

Effect of *Dracocephalum argunense* on Mast-Cell-Mediated Hypersensitivity

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Key Words

Dracocephalum argunense · Immediate-type hypersensitivity · Mast cell · Histamine · Calcium · Inflammatory cytokine · NF- κ B

Abstract

Background: Stimulation of mast cells starts the process of degranulation resulting in release of mediators such as histamine and an array of inflammatory cytokines. In this report, we investigated the effect of an aqueous extract of *Dracocephalum argunense* Fisch. (Labiatae) (DAAE) on immediate-type hypersensitivity and studied its possible mechanisms of action, focusing on histamine release and proinflammatory cytokine expression in mast cells. **Methods:** An in vivo model of systemic and local allergic reaction was investigated. Compound 48/80- or IgE-induced histamine release from mast cells was measured. The expression of proinflammatory cytokines such as TNF- α and IL-6 was measured by RT-PCR and ELISA. The level of intracellular calcium was measured by spectrofluorometry. NF- κ B activation was measured by Western blot, EMSA and luciferase assay. **Results:** DAAE inhibited systemic anaphylaxis, local allergic reactions, and serum histamine release in a dose-dependent manner in mice. DAAE dose-dependently reduced histamine release from mast cells activated by compound 48/80 or IgE. The inhibitory effect of DAAE on histamine release was mediated by the modulation of intracellular calcium. In addition, DAAE decreased TNF- α and

IL-6 gene expression and production in human mast cells stimulated by phorbol-12-myristate-13-acetate (PMA) plus calcium ionophore A23187. The inhibitory effect of DAAE on the TNF- α and IL-6 expression was NF- κ B dependent. **Conclusion:** Our findings provide evidence that DAAE inhibits mast-cell-derived immediate-type hypersensitivity. Taken together, the anti-allergic effect of DAAE in vivo and in vitro suggests a possible clinical use of this agent in immediate-type hypersensitivity.

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Introduction

Mast cells, which are constituents of virtually all organs and tissues, are important mediators of inflammatory responses such as allergy and hypersensitivity. Immediate-type hypersensitivity (anaphylaxis) is an acute and life-threatening allergic reaction that occurs massively and systemically. Anaphylaxis is mediated by histamine release in response to antigen cross-linking of immunoglobulin E (IgE) bound to Fc ϵ RI on mast cells. Mast cell activation both by IgE-dependent and IgE-independent stimuli brings about the process of degranulation that results in the fusion of the cytoplasmic membranes with the plasma membrane. This is accompanied by both the fast external release of granule-associated stored mediators (histamine, chemotactic factors, neutral proteases, proteoglycans, cytokines, etc.) as well as by the generation and release of newly generated mediators, such as products of

arachidonic acid metabolism and, at later times, by the production and release of an array of cytokines [1, 2].

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 [3]. Mast cell degranulation can be elicited by nonimmunologic stimulators such as neuropeptides, basic compounds, complement components, and certain drugs [4]. Compound 48/80 and polymers of basic amino acids, such as substance P, are some of the most potent stimulators of mast cells [5]. Compared with the natural process, a high concentration of compound 48/80 induces almost 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. Immunologic stimulators can also activate mast cells. Mast cell activation is initiated by interaction of multivalent antigen with its specific IgE antibody attached to the cell membrane via the high-affinity receptor FcεRI [6, 7]. Anti-dinitrophenyl (DNP) IgE antibody has been established to induce passive cutaneous anaphylaxis (PCA) reactions as a typical *in vivo* model for immediate hypersensitivity.

The signaling pathway leading to degranulation of mast cells after engagement of the FcεRI receptor has been extensively characterized [2, 8]. 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), nuclear factor-κB (NF-κB), 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 within 30 min after their surface FcεRI is cross-linked with specific antigen [9–12]. The finding that activated mast cells are also a source of several cytokines suggests an additional role of mast cells in late-phase reactions and other persistent inflammatory processes.

Dracocephalum argunense Fisch. (Labiatae) has been used for centuries in traditional oriental medicine. This crude drug is used for the management of pulmonary phthisis, abdominal phthisis, hepatitis and gastritis [13]. In this study, we evaluated the effect of DAAE on systemic reactions, IgE-induced local allergic reactions, and histamine release from mast cells induced by compound 48/80. The intracellular calcium content was investigated to clarify the mechanism by which DAAE inhibited histamine release from mast cells. In addition, the effect of

DAAE on TNF-α and IL-6 expression induced by phorbol-12-myristate-13-acetate (PMA) plus calcium ionophore A23187 and the role of NF-κB in this effect were investigated in human mast cells (HMC-1).

Materials and Methods

Reagents and Cell Culture

Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), *o*-phthaldialdehyde, PMA, calcium ionophore A23187, metrizamide, and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma (St. Louis, Mo., USA). rTNF-α and rIL-6 were purchased from R&D Systems Inc. (Minneapolis, Minn., USA). The human mast cell line (HMC-1) was grown in Iscove's media (Life Technologies, Grand Island, N.Y., USA) supplemented with 10% FBS and 2 mM glutamine at 37°C in 5% CO₂. The passage ranging 4–8 of HMC-1 cells was used throughout the study.

Animals

The original stock of male ICR mice and male Sprague-Dawley rats was 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 a 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.

Preparation of *D. argunense* Aqueous Extract

The plants of *D. argunense* Fisch. were collected in Jeonju, Jeonbuk, South Korea, on the 21th of September, 2004. A voucher specimen (No. 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.

Compound-48/80-Induced Systemic Reaction

Mice were given an intraperitoneal injection of 8 mg/kg, body weight (BW) of the mast cell degranulator, compound 48/80. *D. argunense* aqueous extract (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.

PCA Reaction

An IgE-dependent cutaneous reaction was examined as previously described [14]. The PCA reaction was generated by sensitizing skin with an intradermal injection of anti-DNP IgE followed 48 h later with 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 and 9 ml of a mixture of acetone and phosphoric acid (5:13) based on the method of Katayama et al. [15]. The intensity of absorbance was measured at 620 nm in a spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan).

Preparation of Rat Peritoneal Mast Cells

Rat peritoneal mast cells (RPMC) were isolated as previously described [16]. 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 of serum and RPMC was measured by the *o*-phthalaldehyde spectrofluorometric procedure [17]. The blood from the mice was centrifuged at 400 g for 10 min and the serum was withdrawn to measure histamine content. RPMC were preincubated with DAAE, and then incubated for 10 min with compound 48/80 (5 µg/ml). RPMC suspensions were also 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.

Intracellular Calcium

Fura-2/AM (2 µM, molecular probes, Eugene, Oreg., USA) was used to determine the intracellular calcium following the manufacturer's protocol briefly described as follows: RPMC were preincubated with Fura-2/AM for 30 min at 37°C. After washing the dye from the cell surface, DAAE was pretreated 10 min prior to the compound 48/80 treatment. The fluorescent intensity was recorded using a fluorescent plate reader (Molecular Devices, Sunnyvale, Calif., USA) at an excitation of 340 nm and an emission of 500 nm.

RNA Extraction and mRNA Expression

The total cellular RNA was isolated from the cells (1×10^6 /well in 24-well plate) after stimulation of PMA plus A23187 with or without DAAE using a TRI reagent (Molecular Research Center, Cincinnati, Ohio, USA) according to the manufacturer's protocol. The first strand complementary DNA (cDNA) was synthesized using the Superscript II reverse transcriptase enzyme (Life Technologies). A reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNF-α, IL-6 and β-actin (internal control). The conditions for the reverse transcription and PCR steps were similar to those previously described [18]. The primer sets were chosen by the Primer3 program (Whitehead Institute, Cambridge, Mass., USA). The cycle number was optimized in order to ensure product accumulation in the exponential range. The amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide, documented using a Kodak DC 290 digital camera.

Assay of TNF-α and IL-6 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-ethylbenzothiazoline-6-sulfonic acid) (ABTS) tablet substrates. Optical density was read within 10 min of the addition of the substrate with a 405 nm filter.

Western Blot Analysis

Samples of protein were electrophoresed with 8% SDS-PAGE gel and transferred to nitrocellulose membrane. The nucleus and cytosolic p65 NF-κB, and IκBα was assayed using anti-NF-κB (p65) and anti-IκBα antibodies (Santa Cruz Biotech, Santa Cruz, Calif., USA). Immunodetection was done using an enhanced chemiluminescence detection kit (Amersham).

Electrophoretic Mobility Shift Assay

Nuclear protein (10 µg) was incubated for 20 min at room temperature with 20 µg of BSA, 2 µg of poly (dI-dC) from Pharmacia (Uppsala, Sweden), 2 µg of buffer C (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM PMSF), 4 µg of buffer F (20% Ficoll-400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.5 mM PMSF), and 20,000 cpm of a ³²P-labeled probe encoding the κB consensus sequence (5'-GAT CTC AGA GGG GAC TTT CCG AGA AGA-3') in a final volume of 20 µl. DNA-protein complexes were resolved at 120 V for 2 h in a 5% polyacrylamide gel, dried, and visualized.

Transient Transfection and Luciferase Activity Assay

For transient transfections, HMC-1 cells were seeded at 2×10^6 in a 6-well plate 1 day before transient transfection. The expression vector containing NF-κB luciferase reporter construct (pNF-κB-LUC, plasmid containing NF-κB binding site; Stantagen, Grand Island, N.Y., USA) were transfected with serum- and antibiotic-free Iscove's medium containing 8 µl of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, Calif., USA). After 5 h of incubation, medium was replaced with Iscove's medium containing 10% FBS and antibiotics. Cells were allowed to recover at 37°C for 20 h and subsequently were stimulated as indicated. For the luciferase activity assay, cell lysates were prepared and assayed for luciferase activity using Luciferase Assay System (Promega, Madison, Wisc., USA), according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, N.C., USA). 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.

Table 1. Effect of DAAE on systemic reaction induced by compound 48/80

DAAE treatment g/kg BW	Compound 48/80 8 mg/kg BW	Mortality %
None (saline)	+	100
0.01	+	100
0.05	+	60
0.1	+	20
0.5	+	0
0.5	–	0

Groups of mice (n = 10/group) were intraperitoneally treated with 200 µl of saline or DAAE at various doses 1 h before the intraperitoneal injection of compound 48/80 (8 mg/kg). Mortality (%) within 1 h following compound 48/80 injection is represented as the number of dead mice × 100/total number of experimental mice.

Results

DAAE Inhibits Anaphylactic Reactions Mediated by Immunologic and Nonimmunologic Stimulations

The in vivo model of systemic immediate-type hypersensitivity was first used in order to assess the contribution of DAAE in anaphylactic reactions. Compound 48/80 was used as a model of induction of systemic fatal allergic reactions. 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 mg/kg BW) induced fatal shock in 100% of animals (table 1). When DAAE was intraperitoneally administered at concentrations ranging from 0.01 to 1 g/kg BW for 1 h, the mortality induced by compound 48/80 was dose-dependently reduced. DAAE 0.5 g/kg BW completely inhibited the fatal shock induced by compound 48/80. In addition, the mortality of mice administered DAAE (1 g/kg, BW) 5, 10, 20 or 30 min after compound 48/80 injection increased time dependently (table 2).

The effect of DAAE on serum histamine release induced by compound 48/80 was investigated as well. DAAE was administered at concentrations ranging from 0.01 to 1 g/kg BW 1 h before injection of compound 48/80. Injection of compound 48/80 markedly caused a marked increase in serum histamine release which was inhibited by DAAE treatment in a dose-dependent manner (fig. 1a).

Another way to test the anaphylactic reactions is to induce PCA. 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

Table 2. Time-dependent effect of DAAE on systemic reaction induced by compound 48/80

DAAE treatment g/kg BW	Time min	Compound 48/80 8 mg/kg BW	Mortality %
None (saline)		+	100
1	5	+	0
1	10	+	10
1	20	+	80
1	30	+	100

Groups of mice (n = 10/group) were intraperitoneally treated with 200 µl of saline or DAAE. DAAE (1 g/kg) was given at 5, 10, 20, or 30 min after intraperitoneal injection of compound 48/80. Mortality (%) within 1 h following injection of compound 48/80 is represented as the number of dead mice × 100/total number of experimental mice.

right dorsal skin. As a control, the left dorsal skin 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 intraperitoneally 1 h prior to the challenge with antigen. The administration of DAAE (0.001–1 g/kg BW) showed a dose-dependent inhibition in the PCA reaction (fig. 1b).

DAAE Decreases Histamine Release from RPCM and Intracellular Calcium Levels of RPMC Mediated by Immunologic and Nonimmunologic Stimulation

We evaluated the activity of DAAE on compound-48/80-induced or IgE-mediated histamine release from RPMC. The level of released histamine was hardly detectable in unstimulated cells; however, RPMC released a high level of histamine when stimulated with compound 48/80 (5 µg/ml) or anti-DNP IgE (10 µg/ml) sensitizing and DNP-HSA (1 µg/ml) challenge (fig. 2a). DAAE dose-dependently inhibited both compound-48/80- and IgE-mediated histamine release in a dose-dependent manner. The concentration and duration of DAAE treatment used in these studies had no significant effect on the viability of RPMC (data not shown). To investigate the possible mechanism of DAAE on the reduction of histamine release, we assayed the level of intracellular calcium. Calcium movements across membranes of mast cells are critical to histamine release [19]. Figure 2b shows the stimulation of intracellular calcium when the RPMC were treated with compound 48/80 (5 µg/ml). Preincubation of DAAE (1 mg/ml) with RPMC decreased the intracellular calcium level induced by compound 48/80.

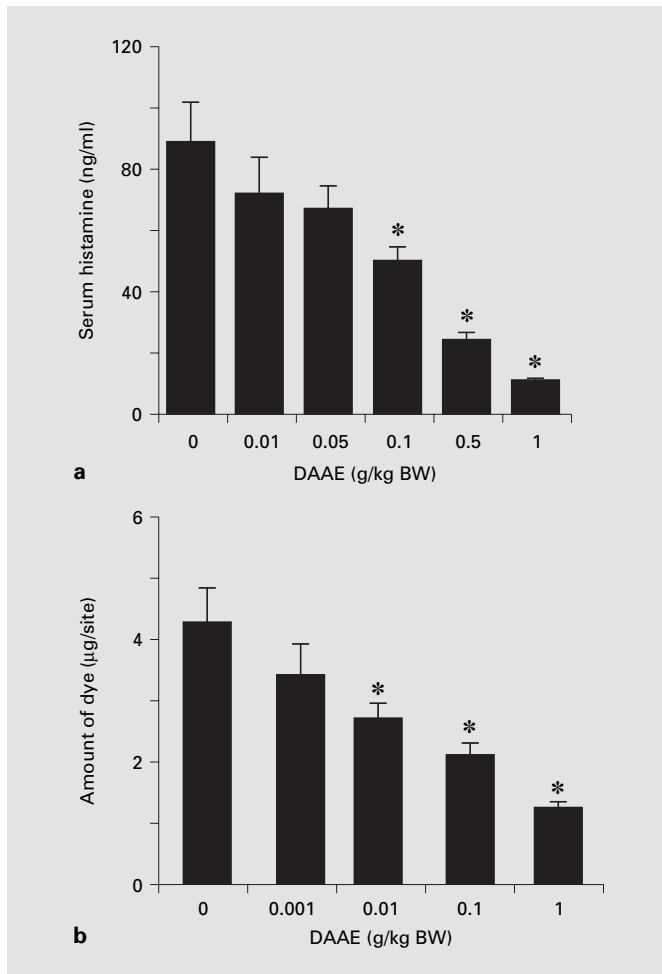


Fig. 1. Effect of DAAE on serum histamine release and PCA reactions. **a** Groups of mice ($n = 10/\text{group}$) were intraperitoneally treated with 200 μl of saline or DAAE at various doses 1 h before the intraperitoneal injection of the compound 48/80 (8 mg/kg). The blood was obtained from the heart of each mouse and histamine content was measured by spectrofluorometry. **b** DAAE was administered 1 h prior to the challenge with antigen. Each amount of dye was extracted as described in Materials and Methods and measured by spectrophotometry. * $p < 0.05$, statistically significant.

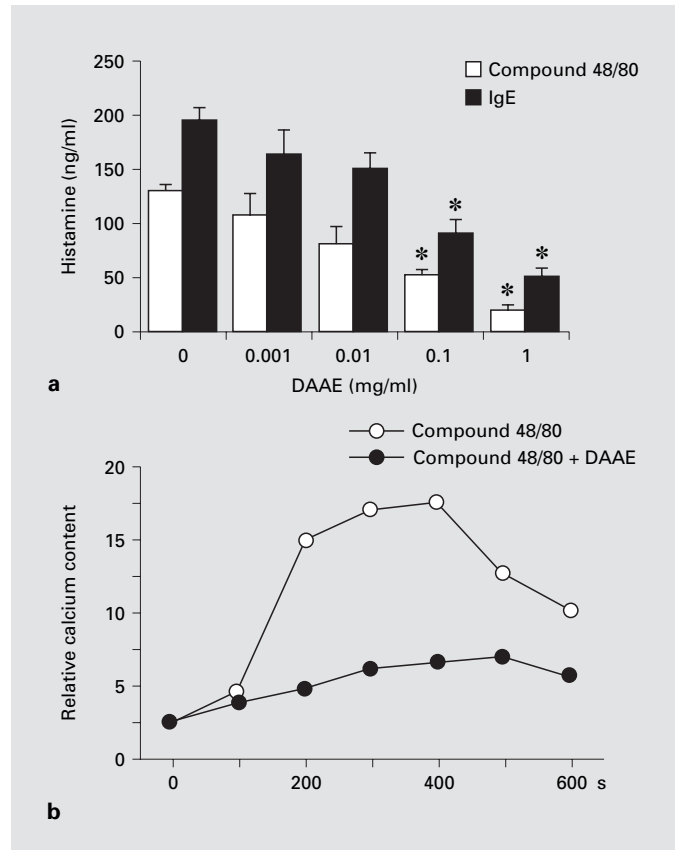


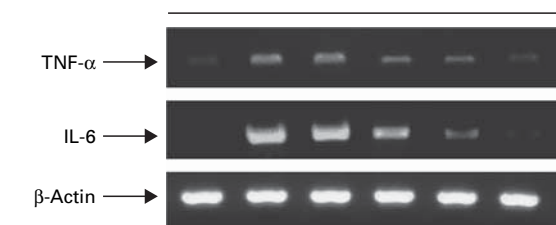
Fig. 2. Effect of DAAE on histamine release and intracellular calcium level from RPMC. **a** Cells were preincubated with DAAE at indicated concentrations for 10 min prior to incubation with either compound 48/80 (5 $\mu\text{g}/\text{ml}$) or DNP-HSA (1 $\mu\text{g}/\text{ml}$). **b** RPMC was stained with Fura-2/AM and then cells were preincubated for 10 min with DAAE (1 mg/ml) before adding compound 48/80 (2 $\mu\text{g}/\text{ml}$), and then for another 10 min with compound 48/80. Each value represents the mean $\pm$ SEM of three independent experiments. * $p < 0.05$, statistically significant.

DAAE Attenuates Proinflammatory Cytokine Expression and Production through the Inhibition of NF- κ B Activation in HMC-1 Cells

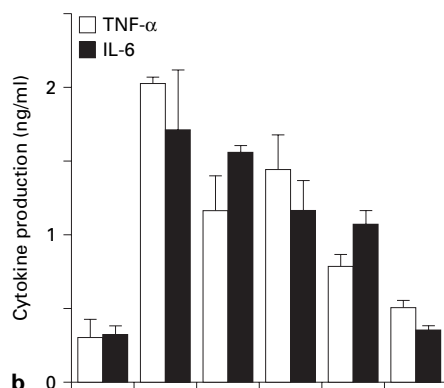
We examined whether DAAE could regulate proinflammatory cytokines such as TNF- α and IL-6 in HMC-1 cells. TNF- α and IL-6 play a major role in triggering and sustaining the allergic response in mast cells. The HMC-1 cell line is useful for studying the cytokine activation pathway [20]. Stimulation of HMC-1 cells with PMA (20 nM) plus A23187 (1 μM) during 4 h induced

the gene expression of both cytokines (fig. 3a). DAAE (0.01–1 mg/ml) dose-dependently inhibited TNF- α and IL-6 gene expression induced by PMA plus A23187. In contrast to TNF- α and IL-6, the level of β -actin mRNA expression remained the same under these conditions. To confirm the correlation of mRNA expression with protein production, we measured the TNF- α and IL-6 production by ELISA. Stimulation of HMC-1 cells with PMA (20 nM) plus A23187 (1 μM) during 16 h induced the production of both cytokines (fig. 3b). Treatment with

PMA + A23187	-	+	+	+	+	+
DAAE (mg/ml)	-	-	0.01	0.1	1	-
PDTC	-	-	-	-	-	+

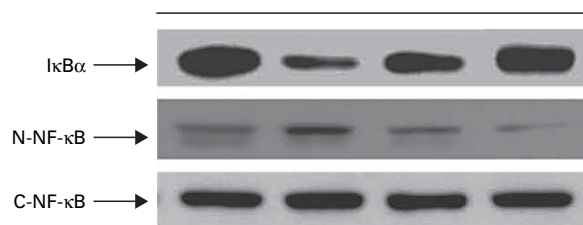


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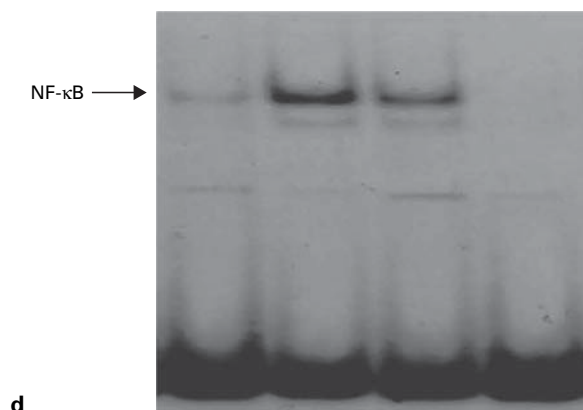


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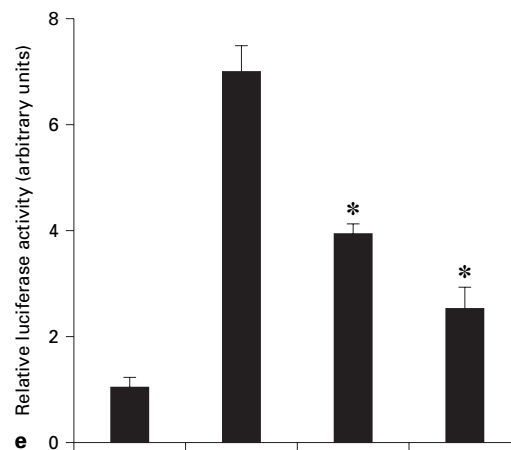
PMA + A23187	-	+	+	+
DAAE (mg/ml)	-	-	1	-
PDTC	-	-	-	+



c



d



e

Fig. 3. Effect of DAAE on proinflammatory cytokine expression and NF- κ B activation in HMC-1 cells. Cells were treated with indicated concentrations of DAAE or PDTC (50 μ M) for 30 min before stimulation with PMA (20 nM) plus A23187 (1 μ M). The expression level of mRNA and production level of TNF- α and IL-6 were determined by RT-PCR (**a**) and ELISA (**b**), respectively. **c** I κ B α degradation and NF- κ B translocation were assayed by Western blot (N-NF- κ B = nucleus NF- κ B; C-NF- κ B = cytoplasmic NF- κ B). **d** Nuclear extracts prepared and incubated with ^{32} P-labeled oligonucleotides corresponding to NF- κ B were subjected to EMSA analysis. **e** For NF- κ B luciferase assay, cells were transiently transfected with the NF- κ B luciferase reporter construct or empty vector. NF- κ B-dependent transcriptional activity was determined by luciferase activity assay. Each bar represents the mean $\pm$ SEM of three independent experiments. * $p < 0.05$, statistically significant.

DAAE also dose-dependently reduced TNF- α and IL-6 production induced by PMA plus A23187. A potent NF- κ B inhibitor, PDTC (50 μ M), almost completely blocked TNF- α and IL-6 expression and production induced by PMA plus A23187.

NF- κ B is an important transcriptional regulator of inflammatory cytokines and plays a crucial role in immune and inflammatory responses. To investigate the intracellular mechanism responsible for the inhibitory effect of DAAE on TNF- α and IL-6 expression, we examined the effect of DAAE on NF- κ B activation using Western blot, electrophoretic mobility shift assay (EMSA) and luciferase reporter assay. We used PDTC as a positive control. Stimulation of HMC-1 cells with PMA plus A23187 for 2 h induced the degradation of I κ B α and nuclear translocation of p65 NF- κ B (fig. 3c). DAAE inhibited the degradation of I κ B α and nuclear translocation of p65 NF- κ B induced by PMA plus A23187. In order to investigate whether NF- κ B/DNA binding was inhibited by DAAE, we performed EMSA. Cells pretreated with either DAAE or PDTC (50 μ M) were subsequently stimulated by PMA plus A23187, and the effect of DAAE and PDTC on binding activity of NF- κ B was examined. Treatment of PMA plus A23187 caused a significant increase in the DNA-binding activity of NF- κ B within 4 h (fig. 3d). In the presence of DAAE, NF- κ B/DNA binding induced by PMA plus A23187 was markedly suppressed. To further confirm the inhibitory effect of DAAE on NF- κ B activation, we examined the effect of DAAE on the NF- κ B-dependent gene reporter assay. HMC-1 cells were transiently transfected with a NF- κ B-luciferase reporter construct or the empty vector. Exposure of cells to PMA plus A23187 increased the luciferase activity in the cells transfected with the NF- κ B-luciferase reporter construct (fig. 3e). DAAE significantly reduced the luciferase activity induced by PMA plus A23187.

Discussion

Numerous reports established that stimulation of mast cells with compound 48/80 or IgE initiates the activation of the 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 [21, 22]. Tasaka et al. [23] reported 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 essen-

tial trigger for the release of the mediator from mast cells. In this sense, anti-allergic agents having a membrane-stabilizing action may be desirable. The results of our study demonstrated that DAAE has anti-allergic properties. DAAE significantly inhibited the systemic allergic reaction induced by compound 48/80. DAAE also inhibited histamine release from mast cells induced by compound 48/80 or mediated by IgE. These results indicate that mast-cell-mediated immediate-type allergic reactions are inhibited by DAAE. Taken together, we could speculate that DAAE might stabilize the lipid bilayer membrane, thus preventing the perturbation induced by compound 48/80. In addition, mice administered DAAE are protected from IgE-mediated PCA, one of the most important in vivo models of anaphylaxis in local allergic reactions. This finding suggests that DAAE might be useful in the treatment of allergic skin reactions.

Intracellular calcium is critical to the degranulation of mast cells. Calcium movements across membranes of mast cells represent a major target for effective anti-allergic drugs, as these are essential events linking stimulation to secretion [8]. The transduction pathways modulating intracellular calcium is modified by ADP-ribosylate G-protein binding protein [24]. Our results showing an attenuation of intracellular calcium in mast cells following DAAE treatment are consistent with other reports. According to these observations, we strongly speculate that decreased intracellular calcium 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 [20, 25]. 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 inflammatory states by stimulating cytokine production in other types of cells [26, 27]. Mast cells are a principal source of TNF- α in human dermis, and degradation of mast cells in the dermal endothelium is abrogated by the anti-TNF- α antibody [28]. IL-6 is also produced from mast cells and its local accumulation is associated with PCA reaction [29]. These reports indicate that reduction of TNF- α and IL-6 from mast cell is one of the key indicators of reduced allergic symptoms. DAAE inhibited the gene expression and production of TNF- α and IL-6 in PMA plus A23187-stimulated HMC-1 cells. Expression of TNF- α and IL-6 gene is dependent on the

activation of transcription factor NF- κ B [30]. To evaluate the mechanisms of the effect of DAAE on the TNF- α and IL-6 expression, we examined the effect of DAAE on the NF- κ B activation. Activation of NF- κ B requires phosphorylation and proteolytic degradation of the inhibitory protein I κ B α , an endogenous inhibitor that binds to NF- κ B in the cytoplasm [31]. In our present study, DAAE blocked the NF- κ B pathway in human mast cells stimulated by PMA and A23187. In addition, PDTC, a potent inhibitor of NF- κ B, reduced TNF- α and IL-6 expression and production induced by PMA plus A23187. These data demonstrate that DAAE attenuates the activation of NF- κ B and downstream TNF- α and IL-6 production.

Several reports show the relation of intracellular calcium and inflammatory cytokine production from mast cells [32, 33]. Depletion of intracellular calcium with various calcium blockers inhibits IgE-induced TNF- α and IL-6 production in mast cells. The NF- κ B pathway mediates these effects. In this study, DAAE decreased the elevated intracellular calcium levels and inhibited the gene expression and protein production of TNF- α and IL-6 in

mast cells. These data suggest that inhibition of intracellular calcium level by DAAE may modulate the inflammatory cytokine production.

Because we used the whole water extract of *D. argu-nense* rather than 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 the immediate-type hypersensitivity is ongoing in our laboratory. However, the results presented in this report give an insight into the mechanism responsible for the anti-allergic activity of DAAE and evidence that DAAE could contribute to the treatment of mast cell-mediated allergic diseases.

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