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(+)-Nootkatone inhibits tumor necrosis factor α/interferon γ-induced production of chemokines in HaCaT cells

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ABSTRACT

Chemokines are important mediators of cell migration, and thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) are well-known typical inflammatory chemokines involved in atopic dermatitis (AD). (+)-Nootkatone is the major component of *Cyperus rotundus*. (+)-Nootkatone has antiallergic, anti-inflammatory, and antiplatelet activities. The purpose of this study was to investigate the effect of (+)-nootkatone on tumor necrosis factor α (TNF-α)/interferon γ (IFN-γ)-induced expression of Th2 chemokines in HaCaT cells. We found that (+)-nootkatone inhibited the TNF-α/IFN-γ-induced expression of TARC/CCL17 and MDC/CCL22 mRNA in HaCaT cells. It also significantly inhibited TNF-α/IFN-γ-induced activation of nuclear factor kappa B (NF-κB), p38 mitogen-activated protein kinase (MAPK), and protein kinase Cζ (PKCζ). Furthermore, we showed that PKCζ and p38 MAPK contributed to the inhibition of TNF-α/IFN-γ-induced TARC/CCL17 and MDC/CCL22 expression by blocking IκBα degradation in HaCaT cells. Taken together, these results suggest that (+)-nootkatone may suppress TNF-α/IFN-γ-induced TARC/CCL17 and MDC/CCL22 expression in HaCaT cells by inhibiting of PKCζ and p38 MAPK signaling pathways that lead to activation of NF-κB. We propose that (+)-nootkatone may be a useful therapeutic candidate for inflammatory skin diseases such as AD.

1. Introduction

Atopic dermatitis (AD) is one of the most common inflammatory skin diseases and is influenced by genetic, environmental, and immunologic factors [1]. AD is estimated to affect approximately 8–25% individuals worldwide, and the incidence of AD continues to increase [2]. Keratinocytes, which are the major group of epidermal cells, play a critical role in the pathogenesis of AD [3]. Epidermal keratinocytes are involved in the recruitment and activation of inflammatory cells, including monocytes, neutrophils, dendritic cells, and T cells, through the production of various inflammatory mediators such as cytokines and chemokines [4]. Stimulation of keratinocytes with tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) leads to the expression of proinflammatory cytokines, chemokines, and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), and these factors play a role in the infiltration of inflammatory cells to the sites of inflammation on the skin [5–7].

Thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) are thought to play important roles in the development of some skin diseases such as AD. TARC/CCL17 is a member of the CC chemokine subfamily and is produced by keratinocytes, endothelial cells, and monocyte-derived dendritic cells, and is essentially expressed in the thymus [8]. TARC/CCL17 is a ligand for CC chemokine receptor 4 (CCR4), which is mostly expressed on Th2 lymphocytes, and TARC/CCL17 selectively regulates the migration of Th2 lymphocytes to the site of inflammation [9]. MDC/CCL22 is a specific ligand for CCR4 [10], and constitutively produced by dendritic cells [11], keratinocytes, and epithelial cells [12]. Previous studies suggest that high serum levels of TARC/CCL17 and MDC/CCL22 reflect the disease progression of AD [13]. Further, the expression levels of TARC/CCL17 and MDC/CCL22 are upregulated by keratinocytes in the lesional areas of patients with AD [14]. Taken together, modulation of TARC/CCL17 and MDC/CCL22 production in keratinocytes may contribute to the pathogenesis of AD.

Abbreviations: AD, atopic dermatitis; IFN-γ, interferon γ; IκBα, inhibitor nuclear factor-kappa B alpha; MAPK, mitogen activated protein kinase; MDC, macrophage-derived chemokine; NF-κB, nuclear factor kappa B; PKC, protein kinase C; TARC, thymus and activation-regulated chemokine; TNF-α, tumor necrosis factor α.

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(+)-Nootkatone, one of the major components in *Cyperus rotundus*, is a well-known sesquiterpenoid; sesquiterpenoids are the largest subclass of terpenoids [15,16]. Terpenoids are found in almost every plant and have various bioactivities such as anti-inflammatory, antioxidant, and antiplatelet effects [17]. (+)-Nootkatone inhibits the expression of inducible nitric oxide synthase (iNOS) and NO production in lipopolysaccharide (LPS)-simulated RAW264.7 cells [18]. In addition, (+)-nootkatone has been shown to inhibit β-hexosaminidase degranulation by inhibiting the initial activation reaction, Lyn phosphorylation, in immuboglobulin E (IgE)-stimulated RBL-2H3 cells [19]. In a previous study, we showed that (+)-nootkatone has considerable antiplatelet activity; it inhibits platelet aggregation and prolongs bleeding time [20]. However, the effects of (+)-nootkatone against AD and the mechanisms underlying these effects remain unknown.

In this study, we investigated whether (+)-nootkatone inhibits Th2-related chemokine (TARC and MDC) production in HaCaT cells, and if so, the mechanism by which (+)-nootkatone downregulates the TNF-α/IFN-γ-induced production of TARC and MDC.

2. Materials and methods

2.1. Cell culture and reagents

The human keratinocyte (HaCaT) cell line was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin, and streptomycin at 37 °C in a 5% CO_2 incubator [12]. High-glucose DMEM, FBS, antibiotics, and trypsin–EDTA were obtained from Invitrogen (Carlsbad, CA). A signaling inhibitor, SN 50, and protein kinase C ζ (PKCζ) pseudosubstrate inhibitor, SP-600125, SB-202190, and PD-98059 were obtained from Calbiochem (La Jollla, CA). Specific antibodies against phospho-PKCζ, PKCζ, phospho-p38, p38, IκBα, p65 and histone H1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). (+)-Nootkatone (⩾99.0%, GC), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody, and anti-actin antibody were obtained commercially from Sigma Aldrich (St. Louis, MO).

2.2. Cell viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, the medium was replaced with serum-free medium containing various concentrations of (+)-nootkatone. After incubating for 24 h, the cells were treated with 50 μL of MTT (5 mg/mL) for 1 h. The formazan precipitate was dissolved in 200 μL of dimethyl sulfoxide (DMSO), and absorbance was measured at 540 nm by using a microplate reader.

2.3. Reverse-transcription polymerase chain reaction

Total RNAs were isolated using the easy-BLUE™ Total RNA Extraction Kit (Intron Biotechnology, Sungnam, Republic of Korea). By measuring the 260/280 nm absorbance ratios the DNA concentration and purity of samples were determined. Complementary DNA (cDNA) was synthesized from total RNA (2 μg) by using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Beams Biotechnology, Sungnam, Republic of Korea). The primers used in this study were as follows: TARC/CCL17, (forward) 5′-CTT CTC TGC AGC ACA TCC-3′ (reverse) 5′-AAG ACC TCT CAA GGC TTT G-3′ and MDC/CCL22, (forward) 5′-AGG ACA GAG CAT GGA TCG CCT ACA GA-3′ (reverse) 5′-TAA TGG CAG GGA GGT AGG GCT CCT GA-3′. Actin primers were used as an internal control. All samples were pre-denatured for 5 min at 94 °C. Polymerase chain reaction (PCR) products were resolved on a 1% agarose gel and visualized using GelDoc™ (Bio-Rad, Hercules, CA) after staining with EcoDye™ DNA staining solution (SolGent, Daejeon, Republic of Korea).

2.4. Preparation of nuclear extract

Briefly, cells were washed twice times with cold PBS and centrifuged at 1500 rpm for 5 min. The pellet was carefully resuspended in 3 pellet volumes of cold buffer containing 20 mM HEPES, pH 7.0, 0.15 mM EDTA, 10 mM KCl, 2.5% Nonidet-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM Na_3VO_4, and 1 mM DTT. After centrifugation at 5000 rpm for 10 min, nuclei were resuspended in 2 pellet volumes of hypertonic cold buffer (10 mM HEPES, pH 7.0, 25% glycerol, 0.4 M NaCl, 0.1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM Na_3VO_4, and 1 mM DTT) and incubated for 30 min at 4 °C. After centrifugation at 14,000 rpm for 15 min, the supernatant (nuclear fraction) were used for Western blot analysis.

2.5. Western blot analysis

Western blot analysis was performed as modified previously [21]. The whole cell and nuclear fraction were electrophoresed on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. The membrane was incubated in anti-p38 (1:1000), anti-phospho-p38 (1:1000), anti-p65 (1:1000), anti-PKCζ (1:1000), anti-phospho-PKCζ (1:1000), anti-histone H1 (1:500), and anti-IκBα (1:1000) at 4 °C followed by incubation with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Bands were detected using enhanced chemiluminescent reagent (Intron Biotechnology), and images were captured using Image-Quant Las 4000 (GE Healthcare, Madison, WI, USA).

2.6. Immunocytochemistry

Cultured confluent HaCaT cells grown on glass coverslips were fixed in 4% paraformaldehyde for 30 min after being exposed to TNF-α/IFN-γ. Cells were incubated with 3% bovine serum albumin-phosphate-buffered saline (BSA-PBS) blocking solution for 1 h and anti-p65 (1:500; Santa Cruz Biotechnology, Inc.) antibody overnight, and then incubated with a secondary antibody labeled with Alexa Fluor 488 (1:1000; Molecular Proves, Eugene, OR) for 2 h. Nuclei were stained using Hoechst 33258 for 10 min. All samples were then observed under a confocal microscope.

2.7. Statistical analysis

All data are presented as mean ± standard error of mean (SEM) of at least three separate experiments. Comparisons between two groups were analyzed using the Student's *t*-test. A p value less than 0.05 was considered to be statistically significant.

3. Results

3.1. (+)-Nootkatone inhibited TNF-α/IFN-γ-induced expression of TARC/CCL17 and MDC/CCL22 in HaCaT cells

To examine the inhibitory effects of (+)-nootkatone, we co-treated HaCaT cells with TNF-α and IFN-γ in the absence or presence of (+)-nootkatone for 6 h and analyzed the expression of TARC/CCL17 and MDC/CCL22 mRNA by performing RT-PCR. We observed that (+)-nootkatone significantly inhibited the TNF-α and IFN-γ-induced mRNA expression of TARC/CCL17 and MDC/CCL22 (Fig. 1A and B). These results show that (+)-nootkatone suppresses

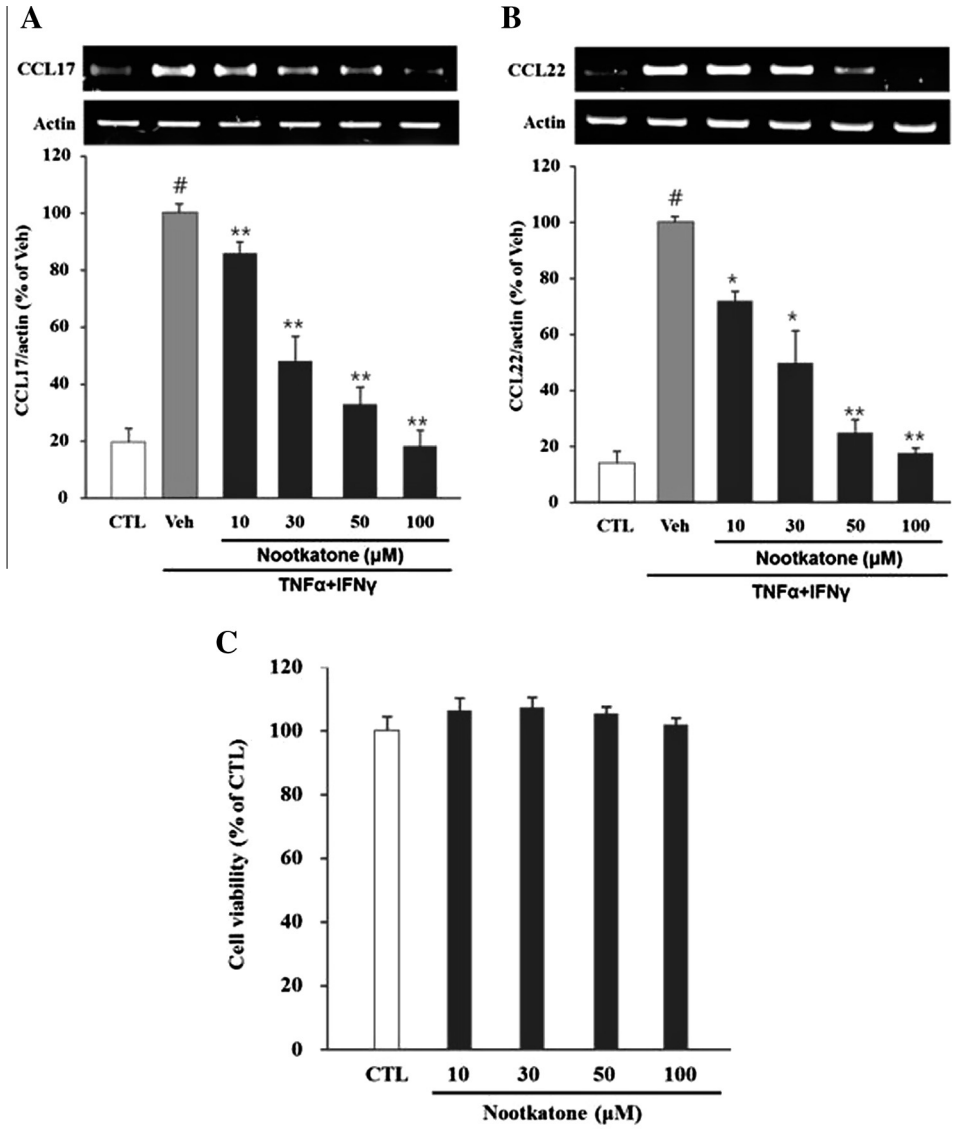

Fig. 1. Effects of (+)-nootkatone on TNF-α/IFN-γ-induced expression of CCL17 and CCL22 in HaCaT cells. (A and B) For analysis of mRNA expression, cells treated with (+)-nootkatone for 1 h were exposed to TNF-α and IFN-γ for 6 h. Total RNA was extracted. TARC, MDC, and β-actin mRNA were analyzed by RT-PCR using specific primers. (C) Cells were treated with various concentration of (+)-nootkatone for 24 h. Cell viability was assessed using MTT assay. Data are presented as the mean ± SD of three experiments. $^{*}p < 0.05$, $^{**}p < 0.001$ compared to the TNF-α and IFN-γ alone.

the TNF-α and IFN-γ-induced chemokine (TARC/CCL17, MDC/CCL22) expression in human keratinocytes.

We measured the cytotoxic effect of (+)-nootkatone on HaCaT cells by using the MTT assay. Treatment with different concentrations of (+)-nootkatone ranging from 10 to 100 μM for 24 h did not have any significant cytotoxic effects (Fig. 1C). Thus, we used (+)-nootkatone at a concentration of 10–100 μM for subsequent experiments.

3.2. (+)-Nootkatone inhibited TNF-α/IFN-γ-induced activation of p38 mitogen-activated protein kinase in HaCaT cells

We investigated the involvement of mitogen-activated protein kinase (MAPK) in TNF-α/IFN-γ-induced TARC/CCL17 and MDC/CCL22 expression in HaCaT cells. The p38 inhibitor significantly decreased the mRNA levels of TNF-α/IFN-γ-induced TARC/CCL17 and MDC/CCL22, whereas the extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK) inhibitors had a very small effect on the mRNA levels of these chemokines (Fig. 2A). We suggested that p38 MAPK mediates the expression of TARC/CCL17 and MDC/CCL22 in TNF-α/IFN-γ-stimulated HaCaT cells. Therefore, we further examined whether (+)-nootkatone inhibits the activation of p38 MAPK-induced TNF-α and IFN-γ in HaCaT cells. (+)-Nootkatone inhibited TNF-α/IFN-γ-induced activation of p38 MAPK in a dose-dependent manner (Fig. 2B). These results suggest that (+)-nootkatone suppresses the expression of TARC/CCL17 and MDC/CCL22 in TNF-α/IFN-γ-stimulated HaCaT cells by inhibiting the p38 MAPK pathway.

3.3. (+)-Nootkatone inhibited TNF-α/IFN-γ-induced activation of PKCζ in HaCaT cells

Further, we examined the involvement of PKC in TNF-α/IFN-γ-induced TARC/CCL17 and MDC/CCL22 expression in HaCaT cells. The PKCζ inhibitor significantly inhibited the mRNA expression levels of TARC/CCL17 and MDC/CCL22 induced by TNF-α and IFN-γ, whereas the PKCα and PKCδ inhibitors had a small effect on the mRNA levels of these chemokines (Fig. 3A). To elucidate the

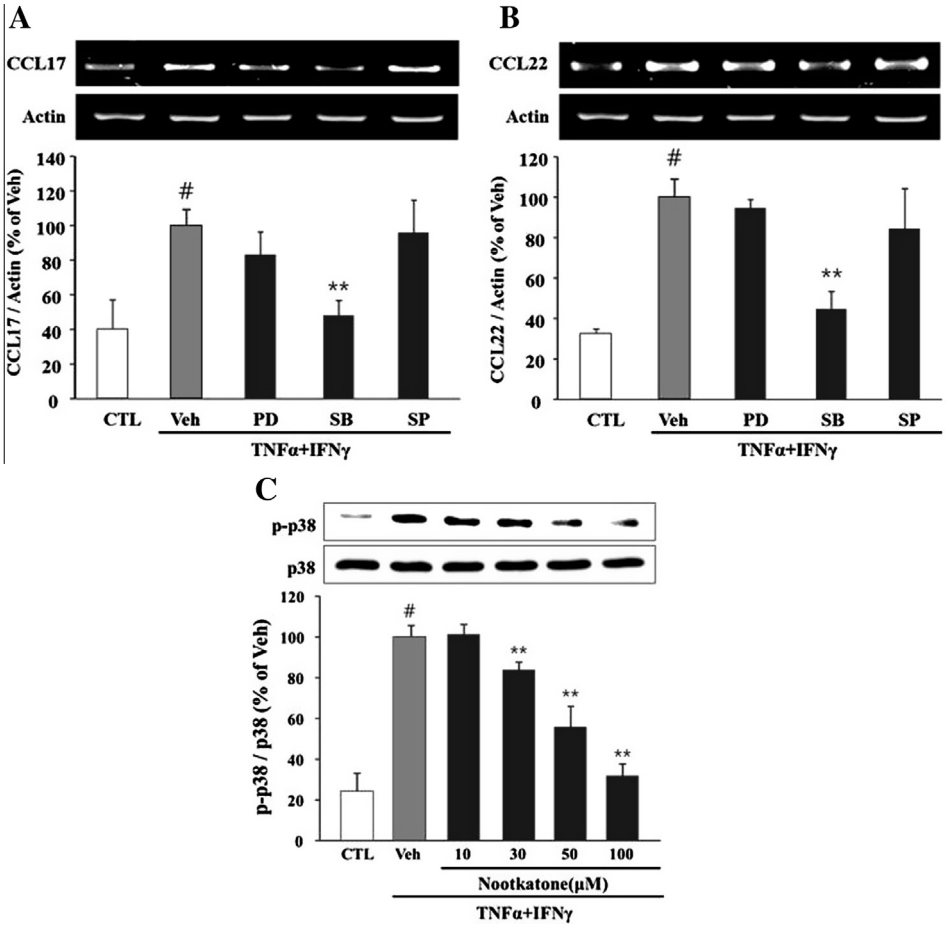

Fig. 2. Roles of MAPKs in TNF-α/IFN-γ-induced expression of TARC and MDC and the effect of (+)-nootkatone on p38 activation in HaCaT cells. (A and B) Cells were pretreated with PD98059 (PD, 20 μM), SB203580 (SB, 20 μM) or SP600125 (SP, 20 μM) for 1 h, and then stimulated with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for 6 h. Total RNA was prepared from cells and mRNA levels of TARC, MDC, and β-actin were analyzed by RT-PCR using specific primers. (C) Cells were pretreated with (+)-nootkatone for 1 h, and then stimulated with TNF-α and IFN-γ for 30 min. Whole cell extracts were prepared and analyzed by Western blot analysis with anti-phospho-p38 or p38 MAPK antibody. Data are presented as the mean ± SD of three experiments. $^{**}p < 0.001$ compared to the TNF-α and IFN-γ alone.

molecular basis of the inhibitory effects of (+)-nootkatone on the expression of TARC/CCL17 and MDC/CCL22 in keratinocytes, we investigated whether (+)-nootkatone inhibits the activation of PKCζ signaling induced by TNF-α and IFN-γ. HaCaT cells were pretreated with (+)-nootkatone for 1 h, and then stimulated with TNF-α and IFN-γ for 15 min. Then, we measured the phosphorylation of PKCζ by Western blot analysis. The results showed that (+)-nootkatone significantly inhibited TNF-α- and IFN-γ-induced phosphorylation of PKCζ (Fig. 3B).

3.4. (+)-Nootkatone inhibited TNF-α/IFN-γ-induced activation of NF-κB in HaCaT cells

Regulation of NF-κB signaling is considered as an important therapeutic target for inflammatory diseases because appropriate activation of NF-κB signaling contributes to inflammatory disorders in humans [22,29]. Therefore, we examined whether (+)-nootkatone affected IκBα degradation and NF-κB activation in TNF-α/IFN-γ-stimulated HaCaT cells. The NF-κB inhibitor, SN 50 significantly decreased the mRNA levels of TNF-α/IFN-γ-induced TARC/CCL17 and MDC/CCL22 (Fig. 4A, B). (+)-Nootkatone effectively reduced degradation of the inhibitory protein IκBα in a dose-dependent manner (Fig. 4C). We performed immunofluorescence microscopy and Western blot to confirm whether treatment with (+)-nootkatone could inhibit nuclear translocation of NF-κB. After pretreatment with (+)-nootkatone, HaCaT cells were treated with TNF-α/IFN-γ for 30 min. Along with the degradation of IκBα, NF-κB p65 proteins translocated from the cytoplasm to the nucleus after TNF-α/IFN-γ stimulation, but this was effectively inhibited by (+)-nootkatone (Fig. 4C, D). These results clearly suggest that the NF-κB signaling pathway is affected by (+)-nootkatone, and thus (+)-nootkatone can be used as an anti-inflammatory agent.

4. Discussion

Many studies have shown that TARC/CCL17 and MDC/CCL22 play a critical role in the migration of lymphocytes to the skin, and a decrease in the production of TARC/CCL17 and MDC/CCL22 in keratinocytes may be an effective target for the treatment of inflammatory skin diseases [23–25]. In this study, we confirmed the effects of (+)-nootkatone on the production of TARC/CCL17 and MDC/CCL22 in TNF-α/IFN-γ-stimulated HaCaT cells, an in vitro model of AD [26]. Our results showed that (+)-nootkatone inhibited the expression of TARC/CCL17 and MDC/CCL22 by blocking the p38 MAPK and PKCζ-NF-κB pathways in HaCaT cells. These findings indicate that (+)-nootkatone might be used clinically as a potential therapeutic agent for inflammatory skin disease such as AD.

AD is a Th2-mediated skin disease in which inflammation is characterized by the infiltration of lymphocytes into the dermis. TARC/CCL17 is one of the functional ligands for CCR4, which is

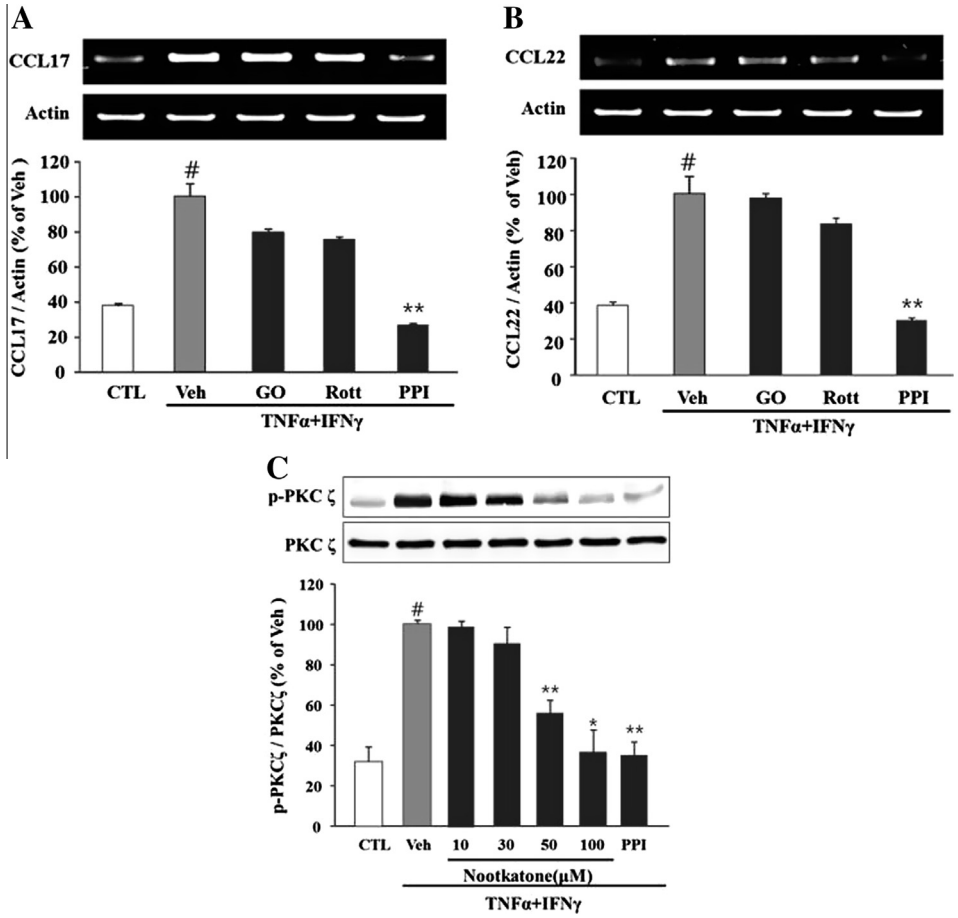

Fig. 3. Roles of PKCs in TNF-α/IFN-γ-induced expression of TARC and MDC and the effect of (+)-nootkatone on PKCζ activation in HaCaT cells. (A and B) Cells were pretreated with Gö6976 (GO, 1 μM), rottlerin (rott, 5 μM) or PKCζ pseudosubstrate inhibitor (PPI, 10 μM) for 1 h, and then stimulated with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for 6 h. Total RNA was prepared from cells and mRNA levels of TARC, MDC, and β-actin were analyzed by RT-PCR using specific primers. (C) Cells were pretreated with (+)-nootkatone for 1 h, and then stimulated with TNF-α and IFN-γ for 15 min. Whole cell extracts were prepared and analyzed by Western blot analysis with anti-phospho-PKCζ or PKCζ antibody. Data are presented as the mean ± SD of three experiments. $^{*}p < 0.05$, $^{**}p < 0.001$ compared to the TNF-α and IFN-γ alone.

preferentially expressed on Th2-type cells, and chemoattracts the CCR4-Th2-type cells to the site of the lesion on the skin [11]. Previous studies have shown that TARC/CCL17 is highly expressed in the basal epidermis of the lesional skin area in NC/Nga mice [14] and in different layers of the lesional epidermis but not in the non-lesional skin of patients with AD [23]. MDC/CCL22 is another important chemokine for infiltration of lymphocytes into the inflammatory sites, and similar to that for TARC, CCR4 is the receptor for MDC/CCL22. High levels of MDC/CCL22 were observed in the monocyte-derived dendritic cells isolated from venous blood of patients with AD [27] and in keratinocytes in the lesional skin of patients with AD [28]. Taken together, the levels of TARC/CCL17 and MDC/CCL22 were significantly higher in AD patients than in healthy control subjects [13]. These preclinical and clinical studies suggest that TARC/CCL17 and MDC/CCL22 are important molecules involved in the pathogenesis of AD. Interestingly, our results show that (+)-nootkatone suppresses TNF-α/IFN-γ-induced expression of TARC/CCL17 and MDC/CCL22 in human keratinocytes.

NF-κB is an important transcription factor activated by various stimuli such as TNF-α and IFN-γ. Upon stimulation, NF-κB in the cytoplasm translocates into the nucleus, where it participates in the expression of many pro-inflammatory genes. Previous studies have shown that the NF-κB signaling pathway is involved in the regulation of TARC/CCL17 and MDC/CCL22 production in HaCaT cells [7,30]. In addition, both promoters of TARC/CCL17 and MDC/CCL22 contain NF-κB-binding sites [29], which indicate that these transcription factors may be involved in the modulation of TARC/CCL17 and MDC/CCL22. In our study, we showed that (+)-nootkatone inhibited the signaling pathways leading to activation of NF-κB. (+)-Nootkatone suppressed TNF-α/IFN-γ-induced NF-κB activation and IκBα degradation. These results indicate that (+)-nootkatone exerts an inhibitory effect on TARC/CCL17 and MDC/CCL22 production by inhibiting the activation of NF-κB.

Previous studies showed that p38 MAPK, but not ERK, and JNK are involved in the TNF-α/IFN-γ-induced expression of TARC/CCL17 and MDC/CCL22 in HaCaT cells [25,30,31]. Our results showed that p38 MAPK mediates the activation of NF-κB and the expression of TARC/CCL17 and MDC/CCL22 in TNF-α/IFN-γ-stimulated HaCaT cells, which is consistent with the findings reported in previous studies. We observed that (+)-nootkatone significantly inhibited the activation of p38 MAPK by stimulation with TNF-α/IFN-γ in HaCaT cells. These results suggest that (+)-nootkatone can inhibit TNF-α/IFN-γ-induced TARC/CCL17 and MDC/CCL22 production in HaCaT cells by inhibiting the p38 MAPK signaling pathway that leads to the activation of NF-κB.

Atypical PKCζ is an important regulator of inflammation that acts through the activation of the NF-κB pathway. PKCζ participates in inflammatory responses to diverse stimuli in vitro and in vivo [32]. In addition, PKC isoforms, namely PKC α, δ, and ζ, were differentially expressed in epidermal keratinocytes [33]. In this study, we confirmed that PKCζ is involved in TNF-α/IFN-γ-induced

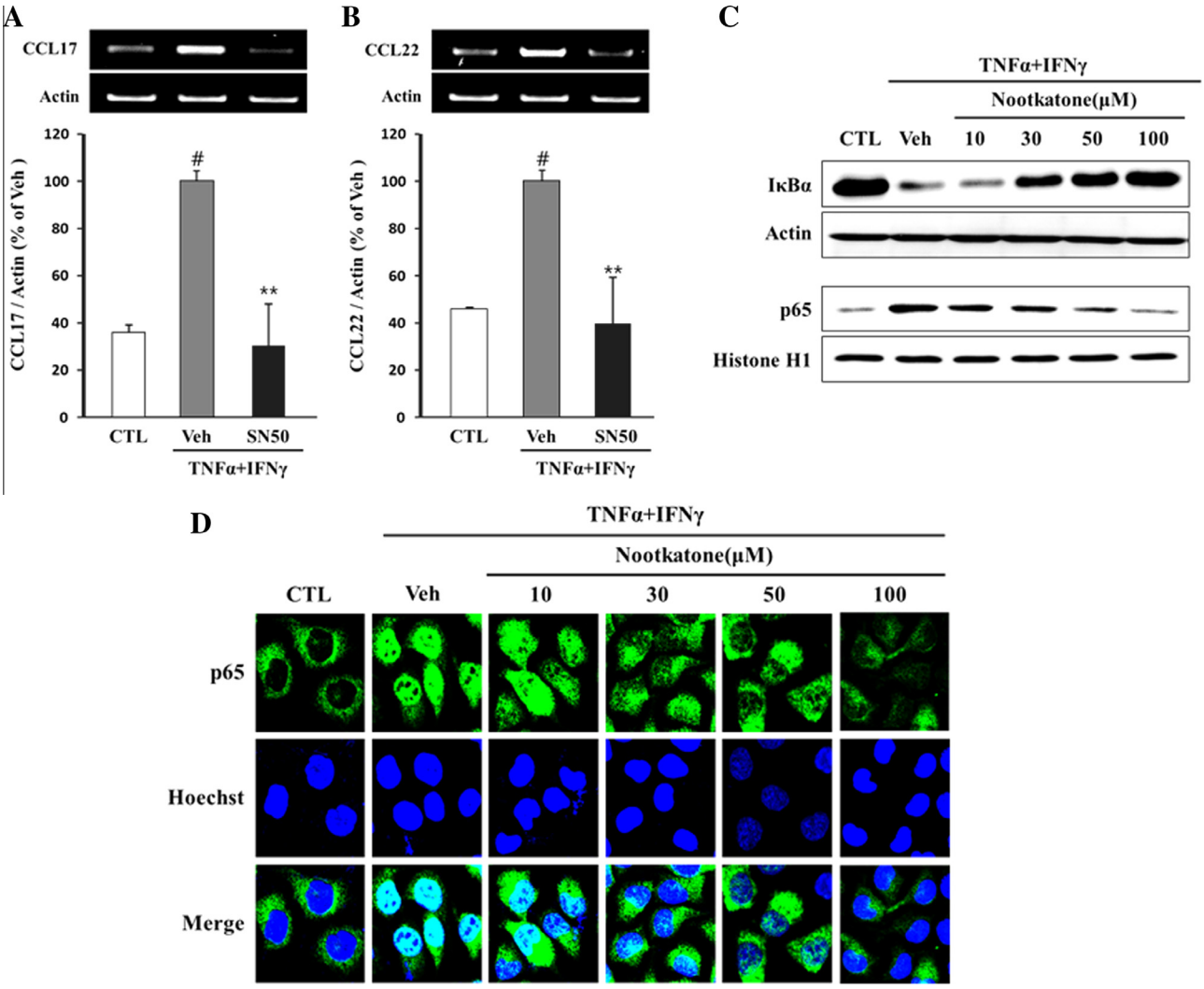

Fig. 4. Effects of (+)-nootkatone on TNF-α/IFN-γ-induced IκBα degradation and NF-κB translocation in HaCaT cells. (A and B) For analysis of mRNA expression, cells treated with SN 50 for 1 h were exposed to TNF-α and IFN-γ for 6 h. Total RNA was extracted. TARC, MDC, and β-actin mRNA were analyzed by RT-PCR using specific primers. (C) Cells were pretreated with (+)-nootkatone for 1 h, and then stimulated with TNF-α and IFN-γ for 30 min. Whole cell and nuclear extracts were prepared and analyzed by Western blot analysis with anti-IκBα or anti-p65 antibody. (D) For NF-κB staining of keratinocytes, cells were pretreated with (+)-nootkatone for 1 h and stimulated with TNF-α and IFN-γ for 30 min. Incubation was stopped and cells were stained with a primary mouse anti-p65 antibody followed by an Alexa 488-labeled goat anti-mouse antibody. Cells that did not react to the stimulus gave a representative cytoplasmic staining pattern, while reactive cells gave a nuclear pattern. Hoechst was used for nuclear staining. Data are presented as the mean ± SD of three experiments. $^{*}p < 0.05$, $^{**}p < 0.001$ compared to the TNF-α and IFN-γ alone.

expression of TARC/CCL17 and MDC/CCL22 in HaCaT cells. Our results showed that PKCζ mediates the expression of TARC/CCL17 and MDC/CCL22 in TNF-α/IFN-γ-stimulated HaCaT cells. In addition, we observed that (+)-nootkatone inhibited the activation of PKCζ in HaCaT cells after treatment with TNF-α/IFN-γ. These results suggest that (+)-nootkatone can inhibit TNF-α/IFN-γ-induced TARC/CCL17 and MDC/CCL22 production in HaCaT cells by inhibiting the PKCζ signaling pathway that leads to the activation of NF-κB.

In conclusion, our study shows that the inhibitory effect of (+)-nootkatone on the expression of Th2 chemokines such as TARC/CCL17 and MDC/CCL22 is mediated by inhibition of the p38 MAPK and PKCζ-NF-κB pathways in HaCaT cells. Further understanding of the mechanism by which (+)-nootkatone modulates the expression of Th2 chemokines would provide a molecular basis for its application in inflammatory skin diseases such as AD.

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