Anti-allergic Effect of Seungmagalgeun-tang through Suppression of NF-κB and p38 Mitogen-Activated Protein Kinase Activation in the RBL-2H3 Cells

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In previous report, Seungmagalgeun-tang (SGT) could exert its anti-inflammatory actions in the BV-2 microglial cells. However, study on the anti-inflammatory effect of SGT in mast cells has not been identified. Therefore, we examined on the anti-inflammatory effect of SGT on the phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187-induced rat basophilic leukemia (RBL-2H3) cells. SGT inhibited the release of β-hexosaminidase and secretion and expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-4 on RBL-2H3 cells, without affecting cell viability. The protein expression level of nuclear factor (NF)-κB (p65) was decreased in the nucleus by SGT. In addition, SGT suppressed the degradation of inhibitory protein IκB-α protein, the activation of p38 mitogen-activated protein kinase (MAPK), and the expressions of cyclooxygenase (COX)-2 mRNA and protein level in RBL-2H3 cells. These results suggest that SGT could be involved anti-allergic effect by control of NF-κB (p65) translocation into the nucleus through inhibition of IκB-α degradation and suppression of COX-2 expression.

Key words : Seungmagalgeun-tang, cytokines, nuclear factor kappa B, Inhibitor kappa B, cyclooxygenase-2, mitogen-activated protein kinases

Introduction

Seungmagalgeun-tang (SGT) is a traditional orient herbal medicine formula consisting of four ingredients. This formula has traditionally been used to treat shang han epidemicdiseases, smallpox and measles, urticaria, etc. by dispelling the superficial muscles, promoting eruption, tonifying the blood and clearing away heat and detoxification in the inflammatory diseases1(2). The mast cell is a tissue-based inflammatory cell of bone marrow origin that responds to the danger signals of innate and acquired immunity with both the immediate and delayed release of inflammatory mediators3. Activated mast cells can produce histamine as well as a wide variety of other inflammatory mediators such as eicosanoids, proteoglycan, proteases, and several pro-inflammatory and chemotactic cytokines4(6). β-hexosaminidase is also stored in secretory granules of mast cells, and is also released concomitantly with histamine when mast cells are immunologically activated3. Tumor necrosis factor (TNF)-α has been suggested to induce tissue damage and it also is considered a major initiator of inflammation5(9). Interleukin (IL)-4 is a multi-functional cytokine that regulates both innate and adaptive immunity. Inappropriate expression is associated with allergic disease, autoimmunity, and failure to clear certain infections6(11). The expression of inflammatory cytokines depends on the activation of a transcription factor, namely nuclear factor-kappa B (NF-κB). The activation involves the phosphorylation, ubiquitination, and degradation of Inhibitor kappa B (IκB), leading to the nuclear migration of NF-κB7. Cyclooxygenase (COX) are two isoforms, COX-1 is expressed constitutively in many types of cells, where it is believed to perform housekeeping activities for normal cellular function, and the other is (COX-2) induced in certain types of cells by a variety of inflammatory stimulants. In general, the inflammatory reactions are associated with an induction of COX-25(9). Some of the mitogen-activated protein kinases (MAPKs) important to mammalian cells include extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), p38, p38 and NF-κB are thought to play an important role of in the regulation of pro-inflammatory molecules on cellular responses8(10). Recent studies in a variety of cultured cells indicate that induction of COX-2 is regulated by the MAPK16(17).
Rat basophilic leukemia (RBL-2H3) cells display properties of mucosal-type mast cells. Therefore, we used RBL-2H3 cells as a model cell line for allergic reactions. In the present study, we examined the anti-allergic effects of the Seungmagalgeun-tang (SGT) on RBL-2H3 cells. We found that SGT inhibited degranulation, TNF-α, IL-4, COX-2 secretion, NF-κB (p65), and MAPKs activations from the PMA plus A23187-induced RBL-2H3 cells.

Materials and Methods

1. Preparation of Seungmagalgeun-tang (SGT)

SGT was purchased from a local oriental herb store, Kwang Myoung Dang (Busan, Korea). Each of the SGT was identified and authenticated by Professor W.S. Ko, College of Oriental Medicine, Dongeui University (Busan, Korea) (Table 1). SGT, a one day dose for human adults were boiled with distilled water at 100°C, and the whole mixture is decoted until the volume is reduced by half. The extract water (400 ml) was filtered through 0.22 μm filter and the filtrate was freeze-dried (yield, 22.20 g) and kept at 4°C through 0.22 μm filter before use.

<table>
<thead>
<tr>
<th>Herbs</th>
<th>Dose</th>
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<tbody>
<tr>
<td>Puerariae radix</td>
<td>8 g</td>
</tr>
<tr>
<td>Paeoniae radix</td>
<td>4 g</td>
</tr>
<tr>
<td>Cimicifugae radix</td>
<td>4 g</td>
</tr>
<tr>
<td>Glycyrrhizae radix</td>
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<tr>
<td>Zingiberis radix</td>
<td>4 g</td>
</tr>
<tr>
<td>Allium</td>
<td>4 g</td>
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</table>

2. Reagents

Phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and β-nitro-phenyl-N-β-D-glucosaminide were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco’s Modified Eagle’s Medium (DMEM) containing L-glutamine (200 mg/L) and FBS were purchased from Hyclone (Logan, UT). TNF ELISA kit (BD OptEIATM Rat TNF ELISA Set), IL-4 ELISA kit (BD OptEIATM Rat IL-4 ELISA Set) and anti-COX-2 monoclonal antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-NF-κB (p65), IκB-α, β-actin, p38, ERK, JNK, and phosphorylated-p38, -ERK, -JNK polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-COX-1 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphatase labeled affinity purified antibody to rabbit and mouse IgG and BCIP/NBT phosphatase substrate were purchased from KPL (Gaithersburg, MD).

3. Cells culture

RBL-2H3 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with 5 % CO2. In all experiments, RBL-2H3 cells were treated for 1 h with the presence of the indicated concentrations of SGT prior to stimulation with 50 nM PMA plus 1 μM A23187 in serum-free DMEM.

4. MTT assay

The cell viability of SGT was assessed using the MTT assay(27) in the remaining cells after Griess reaction. The MTT solution (0.5 mg/ml) was added to each well. After incubation for 4 h at 37°C and 5 % CO2, the supernatant were removed and formed foramazin crystals in viable cells were measured at 540 nm with a microplate reader. The percentage of cell viability was calculated against untreated cells. All experiments were performed in triplicate well.

5. β-hexosaminidase assay

β-hexosaminidase was measured in both supernatant and pellet fractions using a previously reported method(29). Briefly, RBL-2H3 cells (3 × 10^5 cells) were treated for 1 h with the presence of the indicated concentrations of SGT prior to stimulation with 50 nM PMA plus 1 μM A23187 and incubated at 37°C for 50 min. After stimulation, 50 μl of each sample was incubated with 50 μl of 1 mM β-nitro-phenyl-N-β-D-glucosaminide dissolved in 0.1 M citrate buffer, pH 5, in 96 well microtiter plate at 37°C for 1 h. The reaction was terminated with 200 μl/well of 0.1 M carbonate buffer, pH 10.5. The plate was read at 405 nm in an ELISA reader. The inhibition percentage of β-hexosaminidase release was calculated using the following equation:

\[
\text{β-hexosaminidase release(%) = } \frac{A_{405} \text{ of sup.} - A_{405} \text{ of pellet}}{A_{405} \text{ of sup.}} \times 100
\]

where is A_{405} is absorption of measured at 405 nm and sup. is supernatant.

6. Enzyme-linked immunosorbent assay for pro-inflammatory cytokines (TNF-α, IL-4)

Each cytokines concentration in RBL-2H3 cells were measured with commercially available Rat TNF, IL-4 ELISA kit (BD Biosciences), according to the manufacturer’s protocol.
Color development was measured at 450 nm using an automated microplate ELISA reader.

7. Isolation of total RNA from cells and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated as per the manufacture’s instructions. Briefly, cells were lysed additional Trizol reagent (Invitrogen, Carlsbad, CA) and the cell lysate was passed through the pipette several times. 0.2 ml of chloroform was added per 1 ml of Trizol reagent. The tubes were shaken vigorously and incubated at room temperature for 2-3 min. The samples were centrifuged at 14,000 g for 20 min. The aqueous phase was transferred to a fresh tube and RNA was precipitated by the addition of 0.5 ml isopropanol. The RNA pellet was air-dried and resuspended in nuclease-free water. The concentration of RNA was estimated spectrophotometrically. Three microgram RNAs were reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI). Single stranded cDNA was amplified by PCR with primers (Table 2).

PCR amplifications were done in a 20 μl PCR PreMix (Bioneer Co., Korea) containing 10 mM Tris-HCl, 40 mM KCl, 1.5 mM MgCl₂, 250 μM dNTP, 1 unit of Taq polymerase. Amplifications were carried out in a PCR machine (ASTEC PC802) using an initial denaturation at 95℃ for 5 min followed by 28 cycles of denaturation for 40 sec at 95℃, annealing for 50 sec at 55℃ and extension for 40 sec at 72℃. This was concluded with a final extension for 7 min at 72℃. Products were separated in 1 % agarose gels in 0.5× TBE buffer at 100 V for 30 min, stained with ethidium bromide and visualised under UV light. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control to evaluate relative expressions of TNF-α, IL-4 and COX-2.

Table 2. Oligonucleotide primers used for PCR in this study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Oligonucleotide sequences (5' to 3' direction)</th>
<th>Expected size</th>
<th>Accession number</th>
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<tr>
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<td>CCCCCTAGGCTTCTACTCGTC</td>
<td>664 bp</td>
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<td>GGTCCCAAATCAGCTTTT</td>
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<td></td>
<td>AAGCTTTGAAACGCAGTT</td>
<td>332 bp</td>
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</tr>
<tr>
<td></td>
<td>AGTCAGGACTGCAAGTT</td>
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<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>ACTGGTGTGCTCCATACACC</td>
<td>223 bp</td>
<td>S67721</td>
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<td>CAAAGGTGAGACCCCAAGTT</td>
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<td>COX-1</td>
<td>TGACGACAGACAGACAGAGAGAGAGAGA</td>
<td>250 bp</td>
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<td>CATGAAGCCTTTCAAGGAGAGAGA</td>
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<td>COX-2</td>
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<td>269 bp</td>
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</table>

8. Western blot analysis

Cell extracts were prepared by detergent lysis procedure. Cells (5 × 10⁶ cells) were scraped, washed once with PBS and resuspended in lysis buffer. Equal amounts of protein were separated electrophoretically using 10 % SDS-PAGE, and then the gel was transferred to nitrocellulose membranes. Blots were blocked for at least 2 h with 5 % non-fat dry milk. The blot was incubated with NF-κB (p65), IκB-α, β-actin, p38, ERK, JNK, and phosphorylated-p38, -ERK, -JNK polyclonal antibodies and COX-1, COX-2 monoclonal antibodies at 4℃ and secondary antibodies at room temperature were detected by the AP (BCIP/NBT phosphatase substrate) system according to the recommended procedure.

9. Preparation of nuclear extract

The treated cells were washed and centrifuged and then resuspended in hypotonic buffer and incubated on ice for 30 min. After centrifugation at 14,000 rpm, 4℃ for 20 min, the supernatant cytoplasmic protein was collected. The remnants were resuspended in extraction buffer, incubated on ice for 30 min, and then centrifuged (14,000 rpm, 4℃, 20 min), after which the supernatant nuclear extract was collected.

10. Statistical analysis

Data is presented as the mean ± SEM (standard error of mean) of at least three separate experiments. Comparisons between two groups were analyzed using Student’s t-test. P values less than 0.05 considered be statistically significant.

Results

1. SGT effect on the cell viability

The cell viability effect of SGT on RBL-2H3 cells was evaluated by MTT assay. As shown in Fig. 1, SGT concentrations from 0.5 mg/ml to 2.0 mg/ml had no effect on cell survival. These results suggest SGT inhibits PMA plus A23187-induced TNF-α, IL-4 and COX-2 production in RBL-2H3 cells without effect on the cell viability in each condition.

![Fig. 1. Effect of SGT on the cell viability in RBL-2H3 cells. Cell viability was evaluated by MTT assay. Data represent the mean ± SEM of three independent experiments.](Image)
2. SGT inhibited degranulation in RBL-2H3 cells

Inhibitory effects of SGT on the release of β-hexosaminidase from RBL-2H3 cells were evaluated by the methods, as described in Materials and Methods. The release of β-hexosaminidase decreased significantly with all concentrations of SGT (Fig. 2).

3. SGT reduced pro-inflammatory cytokines (TNF-α and IL-4) levels

We examined whether SGT could regulate pro-inflammatory cytokines such as TNF-α and IL-4 in RBL-2H3 cells. Cells were pre-treated with various concentration of SGT and then PMA plus A23187 challenge for 8 h. Treatment with SGT dose-dependently blocked TNF-α and IL-4 secretion induced by PMA plus A23187 in RBL-2H3 cells (Fig. 3A, 3B). Also these cytokines expression decreased significantly by SGT in dose-dependent manner (Fig. 3C). In contrast to TNF-α and IL-4, the level of GAPDH mRNA expression remained the same under these conditions.

4. SGT inhibited the activation of NF-κB (p65) and the degradation of IκB-α

We examined the effects of SGT on NF-κB (p65) expression and IκB-α degradation using Western blot analysis. RBL-2H3 cells were treated with SGT and PMA plus A23187 for 2 h. In the stimulated cells, the expression level of NF-κB (p65) was not changed in the cytoplasm (C-p65) and increased in the nucleus (N-p65). SGT inhibited the PMA plus A23187-induced the nuclear translocation of NF-κB (p65) (Fig. 4A). RBL-2H3 cells were treated with SGT and PMA plus A23187 for 30 min. As shown in Fig. 4B, PMA plus A23187 treatment caused degradation of IκB-α (lane 2). However, SGT inhibited degradation of IκB-α (lane 3).

5. SGT suppressed the expression of COX-2

RT-PCR analyses were performed to assess the effect of SGT on PMA plus A23187-induced COX-1 and COX-2 mRNA
expressions. RBL-2H3 cells were treated with SGT and PMA plus A23187 for 4 h. The accumulation of COX-2 mRNA levels was inhibited significantly by SGT, whereas COX-1 mRNA levels showed no change after such treatment (Fig. 5A). In addition, the expression of protein level of COX-2 was reduced by SGT (Fig. 5B).

6. SGT inhibited the activation of p38 MAPK activation

To determine the mechanisms of effect of SGT on the inflammatory cytokines expressions, we examined the effect of SGT on the MAPKs activation. RBL-2H3 cells were treated with SGT and PMA plus A23187 for 2 h. As shown in Fig. 6, SGT suppressed PMA plus A23187-induced p38 MAPK activation but did not affect the phosphorylation of ERK and JNK.

Discussion

Mast cells play a central role in immediate hypersensitivity reactions and anaphylaxis, by producing and secreting a large spectrum of inflammatory mediators. Activation of mast cells leads to phosphorylation of tyrosine kinase and mobilization of internal Ca²⁺. This is followed by activation of protein kinase C, MAPKs, NF-κB, and releasing of inflammatory cytokines. And activated mast cells can production histamine, as well as a wide variety of other inflammatory mediators and several pro-inflammatory cytokines. Histamine, which is released from mast cells and basophils stimulated by an allergen, is usually determined as a degranulation marker in immediate allergic reactions in vitro experiments. When granulates in mast cells or basophils degranulate, an enzyme β-hexosaminidase is also released along with histamine. Thus, this enzyme activity is used as a marker of mast cell degranulation. The present study showed that SGT pre-treatment profoundly affected PMA plus A23187-induced degranulation of RBL-2H3 cells. These results may suggest SGT have anti-allergic action.

Mast cells concomitantly synthesize and release a variety of cytokines, including IL-3, IL-4, IL-5, IL-13, granulocyte macrophage colony-stimulating factor (GM-CSF) and TNF-α during the late-phase reaction. Several flavones (e.g., luteolin, apigenin, diosmetin and quercetin) obtained from nature have been reported to inhibit the release of TNF-α and IL-4. Similarly, we found that the SGT dose-dependently inhibited TNF-α and IL-4 secretions in activated RBL-2H3 cells. Also mRNA expression of these cytokines significantly suppressed by SGT.

Most of the inflammatory genes over-expressed in asthma, such as those encoding pro-inflammatory cytokines, chemokines, adhesion molecules, and inflammatory enzymes, contains κB sites for NF-κB within their promoter, suggesting that these genes are controlled predominantly by NF-κB. Pro-inflammatory molecules are regulated at the level of transcription and are involved in the inflammatory cascade. In most cells, NF-κB is sequestered in the cytoplasm in an inactive form because of tight association with its inhibitors, the IκBs. Activation of NF-κB is achieved through signal-induced phosphorylation of IκB at specific amino-terminal serine residues by the IκB kinase (IKK) complex. This phosphorylation triggers IκB degradation via the ubiquitin-proteasome pathway, resulting in NF-κB translocation into the nucleus. SGT suppressed translocation into the nucleus of NF-κB and inhibited degradation of IκB-α.

COX-2 is rapidly expressed in several cell types in

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response to growth factors, cytokines, and pro-inflammatory molecules and has emerged as a major player in inflammatory reactions in peripheral tissues26). Pro-inflammatory cytokines are dependent upon NF-κB activation and NF-κB can promote COX-2 gene transcription. COX-2 inhibitors may exert an anti-inflammatory effect by inhibition of NF-κB25. In this study, the expression of COX-2 mRNA and protein was decreased by treatment of SGT. Therefore, SGT may be used as an anti-allergic agent.

The MAPK signaling cascade plays an essential role in the initiation of inflammatory responses28. The exact signaling pathways among three types of MAPKs such as p38, ERK, JNK are unclear. However, p38 MAPK is thought to play an important role in regulation of inflammatory responses. Activation of p38 MAPK is essential for the expression of the pro-inflammatory cytokines15. We determined the effect of SGT on the PMA plus A23187-stimulated activation of MAPKs in RBL-2H3 cells. In previous study, the effect of Gallic acid on PMA plus A23187 simultaneously activated p38, ERK and JNK MAPKs in HMC-1 cells. Gallic acid specially inhibited the activation of p38 MAPK5. We also confirmed that among all three MAPKs, SGT suppressed the activation of p38 MAPK but not ERK or JNK.

In conclusion, SGT inhibited translocation of NF-κB (p65) into the nucleus through regulation of IκB-α degradation. Therefore, SGT suppressed the TNF-α, IL-4, and COX-2 secretions, and inhibited inflammatory cytokines production via p38 MAPK pathway inhibition. Overall SGT may affect anti-allergic action.

Acknowledgement

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References

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