Inhibitory Effect of *Spirodela Polyrhiza* on the Mast Cell-Mediated Immediate Hypersensitivity

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Spirodela polyrhiza(L.) Schleid (Lemnaceae) have been used as a traditional drug in treating urticaria and itching. However, the exact role of *Spirodela polyrhiza* in allergic reaction has not been clarified yet. Type 1 hypersensitivity (immediate hypersensitivity), popularly known as allergy, is a major clinical problem in humans. It has been found that the histamine release from mast cells is an essential step in the pathological process of immediate hypersensitivity. In the present study, the effect of aqueous extract of *Spirodela polyrhiza* (AESP) on immediate hypersensitivity was investigated. AESP inhibited the antigen-induced passive cutaneous anaphylaxis (PCA). AESP *in vitro* exhibited a dose-dependent inhibition of degranulation in RPMC stimulated by compound 48/80. AESP also suppressed the morphological changes and the increase of intracellular free calcium level induced by compound 48/80. These results suggest that inhibitory effect of AESP on immediate hypersensitivity may be mediated through the decrease of intracellular free calcium levels, and AESP importantly contributes to the treatment of anaphylaxis and may be useful for other allergic disease.

Key words : *Spirodela polyrhiza*, PCA, mast cell, degranulation, intracellular free calcium

Introduction

Spirodela polyrhixa has been used in treatment for inflammation, urticaria, and skin disease in Korean traditional medicine[1]. However, the action mechanisms of *Spirodela polyrhixa* on anti-allergic effects have not been fully explained. Hypersensitivity may be classified into four types[2]. One of these, type 1 hypersensitivity, popularly known as allergy, is a major clinical problem in humans. It has been found that the histamine release from mast cells is an essential step in the pathological process of a type 1 hypersensitivity (immediate hypersensitivity)[3], and a IgE-induced passive cutaneous anaphylaxis (PCA) reaction has been established as a typical model for the immediate hypersensitivity[4].

Mast cells have long been thought to play a crucial role in the development of many physiological changes during immediate allergic responses[5]. Upon activation, they secrete an array of pre-formed and newly generated inflammatory mediators, such as histamine, proteases, prostaglandins, leukotrienes, chemokines and cytokines[6-7]. Mast cell degranulation can be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells[8]. Intracellular calcium, cAMP, and histamine release in murine mast cells were effectively stimulated by positively charged substances such as compound 48/80[9]. It is usually supposed that the most potent secretagogues include the synthetic compound 48/80 and polymers of basic amino acids[10]. Compound 48/80 is a mixture of polymers synthesized by condensing N-methyl-p-methoxyphenylamine with formaldehyde[11]. An appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of the anaphylactic reaction[12]. This study attempts to find scientific understanding of the effects of AESP on the immediate-type allergic reactions.

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Materials and Methods

1. Materials

Metrizamide, compound 48/80, anti-DNP-IgE, DNP-HSA, p-nitrophenyl-N-acetyl-β-D-glucosamine were purchased from Sigma Chemical Co. (St. Louis, MO). α-MEM and other tissue culture reagents were purchased from Gibco Life Technologies

(Gaithersburg, MD). *Spirodela polyrhixa* were purchased from a local herb store, Kwang Myoung Dang (Busan, Korea) in February 1998. The herb was identified and authenticated by Professor W. S. Ko, College of Oriental Medicine, Dongeui University (Busan, Korea). The dry herb (300 g) was extracted with distilled water at 100 ℃for 2 h. The extract was filtered through 0.45 μm filter, lyophilized (yield, 2 g) and kept at 4 ℃. The dried extract was dissolved in phosphate buffered saline (PBS) and filtered through 0.22 μm filter before use.

2. Animals

Male Sprague Dawley rats (SPF) between 8 to 9 weeks of age (250-300 g) and male ICR mice (SPF) weighing 20-30 g (6-8 weeks) were purchased from Dae Han Animal Center (DHAC, Korea) and maintained in our laboratory. The animals were housed two to five per cage in a laminar air flow room maintained under a temperature of 22 ± 1 ℃ and relative humidity of 50 ± 10 % throughout the study.

3. Rat peritoneal mast cell culture

Rat peritoneal mast cells (RPMC) were isolated as previously described[13]. Peritoneal lavage was performed by using 40 ml of PBS containing 1 % FBS and 10 U/ml heparin. Cells were then gradient-centrifuged on 22.5 % metrizamide solution and mast cell fraction was resuspended in Tyrode's buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $HgCl_2$, 5.6 mM glucose, 0.1 % BSA) or α -MEM supplemented with 10 % (v/v) FBS, and were used in degranulation experiment or incubated at 37 ℃ in an atmosphere of 5 % CO_2.

4. β-Hexosaminidase assay

The degranulation assays were adapted from those described by Donnellan[14] with some modifications. The cells were aliquoted at 2 X 10^5 cells/tube in microtube. Cells were treated with or without AESP for 10 min and activated with compound 48/80 for 10 min at 37 ℃. The reaction was terminated by rapid cooling in iced water and centrifugation at 5000 rpm for 5 min. Ten microliter aliquots of the activation reaction supernatants and cell lysates were added to each well and incubated with 5 mM p-nitrophenyl-N-acetyl-β-D-glucosamine (P-NAG) in a 0.1 M sodium citrate buffer (pH 4.5) for 40 min at 37 ℃. At the end of the incubation, 150 ml of a stop solution of 0.2 M Glycine, pH 10.7 was added. The absorbance was measured at 405 nm with a Vmax 96-well microplate spectrophotometer (Molecular Devices, Menlo Park, CA). Total β-hexosaminidase activity was determined by lysing cells with 0.5 % (v/v) Triton X-100 after samples had been taken for analysis of released β-hexosaminidase. The percentage β-hexosaminidase activity released was calculated by the formula:

$$\frac{A_{405} \text{ of supernatant}}{A4_{05} \text{ of supernatant} + A_{405} \text{ of pellet}} \times 100\ (\%)$$

5. Passive cutaneous anaphylaxis (PCA)

The passive cutaneous anaphylactic reaction in rats was generated by anti-DNP-IgE and DNP-HSA. The anti-DNP-IgE (100 μg) in physiologic saline (50 μℓ) was injected intradermally into four sites on the shaved dorsal skin of male Sprague Dawley rats. A total of 48 h after sensitization, the rats were challenged with 500 μℓ of saline containing 1 mg of DNP-HSA and 16 mg of Evans blue via the tail vein. 30 min later, the rats were sacrificed, andthe dorsal skin was removed for measurement of the blue area. The amount of leaked dye was then determined colorimetrically after extraction with 1.0 N KOH and a mixture of acetone and phosphoric acid based on the method of Katayama *et al.*[15]. AESP was administered intraperitoneally 1 h before the challenge of antigen.

6. Measurement of intracellular free Ca^{2+}concentration

Changes in intracellular Ca^{2+} concentration were measured using Fluo-3 loaded RPMC. For loading, the cells were washed with Hank's balanced salt solution (HBSS) three times, resuspended in a-MEM with 10 % FBS containing 4 μm Fluo-3 acetoxymethyl ester (Fluo-3/AM), and incubated at 37 ℃ for 1 h. The cells were then washed with HBSS three times and resuspended in Tyrode's buffer with 10% FBS. Cells were placed into a cuvette and the kinetics of intracellular calcium values was determined using a fluorescence spectrometer equipped with argon-ion laser emitting light at 488 nm. (F4500, HITACHI, Japan).

Results

1. Effect of AESP on PCA

To assess the contribution of AESP in allergic reaction, we first used *in vivo* model of PCA (16). As described in the MATERIALS AND METHODS, local extravasation is induced by local injection of anti-DNP IgE followed by an intravenous antigenic challenge. Anti-DNP IgE was injected into 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 i.v. with DNP-HSA containing Evans blue dye. The cutaneous anaphylactic reaction was vest visualized by the extravasation of dye. When drugs were given i.p. to rat, AESP inhibited PCA in a dose dependent manner (Table 1). No

physiological differences by appearance were detected by treatment with AESP.

Table 1. Effect of AESP on PCA in rats

Treatment	A620 of dye	Inhibition
None (saline)	1.686 ± 0.43	-
AESP (200 mg/kg)	1.084 ± 0.37	35.7
(400 mg/kg)	0.685 ± 0.11	59.7
(800 mg/kg)	0.459 ± 0.07	72.8

AESP was administrated intraperitoneally 1 h prior to the challenge with antigen. Each absorbance at 620 nm of dye is presented as the mean ± S.E. of four independent experiments.

2. Effect of AESP on mast cell degranulation

To determine the anti-allergic effect of AESP *in vitro* as well as in vivo, RPMC were isolated and the extent of their degranulation was examined. Compound 48/80 was used as amast cell degranulator. RPMC (5 X 10^5 cells/ml) were pretreated or not with AESP for 10 min and stimulated with or without compound 48/80 (5 μg/ml) for 10 min at 37 ℃. As shown in Fig. 1, degranulation was dramatically reduced by the treatment of AESP in a dose dependent manner with an IC_{50} of 0.4 mg/ml. When RPMC were treated with 1 mg/ml of AESP, the level of degranulation was decreased to basal level. The doses applied in this study did not significantly affect the mast cell numbers or viability, indicating that the inhibitory effect on degranulation was not due to a toxic effect on the mast cells. These results suggest that AESP suppresses immediate hypersensitivity by inhibiting mast cell degranulation.

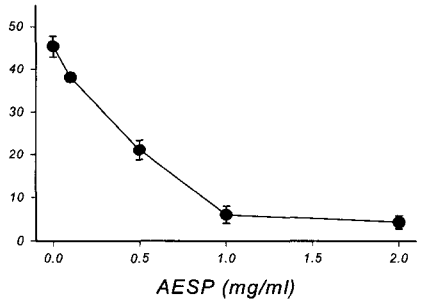

Fig. 1. Effect of AESP on compound 48/80-mediated degranulation from RPMC. RPMC (1 X 10^6 cells/ml) were preincubated with AESP at 37 ℃ for 10 min prior to incubation with compound 48/80 (5 μg/ml). Degranulation was measured by β-hexosaminidase assay. Each data point represents the mean ± S.E. of three experiments.

3. Morphological changes of RPMC

We analyzed morphological changes to clarify the mechanism by which AESP inhibits immediate hypersensitivity. The freshly isolated rat peritoneal mast cells within 10 min of exposure to compound 48/80 (5 μg/ml) showed swelling, disrupted boundary and vacuole, which are interpreted as degranulation. On the other hand, the RPMC with compound 48/80 in the presence of AESP (1 mg/ml) were morphologically similar to the starting ones (Fig. 2).

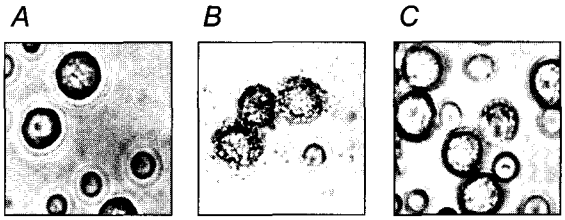

Fig. 2. Morphological changes in isolated RPMC. Isolated RPMC were preincubated at 37 ℃ for 10 min (A). Compound 48/80 (5 μg/ml)-stimulated RPMC were incubated for 10 min in the absence (B) or in the presence (C) of AESP (1 mg/ml). Magnifications were X 200.

4. Effect of AESP on the concentration of intracellular free calcium

The intracellular free calcium has been proposed as an essential trigger for mast cell activation and aggregation of IgE receptor has been known to increase intracellular free calcium level of mast cells. To investigate the effect of AESP on compound 48/80-induced intracellular calcium level, we measured the intracellular free calcium level by using Fluo-3/AM. While the concentration of intracellular free calcium was increased by compound 48/80, AESP inhibited the intracellular free calcium level of mast cell activated by compound 48/80 (Fig. 3). This result indicates that AESP might prevent compound 48/80-induced mast cell degranulation through decrease of intracellular free calcium levels.

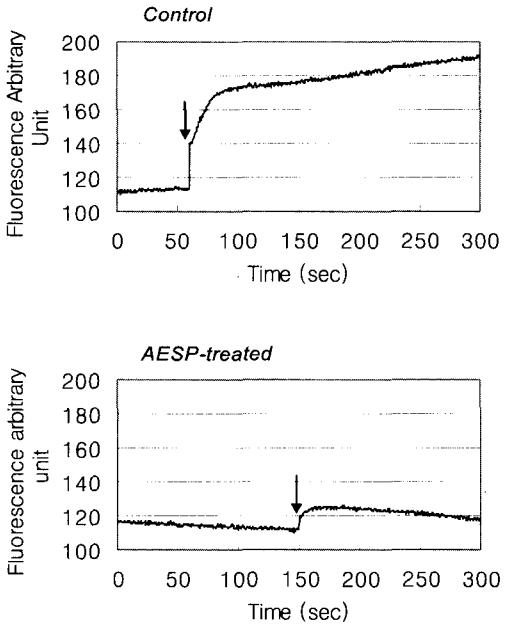

Fig. 3. Effect of AESP on compound 48/80-induced intracellular calcium level. Cells were loaded by incubation with Fluo-3/AM. Intracellular calcium level was measured by fluorescence spectrometer. Cells were treated with AESP (1 mg/ml) or PBS for 30 min and then stimulated with compound 48/80 (5 μg/ml). Arrow indicates the addition of compound 48/80.

Discussion

The application of medicinal herbs dates back to the beginning of civilization, and interestingly, medicinal herbs are still routinely used by most of world's population. Moreover, despite the number of pharmacological agents currently used to prevent the clinical manifestations of allergy, there are still continuing efforts aiming at the development of more potent and more clinically efficacious topical medications to control the worst episodes of the disease.

PCA is a very effective way to test skin allergic reactions, which has been successfully applied by Kim *et al* in murine model with some agents[17,18]. The present study also utilized PCA for testing protection effect from IgE-mediated local allergic reaction. AESP significantly inhibited the PCA reaction. The mechanism of the protection against anti-DNP IgE is suggested only in some particular conditions so far. We just guess AESP may inhibit the initial phase of immediate type allergic reactions, probably through interference with the degranulation system.

The present study showed that AESP pretreatment profoundly affected compound 48/80-induced degranulation of RPMC. These results may suggest AESP have anti-anaphylactic reaction. This suggestion is supported by the study of IgE dependent cutaneous reaction. Study on the compound 48/80 induced histamine release in murine mast cell is still of good use an experimental model[9]. However, we need to remind that compound 48/80 treatment induces degranulation of connective tissue mast cells such as peritoneal mast cells, not mucosal mast cells[19]. Thus the inhibitory effect of AESP against compound 48/80-induced response cannot be applied for describing anything about mucosal type mast cell.

Tasaka *et al.*[20]. 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 essential trigger for the release of the mediators from mast cells. It has been reported that compound 48/80 causes typical morphological changes associated with an increase in volume, which is a function of area and perimeter[21]. Based on these facts we carefully assume that AESP might act on the lipid bilayer membrane preventing the compound 48/80-induced perturbation of membrane. In the present study, we observed that AESP inhibited the compound 48/80-induced morphological change of mast cells. This result suggests that AESP regulates the degranulation of the mast cells by stabilizing membrane fluidity.

It is believed that stimulation of mast cells with compound 48/80 initiates the activation of a signal transduction pathway which leads to histamine release. Some studies report that compound 48/80 and other polybasic compounds are able to activate G proteins[22,23]. And the activation of G proteins has been found to induce a transient rise in intracellular calcium as well as degranulation in RPMC [24]. Calcium increase may be an essential trigger for the release of the mediators by compound 48/80 from the mast cells[25,26]. Our results show that compound 48/80-induced intracellular calcium levels were decreased by AESP treatment. Therefore, we suggest that AESP might prevent immediate type allergic reaction via decrease of intracellular free calcium levels.

This study to reveal the anti-allergic or anti-inflammatory effect of AESP was designed to use an animal model as well as animal cells *in vitro*. Even though the results obtained here are not sufficient to prove the mechanism of AESP in the inhibition of immediate-type allergic reactions, this study could confirm the effect of AESP for clinical uses.

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