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Suppression of Anaphylactic Reaction in Murine by *Siegesbeckia pubescens*

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The aqueous extract of *Siegesbeckia pubescens* (SPAE) inhibited compound 48/80-induced systemic anaphylaxis 100% with the dose of 1.0, 0.5 mg/g body weight (BW) at 1 h before or 5 min, 10 min after intraperitoneal injection of compound 48/80. The passive cutaneous anaphylaxis (PCA) reaction also inhibited to 78.5% by oral administration of SPAE (1.0 mg/g BW). When SPAE pretreated on mice at concentrations ranging from 0.0001 to 1.0 mg/g BW, the serum histamine levels were reduced in a dose-dependent manner. Moreover, SPAE (100-800 μg/ml) dose-dependently inhibited the histamine release from peritoneal mast cells (RPMC) by compound 48/80 (5 μg/ml). Analysis by microscopic appearance observation revealed that SPAE (500 μg/ml) stabilized the RPMC membrane. Therefore, these findings indicate that SPAE inhibits anaphylactic reactions through stabilization of mast cell membrane.

Key words : Siegesbeckia pubescens, Systemic anaphylaxis, Passive cutaneous anaphylaxis, Histamine, Mast cell

INTRODUCTION

The *Siegesbeckia pubescens* (Compositae), well known as 'Tul-Jin-Deuk-Chal' in Korea, has been used for centuries as a traditional medicine. This plant is successfully used for the management of asthma and various allergic disorders. The mast cell has long been thought to play a crucial role in the development of many physiologic changes during anaphylactic and allergic responses (Wasserman *et al.*, 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 type I allergic reactions (Petersen *et al.*, 1996). Mast cell degranulation can be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells (Lagunof *et al.*, 1983). The most potent secretagogues include the synthetic compound 48/80, polymers of basic amino acids (Ennis *et al.*, 1980). The compound is a mixture of polymers synthesized by condensing *N*-methyl-p-methoxyphenyl ethylamine with formaldehyde (Baltzly *et al.*, 1949), and its hypotensive effect was shown by Paton (1951) to be the result of histamine release. Compared with the natural process, a high concentration of compound 48/80 induces almost a 90% release of histamine from mast cells, whereas antigen-antibody-complement-induced histamine release rarely reaches 50% (Johnson and Moran, 1969). Thus, an appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylactic reaction, including allergic conjunctivitis (Allansmith *et al.*, 1989). 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 induce passive cutaneous anaphylaxis (PCA) reactions as a typical model for the type I hypersensitivity (Saito and Nomura, 1989).

In the present study, we showed that the aqueous extract of *Siegesbeckia pubescens* (SPAE) inhibited both compound 48/80-induced systemic anaphylactic shock and anti-IgE antibody-induced cutaneous anaphylactic reaction. We also showed that SPAE inhibited the anaphylactic responses by enhancing the stability of mast cell membrane.

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MATERIALS AND METHODS

Animals

The original stock of BALB/c mice and Wistar rats were purchased from Korean Research Institute of Chemical Technology (Taejeon, Korea), and the animals were maintained in the College of Pharmacy, Wonkwang University. The animals were housed five to ten per cage in a laminar air flow room maintained under the temperature of 22±1°C and relative humidity of 55±10% throughout the study.

Reagents

Compound 48/80, anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), and metrizamide were purchased from Sigma Chemical Co. (St. Louis, MO). The α-minimal essential medium (α-MEM) was purchased from Flow Laboratories (Irvine, UK). Fetal calf serum (FCS) was purchased from Gibco Laboratories (Grand Island, N.Y., USA)

Preparation of SPAE

The plants of *Siegesbeckia pubescens* were collected in Iksan, Korea, on the 9th of July, 1995. A voucher specimen was deposited at the Herbarium of Wonkwang University, and extracted with distilled water at 70°C for 5 h. The extract was filtered through a 0.45 μm filter, lyophilized, and kept at 4°C. 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.

Systemic anaphylaxis

Mice were given an intraperitoneal injection of 8 μg/g body weight (BW) of the mast cell degranulator compound 48/80. SPAE was dissolved in saline and administered by intraperitoneal injection with 0.0001 to 1 mg/g BW at 1 h before the injection of compound 48/80. In time dependent experiment SPAE (0. 5 mg/g BW) was injected intraperitoneally at 1 h before, 5 and 10 min after compound 48/80 injection. Mortality was determined after 1 h induction of anaphylactic shock . After mortality test the blood was obtained from each mouse's heart.

Passive cutaneous anaphylaxis

An IgE-dependent cutaneous reaction was generated by sensitizing skin with an intradermal injection of anti-DNP IgE followed 48 h after with an injection of DNP-HSA into the rat tail vein. The DNP-HSA was diluted in PBS. Rats were injected intradermally with 100 μg of anti-DNP IgE into each of 4 dorsal skin sites that had been shaved 48 h previously. Sites were outlined with a water-insoluble red marker. Forty-eight hours later each rat received an injection of 1 mg of DNP-HSA in PBS containing 4% Evans blue (1:4) via tail vein. SPAE (1.0 mg/g BW) was orally administered 1 h before the challenge. Thirty min after the challenge, the rats were sacrificed, the dorsal skin was removed for measurement of pigment area. The amount of dye was then determined colorimetrically after extraction with 1 ml of 1.0 N KOH and 9 ml of mixture of aceton and phosphoric acid (5:13) based on the method of Katayama *et al.* (1978). The absorbant intensity of extraction was measured at 620 nm in a spectrofluorometer, and the amount of dye was calculated with the measuring-line Evans blue.

Preparation of serum and histamine determination

The serum was withdrawn and histamine content was measured by the o-phthalaldehyde spectrofluorometric procedure of Shore *et al.* (1959). The fluorescent intensity was measured at 438 nm (excitation at 353 nm) in a spectrofluorometer.

Preparation of rat peritoneal mast cells (RPMC)

RPMC were isolated as previously described (Kanemoto *et al.*, 1993). In brief, rats were anesthetized by ether, and 20 ml of Tyrode buffer B (NaCl, glucose, $NaHCO_3$, KCl, NaH_2PO_4) containing 0.1% gelatin (Sigma Chemical Co.) was injected into the peritoneal cavity, and the abdomen was gently massaged for about 90 sec. 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 resuspened in Tyrode buffer B. Mast cells were separated from 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 Tyrode buffer B were layered on 2 ml of 22.5% w/v metrizamide (density, 1.120 g/ml, Sigma Chemical Co.) 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 1ml Tyrode buffer A containing calcium.

Inhibition of histamine release

Purified mast cells were resuspended in Tyrode buffer A containing calcium for the treatment of compound 48/80. Mast cell suspensions (1×10^6 cells/ml) were preincubated for 10 min at 37°C before the addition of compound 48/80. The cells were prein-

cubated with the SPAE preparations (100-800 μg/ml), and then incubated (10 min) with the compound 48/80. The reaction was stopped by cooling 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 cells was released by disrupting the cells with perchloric acid and centrifugation at 400×g for 5 min at 4°C.

Assay of histamine release

The inhibition percentage of histamine release was calculated using the following equation :

$$\% \text{ Inhibition} = \frac{\text{Histamine release without SPAE} - \text{Histamine release with SPAE}}{\text{Histamine release without SPAE}} \times 100$$

Statistical analysis

The results obtained were expressd as means±S.E for the number of animals. The Student's t-test was used to make a statistical comparison between the groups.

RESULTS

Effects of SPAE on compound 48/80-induced systemic anaphylaxis

To assess the contribution of SPAE in allergic reactions, we first used the *in vivo* model of systemic anaphylaxis. We used compound 48/80 (8 μg/g BW) as systemic fatal anaphylaxis inducer. The mortality of mice was determined for 1 h after injection of compound 48/80. As shown in Table I, intraperitoneal injection of 200 μl saline as control 1 h before compound 48/80 injection induced fatal shock in 100% of the each group. When SPAE was pretreated at concentration ranging from 0.0001 to 1 mg/g BW for 1 h, the mortality with compound 48/80 was reduced dose-dependently. Especially, SPAE inhibited compound 48/80-induced anaphylaxis 100% with the dose of 1.0 and 0.5 mg/g BW (Table I). No physiological differences could be detected by the time course of SPAE (2.0 mg/g BW) treatment as the mortality was zero % in all groups (data not shown). Furthermore, the mortality of mice injected intraperitoneally with SPAE (0.5 mg/g BW) 5 min and 10 min after (n=7/groups) compound 48/80 injection was zero % (Table II).

Table I. Effects of SPAE on compound 48/80-induced systemic anaphylaxis

Treatment[a]	Dose (mg/g BW)	Compound 48/80[b] (8 μg/g BW)	Mortality (%)[c]
Saline	-	+	100
SPAE	0.0001	+	100
SPAE	0.001	+	80
SPAE	0.01	+	70
SPAE	0.1	+	30
SPAE	0.5	+	0

[a]Groups of mice were intraperitoneally pretreated with 200 μl saline or SPAE was given at various doses 1 h before (n=10/group) compound 48/80 injection.
[b]Compound 48/80 solution was intraperitoneally given to the group of mice.
[c]Mortality (%) within 1 h following compound 48/80 injection was represented as No. of dead mice×100/total No. of experimental mice.

Effects of SPAE on serum histamine release

The ability of SPAE to influence compound 48/80-induced serum histamine release was investigated. SPAE was given with 0.001 to 1.0 mg/g BW 1 h before (n=7/group) compound 48/80 injection. The serum

Table II. Time-dependent effects of SPAE on compound 48/80-induced systemic anaphylaxis

Treatment[a]	Compound 48/80[b] (8 μg/g BW)	Mortality (%)[c]		
		1 h before	5 min after	10 min after
Saline	+	100	100	100
SPAE	+	0	0	0
SPAE	-	0	0	0

[a]200 μl saline or SPAE (0.5 mg/g BW) was given 1 h before, 5 min after and 10 min after (n=7/group) compound 48/80 injection.
[b]Compound 48/80 solution was given intraperitoneally to the group of mice.
[c]Mortality (%) within 1 h following compound 48/80 injection was represented as No. of dead mice×100/total No. of experimental mice.

Table III. Effects of SPAE on compound 48/80-induced serum histamine release

Treatment[a]	Dose (mg/g BW)[b]	Compound 48/80 (8 μg/g BW)[c]	Inhibition (%)
Saline	-	-	-
Saline	-	+	0
SPAE	0.0001	+	16.5
SPAE	0.001	+	38.2
SPAE	0.01	+	42.8
SPAE	0.1	+	53.9
SPAE	0.5	+	63.2[d]
SPAE	1.0	+	67.5[d]

[a]Groups of mouse were intraperitoneally pretreated with 200 μl saline or SPAE.
[b]Each drug was given with various doses at 1 h before (n=7/group) compound 48/80 injection.
[c]Compound 48/80 solution was intraperitoneally given to the group of mice.
[d]$P<0.05$; significantly different from the saline value.

of mice was uniformly gathered with heart puncture at 15 min after compound 48/80 injection in all groups of mice. The similar results with those of the mortality test were shown when their sera histamine contents were measured (Table III). Inhibition rate was significant ($p<0.05$) at the doses of 1.0, 0.5 mg/g BW.

Effects of SPAE on PCA

Another way to test allergic reactions is to induce PCA (Wershil *et al.*, 1987). As described in the Experimental Procedures, local extravasation is induced by local injection of anti-DNP IgE followed by an intravenous antigenic challenge. Anti-DNP IgE was injected in the right dorsal skin sites. As a control, the left dorsal skin site of these rats was injected with saline alone. After 48 h, all animals were injected intravenously with DNP-HSA injected with Evans blue dye. The cutaneous anaphylactic reaction was best visualized by the extravasation of the dye. Oral administration of SPAE (1.0 mg/g BW) showed marked inhibition rate in PCA reactions (Table IV).

Effects of SPAE on histamine release from RPMC

The inhibitory effects of SPAE on compound 48/80-induced histamine release from PRMC following 10 min preincubation are shown in Table V. SPAE dose-dependently inhibited compound 48/80-induced histamine release at concentrations of 100-800 μg/ml. The standard antiallergic agent, disodium cromoglycate (DSCG), an isoquinoline alkaloid isomeric with tylophorine, was isolated from Indian Tylophora species (Gopalakrishnan *et al.*, 1980).

Table IV. Effect of SPAE on the 48-h PCA in rats

Treatment	Dose (mg/g BW)[a]	Amount of dye (μg/site)[b]	Inhibition (%)
Saline	-	13.99±0.40	-
SPAE	1.0	3.01±0.64[c]	78.5

[a]Drug was administered orally 1 h prior to challenge with antigen (n=3/group).
[b]Each amount of dye represents the mean±S.E. of 4 experiments.
[c]$P<0.05$; significantly different from the saline value.

Table V. Effects of SPAE on compound 48/80-induced histamine release from RPMC[a]

Treatment	Dose (mg/ml)[a]	Inhibition (%)
Saline	-	-
SPAE	0.1	47.88±11.91
SPAE	0.2	52.52±5.77
SPAE	0.4	61.38±10.71[b]
SPAE	0.8	85.22±12.35[b]
DSCG	0.01	33.00±6.85
DSCG	0.10	36.50±7.01
DSCG	0.40	80.10±8.48[b]

[a]RPMC (2×10^5 cells/ml) were preincubated with drug at 37°C for 10 min prior to incubation with compound 48/80 (5 μg/ml) for 10 min.
[b]$P<0.05$; significantly different from the saline value.

Effects of SPAE on microscopic appearance of compound 48/80-induced RPMC membrane

We next analyzed the morphological changes to clarify the mechanism by which SPAE inhibited his-

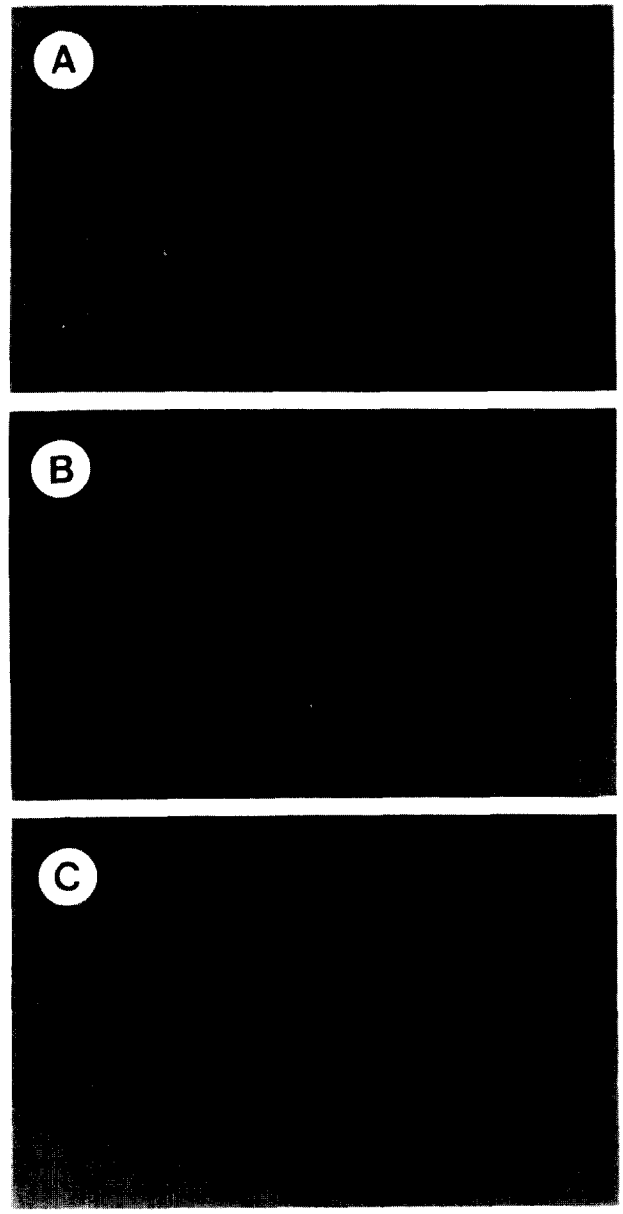

Fig. 1. Visualization of morphological changes in isolated RPMC. Fresh isolated RPMC (A), in the presence of compound 48/80 (B) and in the presence of SPAE (0.8 mg/ml) plus compound 48/80 (C). Magnifications×700.

tamine release from RPMC. The freshly isolated RPMC (Fig. 1A) within 10 min of exposure to compound 48/80 (5 μg/ml), showed swelling and disrupted boundary, and vacuoles, which were interpreted as degranulation (Fig. 1B). On the other hand, the RPMC with compound 48/80 in the presence of SPAE (0.8 mg/ml) were morphologically similar to the starting PRMC (Fig. 1C).

DISCUSSION

The present study showed that SPAE pretreatment profoundly affected compound 48/80-induced systemic anaphylaxis and anti-DNP IgE-induced PCA as well as compound 48/80-induced histmine release from RPMC. We simply speculate that these results indicate that anaphylactic degranulation of mast cells is inhibited by SPAE. This speculation was based on the stabilization of mast cell membrane by SPAE. There is absolutely no doubt that stimulation of mast cells with compound 48/80 initiates the activation of signal-transduction pathway which lead 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 Gi-like and that the activation is inhibited by benzalkonium chloride (Bueb *et al.*, 1990)

Our findings indicated that SPAE might be capable of stabilizing the mast cell membrane (Fig. 1). Tasaka *et al.* (1986) reported that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane (Tasaka *et al.*, 1986). This result indicates that the membrane permeability increase may be essential trigger for the release of the mediator from mast cells. SPAE might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80. Taken together, compound 48/80-induced histamine release from RPMC was inhibited by SPAE. SPAE also protected the IgE-mediated cutaneous anaphylaxis reaction.

In conclusion, the results obtained in the present study provide evidence that SPAE importantly contributes to the prevention or treatment of allergic diseases. The studies on the isolation and characterization of the active chemical constituents are in progress.

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