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Inhibitory Effects of *Syzygium aromaticum* Ethanol Extracts on IgE Mediated RBL-2H3 cell Activation

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IgE 매개 RBL-2H3 세포 활성화에 대한 정향 에탄올 추출물의 억제 효과

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Objectives : In this report, we investigated the effect of ethanol extract of *Syzygium aromaticum*(L.) Merr. & Perry.(SAE) on the RBL-2H3 cell-mediated allergic response and studied its possible mechanisms of action. **Methods :** Cytotoxicity on RBL-2H3 cell was evaluated by MTT assay. Anti-allergic activity of SAE was assessed by β-Hexosaminidase and Histamine secretion, β-Hexosaminidase and Histamine secretion were measured by ELISA assay. Evaluate the mechanisms of effect of SAE on the secretion of degranulate mediators, we examined the effect of SAE on the activation of mitogen-activated protein kinases using western blot analysis. **Results :** SAE had no cytotoxicity on rat basophilic leukemia cell(RBL-2H3). Moreover SAE dose-dependently inhibited RBL-2H3 cell degranulation and histamine release. SAE specifically blocked the IgE-induced p38 mitogen-activated protein kinase activation. **Conclusions :** Our findings provide evidence that *Syzygium aromaticum* ethanol extract inhibits mast cell derived allergic reaction, and also demonstrate the involvement of p38 MAPK phosphorylation.

Key words : *Syzygium aromaticum*, anti-allergic, mitogen-activated protein kinase

Introduction

The flower bud of clove(*Syzygium aromaticum* Merrill et Perry) has been used as a suppression of toothache and halitosis in traditional Korean medicine[1]. Especially, clove oil has been well-known extensive use in dental formulations, mouth washer, and insect repellent. Recently, clove has been widely investigated a biological activity against free radical[2], pathogenic bacteria and virus[3-5]. Eugenol, a major component of clove oil also was previously have been reported on antibacterial[6] and antifungal[7] properties.

Among the inflammatory substances released from granulocytes, histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity[8]. Basophil and mast cell activation are initiated upon interaction of multivalent antigen with its specific IgE antibody attached to the cell membrane via FcεRI[9,10]. To search for degranulation inhibitors from

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herbs, we selected the rat basophilic leukemia cell line, RBL-2H3, as an *in vitro* model. Cells from this line express Fc ε RI complexes and can release chemical mediators in response to the cross-linking of Fc ε RI[11]. This cell line has also been widely used to determine signal transduction from Fc ε RI[12,13].

The signaling pathway leading to degranulation of mast cells and basophil after engagement of the Fc ε RI receptor has been extensively characterized[14,15]. Activation of mast cells leads to phosphorylation of tyrosinase kinase and mobilization of internal calcium. This is followed by activation of protein kinase C, mitogen-activated protein kinase(MAPK).

In this study, we evaluated the effect of SAE on the IgE mediated degranulation activity and activation of MAPK in RBL-2H3.

Materials and Methods

1. Reagents and cell culture

Mouse monoclonal anti-dinitrophenol(DNP) IgE and DNP-labeled human serum albumin were purchased from Sigma(St. Louis, Mo., USA). Histamine ELISA kit was purchased from Cayman(Ann Arbor, Michigan, USA). Rat basophilic leukemia (RBL-2H3) cell line was purchased from ATCC(Manassas, VA, USA). MEM supplemented with 15% heat-inactivate fetal bovine serum, kept in a humidified atmosphere with 5% CO_2 at 37°C. The same medium used for routine subcultivation before cells reached confluence. Cells were counted with a hemocytometer and number of viable cells was determined through trypan blue dye exclusion.

2. Plant material

The authenticated dried flower buds of clove were purchased from a local market in Jegi, Seoul, Korea on August 2010 and identified by Dr. Ee-Hwa Kim in comparison with a voucher specimen deposited at the Department of Korean Medicine, Semyung University(Jecheon, Korea) under number SMU-2436.

3. Preparation of *Syzygium aromaticum* Ethanol Extract (SAE)

The dried flower buds of clove(1 kg) were powdered using a commercial electric stainless steel blender and extracted three times with ethanol(5 L) at room temperature for 24 h. The extract was filtered and concentrated *in vacuo* to obtain the crude ethanol extract(185 g). The crude ethanol extract was placed at 4°C until use.

4. MTT (3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide) colorimetric assay

A colorimetric assay using MTT was performed. Briefly, cells were added onto the 96 well flat-bottomed micro culture plates in the presence or absence of various concentrations of the SAE(in triplicate) and incubated at 37°C in a 5% humidified CO_2 incubator for 12 hours. Then, 10 μl of MTT(5 mg/ml) was added to each well and incubation was continued for a further 2 hours at 37°C. After discarded media, 100 μl/well of DMSO was added into each well. After complete solubilization of the dye, plates were read at 570 nm. The reference wavelength was 690 nm.

5. Assay of anti-allergic activity

The inhibitory activity of the plant extracts against the release of β-hexosaminidase from RBL-2H3 cells was evaluated according to Choi et al[13]. RBL-2H3 cells were grown in MEM supplemented with 15% fetal bovine serum and L-glutamine. Before the experiment, cells were dispensed into 24-well plates at a concentration of 5×10^5 cells per well. The cells were then sensitized by incubation in medium containing 0.5 μg/ml of mouse monoclonal IgE overnight at 37°C in 5% CO_2. They were subsequently washed with 500 μl of siraganian buffer(pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH) and incubated in 180 μl of siraganian buffer containing 5.6 mM glucose, 1 mM $CaCl_2$ and 0.1% BSA for an additional 10 min at 37°C. The cells were then exposed to 20 μl of test material for 20 min, followed by treatment with 20 μl of antigen (DNP-BSA, 1 μg/ml) for 30 min at 37°C to activate the cells and evoke allergic reactions(degranulation). The reaction was

stopped by cooling in an ice bath for 10 min. The reaction mixture was centrifuged at 1,000 rpm for 10 min, and 25 μl aliquots of the supernatant were transferred to a 96-well plate and incubated with 25 μl of substrate (1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide) for 1 h at 37°C. The reaction was stopped by adding 200 μl of 0.1 M Na_2CO_3/$NaHCO_3$. Absorbance was measured using an ELISA plate reader at 405 nm.

Histamine secretion was measured by an enzyme-linked immunosorbent assay(ELISA). RBL-2H3 cells were sensitized with monoclonal mouse IgE. The ELISA was performed by coating 96-well plates with histamine monoclonal antibody. Before use and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 and twice with PBS alone. For the standard curve, r-histamine was added to the reaction buffer. After exposure to the medium, the assay plates were exposed sequentially to biotinylated anti-hiatamine and 2,2'-azino-bis(3-ethylbez thiazoline-6-sulfonic acid. Optical density was read within 10 min of the substrate with a 405 nm.

6. Western Blot Analysis

Cell extracts were prepared by detergent lysis procedure. Samples of protein(50 μg) were electrophoresed using 10% SDS-PAGE, and then transferred to nitrocellulose membrane. The p38 MAPK, ERK, and JNK activation was determined using anti-phospho-p38, -ERK, and -JNK antibodies (Cell Signaling, Beverly, MA, USA).

7. Statistical analysis

The results obtained were expressed as mean±S.E.M. for the number of experiments. Student's t-test was used to make a statistical comparison between the groups. Results with $p<0.05$ were considered statistically significant.

Results

1. Cell viability

To assess the cytotoxicity on RBL-2H3 cell, we peformed MTT assay after cells were exposed to different concentrations of SAE for 24 h. When cells were treated with 10 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml concentrations of SAE, viability of cells were 1.05±0.05, 1.06±0.06, 1.01±0.03, 0.97±0.02, respectively.(viablity of Control=1) But the viability of cells was 0.76±0.07 when treated with 200 μg/ml concentration of SAE, there was significant diffrence with Control(Fig. 1).

2. Assay of anti-allergic activity

1) β-Hexosaminidase release: To determine the extent of degranulation we measured β-Hexosaminidase release. Only challenged with DNP-BSA, release of β-Hexosaminidase was

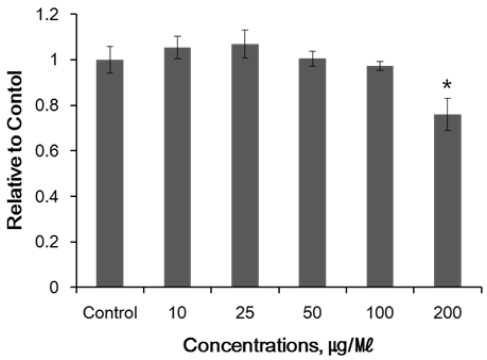

Fig. 1. Effect of SAE on the cell viability of RBL-2H3 cells.
Viability of cells exposed to different concentrations of SAE for 24 h. The values are expressed as mean±SEM from three independent experiments. *Significant difference at <0.05.

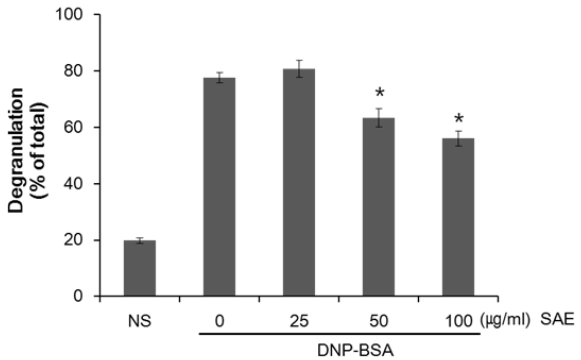

Fig. 2. Effect of SAE on IgE-mediated β-hexosaminidase release from RBL-2H3 cells.
The cells(2×10^4 cells/well) were preincubated with SAE at 37°C for 10 min prior to challenge with DNP-BSA. The values are expressed as mean±SEM from three independent experiments. *Significant difference at <0.05.

77.60±1.88%. When preincubated with SAE(50 μg/ml, 100 μg/ml) at 37°C for 10 min prior to challenge with DNP-BSA, release of β-Hexosaminidase was significantly inhibited (63.29±3.29%, 56.00±2.70%). There was no inhibitory effect preincubated with 25 μg/ml of SAE(80.78±3.07%)(Fig. 2).

2) Histamine release: Similar to the inhibatory effect on β-Hexosaminidase release, when preincubated with SAE(50 μg/ml, 100 μg/ml) at 37°C for 10 min prior to challenge with DNP-BSA, release of histamine was significantly inhibited (53.30±3.26 pg/ml, 39.87±4.16 pg/ml). There was no significant difference at concentration of 25 μg/ml(62.05±2.80 pg/ml) in comparison with only DNP-BSA challenged(60.68 ±4.16 pg/ml)(Fig. 3).

3. Western blot analysis

To evaluate the mechanisms of effect of SAE on the secretion of degranulate mediators, we examined the effect of SAE on the activation of MAPKs. As shown in Fig. 4, SAE attenuated the IgE-induced phosphorylation of p38 MAPK but did not affect the phosphorylation of JNK and ERK.

Discussion

Allergic diseases are increasing rapidly, because of multiple reasons such as pollution, vegetation Changes, and genetic influences[14]. So the discovery of drugs of the treatment of allergic disease is an important subject in human health.

Syzygium aromaticum Merrill et Perry has been used medicinal herb in oriental medicine for many diseases such as suppression of toothache and halitosis[1], and recently clove has been widely investigated and was reported activity against oxidant[2] and pathogenic bacteria/virus[3-5]. The major component of clove oil, Eugenol, also was previously have been reported, namely antibacterial[6], and antifungal[7] properties.

In this study, we investigated the effect of ethanol extract of *Syzygium aromaticum*(L.) Merr. & Perry.(SAE) on the RBL-2H3 cell-mediated allergic response and studied its possible mechanisms of action.

Cytotoxicity on RBL-2H3 cell was evaluated by MTT assay. No cytotoxicity was apparent when the concentration of SAE was less than 100 μg/ml on rat basophilic leukemia cell (RBL-2H3)(Fig. 1).

Stimulation of mast cells starts the process of degranulation resulting in releasing of mediators, such as histamine. β-Hexosaminidase is located in the secretory granules of mast cells where histamine is stored, and is released along with histamine when mast cells are immunologically activated[16]. Therefore, β-hexosaminidase is designated as a 'degranulation marker', and the release of β-hexosaminidase has been used to determine the extent of degranulation and to evaluate anti-allergic activities[17]. We evaluated the ability of SAE to inhibit IgE-mediated β-hexosaminidase and histamine release at concentrations of 50-100 μg/ml(Fig. 2, 3).

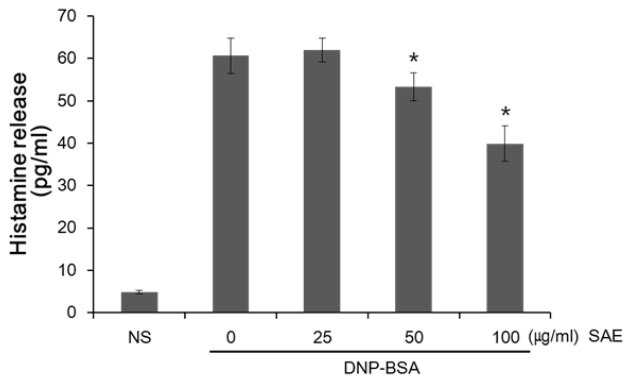

Fig. 3. Effect of SAE on IgE-mediated histamine release from RBL-2H3 cells.
The cells(2×10^4 cells/well) were preincubated with SAE at 37°C for 10 min prior to challenge with DNP-BSA. The values are expressed as mean±SEM from three independent experiments. *Significant difference at <0.05.

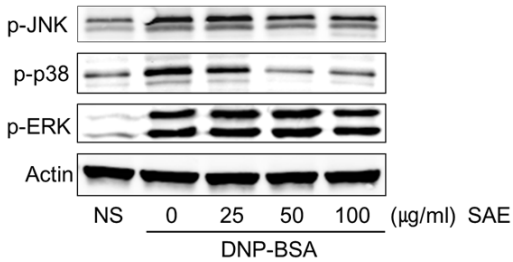

Fig. 4. Inhibitory effect of SAE on MAPKs phosphorylation in RBL-2H3 cells.
After pretreatment of SAE for 30 min, RBL-2H3 cells were stimulated by DNP-BSA 15 min for MAPKs activation. Phosphorylation of MAPKs was analyzed by Western blot.

SAE dose-dependently inhibited RBL-2H3 cell degranulation.

To evaluate the mechanisms of effect of SAE on the secretion of degranulate mediators, we examined the effect of SAE on the activation of MAPKs. SAE attenuated the IgE-induced phosphorylation of p38 MAPK and JNK but did not affect the phosphorylation of ERK(Fig. 4).

The MAPK cascade is one of the important signaling pathways in immune response[18]. MAPK pathways play a crucial role in the regulation of proinflammatory molecules on cellular responses[18,19]. The exact signaling pathways among three types of MAPKs, such as p38, ERK, and JNK, are still unclear; however, p38 MAPK is thought to play an important role in regulation of inflammatory responses.

Although the results discussed above are limited to in vitro anti-allergic activities, the results of this study suggest that flower bud of clove ethanol extract could be a good source for natural anti-allergic agents.

Conclusion

We evaluated the effect of ethanol extract of *Syzygium aromaticum*(L.) Merr. & Perry.(SAE) on the RBL-2H3 cell-mediated allergic response and its possible mechanisms of action as follows:

1. SAE had no cytotoxicity on rat basophilic leukemia cell(RBL-2H3).
2. SAE dose-dependently inhibited RBL-2H3 cell degranulation and histamine release.
3. SAE blocked the IgE-dependent activation of JNK and p38 MAPK, thereby inhibiting allergic responses.

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국문초록

목적 : 본 연구에서는 정향 에탄올 추출물이 RBL-2H3 세포 매개 알레르기 반응에 대해 미치는 영향과 그 작용기전에 대해 연구했다. **방법 :** 정향 에탄올 추출물의 RBL-2H3 세포에 대한 독성 여부는 MTT 분석을 통해 평가했다. 정향 에탄올 추출물의 항알러지 작용은 효소결합면역분석방법(ELISA)을 이용해 β-Hexosaminidase과 Histamine의 분비량을 측정하여 평가하였다. 정향 에탄올 추출물의 작용기전에 대해서는 유사 분열물질-활성화단백질인산화효소(mitogen-activated protein kinase, MAPK)를 western blot 법을 이용하여 측정함으로써 평가하였다. **결과 :** 정향의 에탄올 추출물은 RBL-2H3 세포에 대해 독성을 나타내지 않는 농도에서 RBL-2H3 세포의 탈과립과 히스타민 분비를 유의하게 억제하였으며, p38 MAPK의 활성을 차단하였다. **결론 :** 본 연구의 결과 정향의 에탄올 추출물은 비만세포에서 유래된 알러지 반응을 억제하는 효과가 있으며, 또한 그 작용기전은 p38 MAPK 인산화와 연계되어 있을 것으로 사료된다.