A Novel Pathway for Hormonally Active Calcitriol

Bodo Lehmann Peter Knuschke Michael Meurer

Department of Dermatology, Carl Gustav Carus Medical School, Dresden University of Technology,
Dresden, Germany

Key Words

Calcitriol · Keratinocyte · Microdialysis · UVB ·
Vitamin D₃

Abstract

Calcitriol [$1\alpha,25(\text{OH})_2\text{D}_3$], the hormonally active form of vitamin D₃ (D₃) is produced in both renal and extrarenal tissues. Our findings demonstrate that physiological doses of UVB radiation at 300 nm induce the conversion of 7-dehydrocholesterol (7-DHC) via preD₃ and D₃ into calcitriol in the pmol range in epidermal keratinocytes. The hydroxylation of photosynthesized D₃ to calcitriol is strongly suppressed by ketoconazole, a known inhibitor of cytochrome P450 mixed function oxidases. The UVB-induced formation of calcitriol in human skin is demonstrable in vivo by the microdialysis technique. These results suggest that human skin is an autonomous source of hormonally active calcitriol.

Copyright © 2001 S. Karger AG, Basel

Introduction

Cutaneous vitamin D₃ (D₃) is generated by UVB-induced photolysis of 7-dehydrocholesterol (7-DHC) [1]. Once formed, D₃ is transported to the liver [2, 3] where it is metabolized to calcidiol (25OHD₃) which is

further metabolized in the kidney [4, 5] to calcitriol [$1\alpha,25(\text{OH})_2\text{D}_3$], the hormonal active form of D₃. Except from its calcitropic action, $1\alpha,25(\text{OH})_2\text{D}_3$ has antiproliferative and prodifferentiative effects on epidermal keratinocytes [6–8]. However, the calcitriol concentration required to suppress growth of keratinocytes in vitro are substantially higher than present in blood [6]. On the other hand, it has been shown that cultured keratinocytes can convert exogenous calcidiol (25OHD₃) to calcitriol [9]. The physiological importance of this pathway however is unclear because only trace amounts of calcitriol were synthesized from calcidiol by skin ex vivo [10]. Previously, we were able to demonstrate that in cultures of human keratinocytes biologically inactive D₃ can be converted to $1\alpha,25(\text{OH})_2\text{D}_3$ [11, 12]. Since the skin is both the site of D₃ synthesis and a target organ for $1\alpha,25(\text{OH})_2\text{D}_3$ it is important to know whether UVB-induced photolysis of 7-DHC with consecutive synthesis of D₃ can influence the epidermal production $1\alpha,25(\text{OH})_2\text{D}_3$.

Methods

Cell Culture

HaCaT cells (used with the permission of Prof. N.E. Fusenig of the German Cancer Research Center, Heidelberg) were seeded at a density of 5×10^4 cells/cm² in culture dishes (Ø 30 mm) and grown in DMEM supplemented with 5% (v/v) FCS. Cultures were maintained at 37 °C and 5% CO₂ in air. After synchronization the

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2001 S. Karger AG, Basel
0301-0163/00/0546-0312\$17.50/0

Accessible online at:
www.karger.com/journals/hre

Bodo Lehmann, Department of Dermatology
Carl Gustav Carus Medical School, Dresden University of Technology
Fetscherstrasse 74, D-01307 Dresden (Germany)
Tel. +49 351 458 2692, Fax +49 351 458 4338
E-Mail Bodo.lehmann@mailbox.tu-dresden.de

medium was replaced by serum-free DMEM supplemented with 1.0% (w/v) of highly purified BSA (Sigma). At this time cells were preconfluent (equal to $\approx 0.7 \times 10^6$ cells/dish). Cell numbers and their viability were assessed using a CASY® 1 cell counter (Schärfe System GmbH, Reutlingen, Germany). The viability was always $\geq 93\%$.

Incubation Conditions

7-DHC (final concentration: 25 μM , 30 nmol/dish) dissolved in ethanol (final concentration: 0.83% (v/v)), was added to the cultures. Control experiments were carried out: (1) with cultures in presence of 7-DHC without irradiation; (2) solvent alone with and without irradiation; (3) medium containing 7-DHC in absence of cells with irradiation. Irradiation was followed by 16 h incubation in dark. The medium and detached cells were extracted with methanol:chloroform (1:1). In the chloroform phase pre- D_3 , D_3 , 7-DHC and $1\alpha,25(\text{OH})_2\text{D}_3$ were determined.

UV Irradiation

Monochromatic light (bandwidth 5 nm) was generated by a Dermolum HI monochromator (1-kVA Xe lamp, Müller, Germany). The distance between light source and the bottom of open culture dish ($\varnothing$ 30 mm) was 14 mm. The beam had a diameter of 15 mm. The culture dish was continuously turned during irradiation and the beam was positioned at the radius of the rotating dish. The UVB doses used were adapted to these experimental conditions (effective dose = D_{eff} = calculated dose $\times$ 0.25). Irradiances were measured with a calibrated thermopile.

HPLC Analysis

NP-HPLC. Merck/Hitachi; column: LiChroCART 250-4, Superspher Si 60, 5 μm ; eluent 1 (n-hexane:2-propanol = 95:5 (v/v); flow rate: 0.5 ml/min) for the determination of pre- D_3 , D_3 and 7-DHC; eluent 2 (n-hexane:2-propanol:methanol = 87:10:3 (v/v/v); flow rate: 1.0 ml/min) for fractionation of calcitriol (retention time: 21 min). The peaks of pre- D_3 , D_3 and 7-DHC (retention times: 8.20 min, 10.56 min and 11.58 min respectively) were quantified by UV detection at 265 nm. HPLC fractions containing calcitriol were collected and analyzed for calcitriol using a radioreceptor assay (Nichols Institute). The calcitriol generated was identified by co-chromatography of the ^3H -labeled standard.

RP-HPLC. Rechromatography of calcitriol on a Hibar® column, 250-4, LiChrospher 100RP-18, 5 μm (Merck, Darmstadt, Germany); eluent: methanol:water = 85:15 (v/v), flow rate: 1 ml/min; retention time of synthetic [^3H] $1\alpha,25(\text{OH})_2\text{D}_3$: 10.0 min.

GC-MS Analysis

Dried fractions from HPLC were derivatized to TMS ether derivatives and analyzed by GC-MS. The derivatized sample (1 μl) was directly injected manually into a model 5890/II gas chromatograph equipped with a 25 m $\times$ 0.2 mm HP-1 capillary column (cross-linked methylsiloxane, 0.33 μm) and interfaced with a model 5989A MS Engine (Hewlett-Packard, Palo Alto, CA, USA).

Microdialysis

Investigations were carried out during winter. The microdialysis probe used (CMA 70, CMA/Microdialysis Research AB, Sweden) has a shaft length of 60 mm and a membrane length of 10 mm. The steel shaft of the probe has an outer diameter of 0.64 mm. The diameter at the tip of the probe where the dialysis membrane is located is 0.5 mm. The molecular weight cutoff point for the membrane is

Fig. 1. Relationship between wavelengths of UVB light and the generation of D_3 and $1\alpha,25(\text{OH})_2\text{D}_3$ in HaCaT cells. Cultures preincubated with 25 μM 7-DHC were irradiated at several wavelengths between 285 nm and 320 nm (dose: 30 $\text{mJ} \times \text{cm}^{-2}$) followed by 16 h incubation. Each point represents the mean of two independent experiments.

20 kD. The abdomen was selected as site for insertion of two independent microdialysis probes (UVB irradiation and control, respectively). When local anesthesia at the point of insertion was done, an apheresis needle (1.60 $\times$ 32 mm) was inserted as a guide, as superficially as possible for 15 mm. The probe was inserted through the guide, which was then withdrawn and taped in position. The site of insertion and the area above the tip of the probe were marked. The probe was first flushed with Ringer's solution using a CMA 107 microdialysis pump for 5 min at a flow rate of 15 $\mu\text{l}/\text{min}$. Thereafter the flow was changed to 0.3 $\mu\text{l}/\text{min}$, and the dialysate was fractionated into microvials for 3 h each (control). The second probe was uncovered after 3 h and irradiated with monochromatic UVB at 300 nm (doses: 14 or 28 $\text{mJ} \times \text{cm}^{-2}$, equal to 0.7 or 1.4 MED, respectively). The microvials were changed every 3 h until 24 h after UVB exposure. The content of $1\alpha,25(\text{OH})_2\text{D}_3$ in 50 μl dialysate of each fraction was determined by radioreceptor assay (see above).

Results and Discussion

In vitro experiments using the HaCaT cell line demonstrated the UVB-induced conversion of exogenous 7-DHC via previtamin D_3 (pre- D_3) and D_3 to $1\alpha,25(\text{OH})_2\text{D}_3$ in these cells. The rate of formation of $1\alpha,25(\text{OH})_2\text{D}_3$ in HaCaT cells depends on the UVB wavelength used for irradiation and is very similar to that of D_3 , showing maxima at around 303 nm (fig. 1). This finding points to a close relation between UVB-induced D_3 synthesis and consecutive formation of $1\alpha,25(\text{OH})_2\text{D}_3$.

Fig. 2. Inhibitory effect of ketoconazole and 1,2-dianilinoethane on hydroxylation of D₃ created after irradiation with UVB to 1α,25(OH)₂D₃ in HaCaT cells. Cultures containing 25 μM 7-DHC were irradiated at 300 nm (dose: 30 mJ × cm⁻²). Immediately after irradiation, ethanol (control), ketoconazole (10 μM) or 1,2-dianilinoethane (10 μM) were added to the cultures, and further incubation for 16 h at 37 °C was done in the dark. Concentrations obtained are depicted as relative percent ± SD of control (100 ± 8% equal to 1990 ± 159 fmol calcitriol) of three independent experiments. *** p < 0.001 compared to control.

Calcitriol obtained after NP-HPLC (eluent 2) of extracts of irradiated HaCaT cells (300 nm; dose: 30 mJ × cm⁻²; irradiance: 0.28 mW × cm⁻²) was identical with synthetic standard calcitriol as shown by co-migration with ³H-1α,25(OH)₂D₃ in NP and RP-HPLC systems. No calcitriol was detectable in extracts of unirradiated HaCaT cells. Studies of the TMS derivatives of synthetic and generated calcitriol by GC-MS demonstrated identical retention times as well as full-scan EI mass spectra.

Ketoconazole [13] dose-dependently inhibited the UVB-induced generation of calcitriol in HaCaT cells when added to the culture immediately after irradiation (fig. 2). In contrast, the radical scavenger and antioxidant 1,2-dianilinoethane [14] showed only marginal inhibitory effects on the generation of 1α,25(OH)₂D₃ (fig. 2). This finding points to an enzymatically catalyzed pathway of D₃ to 1α,25(OH)₂D₃ as previously found after exogenous addition of D₃ to cultured keratinocytes [11, 12].

Calculations indicate that only 0.007% of the UVB-irradiated 7-DHC (30 nmol/dish) is converted to 1α,25(OH)₂D₃ (equal to 2.093 fmol/10⁶ cells). For comparison, the normal range of the concentration of 1α,25(OH)₂D₃ in human serum is 68 ± 27 fmol/ml [15].

Fig. 3. Microdialysis probe for cutaneous monitoring of 1α,25(OH)₂D₃ after UVB irradiation of human skin. Skin was irradiated at 300 nm at 14 mJ × cm⁻² or 28 mJ × cm⁻² (equal to 0.7 and 1.4 MED, respectively) and not irradiated (control). Flow rate of dialysis fluid: 0.3 μl/min, volume of fraction: 54 μl/3 h, total duration of microdialysis: 27 h. Calcitriol was determined in 50 μl dialysate. Data are depicted as mean values of two independent experiments.

Finally, we have been able to show in preliminary experiments that the UVB-induced formation of calcitriol at 300 nm in human skin can be demonstrated using the microdialysis technique (fig. 3). Calcitriol concentrations in the dialysate were at a maximum 12–18 h after UVB irradiation and increased with rising UVB doses up to 28 mJ × cm⁻² (equal to 1.4 MED).

It can be concluded from our findings that the metabolism of photosynthesized D₃ to 1α,25(OH)₂D₃ in keratinocytes is obviously catalyzed by P450 mixed function oxidases. Our findings demonstrate the UVB-triggered conversion of 7-DHC to substantial amounts of 1α,25(OH)₂D₃ in HaCaT keratinocytes. In vivo experiments using the microdialysis technique demonstrated cutaneous generation of 1α,25(OH)₂D₃ 12–18 h after the skin was UVB irradiated at 300 nm in therapeutic doses. These findings are of potential importance for the proliferation, differentiation and apoptosis of keratinocytes as well as immunosuppression in skin under influence of UVB. Also, one could speculate whether the therapeutic effect of UVB in hyperproliferative skin diseases such as psoriasis can be partially attributed to the action of epidermally synthesized calcitriol.

References

- 1 Holick MF, MacLaughlin JA, Clark MB, Holick SA, Potts JT: Photosynthesis of previtamin D₃ in human skin and the physiologic consequences. *Science* 1980;210:203–205.
- 2 Ponchon G, Kennan AL, DeLuca HF: Activation of vitamin D by the liver. *Clin Invest* 1969;48:2032–2037.
- 3 Horsting M, DeLuca HF: In vitro production of 25-hydroxycholecalciferol. *Biochem Biophys Res Commun* 1969;36:251–256.
- 4 Holick MF, Schnoes HK, DeLuca HF: Identification of 1,25-dihydroxycholecalciferol, a form of vitamin D₃ metabolically active in the intestine. *Proc Natl Acad Sci USA* 1971;68:177–181.
- 5 Lawson DE, Fraser DR, Kodicek E, Morris HR, Williams DH: Identification of 1,25-dihydroxycholecalciferol, a new kidney hormone controlling calcium metabolism. *Nature* 1971;230:228–230.
- 6 Matsumoto K, Hashimoto K, Nishida Y, Hashiro M, Yoshikawa K: Growth-inhibitory effects of 1,25-dihydroxyvitamin D₃ on normal human keratinocytes cultured in serum-free medium. *Biochem Biophys Res Commun* 1990;166:916–923.
- 7 Hosomi J, Hosoi J, Abe E, Suda T, Kuroki T: Regulation of terminal differentiation of cultured mouse epidermal cells by 1 α ,25-dihydroxyvitamin D₃. *Endocrinology* 1983;113:1950–1957.
- 8 Smith EL, Walworth NC, Holick MF: Effect of 1 α ,25-dihydroxyvitamin D₃ on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. *J Invest Dermatol* 1986;86:709–714.
- 9 Bikle DD, Nemanic MK, Elias P: 1,25-Dihydroxyvitamin D₃ production by human keratinocytes. *J Clin Invest* 1986;78:557–566.
- 10 Bikle DD, Halloran BP, Riviere JE: Production of 1,25-dihydroxyvitamin D₃ by perfused pig skin. *J Invest Dermatol* 1986;102:796–798.
- 11 Lehmann B, Pietzsch J, Kämpf A, Meurer M: Human keratinocyte line HaCaT metabolizes vitamin D₃ and 1 α -hydroxyvitamin D₃ to 1 α ,25-dihydroxyvitamin D₃. *J Dermatol Sci* 1998;18:118–127.
- 12 Lehmann B, Rudolph T, Pietzsch J, Meurer M: Conversion of vitamin D₃ to 1 α ,25-dihydroxyvitamin D₃ in human skin equivalents. *Exp Dermatol* 2000;9:97–103.
- 13 Wilkinson CF, Hetnarski K, Cantwell GP, DiCarlo FJ: Structure-activity relationships in the effects of 1-alkylimidazoles on microsomal oxidation in vitro and in vivo. *Biochem Pharmacol* 1974;23:2377–2386.
- 14 Sietsema WK, DeLuca HF: Retinoic acid 5,6-epoxidase: properties and biological significance. *J Biol Chem* 1982;257:4265.
- 15 Hollis BW: Assay of circulating 1,25-dihydroxyvitamin D involving a novel single-cartridge extraction and purification procedure. *Clin Chem* 1986;32:2060–2063.