



Gallic acid-grafted chitoooligosaccharides suppress antigen-induced allergic reactions in RBL-2H3 mast cells

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ABSTRACT

In this study, a bioactive derivative of chitoooligosaccharides (3–5 kDa) was synthesized via grafting of gallic acid onto chitoooligosaccharides (G-COS) to enhance anti-allergic activity. Hence, G-COS was evaluated for its capabilities against allergic reactions in RBL-2H3 mast cells sensitized with dinitrophenyl-specific immunoglobulin E antibody and stimulated by antigen dinitrophenyl-bovine serum albumin. It was revealed that G-COS exhibited significant inhibition on histamine release and production as well as intracellular Ca^{2+} elevation at the concentration of 200 $\mu\text{g}/\text{ml}$. Likewise, the suppressive effects of G-COS on expression and production of interleukin (IL)-4 and tumor necrosis factor (TNF)- α were evidenced. Moreover, G-COS treatment caused a remarkable blockade on degradation of inhibitory κB - α (I κB - α) protein, translocation of nuclear factor (NF)- κB , and phosphorylation of mitogen-activated protein kinases (MAPKs). Notably, the inhibitory activities of G-COS on allergic reactions were found as a consequence of suppression of Fc ϵ RI expression in antigen-stimulated cells. Accordingly, G-COS was suggested to be a promising candidate of novel inhibitors against allergic reactions.

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1. Introduction

Allergy is considered as a disorder of the immune system in which an exaggerated response occurs when a person is exposed to normally harmless environmental substances, such as animal dander, house dust mites, foods, pollen, insects, and chemical agents (Arshad, 2010; Milián and Díaz, 2004). The initial event responsible for the development of allergic diseases is the generation of allergen-specific CD4⁺ Th2 cells. Once generated, effector Th2 cells produce IL-4, IL-5, IL-9, and IL-13 which cause the production of allergen-specific IgE by B cells (Akdis et al., 2005). Subsequently, allergic reactions are induced upon binding of allergen to IgE, which is tethered to the high-affinity IgE receptor (Fc ϵ RI) on surface of mast cells. Following the aggregation of cell-surface receptors is a cascade of intracellular events, including the increase of intracellular Ca^{2+} level, the release of preformed inflammatory mediators from secretory granules such as histamine and β -hexosaminidase, the generation and secretion of the newly synthesized substances such as leukotrienes, prostaglandins, and cytokines (Galli et al., 2008; Beaven and Metzger, 1993). These mediators are the origination of various pathophysiologic events in acute allergic reactions including airway constriction, mucous

production, and recruitment of other inflammatory cells (Galli et al., 2008). Accordingly, the control of inflammatory mediator production are especially important for the regulation of type I allergic reaction, thus allergic diseases may be managed.

Phenolic compounds are some of the most widespread molecules among plant secondary metabolites (Makoi and Ndakidemi, 2007). These phenolics are rich in some foods and medicinal plants, and considered as natural antioxidants (Li et al., 2010). Among them, gallic acid (GA, 3,4,5-trihydroxy benzoic acid) is particularly abundant in processed beverages such as red wine and green tea (Graham, 1992). It has been reported that gallic acid possesses a wide range of biological activities, including anti-oxidant, anti-inflammatory, anti-microbial, and anti-cancer activities (Kim et al., 2006). Notably, gallic acid has been determined to be a strong inhibitor against histamine release and pro-inflammatory cytokine production in mast cells (Kim et al., 2006).

Chitosan is known as a potential biomaterial due to its biodegradable, non-toxic, and non-allergenic properties, which made it possible to be used in many fields of food, cosmetics, biomedicine, agriculture, and environmental protection (Kim and Rajapakse, 2005). Moreover, chitoooligosaccharides (COS), which are generated via either chemical or enzymatic hydrolysis of chitosan, exhibit various biological activities, such as anti-bacterial, anti-tumor, anti-oxidant, anti-inflammatory, and anti-asthma activities (Kim and Rajapakse, 2005; Aam et al., 2010; Vo and Kim, 2010). In the previous studies, COS have been determined to be effective against

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mast cell degranulation (Vo et al., 2011, 2012). However, the inhibitory effects were only observed at high concentration of COS treatment (1000 µg/ml). Recently, chemical modifications of COS have attracted a great deal of attention because such approach would enhance or add bioactivities while keeping the natural backbone of COS and the biochemical properties of the introduced group. In this respect, gallic acid-grafted derivative of COS has been synthesized to improve their bioactivities. In the present study, we further evaluate whether gallic acid-grafted COS (G-COS) enhance the inhibitory effect against allergic reactions in mast cells.

Rat basophilic leukemia (RBL-2H3) cells display properties of mucosal-type mast cells. These cells act in response to the cross-linking of their IgE-bound FcεRI by multivalent allergens, with the release and generation of several inflammatory mediators (Passante and Frankish, 2009). Thus, RBL-2H3 cells have been commonly and successfully used in *in vitro* studies on binding of IgE to FcεRI receptors and subsequent downstream events. Herein, RBL-2H3 cells were used as a model cell line for evaluating the inhibitory effect of G-COS on antigen-induced allergic reactions.

2. Materials and methods

2.1. Reagents

RBL-2H3 mast cells were purchased from the Korean Cell Line Bank (Seoul, Korean). COS (3–5 kDa) was kindly provided by Kitto Life Co. (Seoul, Republic of Korea). Gallic acid was purchased from the Acros Organics (Morris Plains, NJ, USA). Enzyme immunoassay reagents for cytokine assays were purchased from R&D Systems (Minneapolis, MN, USA). Specific antibodies used for western blot analysis were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). Anti-DNP antibody conjugated with fluorescein isothiocyanate (FITC) was obtained from Invitrogen (Eugene, OR, 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 chemicals for synthesis, dinitrophenyl-bovine serum albumin (DNP-BSA), dimethyl sulfoxide (DMSO), o-phthalaldehyde (OPA), and MTT reagent (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Synthesis of G-COS

G-COS was produced as described by Ngo et al. (2011) (Scheme 1). COS with molecular weight range of 3–5 kDa (2.50 g) was dissolved in a solution of 20 ml distilled water and 40 ml methanol. This mixture was then adjusted to the pH 6.8 with triethylamine to obtain solution A. Gallic acid (0.94 g) was dissolved in 10 ml of methanol and mixed with 10 ml of dicyclohexylcarbodiimide (DCC)–methanol mixture (1.0315 g DCC in 10 ml methanol) to

obtain solution B. The solution B was gradually added to the solution A while stirring at 30 °C and 150 rpm for 5 h. The reaction mixture was then filtered to remove dicyclohexyl urea and kept at 2 °C overnight. Subsequently, the solution was added 90 ml of diethyl ether and mixed well until the precipitate was formed. The precipitate was obtained by filtration through a filter paper and dissolved in 20 ml of distilled water. The un-reacted gallic acid was removed by using dialysis membrane molecular weight cut off below 1 kDa. The solution of G-COS was freeze dried and kept at 2 °C. Stock solution (100 mg/ml) was made in PBS and sterilized by filtration through a 0.2 µm sterilized filter.

2.3. Cell viability assay

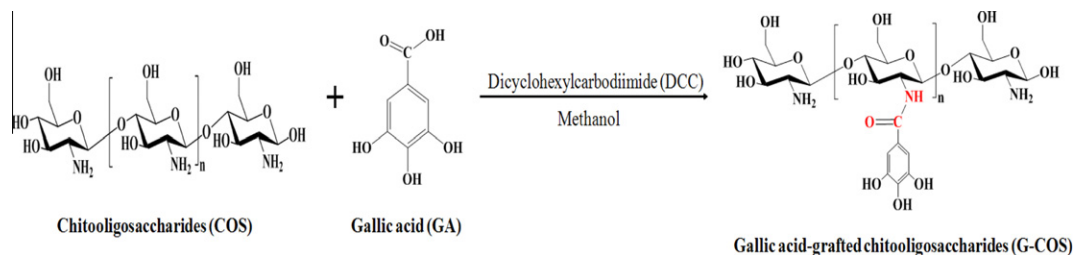
The viability levels of RBL-2H3 cells were determined by MTT assay. In brief, cells (1×10^5 cells/ml) were incubated for 24 h with the indicated concentrations of G-COS (50, 100 or 200 µg/ml). After various treatments, the medium was removed, and the cells were incubated with a solution of 1 mg/ml MTT for 4 h. Finally, the supernatant was removed, and DMSO 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® TecanAustria GmbH, Austria). Viability of cells was quantified as a percentage compared to the blank (no addition of G-COS).

2.4. Histamine release assay

RBL-2H3 cells were seeded into 24-well plates (1×10^5 cells/ml). The cells were treated with different concentrations of G-COS or gallic acid (5 µg/ml) 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 release in the supernatants was determined as previously described (Vo et al., 2011). Histamine release levels were calculated as a percentage compared to control: Release ratio (%) = $(T - B)/(C - B) \times 100$, where *B* is the group without stimulation as well as sample treatment, *C* is the stimulated group without treatment of the tested sample, and *T* is the stimulated group with presence of the tested sample.

2.5. Fluorescent measurement of the intracellular Ca²⁺ level

Intracellular calcium was measured using the calcium reactive fluorescence probe, Fura 2-AM. RBL-2H3 cells were seeded into black 96-wells plates (2×10^5 cells/ml) and treated with different concentrations of G-COS for 1 h before sensitized overnight with DNP-specific IgE antibody (100 ng/ml, final concentration). The cells were then incubated with Fura-2/AM (2 µM, final



Scheme 1. The synthesis of gallate-chitooligosaccharides.

concentration) for 1 h. Subsequently, the cells were washed with Tyrode buffer and stimulated with antigen DNP-BSA (50 ng/ml, final concentration) for 5 min. The Fura-2/AM fluorescence intensity was measured after 2 min of stimulation at an excitation wavelength of 360 nm and an emission wavelength of 528 nm by using a microplate reader (GENios® Tecan Austria GmbH, Austria).

2.6. Fluorescence microscope

RBL-2H3 cells were treated with G-COS (200 µg/ml) for 1 h before sensitized overnight with DNP-specific IgE antibody (100 ng/ml, final concentration). The cells were then incubated with Fura-2/AM (2 µM, final concentration) for 1 h. Subsequently, the cells were washed with Tyrode buffer and stimulated with antigen DNP-BSA (50 ng/ml, final concentration) for 5 min. The medium was then removed and 3% paraformaldehyde was added for 30 min. Fluorescence images were visualized and photographed under fluorescence microscope (CTR 6000, Leica, Wetzlar, Germany).

2.7. Measurement of cytokine production

RBL-2H3 cells were seeded into 24-well plates (2×10^4 cells/ml) and treated with various concentrations of G-COS for 1 h before sensitized overnight with DNP-specific IgE antibody (100 ng/ml, final concentration). The cells were then stimulated with antigen DNP-BSA (50 ng/ml, final concentration) for 24 h. The supernatants were collected, and the productions of IL-4 and TNF- α were quantified by sandwich immunoassays following the protocol of R&D systems.

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

The mRNA expression levels of HDC, IL-4, and TNF- α 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 1X 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 β -actin was used as an internal control. Primer sequences used in this study were as follows: for HDC, sense 5'-GCA-GCA-AGG-AAG-AAC-AAA-ATC-C-3' and antisense 5'-CAA-CAA-GAC-GAG-CGT-TCA-GAG-A-3'; for IL-4, sense 5'-GTA-CTG-TGC-AGC-CCT-GGA-AT-3' and antisense 5'-TTT-AGA-AAC-TGG-GCC-ACC-TC-3'; for TNF- α , sense 5'-ATG-AGC-ACA-GAA-AGC-ATG-ATC-3' and antisense 5'-TAC-AGG-CTT-GTC-ACT-CGA-ATT-3'; for β -actin, sense 5'-AGC-CAT-GTA-CGT-AGC-CAT-CC-3' and antisense 5'-TCC-CTC-TCA-GCT-GTG-GTG-GTG-AA-3'. The amplification was performed by 27 cycles of 95 °C for 45 s (denaturation), 55 °C for 50 s (annealing), and 72 °C for 60 s (extension). The amplified products were analyzed by 1.5% agarose gel electrophoresis. Gels were then stained with 1 mg/ml ethyldium 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 for detection of protein expression. The cells were lysed in RIPA lysis buffer (NP-40, Sigma–Aldrich, USA). Equal amounts of protein were separated on 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. Subsequently, the membranes were incubated with appropriate primary antibodies (diluted 1:1000) for at least 1 h. After washing three times with TBS-T buffer, horseradish peroxidase (HRP)-conjugated IgG secondary antibody (diluted 1:5000) was applied for 1 h. Following three washes in TBS-T buffer, immunoreactive bands were visualized using the electrochemiluminescence (ECL) detection system. In a parallel experiment, nuclear protein was prepared using nuclear extraction reagents according to the manufacturer's protocol (Sigma, St. Louis, MO, USA).

2.10. Flow cytometric assay

Fc ϵ RI expression on the cell surface was measured via flow cytometry. RBL-2H3 cells were treated with different concentrations of G-COS for 1 h before sensitized overnight with DNP-specific IgE antibody (1 µg/ml, final concentration). The cells were then washed, harvested, and re-suspended into cold binding buffer (BD Diagnostic Systems, Cockeysville, MD, USA) containing anti-IgE FITC-conjugated antibody (500 ng/ml, final concentration). The cells were immediately kept on ice for 1 h, washed by centrifugation, and re-suspended into cold binding buffer. Finally, the cells were analyzed by flow cytometry (BD Diagnostic Systems, Cockeysville, MD, USA). The numbers in histograms indicate the percentage of IgE-positive cells.

2.11. Statistical analysis

The statistical differences among groups were assessed by ANOVA with Duncan's multiple range tests. Differences were considered significant at $p < 0.05$. The statistical software package, SAS v 9.1 (SAS Institute Inc., Cary, NC, USA), was used for the analysis.

3. Results and discussion

3.1. Effect of G-COS on histamine release in antigen-stimulated RBL-2H3 mast cells

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 et al., 2003). Activation of mast cells leads to degranulation which causes the release of various preformed mediators (Puxeddu et al., 2003). Among them, histamine is considered as the major mediator of the acute inflammatory and immediate hypersensitivity responses. Its effects are mediated through histamine receptors (H1, H2) causing pain, acute rhinitis, pruritus, and vasodilatation (Jutel et al., 2009). Thus, histamine is the major target for potential anti-allergic drugs and often used as a marker of mast cell degranulation. In this study, we evaluate effect of G-COS on degranulation via measuring histamine release from antigen-stimulated RBL-2H3 mast cells. It was observed that the level of histamine release was reduced by G-COS treatment in a dose-dependent manner (Fig. 1A). Histamine release level upon treatment with 200 µg/ml of G-COS was 46% as compared to control group stimulated with DNP-BSA alone. Meanwhile, histamine release level upon treatment with 200 µg/ml of COS (3–5 kDa) was 96% as determined by Vo et al. (2012). Obviously, G-COS exhibits a stronger inhibitory effect than COS on mast cell degranulation. On the other hand, gallic acid has been shown to reduce 83% histamine release in rat peritoneal mast cells at very low concentration (1.7 µg/ml) (Kim et al., 2006). Moreover, gallic acid (5 µg/ml) displayed a similar inhibitory effect with G-COS

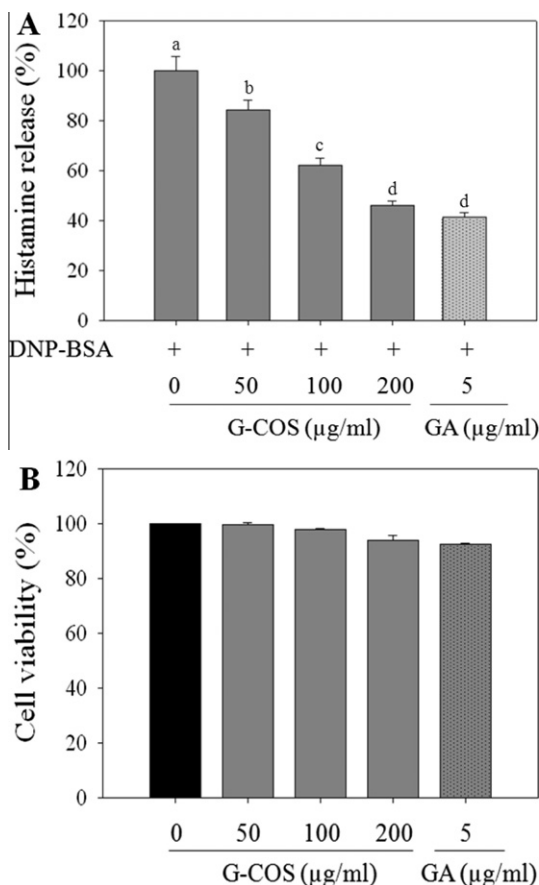


Fig. 1. Effect of G-COS on antigen-induced histamine release and cell viability in RBL-2H3 mast cells. (A) The cells were treated with different concentrations of G-COS or gallic acid (GA, 5 µg/ml) for 1 h and sensitized overnight with DNP-specific IgE antibody before stimulation of antigen DNP-BSA for 60 min. The levels of histamine release were measured via a spectrofluorometric assay. Each determination was made in triplicate, and the data are showed as means $\pm$ SD. ^{a–d}Means with different letters are significantly different ($p < 0.05$). (B) The cells were incubated with the indicated concentration of G-COS for 24 h. Cell viability was demonstrated by MTT method, and the results were expressed as percentage of surviving cells over blank cells (no addition of G-COS). Each determination was made in triplicate, and the data are showed as means $\pm$ SD.

(200 µg/ml) on histamine release from RBL-2H3 cells (Fig. 1A). These results indicate that the grafting of gallic acid onto COS enhances the inhibitory effect of COS against mast cell degranulation.

To exclude the possibility that the inhibition of histamine release was due to cytotoxicity caused by G-COS treatment, MTT assays were performed in RBL-2H3 cells treated with various concentrations of G-COS for 24 h. With the concentrations used in this study (50, 100, or 200 µg/ml), none of the treatments affected cell viability (Fig. 1B). Thus, the inhibitory activity of G-COS on histamine release was not due to any cytotoxic effect on RBL-2H3 cells.

3.2. Effect of G-COS on histamine production in antigen-stimulated RBL-2H3 mast cells

Histamine is largely produced by mast cells and stored in secretory granules. Substantially, L-histidine decarboxylase (HDC) is the enzyme that catalyzes formation of histamine from L-histidine. The increase of HDC expression is recognized as a specific marker for biosynthesis of histamine (Watanabe and Ohtsu, 2002). Therefore, HDC is considered as potential target for control of histamine production in mast cells. In the previous study, Maeyama and

colleagues have determined that HDC expression was induced by the stimulation of higher oligomeric IgE or phorbol myristate acetate (Maeyama et al., 1988). Herein, we have attempted to investigate whether G-COS suppress HDC expression in antigen-stimulated RBL-2H3 cells. As shown in Fig. 2, the mRNA (Fig. 2A) and protein (Fig. 2B) expressions of HDC were markedly increased after 1 h of stimulation as compared to the blank without stimulation. However, the treatment of G-COS reduced the expression level of both HDC mRNA and protein. These results suggested that the suppression of HDC expression by G-COS may contribute to diminish histamine production in the activated mast cells.

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

It is well-known that the activation of mast cells leads to elevation of intracellular Ca^{2+} level (Siraganian, 2003). Meanwhile, the increase in intracellular Ca^{2+} concentration is a necessary and sufficient stimulus for histamine release in mast cells (Aketani et al., 2001). Thus, we also examined whether G-COS alleviate the intracellular Ca^{2+} level in the activated mast cells. As a result, the intracellular Ca^{2+} level was significantly increase in RBL-2H3 cells stimulated with antigen DNP-BSA. Conversely, this increase was alleviated in RBL-2H3 cells pretreated with G-COS before stimulation of antigen DNP-BSA (Fig. 3). Notably, the decrease in intracellular Ca^{2+} level was observed to be effective at concentration of 200 µg/ml of G-COS treatment (Fig. 3A). Further, we have also confirmed the alleviative effect of G-COS on intracellular Ca^{2+} level by using light microscopy. Similarly, these photographs indicated that G-COS (200 µg/ml) exhibited the inhibitory activity against the elevation of intracellular Ca^{2+} in the activated cells (Fig. 3B). Accordingly, G-COS was suggested to be able to inhibit histamine release via inhibition of intracellular Ca^{2+} elevation. This assumption is supported by previous reports that some anti-allergic drugs inhibit mast cell degranulation via inhibition of intracellular Ca^{2+} elevation (Kim et al., 2008; Fischer et al., 1998; Matsubara et al., 2004).

3.4. Effects of G-COS on cytokine generation in antigen-stimulated RBL-2H3 mast cells

The allergic reactions are also characterized by production of various cytokines such as IL-1 β , IL-4, IL-6, IL-8, IL-13, and TNF- α

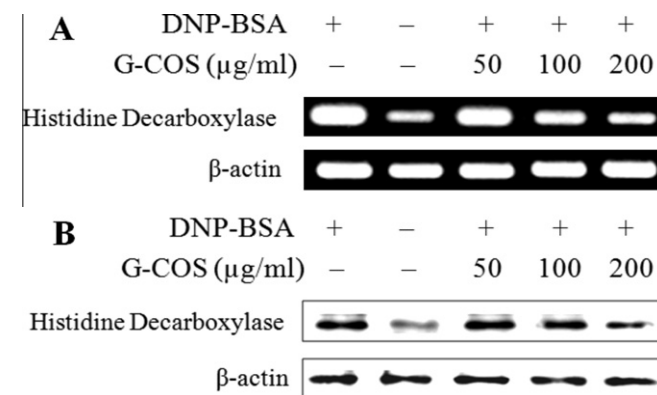


Fig. 2. Effect of G-COS on histamine production in antigen-stimulated RBL-2H3 mast cells. The cells were treated with various concentrations of G-COS for 1 h and sensitized overnight with DNP-specific IgE antibody before stimulation of antigen DNP-BSA for 1 h. The mRNA expression level of histidine decarboxylase (A) was determined via RT-PCR method, while the protein expression level of histidine decarboxylase (B) was assessed by Western blotting analysis. β -actin was used as internal controls.

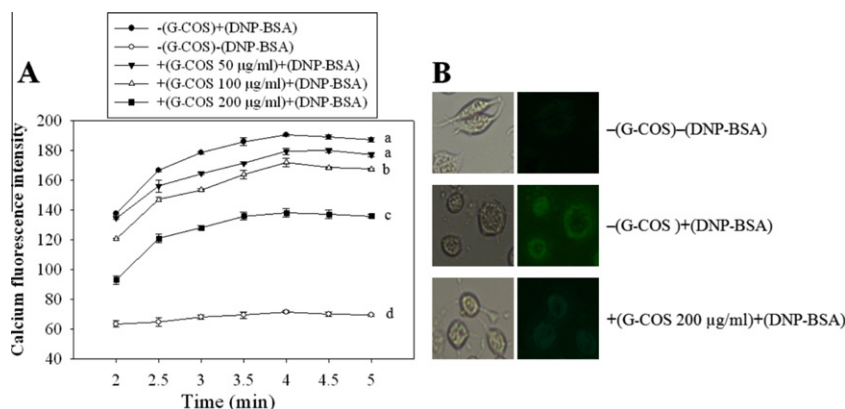


Fig. 3. Effect of G-COS on intracellular Ca^{2+} elevation in antigen-stimulated RBL-2H3 mast cells. (A) The cells were treated with various doses of G-COS for 1 h and sensitized overnight with DNP-specific IgE antibody. The cells were then incubated with Fura-2/AM for 1 h before stimulated with antigen DNP-BSA. The level of intracellular Ca^{2+} was monitored by a spectrofluorometric assay. Each determination was made in triplicate, and the data are showed as means $\pm$ SD. ^{a-d}Means with different letters are significantly different ($p < 0.05$). (B) The representative images of the cells were assessed by using light microscopy (magnification, $\times 20$). The intensity of intracellular calcium was measured in three separate experiments.

from the activated mast cells (Galli et al., 2008). Especially, IL-4 plays a crucial role in the pathogenesis of allergic diseases, such as the induction of IgE synthesis and the development of mast cells (Nabeshima et al., 2005). Meanwhile, TNF- α has many immunologic and inflammatory reactions by causing the production of other cytokines and increasing the expression of adhesion molecules on endothelial cells (Olejnik and Brzezińska-Błaszczuk, 1998). Therefore, modulation of production and expression of IL-4 and TNF- α in mast cells can provide a useful therapeutic strategy for allergic inflammatory disease. In this regard, we have revealed that G-COS induced a dose-dependent suppression on the mRNA (Fig. 4A) and protein (Fig. 4B) expression of IL-4 and TNF- α in antigen-stimulated RBL-2H3 cells. In a parallel experiment, we confirmed the effect of G-COS on the expression of IL-4 and TNF- α through measuring the production of these cytokines in culture supernatants by enzyme-linked immunosorbent assay. As shown in Fig. 4C and D, the levels of cytokine production were increased in the culture supernatants of antigen-stimulated RBL-2H3 cells. The amounts of IL-4 and TNF- α in the stimulated cells were 113 ± 5 and 158 ± 8 pg/ml, respectively, whereas the correlative amounts of these cytokines in the non-stimulated cells were

14 ± 3 and 33 ± 12 pg/ml, respectively. Meanwhile, these increases were considerably diminished in a concentration-dependent manner by G-COS treatment. At the concentration of 200 $\mu\text{g/ml}$, G-COS reduced IL-4 and TNF- α level to 40 ± 11 and 65 ± 3 pg/ml, respectively. As compared to the previous study (Vo et al., 2011), G-COS was also found to be more effective than COS on inhibition of cytokine expression and production at the same concentration (200 $\mu\text{g/ml}$). Herein, the inhibitory effects of G-COS on cytokine generation were suggested to be due to inactivation of mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)- κB . Therefore, the inhibitory effects of G-COS on MAPKs and NF- κB are needed to further elucidate.

3.5. Effects of G-COS on NF- κB and MAPKs activation in antigen-stimulated RBL-2H3 mast cells

NF- κB is a master transcription factor that controls the expression of pro-inflammatory gene products in cells of many different lineages. In normal conditions, p50/p65 dimers of NF- κB (p50/p65) are bound to inhibitory κB (I κB) proteins, and consequently retained within the cytoplasm. Conversely, I κB proteins are rapidly

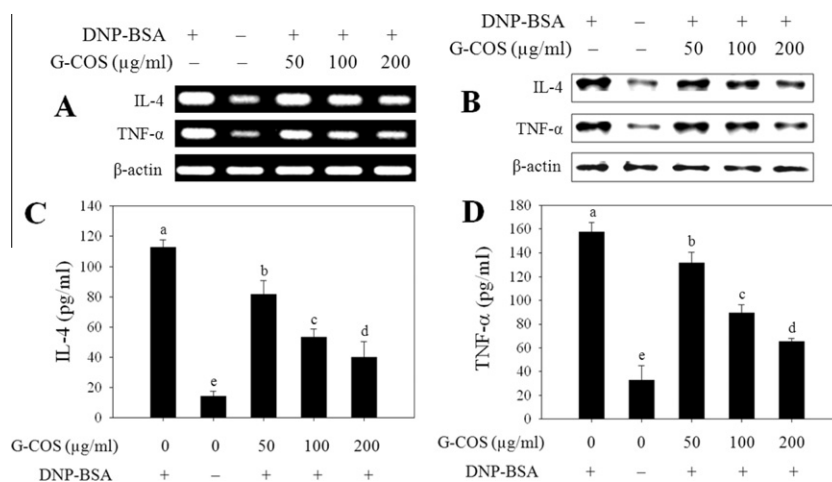


Fig. 4. Effects of G-COS on cytokine expression and production in antigen-stimulated RBL-2H3 mast cells. The cells were treated with various concentrations of G-COS for 1 h and sensitized overnight with DNP-specific IgE antibody before stimulation of antigen DNP-BSA for 24 h. The mRNA transcription levels of IL-4 and TNF- α were determined via RT-PCR (A) while the protein levels were assessed by Western blotting (B). β -actin was used as internal controls. The production levels of IL-4 (C) and TNF- α (D) were quantified in culture media using commercial ELISA kits. Each determination was made in triplicate, and the data are showed as means $\pm$ SD. ^{a-e}Means with different letters are significantly different ($p < 0.05$).

degraded in the activated cells, and the freed NF- κ B is translocated to the nucleus, wherein it binds to target sites and induces the transcriptions of genes (Hayden and Ghosh, 2004). Thus, the blockade of NF- κ B transcriptional activity is considered to be essential to inhibit cytokine generation. Indeed, RBL-2H3 cells stimulated by antigen DNP-BSA induced a remarkable degradation of I κ B- α in the cytoplasm and a rapid increase of p50/p65 subunits in nucleus (Fig. 5A). However, the nuclear translocation of p50/p65 subunits was decreased in RBL-2H3 cells exposed to G-COS. Moreover, G-COS also blocked antigen-induced I κ B- α degradation in a concentration-dependent manner. These results indicated that G-COS inactivated NF- κ B via blocking the degradation of I κ B- α protein, and thus inhibiting the nuclear translocation of p50/p65 subunits.

Substantially, various intracellular signaling pathways are involved in the modulation of NF- κ B activity and inflammatory mediator expression. The MAPKs are a group of signaling molecules that have been known as upstream activators of NF- κ B (Sigala et al., 2011; Arbabi and Maier, 2002). Thus, MAPKs including extracellular signal-regulated kinase (ERK), p38 kinase, and c-jun N-terminal kinase (JNK) are appropriate targets for pharmacological treatment of inflammatory disorders. In the present study, we also evaluate the inhibitory capability of G-COS on MAPKs activation in antigen-stimulated RBL-2H3 cells. Interestingly, G-COS significantly inhibited the increase in phosphorylation of ERK and p38 induced by antigen stimulation. Meanwhile, a moderate inhibition of G-COS on JNK phosphorylation was observed as compared with others (Fig. 5B). According to these results, it was suggested that the inactivation of NF- κ B might be a consequence of inhibition of MAPK signaling pathways. Collectively, these results indicate that the suppressive effects of G-COS on cytokine generation, at least in part, might be derived through regulation of the MAPKs and NF- κ B activation.

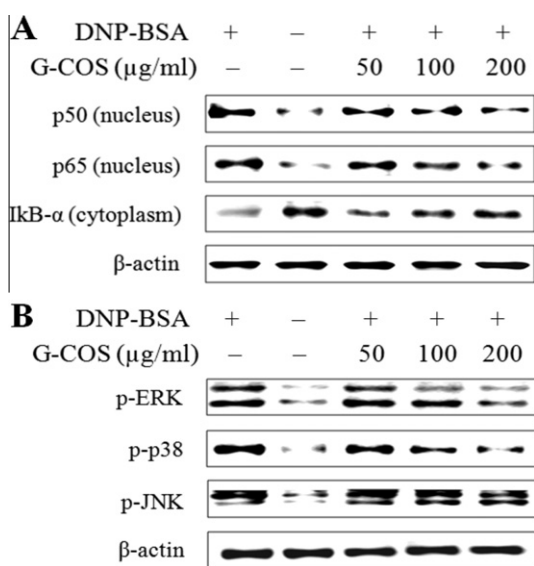


Fig. 5. Effects of G-COS on NF- κ B and MAPKs activation in antigen-stimulated RBL-2H3 mast cells. The cells were treated with different doses of G-COS for 1 h and sensitized overnight with DNP-specific IgE antibody before stimulation of antigen DNP-BSA for 1 h. (A) Cytosolic and nuclear protein extracts were prepared by commercial kit from sigma (St. Louis, MO, USA). The levels of p50/p65 subunits in nuclear protein extract and I κ B- α in cytosolic protein extract were determined by a Western blot analysis. β -actin was used as an internal control. (B) Total cell lysates were subjected to Western blotting to assess the phosphorylation levels of ERK, p38, and JNK protein. β -actin was used as an internal control. The phosphorylation of ERK, p38, and JNK were abbreviated by p-ERK, p-p38, and p-JNK, respectively.

3.6. Effects of G-COS on Fc ϵ RI α chain expression in antigen-stimulated RBL-2H3 mast cells

Obviously, allergic reactions in mast cells including intracellular signaling activation, intracellular Ca^{2+} elevation, histamine release and production, and cytokine generation are mediated by the high-affinity IgE receptor (Fc ϵ RI). Thus, Fc ϵ RI has been well-known to play a central role in the induction and maintenance of allergic reactions (von Bubnoff et al., 2003). It is a tetrameric complex composed of one α chain, one β chain, and two γ chain. Among them, Fc ϵ RI α chain plays an essential role in binding to the Fc portion of IgE with a high affinity (Metzger, 1991). Notably, IgE can potentially enhance the Fc ϵ RI expression of mast cells, and show the enhanced sensitivity and a stronger response to antigenic stimulation (Yamaguchi et al., 1997). Meanwhile, suppression of Fc ϵ RI expression can lead to reduction of the antigen-mediated allergic reactions (Shim et al., 2009; Kageyama-Yahara et al., 2010). In this regard, we have further examined whether G-COS suppress antigen-induced Fc ϵ RI expression in RBL-2H3 mast cells. Indeed, we have determined that G-COS reduced protein expression of Fc ϵ RI α chain in a dose-dependent manner (Fig. 6A). Moreover, the suppressive effect of G-COS on Fc ϵ RI α chain expression was also confirmed via flow cytometry assay. Likewise, the Fc ϵ RI α chain expression on the cell surface was shown to be reduced from 78.8% to 70.6%, 56.1%, and 36.4% at the concentrations of 50, 100, and 200 $\mu\text{g/ml}$ of G-COS treatment, respectively (Fig. 6B). According to Fujimura et al. (2004), ERK activation is associated with expression of Fc ϵ RI in KU812 cells. Moreover, the transcription factor Elf-1 has been previously identified to be essential for the function and expression of Fc ϵ RI α chain (Wang et al., 2008; Nishiyama et al., 1999). Therefore, Fc ϵ RI-suppressive effect of G-COS was suggested to be due to inactivation of ERK activation and blockade of transcription factor Elf-1.

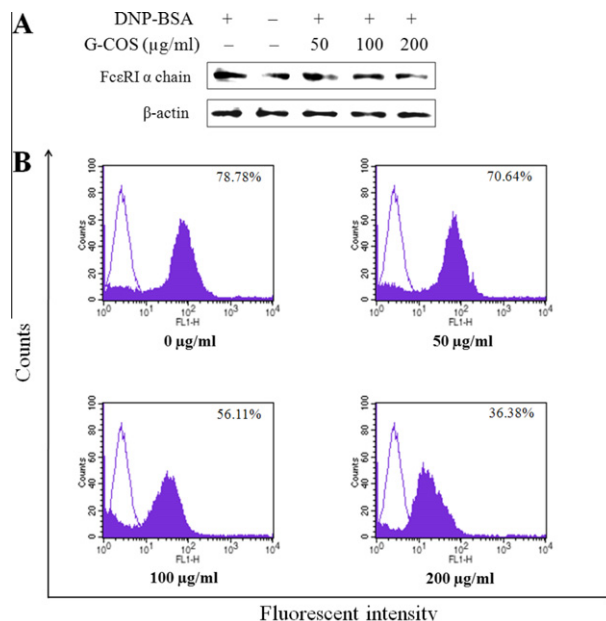


Fig. 6. Effects of G-COS on Fc ϵ RI α chain expression in antigen-stimulated RBL-2H3 mast cells. (A) The cells were treated with various concentrations of G-COS for 1 h and sensitized overnight with DNP-specific IgE antibody before stimulation of antigen DNP-BSA for 24 h. The expression level of Fc ϵ RI α chain was assessed by Western blotting analysis. β -actin was used as an internal control. (B) The treated and sensitized cells were incubated with DNP-BSA and followed by staining with FITC-labeled anti-DNP antibody. The stained cells were then analyzed via flow cytometry. Three independent trials were performed, and the numbers in histograms indicate the percentage of IgE-positive cells.

4. Conclusion

In conclusion, this study has determined that the grafting of gallic acid onto COS enhances the inhibitory effect of COS against allergic reactions in RBL-2H3 mast cells. The inhibitory effects were shown due to suppression of FcεRI expression which might cause the inhibition of intracellular signaling activation and subsequent inhibition of intracellular Ca²⁺ elevation, cytokine generation, and histamine release and production. Our results indicate that G-COS can contribute to suppression of allergic reactions and might be a potential candidate for novel inhibitor of allergic reactions.

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