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Isoegomaketone Ameliorates Atopic Dermatitis via MAPK and STAT Pathway-based Pro-Inflammatory Cytokine Regulation

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Abstract Isoegomaketone (IK), isolated from the radiation-induced mutant cultivar of *Perilla frutescens* var. *crispa*, is a major phytochemical compound that has attracted attention in pharmacological research. In this study, we demonstrated that IK exerts anti-inflammatory and protective effects on human mast cells and in an atopic dermatitis mouse model. IK inhibited tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-8 expression in human mast cells (HMC-1) stimulated with phorbol myristate acetate (PMA) and calcium ionophore A23187 (PMACI). IK significantly reduced the PMACI-induced phosphorylation of ERK and JNK, but not p38. IK also inhibited the PMACI-induced phosphorylation of STAT1 and STAT3. Oral administration of IK in atopic dermatitis mouse model ameliorated skin inflammation severity, as measured by skin thickness and pro-inflammatory cytokine levels such as TNF-α, IL-8, IL-4, and IL-13. These results might suggest that IK is a potent therapeutic agent against skin inflammation and atopic dermatitis.

Key words: Anti-inflammation, Atopic dermatitis, Isoegomaketone, mast cell, *Perilla frutescens*

1. INTRODUCTION

Perilla frutescens (L.) Britt. is an annual herbal plant of the Lamiaceae family commonly found in Asian countries, including South Korea, China, India, and Japan [1]. The plant has various applications, such as in cooking, cosmetics, and products with specific aromatic and medical requirements. The leaves of *P. frutescens* have been used in treating various symptoms, such as cough, cold, depression-related symptoms, vomiting, asthma, fever, chest stuffiness, and indigestion [2]. *P. frutescens* contains various active constituents, such as essential oils, pigments, rosmarinic acid, luteolin, catechin, and ferulic acid [2]. Several studies have demonstrated the physiological effects of herbal extracts/compounds [1-3]. We previously developed a new cultivar of *P. frutescens* var. *cispa*, named "Antisperill," by radiation mutation breeding, which triggered changes in the composition of ingredients toward a higher content of isoegomaketone (IK)—an essential oil in the leaves [4]. In a previous study, we established a method to effectively extract and separate IK from 'Antisperill' using a supercritical carbon dioxide ($SC\text{-}CO_2$) fluid [5], and determined the biological activities of the isolated IK. IK inhibited the production of an inflammatory factor, nitric oxide (NO), in lipopolysaccharide (LPS)-stimulated RAW264.7 cells and BALB/c mice [6]. IK enhanced apoptosis in DLD1, SK-MEL-2 human melanoma, and B16 melanoma cells [7-9]. In addition, it activated Nrf2 via ROS generation, resulting in increased expression of antioxidant enzyme such as heme oxygenase-1 (HO-1), catalase (CAT), glutathione S-transferase (GST), and NADH quinone oxidoreductase (NQO-1) [10]. It inhibited 3T3L-1 differentiation into adipocytes,

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and reduced body weight and visceral fat in a high fat diet mouse model [11]. It also effectively treated skin wounds in human keratinocytes [12]. Futhermore, IK alleviated inflammatory disease-associated symptoms in animal models, reduced arthritis symptoms in a collagen antibody-induced arthritis (CAIA) model [13], and ameliorated dextran sodium sulfate-induced ulcerative colitis in mice [14]. Atopic dermatitis (AD), also called "eczema" and "atopic eczema", is a major chronic and relapsing inflammatory cutaneous disease affecting individuals of all ethnicities and ages, but primarily younger age groups [15]. The proportion of the adult population with AD is 10~20% in developed countries. The etiology of AD is highly complex and multifactorial and includes genetics, nutrient status, lifestyle, and immune and environmental factors [16-18]. Given these complex features, a safe and effective treatment modality for AD remains elusive. Several treatments, such as gels and creams, have been used to treat AD [19]. These treatments reduce inflammation; however, their effectiveness is limited and they have certain side effects. For example, continuous and repeated application of topical glucocorticosteroids results in skin thinning and atrophy [20]. Therefore, there is a need for the development of safe AD treatments. Recently, the use of natural products such as herbs, ginseng extracts, and bee venom for AD treatment has gained considerable interest [21,22].

Mast cells are located in the skin and mucous membrane and play an important role in cutaneous diseases such as AD because they trigger an immune response against invasive pathogens [23]. Mast cells react to external stimuli and activate dendritic cells, macrophages, T cells, and B cells to trigger an immune response; however, inappropriate activation can lead to autoimmune and allergic diseases [24]. In patient with AD and AD-like mouse models, the number of mast cells is increased [25,26]. In addition, skin inflammatory response is lower in mast cell-deficient mice [27]. These results indicate that mast cells are important in atopic diseases. Mast cell-produced interleukin-4 (IL-4) and IL-13 are associated with pruritus, depression, and anxiety symptoms in patient with AD [28].

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) family proteins play an essential role in immune regulation. The JAK-STAT pathway transfers external signals to the intracellular region by binding to specific receptors [29]. In addition, mitogen-activated protein kinase (MAPK) plays a pivotal role in cutaneous inflammation. The activation of this signaling pathway through multiple cytokines, growth factors, and interferons plays essential roles in the cell life cycle, including cell proliferation, differentiation, survival, and apoptosis [30]. MAPKs are comprised of three classes, c-Jun N-terminal kinase (JNK), ERK1/2, and p38. The important roles of the JAK-STAT and MAPK signaling pathway suggest a potential role for their dysregulation in AD. These signaling pathways may be upregulated during skin inflammation, such as in AD, and induce the overexpression of inflammatory mediators, which can adversely affect immune function [31,32]. Therefore, effective therapeutic agents against AD should decrease the overactivation of the MAPK and STAT pathways.

Previously, we demonstrated that IK has anti-inflammatory effects in the LPS-stimulated macrophage cell line RAW264.7 [6]. Based on the importance of macrophages in the pathogenesis of AD [33], we hypothesized that the anti-inflammatory activity of IK would exert therapeutic effects against AD. Specifically, we assessed the anti-inflammatory effects of IK in the human mast cell line HMC-1 and an AD-like mouse model.

2. MATERIALS AND METHODS

2.1. Preparation of isoegomaketone

IK was isolated using the supercritical carbon dioxide ($SC\text{-}CO_2$) extraction from the radiation-induced mutant cultivar of *P. frutescens* var. *crispa* [5]. IK was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, ST. Louis, MO, USA) at a concentration of 1 g mL^{-1} as stock. For oral administration, the IK stock was diffused in PBS containing 0.5% Tween 20 using ultrasonication (300 μm, 15 min).

2.2. Materials

Specific monoclonal antibodies against ERK, JNK, p38, STAT1, STAT3, phospo (p)-STAT1, p-STAT3, p-JNK, p-ERK, and p-p38 were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-β-tubulin monoclonal antibody was obtained from Santa Cruz Biotechnology (Houston, TX, USA). Phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 were purchased from Sigma-Aldrich (USA).

Table 1. Antibody information chart

Name of antibody	Company	Mono/polyclonal	Molecular weight (kDa)	Source	Dilution for WB
JNK	Cell signaling	Mono	46/54	Rabbit	1 : 1,000
p-JNK	Cell signaling	Mono	46/54	Rabbit	1 : 1,000
Erk1/2	Cell signaling	Mono	42/44	Rabbit	1 : 1,000
p-Erk1/2	Cell signaling	Mono	42/44	Rabbit	1 : 1,000
p38	Cell signaling	Mono	40	Rabbit	1 : 1,000
p-p38	Cell signaling	Mono	43	Rabbit	1 : 1,000
STAT1	Cell signaling	Mono	84/91	Rabbit	1 : 1,000
p-STAT1	Cell signaling	Mono	84/91	Rabbit	1 : 1,000
STAT3	Cell signaling	Mono	79/86	Rabbit	1 : 1,000
p-STAT3	Cell signaling	Mono	79/86	Rabbit	1 : 1,000
β-tubulin	Santa cruz	Mono	55	Mouse	1 : 500

2.3. Cell culture

The human mast cell line HMC-1 was obtained from the Korean Cell Line Bank (South Korea) and cultured in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA) and 1% penicillin-streptomycin (Gibco, USA) at 37°C in 5% CO_2 atmosphere.

2.4. Cell viability assay

HMC-1 cells (2×10^5 cells mL^{-1}) were seeded in a 96-well plate and incubated for 24 h at 37°C in a 100 μL volume. The cells were treated with IK at various concentrations (0~50 μM) for 24 h at 37°C. Cell viability was measured using the Ez-Cytox assay kit (Daeilbio, Suwon, South Korea) according to the manufacturer's protocol. Briefly, the cells were incubated with 10 μL of Ez-Cytox reagent for 3 h at 37°C in 5% CO_2 atmosphere, and absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Western blot analysis

The cells were lysed with RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (Sigma-Aldrich), phosphatase inhibitor cocktail 2 (Sigma-Aldrich), phosphatase inhibitor cocktail 3 (Sigma-Aldrich), and phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich). The concentration of the protein lysate was quantified using Bradford reagent (Bio-Rad). Protein samples of equal amounts (50 μg) were separated by 10% SDS-PAGE, transferred to a PVDF membrane blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich) for 1 h at room temperature, incubated with the primary monoclonal antibodies (1 : 1000; JNK, p-JNK, ERK, p-ERK, p38, p-p38, STAT1, p-STAT1, STAT3, and p-STAT3; Cell Signaling Technology) and β-tubulin monoclonal antibody (1:500, Santa Cruz Biotechnology) overnight at 4°C, and finally incubated with secondary antibodies (1 : 10,000) at room temperature (Table 1). A washing step with TBS-T was included in all processes. The membranes were incubated with enhanced chemiluminescence (ECL), and the signals were detected using a Chemiluminescence imaging system (iBright CL1000, Invitrogen).

2.6. Reverse transcription-polymerase chain reaction

Total RNA was isolated from HMC-1 cells and mouse skin tissue using RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Maebashi, Japan) according to the manufacturer's protocol. Quantitative real-time PCR was performed using TB Green Premix Ex Taq II (Takara). Reaction conditions were set according to the manufacturer's instructions using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The primer sequences are shown in Table 2. We used GAPDH as a house keeping gene to compare the relative gene expression in each group.

Table 2. Primer sequences used for reverse transcription-quantitative PCR

Gene	Forward primer sequence (5′→3′)	Reverse primer sequence (5′→3′)
Mouse		
TNF-α	AAGCCTGTAGCCCACGTCGTA	GGCACCACTAGTTGGTTGTCTTTG
IL-8	GACTCTTGCGTCAACTTCAAGG	CAGGCTGTCTTTTGTCAACGA
IL-4	CATCGGCATTTTGAACGAGGTCA	CTTATCGATGAATCAGGCATCG
IL-13	CCAGGTCCACACTCCATACC	TGCCAAGATCTGTGTCTCTCC
GAPDH	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA
Human		
TNF-α	CCCGAGTGACAAGCCTGTAG	GATGGCAGAGAGGAGGTTGAC
IL-6	AGAGTAGTGAGGAACAAGCC	TACATTTGCCGAAGAGCCCT
IL-8	CATACTCCAAACCTTTCCACCCC	TCAGCCCTCTTCAAAAACTTCTCCA
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

2.7. ELISA

TNF-α, IL-8, IL-6, IL-4, and IL-13 cytokine levels were measured using the corresponding ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. HMC-1 cells were pretreated with various concentration of IK for 2 h and incubated for another 24 h. Supernatants were collected for ELISA analysis.

2.8. Animals

A total of 16 5-week-old male BALB/c mice were obtained from G-Bio (Gwangju, South Korea). The mice were housed under conventional conditions at 23 ± 2°C and 60 ± 5% humidity. All experiments were conducted with the approval of the Animal Experimental Committee of the Korea Atomic Energy Research Institute (KAERI, approval no. KAERI-IACUC-2020-020).

2.9. Induction of atopic dermatitis-like mouse model

Mice were randomly divided into four groups: PBS-tween 20 (n = 4), AD (n = 4), AD + IK (5 mg kg^{-1}, n = 4), and AD + IK (10 mg kg^{-1}, n = 4). The dorsal skin of the mice was shaved before applying 0.15% DNFB (100 μL; Sigma-Aldrich) dissolved in acetone/olive oil (3 : 1) on days 1 and 4, then 0.2% DNFB (100 μL) was applied on days 7, 10, and 13. IK administration was started 1 week before the induction of AD and administered for a total of 3 weeks.

2.10. Measurement of serum IgE and skin thickness in atopic dermatitis-like mouse model

After the mice were euthanized, biological samples were collected. Blood samples were collected by orbital sampling and centrifuged to obtain serum. Serum IgE levels were measured using a mouse IgE ELISA kit (R&D Systmes) according to the manufacturer's protocol. The thickness of the dorsal skin tissue was measured using digital caliper (Mitutoyo, Andover, UK).

2.11. Histopathological assessment

The excised skin tissues were obtained and fixed in 10% formalin (Sigma-Aldrich). The skin was embedded in paraffin blocks and were sliced into 5 μm sections. Next, the skin sections were stained with H&E. Histopathological examinations were performed under a light microscope and photographed.

2.12. Statistical analysis

Data are presented as the mean ± SD. Statistically significant differences were calculated using one-way ANOVA with Tukey's post-hoc test in Prism 5 (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Effects of IK on HMC-1 cell viability

Mast cells are major participants in skin inflammation [34]. To determine the therapeutic effects of IK on skin inflammation, we used HMC-1 cells. The structure of IK is shown in Fig. 1A. To determine the nontoxic concentration, HMC-1 cells were treated with a concentration gra-

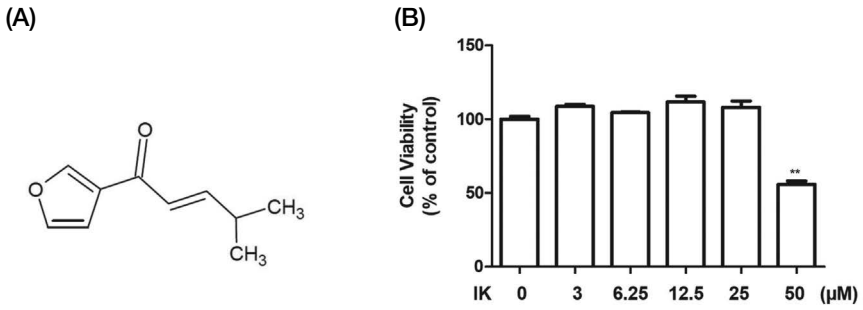

Fig. 1. Chemical structure of IK and its effects on HMC-1 cell viability. (A) Chemical structure of IK. (B) Cell viability determined by an EZ-Cytox cell viability assay kit. Cells were treated with various concentrations of IK for 24 h. Control group were treated with only DMSO. Data are presented as the mean ± SD of four replicates of one representative experiment. $^{**}p < 0.01$ vs control group.

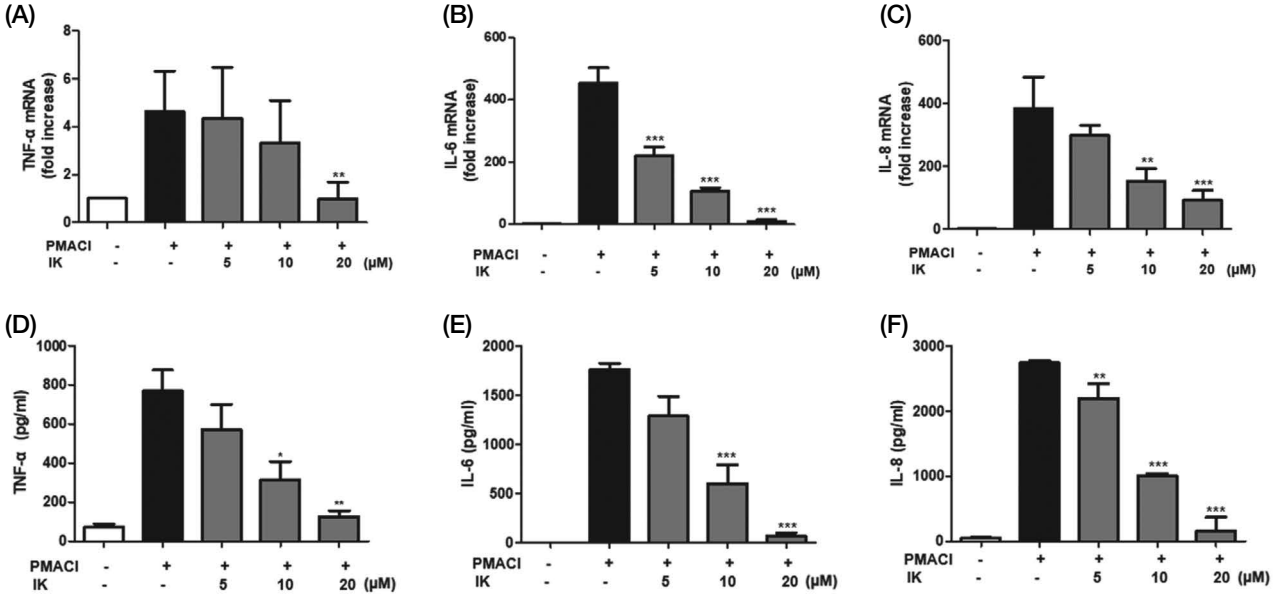

Fig. 2. Effect of IK on PMACI-induced pro-inflammatory cytokine mRNA and protein expression in HMC-1 cells. HMC-1 cells were pretreated with various concentrations (5, 10, and 20 μM) of IK for 2 h and incubated with or without PMACI (20 nM PMA and 1 μM A23187) for another 24 h. The mRNA levels of tumor necrosis factor (TNF)-α (A), interleukin (IL)-6 (B), and IL-8 (C) were determined by RT-PCR. Supernatants were collected, and TNF-α (D), IL-6 (E), and IL-8 (F) production were determined by ELISA. Data are represented as the mean ± SD of triplicate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. PMACI-stimulated group.

dient of IK (0~50 μM) for 24 h. IK did not exhibit cellular toxicity at concentration below 25 μM (Fig. 1B); thus, we conducted the following experiment at an IK concentration of 20 μM or less.

3.2. Effects of IK on pro-inflammatory cytokine expressions in HMC-1 cells

Many immune stimulators induce the expression of pro-inflammatory cytokines in mast cells, which contribute greatly to the pathogenesis of AD [35]. We investigated the anti-inflammatory effects of IK on PMA + A23187 A (PMACI)-induced mast cells, based on the expression of pro-inflammatory cytokines [36]. IK treatment reduced the PMACI-induced mRNA levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-8 in a dose-dependent manner (Fig. 2A-C). These results demonstrate the effective transcriptional regulation of inflammatory mediators in mast cells by IK under inflammatory conditions. An ELISA showed that IK dramatically reduced the expression of secreted TNF-α, IL-6, and IL-8 proteins in PMACI-induced HMC-1 cells (Fig. 2D-F). This demonstrates that IK inhibits the expression of pro-inflammatory cytokines at

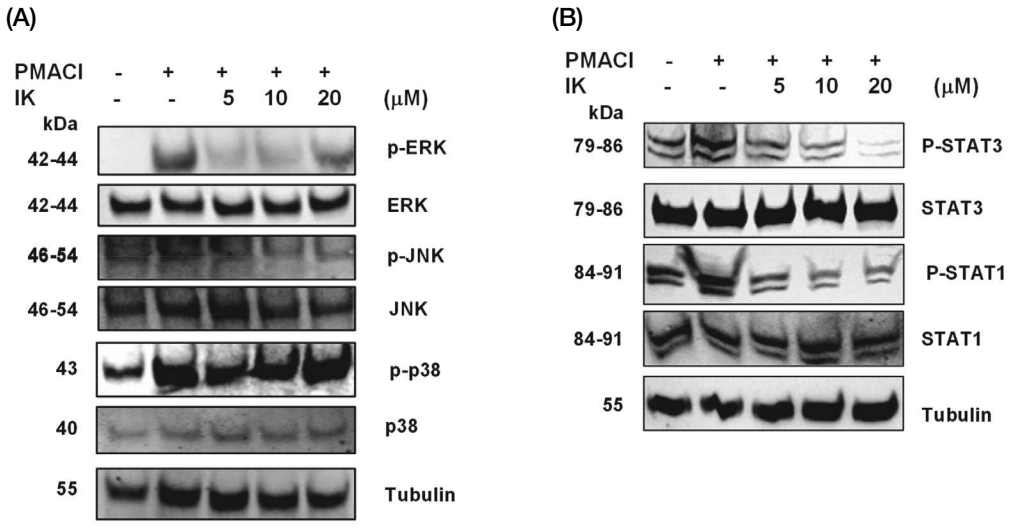

Fig. 3. Effect of IK on PMACI-induced MAPK (A) and STAT (B) signaling in HMC-1 cells. HMC-1 cells were pretreated with various concentrations (5, 10, and 20 μM) of IK for 2 h and incubated with or without PMACI (20 nM PMA and 1 μM A23187) for another 2 h. MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription.

the mRNA and protein levels in HMC-1 cells.

3.3. Effects of IK on inflammatory signaling pathways in PMACI-induced HMC-1 cells

The MAPK and STAT pathways are crucial in the pathogenesis of AD [31,32], and their signaling participates in the production of pro-inflammatory cytokines [35]. We analyzed the effects of IK on the activation of MAPK and STAT pathway-induced PMACI by western blotting. Cells stimulated with PMACI exhibited MAPK phosphorylation (Fig. 3A). IK dramatically reduced the PMACI-induced phosphorylation of ERK and JNK, but inhibition of p38 phosphorylation was unaffected. Subsequently, we investigated whether IK inhibits the activation of STAT-related pathways. IK significantly reduced the PMACI-induced phosphorylation of STAT1 and STAT3 in a dose-dependent manner (Fig. 3B). These results suggest that IK exerts anti-inflammatory effects by inhibiting MAPK and STAT-related pathways, which are essential in the pathogenesis of skin inflammation.

3.4. Protective roles of IK in an atopic dermatitis-like mouse model

To measure the therapeutic effects of IK on AD-like lesions, we used a 2,4 dinitrofluorobenzene (DNFB)-induced AD BALB/c mouse model [37] and orally administered IK (5 and 10 mg kg^{-1}) (Fig. 4A). Repeated application of DNFB solution to the dorsal skin triggered various AD-like symptoms, such as erythema, dryness, thickened skin, and redness. Dorsal images of mice showed that IK alleviated the severity of dorsal inflammation, redness, and loss of skin slough. Hematoxylin and eosin (H&E) staining also showed a dose-dependent protective effect of IK against AD-related erosion and ulcers (Fig. 4B). Dorsal skin thickness (measured to assess inflammation severity) was significantly lower in the IK-treated group than that in the AD group. These results demonstrate that IK regulates the severity of inflammation in DNFB-treated mouse skin (Fig. 4C). Next, we measured IgE levels in mouse serum. Oral treatment with IK reduced DNFB-induced upregulation of IgE levels, though not significantly (Fig. 4D). These results suggest that IK exerts protective effects against AD-like lesions.

3.5. Effects of IK on expression of pro-inflammatory cytokines in mouse dorsal skin

Inflammatory cytokine levels are elevated in patients with AD, reflecting the severity of skin inflammation [38,39]. We compared the relative mRNA levels of TNF-α, IL-8, IL-4, and IL-13 between treatments and found that they increased in the dorsal tissue of DNFB-induced AD-like mice, whereas IK treatment reversed this effect in a dose-dependent manner (Fig. 5). Collectively, these results showed that IK reduced the production of inflammatory cytokines and had protective effects in DNFB-induced AD.

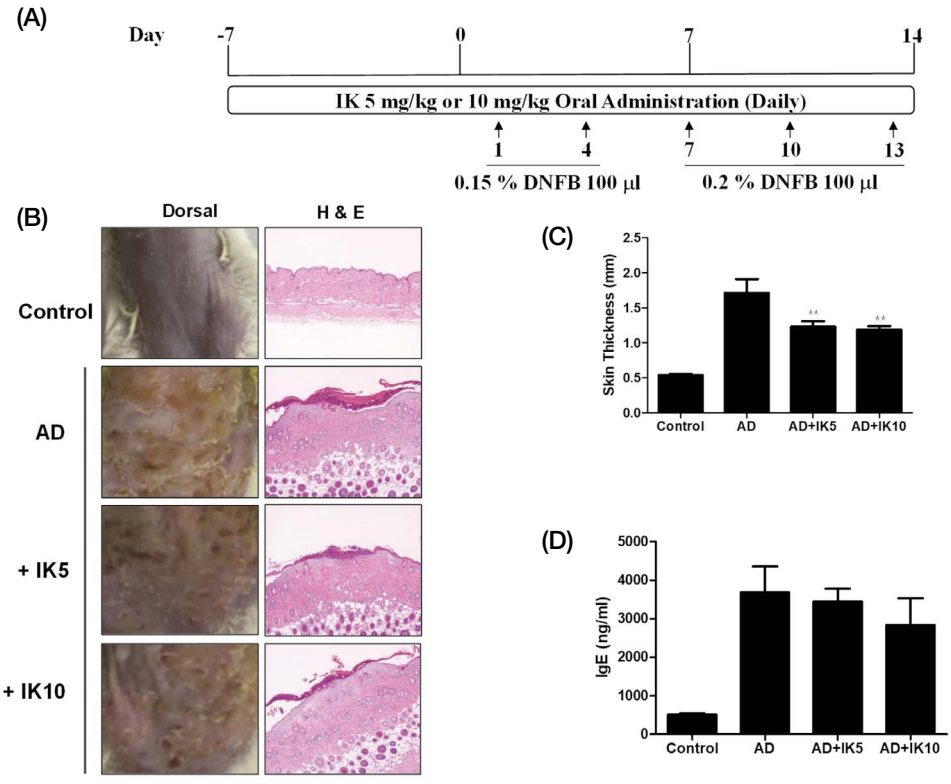

Fig. 4. IK improved the clinical symptoms in DNFB-induced AD mice via oral administration. (A) Schematic representation of the experimental procedures for generating the AD-like mouse model by topical applications of DNFB on the dorsal skin and oral treatment with IK. (B) Phenotypic features of dorsal skin and representative histologic H&E staining of lesioned skin with and without the topical skin sensitizer DNFB and treatment with IK at different concentrations. After the mice were euthanized, the thickness of the dorsal skin (C) was measured using a digital caliper, and the serum IgE levels (D) were measured by ELISA. $^{**}p < 0.01$ vs. DNFB-induced group, n = 4.

4. DISCUSSION

AD is a chronic inflammatory skin disorder that occurs in 2.1~4.9% in adults and 2.7~20.1% in children worldwide [40,41]. IK isolated from Antisperill is a fat-soluble and low-molecular-weight substance that showed anti-inflammatory efficacy in RAW264.7 cells [6]. In animal models of inflammation-related diseases, such as collagen antibody-induced arthritis and ulcerative colitis, oral administration of IK was shown to relieve the symptoms [13,14]. Based on these previous studies, we hypothesized that IK would exert a protective effect in atopic dermatitis.

Mast cells are bone marrow-derived hematopoietic cells and are important immune cells that act through the circulatory system [34]. Mast cells have been reported to participate in a wide range of innate and adaptive immune responses; particularly, they play a major role in allergic responses induced by IgE [42,43]. Mast cells express a high-affinity receptor for IgE (FcεRI) on their surface, which is activated by aggregation or crosslinking with IgE [44]. In AD, mast cells have been reported to be involved in skin barrier dysfunction [45], type 2 inflammation [46], and pruritus [47]. Therefore, before examining whether IK affects AD, we first investigated whether IK suppresses the inflammatory response in human mast cells. PMACI participates in the crosslinking of IgE in mast cells, causing FcεRI aggregation and rapid tyrosine phosphorylation, resulting in mast cells activation and degranulation [48]. The expression of inflammatory cytokines, such as TNF-α, IL-6, and IL-8, increased in HMC-1 cells treated with PMACI and decreased in a dose-dependent manner when treated with IK (Fig. 2). Therefore, IK effectively suppressed the inflammatory responses generated by the activation of mast cells at non-toxic concentrations.

MAPK family members, such as p38, JNK, and ERK, participate in the expression of inflammatory cytokines during aggregation and activation of the mast cell FcεRI [31]. To confirm that the inhibition of inflammatory cy-

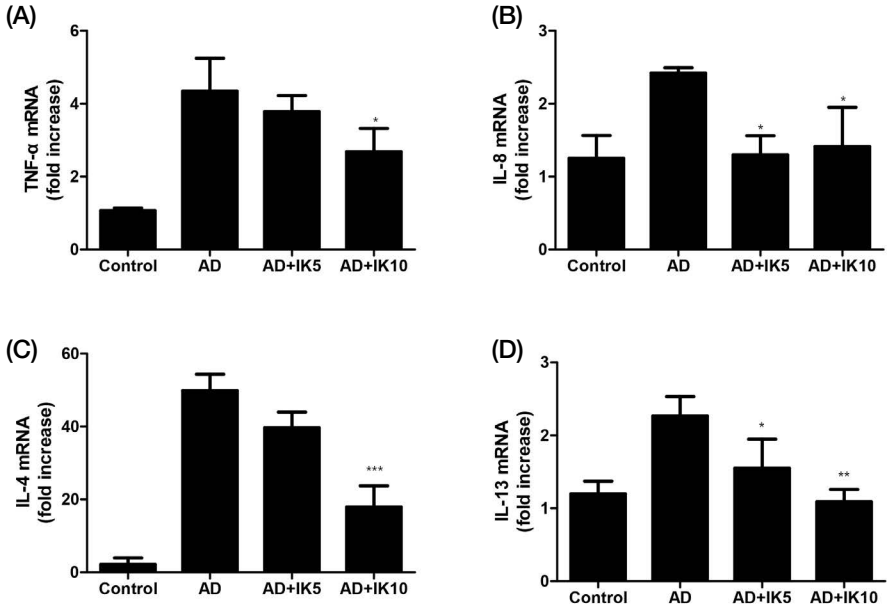

Fig. 5. Oral administration of IK ameliorated the pro-inflammatory cytokine expression in the dorsal skin of DNFB-induced AD mice. TNF-α (A), IL-8 (B), IL-4 (C), and IL-13 (D) mRNA expression measured via quantitative real-time PCR. Data are represented as the mean±SD of triplicate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. DNFB-induced group.

tokine production in PMACI-stimulated HMC-1 cells occurred via the MAPK pathway, we demonstrated that the activities of ERK and JNK were reduced by IK treatment (Fig. 3A). STATs are transcription factors that regulate the inflammatory response. When phosphorylated by an external stimulus, STAT is converted into a dimer that enters the nucleus to participate in the expression of inflammatory genes [49]. STAT1 and STAT3 are closely associated with chronic inflammation [50]. PMACI stimulation of HMC-1 cells enhanced the phosphorylation of STAT1 and STAT3, whereas IK reversed this effect in a dose-dependent manner (Fig. 3B). Therefore, IK suppressed the expression of inflammatory cytokines in PMACI-induced HMC-1 cells by inhibiting the MAPK and STAT pathways.

AD was induced in BALB/c mice using DNFB—a super antigen that can cause inflammation in the skin without haptenization and induces a mainly Th1-biased immune response in BALB/c mice [51]. The symptoms of contact dermatitis include itching, erythema, and blisters, while histopathological findings include spongiosis in the epidermis and a dermal inflammatory reaction with inflammatory cells infiltrating the blood vessels. The histological findings of contact dermatitis generally differ depending on the stage of dermatitis. In the acute stage, more spongiosis is found in the tissue. However, as the chronic stage progresses, spongiosis decreases and acanthosis, keratinization, and crust formation increases. In this study, the repeated DNFB treatment induced a large number of scales and crusts, along with slight hemorrhages, along massive immune cell infiltration (Fig. 4B). Based on this, it is believed that the animal model used in this study embodies the symptoms of moderate to severe, chronic contact dermatitis. In contrast, IK significantly reduced the thickness of the affected skin and tended to suppress the symptoms of contact dermatitis, such as erythema and crusting (Fig. 4B, C). Histopathological analysis revealed a tendency to suppress immune cell infiltration (Fig. 4B). DNFB significantly increased serum IgE levels. Although IK treatment lowered the average serum IgE concentration, the difference was not significant (Fig. 4D). We also confirmed the increased expression of pro-inflammatory cytokines in DNFB-treated skin tissue and the dose-dependent anti-inflammatory effect of IK (Fig. 5). Therefore, the oral administration of IK improved the DNFB-induced AD-like symptoms in mice.

In this study, IK was administered orally to an AD-like mouse model. The first-pass effects, in which the concentration of the drug is reduced through absorption in the intestine and metabolism in the liver, cannot be avoided. Because pharmacokinetic experiments using IK were not conducted in mice, the actual IK concentration in mouse blood remains unknown. Thus, there is a possibility that

transdermal IK administration may be more effective than oral administration in the treatment of AD. IK is a fat-soluble substance with a low molecular weight; therefore, transdermal administration is appropriate. In fact, we found that IK passed through mouse skin tissue. Unlike oral administration, transdermal administration is easy and patient-friendly. Transdermal administration is self-administrative and allows the patient to discontinue use if side effects occur. Other studies that investigated the efficacy of AD treatment using natural products also showed the activities of topical treatment [52]. However, to develop a transdermal agent, efforts are required to overcome IK-associated disadvantage, which can be challenging as IK is a volatile substance with a unique scent of *Perilla frutescens*. Once IK is confirmed to be an effective AD treatment, we aim to conduct further experiment to evaluate transdermal administration. The findings would guide the development of products such as patches, and ointments for treating AD.

5. CONCLUSIONS

This study demonstrated that IK had an anti-inflammatory effect on PMACI-stimulated HMC-1 cells. IK suppressed the mRNA and protein expression of the pro-inflammatory cytokines, TNF-α, IL-6, and IL-8 and inhibited the activation of ERK, JNK, STAT3, and STAT1 but not p38. We confirmed the protective effects of IK in vivo in an AD-like mouse model. Oral administration of IK reduced skin thickness, IgE levels, and inflammatory cytokine expression in DNFB-induced AD. Altogether, these results suggest that IK is a promising candidate for the treatment of skin inflammation, such as in AD.

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