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Ramalin Isolated from *Ramalina Terebrata* Attenuates Atopic Dermatitis-like Skin Lesions in Balb/c Mice and Cutaneous Immune Responses in Keratinocytes and Mast Cells

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Atopic dermatitis (AD) is a chronic inflammatory skin disease that involves eczematous skin lesions with pruritic erythematous papules. In this study, we investigated the mitigating effects of ramalin, a component of the Antarctic lichen *Ramalina terebrata* against AD *in vivo* and *in vitro*. Oral administration of ramalin lessened scratching behaviors and significantly reduced both serum immunoglobulin E and IL-4 levels, and mRNA levels of IL-4 and IL-10 in AD-induced Balb/c mice. *In vitro*, treatment with ramalin produced significantly less inflammatory chemokines and cytokines, including TARC, MCP-1, RANTES, and IL-8 in TNF-α-stimulated HaCaT cells. In addition, ramalin inhibited the activation of nuclear factor-kappa B as well as the phosphorylation of mitogen-activated protein kinases (MAPK). Furthermore, ramalin treatment resulted in decreased production of β-hexosaminidase and proinflammatory cytokines IL-4, IL-6, and TNF-α in 2,4 dinitrophenyl-human serum albumin-stimulated RBL-2H3 cells through blocking MAPK signaling pathways. The results suggest that ramalin modulates the production of immune mediators by inhibiting the nuclear factor-kappa B and MAPK signaling pathways. Taken together, ramalin effectively attenuated the development of AD and promoted the mitigating effects on Th2 cell-mediated immune responses and the production of inflammatory mediators in mast cells and keratinocytes. Thus, ramalin may be a potential therapeutic agent for AD. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: atopic dermatitis; ramalin; Balb/c mice; keratinocyte; mast cell.

INTRODUCTION

Atopic dermatitis (AD) is a disease of relapsing inflammation that occurs predominantly on the skin. AD features eczematous skin lesions with pruritic erythematous papules. In the early stage of AD progression, the skin barrier is damaged by pruritus-induced scratching. This damage results in the upregulated production of inflammatory mediators, which activate various immune cells leading to initiation of the AD inflammatory cycle (Leung and Bieber, 2003).

The infiltration of type 2 helper T cells into skin lesions that occurs in AD is also associated with many skin reactions including crosstalk between keratinocytes and Th2 cells. An intrinsic skin barrier defect as the onset of eczematous lesions allows the heavy influx of Th2 cells to the dermis (Boguniewicz and Leung, 2011). Keratinocytes form the skin barrier against environmental damages such as allergens, microbial toxins or scratching, and play a crucial role in the pathogenesis of inflammatory skin diseases, secreting the proinflammatory mediators as a cellular source of the danger signal (Leung *et al*., 2004). Activated keratinocytes can cause the upregulation of inflammatory chemokines, including thymus and activation-regulated chemokine (TARC)/CCL17, monocyte chemo-attractant protein-1 (MCP-1)/CCL2, and regulated on activation, normal T-cell expressed and secreted (RANTES)/CCL5 (Homey *et al*., 2006). These skin-associated chemokines selectively regulate the migration of T cells to the site of skin lesions and stimulate inflammatory responses (Werfel, 2009). Additionally, interleukin-18 (IL-18) secreted by keratinocytes is involved in the development of a relapsing skin dermatitis with accumulation of Th2 cytokines, systemic increase of immunoglobulin E (IgE) and histamine levels, and increased numbers of mast cells (Konishi *et al*., 2002). In acute AD, Th2-type lymphocytes produce proinflammatory cytokines, which are responsible for IgE production by B cells, such as IL-4, IL-5, IL-10, and IL-13 (Romagnani, 1991). Especially, IL-4 plays an important role in switching to IgE isotype by differentiating B cells and promotes the differentiation of naïve T cells into allergic type Th2 cells. In addition, mast cells are believed to be key effector cells in IgE-dominated atopic inflammation (Kawakami *et al*., 2009). IgE-sensitized mast cells release preformed and newly synthesized cytokines, such as IL-4, IL-6, tumor necrosis factor-alpha (TNF-α), and histamine to attract other mediators of the immune defense system into the involved areas of the body

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(Bradding *et al*., 1994; Tkaczyk *et al*., 2006). In conclusion, AD is caused by complicated inflammatory response associated with a cascade of inflammatory mediators.

Lichens are the symbiotic complex of unique organisms, such as fungi and algae, which are essential constituents of many ecosystems. Lichen and bioactive secondary metabolites extracted from Antarctic flora have various biological activities including antimicrobial, antioxidant, anti-viral, anticancer, anti-genotoxic, antiinflammatory, analgesic, and anti-pyretic activities (Ranković and Kosanić, 2015). These activities have spurred interest in lichen as sources of phytochemical and pharmaceutical agents that have no toxic side effects.

Ramalin is a compound isolated from the Antarctic lichen, *Ramalina terebrata*. Ramalin has a wide range of biological functions including antioxidant, anti-atherosclerotic, and cytotoxic activities (Lee *et al*., 2015; Park *et al*., 2015; Paudel *et al*., 2011). However, the anti-allergic activity of ramalin and its molecular mechanisms remain unclear.

In the present study, we investigated whether oral administration of ramalin has mitigating effects on 1-chloro, 2,4-dinitrochlorobenzene (DNCB)-induced AD-like skin lesions in Balb/c mice and evaluated the anti-atopic effect of ramalin against cutaneous allergic responses regulated by the complicated inflammatory mediators in HaCaT human epidermal keratinocytes and RBL-2H3 rat basophilic leukemia mast cells.

MATERIALS AND METHODS

Preparation of Ramalin. Ramalin (MW 254.1) was isolated from the freeze-dried and ground lichen *Ramalina terebrata* as previously described (Paudel *et al*., 2011). The molecular formula of ramalin [1,(-glutamyl-N'-(2-hydroxyphenyl) hydrazide] (Fig. 1) was determined by $C_{11}H_{15}N_3O_4$ by analysis of its high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) data [m/z 254.1141 $(M+H)^+$; (0.0 mmu)], indicating six degrees of unsaturation. This formula was supported by ^1H and ^{13}C nuclear magnetic resonance spectroscopy data. The purity of the isolated compound generally exceeds 99%.

Animals and treatment. Male Balb/c mice (6 weeks of age) were purchased from Dae-Han Bio Link Co., Ltd. (Chungcheongbuk-do, Korea) and housed under conditions of controlled temperature (23 ± 1 °C), humidity (55 ± 5%), and a 12-hour light/dark cycle with *ad libitum* access to food and water. After acclimation, mice were randomized into five groups ($n = 5$ per group): group 1, normal control group; group 2, vehicle-treated group; group 3, positive control group; and groups 4 and 5, 1 mg/kg and 5 mg/kg ramalin, respectively. To induce AD-like symptoms and skin lesions, 1-chloro, DNCB was used to induce AD (Yun *et al*., 2008). Briefly, 1% DNCB was freshly dissolved in a 4:1 v/v mixture of acetone and olive oil and topically applied on the shaved area of the dorsal surface of mice twice per week for 2 weeks. After inducing the AD-like skin lesions and symptoms, the mice were administered Dulbecco's phosphate buffered saline (vehicle), dexamethasone (positive control), or ramalin (1 and 5 mg/kg) three times per week for 2 weeks. The doses of ramalin were selected considering that there was no clinical pathology in our preliminary toxicity study (data not shown). All animal procedures were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee at Sungkyunkwan University.

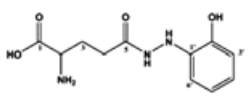

Figure 1. Anti-atopic compound, ramalin isolated from *Ramalina terebrata*.

Cell culture and reagents. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. HaCaT cells acquired from the German Cancer Research Center (DKFZ, Heidelberg, Germany) were grown in Dulbecco's modified eagle's medium (DMEM; Corning Life Sciences, MA, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). RBL-2H3 cells acquired from the American Type Culture Collection (ATCC, VA, USA) were grown in minimum essential medium with Earle's salts (GIBCO, NY, USA) containing 15% FBS, 1% P/S, and 1 mmol/L sodium pyruvate. All reagents were purchased from Sigma-Aldrich Co. (MO, USA) unless otherwise stated.

Measurement of scratching frequency. After completing all treatments, mice were subjected to a scratching behavior frequency test. In brief, mice were placed into cages and scratching behavior was counted for 10 min. The test was repeatedly performed five times for a total of 50 min. Scratching behavior was defined as hind paw movements toward and contacting ears and dorsal skin.

Measurement of immunoglobulin E and cytokine level. Blood samples were collected from the inferior vena cava of mice at the end of the experiment and then centrifuged to separate the serum. Total IgE in serum was quantified by an enzyme-linked immunoassay (ELISA) kit (Biolegend, Inc., CA, USA). The spleens were aseptically removed, and total splenocytes were prepared. Splenocytes (5×10^5 cells/well) were plated at 96-well plates and incubated for 24 h in Roswell Park Memorial Institute medium (RPMI 1640; GIBCO, NY, USA) containing 10% FBS and 1% P/S at 37 °C in a 5% CO_2 humidified incubator. Culture supernatants were collected, and the amount of secreted IL-4 was measured using ELISA. IgE-sensitized RBL-2H3 cells were treated with 0.1, 0.5, and 1 μg/mL ramalin for 20 min, followed by challenge with 0.1 μg/mL 2,4 dinitrophenyl-human serum albumin (DNP-HAS) for 10 min. The levels of TNF-α, IL-6, and IL-4 in cell culture supernatants were quantified by ELISA to the manufacturer's protocol.

Measurement of β-hexosaminidase release. RBL-2H3 cells were primed with 0.5 μg/mL anti-DNP specific

IgE overnight. Primed cells were then washed twice with Siraganian buffer (119 mmol/l NaCl, 5 mmol/l KCl, 0.4 mmol/l $MgCl_2$, 25 mmol/l PIPES, 40 mmol/l NaOH, pH 7.2) and treated with 0.1, 0.5, and 1 μg/mLramalin for 20 min. After incubation, cells were challenged with 0.1 μg/mL DNP-HAS for 10 min, after which 25 μL of each supernatant and substrate (p-nitrophenyl-N-acetyl-β-D-glucosaminide 1 mmol/L in citrate 0.1 mol/L, pH 4.5) were mixed and incubated for 1 h. This reaction was terminated by adding 200 μL of stopping solution (0.1 mol/L Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured using a microplate reader (Molecular Devices, CA, USA) at $\lambda = 405$ nm.

Immunofluorescence assay. Cells grown on coverslips were washed, fixed with 3.7% formaldehyde for 15 min at room temperature, and rinsed with cold phosphate-buffered saline (PBS). After blocking with 5% rabbit serum in 1% bovine serum albumin/0.5% Triton X-100/PBS for 1 h at room temperature, cells were incubated with p65 antibody (Santa Cruz Biotechnology, CA, USA) at 4 °C for overnight. After washing with PBS, cells were incubated with anti-rabbit IgG-fluorescein isothiocyante for 1 h at room temperature in the dark, followed by incubation with Hoechst stain for 5 min. After staining, cells were washed with PBS and coverslips were mounted on slides.

Luciferase reporter assay. Cells were transiently co-transfected with nuclear factor-kappa B (NF-kB) luciferase reporter plasmid and Renilla luciferase reporter plasmid using iN-fect™ *in vitro* transfection reagent (iN-tRON Biotechnology, Inc., Sungnam, Korea) according to the manufacturer's instructions. Following treatment, luciferase activities were measured using a Dual-Glo luciferase reporter assay system (Promega, WI, USA) and NF-kB firefly luciferase activity was normalized to Renilla luciferase activity.

Polymerase chain reaction analysis. The spleen was removed aseptically after completing all treatments, and total splenocytes were prepared. Total RNA was extracted from splenocytes with TRIzol (Invitrogen, CA, USA) according to the manufacturer's instructions, and cDNA was obtained by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc., MA, USA). Reverse transcriptase-polymerase chain reaction was carried out to analyze the level of mRNA transcripts of IL-4 and IL-10. To quantify the amount of mRNA encoding IL-4, TARC/CCL17, MCP-1/CCL2, RANTES/CCL5, and IL-18 in HaCaT or RBL-2H3 cells, quantitative real-time polymerase chain reaction (ABI Prism 7500; Applied Biosystems, CA, USA) was performed on cDNA samples using SYBR green (Enzynomics Co., Ltd., Daejeon, Korea) system. The sequences of the primers are shown in Table 1.

Western blot analysis. Western blot analysis was performed by a modification of the technique described previously (Jang *et al*., 2015). Whole cell lysates were prepared by lysing cells in lysis buffer (150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl, 1 mM phenylmethylsulfonyl fluoride). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then electrotransferred to nitrocellulose membranes. After blotting, membranes were blocked with 5% skim milk for 1 h and were probed with primary and corresponding secondary antibodies. Immunoreactive bands were visualized using chemiluminescence.

Table 1. The sequences of the forward and reverse primers

Genes	Primers for reverse transcriptase PCR
IL-4	Forward: 5-ATGGGTCTCAACCCCCAGCTAGT-3
	Reverse: 5-GCTCTTTACGCTTTCCAGGAAGTC-3
IL-10	Forward: 5-CTGCTCTTACTGACTGGCATGAG-3
	Reverse: 5-GACTCAATACACACTGCAGGTGT-3
GAPDH (mouse)	Forward: 5-CCATGGAGAAGGCTGGGG-3
	Reverse: 5-CCAAGTTGTCATGGATGACC-3
Genes	**Primers for quantitative real time PCR**
TARC/CCL17	Forward: 5-AGGGAGCCATTCCCCTTAGA-3
	Reverse: 5-CCTGGAGCAGTCCTCAGATGTC-3
MCP-1/CCL2	Forward: 5-GCAATCAATGCCCCAGTCA-3
	Reverse: 5-GCCTCTGCACTGAGATCTTCCT-3
RANTES/CCL5	Forward: 5-CATCTGCCTCCCCATATTCCT-3
	Reverse: 5-GCGGGCAATGTAGGCAAA-3
IL-18	Forward: 5-AAGGAAATGAATCCTCCTGA TAACA-3
	Reverse: 5-CCTGGGACACTTCTCTGA AAGAA-3
GAPDH (human)	Forward: 5-TCCCTCAAGATTGTCAGCAA-3
	Reverse: 5-AGATCCACAACGGATACATT-3
IL-4	Forward: 5-TGAGAAGCTGCACCGTGAAT-3
	Reverse: 5-GGCTTTCCAGGAAGTCTTTCAG-3
GAPDH (rat)	Forward: 5-CCTGGAGAAACCTGCCAAGTAT-3
	Reverse: 5-CTCGGCCGCCTGCTT-3

PCR, polymerase chain reaction; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis. All values are expressed as the mean ± S.E.M. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of Ramalin on 2,4-dinitrochlorobenzene-induced Atopic Dermatitis-like Skin Lesions and Symptoms in Balb/c Mice

Atopic dermatitis-like skin lesions and symptoms were induced by the repeated topical application of DNCB on the dorsal skin of the Balb/c mice. Oral administration of ramalin (1 and 5 mg/kg) for 2 weeks significantly mitigated the prognosis of AD-like skin lesions as compared with the control group. In addition, treatment of dexamethasone (10 μM), a well-known therapeutic agent for AD, significantly alleviated DNCB-induced AD-like skin lesions (Fig. 2A). The concentration of dexamethasone was selected

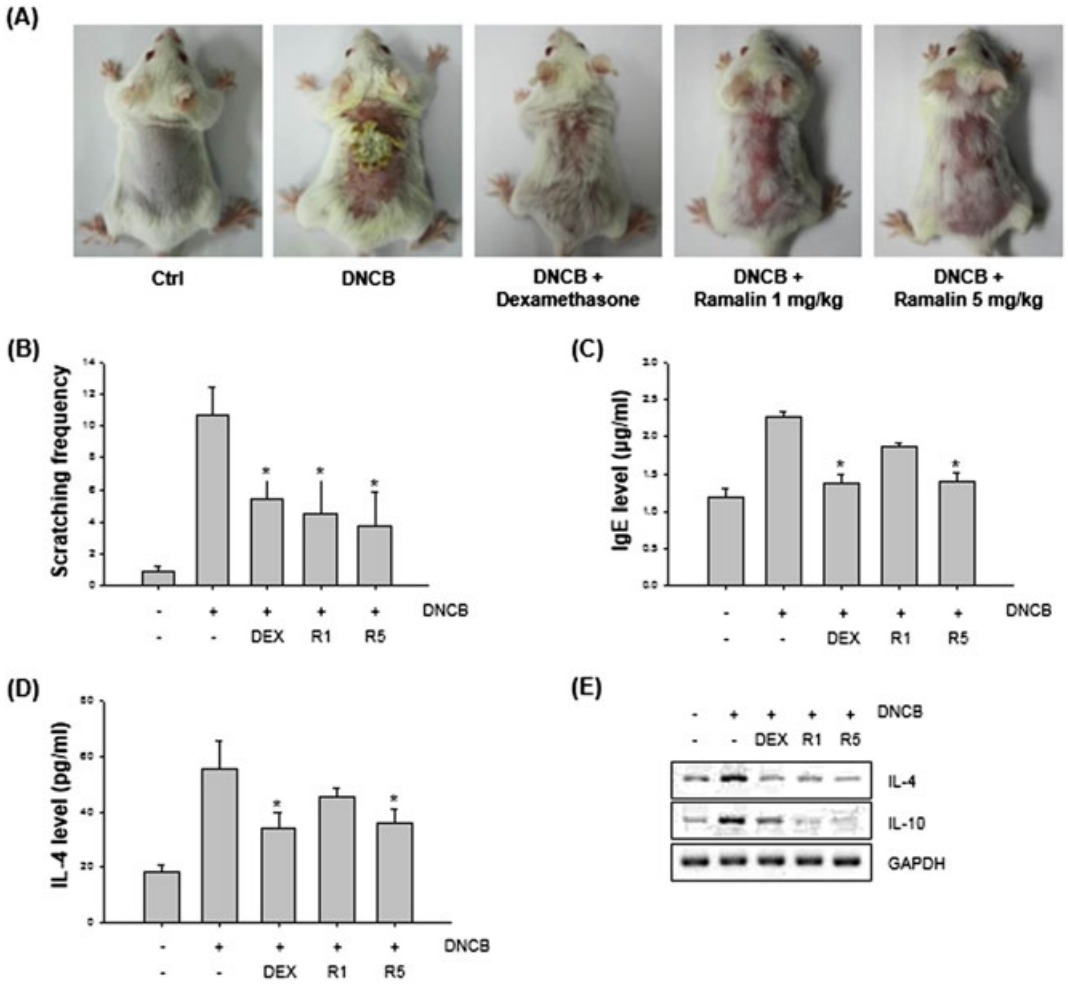

Figure 2. Atopic dermatitis-like skin lesions and symptoms in 2,4-dinitrochlorobenzene (DNCB)-induced mice. (A) Atopic dermatitis-like skin lesions were induced by topical application of DNCB for four times in twice a week, and oral administration of ramalin application improved overall skin condition. Skin features of mice were noted at the end of this experiment. Representative images are from five mice per group. (B) The number of scratching events was counted over 10 min. This measurement was repeated five times (50 min in total). (C) The effect of ramalin on serum level of IgE in DNCB-induced mice. (D) The effect of ramalin on DNCB-induced IL-4 secretion in splenocytes. (E) The inhibitory effect of ramalin on mRNA expression of IL-4 and IL-10 in DNCB-induced mice. Reverse transcription polymerase chain reaction analysis was performed to measure mRNA levels of IL-4 and IL-10. Those experiments were performed after the completion of all treatments. The results illustrated are from a single experiment, and the data are presented as the mean ± S.E.M. $*p < 0.05$ versus only DNCB-induced group. DEX, dexamethasone; R, ramalin. [Colour figure can be viewed at wileyonlinelibrary.com]

based on a previous report (Sohn *et al.*, 2011). Scratching behavior, a typical AD-like symptom, was significantly reduced by ramalin treatment (1 and 5 mg/kg) than with dexamethasone (Fig. 2B). Based on this observation, ramalin could have pharmacological effect on improving skin condition like the effect of dexamethasone. Because typical symptoms of AD are also characterized by high serum level of IgE and overexpression of IL-4 and IL-10, we measured the level of serum IgE and secretion of IL-4. In this study, application of DNCB caused a substantial increase in the lgE level and IL-4 secretion compared with control mice. However, treatment with 1 and 5 mg/kg ramalin reduced the serum level of lgE (Fig. 2C) and secretion of IL-4 (Fig. 2D). In addition, ramalin significantly suppressed DNCB-induced mRNA level of IL-4 as much as dexamethasone and had a more pronounced inhibitory effect on the expression of IL-10 mRNA than dexamethasone in splenocytes (Fig. 2E). These results indicate that ramalin has an anti-allergic effect in AD-induced Balb/c mice.

In Vitro Effects of Ramalin on TNF-α-stimulated HaCaT Cells

In the onset of AD, keratinocytes stimulated by severe scratching behavior produce proinflammatory mediators that cause an imbalanced immune response to various allergens (Leung *et al.*, 2004). Presently, ramalin inhibited formation of skin lesions and symptoms in the mouse model. To explain these findings, we hypothesized that ramalin affects the levels of proinflammatory mediators in keratinocytes. To substantiate this, we determined the effect of ramalin on production of proinflammatory chemokines and cytokine in TNF-α-stimulated HaCaT cells. TNF-α caused a significant increase in the mRNA expression of proinflammatory mediators. However, ramalin treatment

markedly suppressed TNF-α-mediated proinflammatory chemokines (TARC/CCL17, MCP-1/CCL2, and RANTES/CCL5) and cytokine (IL-18) expression in a concentration-dependent manner in HaCaT cells (Fig. 3).

Effect of Ramalin on Nuclear Factor-kappa B and Mitogen-activated Protein Kinases Pathways in TNF-α-induced HaCaT Cells

To investigate the mechanism of antiinflammatory activity of ramalin in TNF-α-stimulated keratinocytes, we examined the activation of NF-kB and IkBα degradation, which are involved in inflammatory responses. Stimulation of HaCaT cells with TNF-α induced the nuclear translocation of p65 NF-kB and degradation of IkBα, whereas treatment of ramalin inhibited the translocation of p65 NF-kB into the nucleus and recovered the expression of IkBα (Fig. 4A, and B). In addition, p65 NF-kB, which is normally present mainly in the nucleus in TNF-α stimulated cells, moved into the cytosol upon treatment with ramalin (Fig. 4C). To further confirm the inhibitory effect of ramalin on NF-kB activation, a NF-kB dependent gene reporter assay was performed (Fig. 4D). Stimulation of the cells with TNF-α resulted in an approximately 2.8-fold increase in luciferase activity, and this increase was suppressed by ramalin. Based on these findings, the present data suggest that ramalin inhibits the expression of proinflammatory chemokines and cytokine by blocking p65 NF-kB. Next, we investigated whether ramalin inhibits the activation of mitogen-activated protein kinases (MAPK) in TNF-α-stimulated HaCaT cells. Phosphorylation of ERK1/2, p38 MAPK, and JNK by TNF-α was markedly decreased in ramalin-treated HaCaT cells (Fig. 4E), implying that ramalin attenduated the TNF-α-induced phosphorylation of MAP kinases in HaCaT cells. In addition, consistent with previous results (Kwon *et al.*, 2011), we observed that the expression of ERK1/2, p38 MAPK, and JNK was inhibited by MAP kinase inhibitors (PD98059, SB203580, and SP600125), in TNF-α-stimulated HaCaT cells (Fig. 4F). Thus, these results indicate that ramalin has an anti-inflammatory effect on the accumulation of allergic modulators by blocking MAPK pathways.

In Vitro Effects of Ramalin on Degranulation, Cytokine Release and Mitogen-activated Protein Kinases Pathways in IgE-sensitized RBL-2H3 Cells

Next, we were interested in determining the effect of ramalin on the production of various inflammatory cytokines in DNP-HAS-treated RBL-2H3 cells, a tumor analog of mucosal mast cells, because mast cells play an important role in the initiation and development of an allergic response. We evaluated the levels of β-hexosaminidase, IL-4, TNF-α, and IL-6 expression. Treatment with ramalin significantly reduced the release of β-hexosaminidase in IgE-sensitized RBL-2H3 cells. In addition, DNP-HAS stimulation significantly induced the secretion of IL-4, TNF-α, and IL-6 in RBL-2H3 cells compared with untreated cells (Fig. 5A). In contrast, ramalin decreased the production of IL-4, IL-6, and TNF-α expression. DNP-HAS-induced IL-4 mRNA expression was also suppressed by ramalin treatment (Fig. 5B). These results indicated that ramalin treatment downregulates

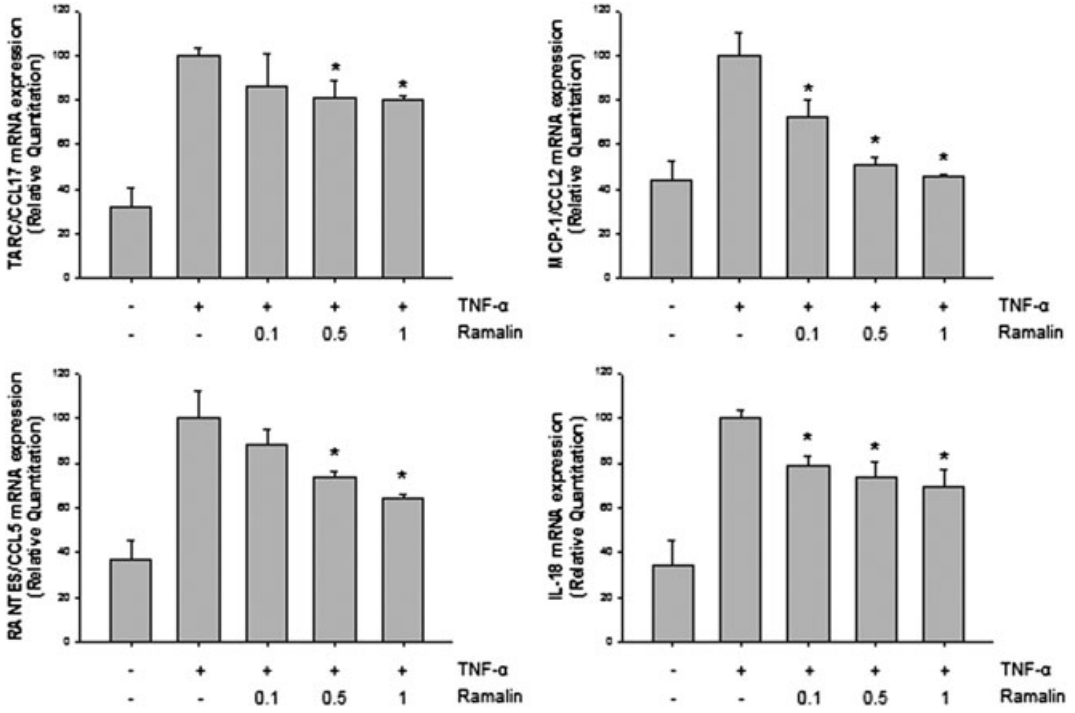

Figure 3. The effect of ramalin on proinflammatory cytokine-induced productions of chemokines and cytokine in HaCaT cells. HaCaT cells were pretreated with 0.1-1 μg/mL ramalin for 2 h then stimulated overnight with 10 ng/mL of TNF-α. The mRNA expression of TARC/CCL17, MCP-1/CCL2, RANTES/CCL5, and IL-18 were quantified by quantitative reverse transcription polymerase chain reaction. All values are presented as the mean ± S.E.M. of three experiments performed in triplicate. The percentages of densitometry are relative to the only TNF-α-induced control (100%). $*p < 0.05$ versus only TNF-α-induced group.

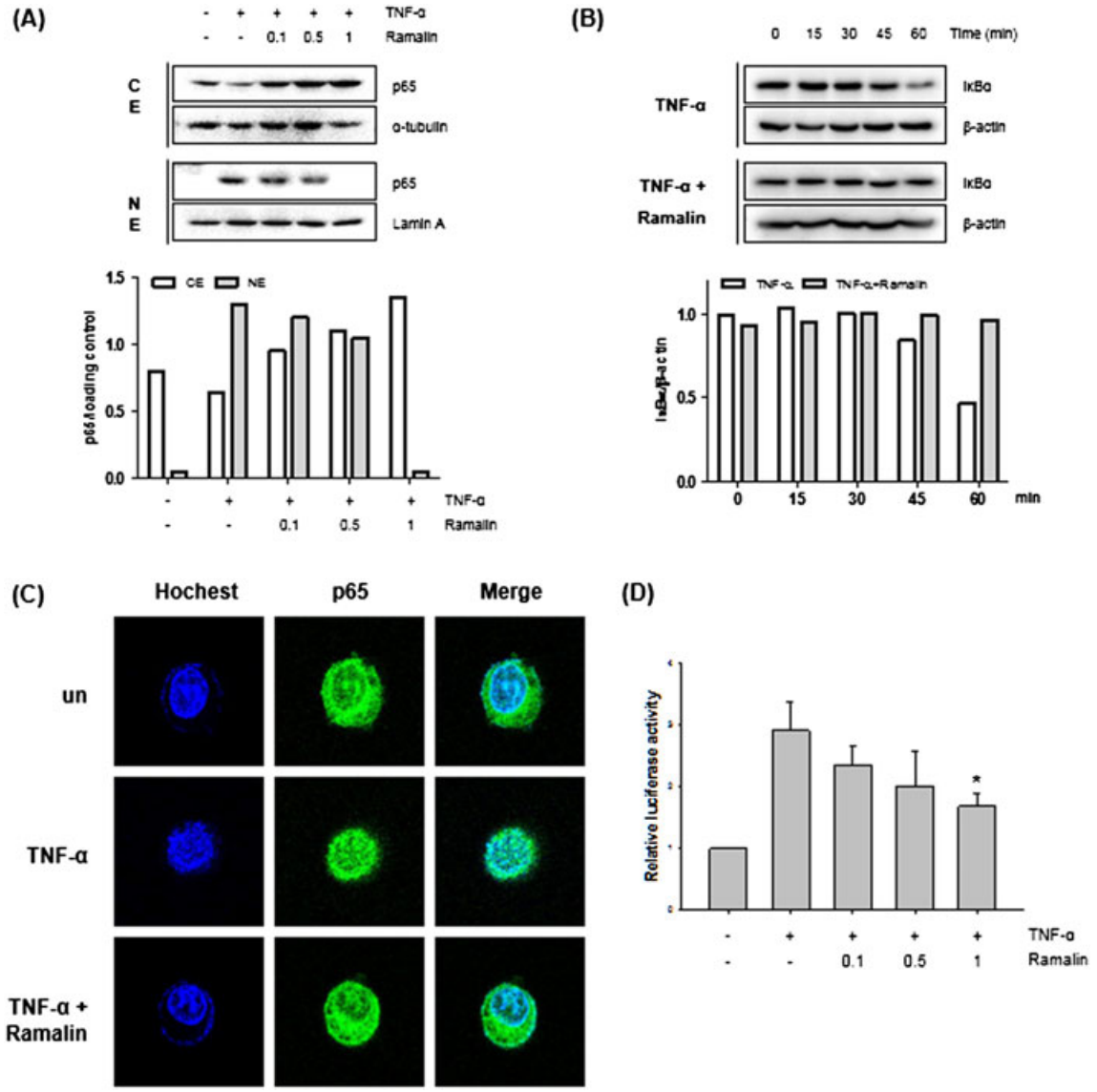

Figure 4. The inhibitory effect of ramalin on TNF-α-stimulated nuclear factor-kappa B and mitogen-activated protein kinases (MAPK) pathways. HaCaT cells were pretreated with 0.1-1 μg/mL ramalin for 2 h then stimulated with 10 ng/mL of TNF-α for 4 h and 30 min. (A) p65 translocation was inhibited by ramalin treatment on the cells. CE, Cytoplasmic Extract; NE, Nuclear Extract. (B) IkBα degradation was inhibited in a time-dependent (0–60 min) manner after TNF-α stimulation of the cells. (C) Inhibition of p65 translocation was determined using immunofluorescence analysis. (D) Inhibition of p65 activity was determined by ramalin treatment on the cells using luciferase assay. All values are presented as the mean ± S.E.M. of three experiments performed in triplicate. $*p < 0.05$ versus only TNF-α- induced group (E, F) MAPKs including ERK1/2, p38, and JNK were assessed by Western blot analysis. (F) HaCaT cells were pretreated with 20 μM ERK inhibitor (PD98059), 20 μM p38 MAPK inhibitor (SB203580), or 20 μM JNK inhibitor (SP600125) for 2 h then stimulated with 10 ng/mLof TNF-α for 30 min. [Colour figure can be viewed at wileyonlinelibrary.com]

the level of IL-4, TNF-α, and IL-6 in IgE-sensitized RBL-2H3 cells. We also examined whether the inhibitory effect of ramalin on Th2-dominant cytokines is mediated by regulating the activation of MAPK pathways in DNP-HAS-induced RBL-2H3 cells. Ramalin significantly inhibited phosphorylation of ERK1/2, p38 MAPK, and JNK in IgE-sensitized RBL-2H3 cells (Fig. 5C). In accordance with previous studies (Koranteng *et al*., 2004; Frossi *et al*., 2007), our results showed that Treatment of MAPK inhibitors (PD98059, SB203580, and SP600125) suppressed the DNP-HAS-stimulated phosphorylation of ERK1/2, p38 MAPK, and JNK (Fig. 5D). Taken together, these results suggest that map kinases are critical in our model, and ramalin inhibits the production of DNP-HAS-induced proinflammatory cytokines by blocking MAPK pathways.

DISCUSSION

Many different approaches have been introduced for the treatment of AD. Recently, lichens as sources of phytochemical and pharmaceutical therapeutic compounds have been explored. Several studies examined the biological activities of several lichen metabolites (Müller, 2001; Ranković and Kosanić, 2015). However, the potential inflammatory effect associated with AD has not been explored. In the present study, we demonstrated that oral administration of ramalin mitigates the AD-like skin condition and symptoms *in vivo*, as well as activation of keratinocytes and mast cells *in vitro*.

Atopic Dermatitis is characterized by pruritus, redness, and swelling of the skin with elevated levels of proinflammatory cytokines, histamine and IgE, and by

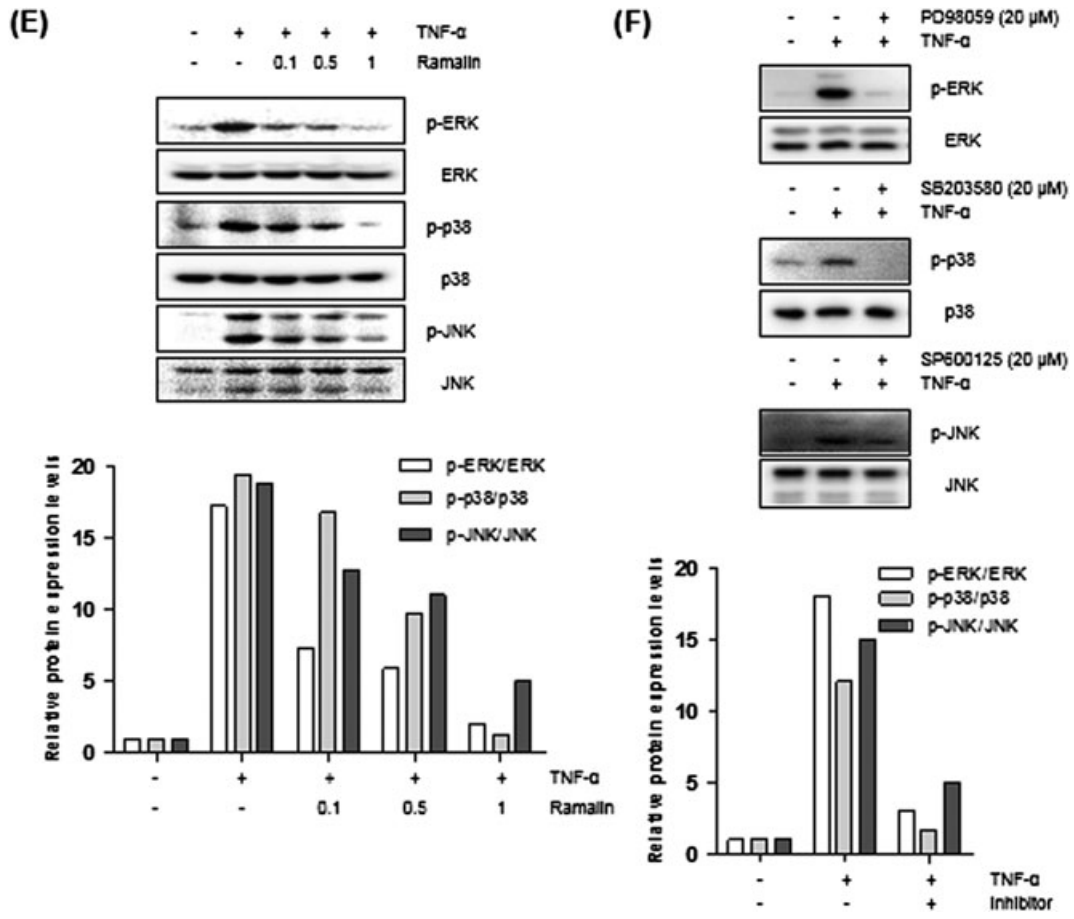

Figure 4. (Continued)

inflammatory cell infiltration, epidermis hypertrophy, and hyperkeratosis (Leung and Bieber, 2003). Patients with AD show superfluous production of IgE, which is the conventional property of skin inflammatory response, corresponding to AD-like skin lesions in induced mouse models (Matsuda *et al*., 1997). Elevated IgE levels are due to increased production of IL-4, an inducer of IgE production. In the present study, Balb/c mice repeatedly exposed to DNCB developed AD-like lesions, exhibited frequent scratching behavior, and had elevated plasma levels of IgE, IL-4, and IL-10. Oral administration of ramalin has beneficial effects in the mice; the inhibitory effects on atopic responses were equivalent to treatment with dexamethasone.

The chronic inflammation and skin damage of the skin that occur in AD involves a complex interplay between environmental, genetic, immunological, and biochemical factors (Elias and Schmuth, 2009). It is well known that mast cells, T cells, and keratinocytes interact with one another in the uppermost dermis of inflamed skin. Keratinocytes stimulated with proinflammatory cytokines are important cellular sources of chemokines, affecting T lymphocyte differentiation and the recruitment of leukocytes to skin lesions (Werfel, 2009). TARC/CCL17 produced by keratinocytes is important in the commencement of AD (Saeki and Tamaki, 2006). In addition, keratinocytes of patients with AD display much higher levels of both MCP-1/CCL2 and RANTES/CCL5 (Giustizieri *et al*., 2001). *In vitro*, RANTES/CCL5 and Th2 chemokine such as TARC/CCL17 are secreted from HaCaT cells after stimulation with cytokine (Vestergaard *et al*., 1999; Vestergaard *et al*., 2000). IL-18 contributes to the spontaneous development of AD-like cutaneous lesions (Konishi *et al*., 2002). In this study, ramalin significantly inhibited the mRNA expression of TARC/CCL17, MCP-1/CCL2, and RANTES/CCL5 in TNF-α-stimulated HaCaT cells, and decreased IL-18 production.

In AD, keratinocytes regulate the expression of inflammatory genes by mediating the dysregulated activity of transcription factors including NF-kB (Girolomoni and Pastore, 2001). It has been reported that MAPKs are required for the regulation of NF-kB and transcriptional activation of cytokines (Schulze-Osthoff *et al*., 1997; Whitmarsh, 2007). Therefore, it is plausible that treatment of ramalin affects the production of mediators of allergic inflammation through NF-kB and MAPK signaling pathways targeting inflammatory responses in the skin. NF-kB is a transcription factor that is involved in the regulation of the expression of chemokines and proinflammatory cytokines (Foo and Nolan, 1999). Dysregulated NF-kB activity is related with inflammatory responses like AD and is important in the physiology and pathology of skin (Bell *et al*., 2003). Moreover, Th2 type chemokines can be induced by TNF-α in keratinocytes, and TNF-α induce the activity of NF-kB (Baldwin, 2001). Presently, ramalin reduced the translocation of NF-kB to the nucleus, degradation of IkBα, and NF-kB promoter activity. Inflammatory dermatitis like skin lesions induced by TNF-α were reportedly mediated through one or more MAPK

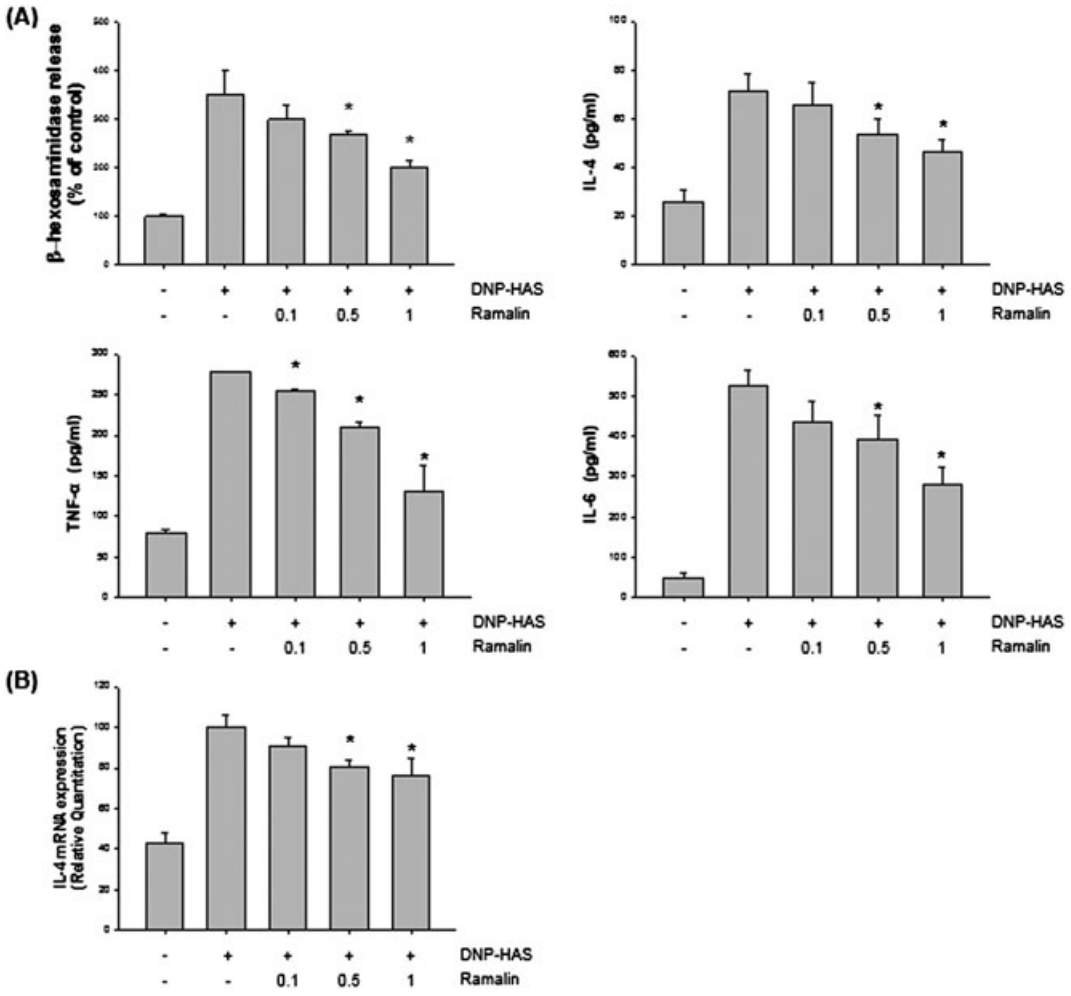

Figure 5. The effect of ramalin on antigen-induced degranulation and cytokine release by regulation of mitogen-activated protein kinases (MAPKs) in RBL-2H3 cells. RBL-2H3 cells were sensitized overnight with 0.5 μg/mL of DNP-specific IgE. These IgE-primed cells were pretreated with 0.1-1 μg/mL ramalin for 1 h then stimulated with 0.1 μg/mL of DNPHSA for 10 min. (A) The release of β-hexosaminidase and concentrations of IL-4, TNF-α, and IL-6 in the supernatant were measured using enzyme-linked immunoassay. (B) The mRNA expression of IL-4 was quantified by quantitative reverse transcription polymerase chain reaction. All values are presented as the mean ± S.E.M. of three experiments performed in triplicate. The percentages of densitometry are relative to the only DNP-HAS-induced control (100%). $*p < 0.05$ versus only DNP-HAS-induced group (C, D) MAPKs including ERK1/2, p38, and JNK were assessed by Western blot analysis. (D) RBL-2H3 cells were sensitized overnight with 0.5 μg/mL of DNP-specific IgE. The IgE-primed RBL-2H3 cells were pretreated with 20 μM ERK inhibitor (PD98059), 10 μM p38 MAPK inhibitor (SB203580), or 10 μM JNK inhibitor (SP600125) for 1 h then stimulated with 0.1 μg/mL of DNP-HSA for 10 min.

signaling pathways in several cell types (Lazennec and Richmond, 2010). Presently, ramalin inhibited the phospholyation of MAPKs. Taken together, the results suggest that ramalin inhibits the production of Th2 lymphocytes-mediated inflammatory mediators by suppressing MAPKs and NF-kB signaling pathways.

Allergen-mediated stimulation increases the level of $CD4^+$ T cell immune mediators, resulting in overproduction of both IgE and cytokines, especially IL-4 (Jin *et al*., 2009). Mast cells sensitized with IgE respond to antigen-initiated activation with the release of histamine, β-hexosaminidase, and several inflammatory mediators including IL-4, IL-5, IL-6, and TNF-α. Furthermore, cytokines secreted by allergen-sensitized mast cells promote intensified acute and chronic allergic reactions (Bradding *et al*., 1994; Tkaczyk *et al*., 2006). IL-4 is believed to mediate the switch of immunoglobulin M to IgE, and promotes the differentiation of naïve T cells into type 2 helper T cells (Yoshimoto *et al*., 2000). TNF-α is produced by activated immune cells (both macrophages and T cells) and is also synthesized and secreted by mast cells in response to IgE challenge (Han and Shikazono, 2009). IL-6 produced by basophils and mast cells is also believed to regulate cutaneous inflammatory and allergic responses (Krüger-Krasagakes *et al*., 1996). In the present study, ramalin treatment concentration-dependently inhibited secretion of β-hexosaminidase and Th2-dominant cytokines in DNP-HAS-stimulated RBL-2H3 cells, suggesting that ramalin suppresses the spontaneously induced atopic reactions by blocking the production of immune mediators in antigen-induced RBL-2H3 cells. MAPKs have been implicated in several signaling events that are important in the inflammatory response (Morrison, 2012), implicating MAPKs as being pivotal in cytokine production. In the study, ramalin inhibited MAP kinases phosphorylation in DNP-HAS-induced RBL-2H3 cells. The data presented here suggest that ramalin reduces the Th2-dominant cytokines in IgE-stimulated mast cells by suppressing the activity of MAPK pathways.

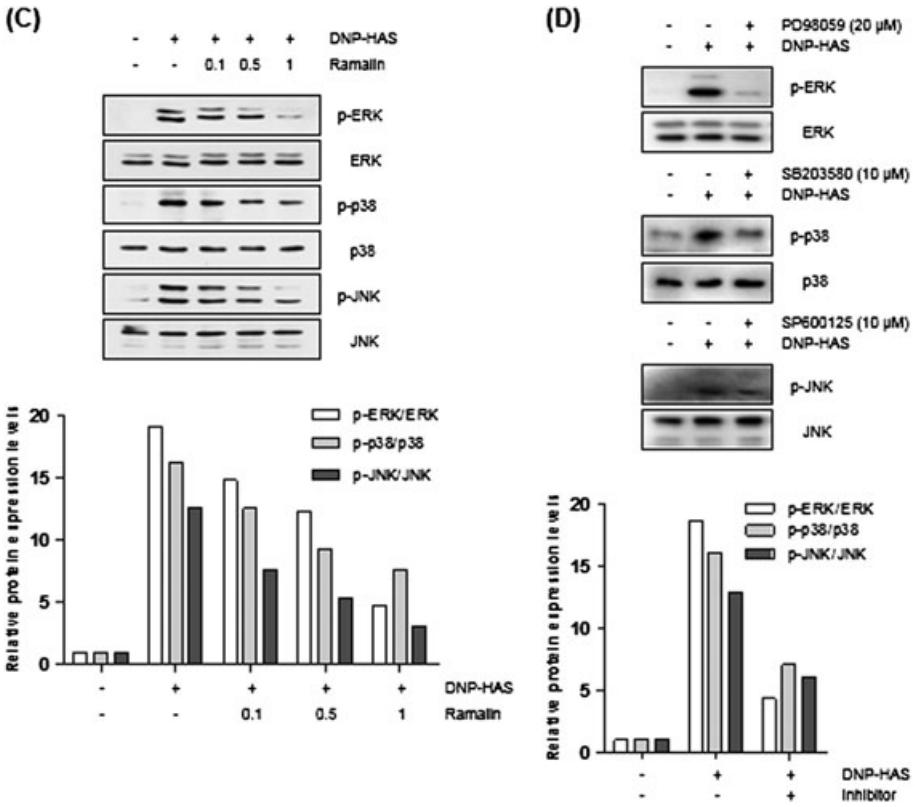

Figure 5. (Continued)

In conclusion, the administration of ramalin inhibits the development of DNCB-induced cutaneous lesions and inflammatory responses in Balb/c mice. In addition, the results of the present study prove that ramalin has the inhibiting effects on release of inflammatory mediators in keratinocytes and mast cells through the suppression of NF-kB and MAPKs signaling pathways. Thus, ramalin may be a potential novel therapeutic agent in the mediation of atopic dermatitis.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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