TNF-α increases the expression and activity of vitamin D receptor in keratinocytes: role of c-Jun N-terminal kinase

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ABSTRACT

Several inflammatory mediators increase calcitriol production by epidermal keratinocytes. In turn calcitriol attenuates the keratinocyte inflammatory response. Since the effect of the in-situ generated calcitriol depends also on the sensitivity to the hormone we studied the effect of inflammatory cytokines on the response of HaCaT human keratinocytes to calcitriol by examining the expression and transcriptional activity of VDR. Treatment with TNF, but not with IL-1β or interferon γ, increased VDR protein level, while decreasing the level of its heterodimerization partner RXRα. This was associated with increased VDR mRNA levels. c-Jun N-terminal kinase, but not P38 MAPK or NFκB, was found to participate in the upregulation of VDR by TNF. The functional significance of the modulation of VDR and RXRα levels by TNF is manifested by increased induction of VDR target gene CYP24A1 by calcitriol. Calcitriol, in turn, inhibited the enhanced expression of VDR by TNF. In conclusion, the inflammatory cytokine TNF increases the response of keratinocytes to calcitriol through upregulation of its receptor VDR, which in turn is subject to negative feedback by the hormone accelerating the return of the keratinocyte vitamin D system to its basal activity. We surmise that the increased generation and sensitivity to calcitriol in keratinocytes play a role in the resolution of epidermal inflammation.

ARTICLE HISTORY
Received 21 October 2015
Revised 22 December 2015
Accepted 28 December 2015

KEYWORDS
c-Jun N-terminal kinase; calcitriol; keratinocytes; tumor necrosis factor; vitamin D receptor

The autocrine/paracrine actions of calcitriol, the active metabolite of vitamin D, have an important role in epidermal homeostasis. It promotes keratinocyte differentiation, modulates their proliferation, protects keratinocytes from damage inflicted by a variety of environmental and pathophysiological stressors and attenuates their inflammatory response.1-7 Epidermal keratinocytes are capable of producing calcitriol from its precursor, 7-dehydrocholesterol. This is a three-step process that includes a photochemical reaction producing vitamin D3, and two successive hydroxylations.5-8 Keratinocytes are also able to degrade calcitriol and are target to its action via their vitamin D receptor (VDR).5 VDR is a nuclear receptor that interacts with vitamin D response elements in the promoters of its target genes as a heterodimer with another nuclear receptor, RXR. Therefore, the epidermis can be viewed as a self-contained vitamin D endocrine system. Epidermal inflammation is a recurrent phenomenon invoked by breaching of the epidermal barrier and by exposure to environmental stressors, infectious agents and infiltrating immune cells.9 Accumulating evidence indicates that the inflammatory milieu affects at least one aspect of the epidermal vitamin D endocrine system, namely, the production of calcitriol. We and others have shown that exposure of keratinocytes to inflammatory triggers and mediators increased the rate of calcitriol production and the expression of CYP27B1 encoding 25-hydroxyvitamin D 1α-hydroxylase, the enzyme responsible for calcitriol synthesis.7,10,11 On the other hand, there is no information on effects of inflammatory mediators on the other aspect of this endocrine system, namely, the response of keratinocytes to VDR ligands. The objective of the present study is to shed light on this subject.

Materials & methods

Materials

MEM, fetal calf serum (FCS), L-glutamine, antibiotics mixture (penstrepnystatin) and trypsin-EDTA solution B were purchased from Biological Industries.
Tissue culture dishes were purchased from Corning Glass Work. 1α,25-dihydroxyvitamin D₃ (calcitriol) was obtained from Teva Pharmaceutical Industries Ltd. BSA fraction V (160069) was purchased from ICN Biomedicals, Inc. BCA Protein Assay Kit (23225) was obtained from Pierce Biotechnology Inc.. Human recombinant tumor necrosis factor α (TNFα) (300-01A), interleukin-1β (IL-1β) (200-01B) and interferon γ (IFNγ) (300-02L) were obtained from PeproTech Inc.. U0126 (BML-EI282-0001) and SB203580 (BML-EI286-0001) were purchased from Alexis Biochemicals. SP600125 (420119) and BMS-345541 (401480) were purchased from Calbiochem. Actinomycin D (A-1410) was purchased from Sigma Chemical Co. Mouse monoclonal anti-VDR (D-6) (sc-13133) and polyclonal rabbit anti RXRα (sc-553) were obtained from Santa Cruz Biotechnology. IRDye 800CW goat anti-mouse IgG (925-32210) and IRDye 800CW goat anti-rabbit IgG Abs (926-32211) were from LICOR. All other reagents are of analytical grade.

**Cell culture**

The human keratinocyte cell line HaCaT was kindly provided by Professor N. Fusenig, German Cancer Research Center, Heidelberg, Germany. Cells were maintained in minimal essential medium containing 0.075 mM calcium supplemented with 10% FCS and antibiotics. In preparation of an experiment HaCaT cells were plated in serum-containing medium at 250,000 cells/3.5 cm Petri dish for western blot analysis, and 500,000 cells/6 cm Petri dish for RNA extraction. 24 hours later the medium was replaced with serum-free medium containing 0.5 mg/mL BSA. Cultures were then treated with calcitriol, various cytokines and inhibitors; ethanol and DMSO, the vehicles of calcitriol and kinase inhibitors, were added to control cultures.

**RNA extraction and quantitative real-time PCR**

Total RNA was isolated and reverse transcribed using random hexamer primers, employing the EZ-RNA total RNA isolation kit (20-400-100) and EZ-First Strand cDNA Synthesis Kit (20-800-50) (Biological Industries) according to the manufacturer’s instructions. Transcribed cDNA was then amplified using TaqMan gene expression assays (Hs00172113 for VDR, and Hs9999902_m1 for the endogenous control gene, ribosomal protein large p-Zero, RPLP0) (Applied Biosystems, Forster City, CA) according to the manufacturer’s instructions by means of the Applied Biosystems Prism 7000 Sequence Detector.

**Western blot analysis**

Preparation of cell extracts, SDS-PAGE (10% polyacrylamide), and protein gel blot analysis were performed as described previously. Blots were imaged using the LI-COR Odyssey Infrared Imager, and LI-COR software was used to calculate integrated intensities of bands (LI-COR Biosciences, Lincoln, NE).

**Results**

**The experimental system**

Our experimental system was the human immortalized non-tumorigenic HaCaT keratinocytes. HaCaT cells can undergo differentiation in vivo and in vitro and serve as a model for proliferating non-tumorigenic epidermal cells. Keratinocytes in primary cultures require exogenous growth factors and other active ingredients to survive and proliferate. These exogenous agents such as pituitary-derived peptides, cAMP-elevating agents, glucocorticosteroids and a variety of growth factors are all known to modulate intracellular signaling pathways involved in the inflammatory response. In contrast to primary keratinocytes, HaCaT cells are able to grow in the absence of serum or other exogenous active ingredients and thus are less subjected to background noise of confounding signals. The inflammatory milieu was simulated by exposure of HaCaT cells to several inflammatory cytokines.

**Protein expression of VDR and RXRα in HaCaT cells treated with TNF, IL-1β or IFNγ**

Treatment of HaCaT cells for 24 hours with the inflammatory cytokine TNF brought about a marked, dose dependent increase in the protein level of VDR (Fig. 1A). This effect was repeatedly observed in 10 independent experiments in which treatment with TNF (10 ng/mL) increased VDR levels 3.0 ± 0.3 fold (mean ± SEM, P< 0.0002). However, the same treatment decreased by 50% the level of the VDR heterodimerization partner,
RXRα (the most abundant RXR isotype in epidermal keratinocytes) (Fig. 1A). It is notable that exposure of HaCaT cells to IL-1β or IFNγ had no effect on the protein cellular levels of VDR and RXRα (Fig. 1B).

**mRNA expression of VDR in HaCaT cells treated with TNF, IL-1β or IFNγ**

To assess whether the effect of TNF was due to increased gene expression, we treated HaCaT cells with TNF for 0–24 hours and assayed VDR mRNA. As can be seen in Figure 2A, VDR mRNA increased in a time dependent manner, attaining maximal levels at 16 hours of exposure. 24 hour exposure to IL-1β or IFNγ had no effect on VDR mRNA levels in accordance with their lack of effect on VDR protein level (Fig. 2B).

In order to assess whether the increase in VDR mRNA levels is at least partially due to increased stability of the transcript, VDR mRNA decay in control and TNF-treated cultures was measured following blockage of mRNA transcription with actinomycin D. As is clearly seen in Figure 2C, VDR mRNA stability was not affected by TNF. This indicates that the effect of TNF on VDR mRNA is probably due to increased transcription rate.

**Functional significance of the modulation of VDR and RXRα levels by TNF**

The opposing effects of TNF on VDR and its heterodimerization partner RXRα leaves unpredictable its impact on VDR ligand activity. The functional significance of these opposing effects was assessed by assaying VDR transcriptional activity (e.g., induction of its target gene, CYP24A1) in control and TNF-treated cells following exposure to calcitriol. As can been seen in Figure 3, 22 hour treatment with TNF increased the responsiveness of HaCaT cells to calcitriol as determined by induction of CYP24A1.

**Involvement of MAPK and NFκB signaling pathways in the upregulation of VDR by TNF**

By using an inhibitor of the c-Jun N-terminal kinase (JNK), SP600125, we found that this pathway is involved in the up regulation of VDR by TNF as evidenced both on the protein and mRNA levels (Fig. 4A-B). Using pharmacological inhibitors (SB203580 and BMS345541) of other signaling
pathways we found that the p38 MAPK and the NFκB signaling pathways are not involved in the upregulation of VDR by TNF. The inhibitor of the ERK pathway, U0126, moderately increased the effect of TNF, indicating that the ERK pathway exerts an inhibitory effect on the upregulation of VDR by TNF (Fig. 4C).

Inhibition of TNF-induced upregulation of VDR by calcitriol

Effects of calcitriol on the constitutive and regulated expression of VDR, reported in many studies, were shown to be cell and tissue context dependent.\textsuperscript{15-20} Therefore, we examined the effect of calcitriol on the levels of VDR and on its upregulation by TNF. HaCaT cells were exposed to calcitriol (100 nM) for 48 hours and to TNF (10 ng/mL) for the last 24 hours and VDR mRNA levels were determined by real-time PCR. As can be seen in Figure 5, treatment with calcitriol as a single agent had no effect on VDR levels, whereas it abrogated the upregulation of VDR by TNF.

Figure 2. TNF selectively increases VDR gene expression without affecting mRNA stability. HaCaT cells were treated with TNF (10 ng/mL) for 0–24 hours (A) or with IL-1β and IFNγ (20 ng/mL) for 24 hours (B). mRNA levels of VDR were quantified by real-time PCR and normalized to RPLP0 mRNA levels. Data are presented as % of control of four independent cultures. The significance of the difference between groups was assessed by unpaired Student’s t-test: cultures treated vs non-treated with TNF (*, P < 0.02; **, P < 0.005). (C), HaCaT cells were treated with TNF (10 ng/mL) for 17 hours and then exposed to actinomycin D (1μg/mL) for 0, 2, 4 and 6 hours before harvesting. mRNA levels of VDR were quantified by real-time PCR and normalized to RPLP0 mRNA. Results are the mean of 3 independent cultures and presented as percent of mRNA level at the time of addition of actinomycin D. SD did not exceed 15% of the mean (not shown).

Figure 3. TNF increases CYP24A1 gene expression induced by calcitriol. HaCaT cells were treated with TNF (10 ng/mL) for 22 hours. Calcitriol was then added to TNF-treated and untreated cultures for 2 hours. mRNA levels of CYP24A1 were quantified by real-time PCR and normalized to RPLP0 mRNA levels. Values for untreated cultures were assigned the arbitrary value of 1. The significance of the difference between groups was assessed by unpaired Student’s t-test: cultures treated vs non-treated with calcitriol (*, P < 0.01); cultures treated vs non-treated with TNF (#, P < 0.01).
The main finding of this study is that exposure of keratinocytes to the ubiquitous inflammatory cytokine tumor necrosis factor α (TNF) brings about a marked increase in the expression of the vitamin D receptor (VDR) with a concurrent decrease in the expression of its heterodimerization partner RXRα. The outcome of these opposing effects was enhanced transcriptional activity of VDR. This upregulation of VDR by TNF is not a general phenomenon, since in other cell types such as smooth muscle cells and kidney epithelial cells the cytokine was shown to reduce rather than increase VDR expression. It is noteworthy that this activity of TNF was not shared by two other main inflammatory cytokines IL-1β and IFN-γ. The increase in VDR expression is evident at both the protein and mRNA levels and is probably due to increased transcription rate. The functional impact of the opposing effects of TNF on the levels of VDR and RXRα turns out to be more efficient transactivation of VDR target gene as illustrated by induction of CYP24A1 by calcitriol. This indicates that under these conditions RXRα level is not rate limiting for VDR activity.

Treatment of keratinocytes with TNF is known to stimulate numerous signaling pathways such as the MAPK pathways and the NFκB pathway. Using specific pharmacological inhibitors we found that the JNK pathway has a major role in the upregulation of VDR by TNF, while the p38 and NFκB pathways do not participate in this process and ERK1/2 seems to slightly inhibit it. The involvement of JNK in VDR expression is in line with previous reports showing that stress-induced and estrogen-induced activation of the JNK pathway brought about increased VDR expression in

**Figure 4.** JNK pathway is partially involved in the upregulation of VDR by TNF. HaCaT cells were treated with SP600125 (SP) (30 μM), SB203580 (SB) (5 μM), U0126 (1 μM) or BMS345541 (BMS) (1 μM) for 30 minutes and then exposed to TNF (10 ng/mL) in the presence of inhibitors for 24 hours. VDR protein levels were determined in cell extracts by protein gel blot analysis (A) and mRNA levels of VDR were quantified by real-time PCR and normalized to RPLP0 mRNA levels (B,C). Data are presented as mean ± SD of four independent cultures. The significance of the difference between groups was assessed by unpaired Student’s t-test: cultures treated vs non-treated with TNF (*, P < 0.05); cultures treated vs non-treated with inhibitor (#, P < 0.05).

**Figure 5.** Calcitriol inhibits the upregulation of VDR by TNF. HaCaT cells were treated with calcitriol (100 nM) for 24 h and then incubated with TNF (10 ng/mL) in the presence of calcitriol for 24h. mRNA levels of VDR were quantified by real-time PCR and normalized to RPLP0 mRNA levels. Data are presented as mean ± SD of four independent cultures. The significance of the difference between groups was assessed by unpaired Student’s t-test: cultures treated vs non-treated with TNF (*, P < 0.01); cultures treated vs non-treated with calcitriol (#, P < 0.001).

**Discussion**

The main finding of this study is that exposure of keratinocytes to the ubiquitous inflammatory cytokine tumor necrosis factor α brings about a marked increase in the expression of the vitamin D receptor VDR with a concurrent decrease in the expression of its
human breast cancer cells and mouse embryonic fibroblasts. However, in breast cancer cells p38 MAPK was also essential for regulated VDR expression. Contrary to our finding the ERK pathway was reported to mediate the upregulation of VDR by estrogen in human breast and colon cancer cells. Taken together these results demonstrate that the MAPK cascades play a role in the regulation of VDR expression although their specific role is context dependent. The regulation of VDR expression by the MAPK pathways has been attributed to the presence of AP-1 binding sites in the VDR promoter.

TNF is considered to be an important mediator of cutaneous inflammation and is involved in the pathogenesis of a variety of inflammatory skin conditions such as psoriasis, allergic and irritant contact dermatitis, cutaneous lupus and chronic photosensitive disease. It is well accepted that calcitriol attenuates the inflammatory response of epidermal keratinocytes. This anti-inflammatory action is at least partially due to inhibition of both TNF production by keratinocytes and its pro-inflammatory activity in keratinocytes. Previous studies have shown that the rate of calcitriol production by keratinocytes increased following exposure to TNF. This increase is associated with elevated expression of 25-hydroxyvitamin D 1α-hydroxylase (CYP27B1). In the present study we demonstrate that not only the production of calcitriol is increased following exposure to TNF, as shown in refs 7 and 10, but also the response of keratinocytes to calcitriol due to upregulation of its receptor VDR. This VDR upregulation by TNF is subject to negative feedback by calcitriol (Fig. 5) suggesting that increased calcitriol levels at later phases of inflammation contribute to the relaxation of the vitamin D response system to its basal activity. We surmise that the increased generation and activity of calcitriol in keratinocytes have a role in the resolution phase of epidermal inflammation. This study highlights the major role of JNK pathway in the mediation of both the keratinocyte inflammatory response to TNF and, as shown here, the upregulation of VDR by the cytokine. We have previously shown that calcitriol inhibits TNF-induced JNK activation in keratinocytes. Taken together we conclude that inhibition of JNK activation underlies the anti-inflammatory activity of calcitriol and is also responsible for timely termination of the increased sensitivity of keratinocytes to the hormone.

**Abbreviations**

CYP24A1 1,25-dihydroxyvitamin D, 24-hydroxylase
IL-1β interleukin-1β
IFNγ interferon γ
JNK c-Jun N-terminal kinase
TNF tumor necrosis factor α
VDR vitamin D receptor

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

This work was performed in partial fulfillment of the requirements for a Ph.D. degree of Ester Ziv, Sackler Faculty of Medicine, Tel Aviv University, Israel.

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