

Ailanthus altissima swingle has anti-anaphylactic effect and inhibits inflammatory cytokine expression via suppression of nuclear factor-kappaB activation

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Abstract *Ailanthus altissima* swingle (ailanthic cortex, AAS) has been used as a traditional medicine for fever, bleeding, infection, and inflammation for many years in Korea. However, its mechanisms have not been examined. In the present study, we investigate the effect of AAS on the mast-cell-mediated allergic and inflammatory reaction using in vivo and in vitro models and elucidate its molecular mechanisms. AAS significantly inhibited compound 48/48-induced edema and systemic anaphylaxis. AAS significantly inhibited passive cutaneous anaphylaxis. AAS inhibited histamine release from rat peritoneal mast cells (RPMCs) in a dose-dependent manner. Moreover,

AAS significantly inhibited production of inflammatory cytokines, tumor necrosis factor (TNF), interleukin (IL)-6, and IL-8 on the phorbol 12-myristate 13-acetate and calcium ionophore A23187 (PMACI)-stimulated human mast cell line, HMC-1 cells. AAS inhibits the IgE or stem cell factor-induced TNF production on RPMCs. In activated HMC-1 cells, the expression level of NF-kappaB/*Rel A* protein increased in the nucleus, whereas the level of NF-kappaB/*Rel A* in the nucleus was decreased by AAS treatment. In addition, AAS inhibited the PMACI-induced IkappaBalpha degradation. In conclusion, the present results indicate that AAS has potent anti-anaphylactic and anti-inflammatory properties.

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Introduction

The *Ailanthus alissima* swingle (ailanthic cortex, AAS) is a perennial herb that is widely distributed throughout Korea, China, and Japan. AAS has been used as a traditional medicine to remove fever, stop bleeding, and destroy worms and anti-inflammatory effect for many years in Korean. However, its mechanisms of action have not been examined. We tested the inhibitory effects of AAS on mast cell mediated-allergic inflammatory reactions.

Mast cells are broadly distributed throughout mammalian tissues and play a critical role in a wide variety of biological responses. Typically, mast cells have been considered not only in the association of immediate-type hypersensitivity but also in late reactions like inflammatory responses, which are mast cell dependent (Metzger et al.

1986; Kemp and Lockey 2002). One of the well-used approaches in the examination of the immunopathological mechanisms of anaphylactic and inflammatory disorders is to elicit the formation of paw edema by injecting various substances into the subplantar tissue of rats or mice (Linardi et al. 2000; Sawynok et al. 2000). In the present study, the author determined the effect of AAS on compound 48/80-induced paw edema.

Mast cells are important producers of mediators of inflammatory responses such as allergy and immediate-type allergic reaction. Immediate-type allergic reaction is mediated by histamine released in response to the antigen cross-linking of immunoglobulin E (IgE) bound to FcεRI on the mast cells. After activation via the FcεRI, the mast cells start the process of degranulation, which results in the releasing of mediators, such as products of arachidonic acid metabolism and an array of inflammatory cytokines (Church and Levi-Schaffer 1997; Miyajima et al. 1997; Galli et al. 2005). Among the inflammatory substances released from mast cells, histamine is one of the best-characterized and most potent vasoactive mediators implicated in the acute phase of immediate hypersensitivity (Cook et al. 2002). Mast cell activation is initiated upon interaction of multivalent antigen with its specific IgE antibody attached to the cell membrane via FcεRI (Metzger et al. 1986; Alber et al. 1991). The release of vasoactive substances is produced locally in naive animals using the IgE of animals. IgE is injected intradermally into naive animals, and the tested substance mixed with a dye (typically Evans blue) is subsequently administered intravenously (usually after 24–72 h). Subsequent to the antigen–antibody reaction, vasoactive substances are released, resulting in a dramatic local increase in vascular permeability (Bazin et al. 1999). As the tested substance is mixed with a dye, the local cutaneous reaction is evidenced by a colored spot (Verdier et al. 1994). In general, immediate-type hypersensitivity reactions that involve urticaria, allergic rhinitis, and asthma are mediated by various chemical mediators released from mast cells (Miescher and Vogel 2002). Histamine, the main content of granules in mast cells, exerts many effects related to the immediate phase of allergic inflammation, including vasodilation, increased vascular permeability, tissue edema, contraction of bronchial and intestinal smooth muscle, and increased mucus production (White 1991; Guo et al. 1997). Compound 48/80 is a well-known histamine releaser (Kim et al. 2003). Antihistamines reduced proinflammatory mediators from inflammatory cells, chemotaxis, and activation of eosinophils in vitro study (Assanasen and Naclerio 2002). 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.

Stem cell factor (SCF) is a crucial growth factor in mast cell biology. It regulates such diverse cellular functions as proliferation, differentiation, survival, adhesion, and release of inflammatory mediators (Irani et al. 1992). SCF also induces the proinflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-8, IL-16, and IL-18 from mast cells (Lorentz and Bischoff. 2001).

In inflammatory process, several proinflammatory cytokines, such as TNF-α, interleukin (IL)-1, IL-4, IL-6, and IL-8, recruit activated immune and inflammatory cells to the site of lesions, thereby amplifying and perpetuating the inflammatory condition (Church and Levi-Schaffer 1997; Metcalfe et al. 1997). Mast-cell-derived proinflammatory cytokines play an important role in the development of acute- and late-phase allergic inflammatory reactions. Therefore, inhibition of the production and production of these mediators is likely to provide a major mechanism to enhance efficacy in the treatment of inflammatory diseases (Kim et al. 2004, 2005a, b).

The signaling pathway leading to degranulation of mast cells after engagement of the FcεRI receptor has been extensively characterized (Metcalfe et al. 1981; Beaven et al. 1984). Activation of mast cells leads to phosphorylation of tyrosine kinase and mobilization of internal calcium. This is followed by activation of protein kinase C and nuclear factor kappaB (NF-κB) and release of inflammatory cytokines. NF-κB is a substantial transcription factor required for the expression of many inflammatory genes (Collart et al. 1990). NF-κB plays a serious role in the expression of cytokines such as TNF and IL-6, which mediate response to inflammatory stimuli in the allergic inflammatory responses (Mukaida 2000). For this reasons, NF-κB is an obvious target of anti-inflammatory treatment (Yamamoto et al. 1999; Kim et al. 2005a,b; Kim and Shin 2006).

In this study, we evaluated the effect of AAS on the systemic and local allergic reaction and histamine release from mast cells. In addition, the effect of AAS on phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 (PMACI)-induced secretion of TNF, IL-6, and IL-8 and the role of NF-κB in this effect were investigated using human mast cells line, HMC-1.

Materials and Methods

Materials. Compound 48/80, PMA, A23187, anti-DNP IgE antibody, DNP-human serum albumin (HSA), and *o*-phthaldialdehyde were purchased from Sigma Chemical Co. (St. Louis, MO). The α-minimal essential medium was purchased from Flow Laboratories (Irvine, UK). Fetal bovine serum (FBS), Iscove's modified Dulbecco's medium

(IMDM), and streptomycin were purchased from Gibco BRL (Grand Island, NY). Avidin peroxidase, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) tablets substrate (ABTS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other reagents were obtained from Sigma Chemical Co. Antihuman TNF/IL-6/IL-8 antibody (Ab), biotinylated antihuman TNF/IL-6/IL-8 antibody (Ab), recombinant human (rh) TNF/IL-6/IL-8, and recombinant SCF were purchased from R&D Systems (Minneapolis, MN). NF- κ B, I κ B- α , β -actin, and histone were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Animals. The original stock of male ICR mice (6 wk, 25–30 g) and male SD rats (7 wk, 250–300 g) were purchased from Da-Mool Science (Daejeon, Chungnam, South Korea). The animals were maintained at the College of Oriental Medicine, Kyung Hee University. The animals were housed five to ten per cage in a laminar air-flow room maintained at a temperature of $22\pm1^{\circ}\text{C}$ and relative humidity of $55\pm1\%$ throughout the study. No animal was used more than once. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in US guidelines (NIH publication 85–23, revised in 1985).

Preparation of AAS. For all experiments, AAS extract was prepared by decocting the herb with boiling distilled water (100 g/l) for 3 h. The decoction was filtered, lyophilized, and kept at 4°C . The yield of dried extract from starting materials was about 10% (w/w). The dried extract was dissolved in phosphate-buffered saline (PBS) and filtered with 0.22- μm syringe filter.

Culture of HMC-1. Human leukemic mast cell line (HMC-1) was grown in IMDM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated FBS at 37°C 5% CO_2 and 95% humidity. HMC-1 cells (1×10^6 cells per milliliter) were treated with AAS (0.01–1.0 mg/ml) for 1 h prior to stimulation with PMACI and incubated for 6 h.

Measurement of paw oedema. To test the inhibitory effect on compound 48/80-induced paw edema in mice, prepared AAS (2 g/kg and 4 g/kg) or the vehicle (DW) was administered by oral route. The mice were intramuscularly anesthetized with ketamine HCl (30 g/kg) and rumpun (6 g/kg). Then, compound 48/80 (1 $\mu\text{g}/\text{site}$) was injected into the subplantar area of the hind paw. The size of edema was assessed by measuring the thickness of the hind paw immediately before and after the intraplantar injection at 15-min intervals. The paw thickness was measured with a digimatic micrometer. The results were expressed as the

difference in thickness (millimeter) of paw before injection of compounds 48/80.

Compound 48/80-induced systemic anaphylactic reaction.

Mice were given an intraperitoneal injection of the mast cell degranulator compound 48/80 (8 mg/kg). AAS dissolved in saline was administered orally 1 h before the injection of compound 48/80. Mortality was monitored for 23 min after induction of an anaphylactic reaction.

PCA reaction. An IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE antibody (Ab) followed by an injection of DNP-HSA into the mice tail vein 48 h later. The DNP-HSA was diluted in PBS. The mice were injected intradermally with 100 ng of anti-DNP IgE Ab into each of three dorsal skin sites that had been shaved. The sites were outlined with a water-insoluble red marker. Forty-eight hours later, each mouse received an injection of 200 μl of the 1:1 mixture of 1 mg/ml DNP-HSA in PBS and 4% Evans blue via the tail vein. AAS was orally administered to mice 1 h before the challenge. The mice were killed 40 min after the intravenous challenge. The dorsal skin of the mouse was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 0.5 ml of 1 N KOH and 4.5 ml of a mixture of acetone and phosphoric acid (with a ratio of 13:5), based on the method outlined by Katayama et al. (1978). The absorbent intensity of the extraction was measured at 620 nm in a spectrofluorometer, and the amount of dye was calculated with the Evans blue measuring line.

Preparation of rat peritoneal mast cells. Rat peritoneal mast cells (RPMCs) were isolated as previously described (Hong et al. 2001). In brief, rats were anesthetized by ether and injected with 30 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO_3 , 2.7 mM KCl, and 0.3 mM Na_2HPO_4) containing 0.1% gelatin into the peritoneal cavity, and the abdomen was gently massaged for about 2 min. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells were aspirated with Pasteur pipette. Thereafter, peritoneal cells were sedimented at $150\times g$ for 10 min at room temperature and resuspended in Tyrode buffer B. To separate mast cells from the major components of rat peritoneal cells, i.e., macrophages and small lymphocytes, suspended peritoneal cells were layered on 2 ml of 22.5% metrizamide (density, 1.12 g/ml) and centrifuged at $400\times g$ for 10 min at room temperature. The cells remaining at the buffer–metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM

CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.1% bovine serum albumin) containing calcium. Mast cells 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. RPMC suspensions (2×10^5 cells per milliliter) were incubated for 15 min at 37°C before the addition of compound 48/80 for stabilization. The cells were incubated with the AAS for 2 h and then incubated for 15 min with compound 48/80 (6 µg/ml). The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at $400 \times g$ for 5 min at 4°C. Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at $400 \times g$ for 5 min at 4°C. The histamine content was measured using the *o*-phthalaldehyde spectrofluorometric procedure of Shore et al. 1959. The fluorescent intensity was measured at 440 nm (excitation at 360 nm) using a spectrofluorometer. The inhibition percentage of the histamine release was calculated using the following equation:

$$\% \text{ Inhibition} = (A - B) \times 100 / A$$

In the equation, *A* is the histamine release without AAS, and *B* is the histamine release with AAS.

MTT assay. MTT assay, as previously described (Jeong et al. 2009a), was used to measure the cell viability. Briefly, 500 µl of HMC-1 cell suspension (3×10^5 cells) was cultured in four-well plates for 24 h after treatment with each concentration of AAS. Fifty microliters of MTT solution (5 mg/ml) was added, and the cells were incubated at 37°C for an additional 4 h. After washing out the supernatant, the insoluble formazan product was dissolved in dimethyl sulfoxide. Then, the optical density of 96-well culture plates was measured using an ELISA reader at 540 nm. The optical density of formazan formed in untreated control cells was taken as 100% viability

Enzyme-linked immunosorbent assay. A modified enzyme-linked immunosorbent assay (ELISA), as previously described (Jeong et al. 2009b), was used to measure the cytokine on tissue protein. The ELISA was performed by coating 96-well plates with 6.25 ng/well of anti-human IL-6, IL-8, and TNF antibody having specificity for IL-6, IL-8, and TNF. Before the subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 (PBST). All reagents and coated wells used in this assay were incubated for 2 h at room temperature. The standard curve was generated from known concentrations of cytokine, as provided by the

manufacturer. After exposure to the medium, the assay plates were exposed sequentially to each of the biotin-conjugated secondary antibodies (IL-6, IL-8, and TNF), avidin peroxidase, and ABTS substrate solution containing 30% H₂O₂. The plates were read at 405 nm. Appropriate specificity controls were included, and all samples were run in duplicate. Production of IL-6, IL-8, and TNF was divided according to the total protein. Protein was determined using a bicinchoninic acid (Sigma).

Preparation of nuclear extract. Nuclear extracts were prepared as described somewhere else (Schoonbroodt et al. 1997). In brief, after stimulation for the indicated times, 3×10^6 cells were washed with ice-cold PBS and centrifuged at $400 \times g$ for 5 min at 4°C resuspended in 100 ml of hypotonic buffer A (10 mM HEPES/KOH, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9). The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 ml of 10% Nonidet P-40. After centrifugation at $400 \times g$ for 10 min at 4°C, the supernatant was acquired in the form of a cytoplasmic fraction. The nuclear extract was subsequently gathered by resuspending the nuclear pellet in 40 ml of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9). After 40 min on ice with sonication and vortexing, the suspension was centrifuged for 15 min at $15,000 \times g$ on 4°C. The nuclear debris was then spin down, and the supernatant was collected as a nuclear extract and stored at -70°C until the exercise. The quantity of protein in the cytoplasmic and nuclear extract was evaluated using a BCA protein assay (Sigma).

Western blot analysis. Nuclear extract combined with an equal volume of sodium dodecyl sulfate sample loading buffer, boiled for 5 min for denaturation. Samples of protein were electrophoresed using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, as described elsewhere (Kim et al. 2004), and then transferred to nitrocellulose membrane. The membrane was blocked in 5% skim milk for 1 h, washed and incubated overnight at 4°C with primary antibodies in 3% bovine serum albumin in PBS. After washing the membranes, the membranes were incubated for 1 h with horseradish peroxidase-linked anti-rabbit immunoglobulin (secondary antibodies). Immunodetection of the protein was done using enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ) and then exposed to X-ray film.

Statistical analysis. The experiments shown are a summary of the data from at least three experiments and statistical

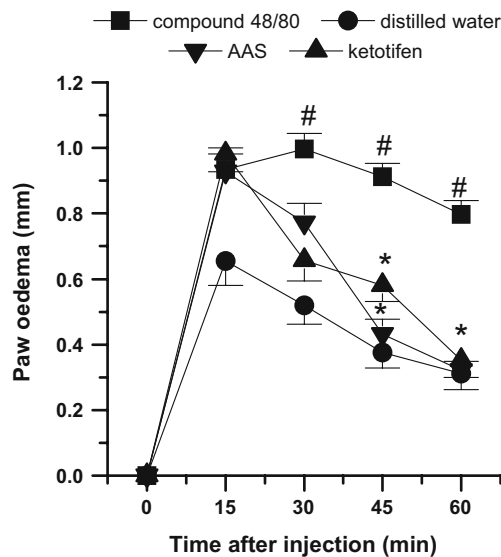


Figure 1. Time-dependence of the anti oedema effect of AAS. Time-course of edema formation induced by subplantar injection of compound 48/80. One microgram compound 48/80 was injected into the subplantar area hind paw of mice (square). An equal volume of DW was injected into the subplantar area hind paw of mice (circle), the effect of pretreatment (i.v.) for 1 h with ketotifen (1 μ g/g, triangle) was shown. AAS (2 mg/ml) was orally administered 1 h before subplantar injection of compound 48/80 (inverted triangle). # P <0.05 compared to distilled water treated value. * P <0.05 compared to compound 48/80-treated value.

analyses were performed using SPSS statistical software (SPSS 11.5, Chicago, IL). Treatment effects were analyzed by one-way ANOVA, offered by Duncan's multiple range tests, and P <0.05 was used to indicate significance.

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

AAS concentration (g/kg) ^a	Compound 48/80 (8 mg/kg) ^b	Mortality (%) ^c
NONE (saline)	+	100
0.01	+	80±5
0.1	+	40±4*
1	+	40±2*
1	–	0±0*

^a The groups of mice (n =20/group) were orally pretreated with 200 ml of saline or AAS extract was given at various doses 1 h before the compound 48/80 injection

^b The compound 48/80 solution was intraperitoneally given to the groups of mice

^c Mortality (%) is presented as the “number of dead mice $\times$ 100/total number of experimental mice.” Each datum represents the mean $\pm$ SEM of four independent experiments

* P <0.05; significantly different from the control value.

Results

Effects of AAS on compound 48/80-induced paw edema.

Mast-cell-derived paw edema induced by the subplantar injection of mast cell degranulator compound 48/80 had a rapid onset. The author measured the thickness of the hind paw after the intraplantar injection at 15-min intervals and showed the increase change of paw thickness as a parameter of edema formation in Fig. 1. After subplantar injection of compound 48/80, edema formation was significantly greater than DW-treated controls and lasted for 60 min. Mice pretreated with AAS and ketotifen (mast cell stabilizer) showed markedly less edema than compound 48/80-treated mice.

Effect of AAS on compound 48/80-induced systemic anaphylaxis.

To assess the contribution of AAS in anaphylactic reactions, the in vivo model of systemic anaphylaxis was used initially. As a non-immunologic stimulator, compound 48/80 (8 mg/kg) was used. After the injection of the compound 48/80, the mice were monitored for 23 min, after which the mortality rate was determined. As shown in Table 1, an oral administration of saline as a control induced a fatal reaction in 100% of each group. When the AAS was orally administered at concentrations of 0.01–1 g/kg 1 h before the compound 48/80 injections, the mortality was reduced (Table 1).

Effect of AAS on PCA in mice.

We examined the anti-allergic activity of AAS using PCA in the rat dorsal skin model. Anti-DNP IgE was injected into dorsal skin sites followed 48 h later with an injection of DNP-HAS containing 4% Evans blue. AAS (1 g/kg, body weight) was orally administered 1 h before injection of antigen. We found that AAS significantly suppressed the mast-cell-dependent PCA reaction at concentration of 1 g/kg (Table 2).

Effects of AAS on histamine release from RPMCs.

To investigate regulatory effects of AAS on histamine release from mast cells, we measured histamine release. The inhibitory effects of AAS on compound 48/80-induced

Table 2. Effect of AAS on 48 h passive cutaneous anaphylaxis in mice

AAS concentration (g/kg) ^a	Amount of dye (μ g/ml) ^b	Inhibition (%)
None (saline)	0.75±0.05	–
1	0.52±0.06	30.36

^a AAS dissolved with distilled water was orally administered to mice 1 h before the challenge

^b Each value indicates means $\pm$ SEM (n =4, male, 6 wk, 25–30 g)

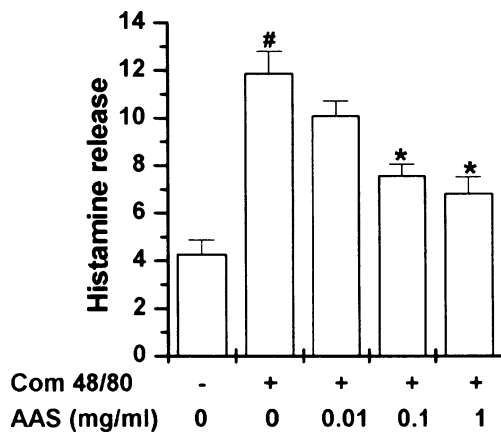
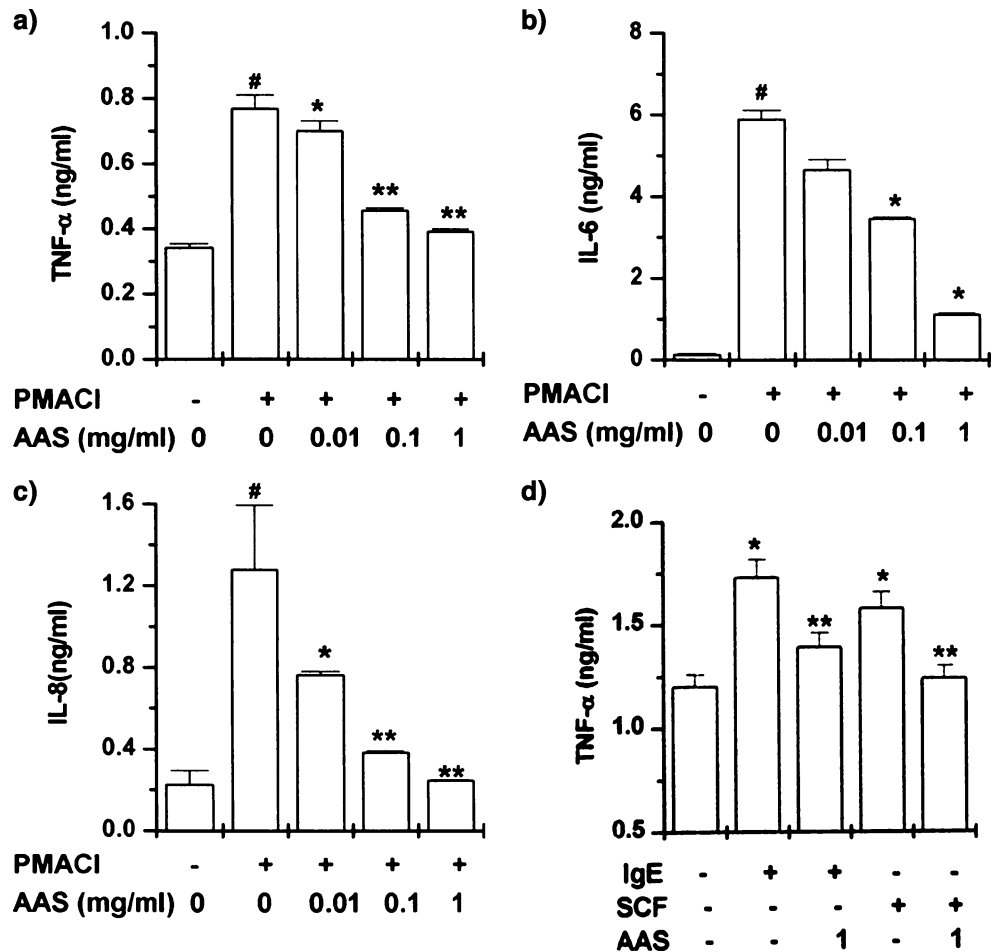


Figure 2. Effect of AAS extract on compound 48/80-induced histamine release from RPMCs. RPMCs (2×10^5 cells) were preincubated with various concentrations of AAS extract at 37°C for 10 min prior to incubation with compound 48/80. Each datum represents the mean $\pm$ SEM of three independent experiments. $^\#P < 0.05$ compared to no stimulation. $^*P < 0.05$ compared to compound 48/80 stimulation. Com 48/80 compound 48/80.

histamine release from RPMCs are shown in Fig 2. AAS extract dose-dependently inhibited compound 48/80-induced histamine release at concentrations of 0.01–1 mg/ml. At the concentration of 1 mg/ml, the inhibition rate reached up to 66.41%. In particular, AAS extract significantly inhibited the compound 48/80-induced histamine release at concentrations of 0.1 and 1 mg/ml. The histamine intensity at the doses of 0.01–1 mg/ml was 10.09 ± 0.63 , 7.56 ± 0.49 , and 6.81 ± 0.71 , respectively. Control and spontaneous values were 11.88 ± 0.92 and 4.26 ± 0.62 .

Effects of AAS on TNF, IL-6, and IL-8 production. In order to examine the inhibitory effect of AAS on PMACI-induced TNF, IL-6, and IL-8 production, the dose response of AAS (0.01–1 mg/ml) was measured in HMC-1 cells. The cells were pre-treated with AAS (0.01–1 mg/ml) for 1 h before PMACI stimulation for 8 h. Culture supernatants were assayed for TNF, IL-6, and IL-8 protein levels using the ELISA method. AAS significantly inhibited PMACI-induced TNF, IL-6, and IL-8 production in a dose-dependent manner (Fig. 3a–c). The maximal inhibition rate of TNF, IL-6, and IL-8 production by AAS (1 mg/ml) was about 87.77%, 82.67%, and 85.3%, respectively. Next, we

Figure 3. Inhibition of TNF, IL-6, and IL-8 production by AAS in activated mast cells. Cells were pretreated with AAS for 1 h and then challenged with PMA (50 nM) plus A23187 (1 μM) for 8 h. TNF, IL-6, and IL-8 concentrations were measured from cell supernatants using the ELISA method (a–c). RPMC (1×10^5) were sensitized with 10 $\mu\text{g/ml}$ anti-DNP IgE for 2 h and then stimulated with 100 ng/ml DNP-HSA for 24 h with or without AAS (1 mg/ml). RPMCs (1×10^5) were treated with AAS (1 mg/ml) for 1 h and then stimulated with SCF (50 ng/ml) for 6 h. The level of TNF in the supernatant was measured by using ELISA method (d). Values are the mean $\pm$ SEM of duplicate determinations from three separate experiments. $^\#P < 0.05$ compared to no stimulation. $^*P < 0.05$, $^{**}P < 0.01$; significantly different from the stimulated group.



investigated the ability of AAS to decrease cytokine production in the presence of IgE or SCF on RPMCs. Inhibition rate of TNF production was observed to be higher than the other inflammatory cytokines production. Therefore, we performed the ELISA method for TNF. As shown in Fig. 3d, AAS (1 mg/ml) also inhibited IgE or SCF-induced TNF production on RPMCs. Cell cytotoxicity by AAS was not observed up to 1 mg/ml (data not shown).

Effect of AAS on activation of NF- κ B. Next, we examined the effect of AAS on PMACI-induced NF- κ B activation, which is known to be important for cytokine expression. Because the suppression of NF- κ B is linked with anti-inflammation, we postulated that AAS mediates its effects at least partly through the suppression of NF- κ B activation. The pretreatment with AAS (0.1 and 1 mg/ml) inhibited the PMACI-induced increase in the nuclear *RelA*/p65 levels in HMC-1 cells (Fig. 4). As a marker of NF- κ B

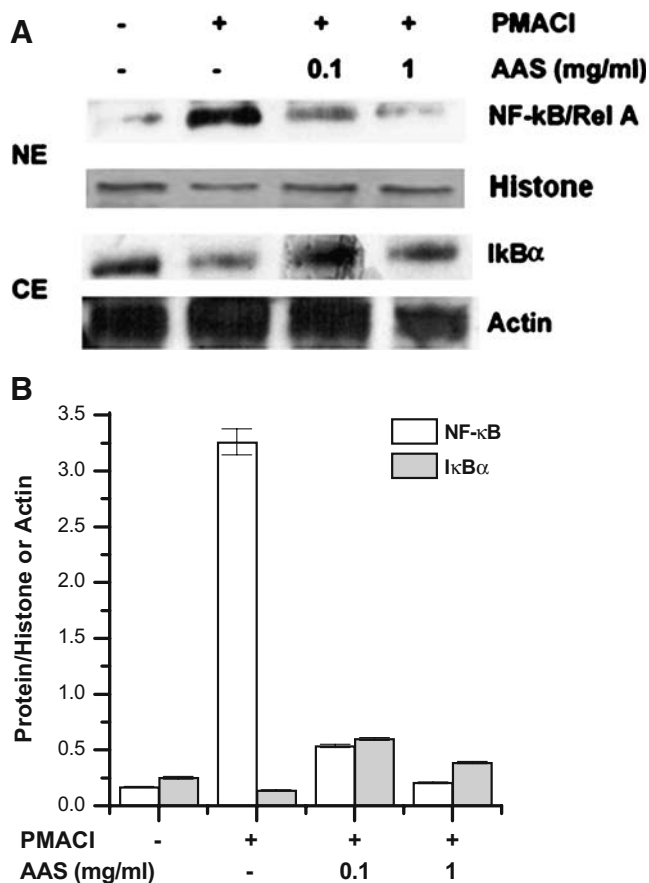


Figure 4. Effect of AAS on the PMACI-induced NF- κ B activation. HMC-1 cells were treated with AAS (0.1–1 mg/ml) for 1 h and then stimulated with PMACI for 2 h. Nuclear protein and cytoplasmic protein was prepared and analyzed for NF- κ B and I κ B α by Western blotting as described in the experimental procedures (A). Results are representative of three independent experiments, and the mean $\pm$ SEM of the band intensities corresponding to the levels of NF- κ B/Histone and I κ B α /Actin (B). NE nuclear extract, CE cytoplasmic extract.

activation, the degradation of I κ B α in cell lysates was detected. Activation and nuclear translocation of NF- κ B are dependent on the phosphorylation of I κ B α , which is then rapidly degraded. We also showed that AAS (0.1 and 1 mg/ml) significantly inhibited the PMACI-induced I κ B α degradation (Fig. 4).

Discussion

Using in vivo and in vitro model, we show that AAS inhibits mast-cell-mediated allergic responses. Mast cells known to be involved in inflammatory reactions, and mast-cell-mediated edema was described in other reports besides induced by compound 48/80. The activation of proteinase-activated receptor-2 (PAR-2), receptor for mast cell tryptase, induced an acute inflammatory response characterized by edema formation, granulocyte infiltration, and increase vascular permeability (Kawabata et al. 1998; Vergnolle et al. 1999). These reports suggest that the activation of PAR-2 induces acute inflammation via mast cell degranulation in rat hind paw (Table 1). Mast cells also can contribute to edema by neurogenic mechanism, which by capsaicin, sensory neurotoxin (Coderre et al. 1989), or adenosine receptor agonists (Linardi et al. 2000), induced an acute paw edema. We clearly demonstrated that AAS decreased compound 48/80 or IgE-induced anaphylactic reactions and PMACI-mediated expression of inflammatory cytokines such as TNF, IL-6, and IL-8. AAS significantly inhibited systemic allergic reaction and histamine release. These results indicate that immediate-type allergic reactions are inhibited by AAS. In addition, AAS-administered mice are protected from IgE-mediated PCA, one of the most important in vivo models of anaphylaxis in local allergic reaction (Jeong et al. 2007). These results indicate that mast-cell-mediated immediate-type allergic reactions are inhibited by AAS.

The intracellular calcium pathways are critical to the degranulation of mast cells. Agents that stimulate an intracellular calcium level have been shown to induce mast cell degranulation (Tasaka et al. 1986). Calcium movements in mast cells represent a major target for effective anti-allergic drugs, as this is an essential event linking stimulation to secretion (Beaven et al. 1984). The transduction pathways modulating intracellular Ca^{2+} are modified by ADP-ribosylates G-protein binding protein (Alfonso et al. 2000). From these observations, we strongly speculate that the decreased intracellular Ca^{2+} is involved in the inhibitory effect of AAS on histamine release, and AAS might have membrane-stabilizing activity through the G-protein. 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 recent studies have shown that compound 48/80 and other polybasic compounds are able, apparently directly, to activate G-proteins (Mousli et al. 1990). Compound 48/80 increases the permeability of the lipid bilayer membrane by causing a perturbation in the membrane. This result indicates 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. AAS might stabilize the lipid bilayer membrane, thus preventing the perturbation being induced by compound 48/80.

The intracellular calcium pathways are critical to the degranulation of mast cells. A reduction of calcium influx has been found in resting RBL cells in contrast to the present observation with human cells, and an increase in tyrosine phosphorylation has been reported as well (Fisher et al. 1997; Hide et al. 1997). The inhibition of intracellular Ca^{2+} elevation could lead to an inhibition of calcium-dependent protein kinase C, which is critical in mast cell activation (Beaven et al. 1984; Saito et al. 1996), and/or an inhibition of exocytosis (Douglas 1974). The effects of calcium flux are suggested to be involved in the inhibition of histamine release. Previous studies have also shown that the immunologic or nonimmunologic stimulation of mast cells increases the intracellular Ca^{2+} levels, a necessary step for mediator secretion (Saito et al. 1996; Kimata et al. 1999). Increased Ca^{2+} induces the release of inflammatory mediators, including TNF, IL-6, and IL-8 (Beaven et al. 1984). Intracellular Ca^{2+} plays an important role in the expression of TNF, IL-6, and IL-8. Mast-cell-derived proinflammatory cytokines, especially TNF, IL-6, and IL-8, have a critical biological role in the allergic reaction. The spectrum of cytokines produced by HMC-1 cells with PMACI stimulation supports the well-recognized role of mast cells' immediate hypersensitivity. TNF and IL-6 play a major role in triggering and sustaining the inflammatory allergic response in mast cells. TNF promotes inflammation, leukocyte infiltration and tissue fibrosis and is thought to be an initiator of cytokine-related inflammatory states via the stimulation of cytokine production (Hide et al. 1997). IL-6 is also produced from mast cells and its local accumulation is associated with a skin allergic reaction (Mican et al. 1992). The human chemokine IL-8 released from mast cells might act on surrounding cells such as neutrophils, T and B cells, and eosinophils and induce migration and activation on inflammatory effector cells (Mukaida 2000). These reports indicate that the reduction of proinflammatory cytokines from mast cells is one of the key indicators of reduced allergic inflammatory symptoms. This study has demonstrated that AAS inhibited the production of TNF, IL-6, and IL-8 in PMACI-stimulated

HMC-1 cells. AAS also inhibited IgE or SCF-induced TNF production on RPMCs. This suggests that the anti-allergic effect of AAS was the result of its ability to decrease the level of TNF, IL-6, and IL-8 production from mast cells.

We also experimented on NF- κ B activation to recognize the mechanism for the effect of AAS on proinflammatory cytokine expression. NF- κ B specifically regulates the production of TNF, IL-6, and many other chemokines, the levels of which were increased in patients with inflammatory diseases (Barnes et al. 1997). The activation of NF- κ B requires phosphorylation and a proteolytic degradation of the inhibitory protein (Azzolina et al. 2003; Song et al. 2006). The NF- κ B-activating pathways, induced by a dietary-related endogenous detergent, deoxycholate, provide mechanisms for the promotion of colon cancer and identify possible new targets for chemoprevention (Payne et al. 2006). In PMACI-stimulated mast cells, AAS decreased the nuclear translocation of p65 NF- κ B. These results demonstrate that AAS inhibits the PMACI-induced TNF, IL-6, and IL-8 production via an attenuation of NF- κ B activation. From these results, it is concluded that AAS down-regulates PMACI-induced NF- κ B activation in mast cells, indicating that it plays an anti-inflammatory role in mast cell-mediated inflammatory diseases.

In summary, our data indicated that AAS extracts inhibited paw edema and vascular permeability induced by compound 48/80 in vivo. The main factors of mast-cell-mediated inflammation, histamine, TNF, IL-6, and IL-8 were investigated in vitro. The treatment of AAS extracts showed a significant inhibitory effect on histamine, TNF, IL-6, and IL-8 release from mast cells. We concluded that AAS was potential for treatment of inflammatory diseases by down-modulating of mast cell activation. In addition, AAS inhibited the NF- κ B pathway. Given these findings, the regulation of the NF- κ B pathway by AAS in HMC-1 cells could form the basis of a new strategy for the treatment of mast-cell-mediated inflammatory allergic diseases.

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