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Inhibitory effects of chitooligosaccharides on degranulation and cytokine generation in rat basophilic leukemia RBL-2H3 cells

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ABSTRACT

Chitooligosaccharides (COS) of three different molecular weight ranges (1–3 kDa, 3–5 kDa and 5–10 kDa) were investigated for their abilities against allergic reactions in RBL-2H3 cells. At the high concentration of 1000 μg/ml, COS with molecular weights of 1–3 kDa greatly attenuated the histamine and β-hexosaminidase release and intracellular Ca^{2+} elevation induced by calcium ionophore A23187, at the rates of 32% and 34% release and 37% elevation, respectively. Furthermore, the inhibitory activities of COS on calcium ionophore A23187 plus phorbol 12-myristate 13-acetate (PMA)-induced production as well as mRNA expression of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, and IL-6 were evidenced. Notably, the suppressive effects on the phosphorylation of extracellular signal-regulated kinases (MEK/ERK) and p38 kinase by COS were confirmed. These results indicate that COS could be used as an inhibitor in regulating mast cell-mediated allergic inflammatory responses.

1. Introduction

Allergies are caused by an exaggerated or hypersensitivity reaction of the immune system to harmless environmental substances (Milián & Díaz, 2004). Allergic rhinitis, asthma and atopic eczema are the commonest causes of chronic ill-health. The prevalence, severity and complexity of these allergic diseases in the population are rapidly rising and considerably adding to the burden of health-care costs (Kay, 2000). Therefore, the knowledge about the pathophysiology of allergic diseases has substantially increased, offering new opportunities for therapeutic intervention. Mast cells, which are located in virtually all organs and tissues, are important mediators in the development of allergic diseases and inflammatory processes (Galli et al., 2005; Puxeddu, Piliponsky, Bachelet, & Levi-Schaffer, 2003). Activation of mast cells leads to degranulation, one of the critical steps in allergic responses causes the release of various mediators such as histamine and an array of cytokines (Shin et al., 2005). These inflammatory mediators are the origination of various pathophysiologic events in acute allergic reactions, such as an increase in vascular permeability, induction of bronchial smooth-muscle contraction or mucus production, and recruitment of inflammatory cells (Li, Lee, Le, Kim, & Kim, 2008). In addition, mast cell activation can provoke the phosphorylation of members of the mitogen-activated protein kinase (MAPK) super-family, including extracellular signal-regulated kinase (MEK/ERK), p38 kinase, and c-jun *N*-terminal kinase (JNK). The function of these enzymes is involved in converting extracellular stimuli to intracellular signals, which in turn, participate in regulation of cytokine expression in mast cell (Hong et al., 2010; Teramoto, Salem, Robbins, Bustelo, & Gutkind, 1997). In *in vitro* experiments, mast cell can also be activated by calcium ionophore A23187 as well as phorbol 12-myristate 13-acetate (PMA), which have been used as direct and convenient reagents for study of the mechanism of allergic reaction (Shin et al., 2005, 2009).

Chitosan and its derivatives have been used as food (Shahidi & Synowiecki, 1991), cosmetics (Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004), and in biomedicine (Enescu & Olteanu, 2008). Further, chitosan has been used as a bioactive material due to its biodegradable, non-toxic, and non-allergenic characteristics (Dutta, Dutta, & Tripathi, 2004; Kurita, 1998). Recent studies have focused on the conversion of chitosan to COS, since COS are not only water-soluble (Yang et al., 2010) and possess higher oral absorption (Chae, Jang, & Nah, 2005) but also have various biological effects, including anti-bacterial, anti-tumor, anti-oxidant, anti-inflammatory, and anti-asthma activities (Aam et al., 2010; Fernandes et al., 2008; Kim & Rajapaksea, 2005). However, the effects of COS for anti-allergic activity remain to be unclear. In the present study, the anti-allergic effects of COS with different molecular weight ranges (1–3 kDa, 3–5 kDa, and 5–10 kDa) were

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evaluated by measuring the effect on degranulation and cytokine generation in rat basophilic leukemia RBL-2H3 cells as a part of our continuous study of COS.

2. Materials and methods

2.1. Reagents

Chemicals and cell culture materials were obtained from the following sources: RPMI-1640 medium, fetal bovine serum (FBS), antibiotics, and antimycotics were obtained from Gibco BRL (Gaithersburg, MD, USA). Enzyme immunoassay reagents for cytokine assays were purchased from R&D Systems (Minneapolis, MN, USA). Oligo $(dT)_{15}$ primer, M-MLV reverse transcriptase, and GoTaq DNA polymerase were purchased from Promega (Madison, WI, USA). Calcium-specific fluorescence probe (Fura-2/AM) was purchased from Tocris Bioscience (Ellisville, MO, USA). All other reagents, including calcium ionophore A23187, phorbol 12-myristate 13-acetate (PMA), *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, hydroxyethyl piperazinylethanesulfonic acid (HEPES), L-glutamine, dimethyl sulfoxide (DMSO), *o*-phthalaldehyde (OPA), and MTT reagent [3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of chitooligosaccharides by ultra filtration membrane bi reaction

Chitooligosaccharides (COS) were kindly donated by Kitto Life Co. (Seoul, Korea). COS were prepared by continuous hydrolysis of chitosan in an ultra filtration (UF) membrane reaction system connected to an immobilized enzyme column, in which chitosanase from *Bacillus sp*. was adsorbed to chitin as a carrier for immobilization, according to our previous method (Jeon & Kim, 2000). A 1% chitosan solution was prepared by dispersing chitosan in a 1:1 ratio with water, followed by dissolving and stirring in 400 ml of lactic acid (1 M) and dilution up to 15 l with water. The pH was adjusted to 5.5 with saturated $NaHCO_3$ solution. Chitosan was hydrolyzed by enzymatic reaction in the reaction system and fractioned by passing through UF membranes with molecular weight cut offs (MWCOs) of 10 kDa, 5 kDa, 3 kDa, and 1 kDa. COS I, COS II, and COS III were named as chitooligosaccharides of 1–3 kDa, 3–5 kDa, and 5–10 kDa, respectively. Stock solution (100 mg/ml) was made in PBS and sterilized by filtration through a 0.2 μm sterilized filter.

2.3. Cell culture and cell viability assay

RBL-2H3 mast cells were purchased from the Korean Cell Line Bank (Seoul, Korean). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES buffer, 100 U/ml of penicillin G, and 100 mg/ml of streptomycin.

The viability levels of RBL-2H3 cells were determined by MTT [3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide] assay. The cells were grown in 96-well plates at a density of 2×10^5 cells/ml. After 24 h, cells were washed with fresh medium and treated with different concentrations of COS samples. After 24 h of incubation, cells were rewashed and treated with MTT solution (1 mg/ml, final concentration) for 4 h. Finally, the supernatant was removed, and DMSO (100 μl) was added to solubilize the formed formazan salt. The amount of formazan salt was determined by measuring the absorbance at 540 nm using a microplate reader (GENios® Tecan Austria GmbH, Austria). Viability of cells was quantified as a percentage compared to blank.

2.4. Histamine release assay

Histamine release was measured by spectrofluorometric assay. RBL-2H3 cells were seeded into 24-well plates (2×10^5 cells/ml). Cells were treated with different concentrations of samples for 12 h. The treated cells were washed by Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, and 1.8 mM $CaCl_2$) and stimulated with calcium ionophore A23187 (1 μM, final concentration) at 37 °C for 30 min. Then, 0.5 N NaOH (40 μl) and 2.5 mg/ml of *o*-phthalaldehyde (20 μl) were added to 100 μl of the supernatant solution and incubated for 30 min. The reaction was terminated by the addition of 3 N HCl (10 μl). The fluorescence intensity was measured at an excitation wavelength of 365 nm and an emission wavelength of 465 nm. The supernatant from the non-stimulated cells was used as a blank while the supernatant from the stimulated cells with calcium ionophore A23187 alone was used as the control. The histamine release levels were calculated as a percentage compared to that of control: histamine release (%) = (fluorescence intensity of tested sample − fluorescence intensity of blank)/(fluorescence intensity of control − fluorescence intensity of blank) × 100.

2.5. β-Hexosaminidase release assay

β-Hexosaminidase assay was performed as described previously with some modifications (Na et al., 2005). Cells were treated with different concentrations of samples for 12 h. The treated cells were washed two times with Tyrode buffer and stimulated with calcium ionophore A23187 (1 μM, final concentration) at 37 °C for 30 min. Aliquots (50 μl) of the supernatants were incubated with 50 μl of 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) at 37 °C for 1 h. At the end of incubation, 250 μl of carbonate buffer containing 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$ (pH 10) was added, and then the absorbance due to formation of *p*-nitrophenol was measured at 410 nm. The supernatant from the non-stimulated cells was used as a blank while the supernatant from the stimulated cells with calcium ionophore A23187 alone was used as a control. The β-hexosaminidase release levels were calculated as a percentage compared to that of control: β-hexosaminidase release (%) = (absorbance of tested sample − absorbance of blank)/(absorbance of control − absorbance of blank) × 100.

2.6. Measurement of intracellular Ca^{2+} levels

Intracellular Ca^{2+} was measured using a calcium reactive fluorescence probe, Fura 2/AM. The cells were seeded in black 96-wells microplates (2×10^5 cells/ml) and incubated with different concentrations of the test samples. The cells were incubated with 2 μM Fura-2/AM for 1 h and then washed two times with Tyrode buffer. The treated cells were stimulated with calcium ionophore A23187 (1 μM, final concentration) at 37 °C for 15 min. Fura-2/AM fluorescence was monitored at an excitation wavelength of 360 nm and an emission wavelength of 528 nm. The supernatant from the non-stimulated cells was used as a blank while the supernatant from the stimulated cells with calcium ionophore A23187 alone was used as a control. Intracellular calcium level (%) = (Fura-2/AM fluorescence of tested sample − Fura-2/AM fluorescence of blank)/(Fura-2/AM fluorescence of control − Fura-2/AM fluorescence of blank) × 100.

2.7. Measurement of cytokine production

For cytokine immunoassays, RBL-2H3 cells (1×10^6 cells/ml) were treated with different concentrations of COS for 12 h and then stimulated with calcium ionophore A23187 (1 μM, final concentration) plus phorbol 12-myristate 13-acetate (PMA) (20 nM, final

Table 1
Gene-specific primer sequences used for reverse transcription-polymerase chain reaction.

Gene	Direction	Sequence
IL-1β	Sense	5′-ATG-GCA-ACT-GTT-CCT-GAA-CTC-AAC-T-3′
	Antisense	5′-TTT-CCT-TTC-TTA-GAT-ATG-GAC-AGG-AC-3′
IL-4	Sense	5′-GTA-CTG-TGC-AGC-CCT-GGA-AT-3′
	Antisense	5′-TTT-AGA-AAC-TGG-GCC-ACC-TC-3′
IL-6	Sense	5′-AGT-TGC-CTT-CTT-GGG-ACT-GA-3′
	Antisense	5′-CAG-AAT-TGC-CAT-TGC-ACA-AC-3′
TNF-α	Sense	5′-ATG-AGC-ACA-GAA-AGC-ATG-ATC-3′
	Antisense	5′-TAC-AGG-CTT-GTC-ACT-CGA-ATT-3′
GAPDH	Sense	5′-TGA-AGG-TCG-GTG-TGA-ACG-GAT-TTG-GC-3′
	Antisense	5′-CAT-GTA-GGC-CAT-GAG-GTC-CAC-CAC-3′

concentration) for 12 h. Supernatants were collected, and production of IL-1β, IL-4, IL-6, and TNF-α was quantified by sandwich immunoassays using the protocol supplied by R&D systems.

2.8. Reverse transcription-polymerase chain reaction analysis

Reverse transcription-polymerase chain reaction (RT-PCR) was used to analyze the expression of cytokine mRNA. Total cellular RNA was isolated using Trizol reagent according to manufacturer's instructions. For cDNA synthesis, total RNA (2 μg) was added to RNase-free water containing oligo (dT), followed by denaturation at 70 °C for 5 min and then cooling. RNA was reverse-transcribed in a master mix containing 1X RT buffer, 1 mM dNTPs, 500 ng of oligo (dT), 140 U of M-MLV reverse transcriptase, and 40 U of RNase inhibitor at 42 °C for 1 h. The resulting cDNA samples were then subjected to PCR analysis in the presence of specific sense and antisense primers. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primer sequences used in this study are listed in Table 1. Amplification of IL-1β, IL-4, IL-6, TNF-α, and GAPDH was performed by 27 cycles at 95 °C for 45 s (denaturation), 55 °C for 50 s (annealing), and 72 °C for 1 min (extension). The amplified products were analyzed by 1.5% agarose gel electrophoresis. Gels were then stained with 1 mg/ml of ethidium bromide and visualized by UV light using AlphaEase® gel image analysis software (Alpha Innotech. San Leandro, CA, USA).

2.9. Western blot analysis

Western blotting was used to analyze protein expression. The treated cells were lysed in RIPA lysis buffer (NP-40, Sigma–Aldrich, USA) at 4 °C for 30 min. Cell lysates (25 μg of protein/sample) were subjected to 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and then blocked in TBS-T buffer (20 mM Tris, pH 7.6, 0.1% Tween 20) containing 5% (w/v) bovine serum albumin (BSA). After washing three times with TBS-T buffer, the membrane was probed with primary antibodies (diluted 1:1000) for at least 1 h. Subsequently, horseradish peroxidase (HRP)-conjugated IgG secondary antibody (diluted 1:5000) was applied for 1 h at room temperature. The immunoreactive proteins were visualized using an enhanced chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences, UK) according to manufacturer's instructions. Western blot bands were visualized using LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

2.10. Statistical analysis

The statistical differences between control and sample groups were assessed by one-way ANOVA with Duncan's multiple range tests. Differences were considered significant at $p < 0.05$. The statistical software package, SAS v9.1 (SAS Institute Inc., Cary, NC, USA), was used for the analysis.

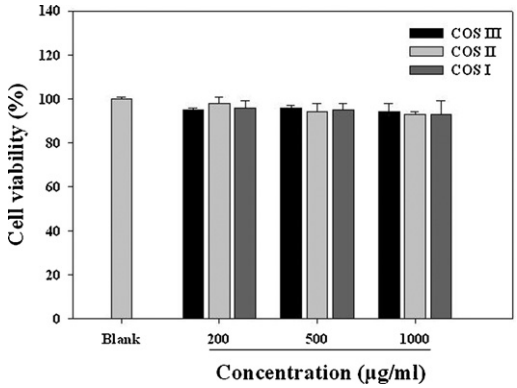

Fig. 1. Cytotoxicity effects of chitooligosaccharides in RBL-2H3 cells. Cytotoxicity levels were assessed by MTT cell viability assays at different concentrations. Cells were incubated with the test samples for 24 h. Each determination was made in triplicate, and the data are expressed as means ± SD. COS I, COS II and COS III are chitooligosaccharides of molecular weight ranges of 1–3 kDa, 3–5 kDa and 5–10 kDa, respectively. Blank: non-treatment group.

3. Results and discussion

3.1. Cytotoxic effects of chitooligosaccharides

In order to investigate the cytotoxic effects, RBL-2H3 cells were cultured with various concentrations of COS. At a concentration of 1000 μg/ml, the lowest cell viability of RBL-2H3 was observed at the ratios of 93%, 93%, and 94% for COS with molecular weight ranges of 1–3 kDa (COS I), 3–5 kDa (COS II), and 5–10 kDa (COS III), respectively (Fig. 1). These COS were evidenced no cytotoxic effects in a concentration range of 200–1000 μg/ml. According to these results, concentrations ranging from 200 to 1000 μg/ml were selected for further experiments.

3.2. Effects of chitooligosaccharides on degranulation

Histamine is considered as the major mediator of the acute inflammatory and immediate hypersensitivity responses. Interactions between histamine and its cell-surface receptors trigger acute symptoms, leading to the development of such symptoms as acute rhinitis, cramping, bronchoconstriction, and cutaneous weal (Jutel, Akdis, & Akdis, 2009). Likewise, the enzyme β-hexosaminidase which has been found to be associated with a variety of inflammatory diseases is also released along with histamine in mast cells (Matsuda, Tewtrakul, Morikawa, & Yoshikawa, 2004; Wendeler & Sandhoff, 2009). Thus, histamine and β-hexosaminidase are major targets for effective anti-allergic drugs and often used as markers of mast cell degranulation (Matsuda et al., 2004). In our study, we evaluated the ability of COS to inhibit calcium ionophore A23187-induced degranulation in RBL-2H3 mast cells. As shown in Table 2, COS were shown to be able to inhibit degranulation via attenuating histamine and β-hexosaminidase releases in calcium ionophore A23187-stimulated RBL-2H3 cells. The attenuation of histamine and β-hexosaminidase releases varied between COS I, COS II, and COS III. Histamine release levels upon treatment with 1000 μg/ml of COS I, COS II, and COS III were 32%, 48%, and 80%, respectively, whereas the levels of β-hexosaminidase release were 34%, 42%, and 72%, respectively. COS I and COS II appear the most effective in suppressing the releases of histamine and β-hexosaminidase. In contrast, COS III exhibited only minor changes as compared to that of the stimulated control. Evidently, calcium ionophore A23187 induces mast cell degranulation by increasing cell-membrane permeability and intracellular Ca^{2+} (Li et al., 2008). Therefore, the increase in membrane permeability may be one of the essential trigger for degranulation from mast cells. In this sense, anti-allergic

Table 2
Effects of COS on histamine and β-hexosaminidase release in calcium ionophore A23187-stimulated RBL-2H3 cells.

	Histamine releases (%)			β-Hexosaminidase releases (%)		
	200 μg/ml	500 μg/ml	1000 μg/ml	200 μg/ml	500 μg/ml	1000 μg/ml
Control	100 ± 1.9*,a	100 ± 1.9[a]	100 ± 1.9[a]	100 ± 1.7[a]	100 ± 1.7[a]	100 ± 1.7[a]
COS I	83 ± 7.5[b]	71 ± 0.8[d]	32 ± 6.7[d]	79 ± 1.3[c]	56 ± 2.8[d]	34 ± 6.7[c]
COS II	91 ± 3.2[ab]	82 ± 1.2[c]	48 ± 5.1[c]	89 ± 2.4[b]	66 ± 2.4[c]	42 ± 3.1[c]
COS III	99 ± 1.1[a]	91 ± 3.3[b]	80 ± 0.7[b]	92 ± 2.0[b]	83 ± 2.4[b]	72 ± 0.5[b]

[a–d]Means with different letters at each concentration are significantly different ($p < 0.05$) by Duncan's multiple range test.
COS I, COS II and COS III are chitooligosaccharides of molecular weight ranges of 1–3 kDa, 3–5 kDa and 5–10 kDa, respectively.
* Means ± SD.

agents having a membrane-stabilizing action may be desirable. It suggests that COS might stabilize the lipid bilayer membrane, thus causing an obstacle for degranulation induced by calcium ionophore A23187.

3.3. Effects of chitooligosaccharides on intracellular Ca^{2+} level

Next, we examined whether COS alleviate the intracellular Ca^{2+} in mast cells. In Table 3, it shows the effects of COS on intracellular calcium ion levels in calcium ionophore A23187-stimulated RBL-2H3 cells. As a result, COS attenuated the increase in intracellular Ca^{2+} level induced by calcium ionophore A23187. Intracellular Ca^{2+} levels upon treatment with 1000 μg/ml of COS I, II, and III were 37%, 48%, and 78%, respectively. Obviously, COS I and II substantially decreased intracellular Ca^{2+} in a dose-dependent manner, while COS III has a slight effect. It known that an increase in the intracellular Ca^{2+} concentration is a necessary and sufficient stimulus for degranulation in mast cells (Neher, 1988). We demonstrated that COS treatment concentration-dependently inhibited intracellular Ca^{2+} in the tested cells. Thus, we have suggested that COS inhibited mast cell degranulation via inhibition of intracellular Ca^{2+}. This assumption is supported by previous report that some anti-allergic drugs inhibit degranulation through blockage of calcium ion influx in mast cell (Matsubara, Masaki, Ohmori, Karasawa, & Hasegawa, 2004).

3.4. Effects of chitooligosaccharides on cytokine expression and production

Mast cells represent potential source of various cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-6, IL-8, and IL-13. Although these inflammatory cytokines possess beneficial effects on host defense, they could trigger pathological conditions. The excessive expression and production of these cytokines alter the local microenvironment and lead to the recruitment of inflammatory cells such as neutrophils and eosinophils eventually (Wang et al., 2007). Therefore, modulation of inflammatory cytokines from mast cells is a one of the key indicators of reduced allergic symptoms. With this regard, we evaluated whether COS suppress the expression and production of cytokines in mast cells. In this assay, a combined stimulation of calcium ionophore A23187 and PMA was used because they are known to induce the generation of inflammatory cytokines (Hong et al., 2010). As a result, stimulation of A23187 plus PMA provoked the mRNA expression of IL-1β, IL-4, IL-6, and TNF-α in RBL-2H3 cells (Fig. 2). Meanwhile, pretreatment of COS dose-dependently inhibited expression of IL-1β, IL-4, IL-6, and TNF-α induced by A23187 plus PMA. Pretreatment of COS I at a concentration of 1000 μg/ml remarkably inhibited the expression of these cytokines, while COS II and COS III showed a moderate effect. Moreover, we confirmed the effect of COS on the expression of IL-1β, IL-4, IL-6, and TNF-α through measuring the production of these cytokines in culture supernatants by ELISA. Similar to mRNA expression, stimulation of A23187 plus PMA also induced the production of IL-1β, IL-4, IL-6, and TNF-α in RBL-2H3 cells (Fig. 3). In contrast, COS treatment substantially inhibited the secretion of these cytokines in the culture medium in a dose-dependent manner. At a concentration of 1000 μg/ml, COS I exhibited the best effect for the production of IL-1β, IL-4, IL-6, and TNF-α with inhibition rates of 56%, 63%, 53%, and 67%, respectively. These results indicate that COS inhibited the gene expression and production of IL-1β, IL-4, IL-6, and TNF-α in A23187 plus PMA-stimulated RBL-2H3 cells. As mentioned in previous studies, intracellular Ca^{2+} are also involved in the activation of TNF-α and IL-gene expressions (Choi, Kang, Koh, Nam, & Friedman, 2007; Shin et al., 2005). Because of the reducing effect of COS on the intracellular Ca^{2+}, we suggest that one possible pathway of the inhibitory effect of COS on the cytokine expression is mediated by the reduction of intracellular Ca^{2+} in RBL-2H3 cells.

Table 3
Effect of COS on the intracellular Ca^{2+} level in calcium ionophore A23187-stimulated RBL-2H3 cells.

	Intracellular Ca^{2+} level (%)		
	200 μg/ml	500 μg/ml	1000 μg/ml
Control	100 ± 3.9*,a	100 ± 3.9[a]	100 ± 3.9[a]
COS I	81 ± 5.0[c]	51 ± 2.8[d]	37 ± 1.8[c]
COS II	87 ± 2.8[bc]	65 ± 4.3[c]	48 ± 4.6[c]
COS III	96 ± 1.8[ab]	88 ± 3.9[b]	78 ± 7.1[b]

[a–d]Means with different letters at each concentration are significantly different ($p < 0.05$) by Duncan's multiple range test.
COS I, COS II and COS III are chitooligosaccharides of molecular weight ranges of 1–3 kDa, 3–5 kDa and 5–10 kDa, respectively.
* Means ± SD.

3.5. Effects of chitooligosaccharides on mitogen-activated protein kinases activation

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that play a significant role in connecting cell-surface receptors to changes in transcriptional programs (Geest & Coffer, 2009). Due to essential roles in regulating the transcriptional activity of various cytokine genes in mast cells, MAPKs are appropriate targets for pharmacological treatment of allergic inflammation. Therefore, to evaluate the mechanisms of action of COS on cytokine expression, we examined the effects of COS on MAPKs activation. According to present results, calcium ionophore A23187 plus phorbol 12-myristate 13-acetate (PMA) induce the phosphorylation of MAPK pathways, including extracellular signal-regulated kinase (MEK/ERK), p38 kinase, and c-jun *N*-terminal kinase (JNK) in RBL-2H3 cells (Fig. 4). Additionally, it was found that COS inhibited the activation of MEK, ERK, and p38 kinase but not of JNK in a dose-dependent manner. Notably, both COS I and COS II exhibited significant inhibition on the phosphorylation of these kinases. These results indicate that the anti-allergic effect of COS, at least in part, might be derived through regulation of the MAPK pathway.

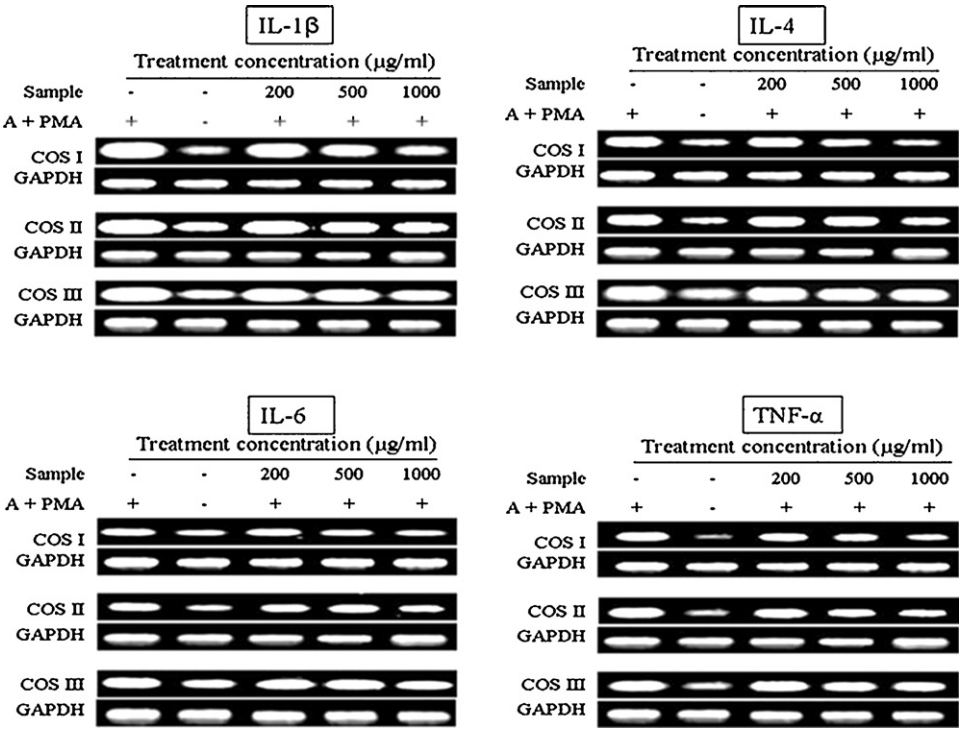

Fig. 2. Effects of chitooligosaccharides on mRNA expression of IL-1β, IL-4, IL-6, and TNF-α in calcium ionophore A23187 plus phorbol 12-myristate 13-acetate (PMA)-stimulated RBL-2H3 cells. The cells were incubated with different concentrations of COS for 12 h and then stimulated with calcium ionophore A23187 (1 μM, final concentration) plus PMA (20 nM, final concentration) for 12 h. Cytokine expression levels were measured by RT-PCR. A + PMA: calcium ionophore A23187 plus phorbol 12-myristate 13-acetate; Control: +A + PMA–COS; Blank: −A − PMA–COS.

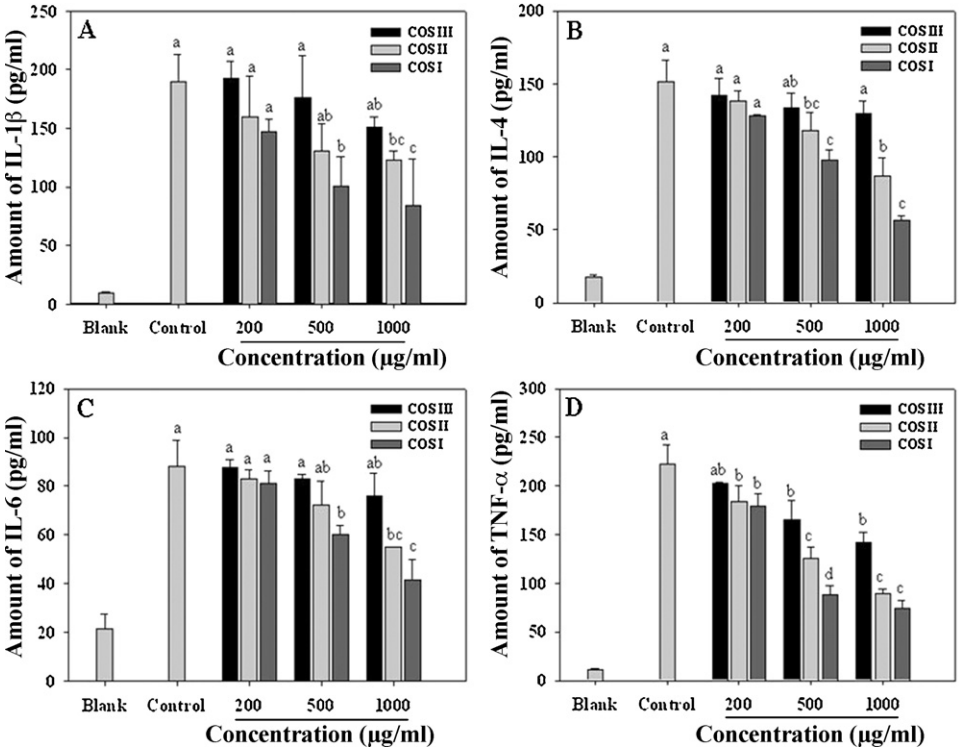

Fig. 3. Effects of chitooligosaccharides on inflammatory cytokine release in calcium ionophore A23187 plus phorbol 12-myristate 13-acetate (PMA)-stimulated RBL-2H3 cells. The cells were treated with different concentrations of COS for 12 h and then stimulated with A23187 (1 μM, final concentration) plus PMA (20 nM, final concentration) for 12 h. The production levels of IL-1β (A), IL-4 (B), IL-6 (C), and TNF-α (D) were determined in cultured media using a commercial ELISA kit. [a–d]Means with different letters at each concentration are significantly different ($p < 0.05$) by Duncan's multiple range test. A + PMA: calcium ionophore A23187 plus phorbol 12-myristate 13-acetate; Control: +A + PMA–COS; Blank: −A − PMA–COS.

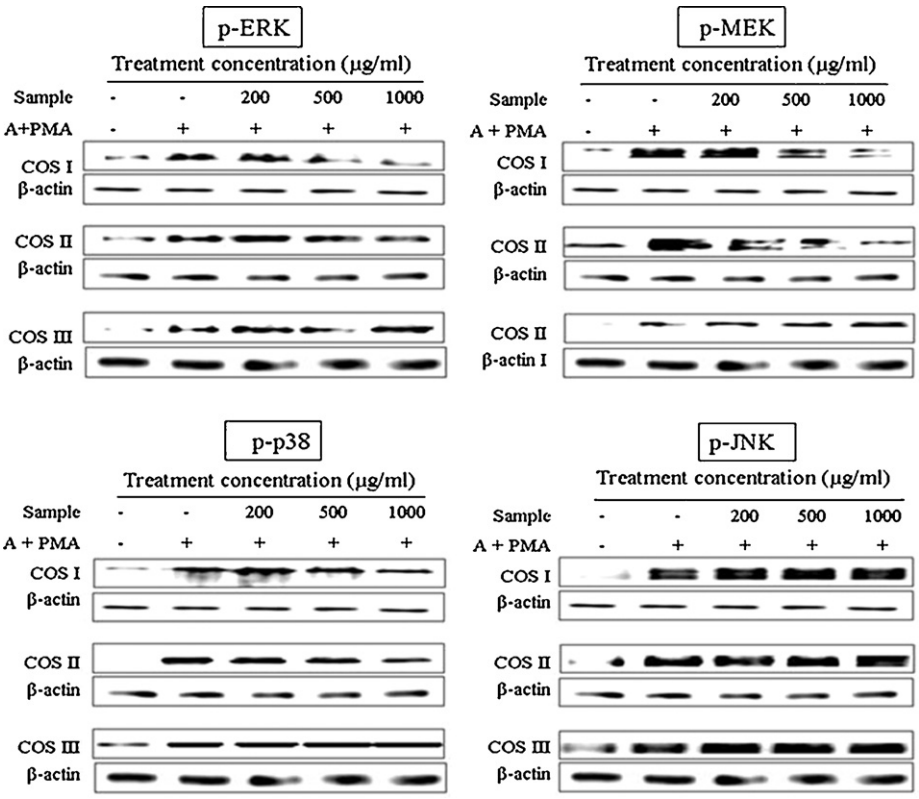

Fig. 4. Effects of chitooligosaccharides on regulation of MAPKs in calcium ionophore A23187 plus PMA-stimulated RBL-2H3 cells. The cells were incubated with different concentrations of COS and then stimulated with A23187 (1 μM) plus PMA (20 nM) for 4 h. The regulation levels were determined by Western blotting. The phosphorylation of extracellular signal-regulated kinase, p38 kinase, and c-jun *N*-terminal kinase were abbreviated by p-ERK/p-MEK, p-p38, and p-JNK, respectively. A + PMA: calcium ionophore A23187 plus phorbol 12-myristate 13-acetate; Control: +A + PMA–COS; Blank: −A − PMA–COS.

4. Conclusion

In all assays performed, COS I possess the highest inhibitory effects on degranulation and cytokine generation of mast cells, followed by COS II and COS III, respectively. Importantly, it remains unknown why 1–3 kDa was the most effective molecular weight for COS activity. However, we suggested that the different molecular weight ranges of COS I, COS II, and COS III might lead to variable absorption, which corresponds to the different inhibitory effects in mast cells. Our results indicate that COS contribute to attenuation of allergic reactions and might be a promising candidate for novel inhibitor of allergic reaction.

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