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Anti-allergic Effect of Aqueous Extract of *Stachys riederi* var. *japonica* Miq. *in vivo* and *in vitro*

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Abstract – The effect of aqueous extract of *Stachys riederi* var. *japonica* Miq. (Labiatae) (SRAE) on the immediate-type allergic reactions was investigated. SRAE was found to exhibit a inhibitory activity on the compound 48/80-induced systemic anaphylaxis in mice. SRAE inhibited the plasma histamine release induced by compound 48/80 in mice. In addition, SRAE also inhibited the passive cutaneous anaphylaxis reaction induced by IgE/anti-IgE in mice. The effect of SRAE on the histamine release from rat peritoneal mast cells (RPMC) was studied. SRAE inhibited the histamine release induced by compound 48/80 in RPMC. To clarify the mechanism of these inhibiting reactions, we investigated the effects of SRAE on cyclic AMP (cAMP). The level of cAMP in human leukemia cell line, HMC-1, when SRAE (1 mg/ml) was added, significantly increased compared with that of basal cells. These resuits indicate that SRAE may be beneficial in the treatment of immediate-type allergic reaction.
Key words: *Stachys riederi*, anaphylaxis, compound 48/80, anti-DNP IgE, histamine, cyclic AMP

Introduction

The *Stachys riederi* var. *japonica* Miq. (Labiatae), well known as Seok-Jam-Pul in Korea, has been used for centuries as traditional medicine. This plant is used for management of hemorrhage, cough and skin diseases. It has contents of caffeic acid, n-methoxybaicalein, palustrine and palustinoside (Bae, 2000). Mast cells have been thought to play a major role in the development of many physiologic changes during immediate-type allergic responses (Kim and Lee, 1999). In general, immediate hypersensitivity, which involves urticaria, allergic rhinitis and asthma, is mediated by various chemical mediators released from mast cells (Wasserman and Marquardt, 1988), Among the preformed and newly synthesized inflammatory substances released on degranulation of mast cells, histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity (Petersen *et al.*, 1996). Mast cells degranulation can be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells (Lagunoff *et al.*, 1983). Compound 48/80 is one of most potent secretagogues of mast cells (Ennis *et al.*, 1980). The compound is a mixture of polymers synthesized by condensing *N*-methyl-*p*-methoxtphenyl ethylamine with formaldehyde (Baltzly *et al.*, 1949), and its hypotensive effect, resulting from histamine release, was shown by Paton (1951). Compared with the natural process, a high concentration of compound 48/80 induces almost a 90% release of histamine from mast cells. Thus, an appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylaxis (Allansmith *et al.*, 1989; Shin *et al.*, 1999). The secretory response of mast cells can also be induced by aggregation of their cell surface-specific receptors for immunoglobulin E (IgE) by the corresponding antigen (Segal *et al.*, 1977; Metzger *et al.*, 1986; Alber *et al.*, 1991). It has been established that the anti-IgE antibody induces passive cutaneous anaphylaxis (PCA) reactions as a typical model for the immediate hypersensitivity. In the present study, the effect of SRAE on compound 48/80-induced systemic anaphylaxis, anti-dinitrophenyl (DNP) IgE antibody-induced local anaphylaxis, and histamine release from RPMC. The cAMP content was also investigated to clarify the mechanism by which SRAE inhibited histamine release from mast cells.

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Materials and Methods

Reagents – Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), α-minimal essential medium (α-MEM), ortho-phthaldialdehyde and metrizamide were purchased from Sigma Chemical Co. (St Louis, MO). cAMP was purchased from Amersham Pharmacia Biotec (UK).

Animals – The original stock of ICR mice and SD rats were purchased from Dae-Han Biolink (Chungbuk), and the animals were maintained in the College of Pharmacy, Woosuk University. The animals were housed five to ten per cage in a laminar air flow room maintained under a temperature of 22±2°C and relative humidity of 55±5% throughout the study.

Preparation of SRAE – The plants of *Stachys riederi* were collected in Wanju, Jeonbuk, Korea, on the 25th of August, 2000. A voucher specimen (number WSP-00-17) was deposited at the Herbarium of the College of Pharmacy, Woosuk University. The plant sample was extracted with distilled water at 70°C for 5 h. The extract was filtered through Whatman No. 1 filter paper and the filtrate was lyophilized, and kept at −4°C. The yield of dried extract from starting crude materials was about 6.2%. The dried extract was dissolved in saline or 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, 0.1% bovine serum albumin) before use.

Compound 48/80-induced systemic anaphylaxis – Compound 48/80-induced systemic anaphylaxis was examined as previously described (Shin *et al.*, 2002). Mice were given an intraperitoneal injection of 0.008 g/kg body weight (BW) of compound 48/80. SRAE (0.005 to 1 g/kg body weight, i.p) was administered 1 h before compound 48/80 (0.008 g/kg) challenge. Mortality was monitored for 1 h after induction of anaphylactic shock. After the mortality test, blood was obtained from the heart of each mouse.

Passive cutaneous anaphylaxis reaction – An 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 tail vein. The anti-DNP IgE and DNP-HSA were diluted in PBS. The mice were injected intradermally with 0.5 μg of anti-DNP IgE into each of two dorsal skin sites that had been shaved 48 h earlier. Each mouse, 48 h later, received an injection of 1 mg of DNP-HSA in PBS containing 4% Evans blue (1:4) *via* the tail vein. SRAE (0.001 to 1 g/kg BW) was orally administered 1 h before the challenge. Then 30 min after the challenge, the mice were sacrificed and the dorsal skin was removed for measurement of pigment area. The amount of dye was then determined colorimetrically after extraction with 1 ml of 1 M KOH and 9 ml of mixture of acetone and phosphoric acid (13:5) based on the method of Katayama *et al.* (1978). The absorbent intensity of the extraction was measured at 620 nm in a spectrophotometer (Shimadzu, UV-1201, Japan) and the amount of dye was calculated with the Evans blue measuring-line.

Preparation of plasma and histamine determination – The blood was centrifuged at 400×g for 10 min. The plasma was withdrawn and histamine content was measured by the ortho-phthaldialdehyde spectrofluorometric procedure of Shore *et al.* (1959). The fluorescent intensity was measured at 438 nm (excitation at 353 nm) in a spectrofluorometer (Shimadzu, RF-5301 PC, Japan).

Preparation of RPMC – RPMC were isolated as previously described (Kanemoto *et al.*, 1993). In brief, rats were anesthetized by ether and injected with 20 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM $NaHCO_3$, 2.7 mM KCl, 0.3 mM NaH_2PO_4 and 0.1% gelatin) into the peritoneal cavity and the abdomen was gently massaged for about 90 seconds. The peritoneal cavity was carefully opened and the fluid containing peritoneal cells was aspirated by a Pasteur pipette. Thereafter, 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.* (1977). In brief, peritoneal cells suspended in 1 ml of Tyrode buffer B were layered on 2 ml of metrizamide (22.5 w/v%) 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 Tyrode buffer A.

Inhibition of histamine release – Purified RPMC were resuspended in Tyrode buffer A for the treatment of compound 48/80. RPMC suspensions (2×10^5 cells/ml) were preincubated for 10 min at 37°C before the addition of compound 48/80 (5 μg/ml). The cells were preincubated with the SRAE preparations, and then incubated (10 min) with the compound 48/80. The cells were separated from the released histamine by centrifugation at 400×g for 5 min at 4°C. Residual histamine in cells was released by disrupting the cells with perchloric acid and centrifugation at 400×g for 5 min at 4°C.

Measurement of cAMP level – The cAMP level was measured according to the method of Peachell *et al.* (1988). In brief, purified mast cells were resuspended in prewarmed (37°C) Tyrode buffer A. Typically, an aliquot of cells (5×10^5 cells/ml) were added to an equivalent volume (50 μl) of prewarmed buffer containing the drug in an Eppendorf tube. The reaction was allowed to proceed for discrete time

intervals, terminated by the addition of ice-cold acidified ethanol (0.9 ml of 86% ethanol/1 M HCl, 99:1) with brief vigorous vortexing and then snap frozen in liquid nitrogen. The sample was later thawed and vortexed, then the debris was sedimented in a centrifuge (400×g at 4°C, for 5 min), and an aliquot (0.9 ml) of the supernatant was removed and evaporated to dryness under reduced pressure. The dried sample was reconstituted in assay buffer (150-200 μl) and stored frozen. The cAMP level was determined by enzyme immunoassay, using a commercial kit (Amersham Pharmacia Biotech).

Statistical analysis – The results obtained were expressed as mean±SEM. Student's *t*-test was used to make a statistical comparison between the groups. Results with $p<0.05$ were considered statistically significant.

Result

Effect of SRAE on compound 48/80-induced systemic anaphylaxis – To assess the contribution of SRAE in anaphylaxis, an *in vivo* model of systemic anaphylaxis was used. Compound 48/80 (0.008 g/kg) was used as a systemic fatal anaphylaxis inducer. After the intraperitoneal injection of compound 48/80, the mice were monitored for 1 h, after which the mortality rate was determined. As shown in Table 1, an intraperitoneal injection of 200 μl saline as a control induced a fatal shock in 100% of each group. When mice were pretreated with SRAE at a concentrations ranging from 0.005 to 1 g/kg BW for 1 h, the mortality with compound 48/80 was reduced dose-dependently.

Effect of SRAE on compound 48/80-induced plasma histamine release – The ability of SRAE to influence compound 48/80-induced plasma histamine release was investigated. SRAE was given from 0.01 to 1 g/kg BW 1 h before (*n*=10/group) compound 48/80 injection. SRAE dose-dependently inhibited compound 48/80-induced plasma histamine release. The inhibition rate of histamine by SRAE was significant at doses of 0.5 and 1 g/kg (Table 2).

Effect of SRAE on anti-DNP IgE-induced passive cutaneous anaphylaxis reaction – Passive cutaneous anaphylaxis reaction is one of the most important *in vivo* models of anaphylaxis in local allergic reaction (Wershil *et al.*, 1987). As described in the experimental procedure, local extravasation was induced by a skin injection of anti-DNP IgE followed by an antigenic challenge. SRAE dose-dependently inhibited PCA reaction (Table 3).

Table 1. Effect of SRAE on compound 48/80-induced systemic anaphylaxis

SRAE treatment (g/kg BW)	Compound 48/80 (0.008 g/kg BW)	Mortality (%)
None (saline)	+	100
0.005	+	100
0.01	+	80
0.05	+	50
0.1	+	40
0.5	+	20
1	+	10
1	–	0

Groups of mice (*n*=10/group) were intraperitoneally pretreated with 200 μl saline or SRAE. SRAE was given at various doses 1 h before the compound 48/80 injection. The compound 48/80 solution was intraperitoneally given to the group of mice. Mortality (%) within 1 h following compound 48/80 injection was represented as the number of dead mice×100/total number of experimental mice.

Table 2. Effect of SRAE on compound 48/80-induced plasma histamine release

SRAE treatment (g/kg, BW)	Compound 48/80 (0.008 g/kg BW)	Amount of histamine (μg/ml)
None (saline)	+	0.186±0.017
0.01	+	0.173±0.019
0.05	+	0.167±0.013
0.1	+	0.140±0.015
0.5	+	0.099±0.011*
1	+	0.061±0.007*

Groups of mice (n=10/group) were intraperitoneally pretreated with 200 μl saline or SRAE. SRAE was given at various doses 1 h before the compound 48/80 injection. The compound 48/80 solution was intraperitoneally given to the group of mice. Each datum represents the mean±SEM of three independent experiments. *p<0.05; significantly different from the saline value.

Table 3. Effect of SRAE on the 48h PCA.

SRAE treatment (g/kg BW)	Anti-DNP IgE plus DNP-HSA	Amount of dye (μg/site)
None (saline)	+	5.481±0.587
0.001	+	4.659±0.501
0.01	+	4.385±0.414
0.1	+	2.741±0.283*
1	+	1.700±0.189*

SRAE was administered orally 1 h prior to the challenge with antigen. Each datum represents the mean±SEM of three independent experiments. *p<0.05; significantly different from the saline value.

Table 4. Effect of SRAE on compound 48/80-induced histamine release from RPMC

SRAE treatment (mg/ml)	Compound 48/80 (5 μg /ml)	Amount of histamine (μg/ml)
None (saline)	+	0.138±0.014
0.001	+	0.124±0.010
0.01	+	0.103±0.018
0.1	+	0.065±0.013*
1	+	0.021±0.006*

The cells (2×10^5 cells/ml) were preincubated with SRAE at 37°C for 10 min prior to incubation with compound 48/80. Each datum represents the mean±SEM of three independent experiments. *p<0.05; significantly different from the saline value.

Table 5. Time-dependent effect of SRAE on cAMP level of HMC-1cells

SRAE treatment (mg/ml)	Incubation time (min)	cAMP content (p mol)
None (saline)	0	3.033±0.408
1	1	6.781±1.008*
	2	4.704±0.358
	3	3.777±0.463

HMC-1 cells (5×10^5 cells/ml) were pretreated with SRAE (1 mg/ml) at 37°C. Each datum represents the mean±SEM of three independent experiments. *$p<0.05$; significantly different from the saline value.

Effect of SRAE on compound 48/80-induced histamine release from RPMC – The inhibitory effect of SRAE on compound 48/80-induced histamine release from RPMC are shown in Table 4. SRAE dose-dependently inhibited compound 48/80-induced histamine release at concentrations of 0.001 to 1 mg/ml. Especially, SRAE significantly inhibited the compound 48/80-induced histamine release at the concentrations of 0.1 and 1 mg/ml.

Effect of SRAE on cAMP level in HMC-1 cells – Finally, the cAMP content was examined to clarify the mechanism by which SRAE inhibits histamine release from mast cells. When HMC-1 cells were incubated with SRAE at a concentration of 1 mg/ml, the cAMP content significantly increased. It peaked at 1 min after SRAE was added, then decreased to basal value about three min later (Table 5).

Discussion

The results of this study demonstrated that SRAE pretreatment profoundly inhibited compound 48/80-induced systemic anaphylaxis and anti-DNP IgE-mediated PCA. SRAE inhibited the plasma histamine levels in mice. SRAE also inhibited the compound 48/80-mediated histamine release from RPMC. It is believed that stimulation of mast cells with compound 48/80 or ati-DNP IgE initiates the activation of signal-transduction pathway, which leads to histamine release. Some recent studies have shown that compound 48/80 and other polybasic compounds are able, apparently directly, to activate G-proteins (Mousli *et al.*, 1990a; Mousli *et al.*, 1990b). The evidence indicates that the protein is G inhibitory-like and that the activation is inhibited by benzalkonium chloride (Bueb *et al.*, 1990). Tasaka *et al.* (1986) reported that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane. This result indicates that the membrane permeability increase may be an essential trigger for the release of the mediators from mast cells. In this sense, anti-allergic agents having a membrane-stabilizing action may be desirable. SRAE might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80. Thus, this data suggest that SRAE is capable of rapidly action on the surface of mast cells and interfere with the generation of the stimulus secretion coupling process. More recently, studies have suggested the importance of chloride channels that provide the driving force for calcium influx during mast cell activation. Nedocromil sodium was shown to block intermediate conductance chloride channels in cultured mucosal-like mast cells and chloride channels may play a functional role in compound 48/80-induced histamine release from mast cells (Lau and Wan, 2000; Levi-Schaffer *et al.*, 2000). SRAE might inhibit compound 48/80-induced histamine release from mast cells by blocking chloride channel. The SRAE orally administered mice were protected from local anaphylaxis, which suggests that SRAE might be useful in the treatment of allergic skin reactions. The release of histamine is known to be depressed by an increase in the intracellular cAMP content due to the activation of adenylate cyclase or inhibition of cAMP phosphodiesterase (Makino *et al.*, 1987). The intracellular cAMP content of the mast cells, when incubated with SRAE (1 mg/ml), increased about 2-fold in comparison with that of basal cells. The several papers of anti-allergic reaction with using HMC-1 cells and RPMC was already reported (Shin *et al.*, 2002; Shin and Lee, 2003).

In conclusion, these results obtained in the present study provide evidence that SRAE may be beneficial in the treatment of allergic diseases. Also, it suggests that SRAE may contain compounds with actions that inhibit mast cell-mediated allergic reactions *in vivo* and *in vitro*. Therefore, further investigation is necessary to clarify unknown anti-anaphylactic constituents that may be more active than the SRAE itself.

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