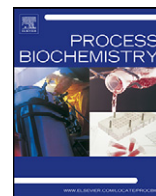




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Short communication

Protective effect of chitosan oligosaccharides against FcεRI-mediated RBL-2H3 mast cell activation

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ABSTRACT

The activation of mast cells by immunoglobulin E-mediated stimuli is considered as a central event in allergic responses. In this regard, chitosan oligosaccharides (COS) of two different molecular weight ranges (1–3 kDa and 3–5 kDa) were investigated for their capabilities against the activation of RBL-2H3 mast cell sensitized with dinitrophenyl-specific immunoglobulin E antibody and stimulated by antigen dinitrophenyl-bovine serum albumin. It was found that COS significantly inhibited RBL-2H3 cell degranulation via attenuating the releases of histamine and β-hexosaminidase. Moreover, the inhibitory activity of COS was accompanied by a reduction in intracellular Ca²⁺ elevation. Notably, the expression of immunoglobulin Fc epsilon receptor I (FcεRI) in RBL-2H3 cells was down-regulated by COS treatment in a dose-dependent manner. The suppressive effect of COS on RBL-2H3 cell activation suggested that COS may be potential candidates of novel inhibitors against allergic reactions.

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1. Introduction

Mast cells are well known for their important role in pathogenesis of allergic diseases such as hay fever, atopic dermatitis, and asthma [1]. Specially, mast cells express high-affinity receptor for IgE (FcεRI), which is responsible for mast cell activation in the initiation of the allergic reactions [2]. The aggregation of complexes of immunoglobulin E (IgE) and FcεRI by multivalent antigen causes a cascade of intracellular events, including the phosphorylation of mitogen-activated protein kinases (MAPKs), the increase of intracellular Ca²⁺ level, and the induction of degranulation [3]. Subsequently, it leads to the release of preformed and newly synthesized inflammatory mediators such as histamine, prostaglandins, leukotrienes, and cytokines [4]. These substances 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 [5]. Therefore, modulation of mast cell activation can provide a useful therapeutic strategy for the control of allergic inflammatory diseases.

During the past decades, chitosan has received considerable attention due to its biodegradable, non-toxic, and non-allergenic properties, which made it possible to be used in many fields including food, cosmetics, biomedicine, agriculture, and environmental protection [6]. Recently, it has been more of interest for converting chitosan to chitosan oligosaccharides (COS), which are not only water-soluble and higher oral absorption profiles [7] but also possess numerous biological activities, such as angiotensin-converting enzyme inhibition, anti-oxidant, antimicrobial, anti-inflammation, anti-cancer, immuno-stimulant, anti-diabetic, anti-Alzheimer's, anti-coagulant [6,8], and anti-asthma [9]. In the previous study, COS have been determined to be effective against calcium ionophore A23187-induced mast cell degranulation [10]. However, the mechanism underlying the inhibitory effect of COS on FcεRI-mediated allergic response remains to be elucidated. Thus, the aim of present study was to further evaluate whether COS suppress mast cell activation induced by IgE-antigen stimulation.

Rat basophilic leukemia (RBL-2H3) cells display properties of mucosal-type mast cells. The RBL-2H3 cells respond with degranulation following crosslinking of their IgE-bound FcεRI by multivalent allergens, with the release of histamine and β-hexosaminidase [11]. Thus, this cell line has been commonly and successfully used in studies on binding of IgE to FcεRI receptors and subsequent downstream events such as degranulation. Likewise, we used RBL-2H3 cells as a model cell line for evaluating the inhibitory effect of COS on FcεRI-mediated cell activation.

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2. Materials and methods

2.1. Reagents

Chemicals and cell culture materials were obtained from the following sources: fetal bovine serum (FBS), antibiotic, and antimycotics were obtained from Gibco BRL (Gaithersburg, MD, USA). Dinitrophenyl-specific immunoglobulin E (DNP-specific IgE) antibody and dinitrophenyl-bovine serum albumin (DNP-BSA) antigen were purchased from Sigma–Aldrich (St. Louis, MO, USA). Anti-FcεRI α, β, and γ chain antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, 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 hydroxyethyl piperazinylthanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *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. COS preparation and cell culture

COS were kindly donated by Kitto Life Co. (Seoul, Korea). COS were prepared as represented in Vo et al. [10]. COS I and COS II were named as chitosan oligosaccharides of 1–3 kDa and 3–5 kDa, respectively. Stock solution (100 mg/ml) was made in PBS and sterilized by filtration through a 0.2 μm sterilized filter.

RBL-2H3 mast cells were purchased from Korean Cell Line Bank (Seoul, Korea). They were cultured in a 5% CO₂ and 37 °C humidified atmosphere 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 penicillin G, and 100 mg/ml streptomycin.

2.3. Histamine and β-hexosaminidase release assays

RBL-2H3 cells were seeded into 24-well plates (2 × 10⁵ cells/ml). Cells were treated with different concentrations of samples for 1 h before sensitized overnight with dinitrophenyl-specific immunoglobulin E (DNP-specific IgE) antibody (100 ng/ml, final concentration). The sensitized cells were washed two times with Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 1 mM MgCl₂, 12 mM NaHCO₃, and 1.8 mM CaCl₂) and stimulated with antigen dinitrophenyl-bovine serum albumin (DNP-BSA) (50 ng/ml, final concentration) for 60 min. Histamine and β-hexosaminidase release in the supernatants was determined as previously described [10]. Histamine or β-hexosaminidase release levels were calculated as a percentage compared to control: Release ratio (%) = $(T - B)/(C - B) \times 100$, where *B* is a group without stimulation and without test sample, *C* is a group with stimulation and without test sample, and *T* is a group with stimulation and with test sample.

2.4. Measurement of intracellular Ca²⁺ level

Intracellular calcium was measured using the calcium reactive fluorescence probe, Fura 2-AM. Briefly, RBL-2H3 cells were seeded in black 96-wells plates (2 × 10⁵ cells/ml). Cells were treated with different concentrations of samples for 1 h before sensitized overnight with DNP-specific IgE antibody (100 ng/ml, final concentration) and then incubated with Fura-2/AM (2 μM, final concentration) for 1 h. Cells were washed with Tyrode buffer and stimulated with antigen DNP-BSA (50 ng/ml, final concentration) for 15 min. The 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 and the supernatant from the stimulated cells with antigen DNP-BSA alone was used as a control. Intracellular calcium level (%) = $(\text{Fura-2/AM fluorescence of tested sample} - \text{Fura-2/AM fluorescence of blank}) / (\text{Fura-2/AM fluorescence of control} - \text{Fura-2/AM fluorescence of blank}) \times 100$.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

FcεRI α, β, and γ chain mRNA levels were determined by RT-PCR. 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 and oligo (dT), denatured at 70 °C for 5 min, and cooled immediately. RNA was reverse transcribed in a master mix containing 1 × RT buffer, 1 mM dNTPs, 500 ng oligo (dT), 140 U M-MLV reverse transcriptase, and 40 U 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. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primer sequences used in this study were as follows: for FcεRI α chain, sense 5'-ATT-GCC-TGA-ATC-ACC-AGC-A-3' and antisense 5'-AGG-GCT-GGG-ATT-ACA-GGC-GTG-3'; for FcεRI β chain, sense 5'-TAG-GGC-CAG-CTG-GTG-TTA-ATG-GCA-3' and antisense 5'-GAT-GAT-TCC-AGC-AGT-GGT-CIT-GCT-3'; for FcεRI γ chain, sense 5'-GCC-ACC-ATG-AAG-GAC-ACT-3' and antisense 5'-ACT-CTG-GTT-GGC-TTC-CTT-CA-3'; for GAPDH, sense 5'-TGA-AGG-TCG-GTG-TGA-ACG-GAT-TTG-GC-3' and antisense 5'-CAT-GTA-GGC-CAT-GAG-GTC-CAC-CAC-3'. The amplifications for FcεRI α, β, and

γ chain and GAPDH were performed by 32 cycles of 95 °C for 45 s (denaturation), 55 °C for 50 s (annealing), and 72 °C for 1 minute (extension). The amplified products were analyzed by 1.5% agarose gel electrophoresis. Gels were then stained with 1 mg/ml ethidium bromide visualized by UV light using AlphaEase® gel image analysis software (Alpha Innotech, San Leandro, CA, USA).

2.6. Western blot analysis

For the western blot analysis of total cellular FcεRI α, β, and γ chain expression, the cells were lysed in RIPA lysis buffer (NP-40, Sigma–Aldrich, USA) at 4 °C for 30 min. Cell lysates (25 μg protein/sample) were subjected to 10% SDS-PAGE gels under the gradient concentration, transferred onto the nitrocellulose membrane and blocked in 5% (w/v) bovine serum albumin (BSA) in TBS-T buffer (20 mM Tris, pH 7.6, 0.1% Tween 20). After washing membrane 3 times with TBS-T buffer, 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) for 1 h at room temperature. The immunoreactive proteins were visualized using the 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).

2.7. Statistical analysis

The statistical differences between control and sample groups were assessed by 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.

3. Results and discussion

3.1. Effect of COS on antigen-induced degranulation in RBL-2H3 mast cells

Mast cells contain numerous prominent cytoplasmic granules staining metachromatically with cationic dyes [12]. Evidently, mast cell activation causes the process of degranulation which is involved in the fusion of the cytoplasmic granule membranes with the plasma membrane. This is accompanied by the fast external release of granule-associated stored mediators [13]. Among the inflammatory substances released from mast cells, histamine and β-hexosaminidase remains the best-characterized and most potent mediator implicated in the majority of the acute symptoms in allergic responses [14,15]. Thus, histamine and β-hexosaminidase are major targets for potential anti-allergic drugs and often used as markers of mast cell degranulation. In our study, we evaluate effects of COS on degranulation via measuring level of histamine and β-hexosaminidase release from antigen-stimulated RBL-2H3 cells. It was observed that histamine and β-hexosaminidase were significantly released from RBL-2H3 cells sensitized with DNP-specific IgE antibody and stimulated by antigen DNP-BSA. Meanwhile, COS were found to be effective against RBL-2H3 cell degranulation by attenuating histamine and β-hexosaminidase release in a dose-dependent manner. Histamine release levels upon treatment with 1000 μg/ml of COS I and COS II were 24% and 45%, respectively (Fig. 1A), meanwhile the levels of β-hexosaminidase release were 29% and 43%, respectively (Fig. 1B). COS I exhibited a significant inhibition of RBL-2H3 degranulation, whereas COS II showed a moderate effect as compared to control. Specially, the inhibitory activity of COS on antigen-induced degranulation was recognized to be stronger than that of COS on degranulation induced by calcium ionophore A23187 in RBL-2H3 cells [10]. Herein, the inhibitory effect of COS on degranulation was suggested to be due to the inhibition of intracellular Ca²⁺ elevation and FcεRI expression in RBL-2H3 cells.

3.2. Effect of COS on intracellular Ca²⁺ elevation in antigen-stimulated RBL-2H3 cells

The FcεRI-cross linking leads to elevation of intracellular Ca²⁺ that is essential for degranulation of mast cells [16]. Since the

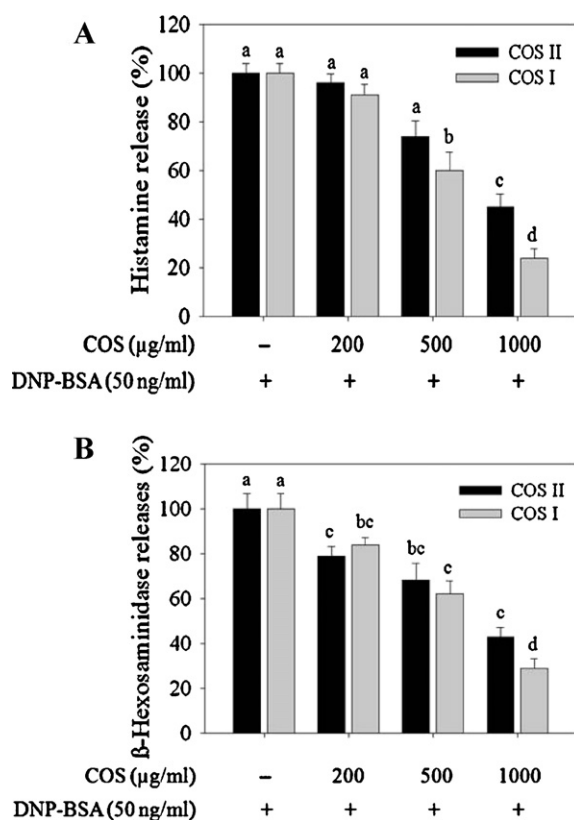


Fig. 1. Effects of COS on histamine (A) and β -hexosaminidase (B) release in RBL-2H3 mast cells. The cells were treated with different concentrations of COS for 1 h and sensitized overnight with DNP-specific IgE antibody before stimulation of antigen DNP-BSA for 60 min. The levels of histamine and β -hexosaminidase release were measured. Each determination was made in triplicate, and the data are showed as means $\pm$ SD. ^{a-d}Means with different letters at each concentration are significantly different ($p < 0.05$).

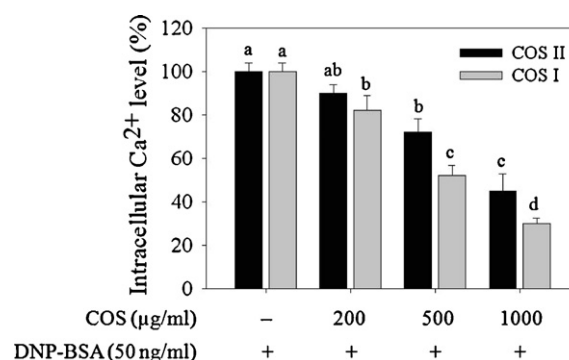


Fig. 2. Effect of COS on intracellular Ca²⁺ elevation in RBL-2H3 mast cells. The cells were treated with various doses of COS for 1 h and sensitized overnight with DNP-specific IgE antibody. The sensitized cells were then incubated with Fura-2/AM for 1 h before stimulated with antigen DNP-BSA for 15 min. The level of intracellular Ca²⁺ was monitored by a spectrofluorometric assay. Data show the means $\pm$ SD of three independent experiments. ^{a-d}Means with different letters at each concentration are significantly different ($p < 0.05$).

importance of intracellular Ca²⁺ in mast cell degranulation is well known, we attempted to determine whether COS alleviate intracellular Ca²⁺ level in antigen-stimulated RBL-2H3 cells. As shown in Fig. 2, COS diminished the increase in intracellular Ca²⁺ level induced by antigen stimulation. Intracellular Ca²⁺ levels upon treatment with 1000 µg/ml of COS I and COS II were 30% and 45%, respectively. Similarly, the COS I was found to be stronger inhibitor than COS II against intracellular Ca²⁺ elevation in RBL-2H3 cells. These results suggested that the inhibitory effect of COS on RBL-2H3 cell degranulation is also due to the suppression of intracellular Ca²⁺ elevation. This assumption is supported by previous studies that some anti-allergic agents inhibit degranulation through blockage of calcium ion influx in mast cell [17,18].

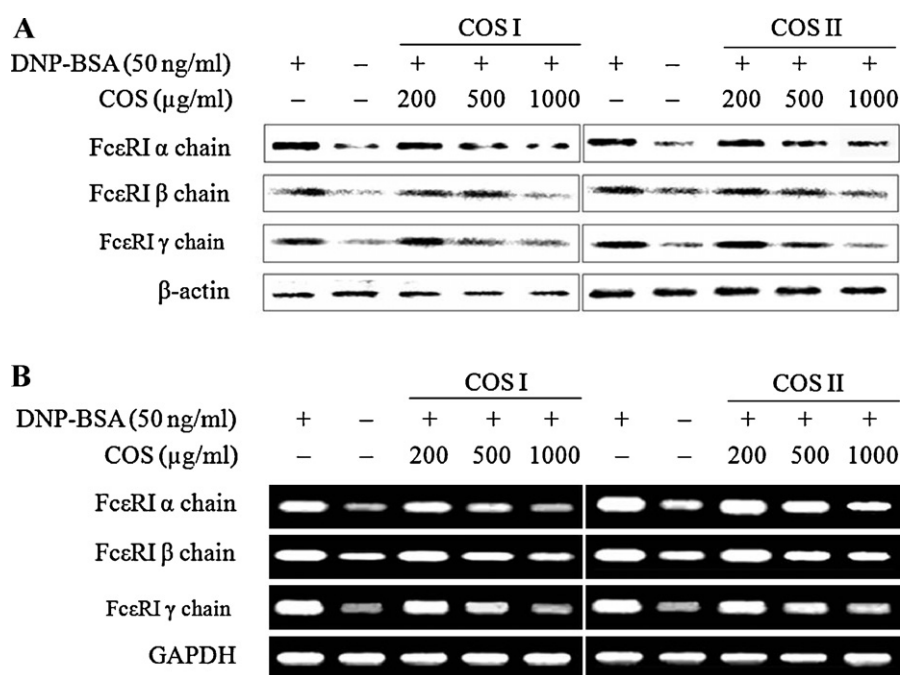


Fig. 3. Effects of COS on protein (A) and mRNA (B) expression level of Fc ϵ RI α , β , and γ chain in RBL-2H3 mast cells. The cells were treated with various concentrations of COS for 1 h and sensitized overnight with DNP-specific IgE antibody before stimulation of antigen DNP-BSA for 24 h. The expression level of Fc ϵ RI mRNA was determined via RT-PCR method, while the expression level of Fc ϵ RI protein was assessed by Western blotting analysis. GAPDH and β -actin were used as internal controls for assays of RT-PCR and Western blot, respectively.

3.3. Effect of COS on antigen-induced FcεRI expression in RBL-2H3 cells

Substantially, allergic responses such as intracellular Ca^{2+} elevation and degranulation in mast cells are initiated by FcεRI activation. Evidently, FcεRI is a tetrameric structure composed of one α chain (FcεRI α), one β chain (FcεRI β), and two disulfide-linked γ chains (FcεRI γ). The FcεRI α chain mostly extends out to the extracellular region and binds to the Fc portion of IgE with high affinity. FcεRI β and γ chains are involved in initiating the downstream signal propagation leading to release of preformed and newly synthesized mediators [19]. Especially, it was reported that IgE can potentially enhance the FcεRI expression of mast cells, and show the enhanced sensitivity and a stronger response to antigenic stimulation [20]. Meanwhile, down-regulation of FcεRI expression can lead to attenuation of the antigen-mediated allergic reactions [21,22]. In this sense, we further investigated whether COS inhibit antigen-induced FcεRI expression in RBL-2H3 cells. Fig. 3 shows the effect of COS on expression level of FcεRI protein (Fig. 3A) and mRNA (Fig. 3B) using Western blot and RT-PCR analysis, respectively. Obviously, the expressions of FcεRI protein and mRNA were significantly augmented after antigen stimulation alone. Conversely, treatment of COS before antigen stimulation resulted in a dose-dependent decrease in FcεRI expression. At the concentration of 1000 μg/ml, COS pronouncedly suppressed protein and mRNA expression of FcεRI α, β, and γ chain in RBL-2H3 mast cells. Herein, the suppressive effect of COS I was also observed to be more efficient than that of COS II. According to Fujimura et al., ERK activation is associated with expression of FcεRI in KU812 cells [23]. In our previous study, COS was evidenced to be able to reduce ERK phosphorylation in RBL-2H3 cells [10]. Thus, it was suggested that FcεRI-suppressive effect of COS may be caused by reduction of the phosphorylated ERK. On the other hand, the expression of FcεRI subunits was also referred to be due to regulation of two transcription factors, GATA-1 and Elf-1 in rodents and other mammals, including humans [24]. Consequently, further studies on the regulation of these transcription factors for FcεRI expression are required for a better understanding of the mechanisms involved in suppression of FcεRI expression by COS.

4. Conclusions

In conclusion, this study showed the inhibitory effect of COS against FcεRI-mediated mast cell activation through down-regulation of FcεRI expression, which might cause reduction of intracellular Ca^{2+} elevation and subsequent inhibition of degranulation in RBL-2H3 cells. As a result, COS may contribute to attenuation of the initial phase of immediate type allergic reactions and serve as potential therapeutic agents for allergic diseases. However, future studies are needed to confirm the anti-allergic property of COS in an animal-mediated allergy model.

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