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Ultraviolet B Suppresses Vitamin D Receptor Gene Expression in Keratinocytes

Stéphane J. Courtois,*,[1] Siegfried Segaert,*,† Hugo Degreef,* Roger Bouillon,† and Marjan Garmyn*,[2]

**Department of Dermatology and †Laboratory for Experimental Medicine and Endocrinology, Katholieke Universiteit Leuven, Campus Gasthuisberg, Onderwijs en Navorsing, Herestraat 49, B-3000 Louvain, Belgium*

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Keratinocytes not only produce vitamin D_3 in response to ultraviolet B light (UVB) and convert 25-hydroxyvitamin D_3 to 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2$D) but also possess the vitamin D receptor (VDR) and respond to 1,25$(OH)_2$D. We characterized the regulation of the expression of the VDR gene in primary human keratinocytes following UVB irradiation. We report a marked dose-dependent down-regulation of the VDR mRNA and protein within a few hours after irradiation. This occurs independently of *de novo* protein synthesis and is not due to a change in the half-life of the VDR mRNA. Interestingly, treatment of the cells with sodium salicylate, caffeic acid phenethyl ester and tosylphenylchloromethylketone inhibited this down-regulation. Our results strongly suggest the existence of a feedback mechanism in that UVB initiates vitamin D synthesis in keratinocytes and at the same time limits VDR abundance. They also provide a rational explanation for the reported lack of any additive effect between 1,25$(OH)_2$D and UVB phototherapy in the treatment of psoriasis. © 1998 Academic Press

Keratinocytes occupy a central and unique position in the vitamin D endocrine system. Firstly, by the action of the UVB component of sunlight they convert provitamin D_3 to previtamin D_3 which is subsequently thermo-isomerised to vitamin D_3 (1). Secondly, the presence of 1α-hydroxylase (2) and possibly 25-hydroxylase (3) in keratinocytes allows them to further convert vitamin D_3 to 1,25$(OH)_2$D, the hormonally active form of vitamin D. Thirdly, keratinocytes express the VDR through which they respond to 1,25$(OH)_2$D with changes in proliferation and differentiation (4-8). VDR is a member of the nuclear receptor superfamily that is activated by 1,25$(OH)_2$D thereby regulating the transcription of target genes (9).

Exposure of keratinocytes to UVB also results in a complex regulation of early genes involved in growth arrest, DNA repair and apoptosis (for review see 10). The ultraviolet transduction pathways involved have nuclear as well as extra-nuclear origin and ultimately lead to the induction of transcription factors (for review see 11).

Since the regulation of VDR is still poorly characterized, we were interested in analyzing the expression of VDR in primary human keratinocytes irradiated with a biologically relevant dose of UVB which initiates cutaneous vitamin D synthesis in vivo. We show a dose-dependent down-regulation of VDR gene expression following irradiation. In order to elucidate the transduction pathway elicited by UVB we tested various inhibitors and found that sodium salicylate (Nasa), caffeic acid phenethyl ester (CAPE) and tosylphenylchloromethylketone (TPCK) were able to markedly counteract this decrease. Our results suggest the existence of a negative feedback mechanism induced by UVB which may result in a decreased responsiveness of the skin to VD.

MATERIALS AND METHODS

Cell culture and reagents. Normal human keratinocytes were isolated from foreskins as described (12) and grown in low-calcium (0.09 mM) Keratinocyte Serum Free Medium (Gibco-BRL) supplemented with bovine pituitary extract and human epidermal growth factor 1-51. NaSa (Sigma) was used at 20 mM in phosphate saline buffer (PBS); CAPE (Calbiochem) was used at 25 μg/ml in 70% ethanol; TPCK (Sigma) was used at 50 μM in absolute ethanol.

Irradiation procedure. Before irradiation, cells were washed with PBS and then irradiated through a film of PBS and refed with their own media immediately thereafter. Any sunscreen effects of the re-

[1] Present address: Laboratoire de Biochimie et de Biologie Cellulaire, Facultés Universitaires Notre-Dame de la Paix, Chaussée de Bruxelles 61, B-5000 Namur, Belgium.

[2] To whom correspondence should be addressed. at Dermatologie, U.Z. Sint-Rafaël, Kapucijnenvoer 33, B-3000 Louvain. Fax: 32-16-337951. E-mail: marjan.garmyn@med.kuleuven.ac.be.

Abbreviations used: 1,25$(OH)_2$D, 1α,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; UVB, ultraviolet B; NaSa, sodium salicylate; CAPE, caffeic acid phenethyl ester; TPCK, tosylphenylchloromethylketone; NAC, N-acetylcysteine; PDTC, pyrrolidinedithiocarbamate; ROI, reactive oxygen intermediates; TNFα, tumor necrosis factor α.

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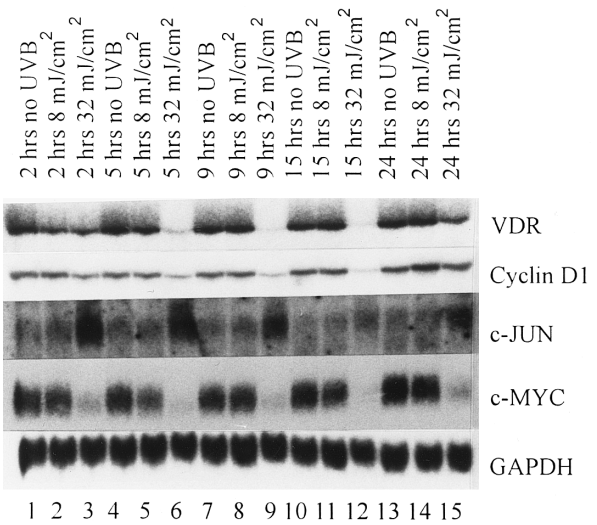

FIG. 1. Expression pattern analysis of primary human keratinocytes following UVB irradiation. Keratinocytes were irradiated with 8 mJ/cm^2 (lanes 2, 5, 8, 11, 14) or 32 mJ/cm^2 (lanes 3, 6, 9, 12, 15) and total RNA was prepared 2 hours (lanes 1-3), 5 hours (lanes 4-6), 9 hours (lanes 7-9), 15 hours (lanes 10-12) or 24 hours (lanes 13-15) post-irradiation. Sequential hybridization was performed with cDNA probes for VDR, cyclin D1, c-Jun, C-Myc and GAPDH as indicated.

agents tested were thus prevented. The UVB source was a parallel bank of three Philips TL20W12 tubes with a peak output around 310 nm.

Northern blot analysis. Northern blot experiments were performed as previously reported (13). The probes used were the human VDR cDNA (American Type Culture Collection) (14); a human C-Myc probe prepared with a RT-PCR amplimer set (Clontech. Laboratories Inc.); the human cyclin D1 cDNA (15); the mouse c-jun cDNA (16). For normalization of even loading, the blots were hybridized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe.

Western blot analyses. Immunoblot analyses were performed as previously reported (13). A mouse monoclonal anti-VDR antibody was used (clone IVG8C11) (17). Equal loading of proteins was verified with a monoclonal anti-actin antibody (Clone C4, 0.2 μg/ml, Boehringer).

RESULTS

UVB-Induced Down-Regulation of VDR in Keratinocytes

Sub-lethal UVB irradiation of human keratinocytes is known to decrease expression of early genes like C-Myc. This was confirmed in our cellular model by a dose-dependent down-regulation of C-Myc expression after irradiation (Fig.1). C-Myc mRNA level remained constant with 8 mJ/cm^2 (Fig.1 lane 2) but decreased as quickly as 2 hours following irradiation with 32 mJ/cm^2 (Fig.1 lane 3) to resume normal levels at only about 24 hours post-irradiation (Fig.1 lane 15). As a proliferation marker, decrease in C-Myc expression should reflect a transient cell cycle arrest. This was confirmed by the dose-dependent down-regulation of the cyclin D1 mRNA level, although more transient than C-Myc since complete re-expression was observed at 24 hours post-irradiation (Fig.1).

Unexpectedly the VDR mRNA level was also strongly decreased in a dose-dependent way, responding to 32 mJ/cm^2 whereas unaffected by 8 mJ/cm^2 (Fig.1). The 32 mJ/cm^2 time-course experiment showed a sustained down-regulation of VDR mRNA level starting at 5 hours post-irradiation (Fig.1 lane 6) and ending at about 24 hours post-irradiation where reappearance was noticed (Fig.1 lane 15). The strong down-regulation of VDR mRNA observed at 5 hours post-irradiation with 32 mJ/cm^2 was comparable to that seen for C-Myc. We observed a dose-dependent up-regulation of the c-Jun mRNA level ruling out a general inhibitory effect of UVB on gene transcription (Fig.1 lane 3). The UVB-induced down-regulation of the VDR mRNA was confirmed at the protein level by immunoblotting (Fig.2). The use of cycloheximide (10 μg/ml) did not hamper the UVB-induced down-regulation of the VDR mRNA (Data not shown). Likewise, no difference in the mRNA decay was observed with the use of actinomycin D (5 μg/ml) without or in combination with UVB (Estimated half-life of the VDR mRNA of 3-4 hours; data not shown). Taken together, these last results suggest that a latent pre-existing factor would be implicated in the transcriptional repression of the VDR gene by UVB.

Signaling Cascade in the UVB-Induced Down-Regulation of VDR in Keratinocytes

In an attempt to elucidate the transduction pathway by which UVB irradiation suppresses VDR expression we tested various agents interfering with signaling cascades. We checked mRNA levels for VDR, C-Myc and cyclin D1 5 hours after 32 mJ/cm^2 UVB, a condition which gives a huge down-regulation of VDR. We could neither demonstrate mediation by growth factor receptor (use of suramin), tyrosine kinase (use of genistein)

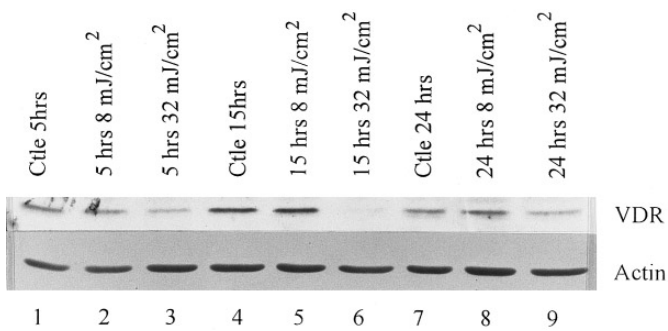

FIG. 2. UVB-induced down-regulation of the VDR protein. Keratinocytes were irradiated with 8 mJ/cm^2 (lanes 2, 5, 8) or 32 mJ/cm^2 (lanes 3, 6, 9) and protein extracts were prepared 5 hours (lanes 1-3), 15 hours (lanes 4-6) or 24 hours (lanes 7-9) post-irradiation. 100 μg of protein samples were subjected to immunoblot analysis with antibodies against VDR and actin.

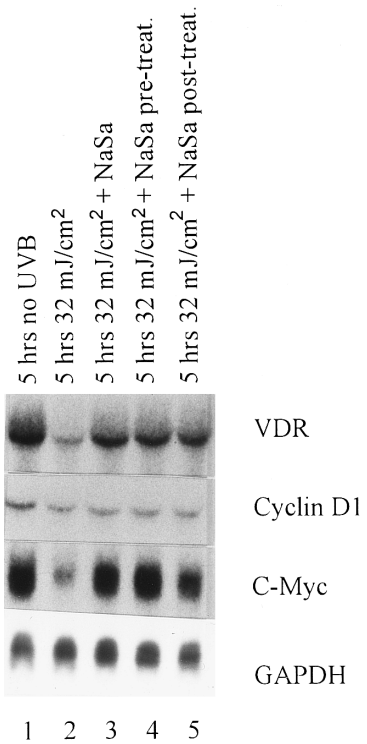

FIG. 3. The UVB-induced down-regulation of VDR is counteracted by sodium salicylate (NaSa). Keratinocytes were irradiated with 32 mJ/cm^2 (lanes 2-5) and total RNA was prepared 5 hours post-irradiation. NaSa 20 mM was added immediately after (lane 3), 30 minutes before (lane 4) or 1 hour after (lane 5) UVB irradiation. The blot (15 μg total RNA) was sequentially probed with VDR, C-Myc, cyclin D1 and GAPDH as indicated.

or protein kinase C (use of staurosporine) activation nor by Mitogen Activated Protein Kinase (use of PD98059 and SB203580) or phosphatidyl-inositol-3 kinase (use of wortmannin) (Data not shown).

Subsequently, we determined whether the aspirin derivative NaSa had any inhibitory effect on UVB-induced VDR down-regulation. It has recently been shown that this compound can inhibit certain UVB signaling pathways (18, 19). Interestingly, 20 mM NaSa markedly reduced UVB-induced VDR down-regulation (Fig.3 lane 3 versus 2). Even when added 1 hour after irradiation, NaSa was still able to significantly counteract the suppression of VDR (Fig.3 lane 5). Strikingly the reduction in inhibition of the C-Myc mRNA level paralleled the VDR expression level in response to NaSa, whereas the UVB-induced down-regulation of cyclin D1 remained unaffected in the presence of NaSa. The counteracting effect of NaSa was not due to its well known inhibitory effect on Tumor Necrosis Factor α (TNFα) which is synthesized and secreted by keratinocytes following UVB irradiation (20). Indeed, treatment of keratinocytes with TNFα for as long as 4 days did not alter the level of expression of VDR (Data not shown). The lack of involvement of TNFα was further confirmed by the fact that conditioned medium from irradiated cells could not decrease VDR expression (Data not shown). CAPE is a structural relative of flavonoids sharing common properties with NaSa. The anti-inflammatory effect of both NaSa and CAPE would, at least partially, result from the prevention of NF-κB activation (21, 22), which is a major mediator of the inflammatory response. We therefore tested the possibility for CAPE to counteract the UVB-induced down-regulation of the VDR. Results of Fig. 4 demonstrate that 25 μg/ml of CAPE efficiently inhibited UVB-induced VDR down-regulation (Fig.4 lane 4 versus 3). Nevertheless, this was observed only in cells pretreated for 2 hours (Fig.4) but not in cells treated with CAPE immediately after irradiation. Moreover as was the case for NaSa, CAPE counteracted the UVB-induced C-Myc down-regulation, but not cyclin D1 down-regulation (Fig.4). The results with NaSa and CAPE could argue for an involvement of NF-κB in the UVB-induced down-regulation of VDR and perhaps also of C-Myc. This view was further supported by treatment with the serine protease inhibitor TPCK which is a commonly used inhibitor for NF-κB activation (23, 24). 50 μM TPCK added immediately after keratinocyte irradiation with 32 mJ/cm^2, markedly limited the down-regulation of both VDR and C-Myc but not cyclin D1 (Fig.5 lane 5 versus 4).

It is generally accepted that NF-κB is an oxidative stress-responsive factor thus sensitive to antioxidants

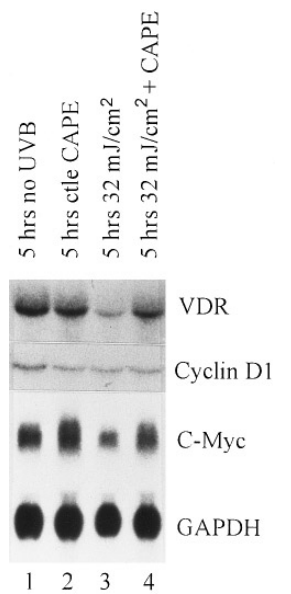

FIG. 4. The UVB-induced down-regulation of VDR is counteracted by caffeic acid phenetyl ester (CAPE). Keratinocytes were irradiated with 32 mJ/cm^2 (lanes 3-4) and total RNA was prepared 5 hours post-irradiation. Keratinocytes were pretreated with CAPE 25 μg/ml for 2 hours prior to UVB irradiation (lane 4). Lane 2 shows addition of CAPE alone. The blot (15 μg total RNA) was sequentially probed with VDR, C-Myc, cyclin D1 and GAPDH as indicated.

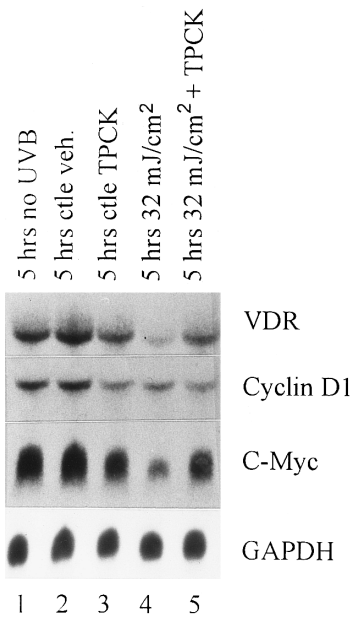

FIG. 5. The UVB-induced down-regulation of VDR is counteracted by tosylphenylchloromethylketone (TPCK). Keratinocytes were irradiated with 32 mJ/cm^2 (lanes 4-5) and total RNA was prepared 5 hours post-irradiation. 50 μM TPCK was added immediately after UVB irradiation (lane 5). Lane 3 shows addition of TPCK alone. The blot (15 μg total RNA) was sequentially probed with VDR, C-Myc, cyclin D1 and GAPDH as indicated.

(25). Since UVB is known to generate reactive oxygen intermediates (ROI) we were interested to evaluate the effect of antioxidant components on the UVB-induced down-regulation of VDR. Pretreatment of keratinocytes with the chemical radical scavengers N-Acetylcysteine (NAC) (10 mM for 30 minutes) or pyrrolidinedithiocarbamate (PDTC) (20 μM for 60 minutes) however did not alter down-regulation of VDR (Data not shown). Therefore the involvement of ROI or ROI-mediated NF-κB activation in the potent UVB-induced suppression is not supported.

DISCUSSION

Our study examined whether the expression of VDR in human primary keratinocytes was altered upon exposure to a biologically relevant dose of UVB. The question was pertinent since keratinocytes synthesize vitamin D_3 in response to sunlight (1), produce 1,25$(OH)_2$D (6) and respond to it (4-8). We observed an early dose-dependent down-regulation in VDR mRNA abundance confirmed at the protein level. It is very tempting to propose the existence of a feedback mechanism in skin consisting of a temporary reduced availability of VDR upon ultraviolet irradiation, thereby probably limiting skin responsiveness to 1,25$(OH)_2$D. This feedback loop could be superimposed to the well known UVB-induced feedback mechanisms affecting the cutaneous vitamin D_3 synthesis consisting of the photodegradation of cutaneous vitamin D_3 and in the UVB-induced melanogenesis acting as a sunscreen thereby decreasing penetration of UVB through the epidermis (1). VDR usually mediates its transcriptional effects as a heterodimer with the retinoid X receptor (26). It has recently been shown that a single exposure to UVB quickly reduces retinoid receptor levels generating a state of functional retinoid deficiency in skin (27). Decreased RXR levels may further contribute to decreased 1,25$(OH)_2$D responsiveness following UVB irradiation. In addition, UVB-induced suppression of VDR may also account for the observed lack of cooperativity between UVB and 1,25$(OH)_2$D in the treatment of the hyperproliferative skin disorder psoriasis (28).

Another possible consequence of the dose-dependent UVB-induced VDR-down-regulation emerges from recent studies suggesting a protective function for VDR against apoptosis (29). Likewise, 1,25$(OH)_2$D-induced metallothionein gene expression in keratinocytes promotes survival and reduces apoptotic keratinocytes formation following UVB injury (30). In line with these observations, our results could be interpreted as a decreased protection against apoptosis.

In osteosarcoma cells a close association was observed between expression of C-Myc and VDR (31). A possible explanation for this is that VDR would act as a natural counter-regulatory signal of oncogenic activation, a process actively involving C-Myc overexpression. VDR would then behave as a protective factor against oncogenesis by modulating cell growth and differentiation. We report that in our primary keratinocytes VDR and C-Myc expression levels also fluctuate in an apparently coordinate way. C-Myc expression is known to be repressed in keratinocytes in response to UVB (32, 33). Concomitantly, a temporary cell cycle arrest takes place which is believed to provide time for DNA repair (13, 34, 35). The observed decrease in cyclin D1 expression is in agreement with this proposal, since its down-regulation is required to allow DNA repair (36). The apparent discrepancy between the beneficial aspects of cell cycle arrest for cell survival and the possibility that a decrease in VDR level could reflect loss of protective function must be viewed in the complex cellular context where fate of the cells depends on the balance between positive and negative signals.

In addition to direct DNA damage, ultraviolet light generates ROI important in eliciting signaling cascades (37). These include growth factor receptor and tyrosine kinase activation (38-41), involvement of Mitogen-Activated Protein Kinases (42, 43) and protein kinases C (44) as well as mediation by membrane phospholipids (45) and sphingolipids (46) or nitric oxide (47). Surprisingly none of the classical mediators investigated seems to play a major role in the down-regulation of VDR. Actually, only treatment of the cells with Nasa, CAPE and TPCK counteracted the UVB effect. It was

intriguing, and still remains unexplainable, that addition of NaSa 1 hour after irradiation was capable of substantially reducing the down-regulation. NaSa and CAPE share anti-inflammatory and anti-carcinogenic properties. Their anti-inflammatory actions could result from interference in the activation pathway of NF-κB (21, 22). This transcription factor is thus a reasonable candidate in mediating transcriptional repression of VDR. Moreover the serine protease inhibitor TPCK, which interferes with NF-κB activation (23, 24), also prevented the down-regulation in response to UVB. In addition, the presence of a consensus binding sequence for NF-κB in the 5′-flanking region of the VDR and C-Myc genes is also in line with its alleged involvement in the transcriptional regulation of these genes (48, 49). While NF-κB activation generally occurs in response to production of ROI (25), antioxidants did not inhibit the UVB-induced VDR down-regulation.

The signaling cascade activated by UVB and leading to repression of VDR and C-Myc expression thus remains elusive although the results reported open new possibilities in UVB elicited cascades.

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