

Atractylone, an active constituent of KMP6, attenuates allergic inflammation on allergic rhinitis *in vitro* and *in vivo* models

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ARTICLE INFO

Article history:

Received 6 June 2016

Received in revised form 7 September 2016

Accepted 8 September 2016

Keywords:

Atractylone

Allergic rhinitis

Mast cells

Caspase-1

Proinflammatory cytokine

Thymic stromal lymphopoietin

ABSTRACT

KMP6 (Pyeongwee-San) is a Korean Medicine used to treat gastrointestinal disorders. Recently, we reported KMP6 had beneficial effects on allergic inflammatory diseases. The aim of this study was to evaluate the effects of atractylone (Atr), a constituent of KMP6, on allergic rhinitis (AR) and to identify the mechanism responsible for these effects. The anti-allergic inflammatory effects of Atr were evaluated on phorbol 12-myristate 13-acetate and calcium ionophore A23187 (PMACI)-stimulated human mast cell line, HMC-1 cells and in an ovalbumin (OVA)-induced AR animal model using Western blotting, quantitative real-time PCR, ELISA, and immunohistochemistry methods. In HMC-1 cells, Atr and KMP6 attenuated PMACI-caused proinflammatory cytokine production and mRNA expression. We found that PMACI induced caspase-1/nuclear factor (NF)- κ B/mitogen activated protein kinases (MAPKs) activation. PMACI-caused caspase-1/NF- κ B/MAPKs activations were attenuated by Atr and KMP6. In AR animal model, Atr and KMP6 reduced AR clinical symptoms and biomarkers including rub scores, total IgE, histamine, prostaglandin D₂, thymic stromal lymphopoietin, interleukin (IL)-1 β , IL-4, IL-5, IL-6, IL-13, tumor necrosis factor- α , cyclooxygenase-2, intercellular adhesion molecule-1, and macrophage inflammatory protein-2. In addition, Atr and KMP6 attenuated eosinophils and mast cells invasions into nasal mucosa tissues and diminished mast cell-derived caspase-1 activation. These results indicate that Atr is an active constituent of KMP6 and a potential therapeutic agent for AR.

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1. Introduction

Allergic inflammatory diseases are dependent upon cutaneous inflammation driven by type 2 helper T cell (Th2)-mediated immunological responses (Buzney et al., 2016). Inflammatory processes associated with Th2 immunity are present in approximately half of those suffering from allergic inflammatory diseases, such as, allergic rhinitis (AR), asthma, and atopic dermatitis (Buzney et al., 2016; Rosati and Peters, 2016). AR is a common disease, and many of AR patients have comorbidities like asthma and chronic rhinosinusitis (Rosati and Peters, 2016). AR is a nasal inflammatory and IgE-mediated disease with clinical symptoms that include rhinor-

rhea, coughing, loss of smell and taste, nasal obstruction, post nasal drainage, sneezing, lacrimation, and nasal irritation (Bousquet et al., 2008). Despite several readily available treatment options, such as, intranasal steroids, antihistamines, leukotriene receptor antagonists, and immunotherapy, 20% of AR patients do not respond well to treatment, and thus, AR continues to present a therapeutic challenge (Steelant et al., 2016). In fact, population surveys have reported that up to 29% of child and 62% of adults sufferers experience partial or only poor relief from pharmacotherapy (Durham and Penagos, 2016).

AR is often accompanied by mast cells and mast cell-derived Th2 cytokines (Oh et al., 2011). Mast cells play a vital role in type 1 hypersensitivity reactions such as those observed in AR. Mast cell mediators have also been implicated in many different disease conditions, such as, mastocytosis, asthma, several cancers, conjunctivitis, psoriasis, and AR (Tsai et al., 2011; Zhang et al., 2015). Mast cell activation can be induced by both IgE and non-immunologic substances, and when activated produce vasoactive and inflammatory molecules via mitogen activated protein kinases

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(MAPKs)/caspase-1/nuclear factor (NF)- κ B signaling cascade pathways (Kim et al., 2016). Mast cell-derived inflammatory molecules, such as, interleukin (IL)-1 β , IL-6, IL-8, thymic stromal lymphopoietin (TSLP), and tumor necrosis factor (TNF)- α , contribute to the influx of immune cells into nasal mucosa tissue of AR, and in particular, the infiltration of neutrophils, eosinophils, and mast cells accelerate inflammatory reactions (Galli and Tsai, 2012). Thus, development efforts tend to focus on the prevention of mast cell mediator production and on blocking the activities of such mediators (Zhang et al., 2015).

KMP6 (Pyeongwee-San) is a Traditional Korean Medicine used to treat gastrointestinal disorders, and recently, we reported KMP6 had ameliorative effects in allergic inflammatory reactions (Han et al., 2012; Oh et al., 2012). KMP6 is composed of *Atractylodes japonica* Koidzumi, *Citrus sunki* Hort. ex Tanaka, *Glycyrrhiza uralensis* Fisch, *Magnolia officinale* Rehder et Wils, *Zingiber officinale* Roscoe, and *Zizyphus jujuba* var. *inermis* (Bunge) Rehder, and has been shown to contain many beneficial natural compounds, such as, hesperidin, glycyrrhizin, magnolol, atractylenolide III, atractylone (Atr), and eudesmol (Jeong et al., 2011). As with the discovery of many medicines in clinical use, natural compound have been served as a very fruitful source of AR. Therefore, the purpose of this study was to examine the effect of Atr on inflammatory reactions using human mast cell line, HMC-1 cells. Subsequently, we confirmed the anti-allergic inflammatory effect of Atr in an ovalbumin (OVA)-induced AR model.

2. Material and methods

2.1. Materials

Dexamethasone (DEX), dimethyl sulfoxide (DMSO), OVA, aluminum hydroxide, phorbol 12-myristate 13-acetate (PMA, protein kinase C activator), A23187, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), bicinechonic acid (BCA), RU486, and other reagents were obtained from Sigma (St. Louis, MO, USA). The caspase-1 assay kit was supplied by R&D Systems Inc. (Minneapolis, MN, USA). Fetal bovine serum (FBS), Iscove's modified Dulbecco's medium (IMDM), and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). Antibodies for c-Kit (mast marker), caspase-1, cyclooxygenase (COX)-2, NF- κ B (p65), phosphorylated (p)I κ B α , p38, pp38, c-Jun N-terminal kinase (JNK), pJNK, Poly (ADP-ribose) polymerase, tubulin, and glyceraldehyde 3-phosphate dehydrogenase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Atr purity 98% was obtained from ChemFaces Biochemical Co., Ltd. (Hubei, China).

2.2. Preparation of KMP6 and Atr

KMP6 was provided by the Korea Bio Medical Science Institute (Seoul, Republic of Korea). KMP6 was boiled with distilled water (DW) at 80 °C for 3 h. The crude extracts were filtered and concentrated *in vacuo* at 60 °C. It was lyophilized. The extract yield of KMP6 was about 21% (w/w). The KMP6 was dissolved in DW and filtered using a 0.22 μ m syringe filter and kept at 4 °C. Atr was prepared by dissolving it in DMSO at 25 mg/ml and then diluting with DW (2.5, 0.25, and 0.025 mg/ml) with reference to the study of Hwang et al. (1996).

2.3. Analyses of Atr in KMP6

The level of Atr in KMP6 was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS; LC: 1290 Infinity, Agilent technologies, USA; MS/MS: API 4000, Applied Biosystems, USA). Briefly, KMP6 was extracted with 0.4 ml acetonitrile, and 5 μ l aliquots of filtered extracts were injected into

Kromasil 100.3.5.-C18 (100 mm $\times$ 2.1 mm; particle size 5 μ m) column. Elution was conducted using an acetonitrile (solvent A)/0.1% formic acid (solvent B) 65:35 (v/v) mixture at a flow rate of 0.2 ml/min. Atr contents in KMP6 were calculated by linear regression. Data were analyzed using Analyst software version 1.4.2, Applied Biosystems, USA. KMP6 was found to contain $\sim$ 715.56 μ g/g of Atr (data not shown).

2.4. Culture of HMC-1 cells

HMC-1 cells were kindly provided by Eichi Morri (Osaka University, Japan). HMC-1 cells were cultured in IMDM supplemented with 100 unit/ml penicillin, 100 μ g/ml streptomycin and 10% heat-inactivated FBS at 37 °C 5% CO₂ and 95% humidity. HMC-1 cells were stimulated with PMA (50 nM) and A23187 (1 μ M) (PMACI).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Cytokines [TSLP, IL-1 β , IL-5, IL-6, IL-8, IL-13, TNF- α , total IgE, IL-4, interferon (IFN)- γ , Inter cellular adhesion molecule-1 (ICAM-1), and macrophage inflammatory protein-2 (MIP-2)] in serum, tissues, and supernatant were measured by an ELISA in accordance with the manufacturer's instructions (Pharmingen, San Diego, CA, USA). The absorbance was measured using an ELISA reader at 405 nm. Recombinant cytokines as controls were loaded, and all samples were run in duplicate. Cytokine levels in the tissues were divided by the total protein. The concentration of protein was calculated using the BCA protein assay method.

2.6. Cell viability assay

To estimate the cell viability, the MTT assay was performed. Briefly, Cells were treated with Atr, KMP6, and DEX for 7 h. MTT (5 mg/ml) was treated and then the cells were cultured at 37 °C for 4 h. The crystallized MTT was dissolved in DMSO and the absorbance measured using an ELISA reader at 540 nm.

2.7. RNA isolation and quantitative real-time PCR

RNA isolation and quantitative real-time PCR were performed as previously described (Kim et al., 2016). Target mRNA levels were normalized versus GAPDH, and data were analyzed using the $\Delta\Delta$ CT method.

2.8. Caspase-1 assay

HMC-1 cells (3×10^6) were treated with Atr (0.25, 2.5, and 25 μ g/ml), KMP6 (0.05 and 0.1 mg/ml), and DEX (100 nM) for 1 h and then stimulated with PMACI for 2 h. Caspase-1 activities were measured using a caspase-1 assay kit.

2.9. Preparation of nuclear and cytosolic extracts

HMC-1 cells (3×10^6) were treated with Atr (0.25, 2.5, and 25 μ g/ml), KMP6 (0.05 and 0.1 mg/ml), and DEX (100 nM) for 1 h and then stimulated with PMACI for 2 h. Briefly, washed cells were resuspended them in 40 ml of a cold hypotonic buffer (10 mM Hepes/KOH, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM PMSF, pH 7.9). Next, we allowed the cells to swell on ice for 15 min; we lysed them gently with 2.5 ml of 10% Non-ide P (NP)-40; and we centrifuged them at 15,000 $\times$ g for 3 min at 4 °C. The supernatant was aliquots (cytosolic protein) and the pellets were gently resuspended in 40 ml of cold saline buffer (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) and then left on ice for 20 min.

After conducting the centrifugation ($15,000 \times g$ for 15 min at 4°C), we froze the aliquots of supernatant containing the nuclear proteins in liquid nitrogen and stored them at -80°C until ready for analysis. Finally, we used the BCA protein assay (Sigma, St. Louis, MO, USA) to measure protein concentrations.

2.10. Western blot analysis

HMC-1 cells (3×10^6) were treated with Atr (0.25, 2.5, and $25 \mu\text{g/ml}$), KMP6 (0.05 and 0.1 mg/ml), and DEX (100 nM) for 1 h and then stimulated with PMACI for 1 or 2 h. Western blot analysis

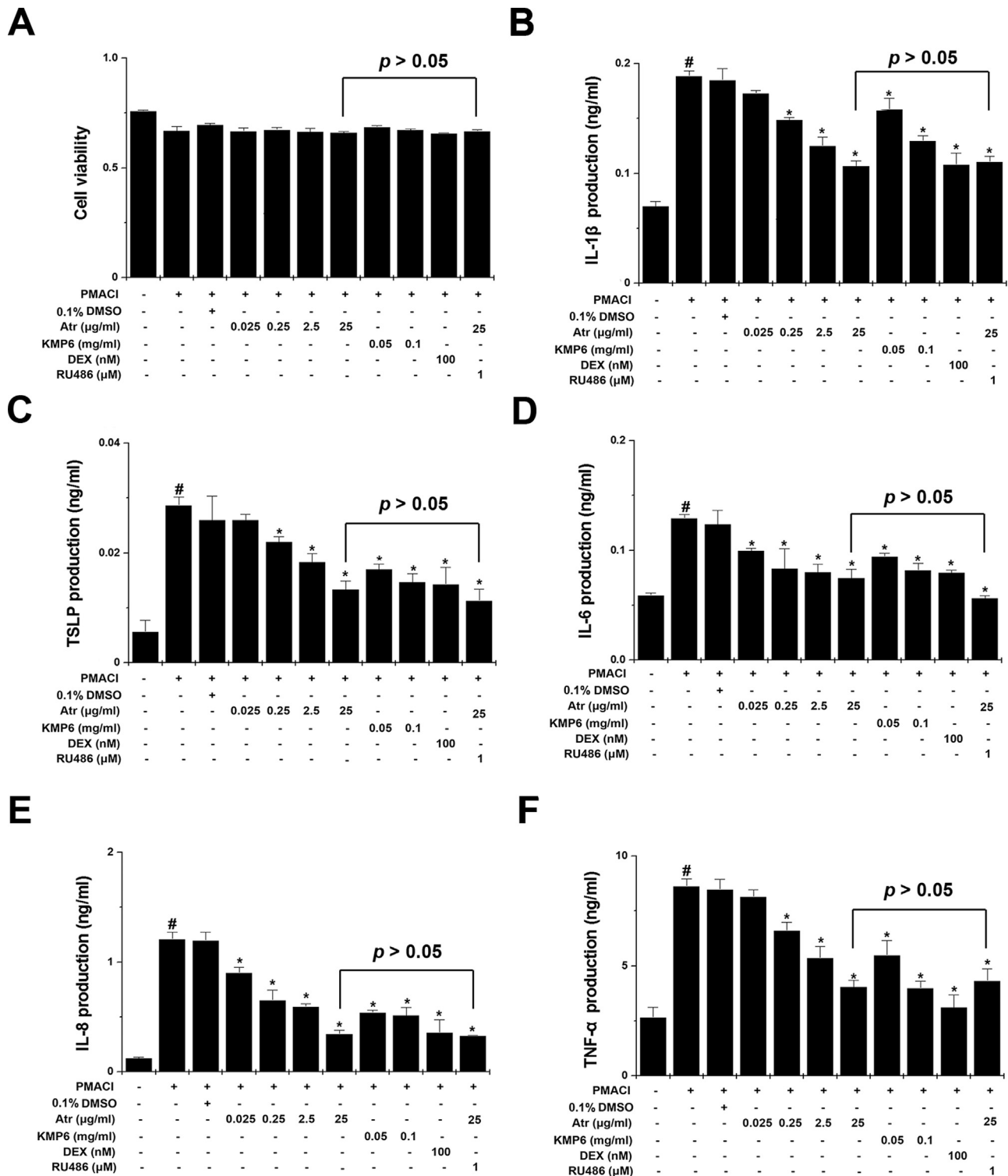


Fig. 1. Inhibitory effect of Atr on inflammatory cytokine production in HMC-1 cells. HMC-1 cells were pretreated with Atr (0.025, 0.25, 2.5, and $25 \mu\text{g/ml}$), KMP6 (0.05 and 0.1 mg/ml), DEX (100 nM), RU486 ($1 \mu\text{M}$), and DMSO (0.1%) for 1 h and then stimulated with PMACI for 7 h. (A) Cell viabilities were evaluated using a MTT assay. (B–F) Cytokine levels were measured by ELISA. # $p < 0.05$; significantly different from unstimulated cells. * $p < 0.05$; significantly different from PMACI-stimulated cells. PMACI, PMA + A23187; Atr, atractylone; DEX, dexamethasone.

was used for cell extracts were prepared by a detergent lysis procedure. Samples were heated at 95 °C for 5 min, and briefly cooled on ice. Protein aliquots were resolved by 10% SDS-PAGE. The resolved proteins were electrotransferred to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycerin, 20% methanol at 25 V. Blots were blocked for 2 h with 6% bovine serum albumin and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) in accordance with the manufacturer's instructions.

2.11. Induction of the AR animal model

We maintained 6-week-old female BALB/c (Charles River Laboratories, Inc., Wilmington, MA, USA) mice under pathogen-free conditions. Mouse care and experimental procedures were performed under approval from the animal care committee of Kyung Hee University [KHUASP (SE)-12-019]. The mice were sensitized on days 1, 5, and 14 by intraperitoneal (i.p.) injection of 100 µg OVA emulsified and 20 mg aluminum hydroxide in a 100 µl phosphate-buffered saline (PBS) and challenged intranasally with 1.5 mg OVA in 2 µl PBS or PBS. Negative control mice were sensitized and challenged with PBS alone. Atr (0.25, 2.5, and 25 mg/kg), KMP6 (0.1 and 0.05 g/kg), DEX (5 mg/kg) or a control vehicle (DW) was administered orally for 10 days before the intranasal (i.n.) OVA challenge. On day 24, nasal symptoms were evaluated by counting the number of nasal rubs that occurred in the 10 min after OVA i.n. provocation at the 10 day mark after the challenge. After rub scoring, the mice were sacrificed and blood, spleen, and nasal mucosa tissues were collected for analyses. The numbers of mice in each group was 5.

2.12. Histamine assay

Serum histamine levels were measured using a histamine assay kit (Oxford Biomedical Research, Oxford, MI, USA).

2.13. Prostaglandin D₂ (PGD₂) assay

Serum PGD₂ levels were measured using a PGD₂ assay kit (Cayman Chemical Com., Ann Arbor, Michigan, USA).

2.14. Histological examinations

Tissue samples were immediately fixed with 10% formaldehyde for 24 h at 20 °C and embedded in paraffin. Sections (4 µm) of nasal mucosa tissues samples were stained with hematoxylin and eosin (H&E, for eosinophils) and alcian blue and safranin O (A&S, for mast cells), and numbers of eosinophils and mast cells on both sides of septal mucosa were counted. For confocal microscopy, after de-waxing and dehydration, sections were blocked with bovine serum albumin and incubated for 60 min with anti-mouse c-Kit and anti-rabbit caspase-1 antibodies. The secondary antibody, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC)-conjugated anti-rabbit IgG (Invitrogen), was then added for 30 min. A mounting medium containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) was used to counterstain DNA. Randomly coded specimens were examined under a confocal laser-scanning microscope by two blinded observers.

2.15. Statistical analysis

In vitro data are representative of three independent experiments conducted in duplicate and *in vivo* data are presented as

the mean ± standard error of mean (SEM). Statistical analyses were performed using SPSS statistical software (SPSS ver. 11.5, USA). Treatment effects were analyzed by one-way ANOVA with Tukey's post hoc test, and differences were considered to be significant when *p* values were <0.05.

3. Results

3.1. Inhibitory effect of Atr on PMACI-caused cytokine production and mRNA expression

To estimate the cytotoxic effect of Atr on HMC-1 cells, cell viabilities were determined using an MTT assay. High concentrations of Atr (25 µg/ml), KMP6 (0.1 mg/ml), and DEX (100 nM) did not affect cell viability (Fig. 1A). PMACI potently induces the production of inflammatory cytokines by mast cells (Kim et al., 2014), and thus, the inhibition of PMACI-caused inflammatory cytokine production is viewed as a means of accessing the therapeutic potentials of agents targeting allergic inflammatory progression. Thus, we investigated whether Atr could regulate proinflammatory cytokine production from PMACI-treated HMC-1 cells. As shown in Fig. 1B–F, the upregulations of TSLP, IL-1β, IL-6, IL-8, and TNF-α by PMACI were significantly and dose-dependently down-regulated by Atr treatment (*p* < 0.05). However, Atr alone did not affect inflammatory cytokine production compared with the media control, and DMSO did not affect inflammatory cytokine production as compared with PMACI stimulation. In addition, the effect of Atr in PMACI-caused inflammatory cytokine mRNA expression was investigated by quantitative real time-PCR. Atr was found to significantly and dose-dependently reduce the PMACI-caused mRNA expressions of TSLP, IL-1β, IL-6, IL-8, and TNF-α (Fig. 2, *p* < 0.05). KMP6 and DEX also significantly decreased PMACI-caused inflammatory cytokine production and mRNA expressions (Figs. 1 and 2, *p* < 0.05). We then investigated whether the modes of action of Atr and DEX are similar. We found that the inhibitory effect of Atr on inflammatory cytokine production was not abolished by the glucocorticoid receptor antagonist RU486 (Fig. 1B–F).

3.2. Inhibitory effect of Atr on PMACI-caused caspase-1/NF-κB/MAPKs activation

The inflammatory cytokine expressions are upregulated by upstream signal transduction pathways, which include the caspase-1, NF-κB, and MAPKs pathways (Oh et al., 2011; Song et al., 2012). First, to estimate the effect of Atr on PMACI-caused caspase-1 activation, we analyzed caspase-1 activities using a caspase-1 assay kit. When cells were stimulated with PMACI, caspase-1 activity increased significantly, but these increases were inhibited by the treatment with Atr (Fig. 3A, *p* < 0.05). Levels of the active form of caspase-1 were also analyzed by Western blotting. Similar to Fig. 3A data, Atr reduced PMACI-caused caspase-1 activation (Fig. 3B & C). As shown in Fig. 2, Atr reduced the mRNA levels of cytokines increased by PMACI. Because the mRNA expressions of inflammatory cytokines are increased by NF-κB activation. Next, we examined whether Atr could regulate NF-κB activation and IκBα phosphorylation in PMACI-treated HMC-1 cells. As shown in Fig. 3D & E, Atr dose-dependently diminished the PMACI-caused NF-κB activation and IκBα phosphorylation. Finally, because the mRNA and protein levels of inflammatory cytokines are known to occur via MAPKs activation, we investigated the effect of Atr on the activation of MAPKs. It was found Atr diminished the PMACI-caused phosphorylations of JNK and p38 (Fig. 3F & G), but did not affect the phosphorylation of ERK (data not shown). In addition, KMP6 and DEX also diminished PMACI-caused caspase-1/NF-κB/MAPKs activation (Fig. 3).

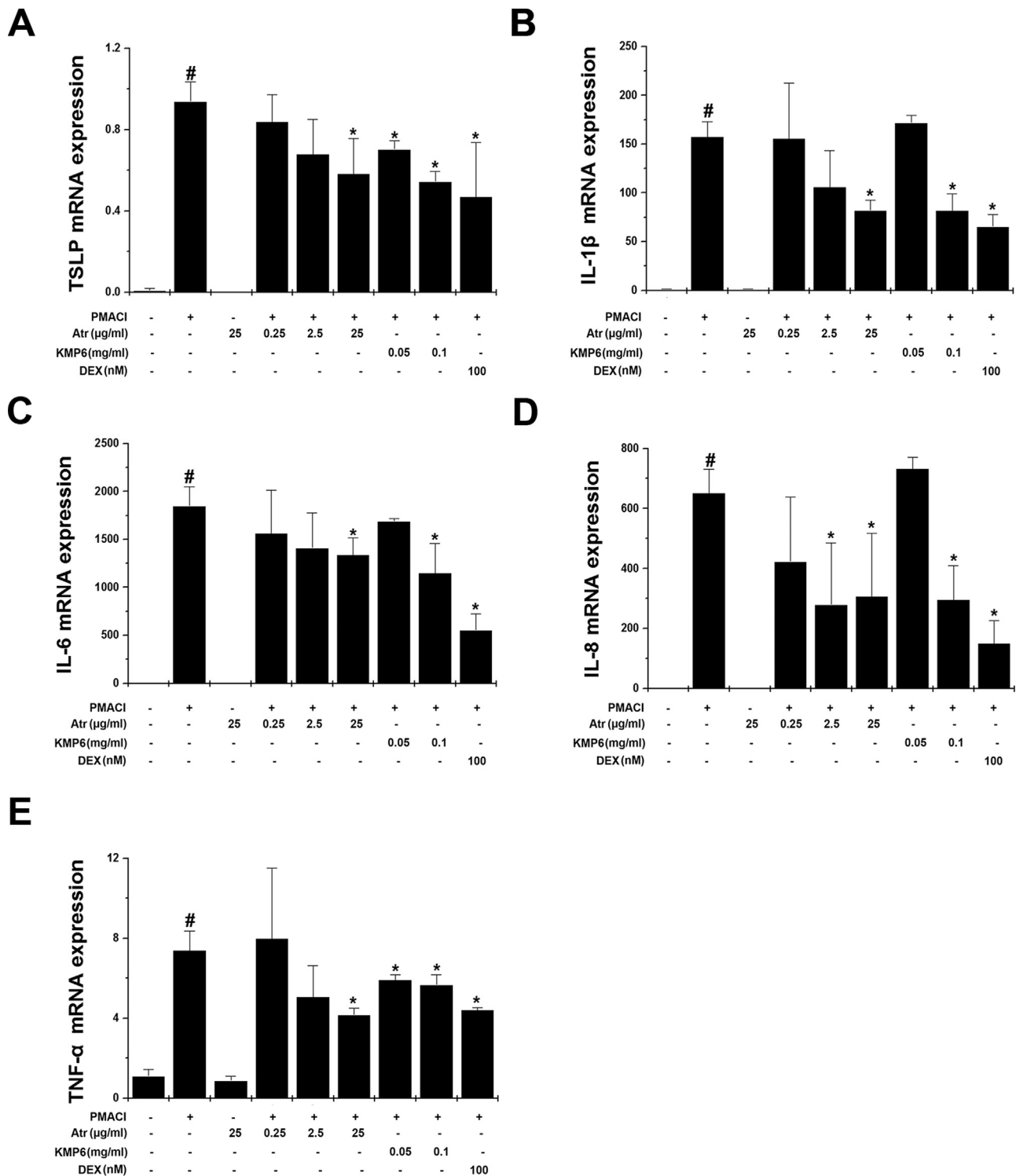


Fig. 2. Inhibitory effect of Atr on inflammatory cytokine mRNA expression in HMC-1 cells. (A–E) HMC-1 cells were pretreated with Atr (0.25, 2.5, and 25 μg/ml), KMP6 (0.05 and 0.1 mg/ml), and DEX (100 nM) for 1 h and then stimulated with PMACI for 6 h. Messenger RNA levels were determined by quantitative real time-PCR. #*p* < 0.05; significantly different from unstimulated cells. **p* < 0.05; significantly different from PMACI-stimulated cells. PMACI, PMA + A23187; Atr, atractylone; DEX, dexamethasone.

3.3. Inhibitory effect of Atr on clinical symptom and biomarkers of AR

We estimated the effect of Atr on AR animal model to confirm the anti-allergic inflammatory effect of Atr. As shown in Fig. 4A, Atr significantly reduced the rub scores compared with the OVA group (*p* < 0.05). Cross-linking of allergen specific IgE antibodies on the

surfaces of mast cells causes them to degranulate and leads to AR (Galli and Tsai, 2012). Histamine and PGD₂ released by mast cell degranulation plays a central role in AR (Takahashi et al., 2012). In a previous study it was shown that DEX reduced serum IgE levels when administered long after OVA sensitization (Chen et al., 2015), and thus, DEX was used as a positive control in the present study. Fig. 4B–D showed that the levels of serum histamine, PGD₂,

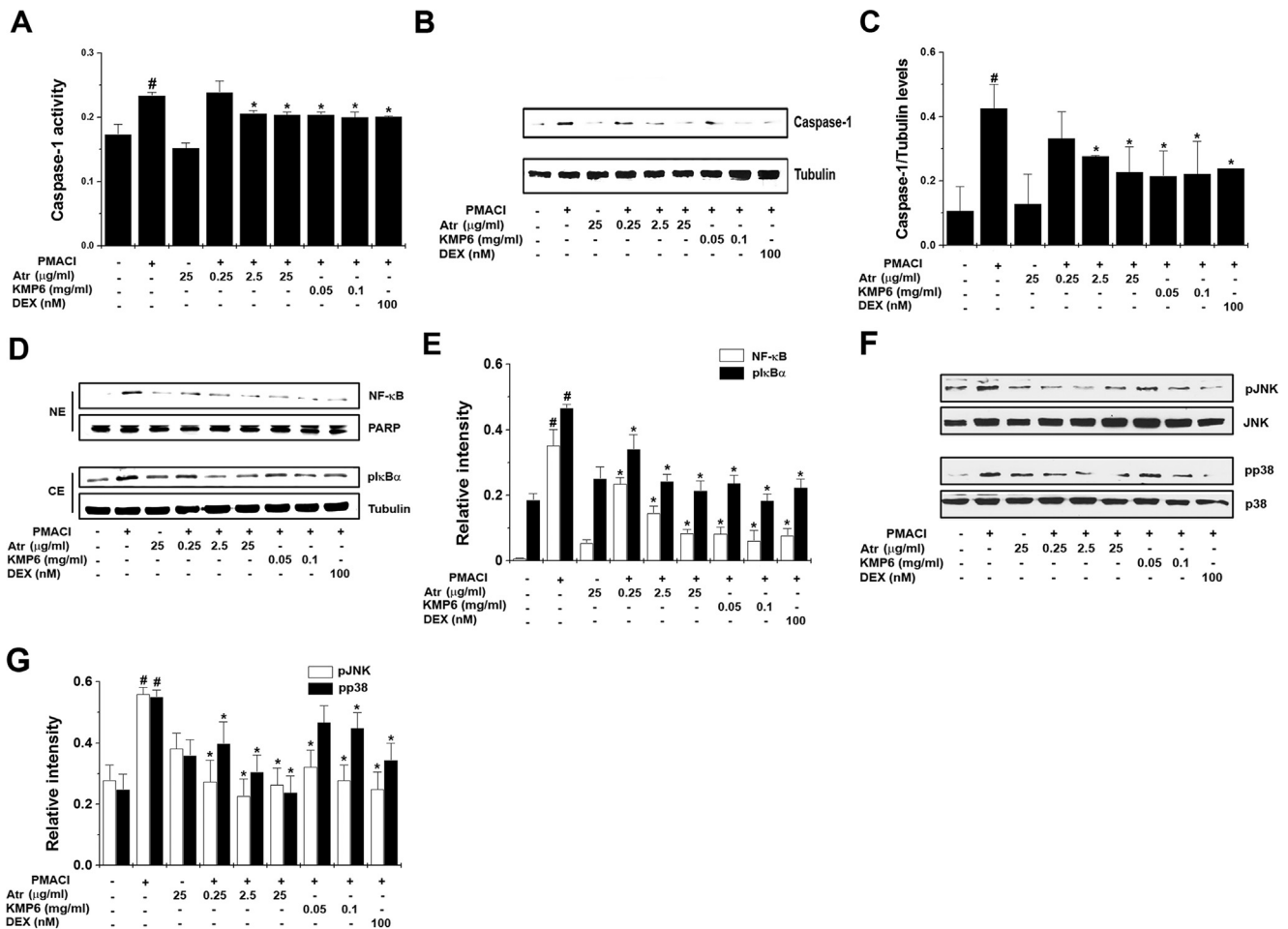


Fig. 3. Inhibitory effect of Atr on caspase-1/NF- κ B/MAPKs activation in HMC-1 cells. HMC-1 cells were pretreated with Atr (0.25, 2.5, and 25 μ g/ml), KMP6 (0.05 and 0.1 mg/ml), and DEX (100 nM) for 1 h and then stimulated with PMACI for 1 or 2 h. (A) Enzymatic activities of caspase-1 were determined using a caspase-1 colorimetric assay. (B) Levels of active caspase-1 were assayed by Western blotting. (C) Protein levels were quantified by densitometry. (D) Nuclear and cytoplasmic proteins were prepared and analyzed for NF- κ B and pI κ B α by Western blotting. (E) Protein levels were quantified by densitometry. (F) Levels of phosphorylated MAPKs were assayed by Western blotting. (G) Protein levels were quantified by densitometry. Results are representative of three independent experiments. Values are mean $\pm$ SEM. # $p < 0.05$; significantly different from unstimulated cells. * $p < 0.05$; significantly different from PMACI-stimulated cells. PMACI, PMA + A23187; Atr, atractylone; DEX, dexamethasone; NE, nuclear extract; CE, cytoplasmic extract.

and total IgE increased by OVA were significantly lowered by the treatment with Atr or DEX ($p < 0.05$). Furthermore, IL-1 β and TSLP, both initiators of inflammatory reactions, were dose-dependently reduced by the treatment with Atr (Fig. 4E & F). To estimate the Th1/Th2 immune reaction in Atr-treated group, we analyzed spleen weight and IL-4 and IFN- γ levels in spleens. As shown in Fig. 4G–I, Atr reduced spleen weights and IL-4 levels, whereas IFN- γ levels were increased by the treatment with Atr compared with the OVA group. The KMP6 or DEX-treated group also exhibited significantly lower rub scores and AR biomarkers levels than OVA group (Fig. 4).

3.4. Inhibitory effect of Atr on Th2 immune responses in the nasal mucosa tissues of AR mice

Th2 cytokines such as TSLP, IL-1 β , IL-5, IL-6, and IL-13 are crucial factors in the pathogenesis of AR (Spencer et al., 2009). Thus, we examined whether Atr affected Th2 cytokine levels in AR mice. Levels of TSLP, IL-1 β , IL-5, IL-6, and IL-13 upregulated by OVA in nasal mucosa tissues were significantly down-regulated by the treatment with Atr (Fig. 5A–E, $p < 0.05$). The inflammatory factors, TNF- α and COX-2, were also significantly down-regulated by the treatment with Atr compared with the OVA group (Fig. 5F & G). As shown in

Fig. 5, Th2 cytokines and inflammatory mediators were also significantly reduced in KMP6 or DEX-treated group ($p < 0.05$).

3.5. Inhibitory effect of Atr on inflammatory cell influx into the nasal mucosa tissues

Eosinophils and mast cells play a central role in the development of AR and infiltrate inflammatory zones in AR mice (Oh et al., 2012). ICAM-1 and MIP-2 are chemotactic molecules for eosinophils and mast cells (Song et al., 2012). The levels of ICAM-1 and MIP-2 in the OVA group were significantly higher than those in the nasal mucosa tissues of the normal control group (Fig. 6A & B, $p < 0.05$). Increased levels of ICAM-1 and MIP-2 were reduced by the treatment with Atr (Fig. 6A & B, $p < 0.05$). To estimate the effects of Atr treatment on the influx of immune cells into the nasal mucosa tissues of AR, we stained the eosinophils and mast cells. In OVA group, numbers of infiltrating cells were approximately 7-fold higher than in normal control group. Eosinophils numbers in the Atr-treated group were significantly decreased compared with the OVA group (Fig. 6C & E). Numbers of mast cells in the nasal mucosa tissues were also significantly lowered by the treatment with Atr (Fig. 6D & F). KMP6 or DEX-treated group also exhibited significantly reduced

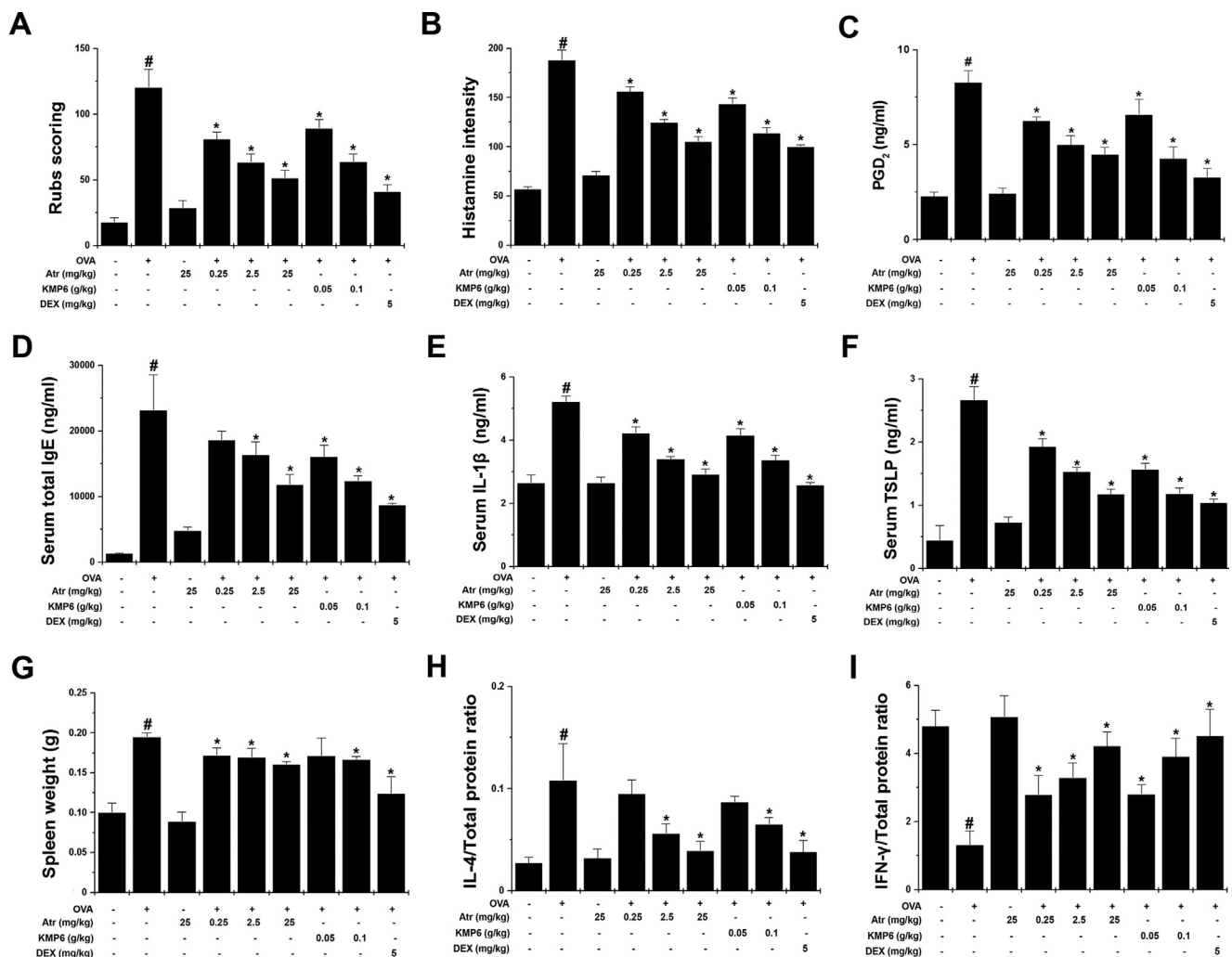


Fig. 4. Inhibitory effect of Atr on AR biomarkers. We sensitized mice on days 1, 5, and 14 by ip injections of 100 μ g OVA emulsified in 20 mg of aluminum hydroxide and we challenged mice with 1.5 mg OVA. Mice received orally Atr (0.25, 2.5, and 25 mg/kg), KMP6 (0.05 and 0.1 g/kg), and DEX (5 mg/kg) before the intranasal OVA challenge for 10 days. (A) The number of the nasal rubs that occurred in the 10 min after the OVA intranasal provocation. (B) Serum was isolated from blood and then assayed about histamine and (C) PGD₂. (D) Total IgE, (E) IL-1 β , and (F) TSLP in the serum were determined by ELISA. (G) Spleen weight. (H) Spleen IL-4 and (I) IFN- γ were determined by ELISA. IL-4 and IFN- γ measured in the tissue homogenate were presented as a ratio to the total protein levels in the tissue. # p < 0.05; significantly different from normal control group. * p < 0.05; significantly different from OVA group. Atr, atractylone; DEX, dexamethasone.

the eosinophils and mast cells infiltration compared with the OVA group (Fig. 6).

3.6. Inhibitory effect of Atr on caspase-1 activation in the nasal mucosa tissues of AR mice

Mast cell-derived caspase-1 is involved in the inflammatory responses of AR (Bae et al., 2011). Thus, we estimated the effect of Atr on caspase-1 activation using a caspase-1 assay kit. OVA-caused caspase-1 activation was significantly reduced by the treatment with Atr (Fig. 7A, p < 0.05). Also, we performed an immunohistochemical study for mast cell and caspase-1 to estimate the effect of Atr on mast cell-derived caspase-1 activation of nasal mucosa tissues. The protein levels of mast cell-derived caspase-1 in the Atr-treated group was significantly decreased compared with the OVA group (Fig. 7B). The KMP6 or DEX also reduced the mast cell-derived caspase-1 activation compared with the OVA group (Fig. 7).

4. Discussion

The present study demonstrated that Atr significantly diminished PMACI-caused inflammatory cytokine production by

blocking caspase-1/NF- κ B/MAPKs signaling pathways. Furthermore, Atr alleviated the clinical symptoms and biomarkers of AR, and diminished Th2 cytokine and chemokine levels, immune cell influx into inflamed mucosal tissue, and mast cell-derived caspase-1 activity.

Mast cells contribute to innate and adaptive immunological reactions. In particular, mast cells play critical roles in allergic inflammatory responses and are known to increase the infiltrations of eosinophils, dendritic cells, and neutrophils (Tsai et al., 2011). Signaling through high affinity receptor for IgE (Fc ϵ RI) on mast cells surface induces mast cell degranulation, inflammatory cytokine secretion, PGD₂, and lipid-derived mediator production (Galli and Tsai, 2012; Oh et al., 2012; Takahashi et al., 2012). Protein kinase C family members and intracellular calcium levels play important roles in IgE-antigen-mediated mast cell activation (Jeong et al., 2002). PMACI (protein kinase C activator and calcium ionophore) stimulates mast cells to produce histamine, TSLP, TNF- α , IL-1 β , IL-5, IL-6, IL-8, and IL-13 via caspase-1/NF- κ B/MAPKs cascade signaling pathways (Oh et al., 2011; Song et al., 2012). These mast cell-derived inflammatory mediators play key roles in the development of AR by inducing Th2 dominant responses (Galli et al., 2008; Miyata et al., 2008; Takahashi et al., 2012), and

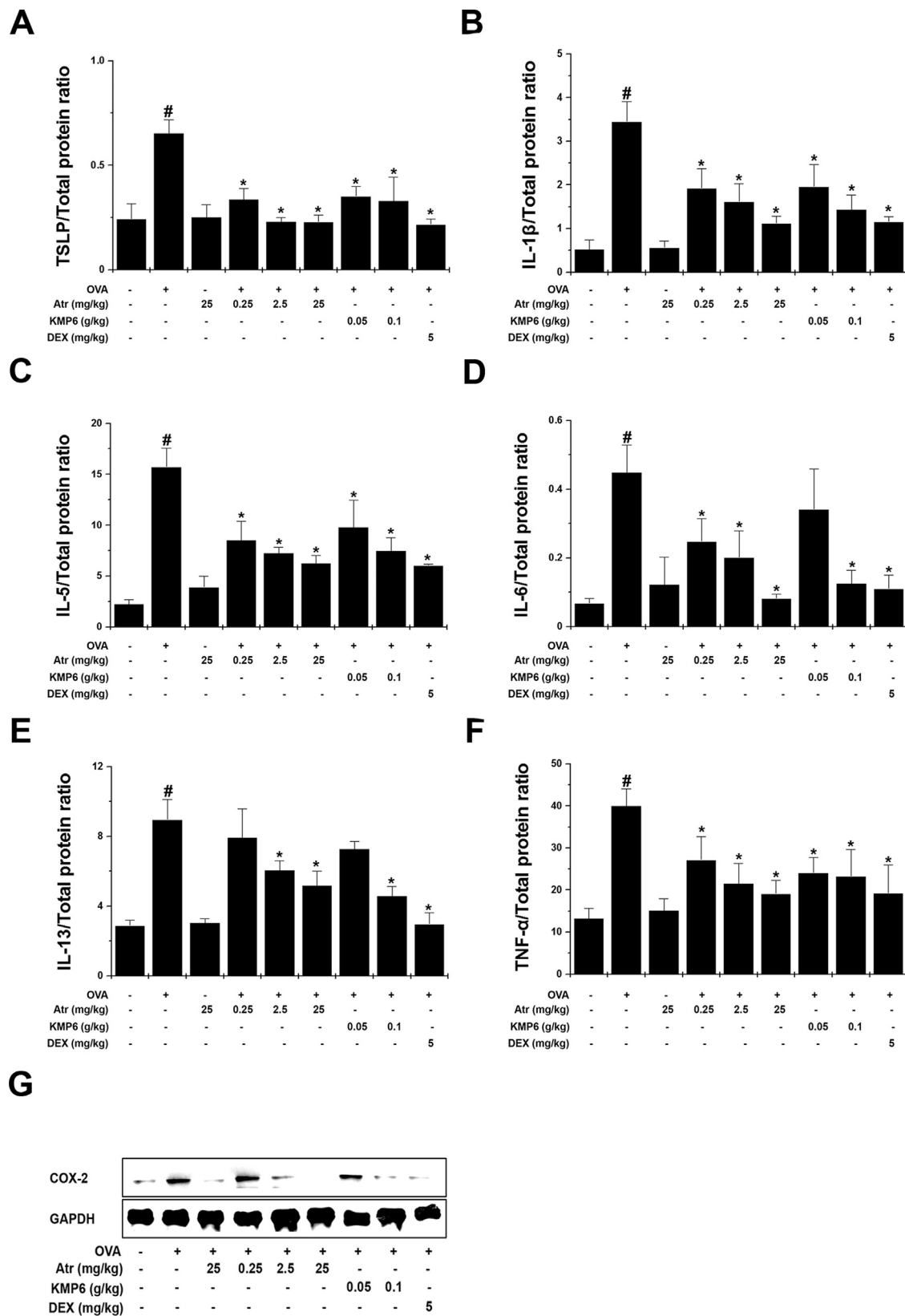


Fig. 5. Inhibitory effect of Atr on inflammatory cytokines of the nasal mucosa tissues. (A), TSLP, (B) IL-1 β , (C) IL-5, (D) IL-6, (E) IL-13, and (F) TNF- α levels were determined by ELISA. All parameters measured in the tissue homogenate were presented as a ratio to the total protein levels in the nasal tissue. (G) Levels of COX-2 were assayed by Western blotting. Results are representative of three independent experiments. [#] $p < 0.05$; significantly different from normal control group. ^{*} $p < 0.05$; significantly different from OVA group. Atr, atracylone; DEX, dexamethasone.

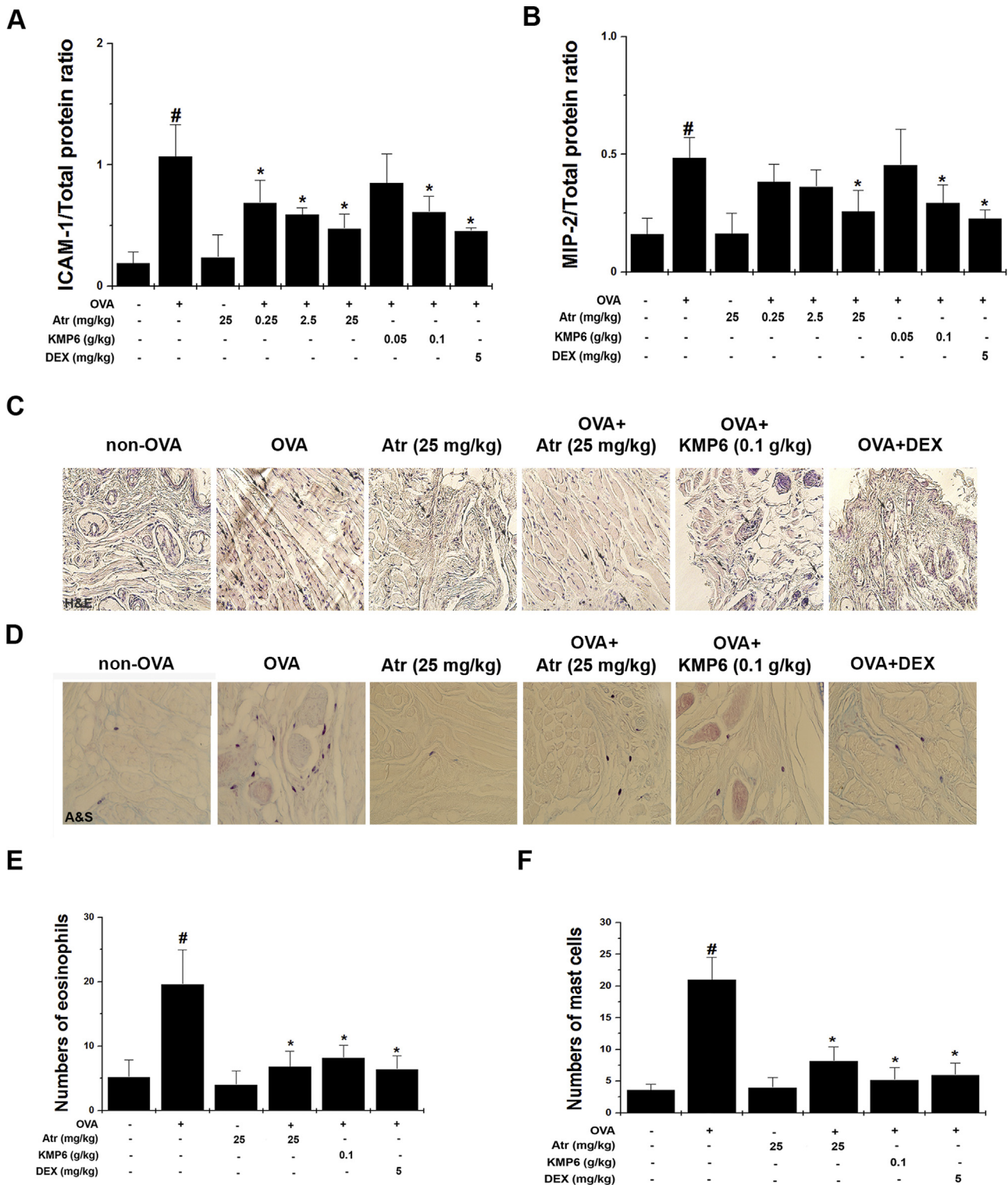


Fig. 6. Inhibitory effect of Atr on infiltration of eosinophils and mast cells into the nasal mucosa tissues. (A) ICAM-1 and (B) MIP-2 levels in the nasal mucosa tissues were determined by ELISA. All parameters measured in the tissue homogenate were presented as a ratio to the total protein levels in the nasal tissue. (C) Nasal mucosa stained with H&E (for eosinophils = arrow) and (D) A&S (for mast cells). (Original magnification $\times 400$, scale bar = 100 μm). (E) Eosinophils and (F) Mast cells numbers were counted by two individuals. Afterwards, five randomly selected tissue sections per mouse were counted. Absolute cell numbers are counted as the mean $\pm$ SEM. # $p < 0.05$; significantly different from normal control group. * $p < 0.05$; significantly different from OVA group. Atr, atracyclone; DEX, dexamethasone.

also act on the vasculature, mucous glands, connective tissue, and smooth muscle cells and induce the accumulation of inflammatory cells (Amin, 2012). The chemokines released by mast cells are able to influence eosinophil function *via* signaling pathways such as MAPKs and NF- κ B (Shakoori et al., 2004). Moreover, the

infiltration of inflammatory cells, including eosinophils, mast cells, basophils, and T cells, results in further releases of leukotrienes and histamine, as well as proinflammatory cytokines, COX-2, ICAM-1, and MIP-2, which maintain allergic reactions and extend inflammatory responses (Fuentes-Beltrán et al., 2009; Fukui et al., 2009). The

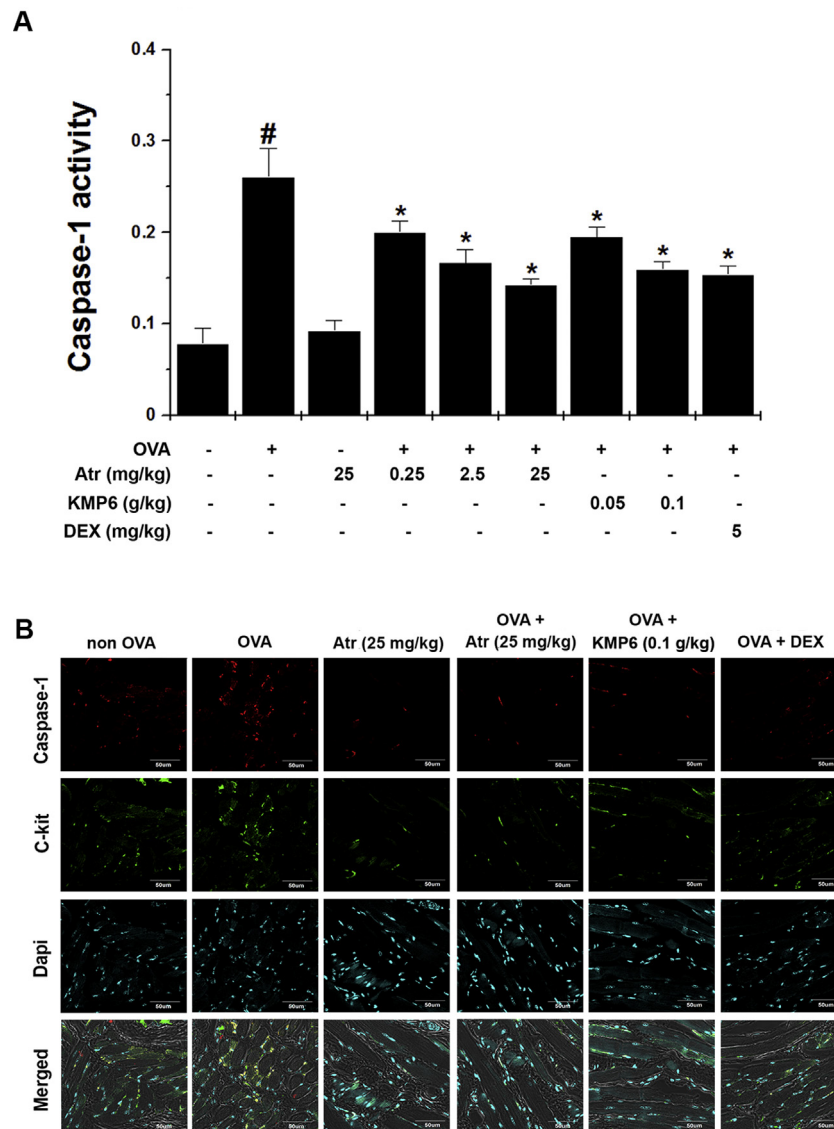


Fig. 7. Inhibitory effect of Atr on caspase-1 activation of the nasal mucosa tissues. (A) Caspase-1 activities in the nasal mucosa tissues were measured using a caspase-1 assay kit. (B) Caspase-1 and c-Kit from the nasal mucosa tissues were assessed by confocal laser-scanning microscopy. Atr, atractylone; DEX, dexamethasone. (Original magnification $\times$ 400, scale bar = 20 μ m).

activation of caspase-1 also up-regulates inflammation by causing the recruitment of immune cells and the secretion of pro-inflammatory cytokines (Faubel et al., 2007). Interestingly, caspase-1 deficient mice were found to exhibit reduced allergic inflammatory reactions (Martin et al., 2013). In the present study, we showed that mast cell-derived caspase-1 activity in the nasal mucosa tissues of AR mice was significantly higher than that of normal mice, which suggests mast cell suppression may provide a means of treating AR. Furthermore, Atr and KMP6 were observed to block PMACI-caused mast cell activation and to alleviate AR reactions, indicating the anti-allergic effects of Atr and KMP6 are due to the suppression of mast cell activation.

It is usually presumed that a correlation exists between mRNA and protein levels. However, Greenbaum et al. (2003) reported this correlation is poor because of the many complicated post-transcriptional mechanisms involved in converting mRNA into protein. In addition, the mRNA expressions of inflammatory cytokines are regulated by various transcription factors, including NF- κ B, activator protein-1, hypoxia inducible factor-1, NF-IL6, and NF-activated T cells (Galien et al., 1996; Jeong et al., 2003;

Rao, 1994; Fiorini et al., 2000). For most cytokines, the compounds have a much greater effect on cytokine protein levels compared to mRNA. In the present study, we observed that the results by Atr in Figs. 1 and 2 implied a similar effect and NF- κ B activation was also partially inhibited by Atr, although the regulatory effect of Atr on various post-transcriptional mechanisms and on activations of other transcription factors are unknown. Therefore, these results indicated that Atr partially regulates cytokine production and mRNA expression. However, further investigation is required to clarify more precisely regulatory effect of Atr on the transcriptional and post-transcriptional mechanisms responsible for the expressions of inflammatory cytokines.

AR develops as a result of complex interactions between environments and genes, and currently affects up to 40% of the populations of industrialized nations. The IgE- Fc ϵ RI -mast-cell pathway acts an important role in the pathogenesis of AR and many anti-allergic drugs target this pathway. Drugs that do so are highly effective at blocking the biological effects of histamine degranulated from mast cells, preventing mast cell activation by blocking IgE, or stabilizing mast-cells (McKay and van Oosterhout, 2005).

Although anti-allergic drugs (antihistamines and steroids) are generally effective, most of these drugs have many serious adverse side-effects and exert only transient effects. For that reasons, many alternative therapeutic strategies have been devised to treat AR. Herbal remedies and contained compounds are broadly used to prevent and cure many diseases, and have few side effects and low cytotoxicities, and thus, much research is being performed in the area. As described above, KMP6 consists of 6 different herbs. In our previous studies, we reported that hesperidin (an active component of *Citrus sunki*), glycyrrhizic acid (an active component of *Glycyrrhiza uralensis*), or atractylenolide III (an active compound of *Atractylodes japonica*) reduced systemic anaphylaxis, atopic dermatitis, or AR by inhibiting caspase-1/MAPKs/NF- κ B signaling pathways in mast cells and in AR animal models (Han et al., 2012; Jeong et al., 2011; Kang et al., 2011; Oh et al., 2012). Interestingly, these compounds are also antioxidants (Hirata et al., 2005; Li et al., 2007; Ojha et al., 2016). Atr, a main sesquiterpene constituent of *Atractylodes japonica*, has been previously reported to have an anti-oxidant effect (Hwang et al., 1996), but the anti-allergic inflammatory effects of Atr have not been previously investigated. Here, we report for the first time Atr, an active compound of KMP6, can regulate AR reactions, and thus, we suggest Atr be considered a new therapeutic for the treatment of AR.

5. Conclusions

The anti-allergic inflammatory activity of Atr and KMP6 were found to be due to the suppression of inflammatory cytokine production by blocking caspase-1/NF- κ B/MAPKs signaling in activated HMC-1 cells. In addition, Atr and KMP6 were observed to improve clinical symptoms and to reduce the expression of AR-associated biomarkers and the recruitment of inflammatory cells in AR mice. Our results show Atr is an active constituent of KMP6 and that it should be considered a potential treatment for AR. However, further studies are needed to determine effects of Atr and its molecular mode of action prior to its clinical use in humans.

Disclosures

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2015R1D1A1A01056607).

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