

Inhibitory effect of *Angelicae dahuricae* radix on mast cell mediated inflammatory responses

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SUMMARY

The *Angelicae dahuricae* radix (ADR) has been used a traditional medicine to treat acne, erythema, headache, toothache, sinusitis, colds, and flu in Korea, Japan and China. Here, we report the effect of ADR on compound 48/80-induced ear-swelling and the phorbol myristate acetate (PMA) plus calcium ionophore A23187-induced inflammatory cytokine secretion in the human mast cell line, HMC-1. ADR dose-dependently inhibited the ear-swelling response induced by intradermal injection of compound 48/80. In *vitro* model, PMA plus A23187 significantly increased interleukin (IL)-1 β , IL-8, granulocyte macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor (TNF)- α secretion compared with media control. We also show that the increased cytokines IL-1 β , IL-8, GM-CSF, and TNF- α level was significantly inhibited by treatment of ADR. In addition, ADR partially blocked PMA plus A23187-induced extracellular signal-regulated kinases phosphorylation. These results suggest that ADR might explain its beneficial effect in the treatment of mast cell-mediated inflammatory diseases.

Key words: *Angelicae dahuricae* radix (ADR); IL-1 β ; IL-8; GM-CSF; TNF- α

INTRODUCTION

The dried roots of *Angelicae dahurica* radix (Umbelliferae) has been listed in a large number of Korean, Chinese, and Japanese herbal prescriptions¹ and is claimed to be effective in the treatment of acne, eruption, and erythema. Biological activities of the components from this plant, such as hepatoprotective activity against tacrine-induced cytotoxicity in Hep G2 cells (Oh *et al.*,

2002), inhibition of compound 48/80-induced histamine release in the mouse peritoneal cavity (Kimura and Okuda, 1997), antimicrobial activity (Kwon *et al.*, 1997), affinity to brain benzodiazepine receptors *in vitro* (Bergendorff *et al.*, 1997), and anti-sepsis (Song *et al.*, 2005), have been reported. In the present study, we investigated pharmacological mechanism of *Angelicae dahurica* radix (ADR) on compound 48/80-induced ear-swelling response and on phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187-stimulated human mast cells, HMC-1.

We used compound 48/80 to activate mast cells, which are known as a potent inducer of degranulation and of the release of histamine and

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other chemical mediators that are responsible for anaphylactic symptoms (Bronner *et al.*, 1987). Studies on the compound 48/80-induced ear-swelling response have been continuously performed on a theoretical basis by Kim *et al.* (Kim *et al.*, 1999; Na *et al.*, 2002).

The HMC-1 should be a useful tool for studying cytokine activation in human mast cells (Sillaber *et al.*, 1993; Nilsson *et al.*, 1995). Mast cells can produce diverse cytokines such as tumor necrosis factor- α (TNF)- α , interleukin (IL)-1, IL-4, IL-6, IL-8, IL-13, granulocyte macrophage colony stimulating factor (GM-CSF), and transforming growth factor (TGF)-1 (Wodnar-Filipowicz *et al.*, 1989; Artuc *et al.*, 1999; Royer *et al.*, 2001; Stassen *et al.*, 2001). These mediators may be pivotal to the genesis of an inflammatory response.

In the present study, we evaluated the effect of ADR on compound 48/80-induced ear-swelling. We also investigated ADR on phorbol 12-myristate 13-acetate (PMA) plus A23187-induced cytokine releases from human mast cells (HMC-1).

MATERIALS AND METHODS

Preparation of ADR

ADR was prepared by decocting the dried prescription of *Angelicae dauricae* radix with boiling distilled water. The duration of decoction was about 2 h. The decoction was filtered, lyophilized and kept at 4°C. The ADR water extract powder was dissolved in sterile saline (100 mg/ml).

Reagents

Fetal bovine serum (FBS), and Iscove's Modified Dulbecco's Medium (IMDM) were purchased from Gibco BRL (Grand Island, NY, USA). Compound 48/80, PMA, A23187, avidin-peroxidase, 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), 3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazolium bromide (MTT), and other reagents were obtained from Sigma (St. Louis, MO, USA). Anti-human IL-1 β /IL-8/GM-CSF/TNF- α antibody (Ab), biotinylated

anti-human IL-1 β /IL-8/GM-CSF/TNF- α Ab, and recombinant human (rh) IL-1 β /IL-8/GM-CSF/TNF- α were purchased from R&D Systems (Minneapolis, MN, USA). Phospho-specific extracellular signal-regulated kinases (p-ERK1/2) and ERK1/2 Ab were purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA, USA).

Animals

The original stock of ICR mice were purchased from the Dae-Han Experimental Animal Center (Eumsung, South Korea), and the animals were maintained in the College of Pharmacy, Wonkwang University. The mice were housed five per cage in a laminar air-flow room maintained at a temperature of 22 \pm 1°C and relative humidity of 55 \pm 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).

Ear-swelling response

Compound 48/80 was freshly dissolved in saline and injected intradermally into the dorsal aspect of a mouse ear using a microsyringe with a 28-gauge hypodermic needle. Ear thickness was measured with a digimatic micrometer (Mitutoyo, Japan) under mild anesthesia. Ear-swelling response represented an increment in thickness above baseline control values. Ear-swelling response was determined 40 min after compound 48/80 or vehicle injection. ADR was administered orally 1 h before the compound 48/80 infection (100 μ g/site). The values obtained would appear to represent the effect of compound 48/80 rather than the effect of the vehicle injection (physical swelling), since the ear-swelling response evoked by physiologic saline returned to almost baseline thickness within 40 min.

Culture of HMC-1 cells

Human mast cell line (HMC-1) were grown in

IMDM medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol and 10% heat-inactivated FBS at 37°C in 5% CO₂. The cells were sensitized with PMA (50 nM) plus A23187 (1 µM) for 8 - 24 h in the absence or presence of ADR.

MTT assay

To test the viability of cells, MTT colorimetric assay was performed as described previously (Kim *et al.*, 2001). Briefly, HMC-1 cells (1×10^6 cells/ml) were incubated for 8 h after stimulation in the absence or presence of ADR (0.01 - 1 mg/ml). 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 cytokine secretion

Secreted IL-1β, IL-8, GM-CSF, and TNF-α level in supernatants from HMC-1 cells was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer's protocol (R&D Systems). Absorption of the avidin-horseradish peroxidase color reaction was measured at 405 nm and compared with serial dilutions of human IL-1β, IL-8, GM-CSF, and TNF-α recombinant as a standard.

Western blot analysis

Cell extracts were prepared by detergent lysis procedure. Cells (2×10^6 cells) were harvested, washed once with PBS, and resuspended in lysis buffer. Samples were vortexed for lysis for a few seconds every 15 min at 4°C for 1 h and centrifuged at 12000 rpm for 5 min 4°C. Samples were heated at 95°C for 5 min, and cooled on ice followed by centrifugation at 12000 rpm for 5 min. 20 µg total proteins were loaded and separated on 12 % SDS-polyacrylamide gels. After electrotransferring onto nitrocellulose membrane (Amersham Pharmacia Biotech UK limited, England) at 4°C, the membrane was blocked with 5% nonfat dry milk in PBST for

1 h. After slightly washing with PBST, membrane was probed with primary Ab for 2 h and washed three times with PBST. Horseradish peroxidase-conjugated secondary Ab was incubated and chemiluminescence detection was performed using ECL detection reagent (Amersham Pharmacia Biotech UK limited, England). Proteins were visualized by fluorography using Agfa X-ray film blue.

Statistical analysis

The results were expressed as mean ± SEM for a number of experiments. Statistical significance was compared between each treated group and control by analysis of variance (ANOVA). Results with $P < 0.05$ were considered statistically significant.

RESULTS

Effect of ADR on ear-swelling response

The fact that intradermal application of compound 48/80 at the dose of 100 µg /site can induce an ear-swelling response in normal mice has been confirmed in previous studies (Na *et al.*, 2002). We choose a concentration of 100 µg/site for compound 48/80-induced optimal ear-swelling response in this experiment. As shown in table 1 when mice were pretreated with ADR for 1 h, the ear-swelling responses derived from compound 48/80 were reduced in dose-dependent manner significantly ($P < 0.05$).

ADR on HMC-1 cells viability

To investigate anti-inflammatory effect of ADR, we performed *in vitro* model. First of all, to test cytotoxic effect of ADR, we performed MTT assay in HMC-1 cells. Fig. 1 shows the viability of cells 8 h incubation after stimulation in the absence or presence ADR (0.01 - 1 mg/ml). Fig. 1 shows ADR does not significantly affect cell viability and has no toxicity on HMC-1 cells (Fig. 1).

Table 1. Effect of ADR on compound 48/80-induced ear-swelling response in mice

ADR (g/kg)	Compound 48/80 (100 µg/site)	Thickness of ear (mm)	Inhibition (%)
None (saline)	+	0.254 ± 0.016	-
0.01	+	0.218 ± 0.005	14.17*
0.1	+	0.165 ± 0.033	35.04*
1	+	0.144 ± 0.020	43.31*

Twenty µl of compound 48/80 (100 µg/site) were applied topically to the ears of mice. The mice $C_{n=b}$ /group were orally administered with the various concentrations (0.01 - 1.0 g/kg) of ADR for 1 h prior to the compound 48/80 application. Each datum represents the means ± SEM of three independent experiments. * $P < 0.05$, Significantly different from the saline value.

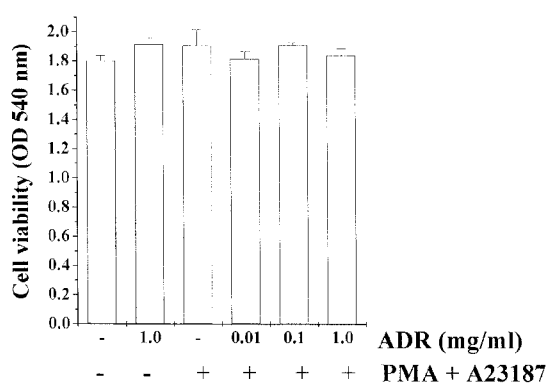


Fig. 1. Effect of ADR on the cell viability in HMC-1 cells. The cell viability was evaluated by MTT assay 8 h after ADR treatment (0.01 - 1 mg/ml) in HMC-1 cells. Data represent the mean ± SEM of three independent experiments.

Effect of ADR on cytokine secretion in HMC-1 cells

To determine whether ADR can modulate PMA plus A23187-induced IL-1 β , IL-8, GM-CSF, and TNF- α secretion, the cells were pretreated with various concentrations of ADR for 30 min prior to PMA plus A23187 stimulation. Culture supernatants were assayed for IL-1 β , IL-8, GM-CSF, and TNF- α secretion levels by ELISA method. PMA plus A23187 significantly enhanced IL-1 β , IL-8, GM-CSF, and TNF- α secretion compared with media control. ADR dramatically decreased IL-1 β and GM-CSF (Fig. 2A and C) by control level. Moreover ADR inhibited IL-8 secretion from PMA plus A23187 stimulated HMC-1 cells (Fig. 2B). ADR slightly inhibited the secretion of TNF- α in

PMA plus A23187-stimulated HMC-1 cells (Fig. 2D). However, the difference between ADR treated group and PMA plus A23187-treated group was not significant.

Effect of ADR on ERK1/2 activation in HMC-1 cells

The induction of most cytokine genes requires activation of the ERK1/2 (Shapiro and Dinarello, 1995). We determined whether ADR regulated ERK1/2 activation. PMA plus A23187 rapidly induced phosphorylation (p) ERK1/2, with no change in total ERK1/2 levels. P-ERK1/2 levels reached a peak at approximately 10 - 20 min after PMA plus A23187 treatment (data not shown). Treatment with 0.1 and 1 mg/ml ADR blocked PMA plus A23187-induced ERK1/2 phosphorylation (Fig. 3).

DISCUSSION

The present study showed that ADR pretreatment profoundly affected compound 48/80-induced ear-swelling response. In addition, the secretion of IL-1 β , IL-8, GM-CSF, and TNF- α in PMA plus A23187-stimulated mast cells was inhibited. Also ADR inhibited PMA plus A23187-induced ERK1/2 activation.

The report that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane indicates that the membrane permeability increase may be

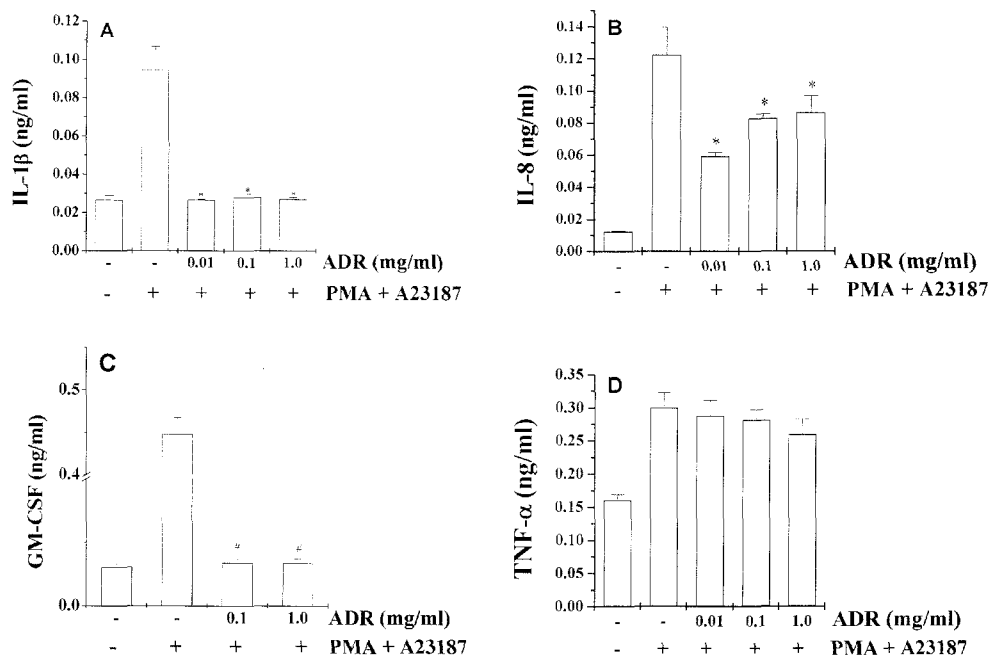


Fig. 2. Inhibition of cytokine secretion by ADR in PMA plus A23187-stimulated HMC-1 cells. The cells were pre-treated with ADR for 30 min and then challenged with PMA plus A23187 for 8-24 h. IL-1 β (A), IL-8 (B), GM-CSF (C), and TNF- α (D) concentrations were measured from cell supernatant using ELISA method. Values are mean \pm SEM of duplicate determinations from three separate experiments. * P < 0.05, # P < 0.001; significantly different from the stimulated group.

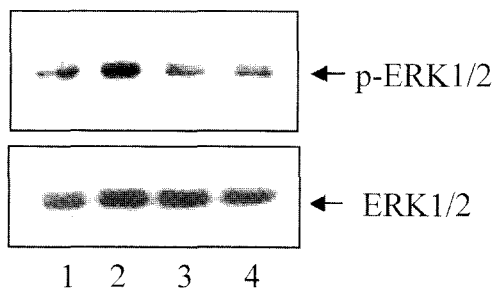


Fig. 3. Effect of ADR on p-ERK1/2 activation. The p-ERK1/2 activation in the absence or presence of ADR (0.1 and 1 mg/ml) for 30 min prior to stimulation was determined by western-blotting. The p-ERK1/2 was investigated after a 10 min incubation following PMA plus A23187 stimulation; lane 1, media control; lane 2, PMA plus A23187 treatment; lane 3, 0.1 mg/ml ADR + PMA plus A23187 treatment; lane 4, 1 mg/ml ADR + PMA plus A23187 treated, respectively. Total levels of ERK1/2 are also shown under the p-ERK panel.

an essential trigger for the release of mediators from mast cells (Tasaka *et al.*, 1986). In present study, ADR inhibited the ear-swelling caused by

compound 48/80 in mice.

HMC-1 cells activated by PMA plus A23187 are useful *in vitro* model system for studying of multi-functional effects of the immune and inflammatory reactions (Hosoda *et al.*, 2002; Shin *et al.*, 2003). We showed that ADR strongly inhibited inflammatory cytokines such as IL-1 β , IL-8, GM-CSF, and TNF- α from PMA plus A23187-stimulated HMC-1 cells.

The pro-inflammatory cytokine IL-1 β is produced and secreted under pathological conditions that are associated with increased pain and hyperalgesia, e.g. during neuropathies, tumor growth or in chronic inflammatory diseases like rheumatoid arthritis (Eastgate *et al.*, 1988; Watkins *et al.*, 1999). IL-8 plays a major role in triggering and sustaining the allergic inflammatory response. IL-8 is the most extensively studied member of the entire chemokine superfamily, with its major actions being as a neutrophil chemoattractant and activator. Free IL-8 has been detected in the sera,

and bronchial tissue of subjects with severe atopic asthma but not in samples from normal subjects (Shute *et al.*, 1997). GM-CSF plays a pivotal role in inflammatory and immunologic processes (Gajewska *et al.*, 2003). Mouse models of asthma and diesel-induced hyper-responsiveness have demonstrated an association between epithelial cell-derived GM-CSF and airway hyper-responsiveness (Lei *et al.*, 1998; Yamashita *et al.*, 2002). TNF- α is an essential cytokine in many pathological conditions such as allergic diseases, rheumatoid arthritis and pulmonary fibrosis (Camussi *et al.*, 1991). TNF- α induced secretion and release of eosinophil chemotactic factors such as eotaxin (Hoeck and Woisetschlager, 2001; Sato *et al.*, 2001) and RANTES (Hashimoto *et al.*, 2001) from fibroblasts and epithelial cells.

The mitogen-activated protein kinase (MAPK) cascade is a major signaling pathway in many cells (Schaeffer and Weber 1999). In mammalian cells, three important groups of kinase pathways compose the MAPK family including the ERK1/2, the p38 MAPK, and the c-Jun NH₂-terminal kinase (JNK1/2). The ERK-cascade appears to mediated signals promoting cell proliferation, differentiation, or survival, whereas the p38 MAPK and JNK cascades appear to be mainly involved in cellular stress responses. And the induction of most cytokine genes requires activation of the ERK1/2 and p38 MAPK. Because the induction of most cytokine genes requires activation of the ERK1/2 (Shapiro and Dinarello 1995), we finally tested the effect of ADR for PMA plus A23187-induced ERK1/2 activation. The present results demonstrate that ADR inhibits PMA plus A23187-induced ERK1/2 phosphorylation in HMC-1 cells.

In conclusion, ADR significantly inhibited compound 48/80-induced ear-swelling response. And ADR had no cytotoxic effect and inhibited the secretion of IL-1 β , IL-8, GM-CSF, and TNF- α in human mast cells. In addition, ADR partially blocked PMA plus A23187-induced ERK1/2 phosphorylation. These results suggested ADR

could inhibit the cytokine secretion through blocking ERK1/2 activity. Further studies for precise mechanism of ADR are needed.

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