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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 Ca^{2+} 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.

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 Ca^{2+} 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 piperazinylethanesulfonic 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% CO_2 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 (2×10^5 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 NaH_2PO_4, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, and 1.8 mM $CaCl_2$) 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 Ca^{2+} 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 (2×10^5 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 (%) = (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.

2.5. 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), denaturated 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-CTT-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 albumine (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 Ca^{2+} elevation and FcεRI expression in RBL-2H3 cells.

3.2. Effect of COS on intracellular Ca^{2+} elevation in antigen-stimulated RBL-2H3 cells

The FcεRI-cross linking leads to elevation of intracellular Ca^{2+} that is essential for degranulation of mast cells [16]. Since the