Regulatory Effect of Inflammatory Reaction by *Anglicae Dahuricae Radix*

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Anglicae dahuricae Radix (ADR), the dried roots of Angelica Dahurica Benth et Hook (Umbelliferae), is a traditional herbal medicine used to treat colds, headache, skin diseases such as acne and possess analgesic, antipyretic and drainage effects. In the present study, the author evaluated the effect of ADR on regulation of inflammatory reaction. ADR reduced the ear-swelling responses derived from compound 48/80 in dose-dependent manner significantly. ADR inhibited the PMA plus A23187-induced productions of IL (Interleukin)-8, IL-1β, granulocyte macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-α from human mast cells (HMC)-1. In addition, ADR blocked PMA plus A23187-induced ERK1/2 phosphorylation. I suggest that ADR regulates inflammatory reaction through inhibition of inflammatory cytokines such as IL-8, IL-1β and GM-CSF.

Key words : *Anglicae Dahuricae Radix*(ADR), Regulation of inflammatory reaction, Human Mast Cell(HMC), inflammatory cytokines

Introduction

Angelica Dahurica (Umbelliferae) is a perennial herb growing to 2.5 m with a hollow stem, large three-branched leaves and umbels bearing many white flower heads. It grows wild in thickets in Korea, Japan, China and Russia and the cultivated herb is mainly from central and eastern regions of China. The *Angelicae Dahuricae Radix* (ADR) has been used a traditional medicine in Korea, Japan and China, where they are classified as a sweat-inducing drug able to counter harmful external influences on the skin, such as cold, heat, dampness and dryness[1]. ADR is also claimed to be effective in the treatment of acne, erythema, headache, toothache, sinusitis, colds and flu[2]. However, it is still unclear how it works on organism.

Mast cells originate from progenitor cells in the bone marrow, circulate as undifferentiated mononuclear cells in the peripheral circulation, and subsequently mature under local influences following migration into tissue. Mast cells are widely distributed throughout the body in both connective tissue and at mucosal surfaces[3]. The role of mast cells as effector cells of immunoglobulin E (IgE)-dependent immediate-type hypersensitivity reactions and anaphylaxis is well understood[4,5]. This activation 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 that further aggravate the ongoing allergic process, and the mediators include histamine, proteases, leukotrienes, prostaglandins such as prostaglandin (PG)D2, Leukotriene (LT) B4, platelet-activating factor, and the cysteinyl leukotrienes LTC4, LTD4, and LTE[6], and different cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-4, IL-6, IL-8, and IL-13, granulocyte macrophage-colony stimulating factor (GM-CSF), and transforming growth factor (TGF)-1[7-12]. The diverse profile of chemical mediators generated and released by activated mast cells leads to plasma extravasation, tissue edema, bronchoconstriction, leukocyte recruitment, and mucosal inflammation, which result from allergen exposure in sensitized hosts. IgE-dependent mast cell activation is a prominent mechanism in a substantial number of diseases treated by allegists, including anaphylaxis, urticaria, angioedema, and acute exacerbations of asthma and rhinoconjunctivitis[6].

The author used compound 48/80 to activate mast cells, which is known as a potent inducer of degranulation and of the release of histamine and other chemical mediators that are responsible for anaphylactic symptoms[13]. Compound 48/80 is one of the most potent secretagogues of mast cells[14]. The compound is a mixture of polymers synthesized by condensing

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N-methyl-p-methoxyphenyl ethylamine with formaldehyde[15], and its hypotensive effect, resulting from histamine release, was shown by Paton[16]. Studies on The compound 48/80-induced ear-swelling response have been continuously performed on a theoretical basis by Kim et al[17,18].

Cytokines, generated by both resident and freshly recruited cells, are responsible for the initiation and coordination of many local processes. Mast cells are also involved in the development of late-phase reactions and influence other chronic inflammatory responses through the generation and production of various cytokines[19]. The human mast cells (HMC)-1 should be a useful tool for studying cytokine activation in human mast cells[20,21]. Although HMC-1 had two point mutation in c-kit resulting in constitutive phosphorylation and activation[22], these cells exhibit a number of phenotypic and functional properties typical for HMC[23]. Because of low expression of FcεR I, HMC-1 cannot be activated by antigen, but the cells can still be activated by treatment with phorbol esters and calcium ionophore.

IL-1β is one of the key mediators of the body's response to microbial invasion, inflammation, immunological reactions, and tissue injury. IL-1β affects a large range of cells and organs. Induction of secondary cytokines, including IL-6, CSF, and chemokines, is involved in many of the in vitro and in vivo actions of IL-1β[24].

IL-8, the first human chemokine to be characterized, is a potent pro-inflammatory cytokine and plays an important role in inflammation[25]. L-8 is CXC-type chemokine that binds to the cellular seven-transmembrane domain G protein-coupled receptors known as CXCR1 and CXCR2. This chemokine acts on inflammatory effector cells such as neutrophils, T-lymphocytes, B-Lymphocytes and eosinophils, inducing migration or release responses[26-29].

GM-CSF, as the name implies, was initially defined by its ability to generate both granulocyte and macrophage colonies from precursor cells as a result of proliferation and differentiation[30]. Upon appropriate stimulation and as for other proinflammatory cytokines, much of the production and action of GM-CSF occurs locally at the site of inflammation. Increased levels of GM-CSF mRNA were observed in skin biopsies from allergic patients with late-phase cutaneous reactions[31]. In inflammatory and noninflammatory arthritis the affected joint fluid contained detectable GM-CSF[32], and it has been detected as part of the erosive inflammatory reaction around orthopaedic implants.

TNF-α has been shown to induce the chemotaxis of neutrophils and T cells, and stimulate the expression of adhesion molecules[33], TNF-α has been found preformed and stored in granules of mast cells or newly synthesized following mast cell activation, and it is a multifunctional cytokine and an important mediator of immune and inflammatory response. Therefore, mast cells are key as effector cells in the early phase allergic inflammation and in diverse immunological and pathological processes[34,35].

The mitogen-activated protein kinase (MAPK) cascade is a major signaling pathway in many cells[36]. In mammalian cells, three important groups of kinase pathways compose the MAPK family including the extracelluar signal-regulated kinases (ERK1/2), the p38 MAPK, and the c-Jun NH_2-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[37].

In the present study, the author evaluated the effect of ADR on compound 48/80-induced ear-swelling. The author also investigated ADR on phorbol 12-myristate 13-acetate (PMA) plus A23187-induced cytokine production from HMC-1 and activation of ERK1/2.

Materials and Methods

1. Preparation of ADR

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

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

3. 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 rats were housed five to ten per cage in a laminar air-flow room maintained at a temperature of 22±1℃ 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).

4. 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. FKME was administered orally 1 h before the compound 48/80 infection (100 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.

5. Culture of HMC-1 cells

Human mast cell line, HMC-1 cells, were grown in IMDM medium supplemented with 100 U/ml penicillin, 100 g/ml streptomycin, 10 M monothioglycerol and 10 heat-inactivated FBS at 37℃ in 5% CO_2.

6. MTT Assay

To test the viability of cells, MTT colorimetric assay was performed as described previously[38]. 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, 0.1, 1 mg/ml). After addition of MTT solution, the cells were incubated at 37℃ for 4 h. The crystallized MTT was dissolved in dimethyl sulfoxide and measured the absorbance at 540 nm.

7. Enzyme-linked immunosorbent assay (ELISA) Method

IL-1β, IL-8, GM-CSF, and TNF-α production was measured by modification of an ELISA as described previously38). HMC-1 cells were cultured with IMDM plus 10% FBS. The cells were sensitized with PMA (50 nM) plus A23187 (1 μM) for 8-24 h in the absence or presence of ADR. The ELISA was performed by coating 96-well plates (Nunc, Denmark) with 1 μg/well of murine monoclonal Ab with specificity for IL-1β, IL-8, GM-CSF, and TNF-α. Before use and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 (PBST). All reagents used in this assay and the coated wells were incubated for 1 h at room temperature. For the standard curve, recombinant human IL-1β, IL-8, GM-CSF, and TNF-α antibody was added to the wells. After exposure to the medium, the assay plates were exposed sequentially to biotinylated anti-human IL-1β, IL-8, GM-CSF, and TNF-α. After 1 hour, the assay plates were exposed to enzyme, avidin-peroxidase, for 30 min. And then substrate, 2,2 ′ -azino-bis (3-ethyl benzthiazoline -6-sulfonic acid) tablet, was added to the wells. Optical density were read within 10 min of the addition of the substrate on a ELISA reader (VersaMax, Molecular Devices) with a 405 nm filter.

8. 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 cytosolic proteins were loaded and separated on 12% SDS-polyacrylamide gels. After electrotransferring onto nitrocellulose membrane (Amersham Parmacia Biotech UK limited, England) at 4℃, 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 1 h and washed three times with PBST. Horseradish peroxidase-conjugated secondary Ab was incubated and chemiluminescence detection was performed using ECL detection reagent (Amersham Parmacia Biotech UK limited, England). Proteins were visualized by fluorography using Agfa X-ray film blue.

9. 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), with post hoc test of the means according to Tukey's method. For all tests, P value less than 0.05 was considered significant.

Results

1. Effect of ADR on ear swelling response

The fact that intradermal application of compound 48/80

at the dose of 100 ㎍/site can induce an ear-swelling response in normal mice has been confirmed in previous studies[18]. The author chose a concentration of 100 ㎍/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 ($P < 0.05$).

Table 1. Effect of ADR on Compound 48/80-induced Ear Swelling Response in Mice

ADR (g/kg)	Compound 48/80 (100 ㎍/site)	Thickness of ear (㎜)	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	+	0.144 ± 0.020	43.31*

Twenty ㎕ of compound 48/80 (100 ㎍/site) were applied intradermally. The mice were orally administered with the various concentrations(0.01, 0.1 and 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.

2. ADR on HMC-1 cells viability

To test cytotoxic effect of ADR, the author 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.

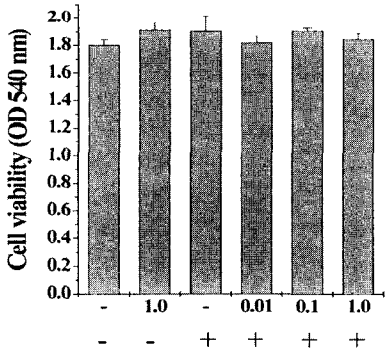

Fig. 1. Effect of ADR on the cell viability in HMC-1 cells The cell viability was evaluated by MTT assay 8 h after ADR treatment (0.01-1 mg/ml) in HMC-1 cells. Data represent the mean ± SEM of three independent experiments.

3. Effect of ADR on IL-1β production in HMC-1 cells

The author examined the inhibitory effect of ADR on the PMA plus A23187-induced production of IL-1β from HMC-1 cells. Culture supernatant was assayed for IL-1β levels by ELISA method. PMA plus A23187 significantly enhanced IL-1β (0.094±0.377 ng/ml) production compared with media control (0.0265±0.053 ng/ml). The IL-1β concentrations of 0.01, 0.1, and 1 mg/ml ADR were 0.027±0.053, 0.028±0.056, and 0.027±0.054 ng/ml, respectively. ADR inhibited the IL-1β production at the level of media control (*P < 0.05).

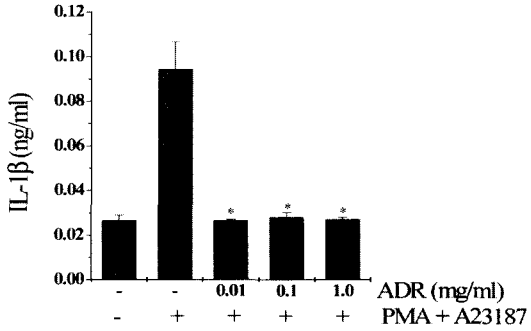

Fig. 2. Inhibition of IL-1β production by ADR in PMA plus A23187-stimulated HMC-1 cells The cells were pre-treated with ADR for 30 min and then challenged with PMA plus A23187 for 8 h. IL-1β concentrations were measured from cell supernatant using ELISA method. Values are mean ± SEM of duplicate determinations from three separate experiments. *P < 0.05 : significantly different from the stimulated group.

4. Effect of ADR on IL-8 production in HMC-1 cells

The author also examined the inhibitory effect of ADR on the PMA plus A23187-induced production of IL-8 from HMC-1 cells. Culture supernatant was assayed for IL-8 levels by ELISA method. PMA plus A23187 significantly enhanced IL-8 (0.122±0.017 ng/ml) production compared with media control (0.012±0.001 ng/ml). The IL-8 concentrations at 0.01, 0.1, and 1 mg/ml ADR pretreated group were 0.059±0.003, 0.083±0.03, and 0.086±0.010 ng/ml, respectively. ADR inhibited IL-8 production significantly (*P < 0.05).

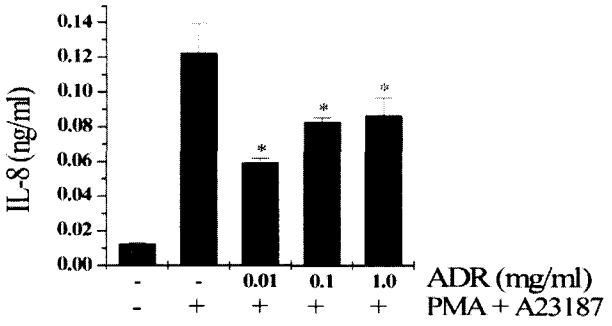

Fig. 3. Inhibition of IL-8 production by ADR in PMA plus A23187-stimulated HMC-1 cells The cells were pre-treated with ADR for 30 min and then challenged with PMA plus A23187 for 8 h. IL-8 concentrations were measured from cell supernatant using ELISA method. Values are mean ± SEM of duplicate determinations from three separate experiments. *P < 0.05 : significantly different from the stimulated group.

5. Effect of ADR on GM-CSF production in HMC-1 cells

Next, the author examined the inhibitory effect of ADR on the PMA plus A23187-induced production of GM-CSF from HMC-1 cells. Culture supernatant was assayed for GM-CSF levels by ELISA method. PMA plus A23187 significantly enhanced GM-CSF (0.448±0.896 ng/ml) production compared with media control (0.031±0.003 ng/ml). The GM-CSF concentrations at 0.1, and 1 mg/ml ADR pretreated group were 0.034±0.068, and 0.033±0.067 ng/ml, respectively. ADR

significantly inhibited GM-CSF production (Fig. 4).

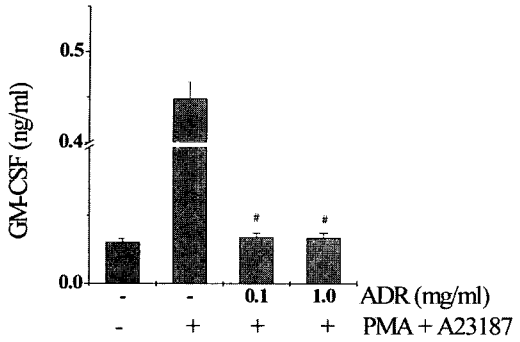

Fig. 4. Inhibition of GM-CSF production by ADR in PMA plus A23187-stimulated HMC-1 cells The cells were pre-treated with ADR for 30 min and then challenged with PMA plus A23187 for 24 h. ADR concentrations were measured from cell supernatant using ELISA method. Values are mean ± SEM of duplicate determinations from three separate experiments. #P < 0.001 ; significantly different from the stimulated group.

6. Effect of ADR on TNF-α production in HMC-1 cells

The author examined the inhibitory effect of ADR on the PMA plus A23187-induced production of TNF-α from HMC-1 cells. Culture supernatant was assayed for TNF-α levels by ELISA method. ADR slightly inhibited the production of TNF-α in PMA plus A23187-stimulated HMC-1 cells. However, the difference between ADR treated group and PMA plus A23187-treated group was not significant (Fig. 5).

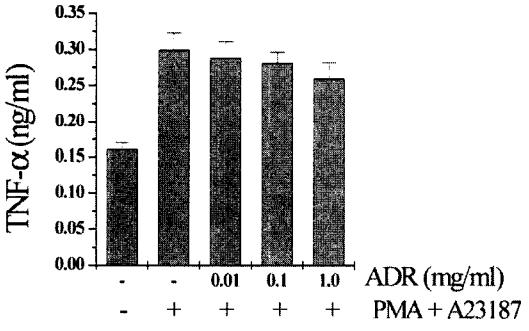

Fig. 5. Inhibition of TNF-α production by ADR in PMA plus A23187-stimulated HMC-1 cells The cells were pre-treated with ADR for 30 min and then challenged with PMA plus A23187 for 8 h. TNF-α concentrations were measured from cell supernatant using ELISA method. Values are mean ± SEM of duplicate determinations from three separate experiments.

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

MAPKs are serin/threonine kinases that become activated by phosphorylation on threonine and tyrosine residues upon extracellular stimuli[36]. PMA plus A23187 rapidly induced phosphorylation of ERK1/2, with no change in total ERK1/2 levels (data not shown). Phospho-ERK1/2 levels reached a peak at approximately 10-20 min after PMA plus A23187 treatment. Treatment with 0.1, and 1 mg/ml ADR blocked PMA plus A23187-induced ERK1/2 phosphorylation (Fig. 6).

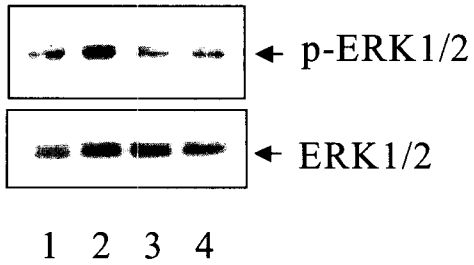

Fig. 6. Effect of ADR on p-ERK1/2 activation The p-ERK1/2 activation in the absence or presence of ADR (0.1, and 1 mg/ml) for 30 min prior to stimulation was determined by western-blotting. The p-ERK1/2 was investigated after a 10 min incubation following PMA plus A23187 stimulation; lane 1, media control; 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.

Discussion

The present study showed that ADR pretreatment profoundly affected compound 48/80-induced ear-swelling response. In addition, the production of IL-1β, IL-8, and GM-CSF in PMA plus A23187-stimulated mast cells was inhibited. ADR inhibited PMA plus A23187-induced ERK1/2 activation. These results indicate that ADR may have inflammatory regulatory activity.

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 an essential trigger for the release of mediators from mast cells[39]. Thus, ADR may stabilize the membrane of mast cells.

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[40,41]. The author showed that ADR inhibited inflammatory cytokines such as IL-1β, IL-8, and GM-CSF from PMA plus A23187-stimulated HMC-1 cells. This means ADR may exhibit the antiinflammatory activity. The pro-inflammatory cytokine IL-1β 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[42,43]. The IL-1 family represents 3 peptides (IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1ra or IL-1γ)[44,45]. Much of the pro-inflammatory activity of IL-1 relates to its ability to induce arachidonate metabolism with many eicosanoids, such as prostaglandin E2 and leukotriene B4, which function as second messengers. IL-1 induces synthesis of additional cytokines including TNF, IL-6, GM-CSF, and (as a positive feedback mechanism) additional IL-1. Finally, like TNF, IL-1 is directly cytotoxic to cancerous and virus-infected cells. TNF and IL-1 share numerous biologic activities, the major distinction being that TNF has no direct

effect on lymphocyte proliferation[46]. IL-8 is the most extensively studied member of the entire chemokine superfamily, with its major actions being as a neutrophil chemoattractant and activator[47]. GM-CSF plays a pivotal role in inflammatory and immunologic processes[48]. Release of GM-CSF in the airway can mediate acute inflammatory responses as well as initiate and perpetuate local immune responses. Elevated levels of GM-CSF have been well demonstrated in bronchoalveolar lavage fluid, endobronchial biopsy, and sputum samples from asthmatics[49-51]. Mouse models of asthma and diesel-induced hyperresponsiveness have demonstrated an association between epithelial cell-derived GM-CSF and airway hyper-responsiveness[52,53]. TNF-α is an essential cytokine in many pathological conditions such as allergic diseases, rheumatoid arthritis and pulmonary fibrosis[54]. TNF-α induced production and release of eosinophil chemotactic factors such as eotaxin[55,56] and RANTES[57] from fibroblasts and epithelial cells.

Because the induction of most cytokine genes requires activation of the ERK1/2[37]. The author finally tested the effect of ADR for PMA plus A23187-induced ERK1/2 activation. In signaling of MAPK pathway, it is important to consider the possible contributions of various factors. These include cell type, the type of stimulus, the duration and magnitude of the response, and the activation of other signaling pathways in the cell. ADR inhibited PMA plus A23187-induced ERK1/2 phosphorylation in HMC-1 cells. The result suggests that ADR regulates inflammatory cytokines through inhibition of ERK1/2 phosphorylation.

Phorbol esters are tumor-promoting compounds originally detected in oil prepared from seeds of Croton tiglium. Some esters, such as PMA or 12-o-tetradecanoyl-phorbol-13-acetate have highly pleiotropic effects on cells in culture and on tissues in vivo and are used frequently in immunology as activators of mast cells in vitro. In many cell types, protein kinase C (PKC) activation assumes a central role in the early stages of signal transduction. PMA, which are thought to directly activate PKC enzymes, can induce histamine release and cytokines from many cell types including human basophils and mast cells[58,59]. A23187 is a widely used ionophore A23187 has the ability to increase the intra-cellular concentrations of cations and is widely used to increase the intra-cellular levels of calcium. Increased intra-cellular calcium contribute to the production of inflammatory cytokines by regulating MAPK activity in HMC-1 cells. Transcription factor nuclear factor-κB is also involved in the up-regulation of cytokines induced by intracellular calcium. So, A23187, calcium ionopore, is used to activate cells in experiment[40,60].

In conclusion, The author demonstrated that ADR could inhibit the inflammatory reaction through blocking ERK1/2 activity.

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