCR amplification products were mixed with 1 μ L loading buffer and separated on a 1% agarose gel. And then, the gels were stained with 3 μ g/mL ethidium bromide and photographed. 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') were used as an internal control.

Statistical Analysis

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

RESULTS

Effects of CTB Glycoprotein on Histamine Degranulation in BPA Stimulated HMC-1 Cells

Results in the Fig. 1 showed that BPA induces 14.85 μ M of histamine degranulation from mast cells. CTB glycoprotein inhibits histamine degranulation. Upon treatment with the CTB glycoprotein (25, 50,

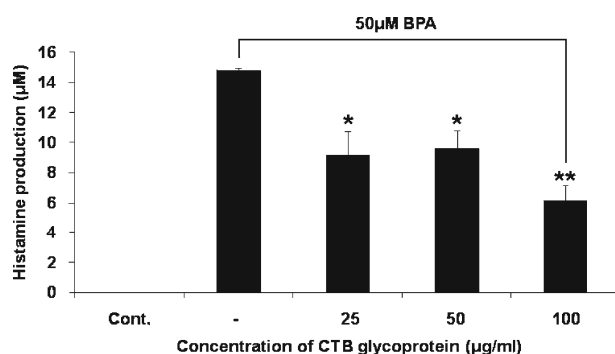


Fig. 1. Effects of CTB glycoprotein on histamine degranulation in BPA stimulated HMC-1 cells. HMC-1 cells were treated with 50 μ M of BPA in the presence of the various concentrations of CTB glycoprotein (25, 50, 100 μ g/mL) for 2 h. Degranulation of histamine was measured at with the excitation set at 360 nm and the emission set at 440 nm. Each bar represents the means $\pm$ SE from triplicate experiments ($n=3$). $^{\#}P<0.05$, compare with control. $*P<0.05$ and $**P<0.01$, compared with treatment of BPA alone.

100 μ g/mL), reduces histamine 5.67, 5.20, 8.69 μ M respectively, compared to BPA treatment alone.

Effect of CTB Glycoprotein on Mitogen-Activated Protein Kinase Inhibition in BPA Stimulated HMC-1 Cells

We examined whether or not CTB glycoprotein modulates the ERK, JNK pathway in BPA stimulated HMC-1 cells. The band intensity of ERK (as a cell proliferation, differentiation and survival marker) and JNK in whole cell protein extracts was augmented by 1.1 at pERK, 0.9 at pJNK in Fig. 2 (lane 2), which treated with BPA alone than in control group (Fig. 2, lane 1). Upon increasing concentration of the CTB glycoprotein (25, 50, 100 μ g/mL), however, its band intensities were gradually attenuated by 1.6, 1.7, and 1.9 at pERK, 1.2, 1.6, and 1.8 at pJNK in BPA-treated groups (Fig. 2, lanes 3, 4 and 5).

Effects of CTB Glycoprotein on Inhibition of AP-1 in BPA Stimulated HMC-1 Cells

Results in western blot showed that whether or not CTB glycoprotein modulates the AP-1 pathway in BPA stimulated HMC-1 cells. The band intensity of c-Jun in AP-1 was augmented by 0.9 in Fig. 3 (lane 2), which treated with BPA alone than in control group (Fig. 3, lane 1). Upon increasing concentration of the CTB glycoprotein (25, 50, 100 μ g/mL), however, its band intensities were gradually attenuated by 1.2, 1.6, and 1.9 in BPA-treated groups (Fig. 3, lanes 3, 4 and 5). On the other hand, NF- κ B and c-fos in AP-1 was not showed the significant difference with control group.

Effects of CTB Glycoprotein on COX-2 Inhibition in BPA Stimulated HMC-1 Cells

We further examined the inhibitory effects of CTB glycoprotein on BPA-induced COX-2 proteins activation using Western blot with whole cell protein extracts. As shown in Fig. 4, CTB glycoprotein was shown to have dose-dependent blocking activities on the activation of pro-inflammatory proteins in BPA stimulated HMC-1 cells. Namely, COX-2 proteins were increased by 0.4 after treatment with BPA (Fig. 4, lane 2). Upon treatment with the CTB glycoprotein (25, 50 μ g/mL), however, the intensities of the COX-2 bands markedly reduced by 0.7 and 1.1, compared to BPA treatment alone (Fig. 4, lanes 3 and 4).

CTB Glycoprotein Inhibits IL-1 β in HMC-1 Cells

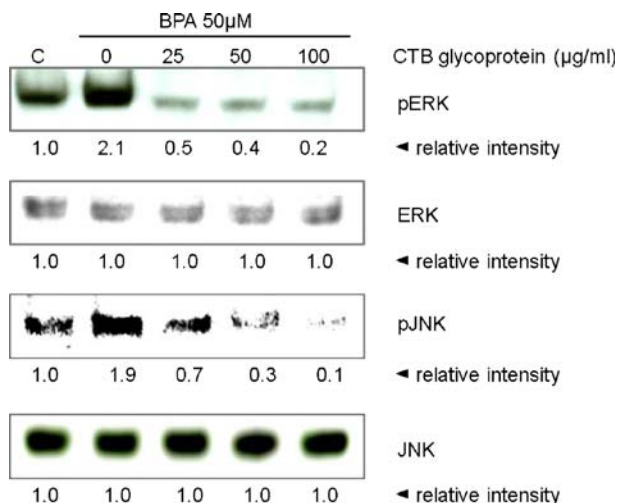


Fig. 2. Effects of CTB glycoprotein on activation of ERK and JNK in BPA stimulated HMC-1 cells. Detection of ERK and JNK, and its phosphor-form was performed by Western blotting using anti-ERK and anti-JNK, and their phosphor-form polyclonal antibodies. The relative intensity of ERK and JNK, and their phosphor-form bands were quantified using Scion Imaging Software (Scion Image Beta 4.02, Maryland, USA), compared to bisphenol A alone. *Lane 1* control, *lane 2* bisphenol A alone, *lane 3* 25 μ g/mL CTB glycoprotein+bisphenol A, *lane 4* 50 μ g/mL CTB glycoprotein+bisphenol A, *lane 5* 100 μ g/mL CTB glycoprotein+bisphenol A. α -Tubulin was used as an internal control.

Effects of CTB Glycoprotein on Inflammatory Cytokine Production in BPA Stimulated HMC-1 Cells

Results in the Fig. 5 showed that expressions of IFN- γ , IL-4, and IL-1 β were generally changed. When HMC-1 cells were treated with BPA, the expression of cytokines increased. However, their expression de-

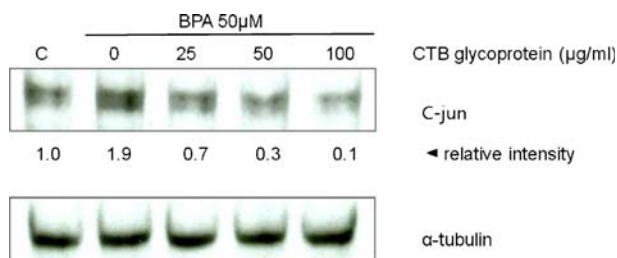


Fig. 3. Effects of CTB glycoprotein on activation of AP-1 in BPA-stimulated HMC-1 cells. Detection of AP-1 proteins was performed by Western blotting using an anti-c-Jun (AP-1) polyclonal antibodies. The relative intensity of AP-1 band was quantified using Scion Imaging Software (Scion Image Beta 4.02, Maryland, USA), compared to bisphenol A alone. *Lane 1* control, *lane 2* bisphenol A alone, *lane 3* 25 μ g/mL CTB glycoprotein+bisphenol A, *lane 4* 50 μ g/mL CTB glycoprotein+bisphenol A, *lane 5* 100 μ g/mL CTB glycoprotein+bisphenol A. α -Tubulin was used as an internal control.

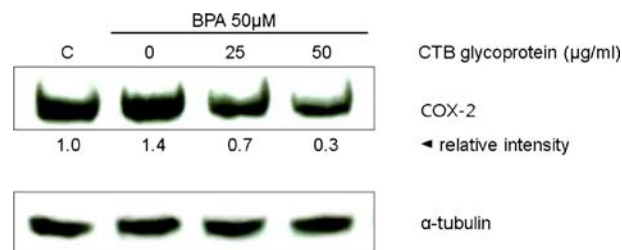


Fig. 4. Effects of CTB glycoprotein on activation of COX-2 in BPA-stimulated HMC-1 cells. Detection of COX-2 proteins was performed by Western blotting using an COX-2 polyclonal antibodies. The relative intensity of COX-2 bands was quantified using Scion Imaging Software (Scion Image Beta 4.02, Maryland, USA), compared to bisphenol A alone. *Lane 1* control, *lane 2* bisphenol A alone, *lane 3* 25 μ g/mL CTB glycoprotein+bisphenol A, *lane 4* 50 μ g/mL CTB glycoprotein+bisphenol A, *lane 5* 100 μ g/mL CTB glycoprotein+bisphenol A. α -Tubulin was used as an internal control.

creased at treatment with CTB glycoprotein (100 μ g/mL) in the presence of BPA (50 μ M). For instance, the relative intensities were augmented by 0.2, 0.1, and 0.1 for IFN- γ , IL-4, and IL-1 β at treated with BPA alone compare to the control while their relative intensities were reduced by 1.0, 1.0, and 0.1 at treatment with CTB glycoprotein in the presence of BPA compare to the BPA alone (Fig. 5a, b).

DISCUSSION

Mast cells are broadly distributed throughout mammalian tissues and play an important role in a wide variety of biological responses. Typically, mast cells have been considered not only in the association of immediate-type hypersensitivity, but also in late reactions like inflammatory responses, which are mast cell dependent [30, 31]. And they play a critical role in allergic inflammation through the immunoglobulin E (IgE) dependent release of including histamine, tryptase, chymase, heparin and some inflammatory cytokines [32, 33]. Through release their proinflammatory mediators, mast cells actively participate in the pathogenesis of inflammation [34]. We showed that CTB glycoprotein reduces mast cell-mediated inflammatory allergic responses by using in HMC-1 cells.

BPA is well-known as a estrogen mimic chemical. The results these experiment showed that BPA stimulates histamine degranulation (Fig. 1) and mitogen activated protein kinase (MAPK) (Fig. 2). And CTB glycoprotein showed the effective decreasing on degranulation of histamine. Also CTB glycoprotein showed

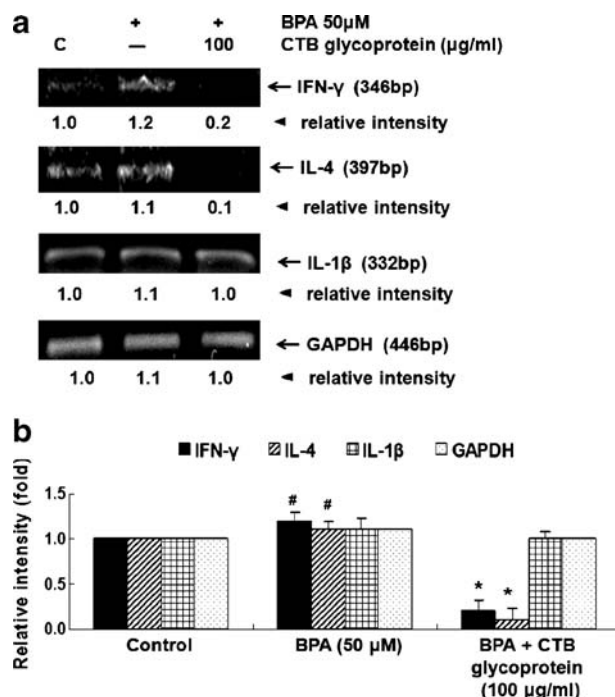


Fig. 5. Effects of CTB glycoprotein on expression of IFN- γ , IL-4, and IL-1 β mRNA in BPA-stimulated HMC-1 cells. (a) Expression of cytokine was determined by RT-PCR using total RNA isolated from HMC-1 cells. (b) The relative intensities of IFN- γ , IL-4, IL-1 β , GAPDH bands were quantified using Scion Imaging Software (Scion Image Beta 4.02, Maryland, USA). In Fig. 5, each bar represents the means $\pm$ SE of triplicate experiments. [#] $P < 0.05$, significant difference compared to the control and bisphenol A treatment alone. ^{*} $P < 0.01$, significant difference compared to the bisphenol A treatment alone and 100 μ g/mL CTB glycoprotein treatment. GAPDH was used as an internal control.

dramatically decreasing effect on ERK, one of the MAPK phosphorylation. ERK family is a common proinflammatory mediator, which activates transcription factors and some downstream protein kinases. However, p38 MAPKs, except ERK and JNK was not showed remarkable decrease compared with BPA stimulation. We suggest that the BPA doesn't affect on the p38 MAPK pathway.

AP-1 activity is stimulated by a complex network of signaling pathways that involves external signal (for example, growth factors) stimulation and the activation of mitogen-activated protein kinase (MAPK) members: extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK). Activated these transcription factors head for nuclear and activate many genes. In this study, the results from Western blotting, we carried out CTB glycoprotein inhibits activation of transcription factors' subunits such as c-Jun and c-fos in AP-1, against

BPA stimulation. Interestingly, only c-Jun increased on the BPA stimulated HMC-1 cells. CTB glycoprotein suppressed c-Jun expression on the BPA stimulated HMC-1 cells.

The results of this study revealed interesting inflammation inhibition effects of CTB glycoprotein in BPA stimulated human mast cells with intracellular signal pathways and secretion of pro-inflammatory mediators, such as COX-2, cytokines. We confirmed the western blot to elucidate the regulation of BPA-induced COX-2, which are sensitive prime components of intracellular signaling pathways responsible for inflammatory events, activation by CTB glycoprotein. Our findings in this study show that CTB glycoprotein suppressed COX-2 activity in HMC-1 cells.

In order to more accurate evaluation of cytokine expression, it was carried out RT-PCR in addition to Western blotting. In RT-PCR experiments, the results in this study were shown to have no significant differences between CTB glycoprotein treatment (25 and 50 μ g/mL) and control. When the HMC-1 cells were treated by BPA, expression of IFN- γ , IL-4, and IL-1 β increased. However the cells were treated with CTB glycoprotein (100 μ g/mL) in the presence of BPA, their expressions significantly decreased compared to BPA treatment alone. Interestingly, in the case of Interleukin-1 β , its expression was not significant difference between treatment with CTB glycoprotein in presence of BPA and BPA alone. It is still not clear why expressions of inflammation-related cytokines (IFN- γ , IL-4, and IL-1 β) were differently, and why IFN- γ was more strong expression than other inflammation-related cytokines (IL-4, and IL-1 β).

In conclusion, our results indicate that CTB glycoprotein has an anti-inflammatory potential due to activated human mast cells (HMC-1) through modulation of pro-inflammatory cytokines such as IFN- γ , IL-4, IL-1 β expression. Hence, we speculate that it is applicable in the prevention and treatment of inflammatory diseases. Further study remains to evaluate the expression of TNF- α and IL-6 by CTB glycoprotein in the presence of BPA at the molecular level and *in vivo* using ELISA.

ACKNOWLEDGEMENT

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund; KRF-2008-521-C00167).

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