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The anti-allergy and anti-inflammatory effect of *Anemarrhenae Rhizoma in vivo* and *in vitro*

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SUMMARY

Anemarrhenae Rhizoma (AR) is used in traditional oriental medicine for various medicinal purposes. However, the exact mechanism that accounts for the anti-allergy and anti-inflammatory effects of the AR is still not fully understood. The aim of the present study is to elucidate whether and how AR modulates the allergic reactions *in vivo*, and inflammatory reaction *in vitro*. In this study, we showed that AR significantly decreased compound 48/80-induced systemic anaphylaxis, paw oedema, and histamine release from preparation of rat peritoneal mast cells. Also, AR inhibited the expression of inflammatory cytokine in PMA plus A23187-stimulated human mast cells (HMC-1). In addition, we showed that anti-inflammatory mechanism of AR is through suppression of nuclear factor-κB activation and IκB-α degradation. These results provided new insight into the pharmacological actions of AR as a potential molecule for therapy of inflammatory allergic diseases.

Key words: *Anemarrhenae Rhizoma*; Allergic reactions; Inflammation; Cytokine; Nuclear factor-κB

INTRODUCTION

Mast cells are widely distributed throughout the body in both connective tissue and mucosal surfaces (Metcalfe *et al.*, 1997). The role of mast cells as effecter cells of immunoglobulin (Ig)E-dependent immediate-type hypersensitivity reactions and anaphylaxis is well understood (Hamelmann *et al.*, 1999; Galli, 2000). Activation of mast cells is elicited through the cross-linking of allergen-specific IgE bound to the high-affinity receptor for IgE, FcεR I, on the cell membrane, which results in the degranulation of mast cells and the release of mediators including histamine, proteases, leukotrienes, prostaglandin (PG)s, and different cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 (Gordon *et al.*, 1990; Kawata *et al.*, 1995; Artuc *et al.*, 1999). Mast cells are also involved in the development of late-phase reactions and influence other chronic inflammatory responses through the generation and secretion of various cytokines (Kruger-Krasagakes and Czarnetzki, 1995). Inflammatory cytokine such as tumor necrosis

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factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-8 induce inflammation and recruit other immune cells including neutrophils and T lymphocytes (Medzhitov and Janeway, 1997). Although these cytokines are beneficial to the host defense, they can also trigger pathological conditions when expreARd in excess. (Beutler, 1995).

·Nuclear factor (NF)-κB is a key transcription factor required for the expression of many inflammatory involved genes including COX-2 and inflammatory cytokines (Jung *et al.*, 2003). In an inactive state, NF-κB is normally sequestered in the cytoplasm of cells where it is bound by a family of inhibitory proteins known as IκB-α (Barnes and Karin, 1997). A variety of stimuli modulate signal transduction pathways to activate the IκB kinases. Stimulation of the activity of these kinases results in phosphorylation, ubiquitination, and degradation of IκB-α, leading to the nuclear translocation of NF-κB (Schaecher *et al.*, 2004).

Anemarrhenae Rhizoma (AR) is used in traditional oriental medicine for various medicinal purposes. However, the exact mechanism that accounts for the anti-allergy and anti-inflammatory effects of the AR is still not fully understood. The aim of the present study was to elucidate whether and how AR modulates the allergic reactions *in vivo*, and mast cell mediated-inflammatory reaction *in vitro*. Therefore, we investigate the mechanisms underlying the pharmacological effects of AR on compound 48/80-induced allergic reactions *in vivo*. Also, to find a possible explanation for the anti-inflammatory mechanisms of AR, we evaluate effects of AR on phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187-induced production cytokine, and NF-κB activation.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS), α-minimal eARntial medium and Iscove's Modified Dulbecco's Medium (IMDM) were purchased from Gibco BRL (Grand Island, NY, USA). Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), 2,2-azino-bis (3-ethylBenzthiazoline-6-sulfonic acid), PMA, metrizamide, A23187, 3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazoliumbromide (MTT) and other reagents were obtained from Sigma (St. Louis, MO, USA). Anti-human TNF-α antibody (Ab), biotinylated anti-human TNF-α Ab, and recombinant human (rh) TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). Anti-human IL-6 and IL-8 Ab, biotinylated anti-human IL-6 and IL-8 Ab, and rh IL-6 and IL-8 were purchased from PharMingen (Sandiego, CA, USA). Abs for anti-human NF-κB, IκB-α, p IκB-α, and actin were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA).

Preparation of AR

AR was obtained from the College of Pharmacy, Wonkwang University (Iksan, Republic of Korea). Extract of AR was prepared by decocting the dried description of herbs with boiling distilled water. The extraction decocted for approximately 3 h has been filtered, lyophilized, and kept a 4°C. The yield of extraction was about 7%. Dilutions were made in saline then filtered through 0.45 μm syringe filter.

Animals

The original stock of ICR mice and Wistar rats were purchased from the Dae-Han Experimental Animal Center (Eumsung, Republic of Korea), and the animals were maintained in the College of Pharmacy, Wonkwang University. The rats were housed five to ten per cage in a laminar air-flow room maintained at a temperature of 22 ± 1°C and relative humidity of 55 ± 10% 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 the US guidelines (NIH publication #85 - 23, revised in 1985).

Compound 48/80-induced systemic anaphylactic reaction

Mice were given an intraperitoneal injection of the

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

Measurement of paw oedema

To test the inhibitory effect on compound 48/80-induced paw oedema in mice, prepared AR or the vehicle (saline, 0.9% NaCl) was administered by oral route. And then compound 48/80 was injected into the subplantar area of the hind paw. The size of oedema was assessed by measuring the thickness of the hind paw immediately before and after the intraplantar injection. The paw thickness was measured with a digimatic micrometer. The results were expressed as the difference in thickness (mm) of paw before injection of compound 48/80.

Preparation of rat peritoneal mast cells (RPMCs)

RPMCs were isolated as previously described (Jippo-Kanemoto *et al.*, 1993). In brief, rats were anesthetized by ether, and injected with 20 ml of Tyrode buffer B (NaCl, glucose, $NaHCO_3$, and NaH_2PO_4) containing 0.1% gelatin into the peritoneal cavity; the abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells were aspirated by Pasteur pipette. Then the peritoneal cells were sedimented at 150×g for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells (i.e. macrophages and small lymphocytes) according to the method described by Yurt *et al* (Yurt *et al.*, 1997). In brief, peritoneal cells suspended in 1 ml of Tyrode buffer B were layered onto 2 ml of 0.225 g/ml metrizamide (density 1.120 g/ml) and centrifuged at room temperature for 15 min at 400×g. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose and 0.1% bovine serum albumine) containing calcium. Mast cell preparation was about 95% pure as aARARd by toluidine blue staining. More than 97% of the cells were viable judged by trypan blue uptake.

Histamine assay

RPMCs suspensions (2×10^5 cells/ml) were preincubated for 10 min at 37°C before the addition of compound 48/80 for stabilization. The cells were preincubated with the AR for 20 min, 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×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×g for 5 min at 4°C. The histamine content was measured by the o-phthalaldehyde spectrofluorometric procedure of Shore *et al* (Shore *et al.*, 1959). The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer. The inhibition percentage of histamine release was calculated using the following equation:

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

Where A is histamine release without AR and B is histamine release with AR.

Culture of HMC-1 cells

Human mast cell line, HMC-1, cells were grown in IMDM medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS at 37°C in 5% CO_2.

MTT assay

To test the viability of cells, MTT colorimetric assay was performed as described previously (Kim *et al.*, 2001). Briefly, HMC-1 cells (1×10^5 cells/ml) were incubated for 8 h after stimulation in the absence or presence of AR. After addition of MTT solution, the cells were incubated at 37°C for 4 h. The crystallized MTT was dissolved in dimethyl sulfoxide and

measured the absorbance at 540 nm.

Assay of cytokines

TNF-α, IL-6, and IL-8 secretion were measured by modification of an enzyme-linked immunosorbent assay (ELISA) as described previously (Na *et al.*, 2002). 96 well plates were coated with 100 ml aliquots of anti-human TNF-α, IL-6, and IL-8 monoclonal Abs at 1.0 μg/ml in PBS at pH 7.4 and were incubated overnight at 4°C. After additional washes, 100 μl of cell medium or TNF-α, IL-6, and IL-8 standards were added and incubated at 37°C for 2 h. After 2 h incubation at 37°C, the wells were washed and then 0.2 μg/ml of biotinylated anti-human TNF-α, IL-6, and IL-8 was added and again incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 30 min at 37°C. Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF-α, IL-6, and IL-8 in serial dilutions.

Preparation of cytoplasmic and nuclear extract

Nuclear and cytoplasmic extracts were prepared as described previously (Schoonbroodt *et al.*, 1997). Briefly, after cell activation for the times indicated cells were washed with ice-cold PBS and resuspended in 60 μl of buffer A (10 mM Hepes/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM PMSF, pH 7.9). The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 μl of 10% Nonide P - 40, and centrifuged at 1200×g for 10 min at 4°C. The supernatant was collected and used as the cytoplasmic extracts. The nuclei pellet was resuspended in 40 μl of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), left on ice for 20 min, and inverted. The nuclear debris was then spun down at 1200×g for 15 min. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen and stored at - 70°C until conducting the analysis.

Western blot analysis

For analysis of the levels of NF-κB, p-IκB-α, and IκB-α, stimulated cells were rinsed twice with ice-cold PBS and were then lysed in ice-cold lysis buffer (1% Triton, 1% Nonidet P- 40, 0.1% SDS, 1% deoxycholate in PBS). Cell lysates were centrifuged at 15,000 × g for 5 min at 4°C; the supernatant was then mixed with an equal volume of 2 × SDS sample buffer, boiled for 5 min, and then separated through 10% SDS-PAGE gels. After electrophoresis, the protein was transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 5% skim milk for 2 h, rinsed, and incubated overnight at 4°C with primary antibodies in PBS/ 0.5% Tween 20. Excess primary antibody was then removed by washing the membranes four times in PBS/0.5% Tween 20, and the membranes were incubated for 1 h with HRP-conjugated secondary antibodies (against mouse, or rabbit). After three washes in PBS/0.5% Tween 20, the protein bands were visualized by an enhanced chemiluminesence assay (Amersham Pharmacia Biotech, NJ, USA) following the manufacturer's instructions.

Statistical analysis

The results were expreARd as mean ± S.E.M. for a number of experiments. Statistical significance was compared between each treated group and control by analysis of variance (ANOVA), with a Tukey *post hoc* test. For all tests, *P* value less than 0.05 was considered significant.

RESULTS

Effect of AR on compound 48/80-induced systemic anaphylaxis

To assay the contribution of AR in anaphylactic reactions, we first used the *in vivo* model of systemic anaphylaxis. As a non-immunologic stimulator, compound 48/80 (8 mg/kg) was used. After the

Table 1. Effect of AR on compound 48/80-induced systemic anaphylactic reaction in mice

AR dose (g/kg)[a]	Compound 48/80 (8 mg/kg)[b]	Mortality (%)[c]
None (saline)	+	100.0
0.01	+	65.2
0.1	+	50.1
1	+	39.2

[a]The groups of mice (n = 5) were orally pretreated with 200 μl of saline or AR 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 × 100/Total number of experimental mice'.

injection of compound 48/80, the mice were monitored for 23 min, after which the mortality rate was determined. The period for observation of mortality was based on the control mice that had died in 23 min by compound 48/80. As shown in Table 1, an oral administration of saline as a control induced a fatal reaction in 100% of each group. When the AR was orally administered at concentrations of 0.01 - 1 g/kg 1 h before compound 48/80 injections, the mortality was reduced (Table 1).

Effect of AR on paw oedema in mice

We investigate the effect of AR on mast-cells-derived paw oedema induced by the subplantar injection of mast cell degranulator compound 48/80. We measured the thickness of the hind paw after the intraplantar injection, and showed the increase change of pawthickness as a parameter of oedema formation. After subplantar injection of compound-48/80 in 30 min, oedema formation was significantly than saline-treated controls. When the AR was orally administered at concentrations of 0.01 - 1 g/kg 1 h before compound 48/80 injections, the oedema was inhibited (Table 2).

Effect of AR on histamine release and degranulation from RPMCs

Histamine has been widely used as a marker of mast cell degranulation *in vitro*, and remains the best-characterized and most potent mediator

Table 2. Effects of AR on compound 48/80-induced Paw oedema in mice

Dose of AR (g/kg)	Paw oedema Thickness (mm)
None	1.87 ± 0.08
0.01	1.80 ± 0.01
0.1	1.67 ± 0.07
1	1.61 ± 0.09*

[a]20 μl of compound 48/80 (100 mg/site) were applied intradermally. The mice were orally administered with the various concentrations (0.01, 0.1 and 1.0 g/kg) of AR for 1 h prior to the compound 48/80 application. Each datum represents the means ± S.E.M. of three independent experiments. *$P < 0.05$, significantly different from the saline value.

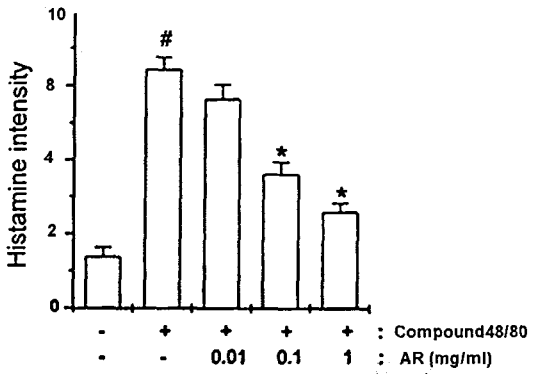

Fig. 1. Effect of AR on histamine release from RPMCs. A) RPMCs (2×10^5 cells) were preincubated with various concentrations of AR at 37°C for 10 min and then incubation with compound 48/80. The histamine content was measured by the spectrofluorometer as described under material and methods. B) The photographs of alcian blue-NFR stained mast cells. 1) blank; 2) Compound 48/80 - stimulated RPMCs; 3) AR (1 mg/ml) + Compound 48/80 - stimulated RPMCs. Magnifications were × 400. All data represent the mean ± S.E.M. of four independent experiments. *$P <$ 0.05, significantly different from the compound 48/80-stimulated cells. #$P < 0.01$, significantly different from the unstimulated cells.

implicated in the acute phase of immediate hypersensitivity (Petersen *et al.*, 1996; Moon *et al.*, 2003). The inhibitory effect of AR on compound 48/80-induced histamine release from RPMCs is shown in Fig. 1. AR dose dependently inhibited compound 48/80-induced histamine release at concentrations of 0.01-1 mg/ml. In particular, AR

significantly inhibited the compound 48/80-induced histamine release at concentrations of 0.1 - 1 mg/ml. Inhibition rate of the histamine release at the doses of 0.1 - 1 mg/ml was about 49.75 % ($P < 0.05$) and 61.18 %($P < 0.05$), respectively.

Effect of AR on cytokine production in PMA plus A23187 - stimulated HMC-1

To assay the effect of AR in PMA plusA23187-induced TNF-α, IL-6, and IL-8 production, the cells were pretreated with various concentrations of AR (0.01 - 1 mg/ml) for 30 min, and then treated with PMA plus A23187 for 8 h. Our results showed that PMA plusA23187 enhanced the secretion of TNF-α, IL-6, and IL-8, and these increase was inhibited by AR in a dose-dependent manner (Fig. 2A). The maximal inhibition rate of TNF-α, IL-6, and IL-8 production by AR (1 mg/ml) was about 59.1% ($P < 0.01$), 71.1% ($P < 0.05$) and 43.1% ($P < 0.05$), respectively. Cell cytotoxicity by AR was not observed (data not shown).

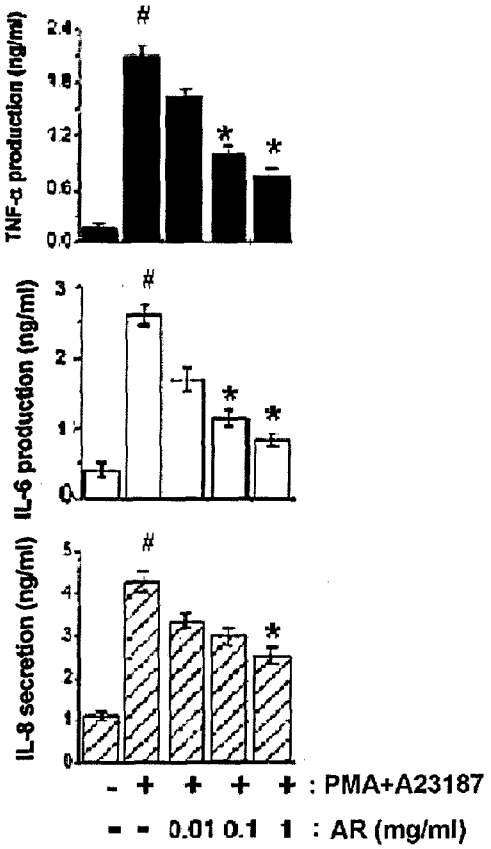

Fig. 2. Effect of AR on cytokines production and expression in PMA plus A23187-stimulated HMC-1 cells. A) 3 × 10^5 HMC-1 cells were treated with AR (0.01 - 1 mg/nl) for 30 min, and then stimulated with PMA (50 nM) plus A23187 (1 μg/ml) for 8 h. Cytokines concentration was measured in cell supernatants using the ELISA method. 1) blank; 2) PMA + A23187; 3) PMA + A23187+0.01 mg/ml AR; 4) PMA + A23187 + 0.1 mg/ml AR; 5) PMA + A23187+1 mg/ml AR. All data represent the mean ± S.E.M. of four independent experiments. *$P < 0.05$, significantly different from the compound 48/80-stimulated cells. #$P < 0.01$, significantly different from the unstimulated cells.

Effect of AR on NF-κB activation and IκB-a degradation in PMA plus A23187-stimulated HMC-1

Since NF-κB activation requires nuclear translocation of *RelA*/p65 subunit of NF-κB, we examined the effect of AR on the cytosolic and nuclear pool of *RelA*/p65 protein by Western blot analysis in HMC-1. As shown in Fig. 3A, we showed that

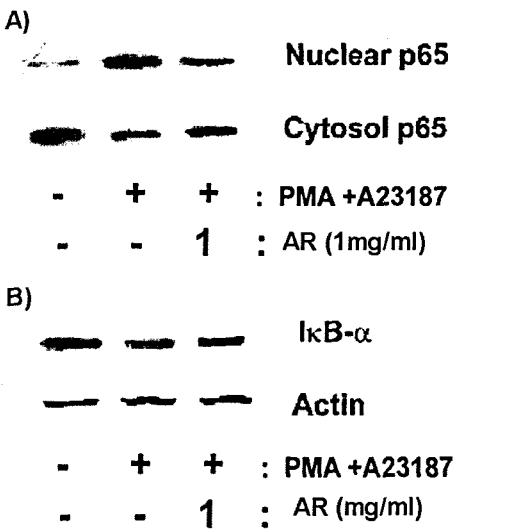

Fig. 3. Effect of AR on NF-κB activation and IκB-a degradation/phosphorylation in PMA plus A23187-stimulated HMC-1 cells.A) 6 × 10^6 HMC-1 cells were preincubated for 30 min with AR (0.01 - 1 mg/ml) and then treated with PMA (50 nM) plus A23187 (1 μg/ml) for 2 h. The cytosolic and nuclear extracts were determined for RelA/p65 by Western blot anlaysis. B) The cytosolic extracts were determined for IκB-α, phospho-IκB-α by Western blot anlaysis. 1) blank; 2) PMA + A23187; 3) PMA + A23187 + AR (1 mg/ml).

PMA plus A23187 treatment considerably increased the nuclear *RelA*/p65 protein level and decreased the cytosolic *RelA*/p65, which is an indication of the nuclear translocation of RelA/p65. Pretreatment of AR (1 mg/ml) inhibited the PMA plus A23187 stimulated increase and decrease of the nuclear and cytosolic *RelA*/p65 levels, respectively. These results suggested that AR blocks the nuclear translocation of the *RelA*/p65 from cytoplasm.

Most agents that activate NF-κB mediate their effects through suppressing IκB-α degradation (Yamamoto *et al.*, 1999). As shown in Fig. 3B, PMA plus A23187 treatment effectively caused an induction of IκB-α degradation in HMC-1. We showed that AR (1 mg/ml) significantly inhibited PMA plus A23187-induced IκB-α degradation.

DISCUSSION

AR, in traditional herb, has been used for the treatment of various diseases. However, the mechanism involved in anti-allergy and anti-inflammatory effect of AR has not been examined. In this study, our findings show that AR inhibited the compound 48/80-induced allergic reactions *in vivo* and inhibited the TNF-α, IL-6, and IL-8 production in PMA plus A23187-simulated HMC-1 cells. In addition, AR inhibited NF-κB activation through suppression of IκB-α degradation.

Mast cells are widely distributed throughout the body in both connective tissue and at mucosal surfaces (Metcalfe *et al.*, 1997). The role of mast cells as effecter cells of IgE-dependent immediate-type hypersensitivity reactions and anaphylaxis is well understood (Galli, 2000; Hamelmann *et al.*, 1993). It was reported that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane indicates, which may be an eARntial trigger for the release of mediators from mast cells (Tasak *et al.*, 1986). In present study, we showed that AR inhibited the systemic anaphylaxis and ear swelling caused by compound 48/80 in mice. Also, AR inhibited histamine release and degranulation from RPMCs. So, it is possible to hypothesize that AR might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80.

Inflammatory cytokines (TNF-α and IL-6) play roles in mediating the progression of many inflammatory diseases. TNF-α from mast cells can orchestrate the migration of neutrophils and T-lymphocytes into tissues, and promote chronicity in inflammatory lesions (Walsh *et al.*, 1995). IL-8 from mast cells act on surrounding cells such as neutrophils, T-lymphocytes, and eosinophils, and activation of inflammatory effector cells (Mukaida, 2000). In this study, we observed that AR inhibited TNF-α, IL-6, and IL-8 production in HMC-1. These results indicated that AR has potential effect on anti-inflammatory response through the regulation of inflammatory cytokine in mast cells, which might explain its beneficial effect in the treatment of mast cell-mediated inflammatory diseases.

NF-κB is required for transcriptions pro-inflammatory molecules, including COX-2 and cytokines such as TNF-α, IL-6 and IL-8. Since these pro-inflammatory molecules are regulated at the transcription level, NF-κB is a critical intracellular mediator of the inflammatory cascade (Ghosh *et al.*, 1998). Therefore, we postulated that AR mediates its effects at least partly through suppression of NF-κB activation. In an inactive state, NF-κB is present in the cytoplasm as a heterotrimer consisting of p50/p65, and IκB-α subunits. In response to an activation signal, the IκB-α subunit is phosphorylated, ubiquitinated, and degraded through the proteosomal pathway, thus exposing the nuclear localization signals on the p50-p65 heterodimer. The p65 is then phosphorylated, leading to nuclear translocation and binding to a specific sequence in DNA, which in turn results in gene transcription. Inhibitors of NF-κB activation mediate their effects through suppressing IκB-α phosphorylation and degradation (Yamamoto *et al.*, 1999). For example, when cells were stimulated with LPS in the presence of sodium calculate or aspirin, the LPS-induced proteolysis of IκB-α was

abolished, which suggests that the observed NF-κB inhibition was mediated through inhibition of the phosphorylation and/or degradation of IκB-α. Previously, we reported that aucubin inhibited the NF-κB activation via phosphorylation and degradation of IκB-α (Jeong *et al.*, 2002). In this study, we showed that AR suppressed NF-κB translocation to the nucleus induced by PMA plus A23187. Also this suppression was mediated through inhibition of IκB-α degradation. Therefore, our results suggest that the anti-inflammatory effect of AR is similar to the mechanism of aucubin. So, it is possible to hypothesize that AR might act as a potent NF-κB inhibitor on the mast cell activation induced by PMA plus A23187.

In conclusion, we have shown that AR can regulate allergy response *in vivo*, and affect the production of inflammatory cytokines through regulation of the NF-κB/IκB-α pathway. These results provided new insight into the pharmacological actions of AR as a potential molecule for therapy of inflammatory allergic diseases.

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