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CYP11A1-derived vitamin D_3 products protect against UVB-induced inflammation and promote keratinocytes differentiation

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Abstract

UVB radiation mediates inflammatory responses causing skin damage and defects in epidermal differentiation. 1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) interacts with the vitamin D_3 receptor (VDR) to regulate inflammatory responses. Additionally, $1,25(OH)_2D_3$/VDR signaling represents a potential therapeutic target in the treatment of skin disorders associated with inflammation and poor differentiation of keratinocytes. Since the protective effect of $1,25(OH)_2D_3$ against UVB-induced skin damage and inflammation is recognized, CYP11A1-derived vitamin D_3-hydroxyderivatives including $20(OH)D_3$, $1,20(OH)_2D_3$, $20,23(OH)_2D_3$ and $1,20,23(OH)_3D_3$ were tested for their anti-inflammatory and skin protection properties in UVB-irradiated human epidermal keratinocytes (HEKn). HEKn were treated with secosteroids for 24 h pre- and post-UVB (50 mJ/cm^2) irradiation. Secosteroids modulated the expression of the inflammatory response genes (*IL-17*, *NF-κB p65*, and *IκB-α*), reducing nuclear-NF-κB-p65 activity and increasing cytosolic-IκB-α expression as well as that of pro-inflammatory mediators, IL-17, TNF-α, and IFN-γ. They stimulated the expression of involucrin (IVL) and cytokeratin 10 (CK10), the major markers of epidermal differentiation, in UVB-irradiated cells. We conclude that CYP11A1-

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AUTHOR CONTRIBUTIONS

Original draft preparation: AC, UP, ATS; Review and editing: AC, ZJ, RCT, WL, CR, UP, ATS; Methodology and investigation: AC, ZJ, TKK, ATS; Formal analysis: AC, ZJ, TKK, ATS; Visualization: AC, RCT, WL, CR, UP, ATS; Project administration: AC, RCT, WL, CR, UP, ATS; Resources: ZJ, TKK, RCT, WL, ATS; Funding acquisition: ATS; Supervision: RCT, WL, CR, UP, ATS; Conceptualization: ATS.

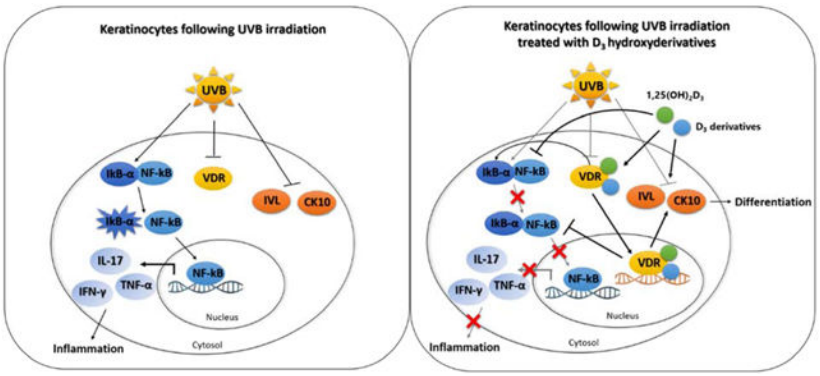
CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

derived hydroxyderivatives inhibit UVB-induced epidermal inflammatory responses through activation of IκB-α expression and suppression of NF-kB-p65 activity and its downstream signaling cytokines, TNF-α, and IFN-γ, as well as by inhibiting IL-17 production and activating epidermal differentiation.

Graphical abstract:

Schematic diagram of the role of $1,25(OH)_2D_3$ (green circle) and CYP11A1-derived D_3 derivatives (blue circle) in the modulation of NF-κB/IκB-α and differentiation biomarkers including involucrin (IVL) and cytokeratin 10 (CK10) through VDR pathway [29, 40].



Keywords

Photobiology; UV Radiation; Keratinocyte Differentiation; Epidermal Structure

INTRODUCTION

UVB plays an important role in inflammatory responses and the regulation of epidermal keratinocytes differentiation [1, 2]. In resting conditions, nuclear factor kappa B (NF-κB) is bound by the inhibitor kappa B (IκB) to prevent translocation of NF-κB to the nucleus [3, 4]. Degradation of IκB-α releases NF-κB, which is then translocated into the nucleus where it transactivates several genes involved in inflammation [3–5]. NF-κB transcriptional activity plays a crucial role in the production of secreted inflammatory cytokines and chemokines including interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-17 (IL-17), interleukin-33 (IL-33), interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-10 (IL-10), interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), intercellular adhesion molecule (ICAM) and enzymes, especially cyclooxygenase-2 (COX-2) [6, 7]. UVB irradiation can affect the NF-κB/IκB complex and transcriptional activity of NF-κB through interfering with transmembrane proteins such as toll-like receptor 4 (TLR4) and cluster of differentiation 14 (CD14), and promotes the phosphorylation of IκB [8, 9]. CYP11A1-derived vitamin D_3-hydroxyderivatives have previously been shown to protect against skin damage through suppression of UVB-induced DNA damage and reactive oxygen species (ROS) levels [10, 11]. Besides skin inflammation, UVB can interfere in the epidermal differentiation process through the induction of oxidative stress [12]. Keratinocytes respond

to UVB exposure to protect the skin against environmental stress with changes in the expression of differentiation-specific markers including keratins and IVL [13].

Vitamin D_3 (D_3) is a promising precursor for therapeutic agents of skin inflammatory responses and in skin damage prevention [14, 15]. The activation of D_3 in the canonical pathway starts with 25-hydroxylation by CYP2R1 or CYP27A1 to produce 25-hydroxyvitamin D_3 ($25(OH)D_3$) and is followed 1α-hydroxylation by CYP27B1 to produce $1,25(OH)_2D_3$ (Figure 1a) [16, 17]. In a non-canonical pathway CYP11A1, a steroidogenic enzyme expressed in the skin [18], hydroxylates D_3 producing $20(OH)D_3$ and $20,23(OH)_2D_3$ which can be hydroxylated by CYP27B1 to produce $1,20(OH)_2D_3$ and $1,20,23(OH)_3D_3$ (Figure 1a) [19–21]. The biologically active $1,25(OH)_2D_3$ interacts with the nuclear vitamin D_3 receptor (VDR) that also controls inflammatory responses [22]. VDR is a member of the nuclear receptor family regulating the biological actions of vitamin D including the expression of genes involved in inflammation and differentiation of keratinocytes [23, 24]. The active heterodimeric form of the VDR can inhibit NF-κB signaling either by induction of IκB expression or by interference with NF-κB DNA binding [25, 26]. An upregulation of VDR can also increase the levels of proteins accompanying differentiation including involucrin (IVL) and keratins [27]. Thus, signaling pathways downstream of the VDR have been proposed to be potential targets for understanding pathogenesis of skin diseases involving epidermal inflammation and differentiation [28–30].

$1,25(OH)_2D_3$ has been shown to have protective effects on skin by inhibiting inflammatory responses [31–33]. Our previous studies on $1,25(OH)_2D_3$ and CYP11A1-derivatived D_3 hydroxyderivatives have demonstrated their protective effects against UVB-induced DNA damage [10] and their ability to stimulate VDR and IVL expression and inhibit the expression of inflammatory genes, in non-irradiated epidermal keratinocytes [34–36]. Moreover, we have reported that the anti-inflammatory activity of $20(OH)D_3$ and $20,23(OH)_2D_3$, which have low calcemic activity compared to$1,25(OH)_2D_3$ [37–39], is mediated through the regulation of the NF-kB complex and IkB-α signaling in non-irradiated keratinocytes [29, 40]. This pathway is believed to protect against UV-induced inflammation [41]. In this study, we aimed to investigate the effects of CYP11A1-derivatived D_3-hydroxyderivatives on inflammatory responses and differentiation in UVB-irradiated HEKn.

MATERIALS AND METHODS

Source of compounds tested

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxy-derivatives of vitamin D_3, including $20(OH)D_3$, $1,20(OH)_2D_3$, $20,23(OH)_2D_3$, and $1,20,23(OH)_3D_3$, were enzymatically synthesized as previously described [35, 42, 43]. In addition, $20(OH)D_3$ was chemically or photochemically synthesized as previously described [44, 45].

Cell culture and treatment

HEKn were isolated from neonatal foreskin and cultured as previously described [29, 46]. Patient consent for experiments was not required because the informed consent laws consider human tissue left over from surgery as discarded material. In this study we used secosteroids at a concentration of 100 nM to test the anti-inflammatory and pro-differentiation effects on cells. This concentration exhibited the most potent activity for photoprotection as well as skin cell proliferation, as tested previously [10, 42]. The D_3 hydroxyderivative (100 nM) or ethanol were diluted in EpiGRO™ keratinocyte media (Millipore, Burlington, MA, USA) mixed with 0.5% BSA. Our previous study evaluated the effects of UVB on skin cell damage at different doses: 25, 50, 75, or 200 mJ/cm^2 [10]. A significant reduction in DNA repair and an induction of oxidant formation was observed in cells with a UVB dose of 50 mJ/cm^2. We thus applied this model in the current study. Cells were treated with vitamin D_3 or its hydroxyderivatives before UVB (50 mJ/cm^2) irradiation (pre-treatment) for 24 h. After irradiation, cells were then immediately incubated in fresh medium supplemented with the vitamin D_3 derivatives, for an additional 24 h (post-treatment) as in [10].

Quantitative RT-PCR for mRNA expression

RNA was isolated from treated cells in 60 mm petri dishes using the absolutely RNA RT–PCR Miniprep kit (Stratagene, La Jolla, CA, USA). PCR reactions were performed in triplicate using a Kapa SYBR Fast qPCR Master Mix (Kapa Biosystems, Boston, MA, USA) as indicated in [10]. Sequences of PCR primer are shown in Table S1. The mRNA levels were normalized relative to the amount of housekeeping genes (β-actin, cyclopilin, or GAPDH mRNA) using the ΔΔCt method. The mean of the fold-change and the heat map were generated using Prism software.

The detection of NF-κB p65 phosphorylation

The NF-κB p65 (Total/Phospho) Human InstantOne™ ELISA Kit (Cat No. 85-86083-11, Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the phosphorylation of NF-κB p65 [29, 47]. An antibody cocktail was pre-coated with a capture antibody reagent, the total-NF-κB p65 or phospho-NF-κB p65 antibody, and a detection antibody reagent (1:1 v/v). Cell lysates, a negative control (cell lysis mix), or a positive control (50 μl/well) mixed with the total-NF-κB or phospho-NF-κB antibody cocktail (50 μl/well) were incubated in the pre-coated plate for 1 h at RT. After washing, stop solutions (100 μl/well) were added and the absorbance measured at 450 nm. The phosphorylation level, normalized by the cell number or the total-NF-κB p65, was calculated relative to the control and data are presented as % of control (mean±SD).

Immunofluorescence analysis of NF-κB p65/IκB-α and IVL

Treated cells in a 96-well plate were fixed in 4% paraformaldehyde and tagged with mouse monoclonal antibody against NF-κB p65 (sc-8008; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal antibody against IκB-α (sc-371; Santa Cruz Biotechnology, Dallas, TX, USA), or mouse monoclonal antibody against IVL (GTX-14504; Genetex, Irvine, CA, USA), overnight at 4°C (1:200 dilution). Plates were then incubated for 1 h with Alexa-

Fluor 488 secondary antibody (Invitrogen Molecular Probes, Eugene, Oregon, USA) diluted 1:300 in blocking solution. Nuclei were stained red with propidium iodide (Vector Laboratories, Burlingame, CA, USA). Stained cells were imaged at 40X magnification and analyzed using Gen5.0 software with a Cytation™ 5 cell imaging reader. The nuclear/cytosolic ratio was calculated from the image intensities of the ImageJ analysis (NIH free download) and presented as % of control (mean±SD) as in [10, 48]. The single color staining represents the expression in cytosol (green), while the dual expression (orange-yellow) represents the expression in both cytosol and nucleus. The cell nuclei were stained red with propidium iodide (red).

The detection of pro-inflammatory cytokines

IL-17, IFN-γ, and TNF-α levels were measured in cell supernatants using RAB0262, RAB022, and RAB1089 ELISA kits, respectively (Sigma-aldrich, St. Louis, MO, USA). Standard or sample cell supernatant was incubated in pre-coated 96-wells plates (100 μl/well) overnight at 4°C. Antibodies (100 μl/well) were then added and plates incubated for 1 h followed by streptavidin (100 μl/well) addition and incubation for 45 min. TMB one-step substrate (100 μl/well) was added and plates incubated for 30 min before adding stop solutions (50 μl/well). Absorbances (450 nm) were measured and cytokine concentrations (pg/ml, mean±SD) calculated from a standard curve.

Western blot analysis of VDR and the proteins involved in inflammation and differentiation

Cytosolic and nuclear fractions were extracted by a nuclear extraction kit (Active motif, Carlsbad, CA, USA). The concentration of protein samples was determined using the Bio-Rad protein Bradford assay kit (BioRad, Hercules, CA, USA). An equal amount of protein (50 μg per well) was loaded into each well of the gel. Proteins were separated using a Mini-PROTEAN® TGX™ gel (BioRad, Hercules, CA, USA) as in [10]. The blot was first probed with anti-VDR Ab, then stripped and reprobed sequentially with antibodies to NFκBp65, IkB-α, IVL, CK10, and beta-actin shown in Figures 1, 3, 4, and 6, respectively. The primary antibodies were as follows: Mouse monoclonal antibody against VDR (sc-13133, Santa Cruz Biotechnology, Dallas, TX, USA; 1:200 dilution), mouse monoclonal antibody against NF-κB p65 (sc-8008, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal antibody against IκB-α (sc-371; Santa Cruz Biotechnology, Dallas, TX, USA), mouse monoclonal antibody against IVL (GTX-14504; Genetex, Irvine, CA, USA), and mouse monoclonal antibody against CK10 (sc-23877; Santa Cruz Biotechnology, Dallas, TX, USA), at 1:1000 dilution. The blot was then incubated for 1 h at RT with the HRP-conjugated secondary antibodies: Goat anti-mouse superclonal™ secondary antibody (A28177, Thermo Fisher Scientific, Waltham, MA, USA) or goat anti-rabbit secondary antibody (ab6721, Abcam, Cambridge, MA, USA), at 1:3000 dilution. Immuno-reactivity was detected using supersignal west pico ECL (BioRad, Hercules, CA, USA). The loading controls, β-actin-peroxidase (A3854, Sigma-aldrich, St. Louis, MO, USA; 1:5000 dilution) and lamin A/C (N-18) (sc6215, Santa Cruz Biotechnology, Dallas, TX, USA; 1:200 dilution) were used for the cytosolic and nuclear fractions, respectively. The protein bands of interest were identified according to their molecular weights (kDa) published in the manufacturers' datasheets, determined relative to the precision plus protein™ kaleidoscope™ standards (BioRad, Hercules, CA, USA). The relevant proteins of interest were detected sequentially with the

blots being reprobed with each antibody type, including those for the loading controls. β-actin-peroxidase served as the loading control for whole cell and cytosolic fractions, while lamin A/C served as the loading control of nuclear fraction [49, 50]. ImageJ analysis of blots were performed from 3–6 independent experiments. To analyze the intensity of relevant bands in the blots, an area was selected around the band at the expected molecular weight (kDa) using Image J software, and the pixel intensity measured. Quantitative data were then imported into Microsoft Excel for calculating the percentage intensity of relevant bands compared to the intensities of controls and the ratio of intensities between the nuclear and cytosolic fractions. For the analysis of whole cell and cytosolic fractions, band intensities measured by ImageJ were normalized relative to the loading control and to the band intensities seen in control cells (ethanol without UVB or ethanol with UVB), and are presented as % of control (mean±SD). For the analysis of the nuclear to cytosolic ratio, the nuclear-NFkB p65 pixel intensities were normalized by dividing the pixel intensity value by that for the Lamin A/C loading control. The expression of cytosolic-NFkB p65 was normalized by dividing its pixel intensity by that for the β-actin loading control. The nuclear to cytoplasmic ratio of NFkB-p65 was then calculated by dividing the normalized value for nuclear expression by that for the cytosol, with final data being presented as fold-change (mean±SD).

STATISTICAL ANALYSIS

Data are expressed as mean ± standard deviation of at least three separate experiments ($n \geq 3$). The significance of non-irradiated controls or individual treatment groups in comparison to the UVB-irradiated groups was evaluated by the student *t*-test, *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 for all experiments, using Prism (GraphPad Software Inc.).

RESULTS AND DISCUSSION

CYP11A1-derived D_3-hydroxyderivatives stimulate VDR expression in UVB-irradiated keratinocytes

UVB irradiation plays a role in skin inflammation through the reduction of VDR expression in mouse skin and cultured keratinocytes [51, 52]. We and others have previously reported that $1,25(OH)_2D_3$ and its analogs have anti-inflammatory effects mediated via upregulation of VDR signaling [32, 53]. Hence, we investigated the effect of CYP11A1-derived $20(OH)D_3$, $1,20(OH)_2D_3$, $20,23(OH)_2D_3$, and $1,20,23(OH)_3D_3$ which bind with high affinity to VDR [37, 54, 55], on VDR expression. We observed a substantial reduction in VDR mRNA and protein levels in UVB (50 mJ/cm^2)-irradiated cells compared to non-irradiated cells (Figure 1b, left panel). All the tested secosteroids significantly reversed the UVB-reduced *VDR* expression at the mRNA level (Figure 1b, black bar chart). In addition, *VDR* expression was significantly increased in cells treated with secosteroids that were not exposed to UVB (Figure 1b, white bar chart). We detected the expression of VDR protein as indicated at 45 kDa as shown in the representative blot (Figure 1c, left panel), which was normalized relative to the β-actin control protein. The summary graph of three-independent western blots (Figure 1c, right panel) shows that the treatment with $1,25(OH)_2D_3$, $20(OH)D_3$

or 1,20(OH)$_2$D$_3$ significantly enhanced VDR protein expression in UVB-irradiated cells, indicating that they can amplify VDR-dependent protective responses.

CYP11A1-derived D$_3$-hydroxyderivatives modulate the expression of inflammatory genes in UVB-irradiated keratinocytes

Since the activation of NF-κB induces the transcription of inflammatory genes including *TLR4*, *COX2*, *ICAM*, *IL-6*, *IL-8*, *IL-17*, *IL-33*, *IL-1α*, *IL-1β*, *CD14*, *NFkB p50*, *NFkB p65 Rel A*, *IkB-α*, *bcl2*, and *BNIP* [5, 6], the expression of the inflammatory genes was determined to investigate the anti-inflammatory role of secosteroids in response to UVB (Figure 2).

The expression of inflammation-related genes in both non-irradiated and UVB-irradiated cells was modulated by the secosteroids under study (Figure 2). A significant reduction in the expression of eleven genes from a total sixteen, including *NFkB p65 (Rel A)*, was observed in non-irradiated cells. The other five genes: *TLR4, COX2, ICAM, NFkB p50*, and *IkB-α* were upregulated in secosteroid- treated cells without prior UVB exposure in comparison to untreated cells (Figure 2a). After UVB exposure (50 mJ/cm^2), most of the inflammatory-targeted genes including *TLR4, COX2, IL-6, IL-8, IL-17, IL-33, IL-1α, IL-1β, IL-10, CD14, NF-kB p50* and *p65, IkB-α, bcl2*, as well as *BNIP*, showed significantly increased expression in UVB-irradiated cells in comparison to non-irradiated cells (Figure 2b, upper map. Nearly all of these genes including *TLR4, IL-6, IL-17, IL-33, NFkB p65 (Rel A), bcl2* and *BNIP* were significantly downregulated by all secosteroids in UVB-irradiated cells (Figure 2b, lower map). However, the expression of some genes such as *IL-1β*, *IL-10, NFkB p50*, and *IkB-α*, was stimulated by some of the secosteroids (Figure 2b, lower map).

We propose that the vitamin D$_3$ derivatives have anti-inflammatory actions following UVB exposure. The mRNA levels of inflammation-related genes, particularly COX2, were increased in treated cells in the absence of UVB exposure. However, the vitamin D$_3$ derivatives slightly inhibited COX2 mRNA levels in cells exposed to UVB, indicating that these compounds do not activate inflammation in cells following UVB irradiation.

D$_3$ hydroxyderivatives down-regulate the phosphorylation of NF-κB p65 in UVB-irradiated keratinocytes

In agreement with previous studies showing that 1,25(OH)$_2$D$_3$, 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ suppressed the expression of the NF-κB p65 subunit in non-irradiated skin cells [29, 40, 47], this study shows that all the secosteroids tested down-regulated *NF-kB p65 (Rel A)* in the non-irradiated control while 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ caused significant down-regulation in the UVB-irradiated group (Figure 2). The level of phospho-NF-κB p65 relative to cell number and total NF-κB p65 levels detected by ELISA were evaluated to assess the activation of NF-κB p65 [56]. In non-irradiated cells, only1,25(OH)$_2$D$_3$ significantly reduced phospho-NF-κB p65 levels/cell number compared to untreated cells (Figure 3a, upper right). A modest decrease (10–20% reduction) in phospho-NF-κB p65 levels/cell number was observed with the other four secosteroids. While treatment with most secosteroids did not cause a significant down-regulation of the phospho-

NF-κB p65/total NF-κB p65 levels (5–15% reduction observed), $1,20(OH)_2D_3$ did significantly suppress their levels compared to the untreated cells (Figure 3a, lower right). UVB exposure (50 mJ/cm^2) caused up-regulation of phospho-NF-κB p65 levels relative to cell number and the total NF-κB p65 levels (Figure 3a, middle panel). $1,25(OH)_2D_3$ and $20(OH)D_3$ significantly decreased phospho-NF-κB p65 levels/cell number in UVB-irradiated cells (Figure 3a, upper left). The UVB induced level of NF-κB p65 (NF-κB p65/total NF-κB p65 ratio) was significantly reduced in cells treated with $1,25(OH)_2D_3$, $20(OH)D_3$, and $1,20(OH)_2D_3$ compared to untreated cells (Figure 3a, lower left). Thus the phosphorylation levels of NF-kB p65 were altered by treatment with some of the secosteroids, presenting their minor effects in phosphorylation levels, as seen in the whole cell fraction.

The effect of D_3 hydroxyderivatives on nuclear translocation of NF-κB p65 in UVB-irradiated keratinocytes

Since the phospho-NF-κB p65 levels assessed by a quantitative immunofluorescence assay (Figure 3a) indicated a protective effect of the secosteroids against the UVB-induced inflammatory response, we evaluated nuclear translocation of NF-κB p65 which is essential for its transcriptional activity, by image-based and western blot analyses [57]. There was a significant increase in the nuclear/cytosolic-NF-κB p65 ratio in irradiated cells compared to non-irradiated control cells (Figure 3b, c). The image-based analysis showed that all secosteroids significantly inhibited the translocation of NF-κB p65 caused by UVB exposure (Figure 3b, Figure S1). A significant reduction in the nuclear/cytosol ratio of NF-κB p65 was also observed in secosteroid-treated cells without UVB irradiation (Figure S2). We observed the expression of NF-kB protein (as indicated at 70 kDa) in both nuclear and cytosol fractions as shown in the representative blot (Figure 3c, left panel), which was normalized relative to lamin A/C and β-actin bands, respectively. The expression of NF-kB is reported as the nuclear to cytosolic ratio with Fig 3c (right panel) summarizing the data from three-independent western blots. The data show that treatment with $1,20(OH)_2D_3$, $20,23(OH)_2D_3$ or $1,20,23(OH)_3D_3$ strongly decreases the nuclear-cytosolic NF-kB p65 ratio in UVB-irradiated cells. Similarly, there is a trend for a decrease (approximately 10% inhibition), although not significant, in the nuclear-cytosolic NF-kB p65 ratio in HEKn treated with $1,25(OH)_2D_3$ and $20(OH)D_3$ in the UVB irradiated cells.

D_3-hydroxyderivatives modulate the activity of the IκB-α in UVB-irradiated keratinocytes

Since IκB-α is involved in the inhibition of NF-κB activity by trapping it in the cytosol [9], alterations to the level of cytosolic-IκB-α would indicate whether the secosteroids affect the inflammatory response through modulating IκB-α activity. While the image-based analysis presented a slight induction, western blot analysis showed a strong reduction (almost 50%) in cytosolic-IκB-α in UVB irradiated cells, indicating that UVB irradiation stimulates the inflammatory response in HEKn (Figure 4b). However, treatment with $1,25(OH)_2D_3$, $20(OH)D_3$, and $1,20(OH)_2D_3$ reversed the decrease in cytosolic IκB-α protein caused by UVB irradiation, as shown in the image-based analysis (Figure 4a, Figure S3). Expression of IkB-α protein at the expected mass of 37 kDa was anlaysed by western blotting of the cytosol fractions, with a representative blot shown in Figure 4b (upper part). Expression was normalized relative to the β-actin control protein. The graph summarizing the data from

three-independent western blots shows that the treatment with $1,25(OH)_2D_3$, $20(OH)D_3$, $1,20(OH)_2D_3$, and $20,23(OH)_2D_3$ significantly enhances cytosolic IκB-α protein levels in comparison to untreated cells (Figure 4b, lower part). Additionally, there was a significant increase in the cytosolic-IκB-α levels in cells treated with $1,25(OH)_2D_3$, $20(OH)D_3$, and $1,20(OH)_2D_3$ without UVB irradiation, as shown in the image-based analysis (Figure S4).

D_3-hydroxyderivatives exert anti-inflammatory effects on UVB-irradiated keratinocytes

Our previous studies on the vitamin D_3 hydroxyderivatives showed that they display anti-inflammatory properties including the inhibition of IFN-γ production in splenocytes incubated *in vitro* [37, 58, 59]. Our current data are consistent with these reports and suggest that the photoprotective effects of compounds under study might include the inhibition of IFN-γ production by keratinocytes. As secosteroids inhibited UVB induced activation and nuclear translocation of NF-kB, we determined if this would lead to inhibition of the expression of NF-kB dependent pro-inflammatory cytokines IL-17, IFN-γ, and TNF-α. UVB exposure (50 mJ/cm^2) significantly increased IL-17, IFN-γ, and TNF-α levels compared to non-irradiated cells (Figure 5). Treatment with $1,25(OH)_2D_3$, $20(OH)D_3$, and $1,20,23(OH)_3D_3$ significantly decreased IL-17 levels in UVB-irradiated cells (Figure 5a). Moreover, there was a significant reduction of IFN-γ levels in cells treated with all secosteroids compared to untreated cells following UVB irradiation (Figure 5b). Interferon (IFN)-γ acts as a crucial biomarker of the immune system in skin cells [60] and its up-regulation has been observed in skin inflammation [61]. IFN-γ expression in treatment groups exhibited the most pronounced change when compared to other cytokines, suggesting that the anti-inflammatory effects of the vitamin D_3 derivatives are, at least in part, mediated through the regulation of IFN-γ. Treatment of HEKn with $1,25(OH)_2D_3$, $1,20(OH)_2D_3$, $20,23(OH)_2D_3$, and $1,20,23(OH)_3D_3$ also significantly suppressed UVB-induced TNF-α levels compared to untreated cells (Figure 5c). Moreover, all secosteroids reduced levels of cytokines including IL-17, TNF-α and IFN-γ in non-irradiated cells (Figure S5). Thus, it is apparent that that $1,25(OH)_2D_3$ and the CYP11A-derived secosteroids not only modulate NF-κB p65 and IκB-α pathways, but also inhibit the production of Th1 and Th17 proinflammatory cytokines.

The long-term inhibitory effects of vitamin D_3 derivatives on inflammation, without reducing DNA damage, could potentially promote carcinogenesis [62, 63]. However, our previous studies using a range of different methods have shown that the vitamin D_3 derivatives under study attenuate direct damage to DNA, stimulate DNA repair, and induce p53 signaling which is a hallmark of the tumor suppressor pathway [10, 11]. Therefore, vitamin D_3 derivatives not only protect against UVB-induced inflammation but also against DNA damage, which implies that they have chemopreventive effects on UVB-induced carcinogenesis. It should also be noted that chronic inflammation can promote malignant transformation [64].

D_3-hydroxyderivatives modulate the expression of genes involved in cell differentiation in UVB-irradiated keratinocytes

Cell differentiation plays an important role in the repair of damaged skin [65]. Gene expression profiling of *IVL*, *LOR*, *FLG*, *TGM1*, and *KRT* (cytokeratin encoded genes)

including *KRT1*, *KRT10*, and *KRT14* was carried out to evaluate the role of the secosteroids under study on epithelial differentiation (Figure 2) [34, 46].

The heat map analysis clearly illustrated a distinct difference in the expression of all genes involved in cell differentiations in HEKn-treated with secosteroids in the presence and absence of UVB. In non-irradiated cells, there was an up-regulation of the expression of five from seven differentiation genes, *IVL, LOR, FLG, TGM1*, and *KRT10*, and down-regulation of *KRT1* and *KRT14* after treatment with secosteroids (Figure 2b, upper map). UVB irradiation significantly suppressed mRNA levels of *IVL, FLG*, and *KRT14* (Figure 2a), but stimulated *TGM1*, *KRT1*, and *KRT10* mRNA expression (Figure 2a). The expression of almost all genes involved in keratinocyte differentiation, including *IVL, LOR, FLG, TGM1, KRT1, KRT10*, and *KRT14*, was significantly upregulated by treatment with secosteroids in UVB-irradiated cells (Figure 2b, lower map). Inversely, there was a down-regulation of *KRT1* and *KRT14* expression in cells treated with 20,23$(OH)_2D_3$ and 1,20,23$(OH)_3D_3$ following UVB irradiation (Figure 2b, lower map).

D_3-hydroxyderivatives stimulate IVL and CK10 protein expression in UVB-irradiated keratinocytes

The D_3 hydroxyderivatives mediated epidermal differentiation through a significant upregulation of *IVL*, *FLG*, and *KRT10* expression in both the non-irradiated control and UVB-irradiated groups. IVL is a protein commonly used as an early stage differentiation marker of human keratinocytes [13, 66]. While UVB did not significantly affected IVL protein expression as measured by immuno-flourescence (Figure 6a), all secosteroids significantly enhanced expression of IVL protein compared to untreated cells (Figure 6a, Figure S6). Moreover, treatment of non-irradiated cells with secosteroids significantly increased IVL levels (Figure S7). IVL protein expression at the expected mass of 68 kDa in shown in a representative western blot (Figure 6b, upper part). IVL band intensity was normalized relative to the β-actin control protein. The graph of summarized data of six-independent western blots shows that there is a marked increase in IVL expression in UVB-irradiated cells treated with secosteroids (except for 1,20,23$(OH)_3D_3$), compared to UVB-irradiated control cells (Figure 6b, lower part).

Previous studies showed that CK10, highly expressed in suprabasal epidermal keratinocytes [67], was downregulated by the proinflammatory cytokines including IL-17 and TNF-α, causing an impairment of epidermal differentiation [68, 69]. Therefore, the effects of secosteroids on CK10 were evaluated. CK10 protein expression at the expected mas of 56 kDa is shown in a representative western blot (Figure 6c, left panel). CK10 band intensity was normalized relative to the β-actin control protein with a graph summarizing the results from three independent experiments being shown in Figure 6c (right panel). While UVB (50 mJ/cm^2) upregulated levels of CK10 compared to non-irradiated cells, the levels of CK10 were further increased in cells treated with 1,25$(OH)_2D_3$, 20$(OH)D_3$, 1,20$(OH)_2D_3$, and 20,23$(OH)_2D_3$ compared with UVB-irradiated untreated cells. Overall the data for IVL and CK10 indicate that the CYP11A1-derivatived secosteroids stimulate epidermal differentiation in UVB treated and untreated cells.

In contrast to the other secosteroids, treatment with 1,20,23(OH)$_3$D$_3$ reduced the expression of genes related to differentiation. However, this effect was not seen at the protein level when tested on the protein markers IVL and CK10. Even though 1,20,23(OH)$_3$D$_3$ could increase VDR mRNA, it could not change VDR protein levels (Figure 1). This suggests that 1,20,23(OH)$_3$D$_3$ has only a weak biological action on the VDR, with minimal effects on downstream targets, including differentiation.

CONCLUDING REMARKS

Here we document that 1,25(OH)$_2$D$_3$ and our synthetic CYP11A1-derived D$_3$-hydroxyderivatives suppress UVB-induced inflammatory responses in keratinocytes by modulating NF-κB/IκB-α and its down-stream signaling pathway, and promote differentiation of keratinocytes. With respect to their mechanism of action, our data indicate that the inhibition of NF-κB signaling by the secosteroids plays a role in inhibition of Th1 and Th17 differentiation directly and indirectly. NF-κBp65 (RelA) binds to enhancer regions within the *Ifng* locus in T cells to promote expression of IFN-γ and drive Th1 differentiation [70]. Similarly, NF-κB is necessary for the efficient expression of retinoic orphan acid receptor (ROR)γ, the key transcription factor necessary for Th17 differentiation [71]. NF-κB is also important for the expression of IL-12 and IL-1β by innate immune cells, cytokines necessary for Th1 and Th17 differentiation, respectively (Reviewed in [5]. This NF-κB regulation may be mediated by the VDR as reported previously in non-irradiated keratinocytes [29, 40]. The downregulation of Th17 responses may be mediated by the reverse agonistic activity of secosteroids on RORs, which are expressed in keratinocytes, [35, 55], and its γ form in particular, since IL-17 is downstream of RORγ [72]. However, indirect involvement of NF-κB acting on RORγ cannot be excluded [5]. With respect to keratinocyte differentiation, the involvement of VDR is expected, however, the contribution of other nuclear receptors including RORα and γ and of the arylhydrocarbon receptor (AhR) is likely since they are expressed and functional in keratinocytes [34, 35, 46, 55]. They may also contribute to the anti-inflammatory effects of the secosteroids. The precise involvement of the above receptors in photoprotective signaling needs further investigations using selective or combined knock-out mice for these and VDR receptors.

The described photoprotective mechanisms of vitamin D$_3$-hydroxyderivatives involving the targeting of nuclear receptors, including VDR, and NF-κB signaling pathways, represent attractive targets for the development of therapeutic agents for skin disorders associated with UVB-induced inflammation and impaired keratinocyte differentiation.

DATA AVAILABILITY STATEMENT

No datasets were generated or analyzed during the current study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS:

1,25(OH)$_2$D$_3$	1α,25-dihydroxyvitamin D$_3$
20(OH)D$_3$	20*S*-hydroxyvitamin D$_3$
1,20(OH)$_2$D$_3$	1α,20*S*-dihydroxyvitamin D$_3$
20,23(OH)$_2$D$_3$	20*S*,23*S*-dihydroxyvitamin D$_3$
1,20,23(OH)$_3$D$_3$	1α,20*S*,23*S*-trihydroxyvitamin D$_3$
CYP	cytochrome P450 enzymes
CYP11A1	cytochrome P450 family 11 subfamily A member 1
HEKn	human epidermal keratinocytes
EtOH	ethanol
UVB	ultraviolet B
NF-κB	nuclear factor kappa B
IκB	inhibitor kappa B
TLR4	toll-like receptor 4
CD14	cluster of differentiation 14
COX-2	cyclooxygenase-2
IL-6	interleukin-6
IL-8	interleukin-8
IL-17	interleukin-17
IL-33	interleukin-33
IL-1α	interleukin-1α, IL-1β, interleukin-1β
IL-10	interleukin-10
IFN-γ	interferon gamma
TNF-α	tumor necrosis factor-alpha
Bcl2	B-cell lymphoma 2

BNIP	B-cell lymphoma 2/adenovirus E1B-19K protein-interacting protein
VDR	vitamin D_3 receptor
IVL	involucrin
CK10	cytokeratin 10
CK1	cytokeratin 1
CK14	cytokeratin 14
KRT	cytokeratin encoded gene
LOR	loricrine
FLG	filaggrine
TGM1	transglutaminase 1

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HIGHLIGHTS

- Vitamin D_3 hydroxyderivatives protect against UVB-induced skin damage
- Vitamin D_3 hydroxyderivatives target NF-κB/IκB inflammatory pathways
- Vitamin D_3 hydroxyderivatives promote keratinocyte differentiation
- Vitamin D_3 hydroxyderivatives are promising compounds for skin photoprotection

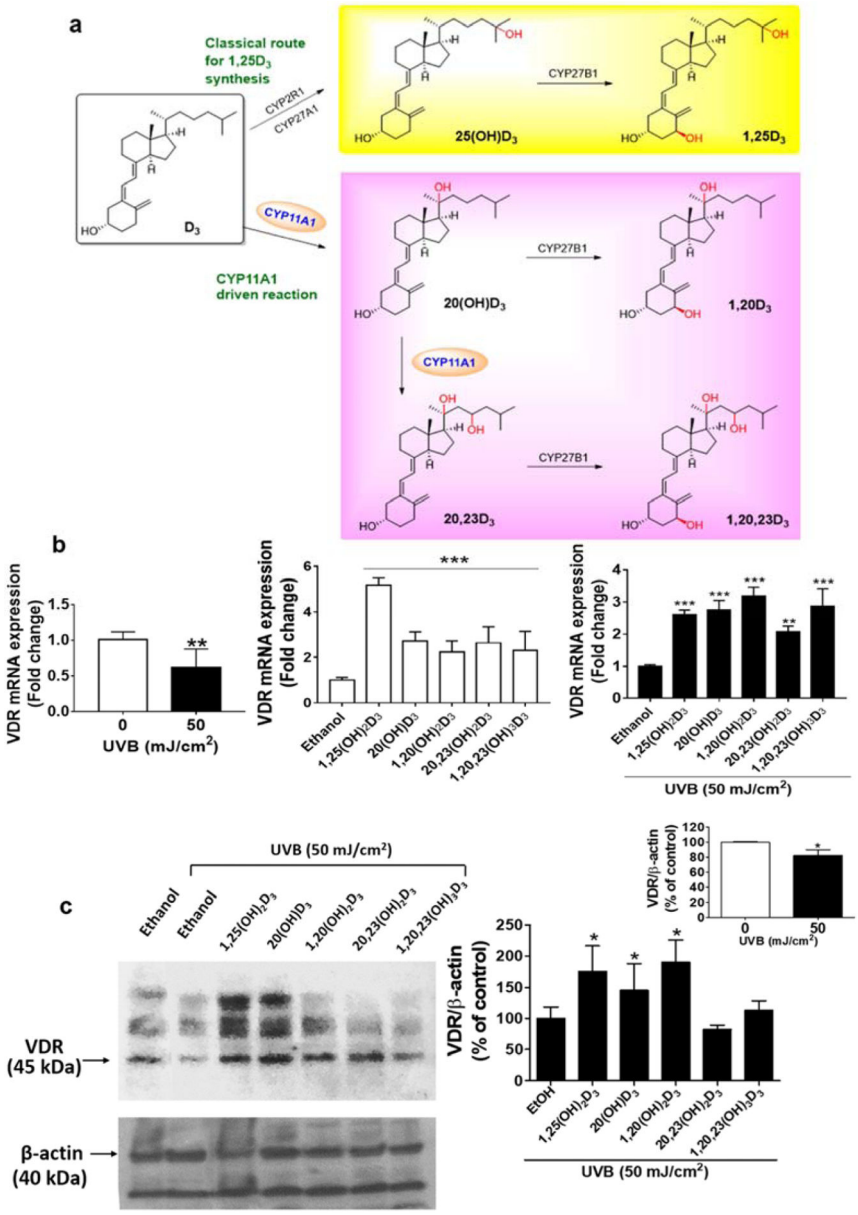

Figure 1.

UVB inhibits, while vitamin D_3 hydroxyderivatives stimulate VDR expression in human keratinocytes. Keratinocytes were pretreated with 100 nM secosteroids (as indicated) or ethanol (solvent control) for 24 h before UVB irradiation (50 mJ/cm^2) and then further incubated with the secosteroids for an additional 24 h. (a) D_3 transformation scheme (b) Real-time RT-PCR analyses of UVB-irradiated cells after treatment are shown with black bar charts while non-irradiated cells are shown with white bar charts. VDR mRNA was normalized relative to β-actin, cyclopilin, and GAPDH mRNA levels. Western blot analyses are in (c). VDR protein, detected at 45 kDa, was normalized relative to β-actin and presented as % of control (mean ± S.D.). All are representative of 3–6 independent experiments. Statistical analyses were done by the student t-test, *P<0.05, **P<0.01, ***P<0.001 for all conditions compared to the untreated control.

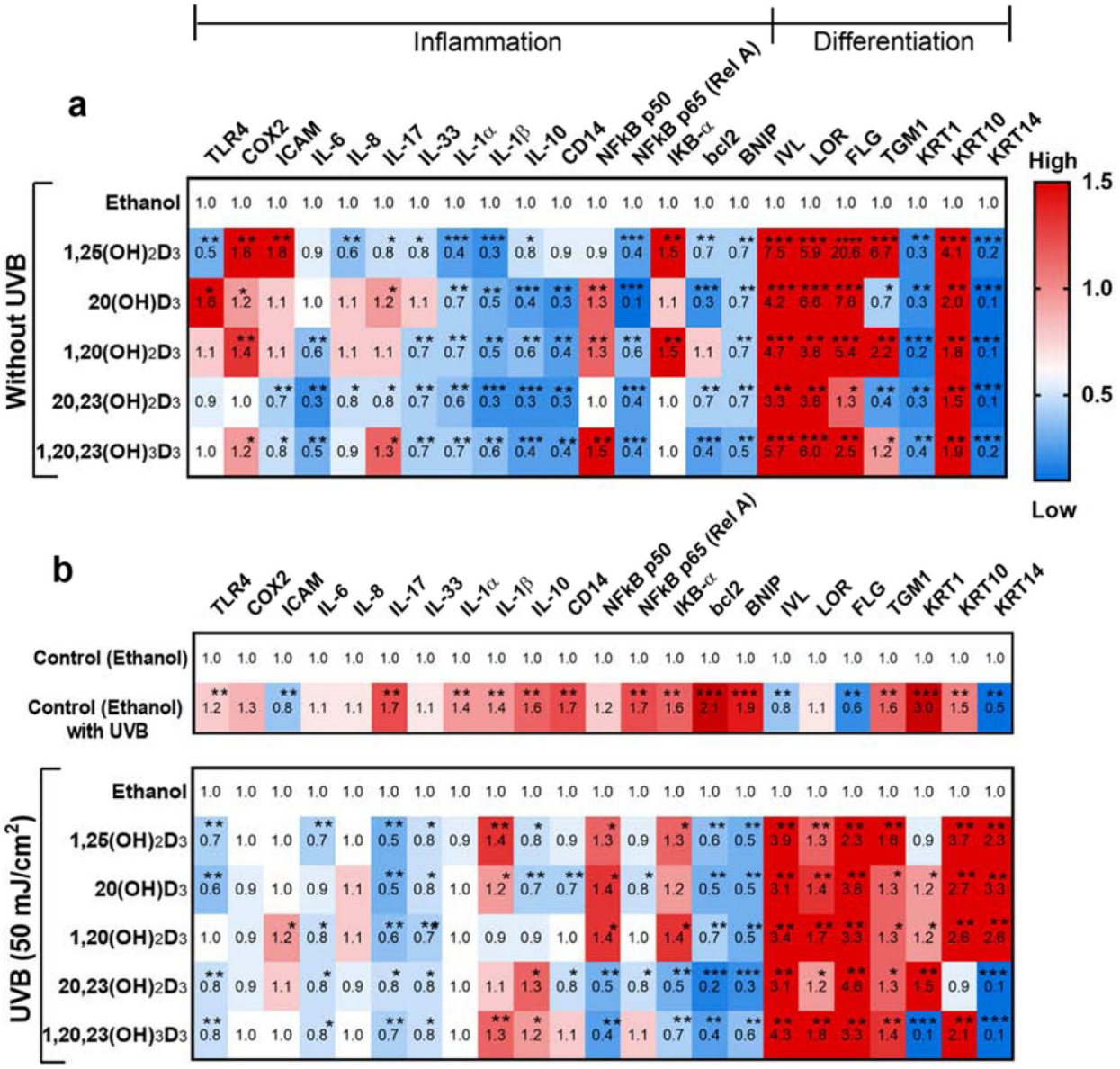

Figure 2.

Heat map analysis of the expression of genes involved in inflammation and differentiation following treatment with the hydroxyderivatives of D_3 with and without UVB irradiation. Treatment with secosteroids was performed as described in materials and methods. Gene expression was normalized relative to β-actin, cyclopilin, and GAPDH mRNA. Cells treated with secosteroids (or ethanol) without (a) or with UVB irradiation (b). (b) Heat-maps of log2 transformed expression ratios for non-irradiated compared with UVB-irradiated cells. Each vertical row represents the same gene product and each horizontal row each sample. The fluorescence range from high (red) to low (blue) is indicated by the colored bar and reflects the degree of fluorescence intensity/gene expression. Statistical analyses was via the student t-test, *P<0.05, **P<0.01, ***P<0.001 for all conditions, n = 3.

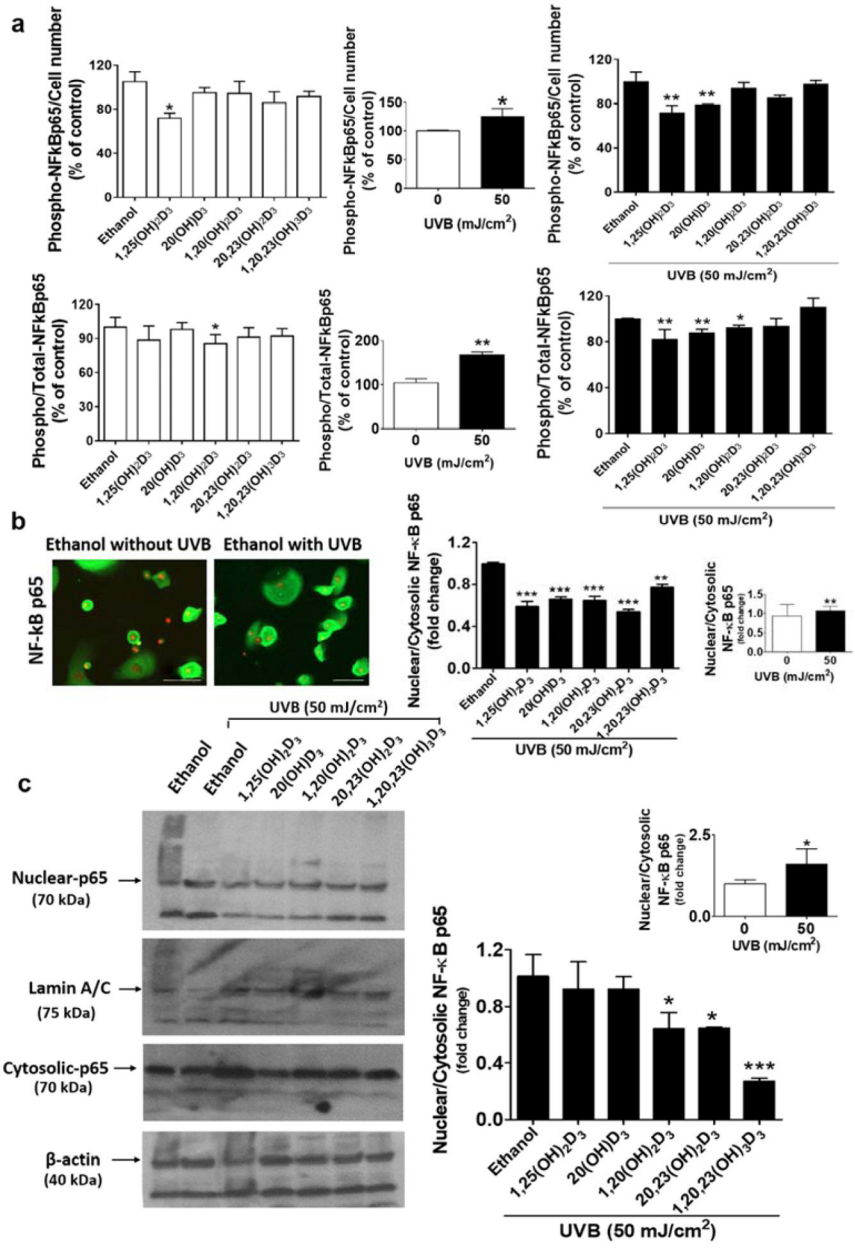

Figure 3.

D_3 hydroxyderivatives protect against UVB-induced increases in NF-κB p65 phosphorylation and nuclear translocation. (a) ELISA tagged with the phosphorylated-NF-κB p65 or total-NF-κB p65 antibody was used to measure the level of phospho-NF-κB p65/cell number and the phospho/total-NF-κB p65 ratio. (b) NF-κB p65-flourescent positive cells analysis. Lower panel: Quantification of immunofluorescent positive cells presented as the fold-change of the nuclear/cytosol ratio (mean ± S.D.). Scale bar = 100 μm, n ≥ 100 cells for each condition. (c) The blot was stripped and reprobed sequentially with antibodies to nuclear and cytosolic-NF-κB p65 (70 kDa). All are representative of 3–6 independent experiments. Lower panel: Quantification of NF-κB p65 protein is presented as the nuclear/

cytosol ratio (mean ± S.D.) and evaluated by the student t-test, *P<0.05, **P<0.01, ***P<0.001 for all conditions compared to the untreated control.

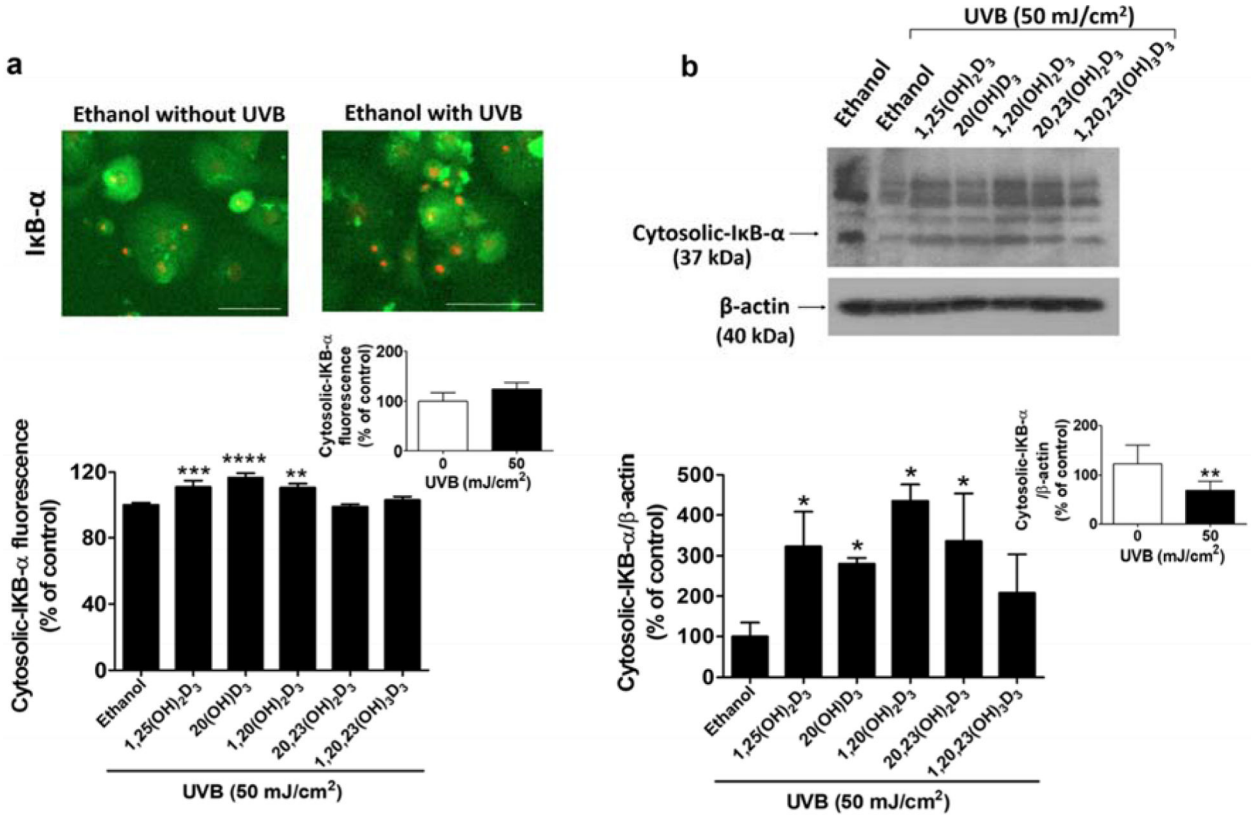

Figure 4.

D_3 hydroxyderivatives stimulate the inhibitory proteins of the κB-α complex (IκB-α) in UVB-irradiated cells. Treatment with secosteroids (100 nM) was performed as described in materials and methods. Fluorescent microscopy of cells stained with anti-cytosolic-IκB-α is presented in (a). Lower panel: Quantification of immunofluorescent positive cells using the Cytation™ 5 cell imaging, n ≥ 100 cells for each condition. Scale bar = 100 μm. Data are presented as the % of control (mean ± S.D.). (b) The blot was stripped and reprobed sequentially with antibodies to cytosolic- IκB-α (37 kDa). All are representative of 3–6 independent experiments. Lower panel: Quantification of IκB-α protein with data being presented as the % of control (mean ± S.D.) and evaluated by the student t-test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 for all conditions relative to the untreated control.

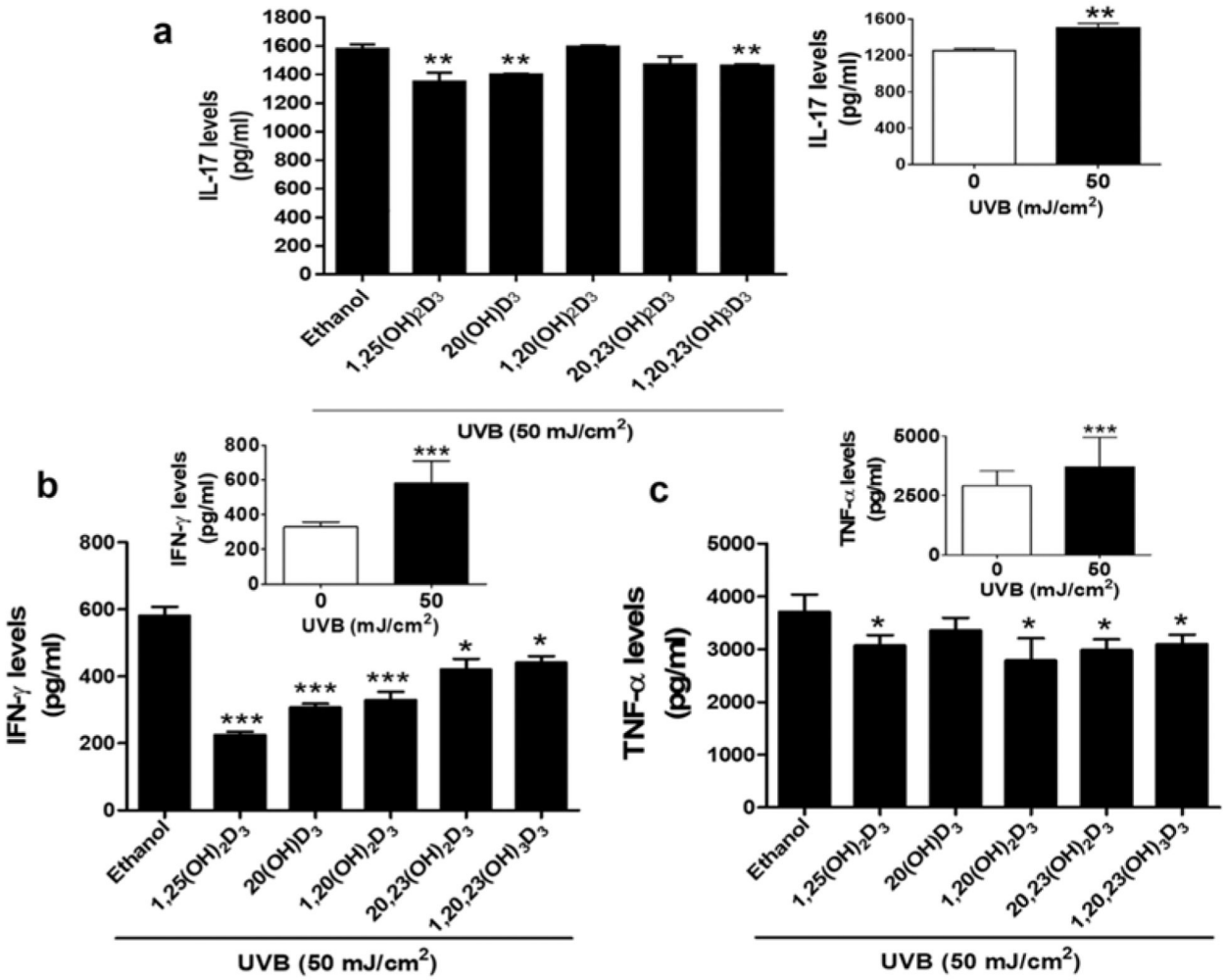

Figure 5.

Anti-inflammatory effect of D_3 hydroxyderivatives on UVB-irradiated keratinocytes. Treatment with secosteroids (100 nM) was performed as described in materials and methods. Cell supernatants were plated using an ELISA assay kit tagged with (a) IL-17, (b) IFN-γ, or (c) TNF-α antibody. The levels of inflammatory cytokines were calculated from the standard curves. Data are presented as the concentration of cytokines (pg/ml). The statistical significance of differences was evaluated by the student t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, for all conditions relative to the untreated control.

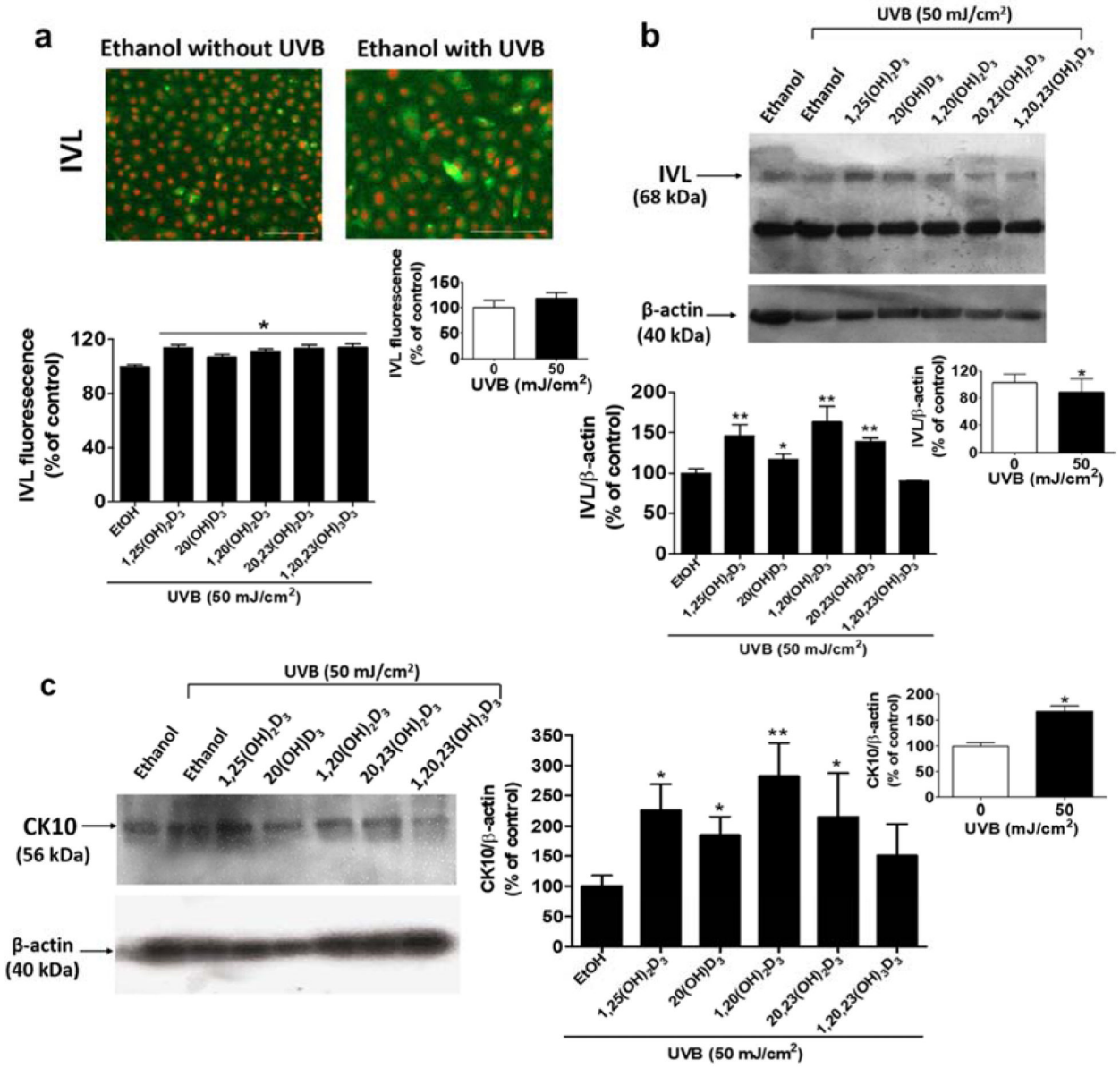

Figure 6.

D_3 hydroxyderivatives modulate the differentiation of UVB-irradiated keratinocytes as measured by the increased expression of involucrin (IVL) and cytokeratin 10 (CK10). IVL-fluorescent positive cells are presented in (a). Lower panel: Quantification of immunofluorescent positive cells using the Cytation™ 5 cell imaging multi-mode reader, n ≥ 100 cells for each condition. Scale bar = 100 μm. (b) The blot was stripped and reprobed sequentially with antibodies to IVL (68 kDa). Lower panel: Quantification of IVL protein. (c) The blot was stripped and reprobed sequentially with antibodies to CK10 (56 kDa). Lower panel: Quantification of CK10 protein. All are representative of 3–6 independent experiments. Data are presented as the % of control (mean ± S.D.) and evaluated by the

student t-test, *P<0.05, **P<0.01, ***P<0.001, for all conditions relative to the untreated control.