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Samsoeum inhibits systemic anaphylaxis and release of histamine, cytokine *in vivo* and *in vitro*

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SUMMARY

Samsoeum (SSE) is used in traditional oriental medicine for various medicinal purposes. However, the exact mechanism that accounts for the anti-allergy and anti-inflammatory effects of the SSE is still not fully understood. The aim of the present study is to elucidate whether and how SSE modulates the allergic reactions *in vivo*, and inflammatory reaction *in vitro*. In this study, we showed that SSE significantly decreased compound 48/80-induced systemic anaphylaxis, ear-swelling response, histamine release from preparation of rat peritoneal mast cells and anti-dinitropheny IgE-induced passive cutaneous reaction. Also, SSE inhibited the expression of inflammatory cytokine and cyclooxygenase-2 in PMA plus A23187-stimulated human mast cells (HMC-1). In addition, we showed that anti-inflammatory mechanism of SSE is through suppression of nuclear factor-κB activation and IκB-α phosphorylation/degradation in HMC-1. These results provided new insight into the pharmacological actions of SSE as a potential molecule for therapy of inflammatory allergic diseases.

Key words: Samsoeum; Allergic reactions; Inflammation; Cyclooxygenase-2; Nuclear factor-κB

INTRODUCTION

Mast cells are widely distributed throughout the body in both connective tissue and mucosal surfaces (Metcalfe *et al*., 1997). The role of mast cells as effecter cells of immunoglobulin (Ig) E-dependent immediate-type hypersensitivity reactions and anaphylaxis is well understood (Hamelmann *et al*., 1999; Galli, 2000). Activation of mast cells is elicited through the cross-linking of allergen-specific IgE bound to the high-affinity receptor for IgE, FcεRI, on the cell membrane, which results in the

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degranulation of mast cells and the release of mediators including histamine, proteases, leukotrienes, prostaglandin (PG)s, and different cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 (Gordon *et al*., 1990; Kawata *et al*., 1995; Artuc *et al*., 1999). Mast cells are also involved in the development of late-phase reactions and influence other chronic inflammatory responses through the generation and secretion of various cytokines (Kruger-Krasagakes and Czarnetzki, 1995). Inflammatory cytokine such as TNF-α, IL-1, IL-6, and IL-8 induce inflammation and recruit other immune cells including neutrophils and T lymphocytes (Medzhitov and Janeway, 1997). Although these cytokines are beneficial to the host defense, they can also trigger pathological conditions when expressed in excess (Beutler, 1995). Cyclooxygenase (COX)-2 is an inducible enzyme found at low concentration in healthy tissues, but it is up regulated in response to tissue damage during inflammation. There was reported that COX-2, one of the major mediators of the inflammatory reaction, is also strongly induced in activated monocytes/macrophages (Meade *et al*., 1993).

Nuclear factor (NF)-κB is a key transcription factor required for the expression of many inflammatory involved genes including COX-2 and inflammatory cytokines (Jung *et al*., 2003). In an inactive state, NF-κB is normally sequestered in the cytoplasm of cells where it is bound by a family of inhibitory proteins known as IκB-α (Barnes and Karin, 1997). A variety of stimuli modulate signal transduction pathways to activate IκB kinases. Stimulation of the activity of these kinases results in phosphorylation, ubiquitination, and degradation of IκB-α, leading to the nuclear translocation of NF-κB (Schaecher *et al*., 2004).

Samsoeum (SSE) is used in traditional oriental medicine for various medicinal purposes. However, the exact mechanism that accounts for the anti-allergy and anti-inflammatory effects of the SSE is still not fully understood. The aim of the present study was to elucidate whether and how SSE modulates the allergic reactions *in vivo*, and mast cell mediated-inflammatory reaction *in vitro*. Therefore, we investigate the mechanisms underlying the pharmacological effects of SSE on both compound 48/80-induced and anti-dinitropheny (DNP) IgE-induced allergic reactions *in vivo*. Also, to find a possible explanation for the anti-inflammatory mechanisms of SSE, we evaluate effects of SSE on phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187-induced production and expression of cytokine, COX-2 expression, and NF-κB activation.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS), α-minimal essential medium and Iscove's Modified Dulbecco's Medium (IMDM) were purchased from Gibco BRL (Grand Island, NY, USA). Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), 2,2-azino-bis (3-ethylBenzthiazoline-6-sulfonic acid), PMA, metrizamide, A23187, 3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazoliumbromide (MTT) and other reagents were obtained from Sigma (St. Louis, MO, USA). Anti-human TNF-α antibody (Ab), biotinylated anti-human TNF-α Ab, and recombinant human (rh) TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). Anti-human IL-6 and IL-8 Ab, biotinylated anti-human IL-6 and IL-8 Ab, and rh IL-6 and IL-8 were purchased from PharMingen (Sandiego, CA, USA). Abs for anti-human NF-κB, IkB-α, p IκB-α, and actin were purchased from Santa Cruz Biotech. (Santa Cruz, CA, USA).

Preparation of SSE

SSE which is a mixture of 12 traditional drugs as shown in Table 1 was obtained from the College of Pharmacy, Wonkwang University (Iksan, South Korea). Amounts of the 12 traditional drugs studied in this work were shown in Table 1. Extract of SSE was prepared by decocting the dried description of herbs with boiling distilled water. The extraction decocted for approximately

Table 1. The ratio of the component in SSE

Components	Ratio
1. Ginseng Radix (*Panax ginseng C.A.* M_{EYER})	4
2. Perillae Folium (*Perilla frutescens (L.)* $B_{RITT.}$ var. acuta ($T_{HUNB.}$) K_{UDO})	4
3. Peucedani Radix (*Peucedanum decursivum* ($M_{IQ.}$) M_{AXIM})	4
4. Pinelliae Tuber (*Pinellia ternata* ($T_{HUNB.}$) $B_{REIT.}$)	4
5. Puerariae Radix (*Pueraria thunbergiana* ($S_{IEB.}$ et $Z_{UCC.}$) $B_{ENTH.}$)	4
6. Hoelen (*Poria cocos* W_{olf})	4
7. Aurantii nobilis Pericarpium (*Citrus unshiu* M_{ARCOR})	3
8. Platycodi Radix (*Platycodon grandiflorum* ($J_{ACQ.}$) A. DC.)	3
9. Glycyrrhizae Radix (*Poncirus trifoliata* (L.) $R_{AF.}$)	3
10. Zingiberis Rhizoma (*Glycyrrhiza uralensis* $F_{ISCH.}$)	3
11. Zizyphi inermis Fructus (*Zingiber officinale* $R_{OSC.}$)	3
12. Ponciri Fructus (*Ziziphus jujuba* $M_{ILL.}$ *var. intermis* (B_{UNGE}) $R_{EHD.}$)	3

3 h has been filtered, lyophilized, and kept a 4 °C. The yield of extraction was about 12%. Dilutions were made in saline then filtered through 0.45-mm syringe filter.

Animals

The original stock of ICR mice and Wistar rats were purchased from the Dae-Han Experimental Animal Center (Eumsung, South Korea), and the animals were maintained in the College of Pharmacy, Wonkwang University. The rats were housed five to ten per cage in a laminar air-flow room maintained at a temperature of 22 ± 1 °C and relative humidity of 55 ± 10% throughout the study. No animal was used more than once. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the US guidelines (NIH publication #85-23, revised in 1985).

Compound 48/80-induced systemic anaphylactic reaction

Mice were given an intraperitoneal injection of the mast cell degranulator compound 48/80 (8 mg/kg). SSE was dissolved in saline and administered orally 1 h before the injection of compound 48/80. Mortality was monitored for 23 min after induction of anaphylactic reaction.

Ear-swelling response

Compound 48/80 was freshly dissolved in saline and injected intradermally into the dorsal aspect of a mouse ear using a microsyringe with a 29-gauge hypodermic needle. Ear thickness was measured with a digimatic micrometer (Mitutoyo, Japan) under mild anesthesia. Ear-swelling response represented an increment in thickness above baseline control values. Ear-swelling response was determined 40 min after compound 48/80 or vehicle injection. SSE was administered orally 1 h before the compound 48/80 injection (100 mg/site). The values obtained would appear to represent the effect of compound 48/80 rather than the effect of the vehicle injection (physical swelling), since the ear-swelling response evoked by physiologic saline returned to almost baseline thickness within 40 min.

Passive cutaneous reaction (PCA)

IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the mice tail vein. The DNP-HSA was diluted in phosphate-buffered saline (PBS). The mice were injected intradermally with 100 ng of anti-DNP IgE into each of three dorsal skin sites. The sites were outlined with a water-insoluble red marker. Forty-eight hours

later, each mouse received an injection of 200 ml of the 1 : 1 mixture of 1 mg/ml DNP-HSA in PBS and 4% Evans blue via the tail vein. One hour before this injection, SSE was administered orally. The mice were sacrificed 40 min after the intravenous challenge. The dorsal skin of the mouse was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 0.5 ml of 1.0 mol/l KOH and 4.5 ml of a mixture of acetone and phosphoric acid (with the ratio of 5 : 13). The absorbent intensity of the extraction was measured at 620 nm in a spectro- fluorometer, and the amount of dye was calculated with the Evans blue measuring-line.

Preparation of rat peritoneal mast cells (RPMCs)

RPMCs were isolated as previously described (Jippo-Kanemoto *et al*., 1993). In brief, rats were anesthetized by ether, and injected with 20 ml of Tyrode buffer B (NaCl, glucose, $NaHCO_3$, and NaH_2PO_4) containing 0.1% gelatin into the peritoneal cavity; the abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells were aspirated by Pasteur pipette. Then the peritoneal cells were sedimented at 150 × g for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells (i.e. macrophages and small lymphocytes) according to the method described by Yurt *et al* (1997). In brief, peritoneal cells suspended in 1 ml of Tyrode buffer B were layered onto 2 ml of 0.225 g/ml metrizamide (density 1.120 g/ml) and centrifuged at room temperature for 15 min at 400 × g. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose and 0.1% bovine serum albumine) containing calcium. Mast cell preparation was about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable judged by trypan blue uptake.

Histamine assay

RPMCs suspensions (2×10^5 cells/ml) were preincubated for 10 min at 37 °C before the addition of compound 48/80 for stabilization. The cells were preincubated with the SSE for 20 min, and then incubated for 15 min with compound 48/80 (6 mg/ml). The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at 400 × g for 5 min at 4 °C. Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at 400 × g for 5 min at 4 °C. The histamine content was measured by the o-phthalaldehyde spectrofluorometric procedure of Shore *et al.* (1959). The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer. The inhibition percentage of histamine release was calculated using the following equation:

$$\% \text{ Inhibition} = (A - B) \times 100/A$$

Where A is histamine release without SSE and B is histamine release with SSE.

Nuclear fast red (NFR) staining

In order to compare the status of mast cells before or after the addition of SSE would make it clear whether SSE affects the degranulation process or not, alcian blue-NFR staining was performed. Mast cells were centrifuged with cytospin at 28 g for 5 min and then fixed with Carnoy's solution. Cells were stained with 1% alcian blue and NFR. They were then rinsed in distilled water and gradually dehydrated in a series of 80%, 90%, 95% and 100% alcohol. The slides were cleared in xylene and mounted with mounting medium.

Culture of HMC-1 cells

Human mast cell line, HMC-1, cells were grown in IMDM medium supplemented with 100 IU/ml

penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated FBS at 37 °C in 5% CO_2.

MTT Assay

To test the viability of cells, MTT colorimetric assay was performed as described previously (Kim *et al*., 2001). Briefly, HMC-1 cells (1×10^5 cells/ml) were incubated for 8 h after stimulation in the absence or presence of SSE. After addition of MTT solution, the cells were incubated at 37 °C for 4 h. The crystallized MTT was dissolved in dimethyl sulfoxide and measured the absorbance at 540 nm.

Assay of cytokines

TNF-α, IL-6, and IL-8 secretion were measured by modification of an enzyme-linked immunosorbent assay (ELISA) as described previously (Na *et al*., 2002). 96 well plates were coated with 100 ml aliquots of anti-human TNF-a, IL-6, and IL-8 monoclonal Abs at 1.0 mg/ml in PBS at pH 7.4 and were incubated overnight at 4 °C. After additional washes, 100 ml of cell medium or TNF-α, IL-6, and IL-8 standards were added and incubated at 37 °C for 2 h. After 2 h incubation at 37 °C, the wells were washed and then 0.2 mg/ml of biotinylated anti-human TNF-α, IL-6, and IL-8 was added and again incubated at 37 °C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 30 min at 37 °C. Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF-α, IL-6, and IL-8 in serial dilutions.

RNA isolation and RT-PCR

Total RNA was isolated from HMC-1 according to the manufacturers specifications using an easy-BLUE RNA extraction kit (iNtRON Biotech, Seoul, South Korea). The concentration of total RNA in the final elutes was determined by spectrophotometry. Total RNA (2.0 mg) was heated at 65 °C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37 °C using a cDNA synthesis kit (AmershamPharmacia, NJ, USA). RT-PCR was carried out with 1ml of a cDNA mixture, in 20 ml final volume with 2.5 mM $MgCl_2$, 200 mM dNTPs, 25 pM of cytokine primers, and 2.5 U of Taq DNA polymerase in the reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9, and 0.1% Triton X-100). PCR was performed with the following primers for human TNF-a (5'-CAC CAG CTG GTT ATC TCT CA-3'; 5'-CGG GAC GTG GAG CTG GCC GAG GAG-3'), IL-8 (5'-CGA TGT CAG TGC ATA AAG ACA-3'; 5'-TGA ATT CTC AGC CCT CTT CAA AAA-3'), IL-6 (5'-GAT GGA TGC TTC CAA TCT GGA T-3'; 5'-AGT TCT CCA TAG AGA ACA ACA TA -3'), COX-2 (5'-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3'; 5'- AGA TCA TCT CTG CCT GAG TAT CTT- 3'), and GAPDH (5'-CAA AAG GGT CAT CAT CTC TG -3'; 5'-CCT GCT TCA CCA CCT TCT TG-3') were used to verify if equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 60 °C for TNF-a and IL-8, 50 °C for IL-6, 55 °C for COX-2 and 62 °C for GAPDH, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Preparation of cytoplasmic and nuclear extract

Nuclear and cytoplasmic extracts were prepared as described previously (Schoonbroodt *et al*., 1997). Briefly, after cell activation for the times indicated cells were washed with ice-cold PBS and resuspended in 60 ml of buffer A (10 mM Hepes/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM PMSF, pH 7.9). The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 ml of 10% Nonide P-40, and centrifuged at 1200 × g for 10 min at 4 °C. The supernatant was collected and used as the cytoplasmic extracts. The nuclei pellet was resuspended in 40 ml of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM

PMSF, pH 7.9), left on ice for 20 min, and inverted. The nuclear debris was then spun down at 1200 × g for 15 min. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen and stored at -70 °C until conducting the analysis.

Western blot analysis

For analysis of the levels of NF-κB, p-IκB-α, and IκB-α, stimulated cells were rinsed twice with ice-cold PBS and were then lysed in ice-cold lysis buffer (1% Triton, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholate in PBS). Cell lysates were centrifuged at 15,000 × g for 5 min at 4 °C; the supernatant was then mixed with an equal volume of 2 × SDS sample buffer, boiled for 5 min, and then separated through 10% SDS–PAGE gels. After electrophoresis, the protein was transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 5% skim milk for 2 h, rinsed, and incubated overnight at 4 °C with primary antibodies in PBS/0.5% Tween 20. Excess primary antibody was then removed by washing the membranes four times in PBS/0.5% Tween 20, and the membranes were incubated for 1 h with HRP-conjugated secondary antibodies (against mouse, or rabbit). After three washes in PBS/0.5% Tween 20, the protein bands were visualized by an enhanced chemiluminesence assay (Amersham Pharmacia Biotech, NJ, USA) following the manufacturer's instructions.

HPLC analysis

The Alliance 2695 HPLC system consisted of a pump (Waters Assoc., USA: 501 HPLC pump), a 2996 PDA detector (Water Assoc., USA: 2996 PDA detector), an autosampler (Water Assoc., USA: 746 computing integrator). A YMC-Pack ODS-AQ 303 colum (4.6 mm × 250 nm, 5 m) was used. Buffer (25 mM/L phosphoric acid, pH 2.25): Acetonitrile (80:20) was used as the mobile phase. Detection of the peaks was made at 210 nm and the sensitivity was set of 1.0 AUFs. The injection volume was 20 ml and flow rate was 1.0 ml/min. Standard solution was prepared by dissolving in distilled water (10 mg/100 ml). The solution was filtered through 0.45 ml membrane filter and applied to HPLC.

Statistical analysis

The results were expressed as mean ± S.E.M. for a number of experiments. Statistical significance was compared between each treated group and control by analysis of variance (ANOVA), with a Tukey *post hoc* test. For all tests, *P* value less than 0.05 was considered significant.

RESULTS

Effect of SSE on compound 48/80-induced systemic anaphylaxis

To assess the contribution of SSE in anaphylactic reactions, we first used the *in vivo* model of systemic anaphylaxis. As a non-immunologic stimulator, compound 48/80 (8 mg/kg) was used. After the injection of compound 48/80, the mice were monitored for 23 min, after which the mortality rate was determined. The period for observation of mortality was based on the control mice that had died in 23 min by compound 48/80. As shown in Table 2, an oral administration of saline as a control induced a fatal reaction in 100% of each group. When the SSE was orally administered at concentrations of 0.01 - 1 g/kg 1 h

Table 2. Effect of SSE on compound 48/80-induced systemic anaphylactic reaction in mice

SSE dose (g/kg)[a]	Compound 48/80 (8 mg/kg)[b]	Mortality (%)[c]
None (saline)	+	100.0
0.01	+	50.0
0.1	+	33.3
1	+	16.7

[a]The groups of mice (n = 5) were orally pretreated with 200 μl of saline or SSE was given at various doses 1 h before the compound 48/80 injection. [b]The compound 48/80 solution was intraperitoneally given to the groups of mice. [c]Mortality (%) is presented as the 'Number of dead mice × 100/Total number of experimental mice'.

Table 3. Effects of SSE on compound 48/80-induced ear swelling response, and on PCA in mice

Dose of SSE (g/kg)	Ear swelling response[a]		PCA[b]	
	Thickness of ear (mm)	Inhibition (%)	Amount of dye (μg/site)	Inhibition (%)
None	0.293 ± 0.002		2.811 ± 0.275	
0.01	0.247 ± 0.010*	15.60	2.453 ± 0.538	12.70
0.1	0.199 ± 0.007*	32.12	2.013 ± 0.242*	28.40
1	0.135 ± 0.009*	53.76	1.880 ± 0.354*	32.74

[a]20 ml of compound 48/80 (100 mg/site) were applied intradermally. The mice were orally administered with the various concentrations (0.01, 0.1 and 1.0 g/kg) of SSE for 1 h prior to the compound 48/80 application. Each datum represents the means ± S.E.M. of three independent experiments. *P < 0.05, significantly different from the saline value. [b]Saline or SSE was applied orally 1 h prior to the challenge with antigen. Each amount of dye represents the means ± S.E.M. of three independent experiments. *P < 0.05, significantly different from the saline value.

before compound 48/80 injections, the mortality was reduced (Table 2).

Effect of SSE on ear swelling response and PCA in mice

To investigate the effect of SSE on allergy response *in vivo,* we performed the ear swelling and PCA in mice. We choose a concentration of 100 mg/site for compound 48/80 induced optimal ear-swelling response in this experiment. As shown in Table 3a, when mice were pretreated with SSE for 1 h, the ear swelling response derived from compound 48/80 was reduced in dose-dependent manner (P < 0.05). Inhibition rate of SSE (0.01-1 g/kg) on ear-swelling response was about 15.60% (P < 0.05), 32.12% (P < 0.05), and 53.76% (P < 0.05), respectively.

PCA is one of the most important *in vivo* models of anaphylaxis in local allergic reactions (Wershil *et al*., 1987). When SSE was orally administered to the mouse, the PCA reaction was inhibited in a dose-dependent manner (Table 3b). Inhibition rate of SSE (0.01 - 1 g/kg) on PCA reaction was about 12.70% (P > 0.05), 28.40% (P < 0.05), and 32.74% (P < 0.05), respectively.

Effect of SSE on histamine release and degranulation from RPMCs

Histamine has been widely used as a marker of mast cell degranulation *in vitro*, and remains the best-characterized and most potent mediator implicated in the acute phase of immediate hypersensitivity (Petersen *et al*., 1996; Moon *et al*., 2003). The inhibitory effect of SSE on compound 48/80-induced histamine release from RPMCs is shown in Fig. 1A. SSE dose dependently inhibited

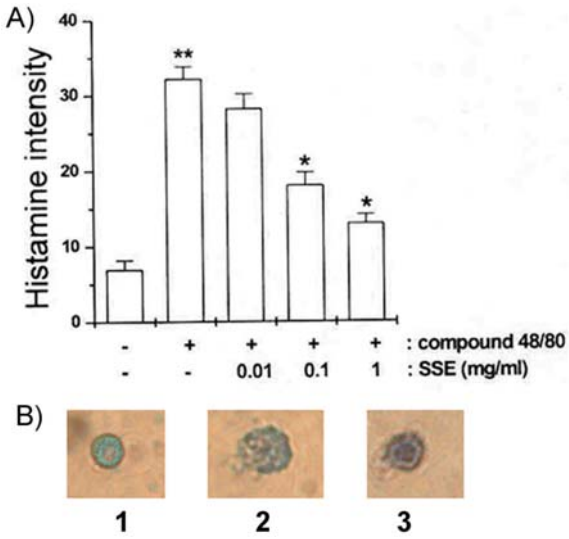

Fig. 1. Effect of SSE on histamine release and degranulation from RPMCs. A) RPMCs (2×10^5 cells) were preincubated with various concentrations of SSE at 37 °C for 10 min and then incubation with compound 48/80. The histamine content was measured by the spectrofluorometer as described under material and methods. B) The photographs of alcian blue-NFR stained mast cells. 1) blank; 2) Compound 48/80 - stimulated RPMCs; 3) SSE (1 mg/ml) + Compound 48/80 - stimulated RPMCs. Magnifications were × 400. All data represent the mean ± S.E.M. of four independent experiments. *P < 0.05, significantly different from the compound 48/80-stimulated cells. **P < 0.01, significantly different from the unstimulated cells.

compound 48/80-induced histamine release at concentrations of 0.01-1 mg/ml. In particular, SSE significantly inhibited the compound 48/80-induced histamine release at concentrations of 0.1 - 1 mg/ml. Inhibition rate of the histamine release at the doses of 0.1 - 1 mg/ml was about 43.75 % ($P < 0.05$) and 59.68 %($P < 0.05$), respectively.

The photographs of alcian blue-NFR stained-RPMCs are shown in Fig. 1B. Compound 48/80-stimulated RPMC in the absence SSE was extensively degranulated compared with SSE-treated cell, which is correlated with an inhibition of histamine release. The results indicate that SSE inhibits the compound 48/80-induced degranulation from mast cells.

Effect of SSE on cytokine production and expression in PMA plus A23187-stimulated HMC-1

To assess the effect of SSE in PMA plusA23187-induced TNF-α, IL-6, and IL-8 production, the cells were pretreated with various concentrations of SSE (0.01 - 1 mg/ml) for 30 min, and then treated with PMA plus A23187 for 8 h. Our results showed that PMA plus A23187 enhanced the secretion of TNF-α, IL-6, and IL-8, and these increase was inhibited by SSE in a dose-dependent manner (Fig. 2A). The maximal inhibition rate of TNF-α, IL-6, and IL-8 production by SSE (1 mg/ml) was about 47.1% ($P < 0.01$), 63.1% ($P < 0.05$) and 41.1% ($P < 0.05$), respectively.

To investigate effect of SSE on TNF-α, IL-6, and IL-8 expression, we performed RT-PCR. The cells were pretreated with various concentrations of SSE for 30 min, and then treated with PMA plusA23187 for 4 h. We showed that PMA plus A23187 induced the TNF-α, IL-6, and IL-8 expression, but pretreatment of SSE decreased the TNF-α, IL-6, and IL-8 mRNA level in a dose-dependent manner (Fig. 2B). Cell cytotoxicity by SSE was not observed (data not shown).

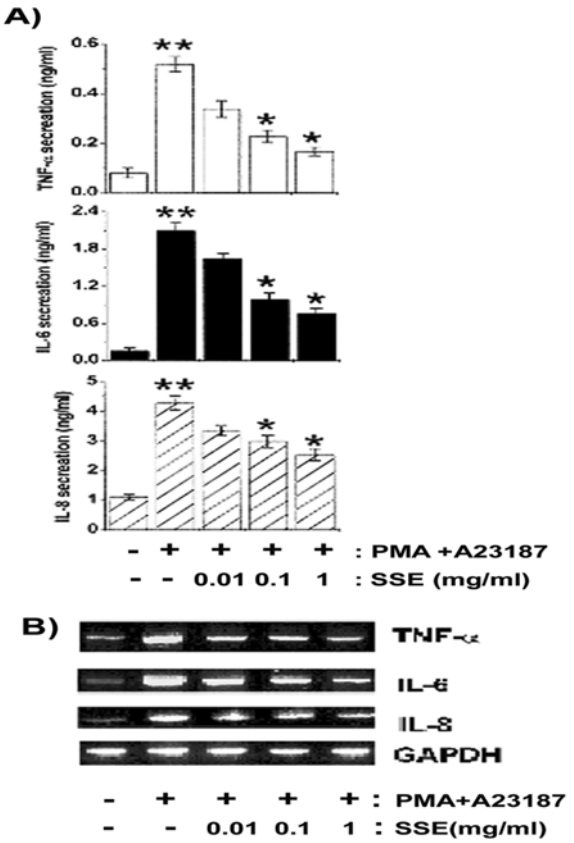

Fig. 2. Effect of SSE on cytokines production and expression in PMA plus A23187-stimulated HMC-1 cells. A) 3×10^5 HMC-1 cells were treated with SSE (0.01 - 1 mg/ml) for 30 min, and then stimulated with PMA (50 nM) plus A23187 (1 mg/ml) for 8 h. Cytokines concentration was measured in cell supernatants using the ELISA method. B) 5×10^6 HMC-1 cells were treated with SSE (0.01-1 mg/ml) for 30 min, and then stimulated with PMA (50 nM) plus A23187 (1 mg/ml) for 4 h. The total RNA was assayed by RT-PCR analysis. 1) blank; 2) PMA + A23187; 3) PMA + A23187 + 0.01 mg/ml SSE; 4) PMA + A23187 + 0.1 mg/ml SSE; 5) PMA + A23187 + 1 mg/ml SSE. All data represent the mean ± S.E.M. of four independent experiments. *$P < 0.05$, significantly different from the PMA plus A23187-stimulated cells. **$P < 0.01$, significantly different from the unstimulated cells.

Effect of SSE on COX-2 expression in PMA plus A23187-stimulated HMC-1

To determine the effect of SSE on COX-2 expression induced by PMA plus A23187, RT-PCR was performed. As shown in Fig. 3A, COX-2 mRNA expression was increased by PMA plus and A23187 for 12 h, and pretreatment of SSE (0.01 - 1 mg/ml) inhibited the COX-2 mRNA level in dose

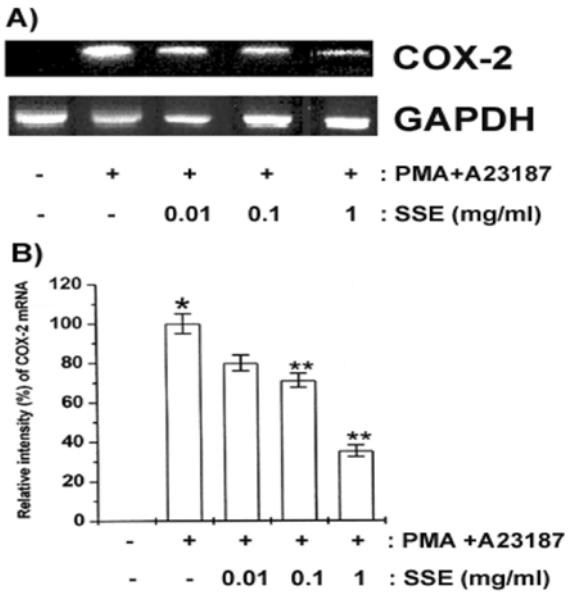

Fig. 3. Effect of SSE on COX-2 expression in PMA plus A23187-stimulated HMC-1 cells. A) Cells (5×10^6 cells/ml) were pretreated with SSE (0.01-1 mg/ml) for 30 min, and then stimulated with PMA (50 nM) plus A23187 (1 mg/ml) for 12 h. Then, COX-2 mRNA level was assayed by RT-PCR. B) The relative mRNA amounts were estimated by an image analyzer. 1) blank; 2) PMA + A23187; 3) PMA + A23187 + 0.01 mg/ml SSE; 4) PMA + A23187 + 0.1 mg/ml SSE; 5) PMA + A23187 + 1 mg/ml SSE. Results show a representative of four experiments, and the mean ± S.E.M. of the band intensities corresponding to the levels of COX-2. *P < 0.05, significantly different from the unstimulated cells. **P < 0.01, significantly different from the PMA plus A23187-stimulated cells.

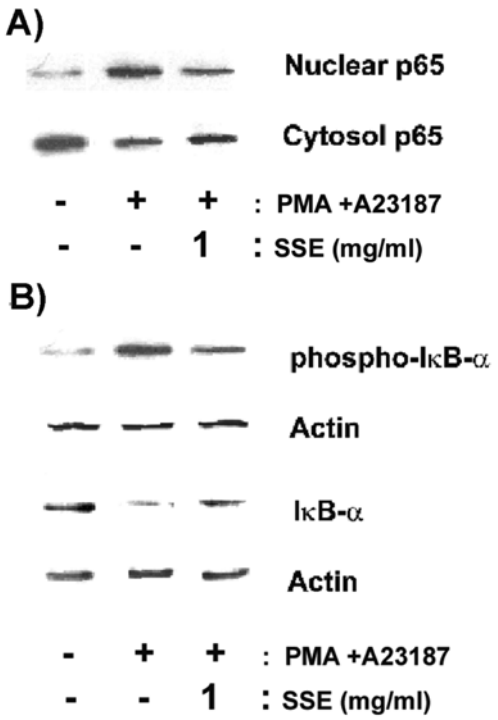

Fig. 4. Effect of SSE on NF-kB activation and IkB-a degradation/phosphorylation in PMA plus A23187-stimulated HMC-1 cells. A) 6×10^6 HMC-1 cells were preincubated for 30 min with SSE (0.01 - 1 mg/ml) and then treated with PMA (50 nM) plus A23187 (1 mg/ml) for 2 h. The cytosolic and nuclear extracts were determined for RelA/p65 by Western blot anlaysis. B) The cytosolic extracts were determined for IκB-α, phospho-IκB-α by Western blot anlaysis. 1) blank; 2) PMA + A23187; 3) PMA + A23187 + SSE (1 mg/ml).

dependent manner. The relative mRNA amounts were estimated by using image analyzer (VILBER LOURMAT FC-26WL, France) (Fig. 3B).

Effect of SSE on NF-κB activation and IkB-α phosphorylation and degradation in PMA plus A23187-Stimulated HMC-1

Since NF-κB activation requires nuclear translocation of *RelA*/p65 subunit of NF-κB, we examined the effect of SSE on the cytosolic and nuclear pool of *RelA*/p65 protein by Western blot analysis in HMC-1. As shown in Fig. 4A, we showed that PMA plus A23187 treatment considerably increased the nuclear *RelA*/p65 protein level and decreased the cytosolic *RelA*/p65, which is an indication of the nuclear translocation of RelA/p65. Pretreatment of SSE (1 mg/ml) inhibited the PMA plus A23187 stimulated increase and decrease of the nuclear and cytosolic *RelA*/p65 levels, respectively. These results suggested that SSE blocks the nuclear translocation of the *RelA*/p65 from cytoplasm.

Most agents that activate NF-κB mediate their effects through suppressing IκB-α phosphorylation and degradation (Yamamoto *et al*., 1999). As shown in Fig. 4B, PMA plus A23187 treatment effectively caused an induction of IκB-α phosphorylation, and degradation in HMC-1. We showed that SSE (1 mg/ml) significantly inhibited PMA plus A23187-

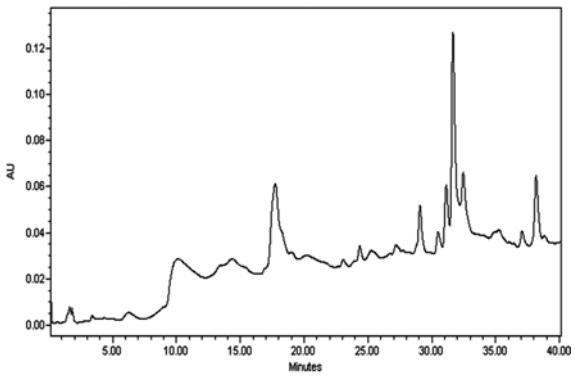

Fig. 5. HPLC Chromatogram of the SSE. The solution of SSE was prepared by dissolving in distilled water (10 mg/100 ml). The injection volume was 20 ml and the detection was made at 254 nm.

induced IκB-α phosphorylation and degradation.

Characterization of the principal components of SSE

The components of SSE were analyzed by HPLC. The SSE can be efficiently resolved by the applied method and were identified according to the retention times of the respective standards. Chromatogram of the SSE is shown in Fig. 5. Peaks of the principal components have not yet been identified in this study.

DISCUSSION

SSE is an oriental medication water-extracted from the herb medicines, which consists of 12 different herbs. Other studies reported that each medicine herb has different effects. For example, Ginsenosides isolated from the root of Ginseng Radix had the inhibitory activity on beta-hexosaminidase release from RBL-2H3 cells and on the PCA reaction (Choo *et al*., 2003), saponins of ginseng root has inhibitory effects on IL-1β and IL-6 gene expression in chronic inflammation model of aged rats (Yu and Li, 2000), Platycodin D, isolated from the root of Platycodi Radix has anti-inflammatory effect through inhibition of PGE_2 production in rat peritoneal macrophages (Kim *et al*., 2001), and 6-Gingerol, one of the major components of fresh Zingiberis Rhizoma, protects HL-60 cells from oxidative stress (Wang *et al*., 2003). SSE composed on the basis of the theory of Korean medicine to maximize its efficacy. However, the mechanism involved in anti-allergy and anti-inflammatory effect of SSE has not been examined. In this study, our findings show that SSE inhibited the compound 48/80-induced and IgE+Ag-induced allergic reactions *in vivo* and inhibited the TNF-α, IL-8, and COX-2, expression in PMA plus A23187-simulated HMC-1 cells. In addition, SSE inhibited NF-κB activation through suppression of IκB-α phosphorylation and degradation.

Mast cells are widely distributed throughout the body in both connective tissue and at mucosal surfaces (Metcalfe *et al*., 1997). The role of mast cells as effecter cells of IgE-dependent immediate-type hypersensitivity reactions and anaphylaxis is well understood (Hamelmann *et al*., 1993; Galli, 2000). It was reported that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane indicates, which may be an essential trigger for the release of mediators from mast cells (Tasak *et al*., 1986). In present study, we showed that SSE inhibited the systemic anaphylaxis and ear swelling caused by compound 48/80 in mice. Also, SSE inhibited histamine release and degranulation from RPMCs. So, it is possible to hypothesize that SSE might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80. PCA is a very effective way to test skin allergic reactions, which has been successfully applied in murine model with oriental medicine (Kim *et al*., 2000). In this study, we utilized PCA for testing protection effect from IgE-mediated local allergic reaction. We showed that SSE inhibited PCA response in dose dependent manner. This result suggested that SSE inhibits the initial phase of immediate type allergic reactions, probably through interference with the degranulation.

As mast cells contain potent mediators including

PGs and multifunctional cytokines, they contribute to the pathogenesis of chronic inflammatory disease. Therefore, mast cell activation significantly contributes to the initiation of exacerbation of inflammation. Inflammatory cytokines (TNF-α and IL-6) play roles in mediating the progression of many inflammatory diseases. TNF-α from mast cells can orchestrate the migration of neutrophils and T-lymphocytes into tissues, and promote chronicity in inflammatory lesions (Walsh *et al*., 1995). IL-8 from mast cells act on surrounding cells such as neutrophils, T-lymphocytes, and eosinophils, and activation of inflammatory effector cells (Mukaida, 2000). COX-2, one of the major mediators of the inflammatory reaction is also strongly induced in activated monocytes/macrophages. Several recent studies demonstrated that PGD_2, which is the COX-2 metabolite released from activated mast cells, is also essential for the pathogenesis of eosinophilic airway inflammation (Bochenek *et al*., 2004). Previously, it has been reported that COX-2 inhibitors abolished PGD_2 synthesis and attenuated eosinophil accumulation in the airways inflammation (O'Sullivan *et al*., 1998; Oguma *et al*., 2002). In this study, we observed that SSE inhibited TNF-α, IL-6, IL-8, and COX-2 expression in HMC-1. These results indicated that SSE has potential effect on anti-inflammatory response through the regulation of inflammatory genes in mast cells, which might explain its beneficial effect in the treatment of mast cell-mediated inflammatory diseases.

NF-κB is required for transcriptions pro-inflammatory molecules, including COX-2 and cytokines such as TNF-α, IL-6 and IL-8. Since these pro-inflammatory molecules are regulated at the transcription level, NF-κB is a critical intracellular mediator of the inflammatory cascade (Ghosh *et al*., 1998). Therefore, we postulated that SSE mediates its effects at least partly through suppression of NF-κB activation. In an inactive state, NF-κB is present in the cytoplasm as a heterodimer consisting of p50/ p65, and IκB-α subunits. In response to an activation signal, the IκB-α subunit is phosphorylated, ubiquitinated, and degraded through the proteosomal pathway, thus exposing the nuclear localization signals on the p50-p65 heterodimer. The p65 is then phosphorylated, leading to nuclear translocation and binding to a specific sequence in DNA, which in turn results in gene transcription. Inhibitors of NF-κB activation mediate their effects through suppressing IκB-α phosphorylation and degradation (Yamamoto *et al*., 1999). For example, when cells were stimulated with LPS in the presence of sodium calculate or aspirin, the LPS-induced proteolysis of IκB-α was abolished, which suggests that the observed NF-κB inhibition was mediated through inhibition of the phosphorylation and /or degradation of IκB-α. Previously, we reported that aucubin inhibited the NF-κB activation via phosphorylation and degradation of IκB-α (Jeong *et al*., 2002). In this study, we showed that SSE suppressed NF-κB translocation to the nucleus induced by PMA plus A23187. Also this suppression was mediated through inhibition of IκB-α phosphorylation and degradation. Therefore, our results suggest that the anti-inflammatory effect of SSE is similar to the mechanism of aucubin. So, it is possible to hypothesize that SSE might act as a potent NF-κB inhibitor on the mast cell activation induced by PMA plus A23187.

In conclusion, we have shown that SSE can regulate allergy response *in vivo*, and affect the expression of inflammatory genes through regulation of the NF-κB/IκB-α pathway. These results provided new insight into the pharmacological actions of SSE as a potential molecule for therapy of inflammatory allergic diseases.

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