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ORIGINAL ARTICLE

Alleviation of Atopic Dermatitis Lesions by a Benzylideneacetophenone Derivative *via* the MAPK Signaling Pathway

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Abstract— This experiment was conducted to investigate the effects of a benzylideneacetophenone derivative ((2E)-3-(4-hydroxy-3-methoxyphenyl)phenylpro-2-en-l-one (JC3)) on trimellitic anhydride (TMA)–induced atopic dermatitis (AD)–like symptoms in mice. To induce AD, the dorsal skins of mice were treated with 5% TMA on day 0 and both ears were treated with 5% TMA on day 5 and with 2% TMA from day 6 to day 14. JC3 (1, 5, 10 mg/kg, i.p.) was treated once daily from day 9 to day 14 before TMA treatment. Histological analysis was performed and auricular lymph node weights, ear thicknesses, skin water contents, scratching behaviors, and serum immunoglobulin (IgE) and IFN-γ, and interleukin-4 (IL-4) levels in serum and ear tissues were determined. In addition, the anti-AD activity of JC3 was investigated on phorbol 12-myristate 13-acetate (PMA)–stimulated human mast cells (HMC-1 cells) derived from patients. Levels of TNF-α, IL-4, and mitogen-activated protein kinase (MAPK) were investigated after treating cultured cells with JC3. Treating mice with JC3 (10 mg/kg) significantly decreased ear thicknesses, lymph node weights, skin scores, skin water contents, scratching behavior, and IFN-γ, IL-4 cytokine levels, and serum IgE levels. Moreover, treatment with JC3 (10 mg/kg) significantly decreased serum and ear tissues levels of IFN-γ and IL-4 in AD mice. Furthermore, treatment with JC3 at 10 μg/ml reduced TNF-α and IL-4 levels and decreased MAPK phosphorylation in the HMC-1 cells. The results of this study provide a molecular basis for developing new therapeutics for the treatment of various inflammatory diseases, such as, eczema, asthma, and AD.

KEY WORDS: atopic dermatitis; benzylideneacetophenone derivative; trimellitic anhydride; interleukin-4; mitogen-activated protein kinase.

INTRODUCTION

Atopic dermatitis (AD) is a skin disease that causes severe recurrent itching [21, 30]. Although previous studies have addressed the pathogenesis of AD, its etiology is not yet understood. AD is characterized by eczema, dermatitis and itching, elevated levels of serum immunoglobulin E (IgE), and the infiltrations of immune cells, such as mast cells, eosinophils, neutrophils, and skin lymphocytes [26]. Recently, some authors have suggested the symptoms of AD are associated with elevated levels of type 2 helper T cell type 2 (Th2) cytokines, such as interleukin (IL)-4, IL-5, and IL-13, and the release of IgE from B cells and its binding to mast cells (MCs), which are the major effectors of IgE-mediated hypersensitivity [13]. Infiltration of the upper dermis by mast cells is a characteristic of atopic skin [25]. Th2 cells mediate IgE-dependent

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mast cell activation and degranulation [15], and immune responses mediated by Th2 cytokines are central to the pathogenesis of AD, because IgE upregulation, a primary cause of AD, is regulated by the Th2 cytokine, IL-4 [14].

Various simple and highly reproducible models, such as transgenic mice and hapten and allergen induction, can be used to induce AD, and trimellitic anhydride (TMA) is a known sensitizer that induces T cell–dependent contact hypersensitivity in mice, T cell infiltration, Th2 cytokine production, and IgE release [22]. In TMA-induced atopic dermatitis models, mice are sensitized by application to dorsal skin and T cell–dependent AD is induced by applying it to ears. In such models, AD-like inflammation can easily be rated by measuring ear thicknesses. For these reasons, the effects of TMA-induced inflammation on immune cell infiltration, cutaneous cytokine profiles, and serum IgE levels have been intensively studied [6, 19].

AD is commonly treated with steroids, anti-histamines, and immunosuppressive agents, but the repeated use of these agents can create susceptibility to infection, adrenal suppression, and skin atrophy [4]. Accordingly, it has been suggested natural medicines might be more effective and have fewer side effects [18].

Yakuchinone B is a component of the seeds of *Alpinia oxyphylla* and a conjugated 1,4-enone (a type of natural chalcone with wide-ranging biological activities) containing a phenyl ring [28]. According to studies, yakuchinone B has significant antitumor [5], antiviral [16], and anti-inflammatory [23] activities, and the benzylideneacetophenone derivative JC3 (JC3) was synthesized by structurally modifying yakuchinone B with a view toward developing a novel anti-inflammatory agent [17].

The physiological effects of JC3 are not well understood and no study has yet investigated the anti-AD effects of JC3 in an animal model of AD. In the present study, we evaluated the ability of JC3 on tetramethylammonium (TMA)-induced AD mouse model and PMA-stimulated human mast cells (HMC-1 cells).

MATERIALS AND METHODS

Isolation and Culture of HMC-1 Cells

HMC-1 cells (a human mast cell line) were obtained from the Korea Collection for Type Culture (Daejon). HMC-1 cells were grown in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum and 1% (*v*/*v*) penicillin/streptomycin in a 5% CO_2/95% air humidified atmosphere at 37 °C.

Animals

Twelve-week male Balb/c mice weighing 28–30 g were obtained from Samtaco Animal Co. (Osan, South Korea) and housed in a limited-access rodent facility at up to five mice per polycarbonate cage at 22 ± 2 °C and a RH of 55 ± 15%. Cages were lit using artificial light for 12 h each day. Sterilized drinking water and standard chow were supplied *ad libitum* during the experiments. All experimental procedures were conducted in accordance with the animal care guidelines issued by the Animal Care and Use Committee of Ewha Womans University. Animals were acclimatized for 7 days before experiments.

Reagents

Trimellitic anhydride (TMA, 98%), phorbol 12-myristate 13-acetate (PMA), and isopropyl myristate (98%) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). JC3 was synthesized as described previously [17]. The 4-hydroxy-3-methoxy cinnamaldehyde was protected with tert-butyldimethylsilyl trifluoromethanesulfonate in the presence of 2,6-lutidine or 2-(trimethylsilyl)ethoxymethyl chloride (SEM–Cl)/N,N-diisopropylehylamine to form aldehydes in 95% yields. JC3 was fully identified by infrared and NMR spectroscopy and high-resolution mass spectroscopy [17].

Experimental Schedule and Treatments

AD-like skin lesions were generated by the repeated application of TMA to both sides of both ears of Balb/c mice [20]. Sensitization was induced by applying 5% TMA to dorsal skin subsequently applying 5% TMA to both ears on day 5 and 2% TMA once daily from day 6 to day 14. JC3 (1, 5, or 10 mg/kg, i.p.) was treated 30 min before 2% TMA application daily from days 9 to 14 (Fig. 1).

Experimental Groups

Mice were randomly divided into five experimental groups as follows: non-treated normal group (NOR); vehicle-treated and TMA-induced atopic group (AD); 1 mg/kg JC3-treated and TMA-induced atopic group (AD+JC3-1); 5 mg/kg JC3-treated and TMA-induced atopic group (AD+JC3-5); 10 mg/kg JC3-treated and TMA-induced atopic group (AD+JC3-10).

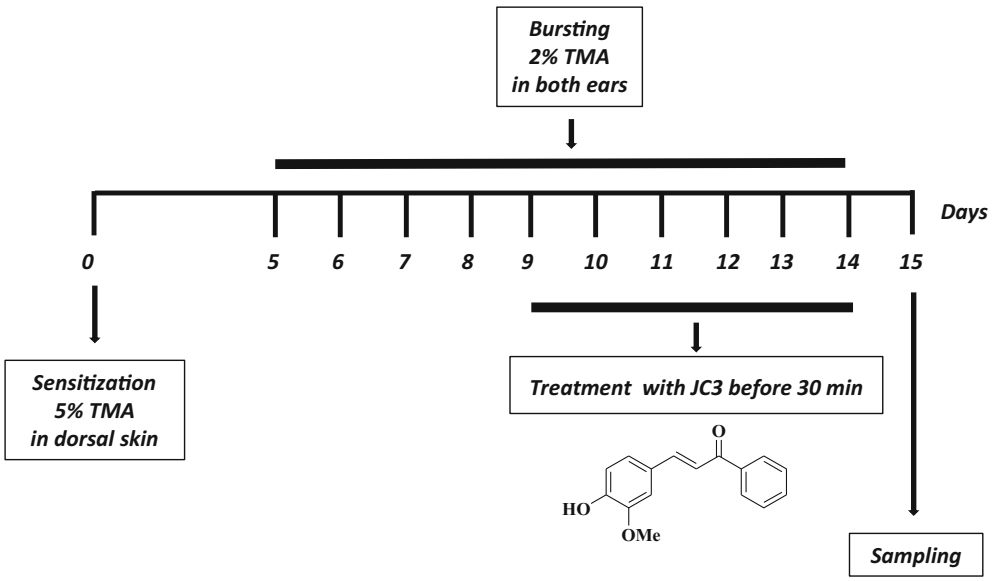

Fig. 1. Schematic diagram of *in vivo* experimental schedules and chemical structure of JC3.

Ear Skin Investigations

Ear skin in each experimental group was photographed using a digital camera (Canon 20D; Canon Inc., Tokyo) to document changes in atopic symptoms and tissue appearances. For hematoxylin and eosin histochemistry, ear skin tissues were sliced, fixed in 10% paraformaldehyde overnight, dehydrated in 99% ethanol, embedded in paraffin, and sectioned at 6 μm (Finesse 325; Thermo Shandon Co., UK). All slides (× 100 magnification) were observed and photographed using a microscope equipped with a digital camera (BX51; Olympus, Tokyo, Japan), and images were analyzed using the DP2-BSW software package (Olympus).

Measurement of Ear Thickness and Auricular Lymph Node Weight Measurements

Ear thickness and auricular lymph node weights of all 10 mice in each experimental group were measured. Ear thickness was measured using a dial thickness gauge (Ozaki Seisakusho Co., Tokyo, Japan) and weights of auricular lymph nodes were measured using a digital balance (Mettler-Toledo Inc., Columbus, OH, USA).

Water Contents

Skin water contents were measured using a Corneometer® (Model CM 825; Courage and Khazaka, Cologne, Germany). Skin areas with the highest coefficient of insulation were chosen for measurements, indicating that the water contents by this protocol are estimating values showing the relative amount of water content. The average of the three measurements for each area was calculated.

Scratching Behavior

Mouse behaviors were recorded for 10 min using a video camera. To prevent disturbance during recording, no one was allowed in the observation room. Numbers of scratching episodes for 10 min were counted by investigators blinded to group classifications and treatments. Scratching with hind paws for ≥ 1 s was defined as one scratching episode.

RT-PCR

After sacrifice on day 15, ear tissues were removed quickly and stored at − 80 °C until required. Total RNA was isolated from ear tissue using TRIzol® Reagent (Invitrogen Co., Carlsbad, CA, USA), according to the supplier's instructions. Complementary DNA (cDNA) was synthesized from total RNA using reverse transcriptase (Takara Co., Shiga, Japan), and the expression levels of the mRNAs of IFN-γ and IL-4 mRNAs were determined by reverse transcription polymerase chain reaction (RT-PCR), which was performed using a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA, USA). All primers were designed using

published mRNA sequences and primer design software (Primer3; Whitehead Institute for Biomedical Research, Cambridge, MA, USA; www.genome.wi.mit.edu). PCR products were separated on 1.2% agarose gels and stained with ethidium bromide, and band densities were measured using an image analysis system (i-Max™; CoreBio System Co., Seoul). cDNA expression levels were determined by calculating the densities of IFN-γ and IL-4 bands relative to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences and annealing temperatures are listed in Table 1.

ELISA

Serum was obtained by centrifugation at 6500 rpm for 20 min, and HMC-1 cells were centrifuged under the same conditions to obtain cell pellets. All samples were stored at − 70 °C until used. Serum IFN-γ, TNF-α, IL-4 (R&D Systems Inc., MN, USA), IgE (Bethyl Laboratories Inc., TX, USA), and MAPK (RayBiotech Inc., GA, USA) levels were measured using ELISA kits. Each antibody was diluted with coating buffer and coated on the surfaces of microplate wells at 4 °C overnight. Wells were then washed three times with washing buffer and 50-μl aliquots of samples were added and allowed to stand for 1 h. Plates were then washed twice, treated with 100 μl of avidin-horseradish peroxidase (HRP)–conjugated antibody, rewashed, and treated with tetramethylbenzidine (TMB) solution for 30 min in the dark. After stopping the reaction with 50 μl of stop solution, absorbance was measured at 450 nm using an ELISA reader (MutiRead 400; Authos Co., Austria).

Statistical Analysis

All measurements were performed by an investigator unaware of experimental details. Results are expressed as means ± standard errors (SEs). Experimental data were analyzed by one-way ANOVA using SPSS version 13.0 (IBM, Chicago, USA). Statistical differences between groups were analyzed using Tukey's *post hoc* test. p values of less than 0.05 were considered statistically significant.

RESULTS

Effects of TMA on the Ears of AD Mice

To determine the effects of JC3 on atopic symptoms, macroscopic clinical responses, morphological changes, ear thicknesses, and lymph node weights were measured in mice treated with or without JC3. Atopic skin symptoms, such as edema, crusting, and excoriation, were exacerbated by TMA, as indicated when the AD group was compared with the NOR group (Fig. 2a). Atopic symptoms were significantly suppressed by JC3 in the AD+JC3-10 group (Fig. 2b), and this suppressive effect of JC3 on the atopic symptoms occurred in a dose-dependent manner. Ear skin thickness (a simple indicator of inflammatory response) was used as an indicator of TMA-induced cutaneous inflammation. Mean ear thickness was significantly lower in the AD+JC3-10 group than in the AD group ($p < 0.01$), and significantly lower in the AD+JC3-10 group than in the AD+JC3-5 group (Fig. 3a). In TMA-treated mice, proliferations of lymph node cells and infiltrations of immune cells into lymph nodes significantly increase lymph node weights (Fig. 3b), but JC3 administration dose-dependently inhibited these increases in a dose-dependent manner (by up to 63.4% at a dose of 10 mg/kg ($p < 0.001$).

Water Contents in Ear Skin and Scratching Behaviors of TMA-Induced AD Mice

Water content in ear skin of mice in the AD group was significantly lower than that in the NOR group ($p < 0.01$) (Fig. 3c), and water content in ear

Table 1. Nucleotide Sequences of PCR Primers and Operating Conditions

Gene		Nucleotide sequence	PCR condition
GAPDH	Sense	5′-AACTTTGGCATTGTGGAAGG-3′	94 °C, 30 s
	Antisense	5′-ACACATTGGGGGTAGGAACA-3′	
IFN-γ	Sense	5′-ACATGAAAATCCTGCAGAGC-3′	58 °C, 30 s
	Antisense	5′-TGGGTTGTTGACCTCAAACT-3′	
IL-4	Sense	5′-TCAACCCCCAGCTAGTTGTC-3′	72 °C, 30 s
	Antisense	5′-TGTTCTTCGTTGCTGTGAGG-3′	

T, thymine; *A*, adenine; *C*, cytosine; *G*, guanine; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *PCR*, polymerase chain reaction; *IL*, interleukin; *IFN*, interferon gamma

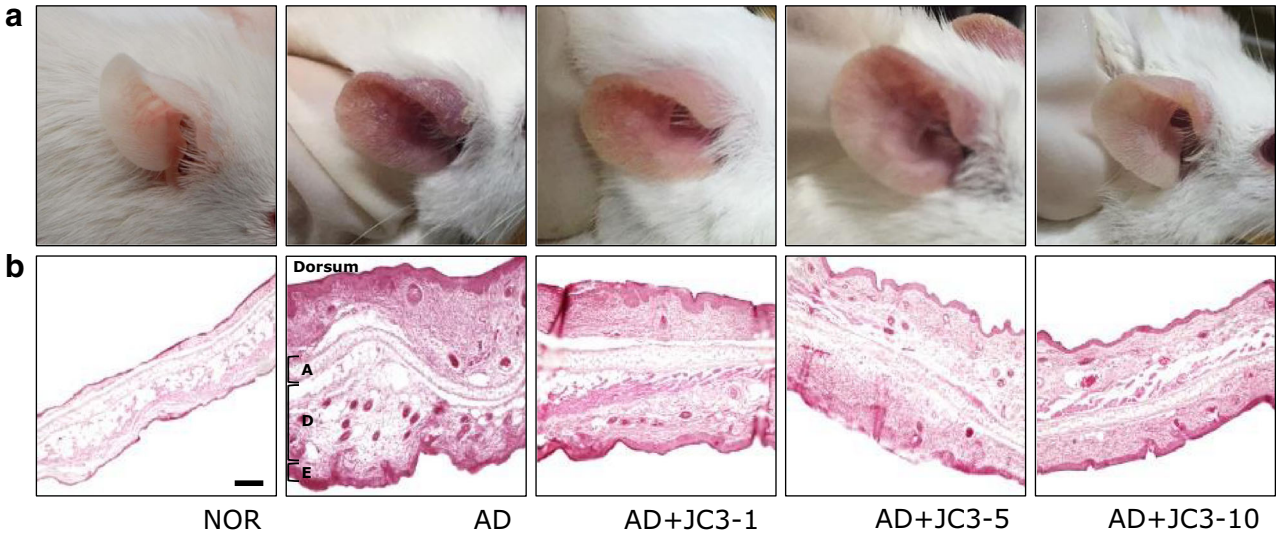

Fig. 2. Representative images of mouse ears (**a**) and their histological sections (**b**) in TMA-induced AD mice. NOR, non-treated group; AD, TMA-treated control group; AD+JC3-1, TMA-induced and 1 mg/kg JC3-treated groups; AD+JC3-5, TMA-induced and 5 mg/kg JC3-treated group; AD+JC3-10, TMA-induced and 10 mg/kg JC3-treated group. (A) auricular cartilage; (D) dermis; (E) epidermis in AD group of **b**. The scale bar in **b** represents 100 μm.

skin of mice was significantly higher in the AD+JC3-10 group than in the AD group ($p < 0.05$). To evaluate the anti-atopic activity of JC3, we examined its effects on scratching behavior (Fig. 3d). Mice in the AD group vigorously scratched lesioned skins with hind paws, and this behavior increased from experimental day 2 throughout the remainder of the 14-day experimental period (data not shown). No changes in scratching behavior were observed in the NOR group, and this behavior was significantly less in the AD+JC3-10 group than in the AD group ($p < 0.001$). Furthermore, scratching behavior was more inhibited in the AD+JC3-10 group than in the AD+JC3-5 group.

The Levels of IFN-γ, IL-4, and IgE in Serum of TMA-Induced AD Mice

Serum IFN-γ, IL-4, and IgE levels are important aspects of the diagnosis of AD, and therefore, we investigated whether JC3 administration decreased serum IFN-γ, IL-4, and IgE levels in mice with TMA-treated AD (Fig. 4). Marked increases in serum IFN-γ, IL-4, and IgE levels were observed in the AD group as compared with the NOR group, and as was expected, JC3 administration reduced these levels. In the AD+JC3-10 group, IL-4 and IgE levels were reduced significantly (IL-4 ($p < 0.01$) and IgE ($p < 0.01$)) (Fig. 4c, d), but IFN-γ levels were not.

TNF-α, IL-4, and MAPK Signaling Pathways in HMC-1 Cells

We also investigated whether JC3 treatment decreased TNF-α and IL-4 levels in PMA-treated HMC-1 cells (Fig. 5). Markedly higher TNF-α and IL-4 levels were observed in the CON group than in the NOR group. As was expected, JC3 treatment (1, 5, 10 μg/ml) reduced the levels of both cytokines. In particular, TNF-α and IL-4 levels were significantly lower in the CON+JC3-10 group ($p < 0.01$ and $p < 0.01$, respectively) (Fig. 5a, b) than in the CON group. Finally, we examined the involvement of MAPK signaling on the effect of JC3 by ELISA, because MAPK pathways have important functions as mediators of cellular responses to extracellular signals in AD [9]. To determine whether and how MAPK cell signaling pathways are involved in the inhibitory effect of JC3, we analyzed the phosphorylations of p38, extracellular signal–regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) in HMC-1 cells pretreated with JC3 and then treated with PMA (Fig. 6). PMA treatment markedly induced the phosphorylations of p38, ERK, and JNK (Fig. 6a–c, respectively), and treatment with 10 μg/ml JC3 significantly inhibited the PMA-induced phosphorylation of MAPKs.

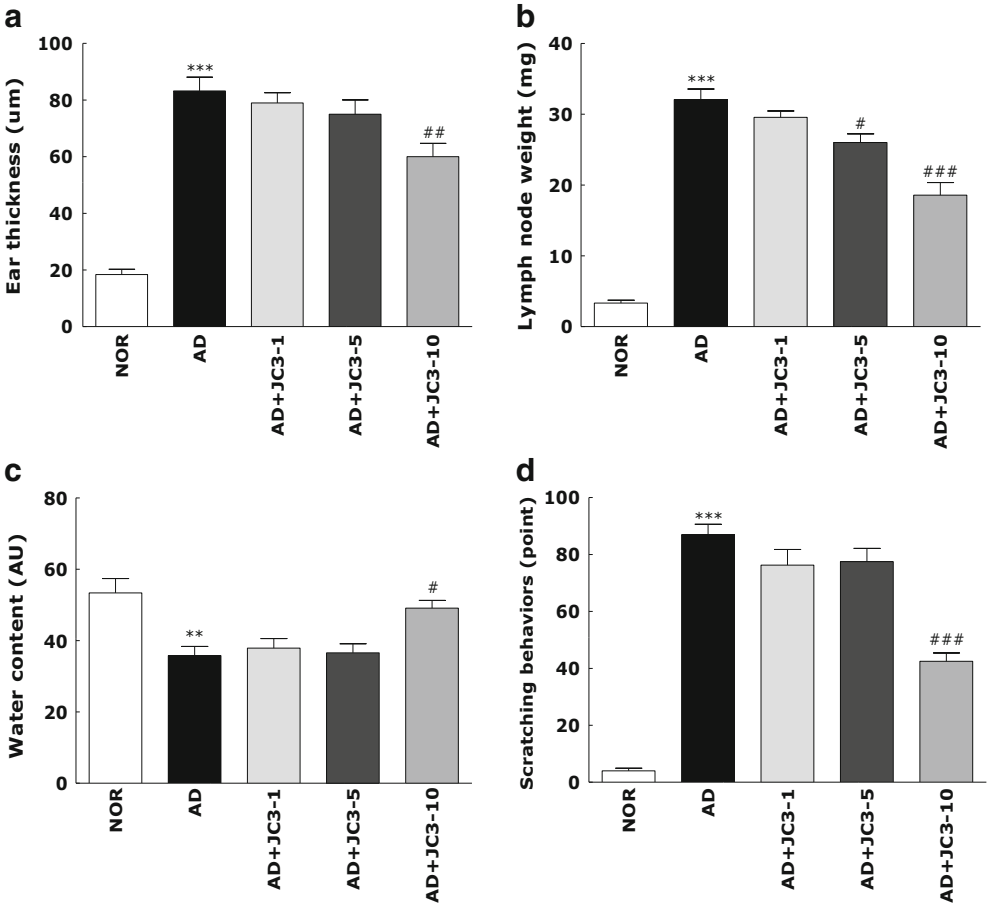

Fig. 3. Measurements of ear thicknesses (**a**), lymph node weights (**b**), skin water contents (**c**), and scratching behaviors (**d**) of TMA-induced AD mice. JC3 was treated 30 min before TMA. Ear thicknesses were measured using a dial thickness gauge and auricular lymph node weights were measured using a digital balance. Skin water contents were measured using a Corneometer® CM 825. Scratching behavior was recorded using a video camera for 10 min. Mice generally scratched several times with their hind paws for ≥ 1 s, and these were counted as one episode of scratching. Results are presented as means ± standard errors. The analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* the NOR group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ *vs.* the AD group.

DISCUSSION

Yakuchinone B is a constituent of the seeds of *Alpina oxyphylla*, a member of the ginger family (Zingiberaceae), which is commonly used in folk medicine. Yakuchinone B has a variety of biological effects, which include anti-inflammatory activity [24], as is demonstrated by its strong inhibitory effect on prostaglandin biosynthesis *in vitro* [10]. In a previous *in vitro* study, it was shown that JC3, a benzylideneacetophenone, exhibited free radical scavenging activity and suppressed lipopolysaccharide (LPS)-induced nitric oxide (NO) production and excitotoxicity in cultured cortical neurons [17], and thus, demonstrated JC3 has potent anti-inflammatory and antioxidant effects. Based on these results, we investigate the effects of JC3 whether it has therapeutic potential for the treatment of AD.

To investigate the anti-dermatitis activities of JC3, we produced a chronic TMA-induced T cell–dependent skin inflammation model in mouse ears using the protocol devised by Schneider *et al.* [20]. In this model, repeated challenge with TMA over 14 days after sensitization caused atopic dermatitis–like skin symptoms, such as skin thickening, changes in skin morphology, and immune cell infiltration into the skin [1]. Eosinophils, mast cells, and $CD4^+$ T cells mainly infiltrate the dermis whereas $CD8^+$ T

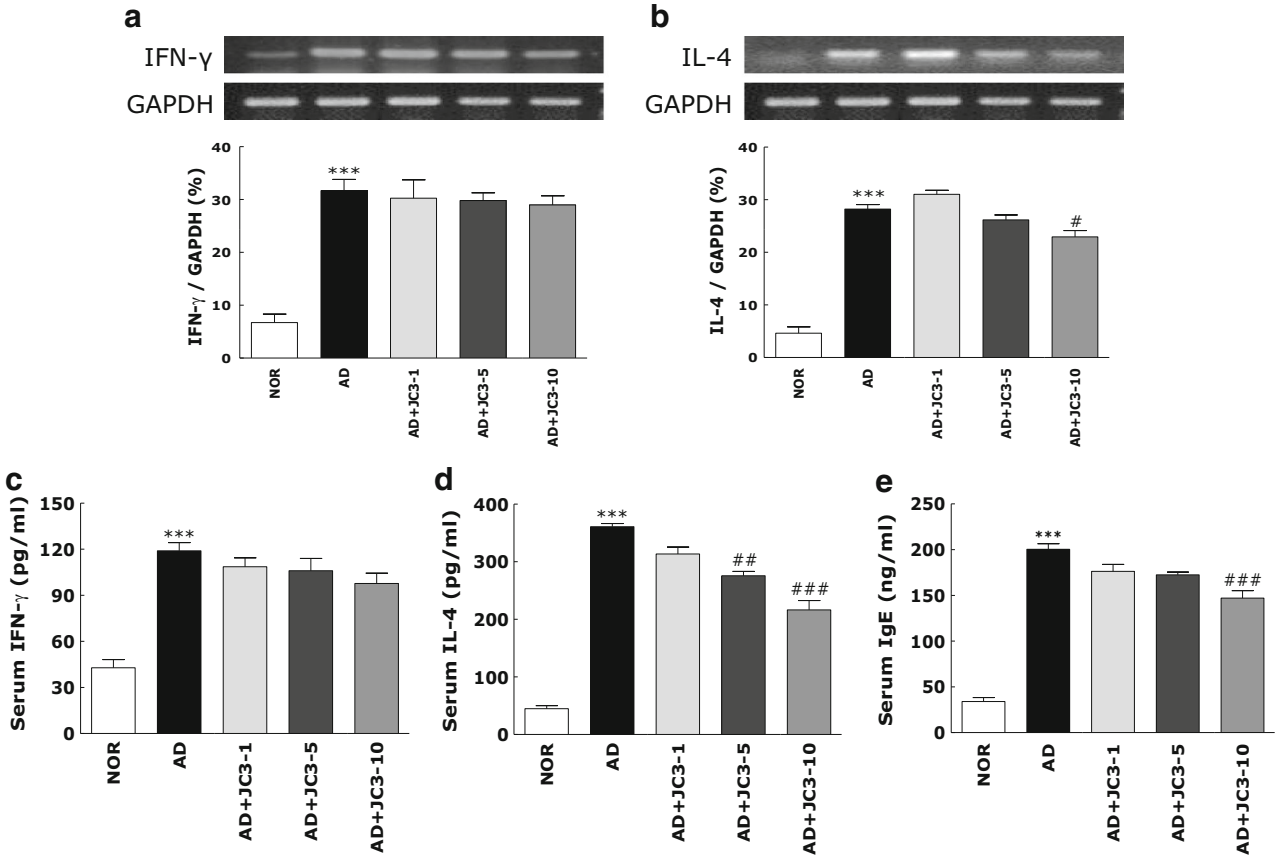

Fig. 4. The levels of IFN-γ (**a** tissue and **c** serum), IL-4 (**b** tissue and **d** serum), and IgE (**e**, serum) in TMA-induced AD mice. JC3 was treated 30 min before TMA treatment. Ear skin tissues and serum were collected from five mice randomly selected from each experimental group. The levels of IFN-γ and IL-4 were measured in ear tissue using RT-PCR. The levels of IFN-γ, IL-4, and IgE in serum were measured in serum by ELISA. Results are presented as means ± standard errors. Data analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. $^{***}p < 0.001$ *vs.* the NOR group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs.* the AD group.

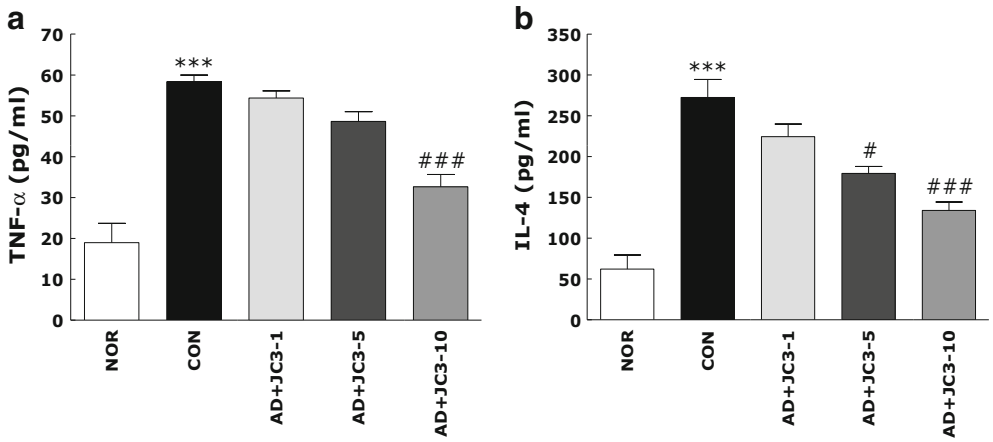

Fig. 5. The levels of TNF-α (**a**) and IL-4 (**b**) levels in HMC-1 cells after treatment with PMA and JC3 (1, 5, 10 μg/ml). HMC-1 cells were harvested at 24 h after vehicle (medium, NOR) or PMA treatments. JC3 was added 30 min before PMA and cells were harvested 24 h later. $^{***}p < 0.001$ *vs.* non-treated HMC-1 cells (NOR); $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ *vs.* PMA-treated HMC-1 cells.

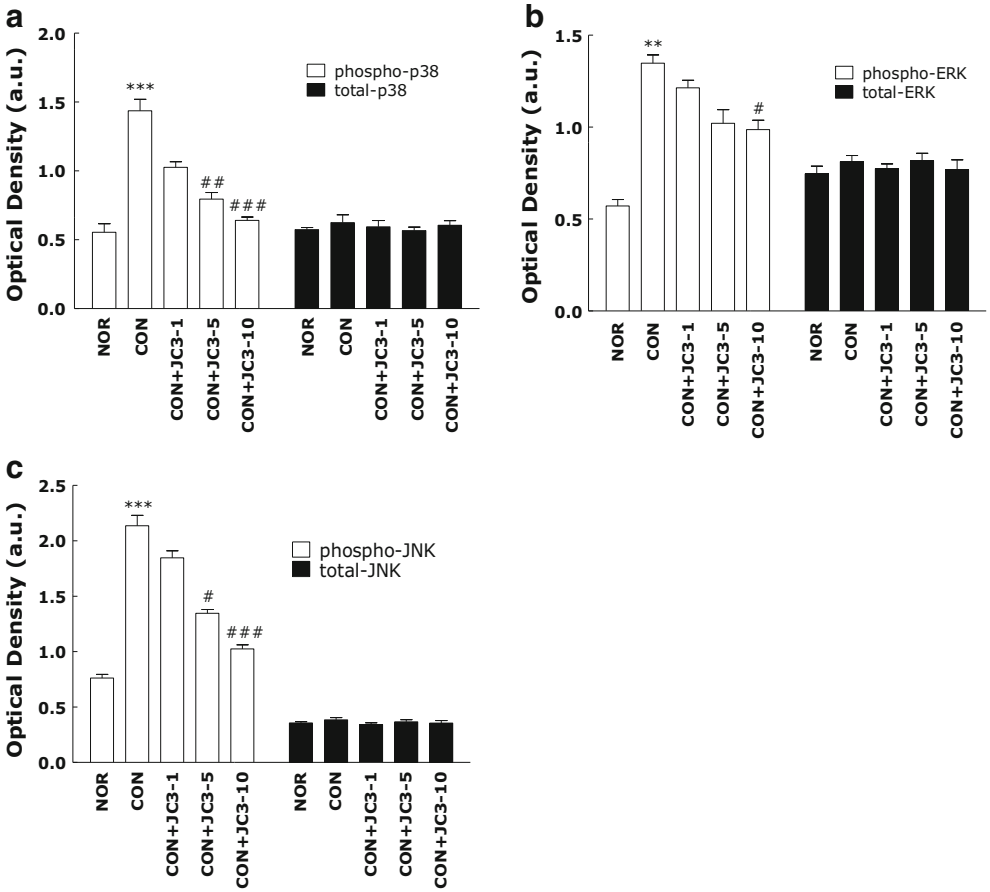

Fig. 6. Inhibitory effect of JC3 on the phosphorylations of p38 (a), ERK (**b**), and JNK (**c**) MAPKs in HMC-1 cells previously stimulated with PMA. In the experiments, HMC-1 cells were harvested at 24 h after vehicle (medium, NOR) or PMA treatments. JC3 (1, 5, 10 μg/ml) was added 30 min before PMA and cells were harvested. MAPK levels were measured in serum by ELISA. Results are presented as means ± standard errors. Optical density on 450 nm. The analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. $^{***}p < 0.001$ *vs.* non-treated HMC-1 cells (NOR); $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs.* PMA-treated HMC-1 cells.

cells tend to infiltrate the epidermis [12]. TMA directly triggered a T cell–mediated contact hypersensitivity reaction and also caused distinct changes in the innate and adaptive immune systems, as indicated by increases in the secretions of Th1 and inflammatory cytokines and the productions of Th2 cytokines and IgE, respectively.

In the present study, the induction of AD led to an increase in epidermal hyperplasia, the dermal infiltration of inflammatory cells, and increases in ear thicknesses and lymph node weights, and JC3 significantly improved these features. Notably, the administration of 10 mg/kg JC3 resulted in ear appearances similar to those seen in the NOR group, and normalized water content, scratching behavior, and inflammatory cytokine expressions. It has been suggested that ear thickness and lymph node weight measurements provide basic means of evaluating atopic skin disease [11]. Our findings show skin inflammation due to the overexpressions of inflammatory cytokines induces ear edema, which is consistent with previous findings [27].

Itching is one of the most relevant symptoms associated with AD-like skin diseases and can be exacerbated by inflammation, metabolic diseases, infection, psychiatric diseases, stress, and other factors [2]. The inflammatory skin environment lowers the threshold for itch stimuli and causes sensitization. Itching is the chief concern in an animal model of AD, because scratching exacerbates skin thickening, cracking, drying, and inflammation. In the

present study, the AD group exhibited more scratching behavior than the NOR group, and treatment with JC3 dramatically and dose-dependently reduced scratching behavior.

Over the past several decades, inflammation and the immune response have been implicated as key players in the pathogenesis of AD. In fact, the productions of cytokines in response to AD are widely recognized as a central mediator of cutaneous AD lesions [8]. IFN-γ has a wide variety of biological effects in man, whereas IL-4 can amplify IgE-induced signals in MCs by upregulating FceRI expression on cell surfaces and providing a receptive environment for eosinophil recruitment due to its presence in local tissues [29]. We found that levels of IFN-γ and IL-4 were significantly higher in the AD group than in the NOR group, and treatment with 10 mg/kg JC3 reduced IFN-γ and IL-4 expressions in the AD group. More specifically, we observed a greater decrease in IL-4 mRNA expression, which suggests IL-4 is important factor in AD. In the present study, various biomarkers that contribute to the pathogenesis of allergic and inflammatory symptoms in AD-like diseases were analyzed to evaluate the effectiveness of JC3 against AD.

To further understand the intracellular signaling pathways underlying the medicinal efficacy of JC3 and MAPK, signaling pathways were evaluated, because MAPK signaling cascades are integral to the activation, proliferation, degranulation, and migration of various immune cells. MAPK signaling modules are divided into three groups, that is, ERK, JNK, and p38 MAPK. Furthermore, it was recently reported the development of pharmacological inhibitors targeting MAPK may provide an attractive strategy for the treatment of allergic diseases [7]. Azzolina *et al.* reported that the induction of TNF-α expression and histamine exocytosis following exposure of rat peritoneal mast cells to substance P requires activation of the p38 and JNK MAPK pathways [3]. In the present study, JC3 prevented the phosphorylations of p38 and JNK MAPKs but did not affect the phosphorylation of ERK in AD.

In vivo experiments showed that JC3 inhibited the TMA-induced AD in mice. Moreover, JC3 was found to significantly reduce the symptoms of AD by modulating p38 and ERK MAPK cell signaling and by affecting the intrinsic signaling associated with atopic inflammation. Thus, we conclude JC3 may be a useful treatment for AD. Moreover, our results provide a molecular basis that might aid the developments of new approaches to the treatment of inflammatory and allergic skin diseases.

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