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UVB-Induced Production of 1,25-Dihydroxyvitamin D_3 and Vitamin D Activity in Human Keratinocytes Pretreated With a Sterol Δ^7-Reductase Inhibitor

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Abstract The skin fulfills an important role in the vitamin D photo-endocrine system. Epidermis is not only the site of vitamin D_3 photoproduction. In addition, epidermal keratinocytes contain the vitamin D receptor (VDR) and possess 25-hydroxylase and 1α-hydroxylase activity indicating that all components of the vitamin D system are present. We investigated whether these components cooperate in inducing vitamin D activity upon treatment with physiological UVB doses. Upon irradiation, 24-hydroxylase mRNA was induced in keratinocytes pretreated with a sterol Δ^7-reductase inhibitor (BM15766) whereby the 7-dehydrocholesterol content increased by 300-fold. Transfection experiments with a vitamin D response element containing construct confirmed VDR-dependent gene activation. Furthermore, the UVB-dependent induction of 24-hydroxylase was blocked by the cytochrome-P450 inhibitor ketoconazole. The 24-hydroxylase inducing photoproduct was transferable to unirradiated keratinocytes by medium and cellular homogenates of UVB-irradiated, BM15766-pretreated cells and was identified as 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] by high-performance liquid chromatography with tandem mass spectrometric detection. Addition of vitamin D binding protein blunted UVB-induced 24-hydroxylase suggesting the possibility of a paracrine or autocrine role for 1,25$(OH)_2D_3$. In conclusion, epidermal keratinocytes can produce vitamin D_3, convert it to 1,25$(OH)_2D_3$ and respond to it upon UVB irradiation in the absence of exogenous 7-dehydrocholesterol and therefore contain a unique and complete photo-endocrine vitamin D system. J. Cell. Biochem. 98: 81–92, 2006.

Key words: vitamin D_3; 1,25-dihydroxyvitamin D_3; keratinocyte; ultraviolet B

The skin fulfils an important role in the vitamin D endocrine system as the site of vitamin D_3 photoproduction thus representing the most important source of vitamin D_3 [Holick, 1988]. The steroidal vitamin D_3 precursor 7-dehydrocholesterol [7-DHC or provitamin D_3] is present in ample quantities in the epidermis and undergoes a photochemical ring opening by the action of ultraviolet B (UVB) photons during sun exposure. However, the resulting secosteroid previtamin D_3 is unstable and isomerizes to vitamin D_3 by thermal energy [Holick et al., 1980]. Via passive diffusion, vitamin D_3 subsequently reaches the capillary bed in the dermis whereupon it leaves the skin bound to vitamin D binding protein (DBP) and is transported throughout the body for storage or further metabolism [Rosenstreich et al., 1971; Mawer et al., 1972]. Indeed, contrary to what its name suggests, vitamin D_3 is a prohormone and needs conversion to active vitamin D_3 or 1α, 25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] in order to exert its physiological effects. The first step is the hepatic formation of 25-hydroxyvitamin D_3 [25OHD_3] by the vitamin D_3 25-hydroxylase (CYP27A1 and the recently identified CYP2R1) [Blunt et al., 1968; Cheng et al., 2003, 2004]. 25-hydroxylase activity has also been observed in intestine, adrenal, lung, kidney [Andersson

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et al., 1989; Usui et al., 1990], bone [Ichikawa et al., 1995], skin [Lehmann et al., 1999], macrophages [Hansson et al., 2003], and in numerous other tissues and cell types [Cheng et al., 2003]. The 1α-hydroxylation of $25OHD_3$ subsequently occurs in kidney by the $25OHD_3$ 1α-hydroxylase (CYP27B1) [Lawson et al., 1971] but is also possible in bone [Howard et al., 1981], placenta [Delvin et al., 1985], epidermal keratinocytes [Bikle et al., 1986b], testes [Fu et al., 1997], prostate [Schwartz et al., 1998], macrophages [Monkawa et al., 2000], brain, intestine, adrenal medulla [Zehnder et al., 2001], cervix [Friedrich et al., 2002], and parathyroid glands [Segersten et al., 2002].

$1,25(OH)_2D_3$ is the physiological vitamin D_3 metabolite with the highest affinity for the vitamin D receptor (VDR), a ligand activated transcription factor that regulates the expression pattern of genes involved in bone mineralization and intestinal calcium transport. However, the presence of the VDR in almost every cell type reflects a much broader action spectrum of $1,25(OH)_2D_3$. Indeed, in addition to its well-known calciotropic effects it also displays important immunomodulary, antiproliferative, and prodifferentiating effects [Bouillon et al., 1995], among others in epidermal keratinocytes [Bikle and Pillai, 1993; Segaert et al., 1997, 2000a,b; Segaert, 1998]. These properties have led to the application of $1,25(OH)_2D_3$ or synthetic analogs in the treatment of hyperproliferative skin diseases such as psoriasis [van de Kerkhof, 1995]. Moreover, VDR or $1,25(OH)_2D_3$ have been implicated in hair cycling [Xie et al., 2002] and the maintenance of epidermal barrier function [Bikle et al., 2004] and pharmacological doses of $1,25(OH)_2D_3$ were recently shown to act as a photoprotective agent [De Haes et al., 2003, 2005]. Therefore, skin is not only a source of vitamin D_3, it is also an important target tissue of $1,25(OH)_2D_3$. Finally the skin is also able to fully activate vitamin D_3 to $1,25(OH)_2D_3$ as both CYP27A1 [Lehmann et al., 1999; Schuessler et al., 2001] and CYP27B1 [Bikle et al., 1986a] are expressed and functional in epidermal keratinocytes. It therefore appears that all components of the vitamin D system are present in the epidermis. Indeed, epidermal keratinocytes induce 24-hydroxylase mRNA upon stimulation with $25OHD_3$ or vitamin D_3 [Schuessler et al., 2001], the transformed keratinocyte cell line HaCaT and in vitro human skin equivalent convert exogenous 7-DHC to $1,25(OH)_2D_3$ upon UVB irradiation [Lehmann et al., 2000, 2001] and recently UVB-induced synthesis of $1,25(OH)_2D_3$ in human skin in vivo was shown [Lehmann et al., 2003].

It remains unclear whether photosynthesized $1,25(OH)_2D_3$ is able to exert vitamin D effects in the epidermis. Therefore, we investigated whether the components of the epidermal vitamin D system cooperate and join in inducing vitamin D activity in cultured human epidermal keratinocytes upon treatment with physiological doses of UVB.

MATERIALS AND METHODS

Cell Culture

Human primary keratinocytes were isolated from foreskins of young donors (less than 6 years) as described [Kitano and Okada, 1983]. Keratinocytes were grown in Keratinocyte Serum Free Medium (Life Technologies, Paisley, Scotland) supplemented with bovine pituitary extract (50 μg/ml) and human epidermal growth factor (5 ng/ml). Third- to fifth-passage cells were used. The spontaneously immortalized human keratinocyte cell line HaCaT was cultured in the same medium as the normal keratinocytes.

$1,25(OH)_2D_3$ (gift from Dr. J.P. van de Velde, Solvay, Weesp, The Netherlands), was used from a stock in absolute ethanol. BM15766 (kind gift from Boeringher–Mannheim, Mannheim, Germany) was dissolved in DMSO. Final ethanol or DMSO concentrations never exceeded 0.05% and did not affect experiments. DBP was prepared as described [Van Baelen et al., 1980]. Treatment with BM15766 started 24 h before irradiation. Cells were washed with PBS twice and irradiated through a thin film of PBS with physiologically relevant UVB doses (up to 30 mJ/cm^2) as measured with an IL 700 A Research Radiometer (International Light, Newburyport, MA). Three Philips TL20W12 tubes with an emission spectrum between 270 and 400 nm were used as the UVB source. After irradiation, fresh medium was provided to the cells. Non-irradiated control dishes were handled identically but placed under a dark cloth adjacent to the UV beam. When used for transfer experiments, 1.5 ml medium was added to the p100 dishes after irradiation. Pooled media from three dishes were transferred to control receptor keratinocytes 18 h after UVB irradiation. Remaining cells were washed twice

with PBS, scraped in 1.5 ml fresh medium with a rubber policeman and the pooled cellular homogenates were transferred to receptor keratinocytes as well.

Northern Blot Analysis

Total RNA was isolated using a commercial kit (RNeasy, Qiagen GmbH, Hilden, Germany). Northern blot analysis was performed as described [Segaert et al., 1997] on 12 μg of total RNA. Human 1α,25-dihydroxyvitamin D_3 24-hydroxylase (CYP24) cDNA [Chen et al., 1993] and a small proline rich protein 2 (SPR2) probe [Gibbs et al., 1993] were labeled with α^{32}P-dCTP by the random priming method (RediPrime II, Amersham Biosciences, Freiburg, Germany). For loading control we used a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantification of bands was established using a laser densitometric scanner (Pharmacia Biotech, Inc., Piscataway, NJ). Experiments were carried out in duplicate and one representative experiment is shown.

Transient Transfection and VDR-Mediated Expression Assay

HaCaT cells were transiently transfected using the cell line Nucleofector kit V according to the manufacturer's instructions (Amaxa Biosystems, Köln, Germany). Briefly, 2.5×10^6 cells were suspended in 100 μl cell line V solution and 7.5 μg of luciferase reporter plasmid containing the rat ANF DR3-type Vitamin D response element (VDRE) (kind gift of C. Carlberg, Kuopio, Finland) [Quack et al., 1998] or minimal thymidine kinase reporter plasmid [Schoenmakers et al., 2000] and 0.375 μg β-galactosidase control plasmid were added. After nucleofection (program U20), cells were seeded at a density of 0.6×10^6 cells per well. Treatment with BM15766 started 24 h later and lasted for 24 h when cells were irradiated. Cells were harvested in reporter lysis buffer (Promega, Southampton, UK) 24 h after irradiation. Aliquots of 10 μl of cleared lysate were assayed for luciferase activity using a luciferase reporter assay kit from Promega (Southampton, UK) and a Berthold Microlumat LB 96P luminometer. The activity of β-galactosidase (Galacto-light plus, Applied Biosystems, Warrington, UK) was used to normalize for transfection efficiencies. Transfection efficiency, as determined by green fluorescent protein (GFP) expression in parallel plates 24, 48, and 72 h after nucleofection, varied between 50% and 70%.

Analysis of 7-DHC and 1,25(OH)$_2$D$_3$

Keratinocytes were seeded in seven-fold, treated with BM15766 and irradiated as described except that 1 ml medium was added after irradiation. Twenty-four hours after BM15766 treatment or 24 h after irradiation, media were pooled, remaining cells were washed twice with PBS and scraped in 1 ml fresh medium with a rubber policeman. The concentration of 7-DHC and 1,25(OH)$_2$D$_3$ in pooled media and cellular homogenates was determined by high-performance liquid chromatography with tandem mass spectrometric detection (LC/MS/MS). However, due to the difference in polarity of the two compounds separate methods were used. For 7-DHC 1 volume of sample was protein precipitated with 2 volumes of acetonitrile. After centrifugation the supernatant was extracted with 4 volumes of t-butylmethylether. The organic phase was isolated and evaporated. The residue was reconstituted in 200 μl of methanol 1 M ammonium acetate-water (500:2:500) before the LC/MS/MS analysis. The analytical column was a Symmetri C_8 50×2.1 mm (3.5 μm particle size)(Waters, Hedehusene, Denmark). The mobile phase was a linear gradient from 75% to 99% methanol with a constant concentration of 2 mM ammonium acetate with a flow-rate of 0.3 ml/min. The injection volume was 100 μl. The mass detector was a PE/Sciex API 3000 Mass Spectrometer using a Heated Nebulizer ion source (Concord, Ontario, Canada). The ion source was operated in the positive atmospheric pressure chemical ionization mode at 400°C. For the analysis of 1,25(OH)$_2$D$_3$ 1 volume of sample was protein precipitated with 2 volumes of acetonitrile. The supernatant was diluted with water and loaded onto a Isolute^{TM} MF C_{18} SPE column (100 mg) (IST,Mid-Glamorgan, UK). Following a washing procedure the analyte was eluated with heptane-2-propanol (93:7). The organic phase was evaporated and the residue was reconstituted as above. The same LC conditions and mass detector were used but with a TurboIon Spray ion source. The ion source was operated in the positive electrospray ionization mode at 350°C.

Statistical Analysis

Data were expressed as mean ± SEM. One-way ANOVA was used to compare luciferase

activity of the different conditions in transfection experiments. To determine differences from control, Tukey–Kramer multiple comparison test was applied. *P*-values of 0.05 or less were considered statistically significant.

RESULTS

UVB Induces 24-Hydroxylase mRNA in Keratinocytes Pretreated With a Sterol Δ^7-Reductase Inhibitor

24-Hydroxylase is considered as one of the most sensitive $1,25(OH)_2D_3$-responsive genes and its transcription is often used as a marker for the presence of active vitamin D metabolites [Makin et al., 1989]. Therefore, production of active vitamin D metabolites in keratinocytes upon UVB irradiation might be reflected in the induction of 24-hydroxylase mRNA. However, we could not detect any 24-hydroxylase mRNA by Northern blot 24 and 48 h after irradiation with physiological UVB doses (10 or 20 mJ/cm^2) (Fig. 1A). As UVB-induced vitamin D_3 photoproduction is a completely non-enzymatic photochemical process not subjected to regulation other than substrate availability and intensity of UVB irradiation [Norman, 1998], we assumed that the low 7-DHC content in cultured keratinocytes could be a limiting factor [Nemanic et al., 1985]. In order to increase the endogenous 7-DHC stores and therefore the amount of vitamin D_3 produced upon UVB irradiation, BM15766, a sterol Δ^7-reductase inhibitor, was added to the keratinocytes [Shefer et al., 1998]. Indeed, an approximately 300-fold increase in endogenous 7-DHC content was observed 24 h after incubation with 10^{-5} M BM15766 as measured by LC/MS/MS (Table I) and a marked induction of 24-hydroxylase mRNA by about 50-fold was seen in BM15766-treated keratinocytes 24 and 48 h after irradiation (Fig. 1A). BM15766 treatment (or the increased 7-DHC content) did not affect UVB-induced gene activation by itself as the induction profile of SPR2, an UVB-induced differentiation marker in keratinocytes [Gibbs et al., 1990], was identical in BM15766-treated and BM15766-untreated conditions (Fig. 1A). Conversely, BM15766 treatment was essential for UVB-induced 24-hydroxylase mRNA induction as this was only seen in keratinocytes with high intracellular 7-DHC content (Table I). Time course studies revealed that the induction of 24-hydroxylase mRNA in BM15766-pretreated keratinocytes started from 8 h after UVB irradiation and gradually increased with increasing time after irradiation (Fig. 1B).

UVB Induces VDR-Mediated Transcription in HaCaT Cells Pretreated With a Sterol Δ^7-Reductase Inhibitor

To investigate the involvement of the VDR in the UVB-induced 24-hydroxylase transcription, HaCaT cells, which similarly induce 24-hydroxylase mRNA upon UVB irradiation when pretreated with BM15766 (data not shown), were transiently transfected with a VDRE-luciferase reporter construct. This resulted in a dose-dependent induction of luciferase activity upon $1,25(OH)_2D_3$ stimulation and, as

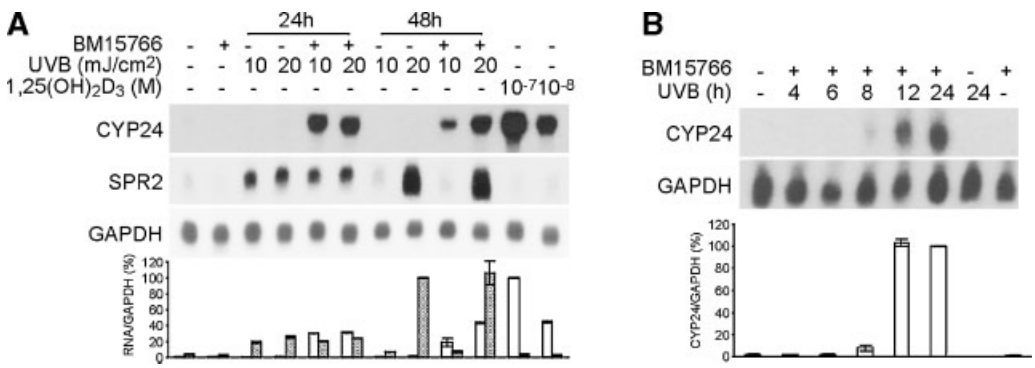

Fig. 1. UVB induces 24-hydroxylase mRNA expression in epidermal keratinocytes pretreated with BM15766. Cells were treated for 24 h with BM15766 10^{-5} M before exposure to the indicated UVB-dose (**A**) or to a UVB dose of 20 mJ/cm^2 (**B**). Cells were harvested at the indicated time periods after irradiation and total RNA was isolated. 12 μg RNA was used for Northern blot analysis. Blots were hybridized with the indicated radiolabeled probes and bands were quantified. The ratio of CYP24 to GAPDH (white) and SPR2 to GAPDH (gray) were plotted as a percentage of positive control (A (CYP24): $1,25(OH)_2D_3$ 10^{-7} M; A (SPR2): UVB 20 mJ/cm^2 48 h; B: BM15766 + UVB 20 mJ/cm^2 24 h). Data represented are mean ± SEM of at least two independent experiments.

TABLE I. 7-DHC Content (pmol/10^6 Cells) in Medium and Cellular Homogenates of Control and BM15766-Treated (10^{-5} M) Epidermal Keratinocytes*,

	Medium	Homogenate
Control	—[a]	9.39
BM15766	102.95	2605.03

*7-DHC content was measured by LC/MS/MS 24 h after starting the BM15766-treatment.
**Results of one representative experiment.
[a]<7.8 pmol/10^6 cells.

shown in Figure 2, a significant induction of luciferase activity was seen upon UVB irradiation of BM15766-pretreated HaCaT cells.

24-Hydroxylase Inducing Activity Is Ketoconazole Sensitive and Is Transferable to Non-Irradiated Keratinocytes

Vitamin D-dependent gene activation usually, although not exclusively, reflects the presence of active vitamin D metabolites acting as a VDR-ligand [Chen et al., 1994; Pascussi et al., 2005]. As the enzymes responsible for vitamin D activation and catabolism are cytochrome P450 [CYP450] enzymes [Bikle et al., 1986a; Lehmann et al., 1999, 2001; Schuessler et al., 2001], we used ketoconazole as a universal CYP450 inhibitor in our experiments.

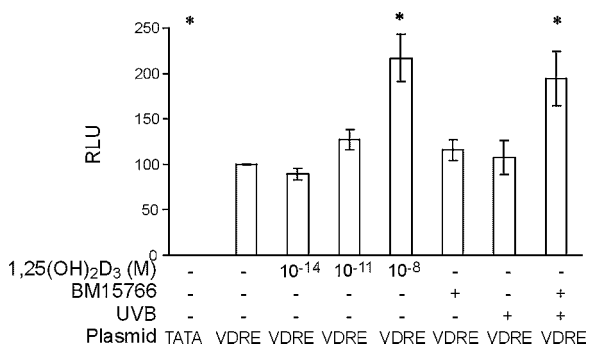

Fig. 2. UVB induces VDR-mediated gene activation in HaCaT cells pretreated with BM15766. Cells were transfected with either VDRE-driven luciferase reporter construct (VDRE) or minimal reporter construct (TATA). Treatment with BM15766 10^{-5} M lasted for 24 h whereupon cells were irradiated with UVB (20 mJ/cm^2). Twenty-four hours after irradiation or 6 h after $1,25(OH)_2D_3$ stimulation, cells were harvested with reporter lysis buffer and the lysates were analyzed for luciferase activity (RLU, relative luciferase activity). Data represented are mean ± SEM of at least three independent experiments performed in duplicate. *$P < 0.05$ versus control.

Addition of ketoconazole prior to or just after UVB irradiation abrogated the 24-hydroxylase expression. When added 2 h after irradiation, the inhibition was incomplete and when added 5 h after irradiation or later, the induction of 24-hydroxylase mRNA was even enhanced (Fig. 3A). This time-dependent effect of ketoconazole suggests that the 24-hydroxylase inducing activity (presumably active vitamin D metabolites) depends on CYP450 enzymes both for its synthesis as well as for its degradation. When added prior to or shortly after irradiation, ketoconazole blocks the formation of active vitamin D metabolites. The enhancing effect on 24-hydroxylase induction when ketoconazole is added (from 5 h) after irradiation suggests that CYP450-dependent catabolism predominates CYP450-dependent synthesis is at this moment.

To further investigate the presence of a soluble factor capable of inducing 24-hydroxylase, transfer experiment were performed in which conditioned (from BM15766-pretreated UVB-irradiated cells) or unconditioned (from untreated cells) medium or cellular homogenates were transferred to control keratinocytes. Significant 24-hydroxylase mRNA induction was seen both after transfer of conditioned medium and cellular homogenates (Fig. 3B). This effect was comparable to the 24-hydroxylase mRNA induction in irradiated BM15766-pretreated keratinocytes (Fig. 3B).

Identification of $1,25(OH)_2D_3$ in UVB-Irradiated Keratinocytes Pretreated With a Sterol Δ^7-Reductase Inhibitor

In cellular homogenates of BM15766-pretreated keratinocytes irradiated with 20 mJ/cm^2 UVB, marked amounts of $1,25(OH)_2D_3$ (166 fmol/10^6 cells) were detected by LC/MS/MS (Table II). In medium of irradiated BM15766-pretreated keratinocytes, small quantities of $1,25(OH)_2D_3$ that only hardly exceed the detection limit of 7.8 fmol/10^6 cells were measured. No $1,25(OH)_2D_3$ was found in control medium, though small amounts of $1,25(OH)_2D_3$ were found in control cellular homogenates. However, UVB treatment resulted in an about 10-fold increase in $1,25(OH)_2D_3$ content in cellular homogenates. Increasing the UVB dose to 30 mJ/cm^2 did not affect the $1,25(OH)_2D_3$ content in cellular homogenates or medium of BM15766-pretreated keratinocytes.

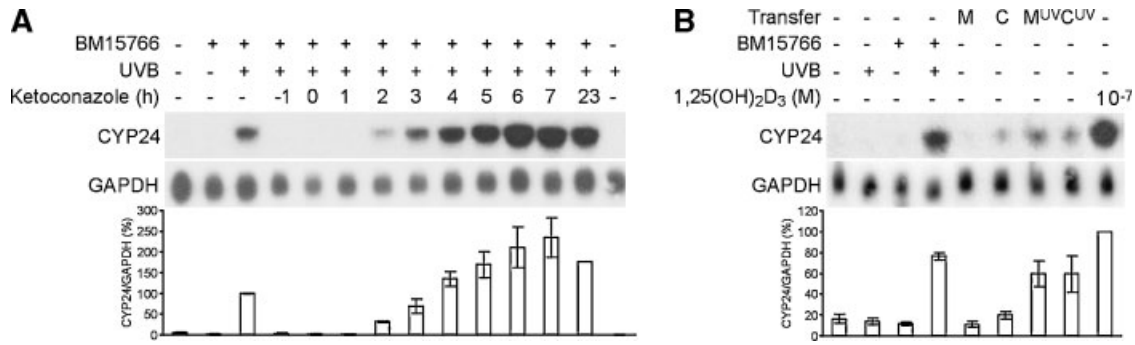

Fig. 3. 24-hydroxylase inducing activity is ketoconazole sensitive and is transferable to non-irradiated keratinocytes. Cells were treated for 24 h with BM15766 10^{-5} M before exposure to a UVB dose of 20 mJ/cm^2. Ketoconazole 10^{-5} M was added at the indicated time before or after irradiation (**A**) or cells were incubated with unconditioned (from control keratinocytes) medium (M) or cellular homogenates (C) or with conditioned (from BM15766-treated, UVB-irradiated keratinocytes) medium (M^{UV}) or cellular homogenates (C^{UV}) (**B**). Twenty-four hours after irradiation, 6 h after transfer or 6 h after 1,25$(OH)_2D_3$ stimulation, cells were harvested and total RNA was isolated. 12 μg RNA was used for Northern blot analysis. Blots were hybridized with the indicated radiolabeled probes and bands were quantified. The ratio of CYP24 to GAPDH was plotted as a percentage of positive control (A: BM15766 + UVB; B: 1,25$(OH)_2D_3$ 10^{-7} M). Data represented are mean ± SEM of at least two independent experiments.

Vitamin D Binding Protein Abrogates 24-Hydroxylase mRNA Induction in UVB-Irradiated Keratinocytes Pretreated With a Sterol Δ^7-Reductase Inhibitor

We present evidence that UVB irradiation induces production of 1,25$(OH)_2D_3$ and activation of vitamin D-dependent genes. Since all these processes may occur intracellularly within the keratinocyte, this could reflect an entirely intracrine pathway. However, addition of vitamin D binding protein (DBP), a serum protein with very high and specific binding affinity for vitamin D metabolites (K_a, 1,25$(OH)_2D_3$ 1.5×10^{-7} M, K_a, 25OHD_3 5×10^{-8} M) [Bouillon et al., 1980], to the culture medium at a physiological concentration (300 μg/ml) immediately after UVB irradiation markedly attenuated 24-hydroxylase mRNA induction (Fig. 4). DBP does not affect VDR-activation as epidermal keratinocytes lack receptors for DBP [Nykjaer et al., 2001] and as passive diffusion through the plasma membrane is quantitatively insignificant [Nykjaer et al., 2001]. Therefore, the blocking effect of DBP on UVB-induced 24-hydroxylase refers to secretion of 1,25$(OH)_2D_3$ into the medium before exerting vitamin D effects on the BM15766-pretreated UVB-irradiated keratinocytes. Indeed, only small amounts of 1,25$(OH)_2D_3$ were found in the medium of BM15766-pretreated UVB-irradiated cells (Table II) and addition of DBP resulted in a five- to seven-fold increase in medium 1,25$(OH)_2D_3$ content of irradiated BM15766-pretreated keratinocytes (Table II) without considerably affecting the 1,25$(OH)_2D_3$ content in cellular homogenates. The latter effect can be explained by a reduced 1,25$(OH)_2D_3$ availability in the medium by DBP [Bikle and Gee, 1989] resulting in an increased concentration gradient and therefore in facilitated diffusion to the culture medium. Hence, these results feed the contention that photoproduced 1,25$(OH)_2D_3$ mainly acts as an autocrine or paracrine rather than as an intracrine factor.

TABLE II. Effect of BM15766-Treatment, UVB-Irradiation and DBP on the 1,25$(OH)_2D_3$ Content (fmol/10^6 cells) in Medium and Cellular Homogenates of Epidermal Keratinocytes*,

	Medium	Homogenate
Control	—[a]	18.72
BM15766[b]	—	12.96
UVB 20 mJ/cm^2	—	17.28
BM15766 + UVB 20 mJ/cm^2	10.08	165.61
BM15766 + UVB 20 mJ/cm^2 + DBP[c]	74.88	171.37
BM15766 + UVB 30 mJ/cm^2	10.08	167.05
BM15766 + UVB 30 mJ/cm^2 + DBP	50.4	164.17

*1,25$(OH)_2D_3$ content was measured by LC/MS/MS 24 h after irradiation or 48 h after BM15766-treatment.
**Results of one representative experiment.
[a]<7.8 fmol/10^6 cells.
[b]BM15766 treatment (10^{-5} M) lasted 24 h, whereupon the cells were rinsed twice with PBS and provided fresh medium.
[c]DBP (300 μg/ml) was added immediately after irradiation.

DISCUSSION

We report the presence of a complete and functional photo-endocrine vitamin D system in

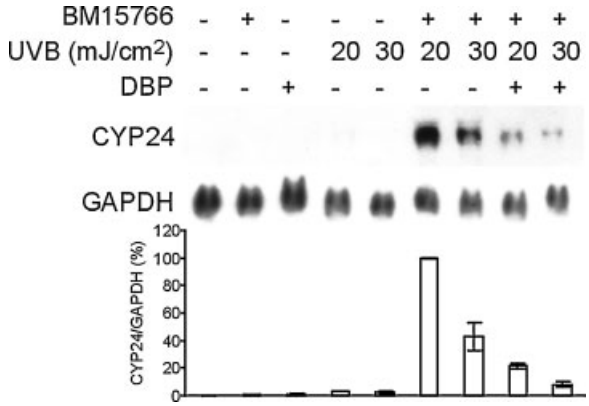

Fig. 4. DBP abrogates 24-hydroxylase mRNA induction in UVB-irradiated keratinocytes pretreated with a sterol Δ^7-reductase inhibitor. Cells were treated for 24 h with BM15766 10^{-5} M before exposure to a UVB-dose of 20 mJ/cm^2. Immediately after irradiation, 300 μg/ml DBP was added. Cells were harvested 24 h after irradiation or 6 h after 1,25$(OH)_2D_3$ stimulation and total RNA was isolated. 12 μg RNA was used for Northern blot analysis. Blots were hybridized with the indicated radiolabeled probes and bands were quantified. The ratio of CYP24 to GAPDH was plotted as a percentage of positive control (BM15766 + UVB 20 mJ/cm^2). Data represented are mean ± SEM of at least two independent experiments.

epidermal keratinocytes. Epidermal keratinocytes are able to produce vitamin D_3 from endogenous 7-DHC upon UVB irradiation in physiologically relevant doses, activate it to 1,25$(OH)_2D_3$ and respond to it inducing the transcription of vitamin D-dependent genes. This sequence of events requires sufficient amounts of 7-DHC, the activity of the vitamin D metabolizing hydroxylases and the presence of a functional VDR.

UVB-induced vitamin D activity and production of 1,25$(OH)_2D_3$ were only seen after increasing the endogenous 7-DHC content in the epidermal keratinocytes in vitro. We preferred the use of a specific sterol Δ^7-reductase inhibitor to the addition of exogenous 7-DHC [Lehmann et al., 2000, 2001] as such approach is a better representation of the physiological situation in epidermis in vivo. Indeed, it is assumed that the 7-DHC accumulation in human epidermis in vivo is a consequence of reduced sterol Δ^7-reductase activity in epidermal keratinocytes in vivo [Nemanic et al., 1985] resulting in adequate 7-DHC amounts to support (active) vitamin D_3 synthesis in vivo [Lehmann et al., 2003]. For vitamin D_3 photoproduction, a sufficient 7-DHC pool and UVB-irradiation of appropriate wavelength (295–305 nm) are necessary [Holick et al., 1980; Takada, 1983; Norman, 1998; Lehmann et al., 2000]. For further activation of vitamin D_3 to 1,25$(OH)_2D_3$, the physiological ligand of the VDR, vitamin D hydroxylating enzymes are necessary. However, epidermal keratinocytes contain both the mitochondrial CYP27A1 and CYP27B1 [Bikle et al., 1986a; Lehmann et al., 1999; Schuessler et al., 2001] and express mRNA of the microsomal CYP2R1 (data not shown). The presence of a functional VDR represents the last prerequisite for a complete and functional photo-endocrine vitamin D system. This condition is also fulfilled in skin as epidermal keratinocytes express the VDR and respond to 1,25$(OH)_2D_3$ [Bikle and Pillai, 1993; Segaert et al., 1997, 2000a,b; Segaert, 1998].

We demonstrate that epidermal keratinocytes pretreated with the specific sterol Δ^7-reductase inhibitor BM15766 induce 24-hydroxylase, one of the most sensitive 1,25$(OH)_2D_3$-responsive genes [Makin et al., 1989; Chen et al., 1994] upon irradiation with a physiological UVB-dose. Transfection experiments in which a VDRE containing luciferase reporter construct was introduced in epidermal keratinocytes revealed the involvement of the VDR and therefore, UVB-induced 24-hydroxylase reflects UVB-induced vitamin D activity. Indeed, 24-hydroxylase induction represents a negative feedback mechanism in 1,25$(OH)_2D_3$-dependent gene expression, reducing the availability of VDR ligand [Chen et al., 1994]. The UVB-induced 24-hydroxylase mRNA expression started from 8 h after UVB irradiation, which is completely in line with the production of active vitamin D metabolites in keratinocytes upon UVB irradiation. Earlier experiments in keratinocytes demonstrated that considerable amounts of vitamin D_3 are formed already at 3 h after UVB irradiation [Lehmann et al., 2000] and that 24-hydroxylase mRNA is induced as early as 3–5 h after addition of vitamin D_3 depending on the dose administered [Schuessler et al., 2001]. In analogy, significant quantities of 1,25$(OH)_2D_3$ are found between 5 and 10 h after irradiation of 7-DHC-supplemented keratinocytes [Lehmann et al., 2000]. Furthermore, keratinocytes were shown to induce 24-hydroxylase mRNA as early as 1 h after addition of 1,25$(OH)_2D_3$ [Chen et al., 1994]. Together with the enhancing effect of ketoconazole on 24-hydroxylase induction from 5 h after irradiation, these data fit with the production of active vitamin D metabolites already 5–7 h following

UVB irradiation of keratinocytes. Twenty-four hours after irradiation (the time at which we performed measurements), we detected marked amounts of $1,25(OH)_2D_3$ in both medium and cellular homogenates of BM15766-pretreated UVB-irradiated keratinocytes confirming that UVB-induced vitamin D activity was the result of vitamin D_3 photoproduction, subsequent conversion to $1,25(OH)_2D_3$ and VDR activation.

The photoproduced $1,25(OH)_2D_3$ quantities in vitro are very minute though. We measured $1,25(OH)_2D_3$ quantities of 175–177 fmol/10^6 cells in cellular homogenates and medium of BM15766-pretreated cells after UVB irradiation, data that correspond to earlier experiments obtained in UVB-irradiated and 7-DHC-supplemented HaCaT cells [Lehmann et al., 2000]. This implies that only 0.005% of the intracellular 7-DHC is converted into $1,25(OH)_2D_3$ 24 h after irradiation with a physiological UVB-dose. Although very small, these amounts are in line with earlier experiments: only 33%–37% of 7-DHC is converted into vitamin D_3 [Lehmann et al., 2000; Obi-Tabot et al., 2000]; about 0.3% of vitamin D_3 is converted into $25OHD_3$ [Schuessler et al., 2001] and 5%–10% of $25OHD_3$ is converted to $1,25(OH)_2D_3$ in keratinocytes in unsaturated conditions [Bikle and Pillai, 1988; Lehmann, 1997]. Taken all together, this results in a similar $1,25(OH)_2D_3$ production rate.

Despite the rather low production of active vitamin D, vitamin D activity in the form of 24-hydroxylase induction and transactivation of a VDRE containing construct was clearly observed in UVB irradiated keratinocytes containing sufficient 7-DHC. Similarly, the in vivo $1,25(OH)_2D_3$ photoproduction is limited [Lehmann et al., 2003] and therefore might only contribute to physiological skin effects such as epidermal barrier homeostasis [Bikle et al., 2004] and hair follicle physiology [Li et al., 1997; Yoshizawa et al., 1997; Malloy et al., 1999; Xie et al., 2002] rather than to pharmacological effects such as keratinocyte differentiation [Bikle and Pillai, 1993], photoprotection [De Haes et al., 2003] and immunoregulation [Dam et al., 1996]. For the same reason, cutanous $1,25(OH)_2D_3$ photoproduction probably does not contribute to the systemic effects of $1,25(OH)_2D_3$. Indeed, the low $1,25(OH)_2D_3$ levels in hepatectomized or nephrectomized animals suggest that epidermal $1,25(OH)_2D_3$ production cannot compensate for the lost 1α-hydroxylase activity in these animals and therefore that epidermal $1,25(OH)_2D_3$ photoproduction maximally accounts for a small fraction of total systemic $1,25(OH)_2D_3$ levels.

On the other hand, it is possible that the UVB-induced $1,25(OH)_2D_3$ photoproduction and VDR-mediated gene activation represent one aspect of the broad array of UVB effects on human skin. UVB irradiation induces changes that may lead to acute effects including erythema, tanning, and immune suppression and long term adverse effects such as photoaging and photocarcinogenesis [Gilchrest et al., 1996; Taylor and Sober, 1996; Clydesdale et al., 2001; Matsumura and Ananthaswamy, 2004]. At the cellular level, these are the result of DNA damage, generation of reactive oxygen species (ROS) or modifications in the expression pattern of several genes [Sesto et al., 2002]. Considering the time profile of the UVB-induced vitamin D activity, some of the late UVB-regulated gene expression changes might be mediated via VDR activation by its photoproduced ligand $1,25(OH)_2D_3$ even though photoproduced $1,25(OH)_2D_3$ amounts are limited. Probably the concentration of photosynthesized $1,25(OH)_2D_3$ is too small to contribute to the therapeutic effect of UVB therapy or climatotherapy for psoriasis but it was very recently shown that small-band UVB is capable of inducing calcitriol synthesis in organotypic keratinocyte cultures [Lehmann et al., 2005]. In addition, UVB-induced vitamin D activity can explain the lack of additive effect between oral or topical $1,25(OH)_2D_3$ and UVB phototherapy in the treatment of psoriasis [Prystowsky et al., 1996] as UVB-induced 24-hydroxylase might result in catabolism of exogenous $1,25(OH)_2D_3$. However, UVB-induced down-regulation of the VDR limiting $1,25(OH)_2D_3$ responsiveness [Courtois et al., 1998] might represent a more reasonable explanation for this reported lack in additive effect.

Our experiments show evidence for a paracrine or autocrine role rather than a solely intracrine role for the photoproduced $1,25(OH)_2D_3$ by the fact that DBP blocked UVB-induced vitamin D activity. Therefore, the in vivo photoproduced $1,25(OH)_2D_3$ in the epidermis might contribute to vitamin D effects in skin regions not exposed to UVB irradiation like the deeper layers of the dermis or skin covered by clothes or hair, as depicted in Figure 5.

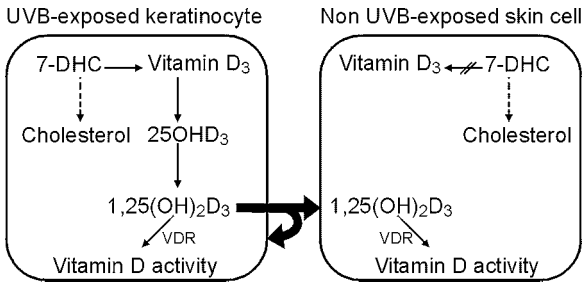

Fig. 5. Schematic representation of the cutanous photo-endocrine vitamin D system. Upon exposure to UVB irradiation, vitamin D_3 photoproduction can take place in epidermal keratinocytes that in vivo contain high 7-DHC stores, presumably due to a decreased activity of the sterol Δ^7-reductase. Owing to their vitamin D_3 25-hydroxylase and $25OHD_3$ 1α-hydroxylase activity, epidermal keratinocytes can convert photoproduced vitamin D_3 into $1,25(OH)_2D_3$ leading to VDR transactivation and hence resulting in UVB-induced vitamin D activity. Besides, photoproduced $1,25(OH)_2D_3$ might act as an autocrine or paracrine factor inducing vitamin D activity in neighboring skin cells (epidermal keratinocytes, dermal fibroblasts or other skin cells) not exposed to UVB irradiation.

At present, a discussion concerning vitamin D_3 deficiency is ongoing [Holick, 2003; Zittermann, 2003; Holick, 2004; Lips, 2004; Vieth, 2004; Dawson-Hughes et al., 2005]. Vitamin D_3 deficiency is a major unrecognized health problem. It not only causes rickets in infants and osteomalacia in adults; subclinical vitamin D_3 deficiency is associated with an increased risk of several cancers (breast, colon prostate), osteoporosis, cardiovascular disease, autoimmune diseases (multiple sclerosis, rheumatoid arthritis, type I diabetes mellitus), schizophrenia, and muscle weakness [Holick, 2001, 2004]. One way to restore vitamin D status is the intake of vitamin D supplements, but increased sun exposure is also recommended as very minute UVB-doses are sufficient to maintain adequate vitamin D levels [Holick, 2001]. However, excessive UVB exposure is harmful and results in an increased risk of melanoma and nonmelanoma skin cancers [Kricker et al., 1995; Taylor and Sober, 1996; Matsumura and Ananthaswamy, 2004]. Therefore, the optimal UVB exposure ensures appropriate vitamin D status without risking skin diseases and depends on skin type, clothing, latitude, season, and time of the day. In addition, cutanous vitamin D_3 photoproduction is reduced in elderly people, presumably by a reduced 7-DHC content, whereby this population is at risk for vitamin D_3 deficiency [MacLaughlin and Holick, 1985]. Our experiments show that inhibition of the sterol Δ^7-reductase and increasing the intracellular 7-DHC content is crucial for UVB-induced vitamin D activity in epidermal keratinocytes in vitro. Therefore, topical or systemic use of a sterol Δ^7-reductase inhibitor on sun exposed skin regions might represent a strategy to optimize vitamin D_3 photoproduction resulting not only in increased UVB-induced vitamin D activity in skin, but also in an improved vitamin D status [Nemanic et al., 1985; Morris, 1999].

In conclusion, we show that epidermal keratinocytes are able to produce vitamin D_3 and convert it to $1,25(OH)_2D_3$ upon UVB irradiation and in the presence of sufficient endogenous 7-DHC stores. In addition, they respond to the photoproduced $1,25(OH)_2D_3$ inducing VDR-mediated transcription in a paracrine or autocrine way. Therefore, epidermal keratinocytes contain a unique and complete photo-endocrine vitamin D system.

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