



Inhibitory effect of phytoglycoprotein (24 kDa) on allergy-related factors in compound 48/80-induced mast cells *in vivo* and *in vitro*

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ABSTRACT

Mast cells are involved in immediate allergic reactions such as asthma, allergic rhinitis, and atopic dermatitis. Allergic reactions caused by extracellular allergens such as xenobiotics may become a critical problem in living circumstances. Recently, we isolated and purified glycoprotein from *Zanthoxylum piperitum* DC fruit (ZPDC), and demonstrated that ZPDC glycoprotein (5–20 mg/kg, 25–100 µg/ml) has an inhibitory effect on allergy-related mediators in the compound 48/80-treated BALB/c and human mast cells (HMC-1 cells). Our results obtained from this study showed that ZPDC glycoprotein (10 mg/kg) inhibited interleukin-4 (IL-4), immunoglobulin E (IgE), and histamine are released in mouse serum. Also, ZPDC glycoprotein (50 µg/ml) attenuated the degranulation of mast cells, intracellular Ca^{2+} levels, and the activities of phosphorylation of p38 mitogen-activated protein kinase (MAPK), nuclear factor (NF)-κB (p50 and p65), and cyclooxygenase-2 (COX-2) in the HMC-1 cells. Taken together, we speculate that the ZPDC glycoprotein might be one component found in natural products that has the ability to prevent dysfunction in the immune system caused by several different allergens.

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1. Introduction

Allergies such as asthma, allergic rhinoconjunctivitis, anaphylaxis and atopic dermatitis have been well known as immediate reactions following contact with certain exogenous allergens [1]. An anaphylactic reaction is also caused by the release of inflammatory mediators (like histamine) from mast cells [2]. Mast cells are mononuclear and granule-containing secretory cells. They are important in the development of many physiological changes during allergic and anaphylactic responses. It has been reported that allergies are associated with the T-helper (Th) 2 type immune response, i.e. activation of interleukin (IL)-4 and immunoglobulin (Ig)-E synthesis [3].

It has been reported that the degranulation of mast cells can be elicited by non-immunologic stimulators such as neuropeptides, basic compounds, complement components, and certain drugs [4]. It is well known that compound 48/80 (a condensation product of N-methyl-p-methoxyphenethylamine with formaldehyde) has the potential to secrete allergy-related factors from mast cells as stimulators [5]. The compound 48/80 has been used to study allergies and anaphylaxis, because it can vigorously activate the release of histamine via the mechanism of cellular exocytosis.

It has been postulated that mast cells, after the activation of non-immunological stimuli, release various performed mediators including histamine and β-hexosaminidase, which subsequently leads to the phosphorylation of tyrosine kinase, the mobilization of internal Ca^{2+} , the activation of mitogen-activated protein kinases (MAPKs), nuclear factor (NF)-κB, and cyclooxygenase (COX)-2 in the signal pathway [6,7].

In Asian countries, herbal plants have been used for many years as traditional folk medicine for healing immune dysfunction (or immune disorder). It has been reported that *Zanthoxylum piperitum* DC (ZPDC) is one of the spices that is used to produce a fresh flavor and to avoid unpleasant fishy and meaty odors [8]. It has been used to heal diarrhea and abdominal pain in Korea [9]. Recently, we isolated glycoprotein (24 kDa) from ZPDC fruit, which consists of a carbohydrate moiety (18%) and a protein moiety (82%). We have reported that the glycoprotein has strong characteristics relating to anti-oxidation, cytoprotection, and anti-inflammation [10]. Because it has been reported that most glycoproteins originating from plants have a modulation characteristic relating to immune-related factors such as anti-inflammation, anti-apoptosis, and anti-tumor [11–13], it is important to determine whether ZPDC glycoprotein can contribute to the inhibition of allergy-related factors, and to verify whether ZPDC glycoprotein inhibits allergies caused by compound 48/80. The evaluation of allergy-related factors such as mortality, IgE, IL-4 and histamine in mouse serum was conducted first, because the compound 48/80 can't stimulate any cytokines in mast cells and evaluated allergy-related factors (p38 MAPK, NF-κB, and COX-2) in

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HMC-1 cells. The reason behind using HMC-1 cells is that other allergy-related factors (p38 MAPK, NF- κ B, and COX-2) from mice cannot be evaluated, because it is very difficult to collect mast cells from mouse serum. Due to the fact that the mouse serum is collected in a small volume, only a small number of mast cells are able to be isolated. So far, nobody has studied the mechanism (possible signaling pathway) of the mast cell-mediated allergy model using compound 48/80.

Therefore, we evaluated the levels of IL-4, IgE and histamine in the compound 48/80-treated BALB/c serum, and the degranulation of mast cells, the activation of p38 MAPK, NF- κ B (p50 and p65), and COX-2 in the compound 48/80-stimulated HMC-1 cells.

2. Materials and methods

2.1. Reagents

All plastic materials were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ). Compound 48/80, penicillin G and streptomycin were 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.

2.2. Preparation of ZPDC glycoprotein

Fruits of *Z. piperitum* DC (ZPDC) were obtained in October 2006 from Na-Ju in the Chonnam province of South Korea, and glycoprotein was isolated and purified as described previously [10]. The ZPDC was cut into small pieces and soaked in 99% ethanol at 4 °C for 3 months in a dark basement. The extract was filtered through Whatman filter paper (No. 2) and then evaporation was performed to remove the 99% ethanol using a rotary evaporator (B465; Buchi, Flawil, Switzerland). After the evaporation of ethanol, the remaining solution was dried with a freeze-dry (SFDS06; Sam won, Seoul, Korea) to make a fine powder. The dried powder (5 g) was re-dissolved in distilled water and precipitated with 80% ammonium sulfate to obtain a protein. Following these steps, this sample was dialyzed with membrane (MWCO 6000–8000) to obtain protein with a molecular weight of more than 8 kDa and then centrifuged with a Microcon (Millipore, MWCO 30,000) according to the manufacturer's protocol (Amicon Inc., MA, U.S.A.). The final amount of ZPDC glycoprotein was 3.5 mg (0.07%) from the initial amount (5 g) of dried ZPDC powder. To characterize the sample, SDS-PAGE was carried out loading the sample of protein (1 mg/10 μ l) containing 0.1% SDS, using a 18% polyacrylamide mini-gel and Mini-PROTEIN II electrophoresis cell (Bio-Rad) at 110 V, 30 mA for 2.5 h. The ZPDC glycoprotein on the gel was stained with Schiff's reagent [14], which is a specific staining reagent for glycoprotein through a redox-reaction. The sample of glycoprotein on the gel revealed one band with 24 kDa, consisting of carbohydrate content (18%) and protein content (82%) [10].

2.3. Animals and cell culture

Female BALB/c mice (25 g, aged 6 weeks) were purchased from Daehan Lab (Animal Research Center Co., Ltd, Daejeon, South Korea) and housed at the experimental animal room of the Veterinary College of Chonnam National University according to the guiding principles in the use of animals in toxicology, adopted by the Society of Toxicology in 1989. All mice were handled under optimal conditions to minimize pain or discomfort and were fed a commercial diet and water *ad libitum* for at least 1 week prior to the injection of compound 48/80 in order to minimize experimental stress. HMC-1 cells (human mast cell line) were grown in Iscove's media supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin at 37 °C in an atmosphere containing 5% CO₂. The medium was renewed twice each week. The

number of cells (1×10^6 cells/ml) was divided into 35 mm culture dishes or 6-well flat bottom plates. To measure the cytotoxicity of the ZPDC glycoprotein, viability was evaluated using the MTT method, as reported previously [15]. Namely, the cells were treated with 5 μ g/ml compound 48/80, or co-treated with 5 μ g/ml compound 48/80 in the presence of ZPDC glycoprotein (25–100 μ g/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% CO₂ 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).

2.4. Compound 48/80-induced systemic reaction

The compound 48/80-induced systemic reaction was examined as previously described [16]. Mice were injected intraperitoneally with the concentration of 0.008 g/kg body weight (BW) of compound 48/80. ZPDC glycoprotein was dissolved in saline and administered orally in 5 to 20 mg/kg BW 1 h prior to the injection of compound 48/80 ($n = 6$ /group). Mortality was monitored for 1 h after the induction of anaphylactic shock.

2.5. Sample collection

Blood samples were collected from anesthetized mice by orbital sinus or maxillary vein-puncture. Samples were collected into 1.5 ml centrifuge tubes, allowed to clot, and centrifuged at 10,000 rpm at 4 °C for 5 min. The serum was transferred to a new tube and stored at –20 °C until testing.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The amounts of IgE and IL-4 were determined by sandwich ELISA using monoclonal antibodies specific to IgE and IL-4 (BD Biosciences, San Diego, SA, USA). ELISA plates were coated with rat anti-mouse IgE and IL-4, respectively. After coating, serially diluted serum was added to the plates and incubated overnight at 4 °C. Murine recombinant IgE and IL-4 were used as a standard for the quantization of IgE and IL-4 in the serum. After washing, biotinylated anti-mouse IgE and IL-4 were added, respectively. The plates were washed and 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).

2.7. Inhibition of β -hexosaminidase and histamine release

β -Hexosaminidase in the supernatant was measured using a previously reported method [17]. In brief, cells were treated with 5 μ g/ml compound 48/80 in the absence or presence of ZPDC glycoprotein (25, 50 and 100 μ g/ml) for 30 min. After 30 min, degranulation was terminated by placing the cells on ice. To determine the amount of β -hexosaminidase activity released by the cells, 20 μ l of each sample was incubated with 20 μ l of 1 mM p-nitrophenyl-N-acetyl- β -D-glucosaminide dissolved in a 0.1 M citrate buffer, pH 4.5, in a 96-well microtiter plate at 37 °C for 1 h. The reaction was terminated with 200 μ l/well of 0.1 M carbonated buffer, pH 10.0. Absorbance was measured at 410 nm with a MicroReader (Hyperion, Inc., USA).

The amount of histamine was assayed using a fluorometric assay [18]. To determine the amounts of total cellular histamine, HMC-1 cells (1×10^6 cells) were suspended and sonicated in a Tyrode buffer. Then, the cell lysates were centrifuged at 10,000 $\times$ g for 10 min and the supernatants were harvested. Afterwards, 2 ml of the supernatant was mixed 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 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 to 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 quantified by comparison to a standard curve generated by coupling histamine dihydrochloride to o-phthalaldehyde.

2.8. Detection of intracellular Ca²⁺ mobilization

Determination of intracellular Ca²⁺ was performed as described previously [19]. In brief, cells were rinsed with a bath solution (140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM glucose, 5.5 mM HEPES, pH 7.5) and then pre-incubated in a bath solution containing 1 μM Fura-2/AM (Molecular probes, Eugene, OR, U.S.A.) with 5% CO₂ at 37 °C for 45 min and shaken gently. After shaking, cells were treated with ZPDC glycoprotein (25–100 μg/ml), compound 48/80 (5 μg/ml) and A23187 (1 μM) as a positive control. Fluorescence intensity was measured at an emission wavelength of 510 nm and an excitation wavelength of 340 nm and 380 nm using a fluorescence microplate reader (Dual Scanning SPECTRAmax, Molecular Devices Corporation, Sunnyvale, CA, USA). The fluorescence ratio (340/380) was calculated as an indicator of intracellular Ca²⁺ intensity.

2.9. Preparation of cell extracts

Preparation of cell extract was basically as described elsewhere [20]. Briefly, the cells were treated with compound 48/80 (5 μg/ml) in the presence of ZPDC glycoprotein (25, 50 and 100 μg/ml) for the indicated times. Then cells were washed in 1 ml of ice-cold PBS after the medium was removed, and scraped into 300 μl of ice-cold buffer C (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% NP-40, 25% glycerol) containing a phosphatase inhibitor cocktail. For lysis, the cells were freeze-thawed, shaken for 30 min at 4 °C, and centrifuged at 14,000 × g for 30 min. The supernatant was designated as a whole extract for Western blotting of p38 MAPK.

To prepare nuclear extracts for Western blotting of NF-κB (p50 and p65), after cell activation for the times indicated, cells were re-suspended in 400 μl of ice-cold hypotonic buffer D (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), left on for 15 min and then centrifuged at 3000 × g for 5 min at 4 °C. Pelleted nuclei were gently re-suspended in 150 μl of NE buffer E (20 mM HEPES, pH 7.5, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1.5 mM MgCl₂, 0.5% NP-40, 25% glycerol) containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany) and centrifuged at 12,000 × g for 15 min at 4 °C. The amount of protein was measured by the method of Lowry [21]. The cellular proteins were stored at –70 °C prior to use.

2.10. Western blot analysis

Cellular proteins were separated in a 10% polyacrylamide mini-gel at 100 V for 2 h at room temperature using a mini-protein II electrophoresis cell (Bio-Rad). After electrophoresis, the proteins were transferred 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% (v/v) Tween-20) containing 5% non-fat dry milk. The membranes were subsequently incubated for 2 h at room temperature with primary antibodies (p38 MAPK, phospho-p38 MAPK, NF-κB p50, NF-κB p65, COX-2 and α-tubulin) in TBS-T solution. After three separate washes with TBS-T, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG and anti-rabbit IgG (1:10,000; Cell Signaling Technology, MA, USA) in TBS-T solution.

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, Frederick, MD, USA).

2.11. Statistical analysis

All experiments were carried out in triplicates, and data are expressed as means ± S.E. One-way analysis of variance (ANOVA) and Duncan tests were carried out to determine significant differences of multiple comparisons (Statistical Package of the Social Sciences program, version 11.0).

3. Results

3.1. Inhibitory effect of ZPDC glycoprotein on compound 48/80-induced systemic reaction

After the intraperitoneal injection of compound 48/80 (0.008 g/kg, BW), the mice were monitored for 1 h, and then the mortality rate was determined. As shown in Table 1, 100% of the mice injected with compound 48/80 underwent a fatal shock. However, when the ZPDC glycoprotein was pre-administered orally at concentration (5–20 mg/kg, BW) 1 h prior to compound injection, the mortality rate after treatment with compound 48/80 was reduced in a dose-dependent manner.

3.2. Inhibitory effect of ZPDC glycoprotein on IL-4 and IgE production in compound 48/80-treated BALB/c mice

Levels of IL-4 and IgE production increased after treatment with compound 48/80 alone compared to the control. However, when the mice were orally pre-administered with the ZPDC glycoprotein in the presence of compound 48/80, levels of IL-4 and IgE production after treatment with compound 48/80 decreased significantly in the presence of ZPDC glycoprotein in a dose-dependent manner. For instance, the amount of IL-4 was 2.24 pg/ml after treatment with compound 48/80 alone, as shown in Fig. 1A. However, when the mice were orally pre-administered with the ZPDC glycoprotein prior to the injection of compound 48/80, amounts of IL-4 decreased by 1.37 ng/ml at 20 mg/kg (BW) of ZPDC glycoprotein in the presence of compound 48/80, compared to the compound 48/80 treatment alone. With respect to IgE production, when the mice were treated with compound 48/80 alone, levels of IgE increased, compared to the control (222.15 ng/ml). However, when the mice were pre-administered with the 10 mg/kg (BW) of ZPDC glycoprotein in the presence of compound 48/80, IgE production decreased significantly in a dose-dependent manner. The values of IgE decreased by 135 ng/ml compared with the compound 48/80 treatment alone (Fig. 1B).

Table 1

Effect of ZPDC glycoprotein on compound 48/80-induced systemic reaction.

ZPDC glycoprotein treatment (mg/kg BW)	Compound 48/80 (8 mg/kg BW)	Mortality (%)
None (saline)	–	0
None (saline)	+	100.0
5	+	88.0
10	+	45.1
20	+	32.5

Groups of mice (n=6/group) were orally administered with either 100 μl saline or ZPDC glycoprotein. ZPDC glycoprotein was orally administered to mice in various doses (5–20 mg/kg) 1 h prior to the injection of compound 48/80. The compound 48/80 solution was intraperitoneally injected to the mice in each group. Mortality (%) within 1 h after compound 48/80 injection was calculated as following equation: (the number of dead mice/total number of mice) × 100 obtained from triplicate experiments, separately. BW: body weight.

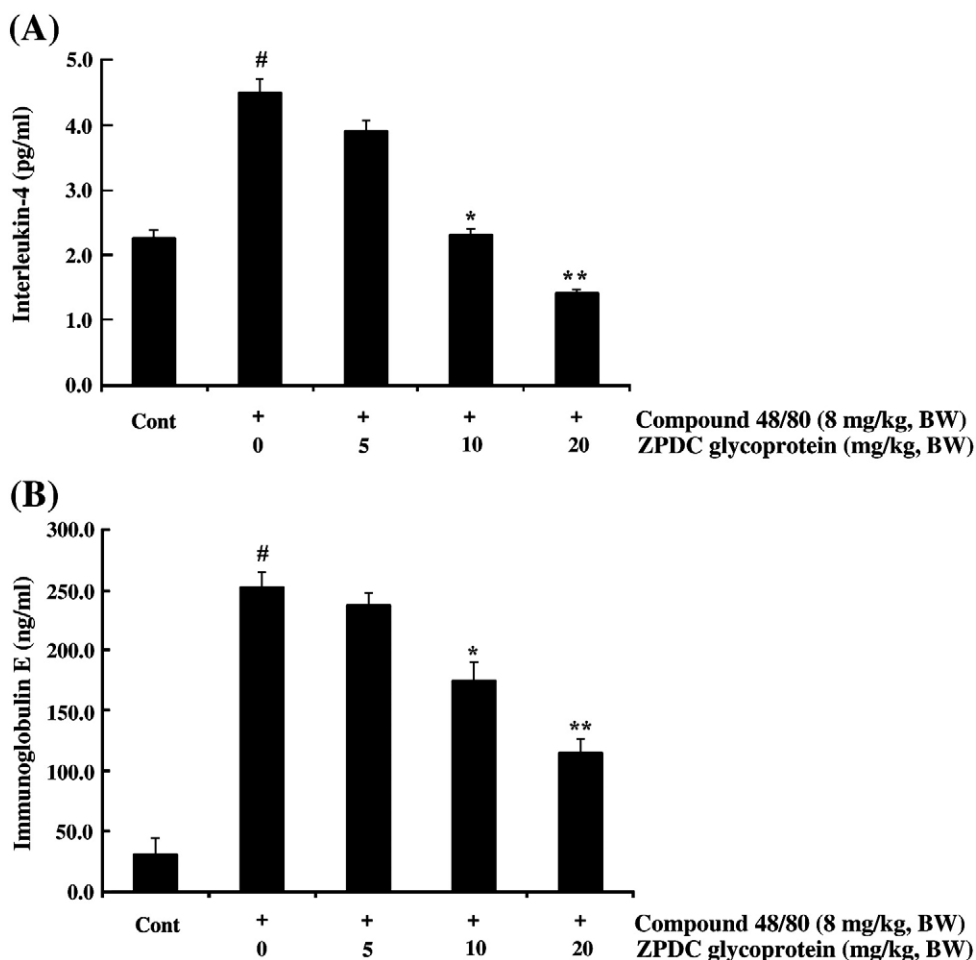


Fig. 1. Inhibitory effect of ZPDC glycoprotein on the amount of IL-4 and IgE production in compound 48/80-treated BALB/c mice. The amounts of IL-4 (A) and IgE (B) were determined by sandwich ELISA using monoclonal antibodies specific to IL-4 and IgE (BD Biosciences, San Diego, CA, USA). All data are the means $\pm$ S.E. from triplicate experiments ($n=6$), separately. # represents a significant difference between treatments and control, $P<0.05$. * and ** indicate a significant difference between compound 48/80 treatment alone and treatment of the ZPDC glycoprotein in the presence of compound 48/80, $P<0.05$ and $P<0.01$, respectively. BW: body weight.

3.3. Inhibitory effect of ZPDC glycoprotein on histamine release in compound 48/80-treated BALB/c mice

Histamine release of mouse serum increased after treatment with compound 48/80 alone compared to the control. However, in the mice

that were pre-administered with the ZPDC glycoprotein in the presence of compound 48/80, histamine release decreased significantly after treatment with compound 48/80 in the presence of ZPDC glycoprotein (5–20 mg/kg, BW) in a dose-dependent manner. For instance, histamine release decreased 0.20- and 0.25-fold after the

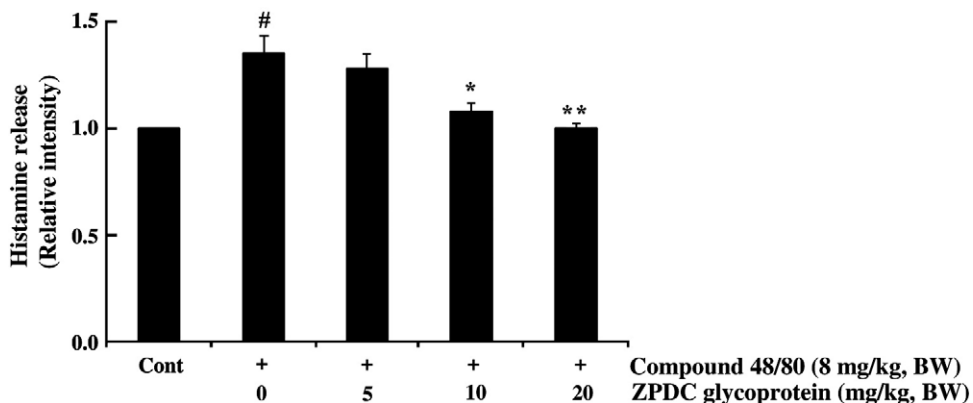


Fig. 2. Inhibitory effect of ZPDC glycoprotein on histamine release in compound 48/80-treated BALB/c mice. Groups of mice ($n=6$ /group) were orally administrated with saline or ZPDC glycoprotein. ZPDC glycoprotein was pre-administrated at the indicated different doses prior to 1 h of the injection of compound 48/80. The compound 48/80 solution (8 mg/kg) was intraperitoneally injected to the mice in the each group. After injection of the compound 48/80 solution, the mouse serum samples were taken within 1 h and the histamine content was evaluated using a fluorescent microplate reader (Dual Scanning SPECTRAmax, Molecular Devices Corporation, Sunnyvale, CA, USA). 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 compound 48/80 treatment alone and the ZPDC glycoprotein treatment in the presence of compound 48/80, $P<0.05$ and $P<0.01$, respectively. BW: body weight.

treatment with compound 48/80 in the presence of ZPDC glycoprotein (10 and 20 mg/kg, BW), as shown in Fig. 2.

3.4. Inhibitory effect of ZPDC glycoprotein on degranulation of mast cell and intracellular Ca^{2+} mobilization in compound 48/80-stimulated HMC-1 cells

In order to determine whether ZPDC glycoprotein can modulate the degranulation of mast cells stimulated by compound 48/80, HMC-

1 cells stimulated by compound 48/80 were incubated with the ZPDC glycoprotein (25, 50 and 100 $\mu\text{g}/\text{ml}$) for 30 min. When the cells were treated with compound 48/80 (5 $\mu\text{g}/\text{ml}$), the release of β -hexosaminidase and histamine increased 1.90- and 1.35-fold compared to the control. However, when HMC-1 cells were treated with compound 48/80, such levels decreased 0.91- and 0.35-fold, respectively, in the presence of 100 $\mu\text{g}/\text{ml}$ ZPDC glycoprotein (Fig. 3A and B).

With respect to the mobilization of Ca^{2+} in the cells, when the HMC-1 cells were treated with 5 $\mu\text{g}/\text{ml}$ compound 48/80, the amount

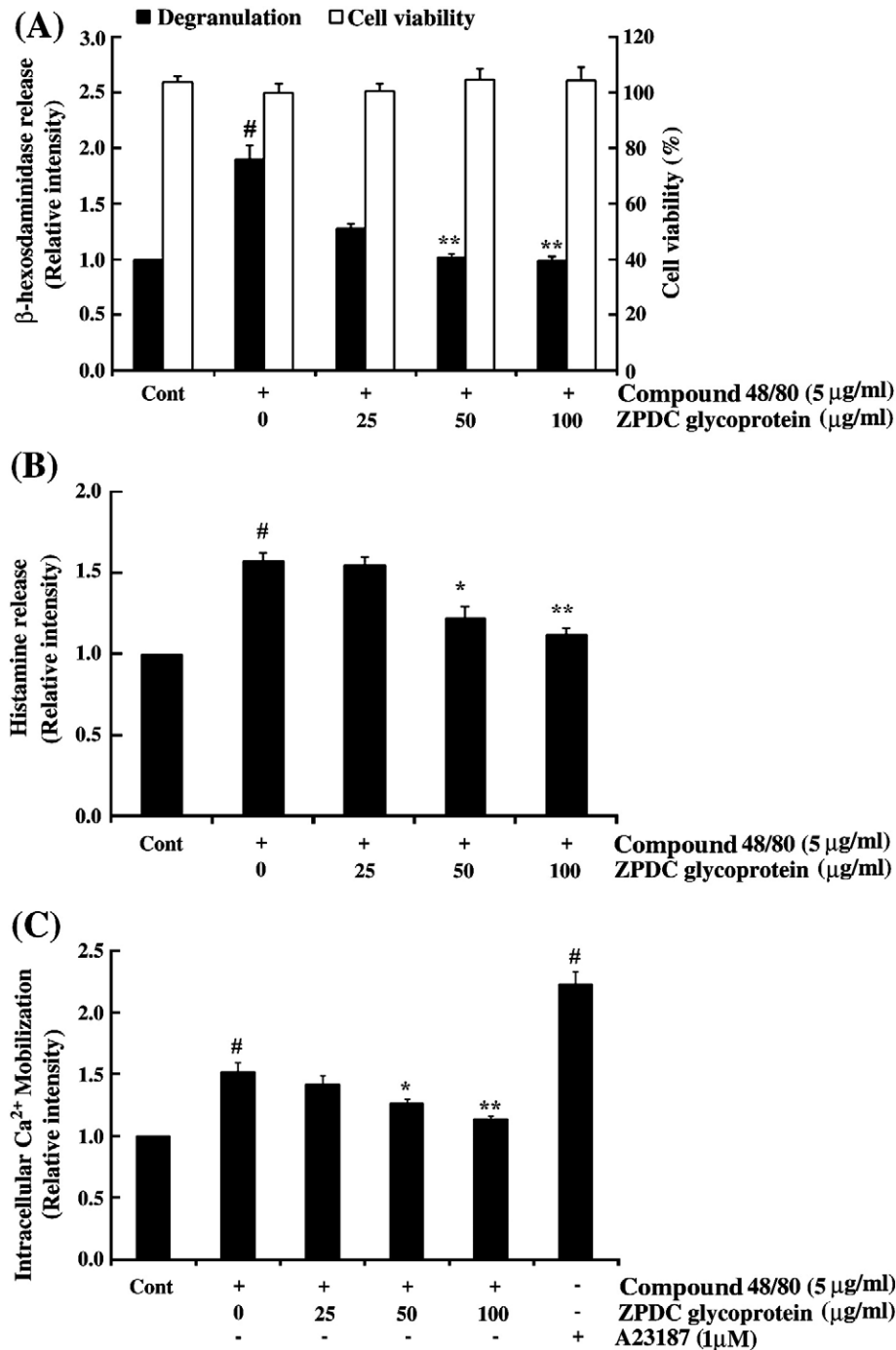


Fig. 3. Inhibitory effect of ZPDC glycoprotein on degranulation of mast cells and mobilization of intracellular Ca^{2+} in the compound 48/80-stimulated HMC-1 cells. HMC-1 cells were treated with compound 48/80 (5 $\mu\text{g}/\text{ml}$) for 30 min in the absence or presence of the ZPDC glycoprotein (25, 50 and 100 $\mu\text{g}/\text{ml}$). The level of β -hexosaminidase (degranulation) and histamine release was measured from the cell culture media (supernatant) (A, B). Changes in intracellular Ca^{2+} mobilization were analyzed with Fura-2/AM, as described in Materials and methods. A23187 (Ca^{2+} ionophore) was used as a positive control (C). All data are the means $\pm$ S.E. from triplicates, separately. [#] represents a significant difference between compound 48/80 treatment alone and control, $P < 0.05$. * and ** indicate significant differences between compound 48/80 treatment alone and the ZPDC glycoprotein treatment in the presence of the compound 48/80, $P < 0.05$ and $P < 0.01$, respectively.

of intracellular Ca^{2+} increased 1.52-fold compared to the control. However, when the cells were treated with compound 48/80 in the presence of ZPDC glycoprotein (50 and 100 $\mu\text{g}/\text{ml}$), such amounts decreased significantly in a dose-dependent manner. For example, the value of intracellular Ca^{2+} decreased 0.19- and 0.33-fold at 50 and 100 $\mu\text{g}/\text{ml}$ ZPDC glycoprotein compared to the compound 48/80 (5 $\mu\text{g}/\text{ml}$) treatment alone (Fig. 3C).

3.5. Inhibitory effect of ZPDC glycoprotein on activity of p38 MAPK in compound 48/80-stimulated HMC-1 cells

To determine whether ZPDC glycoprotein inhibits the phosphorylation of p38 MAPK in the compound 48/80-treated HMC-1 cells, activity of p38 MAPK was determined using Western blot analysis. As shown in Fig. 4, activity of p38 MAPK was augmented after treatment with 5 $\mu\text{g}/\text{ml}$ compound 48/80 for 30 min, compared to the control. For instance, the relative band-intensities of p38 MAPK were augmented 1.28-fold after treatment with compound 48/80, compared to the control. However, when the cells were treated with compound 48/80 in the presence of ZPDC glycoprotein (25, 50 and 100 $\mu\text{g}/\text{ml}$) for 30 min, relative band-intensities were markedly reduced in a dose-dependent manner. That is, the band-intensities of p38 MAPK after treatment with compound 48/80 (5 $\mu\text{g}/\text{ml}$) significantly decreased 0.21- and 0.47-fold in the presence of ZPDC glycoprotein (50 and 100 $\mu\text{g}/\text{ml}$), compared to the compound 48/80 treatment alone.

3.6. Inhibitory effect of ZPDC glycoprotein on activity of NF- κB in compound 48/80-stimulated HMC-1 cells

To evaluate the effect of ZPDC glycoprotein on the activity of NF- κB (p50 and p65) using the Western blot analysis, the cells were treated with compound 48/80 (5 $\mu\text{g}/\text{ml}$) for 60 min, the activity of NF- κB was markedly increased compared to the control. However, when the cells

were treated with compound 48/80 in the presence of ZPDC glycoprotein (25, 50 and 100 $\mu\text{g}/\text{ml}$), its activity was significantly decreased a dose-dependent manner. For instance, the relative intensities of NF- κB (p50 and p65) were augmented 2.22- and 3.49-fold at 5 $\mu\text{g}/\text{ml}$ compound 48/80, compared to the control. However, they were diminished 0.77- and 0.76-fold for NF- κB (p50 and p65) after the treatment with compound 48/80 in the presence of ZPDC glycoprotein (100 $\mu\text{g}/\text{ml}$), separately, compared to compound 48/80 treatment alone (Fig. 5, lane 5).

3.7. Inhibitory effect of ZPDC glycoprotein on activation of COX-2 in compound 48/80-stimulated HMC-1 cells

To examine the activity of COX-2 proteins, Western blot analysis was carried out in whole cell protein extracts. As shown in Fig. 6, ZPDC glycoprotein inhibited activities of COX-2 in compound 48/80-stimulated HMC-1 cells. For instance, in cells that were treated with compound 48/80 (5 $\mu\text{g}/\text{ml}$) for 60 min, the activity of COX-2 was markedly increased compared to the control. However, when the cells were treated with compound 48/80 in the presence of ZPDC glycoprotein (50 and 100 $\mu\text{g}/\text{ml}$), such activity decreased significantly in a dose-dependent manner. For instance, the relative intensities of COX-2 were augmented 2.33-fold after treatment with 5 $\mu\text{g}/\text{ml}$ compound 48/80, compared to the control (Fig. 6, lane 2). However, such activity was diminished 0.83- and 0.82-fold for COX-2 after the treatment with compound 48/80 in the presence of ZPDC glycoprotein (50, 100 $\mu\text{g}/\text{ml}$) separately, compared to compound 48/80 treatment alone (Fig. 6, lanes 4 and 5).

4. Discussion

Allergies are results of inappropriate immune responses, often to common antigens such as plant pollen, food, or animal dander. The sources of allergic occurring can be easily found from fast foods and

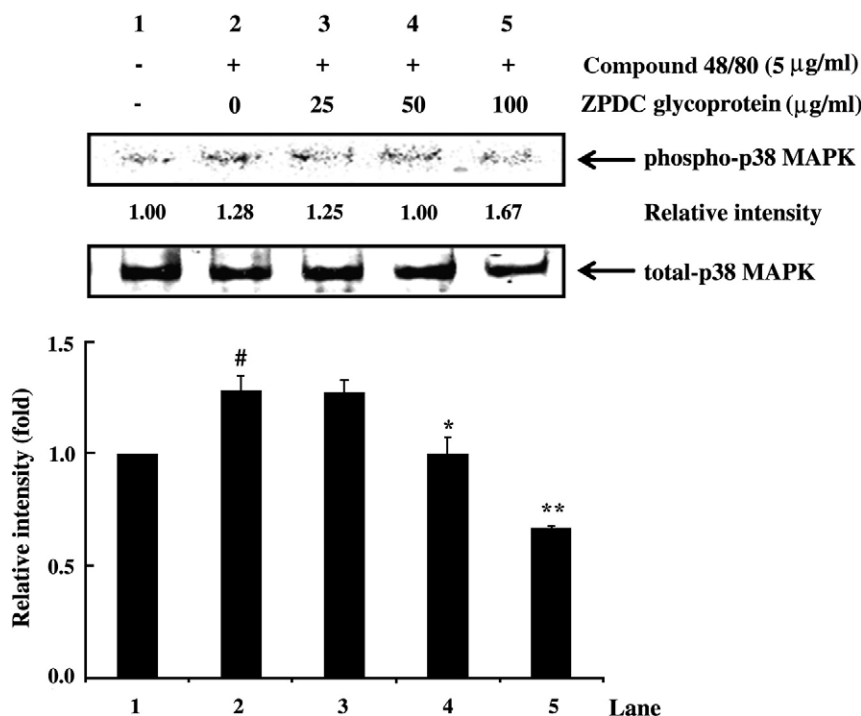


Fig. 4. Inhibitory effect of ZPDC glycoprotein on the activity of p38 MAPK in compound 48/80-stimulated HMC-1 cells. HMC-1 cells were treated with compound 48/80 (5 $\mu\text{g}/\text{ml}$), or co-treated with the ZPDC glycoprotein (25, 50 and 100 $\mu\text{g}/\text{ml}$) and compound 48/80 for 30 min. The relative intensities of bands obtained from Western blot were calculated using Scion Imaging Software (Scion Image Beta 4.02, Frederick, MD, USA). All data are the means $\pm$ S.E. from triplicates, separately. # represents a significant difference between compound 48/80 treatment alone and control, $P < 0.05$. * and ** indicate a significant differences between compound 48/80 treatment alone and the ZPDC glycoprotein treatment in the presence of the compound 48/80, $P < 0.05$ and $P < 0.01$, respectively. Lane 1, control; lane 2, compound 48/80 alone; lane 3, 25 $\mu\text{g}/\text{ml}$ ZPDC glycoprotein in the presence of the compound 48/80; lane 4, 50 $\mu\text{g}/\text{ml}$ ZPDC glycoprotein in the presence of the compound 48/80; lane 5, 100 $\mu\text{g}/\text{ml}$ ZPDC glycoprotein in the presence of the compound 48/80.

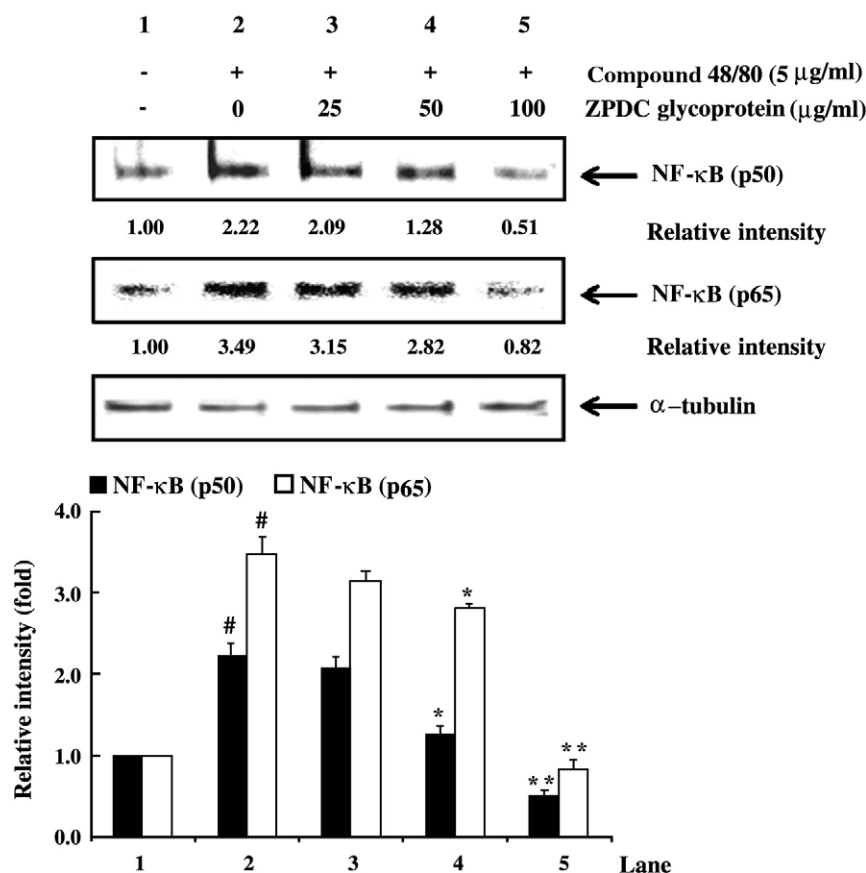


Fig. 5. Inhibitory effect of ZPDC glycoprotein on the activity of NF- κ B in compound 48/80-treated HMC-1 cells. HMC-1 cells were treated with compound 48/80 (5 μ g/ml), or co-treated with the ZPDC glycoprotein (25, 50 and 100 μ g/ml) and compound 48/80 for 60 min. The relative intensities of bands obtained from Western blot were calculated using Scion Imaging Software (Scion Image Beta 4.02, Frederick, MD, USA). All data are the means $\pm$ S.E. from triplicates, separately. # represents a significant difference between compound 48/80 treatment alone and control, $P < 0.05$. * and ** indicate a significant differences between compound 48/80 treatment alone and the ZPDC glycoprotein treatment in the presence of the compound 48/80, $P < 0.05$ and $P < 0.01$, respectively. Lane 1, control; lane 2, compound 48/80 alone; lane 3, 25 μ g/ml ZPDC glycoprotein in the presence of the compound 48/80; lane 4, 50 μ g/ml ZPDC glycoprotein in the presence of the compound 48/80; lane 5, 100 μ g/ml ZPDC glycoprotein in the presence of the compound 48/80. α -Tubulin was used as an internal control.

preservatives, and chemicals which are used for production of children's toys and interior house holders. Nowadays, immune dysfunction (allergy) has gradually raised a huge problem in the front of immune diseases. Thus, it is important to figure out a solution for allergies by using the experimental model with compound 48/80 in mast cells. Anaphylactic allergic reactions in immediate-type allergic reactions caused by compound 48/80, which promotes the release of histamine and activates G-proteins, is a life-threatening syndrome exhibition of releasing β -hexosaminidase, histamine and various cytokines from mast cells. Mast cells exist in the human body and are stimulated via the IgE receptor [22]. In the present study, we firstly confirmed the occurrence of allergic reactions caused by compound 48/80 through the inducement of the release of histamine, β -hexosaminidase, and IgE in the mast cells. After that, we verified that ZPDC glycoprotein decreases allergic reactions induced by compound 48/80 *in vitro* and *in vivo*. The results obtained from this study showed that ZPDC glycoprotein protects from mortality caused by compound 48/80 in BALB/c mice in a dose-dependent manner (Table 1), and that it inhibits the activity of IL-4 and IgE production in compound 48/80-treated BALB/c mouse serum (Fig. 1). It has been reported that the mast cell-mediated allergy reaction is associated with an increase in Th2 cytokines and production of IgE. IL-4 (one of the Th2 cytokines) promotes B cell differentiation to plasma cells that secrete antigen-specific IgE, and also contributes to the proliferation of mast cells [23]. It has been shown that IgE binds to receptors on the surface of mast cells and stimulates the release of various mediators that promote human immediate hypersensitivity disease, mucus

secretion, and increased vascular permeability [24]. It has been reported that compound 48/80 is well known as a potential agent for histamine release from mast cells, with a subsequent depletion of tissue histamine [25]. As shown in Fig. 2, ZPDC glycoprotein inhibited histamine release in mouse serum in a dose-dependent manner. Thus, the results of this study assume that ZPDC glycoprotein has an inhibitory effect in allergic reactions caused by compound 48/80.

To further understand the inhibition mechanism associated with the ZPDC glycoprotein, the activities of allergy-related signals were evaluated using Western blot analysis. It has been postulated that MHC-1 cells participate in allergic diseases and inflammatory disorders [26], and that histamine release and β -hexosaminidase assay has been widely used to assess the degranulation of mast cells [27]. As shown in Fig. 3, the results indicated that ZPDC glycoprotein suppresses degranulation in HMC-1 cells treated by compound 48/80 in a dose-dependent manner. Intracellular Ca^{2+} is one of the critical functional factors for the degranulation in mast cells and stimulates the activation of certain intracellular signal factors as a second messenger through Ca^{2+} movements across the membranes of mast cells. Ca^{2+} movements can be produced by effective anti-allergic drugs or allergy-linked stimulators [3,28]. Our results indicated that ZPDC glycoprotein attenuates the mobilized amount of intracellular Ca^{2+} in mast cells, suggesting the prevention of the degranulation of mast cells (Fig. 3).

It has been reported that MAPK and NF- κ B regulate the expression of many immune- and inflammatory-related genes and are critical factors of the important signaling pathways in immune response [29].

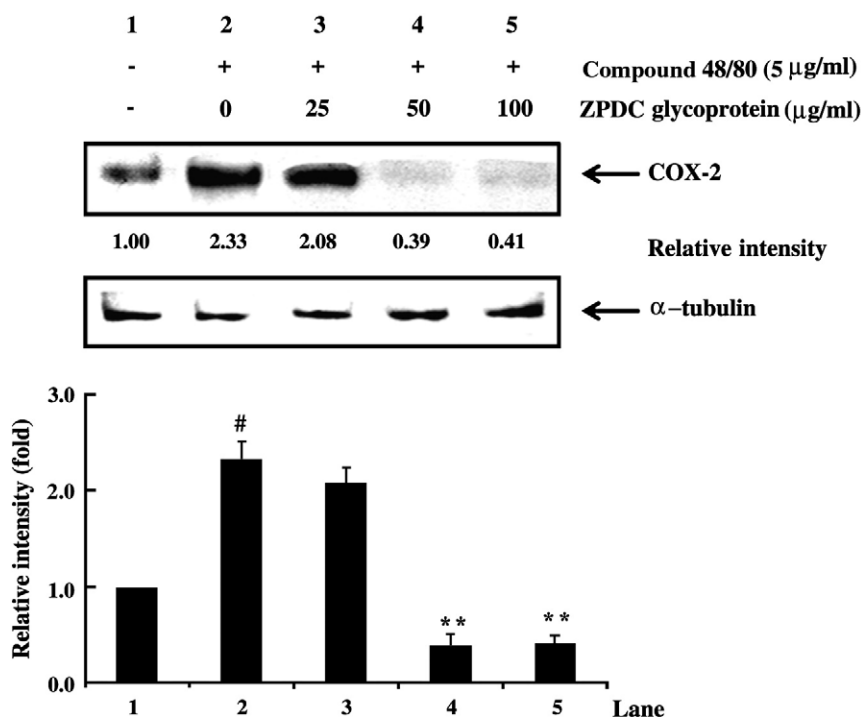


Fig. 6. Inhibitory effect of ZPDC glycoprotein on the activation of COX-2 in compound 48/80-treated HMC-1 cells. Cells were treated with compound 48/80 (5 µg/ml) or co-treated with the ZPDC glycoprotein (25, 50 and 100 µg/ml) and compound 48/80 for 60 min. The relative intensities of bands obtained from Western blot were calculated using Scion Imaging Software (Scion Image Beta 4.02, Frederick, MD, USA). All data are the means $\pm$ S.E. from triplicates, separately. [#] represents a significant difference between compound 48/80 treatment alone and control, $P < 0.05$. ^{**} indicates significant differences between compound 48/80 treatment alone and the ZPDC glycoprotein treatment in the presence of the compound 48/80, $P < 0.01$, respectively. Lane 1, control; lane 2, compound 48/80 alone; lane 3, 25 µg/ml ZPDC glycoprotein in the presence of the compound 48/80; lane 4, 50 µg/ml ZPDC glycoprotein in the presence of the compound 48/80; lane 5, 100 µg/ml ZPDC glycoprotein in the presence of the compound 48/80. α -Tubulin was used as an internal control.

The expression of inflammatory cytokines is regulated by MAPKs which are divided into three types, such as p38, extracellular signal-regulated kinase (ERK), and c-jun N-terminal kinase (JNK). In particular, p38 MAPK is known to be necessary to express pro-inflammatory cytokines [30]. Recent studies have suggested that the NF- κ B activation needs to activate p38 MAPK in HMC-1 cells [31,32]. As shown in Figs. 4 and 5, activation of p38 MAPK and NF- κ B occurred within 30 min and 60 min, respectively, after treatment with compound 48/80 in the HMC-1 cells. However, such activity diminished after treatment with ZPDC glycoprotein. It has been reported that NF- κ B is one of the transcription factors for the expression of inflammation-related genes including COX-2 [33] and is frequently composed of a p50 and a p65 subunit. Under basal conditions, it is retained in the cytoplasm bound to an inhibitory subunit I κ B. During a response to inflammatory stimulators, the p65 subunit dissociates from the I κ B subunit and translocates from the cytoplasm to the nucleus, where it dimerizes with the p50 subunit and interacts with specific target genes, such as COX-2 leading to increased inflammatory processes [34,35]. Because of its central role in regulating inflammatory responses, a pharmacological inhibition of NF- κ B activation could be beneficial in the treatment of inflammation [36].

With respect to the COX-2 expression, our finding indicated that the ZPDC glycoprotein has an inhibitory effect on the expression of COX-2. Interestingly, it has been reported that HMC-1 cells are useful cells for studying cytokine activation pathways [37]. However, they cannot be expressed as pro-inflammatory cytokines by compound 48/80 in the HMC-1 cells, specifically [38]. As shown in Fig. 6, COX-2 was activated within 60 min in the compound 48/80-treated HMC-1 cells but COX-2 amounts diminished after treatment with ZPDC glycoprotein. Generally, COX exists as two isoforms (constitutive COX-1 and inducible COX-2) and COX-2 relates to inflammation and pain. Specifically, COX-2 is barely detectable under normal physiological

conditions, but is rapidly and transiently induced by pro-inflammatory mediators. Also, COX-2 is an important factor for anaphylactic reactions induced by compound 48/80 [39].

In conclusion, the results in the current study indicated that ZPDC glycoprotein inhibits IL-4, IgE levels and histamine release in BALB/c mouse serum, and attenuates degranulation, intracellular Ca^{2+} levels, the activation of p38 MAPK, NF- κ B (p50 and p65) and COX-2 in HMC-1 cells. Thus, the results obtained from this study indicate that ZPDC glycoprotein might be used to prevent dysfunction of the immune system caused by allergic responses. Still, problem remains to elucidate what kind of signal factors are modulated by ZPDC glycoprotein from membrane receptor to gene expression of allergy-related mediators, systematically.

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