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ORIGINAL ARTICLE

Calcitriol May Down-Regulate mRNA Over-Expression of Toll-Like Receptor-2 and -4, *LL-37* and Proinflammatory Cytokines in Cultured Human Keratinocytes

Mi Sook Jeong, Ji-Yun Kim, He In Lee, Seong Jun Seo

Department of Dermatology, Chung-Ang University Hospital, Seoul, Korea

Background: Although vitamin D analogs have been used in the topical treatment of psoriasis, their mechanisms of action are not well understand. Calcitriol, the hormonally active vitamin D3 metabolite, has been demonstrated to exert immunomodulatory effects in the skin by down-regulating the expression of Toll-like receptors (TLRs) and proinflammatory cytokines. **Objective:** We investigated the effects of calcitriol on the expression of *TLR2*, *TLR4*, antimicrobial peptide *LL-37*, and proinflammatory cytokines in cultured human keratinocytes. **Methods:** The mRNA expression levels of *TLR2*, TLR4, tumor necrosis factor α (*TNF-*α), interleukin (*IL*)-1β and *LL-37* in cultured human keratinocytes were measured by real-time polymerase chain reaction (PCR) and reverse transcription (RT). Furthermore, we measured supernatant *TNF-*α levels by an enzyme- linked immunosorbent assay (ELISA) to confirm the effects of calcitriol on *TLR2* and *TLR4*. **Results:** As measured by RT-PCR and real-time PCR, calcitriol was found to suppress the lipopolysaccharide- and ultraviolet B radiation-mediated induction of expression of TLRs, *LL-37* and proinflammatory cytokines such as *TNF-*α and *IL-1*β in normal human keratinocytes. The supernatant *TNF-*α levels measured by ELISA were also suppressed after treatment with calcitriol. **Conclusion:** Calcitriol may down- regulate inflammatory stated over-expression of *LL-37* and proinflammatory cytokines. **(Ann Dermatol 26(3) 296～302, 2014)**

-Keywords-
Calcitriol, *TLR2*, *TLR4*

INTRODUCTION

The hormonally active vitamin D3 metabolite calcitriol (also known as 1,25-dihydroxyvitamin D3 or 1,25$(OH)_2$D3) has immunomodulatory effects in the skin in addition to its roles in bone metabolism, calcium homeostasis, cell differentiation, proliferation. Calcitriol also cause changes in cytokine expression, suppresses keratinocyte proliferation, promotes keratinocyte differentiation, and induces the expression of antimicrobial peptides (AMPs) such as human β-defensin 3 (HBD-3) and the human cathelicidin family member *LL-37*.

There is convincing evidence that vitamin D3 directly regulates AMP gene expression in human skin[1,2]. The promoter regions of the human cathelicidin AMPs (*CAMP*) and defensin 2 genes contain consensus vitamin D responsive elements (VDREs) that mediate calcitriol-dependent gene expression[3], and vitamin D has been shown to enhance the expression of *LL-37* in cultured human keratinocytes *in vivo*[1]. Induction of *CAMP* expression in keratinocytes and monocytes is mediated by Toll-like receptors (TLRs)[4,5]. However, vitamin D3 was shown to down-regulate the expression of *TLR2* and *TLR4* in a human monocyte *in vitro* model[6], to dose-dependently suppress the protein and mRNA levels of *TLR2* and *TLR4* in monocytes[7]. Vitamin D-induced *LL-37* up-regulation would therefore be expected to worsen inflammation in psoriasis; however, vitamin D analogs have long been used in

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Corresponding author: Seong Jun Seo, Department of Dermatology, Chung-Ang University Hospital, 102 Heukseok-ro, Dongjak-gu, Seoul 156-755, Korea. Tel: 82-2-6299-1538, Fax: 82-2-6264-0129, E-mail: drseo@cau.ac.kr

the topical treatment of psoriasis. While one study demonstrated that the vitamin D analog calcipotriol suppressed the expression of *HBD-2* and *LL-37* induced by lipopolysaccharide (LPS) or ultraviolet B (UVB) irradiation in cultured human keratinocytes[8], the molecular effects of vitamin D on TLRs and AMP such as *LL-37* have not been elucidated in keratinocytes.

For this reason, we sought to determine the effects of calcitriol on the expression of *TLR2*, *TLR4*, *LL-37* in cultured human keratinocytes. Furthermore, we performed cytokine analysis for tumor necrosis factor α (*TNF-*α) and interleukin 1β (*IL-1*β) to confirm the effects of calcitriol on *TLR2* and *TLR4*.

MATERIALS AND METHODS

Cell isolation and culture

For harvesting normal human keratinocytes (NHKs), neonatal foreskin was obtained from neonatal circumcision specimens and primary culture was carried out. Briefly, neonatal foreskin was chopped into 1-mm pieces and trypsinized at room temperature overnight. After vortexing the sample vigorously and incubating for 5 minutes, the supernatant was plated in 25-cm^2 culture flasks and incubated in 5% CO_2 at 37°C in keratinocyte growth medium (KGM; Clonetics, East Rutherford, NJ, USA) containing growth supplements and a calcium concentration of 0.03 mM. After 4 passages, cultured keratinocytes were plated at 2×10^5 cells/ml in a standard flat-bottomed plate. The keratinocytes were starved overnight in keratinocyte basal medium supplemented with serum-free KGM .

The cells were divided into six groups as follows: negative control group, LPS-treated group (5 μg/ml; Sigma, St. Louis, MO, USA), UVB-irradiated group (20 mJ/cm^2), calcitriol-treated group (10 nM; Sigma), LPS plus calcitriol-treated group, and UVB plus calcitriol-treated group. The LPS plus calcitriol-treated group was treated with 10 nM calcitriol 30 minutes after the LPS treatment, and the UVB plus calcitriol-treated group was treated with 10 nM calcitriol 30 minutes after the UVB irradiation and incubated for 24 hours.

Ultraviolet B irradiation

The UVB irradiation of 20 mJ/cm^2, which was chosen based on preliminary data, was delivered with a Philips TL 20W/12 (Philips, Eindhoven, Netherlands) fluorescent bulb emitting 280 to 320 nm wavelengths with a peak at 313 nm. Before UVB irradiation, the medium was removed and replaced with phosphate-buffered saline. Irradiation output was monitored with a Waldmann UV-meter (Waldmann, Villigen-Schwenningen, Germany).

Preparaton of primers

Polymerase chain reaction (PCR) primers were designed based on Gene Bank (www.ncbi.nlm.nih.gov) data using a DNA synthesizer (Pharmacia; Bjorkgatan, Uppsala, Sweden). The sequences were as follows:

TLR-2 (298bp)
5′- GGC CAG CAA ATT ACC TGT GT-3′ (sense) and
5′- TTC TCC ACC CAG TAG GCA TC-3′ (anti-sense);
TLR-4 (167bp)
5′- TGA GCA GTC GTG CTG GTA TC-3′ (sense) and
5′- CAG GGC TTT TCT GAG TCG TC-3′ (anti-sense);
TNF-α (219bp)
5′- CAG AGG GCC TGT ACC TCA TCT GA-3′ (sense) and
5′- GGA AGA CCC CTC CCA GAT AG-3′ (anti-sense);
IL-1β (205bp)
5′- GGG CCT CAA GGA AAA GAA TC -3′ (sense) and
5′- TTC TGC TTG AGA GGT GCT GA-3′ (anti-sense);
LL-37 (183bp)
5′- GCT AAC CTC TAC CGC CTC CT -3′ (sense) and
5′- GGT CAC TGT CCC CAT ACA CC-3′ (anti-sense);
GAPDH (238bp)
5′- GAG TCA ACG GAT TTG GTC GT-3′ (sense) and
5′-TTG ATT TTG GAG GGA TCT CG-3′ (anti-sense).

Reverse transcription-polymerase chain reaction

Total RNA was isolated from one dish of cultured keratinocytes using 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After 5 minutes at room temperature, 0.2 ml of chloroform/ml of TRIzol reagent was added, tubes were shaken vigorously by hand for 15 seconds, incubated at 15°C to 30°C for 3 minutes. The mixtures were centrifuged at 12,000 rpm (14,000 *g*) at 4°C for 15 minutes, the upper aqueous phase was transferred to a fresh tube, and an equal amount of 2-propanol was added. After mixtures were incubated at 4°C for 15 minutes, they were centrifuged at 12,000 rpm at 4°C for 15 minutes. The supernatant was removed, and RNA pellets were washed with 500 μl of 70% ethanol, centrifuged at 12,000 rpm at 4°C for 5 minutes, briefly dried. The purified RNA was dissolved in 30 μl of diethyl pyrocarbonate-distilled water. Three micrograms of total cellular RNA was reverse transcribed at 42°C for 30 minutes in a 20 μl volume containing 1 μl of reverse transcriptase (TaKaRa; Shiga, Japan), 2 μl of 10× buffer, 2 μl of 10 mM dNTP, 1 μl of oligo dT primer solution, 0.5 μl of RNase inhibitor and 4 μl of 25 mM $MgCl_2$. Two microliters of each resulting cDNA sample was amplified by PCR in 25 μl containing 2.5 μl of 10× buffer, 2.5 μl of 25 mM $MgCl_2$ and 0.75 μl of 10 pmol primer solution.

Thermal cycle profiles were conducted using the follo-

wing conditions: 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, 59°C for 1 minute, 72°C for 1 minute and a final extension step of 72°C for 10 minutes.

Electrophoresis

The PCR products were run on a 1.5% agarose gel, separated by electrophoresis for 15 minutes at 100 volts, and visualized by UV transillumination.

Real-time polymerase chain reaction

RNA was isolated using TRIzol Reagent (Invitrogen), and cDNA was synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, California, MD, USA). The primer sequences for *GAPDH*, *TLR2*, *TLR4*, *TNF-α*, *IL-1β* and *LL-37* were the same as those described above. The purity and quantity of each sample were determined by UV absorption and gel electrophoresis. Real-time PCR of target cDNA was conducted for *TLR2, TLR4, TNF-α*, *IL-1β* and *LL-37*, and normalized to GAPDH gene expression. All SYBR Green reactions used SsoFast™ EvaGreen (BioRad, Mississauga, ON, Canada). Real-time PCR amplification was performed on a CFX96™ Real-Time

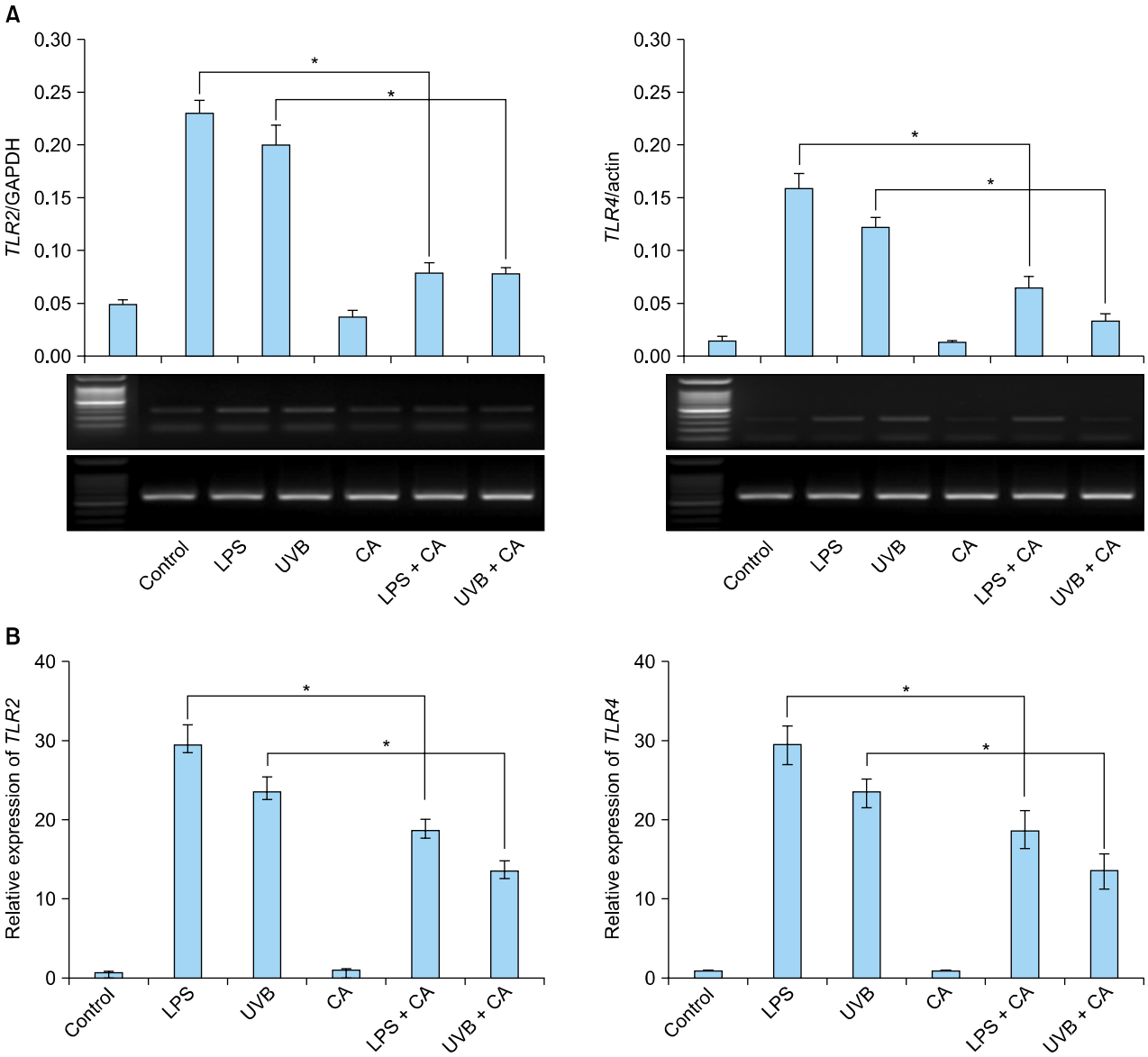

Fig. 1. The expression of *TLR2* and *TLR4* mRNA in keratinocytes increased in response to LPS or UVB irradiation. The increased *TLR2* and *TLR4* mRNA expression was down-regulated upon treatment with 10 nM calcitriol. (A) Reverse transcription-polymerase chain reaction. (B) Real-time polymerase chain reaction. TLR: Toll-like receptor, LPS: lipopolysaccharide, UVB: ultraviolet B, CA: calcitriol. $*p < 0.05$.

System (BioRad).

Enzyme-linked immunosorbent assay

Cell culture supernatants were collected after drug treatment, centrifuged to remove cellular components, and stored at −80°C. To determine the amount of *TNF-α* in each supernatant, antibodies directed against human *TNF-α* were used as the capture and detection antibodies. The fluorescent substrate horseradish peroxidase-avidin (R&D Systems, Baltimore, MD, USA) was used for color development. The amount of cytokine in the test sample was determined from standard curves established with serial dilutions of recombinant human *TNF-α* (R&D Systems). *TNF-α* concentrations were measured with the SpectraMax 340PC384 System (Molecular Devices, Wokingham, UK).

Statistical analysis

Data are presented as mean±standard deviation. Multiple comparisons were adjusted according to ANOVA. *p*-values are two-sided and $p<0.05$ was considered statistically significant. All statical analyses were performed by using a Sigma Plot for Windows (Systat software Inc., San Jose, CA, USA).

RESULTS

Increased *TLR2* and *TLR4* mRNA expression is suppressed by calcitriol

Calcitriol may act through various pattern recognition receptors[9]. Hence, we investigated whether various pattern recognition receptors such as *TLR2* and *TLR4* could be influenced by calcitriol in NHKs. Because there was no change in the expression of *TLR2* and *TLR4* when NHKs were treated with calcitriol (10 nM), the keratinocytes were stimulated with LPS (5 μg/ml) or UVB irradiation (20 mJ/cm^2). As expected, mRNA levels of *TLR2* and *TLR4* were found to be increased as much as 30 times (Fig. 1). This effect was suppressed when the cells were treated with calcitriol (10 nM) prior to stimulation with LPS or UVB irradiation (20 mJ/cm^2) (Fig. 1). These differences were statistically significant relative to the control group ($p<0.05$).

Calcitriol suppresses over-expressed proinflammatory cytokines

We investigated the effects of calcitriol on the immune response by stimulating keratinocytes in the presence of 10 nM calcitriol and measuring the cytokine levels in the cells and supernatant. The expression of *TNF-α* mRNA in NHKs was up-regulated 24 hours after stimulation with LPS or UVB irradiation. This effect was diminished upon treatment with calcitriol (Fig. 2). The levels of supernatant *TNF-α* were also up-regulated upon stimulation with LPS or UVB irradiation. However, the amount of secreted *TNF-α* was reduced after calcitriol treatment (Fig. 3). The expression of *IL-1β* mRNA in NHKs was also up-regulated upon stimulation with LPS or UVB irradiation, and this effect was diminished after treatment with calcitriol (Fig. 4).

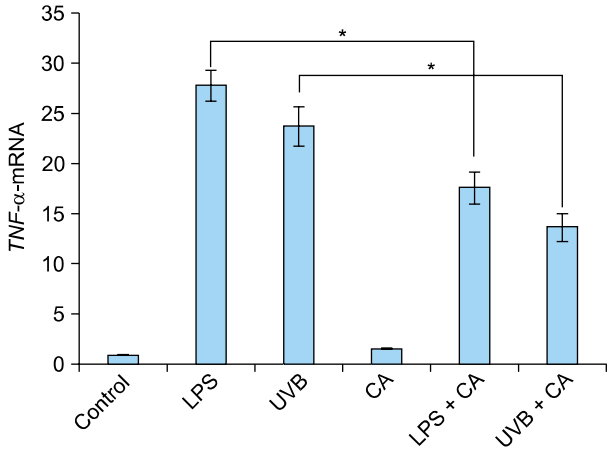

Fig. 2. The expression of *TNF-α* mRNA in keratinocytes increased in response to LPS or UVB irradiation. The increase in *TNF-α* mRNA expression was suppressed upon treatment with calcitriol. Expression levels of *TNF-α* mRNA was measured by real-time polymerase chain reaction. TNF: tumor necrosis factor, LPS: lipopolysaccharide, UVB: ultraviolet B, CA: Calcitriol. *$p<0.05$.

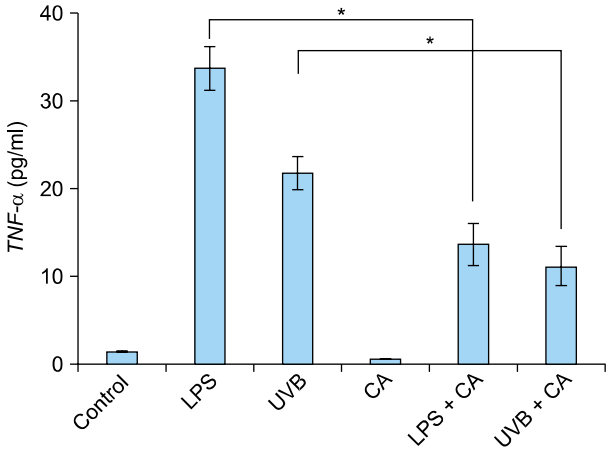

Fig. 3. The levels of secretory cytokine *TNF-α* in the supernatant increased in response to LPS or UVB irradiation. The increased *TNF-α* was down-regulated upon treatment with calcitriol. The expression level of *TNF-α* was quantified by enzyme-linked immunosorbent assay. TNF: tumor necrosis factor, LPS: lipopolysaccharide, UVB: ultraviolet B, CA: Calcitriol. *$p<0.05$.

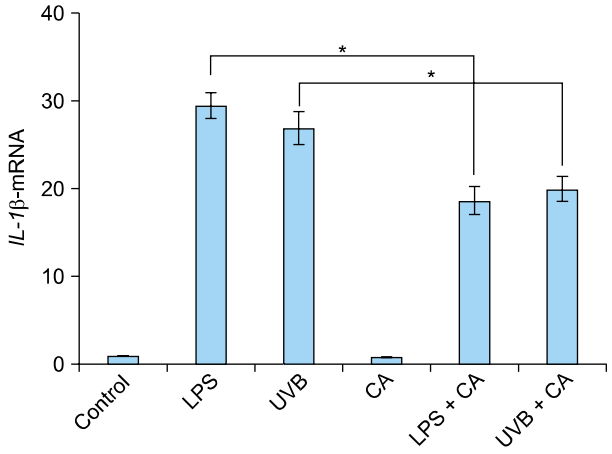

Fig. 4. The expression of *IL-1β* mRNA in keratinocytes increased in response to LPS or UVB irradiation. The increase in *IL-1β* mRNA expression was suppressed upon treatment with calcitriol. Expression levels of *IL-1β* mRNA were measured by real-time polymerase chain reaction. IL: interleukin, LPS: lipopolysaccharide, UVB: ultraviolet B, CA: Calcitriol. $*p<0.05$.

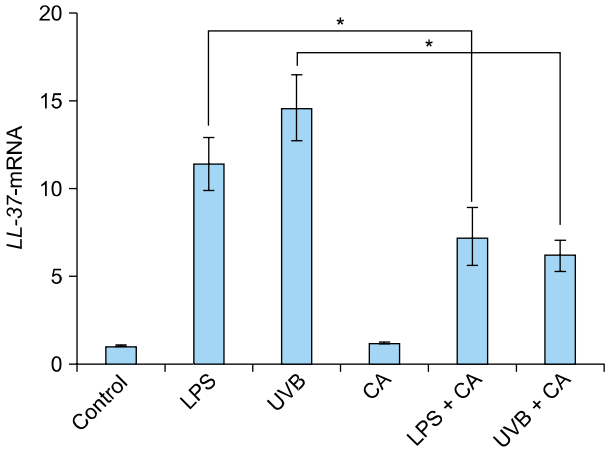

Fig. 5. The expression of *LL-37* mRNA in keratinocytes increased in response to LPS or UVB irradiation. The increase in *LL-37* mRNA expression was suppressed upon treatment with calcitriol. Expression levels of *LL-37* mRNA were measured by real-time polymerase chain reaction. LPS: lipopolysaccharide, UVB: ultraviolet B, CA: Calcitriol. $*p<0.05$.

Increased *LL-37* mRNA expression is suppressed by calcitriol

LL-37 mRNA expression was not detected in the cultured keratinocytes of the unstimulated controls. *LL-37* mRNA expression was increased in the UVB-irradiated and LPS stimulated groups, and decreased after treatment with calcitriol (Fig. 5). These effects were statistically significant when compared with the results of the control group ($p<0.05$).

DISCUSSION

In this study, we investigated the effect of the vitamin D analog calcitriol on the expressions levels of *TLR2*, *TLR4*, and *LL-37* in cultured human keratinocytes. The mRNA levels *TLR2* and *TLR4* were up-regulated in keratinocytes stimulated with LPS or UVB irradiation, while this effect was diminished after treatment with calcitriol. TLRs play important roles in the innate immune response to microbial infection. Dysregulation of TLR signaling is linked to a number of disease conditions, and possible roles for TLRs in innate immunity activation in psoriasis have been investigated. Recent studies have suggested that *TLR2*, *TLR4*, and $\gamma\ \delta$ T-cell receptors may recognize heat shock protein 60 as a ligand and consequently activate the immune system[10-12].

TLRs signal via the transcription factor nuclear factor-κB, which regulates the transcription of proinflammatory cytokines such as *TNF-α*, *IL-1* and *IL-6*. For this reason, we performed cytokine analysis for *TNF-α* and *IL-1β* to confirm the effects of calcitriol on TLRs. The expression levels of *TNF-α* and *IL-1β* in keratinocytes were up-regulated upon stimulation with LPS and UVB irradiation. This effect was diminished after treatment with calcitriol.

Secretion of *TNF-α* was also suppressed after treatment with calcitriol. *TNF-α* is a pluripotent but predominantly proinflammatory cytokine, and is a major factor in the early steps of the innate immune response. *TNF-α* production is induced by LPS/lipoteichoic acie stimulation through *TLR2*- and *TLR4*-dependent pathways. Therefore, *TNF-α* production might be inhibited upon TLR inhibition. Calcitriol would be expected to suppress *TNF-α* synthesis through the down-regulation of *TLRs* in keratinocytes. Our results imply that calcitriol is able to modify the cytokine response towards an anti-inflammatory profile. This effect of calcitriol on the expression in *TNF-α* in keratinocytes could be one mechanism of its of action mechanisms in the treatment of psoriasis.

The link between TLR activation and the expression of AMPs is clearly established[5]. Kumar et al.[13] demonstrated that expression of *HBD2* is regulated by *TLR2*-dependent pathways. Recently, the immunomodulatory effect of calcitriol through down-regulation of *TLR2* and *TLR4* expression was demonstrated in a human monocyte *in vitro* model[6]. Do et al.[7] showed that vitamin D3 was found to dose-dependently suppress the protein and mRNA levels of *TLR2* and *TLR4*. Based on these results, calcitriol would be expected to suppress TLRs, and in turn, TLR-mediated AMP expression in keratinocytes. The present study also showed that *LL-37* induction was suppressed after treatment with calcitriol. Although we did not confirm the pathway by which calcitriol affected AMP expression, our

results suggest a possible mechanism in which calcitriol suppresses TLR activation and in turn decreases TLR-mediated AMP expression in keratinocytes. These results suggest that calcitriol may exert its therapeutic effect on psoriasis by regulating *TLR2*- and *TLR4*-mediated AMP expression in keratinocytes.

Several recent reports suggest a connection between vitamin D3 and AMP expression in keratinocytes. In the presence of vitamin D, human keratinocytes upregulate *LL-37* expression in response to *TLR2* and IL-17 signaling[4,14]. Vitamin D analogs activate vitamin D receptor, which in turn would be expected to bind the VDREs in the promoter regions of cathelicidin genes and thus increase *LL-37* expression[3]. Increased *LL-37* would then aggravate inflammation by binding self-DNA and activating plasmacytoid dendritic cells in psoriasis[15,16]. However, in reality, opposite is true: indeed, vitamin D analogs are a mainstay in the topical treatment of psoriasis. To date, mechanisms that could explain this paradoxical effect of vitamin D analogs on psoriasis are not completely understood. Recent publications highlight the role of dysregulated AMP expression in the pathogenesis of psoriasis. HBD and *LL-37* levels are greatly increased in the keratinocytes of psoriatic plaques[17]. It was demonstrated that *LL-37* is able to suppress the induction of apoptosis in keratinocytes[18]. Therapeutic approaches to restore normal *LL-37* expression in keratinocytes may prove beneficial for the treatment of psoriasis. Our previous study[8] demonstrated that calcipotriol decreased the expression of HBD-2 and *LL-37* induced by UVB and LPS in cultured human keratinocytes. The present study demonstrated that calcitriol suppress the induction of *LL-37* and proinflammatory cytokines in addition to *TLR2* and *TLR4* upon LPS and UVB radiation treatment of cultured human keratinocytes.

In conclusion, calcitriol was found to suppress the LPS- and UVB-mediated induction of *TLR2* and *TLR4* in human keratinocytes. This study suggests that calcitriol modulates the expression of AMPs or *TNF-α* in chronic inflammatory skin diseases associated with overexpression of these factors. Although our experimental results are in conflict with the current understanding of vitamin D effects on AMPs and proinflammatory cytokines, our results support the application of calcitriol in therapy for psoriasis and the possibility for alternative pathways of vitamin D analogs activity in psoriasis patients.

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