Action of *Dracocephalum argunense* on Mast Cell-Mediated Allergy Model

Sang-Hyun Kim, a,b Sung-Hyun Kim, b,c Sung-Hwa Kim, d Jin-Young Moon, b Won-Hwan Park, b Cheorl-Ho Kim, d and Tae-Yong Shin* e

a Department of Pharmacology, Kyungpook National University Medical School; Daegu, 700–422, South Korea; b Cardiovascular Medical Research Center and Dongguk University College of Oriental Medicine; Kyung-Ju, 780–714, South Korea; c College of Pharmacy, Woosuk University; Jeonju, 565–701, South Korea; and d Department of Biological Science, SungKyunKwan University; Sejong, 440–746, South Korea.

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Dracocephalum argunense Fisch. (Labiatae) (DAAE) has been used for centuries as traditional oriental medicine. This crude drug is used for the management of pulmonary phthisis, abdominal phthisis, hepatitis and gastritis. Mast cells, which are constituents of virtually all organs and tissue, are important mediators of inflammatory responses such as allergy and hypersensitivity. Immediate-type hypersensitivity, anaphylaxis, is mediated by histamine release in response to antigen cross-linking of immunoglobulin E (IgE) bound to FceRI on mast cells. Mast cell activation causes the process of degranulation that result in releasing of mediators, such as histamine. Among the inflammatory substances released from mast cells, histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity. Mast cell activation is initiated upon interaction of multivalent antigen with its specific IgE antibody attached to the cell membrane via FcεRI. Anti-dinitrophenyl (DNP) IgE antibody and antigen have been established to induce passive cutaneous anaphylaxis (PCA) reactions as a typical *in vivo* model for immediate hypersensitivity. Mast cell degranulation also can be elicited by non-immunologic stimulators such as neuropeptides, basic compounds, complement components, and certain drugs. Compound 48/80 and polymers of basic amino acids, such as substance P, are some of the most potent stimulators of mast cells. Thus, an appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylactic allergic reaction.

The signaling pathway leading to degranulation of mast cells after engagement of the FceRI receptor has been extensively characterized. Activation of mast cells leads to phosphorylation of tyrosine kinase and mobilization of internal calcium. This is followed by activation of protein kinase C, mitogen-activated protein kinase (MAPK), and releasing of inflammatory cytokines. Although mast cells also store small amounts of cytokines in their granules, these cells dramatically increase the production of tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and other cytokines after their surface FceRI are cross-linked with specific antigen.

In this study, we evaluated the effect of DAAE on the compound 48/80-induced systemic reaction, IgE-mediated local allergic reaction, and histamine release from mast cells. The cAMP level was investigated to clarify the mechanism by which DAAE inhibited histamine release from mast cells. In addition, the effect of DAAE on phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187-induced p38 mitogen-activated protein kinase (MAPK) activation. DAAE decreased the secretion of pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-6 in mast cells. Our findings provide evidence that DAAE inhibits mast cell derived allergic reactions, and involvement of cAMP for histamine release and p38 MAPK for pro-inflammatory cytokine secretion in these effects.

**Key words** *Dracocephalum argunense*; mast cell; histamine; cAMP; inflammatory cytokine; p38 MAPK

**Materials and Methods**

**Reagents and Cell Culture** Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), o-phthalaldehyde, PMA, calcium ionophore A23187, and metrizamide were purchased from Sigma (St. Louis, MO, U.S.A.). SB 203580 was purchased from Calbiochem (La Jolla, CA, U.S.A.). rTNF-α and rIL-6 were purchased from R&D Systems Inc. (Minneapolis, MN, U.S.A.). The human mast cell line (HMC-1) was grown in Iscove’s media (Life Technologies, Grand Island, NY, U.S.A.) supplemented with 10% FBS and 2 mM glutamine at 37 °C in 5% CO2.

**Preparation of DAAE** The plants of *Dracocephalum argunense* Fisch. were collected in Jeonju, Jeonbuk, South Korea, on the 21st of September, 2004. A voucher specimen (number WSP-00-40) was deposited at the Herbarium of the College of Pharmacy, Woosuk University. The plant sample was extracted with purified water at 70 °C for 5 h. The extract...
was filtered through Whatman No. 1 filter paper, and the filtrate was lyophilized. The yield of dried extract from starting crude materials was about 20.6%. The dried extract was dissolved in saline or Tyrode buffer (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.1% bovine serum albumin) before use.

**Animals** The original stock of male ICR mice and male Sprague–Dawley rats were purchased from Dae-Han Experimental Animal Center (Daejeon, Korea). The animals were housed 5 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. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

**Compound 48/80-Induced Systemic Anaphylaxis** Mice were given an intraperitoneal injection of 8 mg/kg body weight (BW) of the mast cell degranulator, compound 48/80. DAAE was dissolved in saline and administered 1 h before the injection of compound 48/80 (n=10/group). Mortality was monitored for 1 h after induction of anaphylactic shock. After the mortality test, blood was obtained from the heart of each mouse to measure serum histamine content.

**Passive Cutaneous Anaphylaxis (PCA)** An IgE-dependent cutaneous reaction was examined as previously described. Pigs were intradermally injected with 0.5 μg of anti-DNP IgE. After 48 h, each mouse received an injection of DNP–HSA into the mouse tail vein. The mice were injected intradermally with 0.5 μg of anti-DNP IgE. After 48 h, each mouse received an injection of 1 μg of DNP–HSA containing 4% Evans blue (1:4) via the tail vein. Thirty minutes after the challenge, the mice were killed and the dorsal skin was removed for measurement of the pigment area. The amount of dye was determined colorimetrically after extraction with 1 ml of 1 M KOH for 10 min and the serum was withdrawn to determine colorimetrically.

**Preparation of Rat Peritoneal Mast Cells (RPMC)** RPMC were isolated as previously described. In brief, the peritoneal cells were suspended in Tyrode buffer, layered on 2 ml of metrizamide (22.5 w/v%), and centrifuged for 15 min at 400 g. The cells that remained at the buffer–metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer. Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue uptake.

**Histamine Assay** Histamine content from serum and RPMC were measured by the o-phthalaldehyde spectrophotometric procedure. The blood from the mice was centrifuged at 400 g for 10 min and the serum was withdrawn to measure histamine content. Purified RPMC were cultured with α-minimum essential media (Life Technologies) supplemented with 10% FBS at 37 °C in 5% CO₂. RPMC suspensions (2×10⁵ cells/ml) were sensitized with anti-DNP IgE (10 μg/ml) for 16 h. The cells were preincubated with DAAE at 37 °C for 10 min prior to the challenge with DNP–HSA (1 μg/ml). The cells were separated from the released histamine by centrifugation at 400 g for 5 min at 4 °C.

**cAMP Assay** The cAMP level was measured followed by the procedure of Peachell et al. In brief, RPMC were resuspended in prewarmed Tyrode buffer. An aliquot of cell was added to an equivalent volume of prewarmed buffer containing DAAE. The reaction was allowed to proceed for indicated time and terminated by the addition of ice-cold acidified ethanol. The sample was reconstituted in assay buffer and cAMP level was determined by enzyme immunoassay using a commercial kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

**Western Blot Analysis** Cell extracts were prepared by detergent lysis procedure. Samples of protein (50 μg) were electrophoresed using 12% SDS-PAGE, as described elsewhere 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, U.S.A.). Immuno detection was done using enhanced chemiluminescence detection kit (Amersham).

**Cytokine Secretion** TNF-α and IL-6 secretion were measured by modification of an enzyme-linked immunosorbent assay (ELISA). HMC-1 cells were sensitized with PMA (20 nm) plus A23187 (1 μM). The ELISA was performed by coating 96-well plates with 6.25 ng/well of monoclonal antibody with specificity for TNF-α and IL-6 respectively. 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, rTNF-α and rIL-6 were added to the serum which was previously determined to be negative to endogenous TNF-α and IL-6. After exposure to the medium, the assay plates were exposed sequentially to biotinylated anti-human TNF-α or IL-6, and IL-6, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) tablet substrates. Optical density was read within 10 min of the addition of the substrate with a 405 nm filter.

**Statistical Analysis** Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, U.S.A.). Treatment effects were analyzed using one way analysis of variance followed by Duncan’s Multiple Range test. A value of p<0.05 was used to indicate significant differences.

**RESULTS**

**DAAE Attenuates in Vivo Model of Allergic Responses** The systemic allergic model (anaphylaxis) was used in order to assess the contribution of DAAE in anaphylactic reaction. Compound 48/80 was used as a model of induction of systemic fatal allergic reaction. After the intraperitoneal injection of compound 48/80, the mice were monitored for 1 h, after which the mortality rate was determined. Compound 48/80 (8 μg/g) induced fatal shock in 100% of animals (Fig. 1A). When DAAE was anally administered at concentrations ranging from 0.001—1 mg/g for 1 h before the compound 48/80 injection, the mortality with compound 48/80 was dose-dependently reduced. DAAE completely inhibited the compound 48/80-induced fatal shock at 1 mg/g. The effect of DAAE on the compound 48/80-induced serum histamine release was investigated. DAAE was administered at concen-
trations ranging from 0.001—1 mg/g 1 h before the compound 48/80 injection. Injection of compound 48/80 caused marked increase of serum histamine release, and this induction was inhibited by DAAE treatment in a dose-dependent manner (Fig. 1B). To confirm the anti-allergic effects of DAAE in in vivo model, we used passive cutaneous anaphylaxis (PCA). PCA is one of the most important in vivo models of local allergic reaction.21) Local extravasation is induced by a 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 mice was injected with saline alone. After 48 h, all animals were injected intravenously with DNP–HSA plus Evans blue dye. DAAE was administered anally 1 h prior to the challenge with antigen. The administration of DAAE (0.001—1 mg/g) showed a dose-dependent inhibition in the PCA reaction (Fig. 2).

**DAAE Decreases IgE-Mediated Histamine Release in Mast Cells** To study the effect of DAAE on mast cell-mediated allergic responses, we evaluated the ability of DAAE on IgE-mediated histamine release from RPMC. The released histamine level was hardly detectable in unstimulated cells; however, RPMC released a high level of histamine when sensitized with anti-DNP IgE (10 μg/ml) and challenged with DNP–HSA (1 μg/ml) (Fig. 3). Treatment of DAAE (0.001—1 mg/ml) dose dependently inhibited IgE-mediated histamine release. The concentration and duration of DAAE treatment used in these studies had no significant effect on the viability of RPMC measured by MTT assay (data not shown). The cAMP pathway is critical to the regulation of mast cell degranulation. An increase of cAMP is known to precede the inhibition of histamine release from mast cells in response to stimulation of IgE receptors.22) To investigate the mechanisms of DAAE on the reduction of IgE-induced histamine release from mast cells, we assayed the intracellular cAMP levels. The level of cAMP was not changed in unstimulated cells. When RPMC were incubated with DAAE at 1 mg/ml, the cAMP content increased at 1—2 min and decreased to basal levels from 3 min (Fig. 4).

**DAAE Inhibits Pro-Inflammatory Cytokine Secretion and Selectively Decreases p38 MAPK Activation** To evaluate the impact of DAAE on downstream mast cell signaling, we examined the effect of DAAE on the MAPKs. Ac-
tivation of MAPKs result from phosphorylation of specific tyrosine and threonine residues. Assessment of the activation of p38, ERK, and JNK was determined by Western blot in human mast cell line (HMC-1). Treatment of PMA (20 nM) plus A23187 (1 μM) activated all three types of MAPKs at 15—30 min in HMC-1 cells (data not shown). DAAE specifically attenuated the PMA plus A23187-induced p38 MAPK activation in a dose-dependent manner but did not affect the phosphorylation of ERK and JNK (Fig. 5).

Next, we examined whether DAAE could regulate pro-inflammatory cytokines such as TNF-α and IL-6 in HMC-1 cells. TNF-α and IL-6 play a major role in triggering and sustaining the allergic response in mast cells. In addition, the production of TNF-α and IL-6 is regulated by p38 MAPK.15,24) Secretion of TNF-α and IL-6 was measured by ELISA. Stimulation of HMC-1 cells with PMA (20 nM) plus A23187 (1 μM) during 16 h induced the secretion of both cytokines (Fig. 6). Treatment of DAAE dose-dependently reduced PMA plus A23187-induced TNF-α and IL-6 secretion. A specific p38 MAPK inhibitor, SB 203580 (10 μM), abolished PMA plus A23187-induced TNF-α and IL-6 secretion.

DISCUSSION

Using in vitro and in vivo model, we show that DAAE regulates mast cell-mediated allergic responses. In the present study, we clearly demonstrated that DAAE decreased compound 48/80 or IgE-induced histamine release and PMA plus calcium ionophore-mediated pro-inflammatory cytokine secretion.

Numerous reports established that stimulation of mast cells with compound 48/80 or IgE initiates the activation of signal-transduction pathway, which leads to histamine release. Several studies have shown that compound 48/80 and other polybasic compounds are able, apparently directly, to activate G-proteins.25,26) Tasaka et al.27) report that compound 48/80 increases the permeability of the lipid bilayer membrane by causing a perturbation of the membrane. These reports indicate that the increase in membrane permeability may be an essential trigger for the release of the mediator from mast cells. In this sense, anti-allergic agents having a membrane-stabilizing action may be desirable. In the present study, DAAE significantly inhibited systemic allergic reaction and histamine release from mast cells. These results indicate that mast cell-mediated immediate-type allergic reactions are inhibited by DAAE. In addition, DAAE administered mice are protected from IgE-mediated PCA, one of the most important in vivo models of anaphylaxis in local allergic reaction. This finding suggests that DAAE might be useful in allergic skin reactions.

cAMP pathway is critical to the degranulation of mast cells. Agents that stimulate an intracellular cAMP level have been shown to reduce mast cell degranulation. An increase of cAMP is believed to precede the inhibition of histamine release from mast cells in response to stimulation of IgE receptors or compound 48/80.22,27,28) The release of histamine is known to be depressed by an increase in intracellular cAMP content due to the activation of adenylate cyclase or inhibition of cAMP phosphodiesterase.29) The intracellular cAMP content of the mast cells, when incubated with DAAE, increased in comparison with that of basal cells. These results suggest that the effects of DAAE on the allergic reaction may be associated with an increase in the intracellular cAMP content of the mast cells. Our result showing an enhancement of cAMP in mast cells following DAAE treatment is consistent with other reports. According to these observations, we speculate that increased cAMP might be involved in the inhibitory effect of DAAE on histamine release.

HMC-1 cell line is a useful cell for studying cytokine activation pathways.30,31) The spectrum of cytokines produced by HMC-1 cells with PMA plus A23187 stimulation supports the well-recognized role of mast cells in immediate-type hypersensitivity. TNF-α and IL-6, play a major role in triggering and sustaining the allergic inflammatory response in mast cells. TNF-α promotes inflammation, leukocyte infiltration,
granuloma formation and tissue fibrosis and is thought to be an initiator of cytokine related inflammation states by stimulating cytokine production.\(^{32,33}\) IL-6 is also produced from mast cells and its local accumulation is associated with PCA reaction.\(^{41}\) These reports indicate that reduction of TNF-\(\alpha\) and IL-6 from mast cell is a one of the key indicator of reduced allergic symptom. In the present study, DAAE inhibited the secretion of TNF-\(\alpha\) and IL-6 in PMA plus A23187-stimulated HMC-1 cells. These results suggest that DAAE reduces the allergic responses through decreasing the pro-inflammatory cytokine production.

The MAPK cascade is one of the important signaling pathways in immune responses.\(^{23}\) The expression of TNF-\(\alpha\) and IL-6 is regulated by MAPKs. 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. Activation of p38 MAPK is essential for the expression of the pro-inflammatory cytokines.\(^{35,36}\) According to the present results, PMA plus A23187 simultaneously activated all three MAPKs in HMC-1 cells. Among the MAPKs, DAAE specifically inhibited the activation of p38 MAPK but not of ERK or JNK. Furthermore, the specific p38 MAPK inhibitor, SB 203580, decreased TNF-\(\alpha\) and IL-6 production. These data suggest that DAAE has the inhibitory activity on p38 MAPK activation and downstream TNF-\(\alpha\) and IL-6 production.

Because we used the whole water extract of \(D.\ argunense\) not a purified component, the active components that are responsible for the biological effect are not clear at this time. The effort for identify active components from DAAE in allergic reaction is ongoing in our laboratory. However, the results presented in this report give an insight into the mechanism responsible for anti-allergic activity of DAAE and evidence that DAAE could contribute to the prevention or treatment of mast cell-mediated allergic diseases.

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