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Inhibition of the 1,25-Dihydroxyvitamin D_3-Induced Increase in Vitamin D Receptor (VDR) Levels and Binding of VDR-Retinoid X Receptor (RXR) to a Direct Repeat (DR)-3 Type Response Element by an RXR-Specific Ligand in Human Keratinocyte Cultures

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ABSTRACT. The biological active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), mediates most of its actions through the intracellular vitamin D receptor (VDR). VDR binds to vitamin D responsive elements (VDREs) in the promoter region of responsive genes and regulates transcription. Usually the VDREs consist of a direct repeat of two hexanucleotides spaced by three nucleotides (DR-3), to which VDR preferentially binds as a heterodimer with the retinoid X receptor (RXR). In the present study, we examined the effect of $1,25(OH)_2D_3$ and a specific ligand for RXR, CD2809, on VDR and RXR levels in cultured human keratinocytes and on the binding of RXR-VDR to a DR-3 type response element. Incubation with $1,25(OH)_2D_3$ increased VDR levels as determined by Western blotting, increased VDR-RXR binding to a DR-3 type response element as determined by the electromobility shift assay (EMSA), and induced the 25-OH-D_3 24-hydroxylase (24-hydroxylase) gene, containing a DR-3 type response element. CD2809 caused a slight decrease in RXRα levels, but had no effect on VDR levels. Addition of both CD2809 and $1,25(OH)_2D_3$ decreased VDR levels as well as the VDR-RXR binding levels to the DR-3 type response element, compared to $1,25(OH)_2D_3$ alone. In conclusion, an RXR-specific ligand interferes with the $1,25(OH)_2D_3$-induced stimulation of VDR levels and VDR-RXR binding to DNA in keratinocyte cultures. It is therefore possible that RXR-specific ligands may counteract certain biological actions of vitamin D_3. BIOCHEM PHARMACOL **55**;6:767–773, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. human cultured keratinocytes; 1,25-dihydroxyvitamin D_3; vitamin D receptor; retinoid X receptor; vitamin D responsive element; h-24-hydroxylase

The epidermis is a target tissue for the active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$).† The vitamin D receptor (VDR) can be detected in epidermal keratinocytes [1] and is also expressed in cultured human keratinocytes [2]. Furthermore, $1,25(OH)_2D_3$ has been shown to inhibit proliferation [2], to promote differentiation [2], and to modulate cytokine formation [3] in keratinocyte cultures.

$1,25(OH)_2D_3$ exerts its effects by both genomic and nongenomic mechanisms. The genomic actions of $1,25(OH)_2D_3$ are mediated through the vitamin D receptor (VDR), which is a member of the steroid/thyroid superfamily of ligand-induced hormone receptors. VDR regulates transcription by binding to specific sequences in the promoter regions of vitamin D responsive genes (VDREs) and thereafter possibly contacts the basal transcription machinery by a direct interaction with general transcription factor IIB [4, 5]. VDR preferentially binds to VDREs as a heterodimer with the retinoid X receptor (RXR), which is a member of the same family of hormone receptors [6, 7]. Most natural VDREs consist of a direct repeat spaced by three nucleotides (DR-3) [8]. This includes the human 25-OH-D_3 24-hydroxylase (h-24-hydroxylase) vitamin D response element [9]. The h-24-hydroxylase gene is the only vitamin D responsive gene in human keratinocytes in which a VDRE has been identified. VDR is also able to form homodimers as well as heterodimers with the retinoid receptor RAR and the T_3 receptor [10]. The heterodimers form a complex system for crosstalk between the ligands for the different receptors. Knowledge about the molecular crosstalk between $1,25(OH)_2D_3$ and retinoids in keratinocyte cultures may, therefore, provide important information concerning the combination of vitamin D_3 analogs and retinoids for the therapy of skin diseases.

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† *Abbreviations:* $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; DR, direct repeat; EMSA, electromobility shift assay; FCS, fetal calf serum; h-24-hydroxylase, human 25-OH-D_3 24-hydroxylase; KGM, keratinocyte growth medium; RPA, ribonuclease protection assay; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D responsive element.

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In cultured human keratinocytes, we have previously shown that VDR is upregulated by $1,25(OH)_2D_3$ and that this upregulation may involve an increased message as well as receptor stabilization [11]. It is, however, unknown whether the VDR increase is accompanied by an increased binding to VDRE. Thus, a limited availability of RXR might interfere with the formation of VDR-RXR heterodimers and thereby the binding to a DR-3 type VDRE. In normal epidermis, RXRα is the predominant form of RXR (90%), although small amounts of RXRβ are also present [12].

The purpose of the present study was to determine the effects of $1,25(OH)_2D_3$ on VDR and RXRα levels and the binding of VDR-RXR to a DR-3 type response element. A further aim was to determine whether the RXR-specific ligand, CD2809, had any costimulatory effect on receptor levels and VDR-RXR heterodimer binding to DNA.

MATERIALS AND METHODS

Materials

Culturing flasks were purchased from Nunc. Keratinocyte growth medium, gentamicin and PBS (−Ca and −Mg) were purchased from Gibco BRL, Life Technologies. $1,25(OH)_2D_2$ was provided by Leo Pharmaceutical Products. CD2809, provided by Galderma, is a specific ligand to RXR with K_D values >1.7 μM for RXRα, RXRβ, and RXRγ and an affinity constant (AC_{50}) value ~50 nM. Monoclonal 9A7γ rat anti-VDR antibody was purchased from Chemicon. Polyclonal anti-VDR rabbit antibody was provided by Rajiv Kumar (Mayo Clinic), and the RXR antibodies by C. Rochette-Egly and P. Chambon, Strasbourg, France. The horse-radish-peroxidase (HRP) conjugated rabbit anti-rat antibody, swine anti-rabbit antibody, and goat anti-mouse antibody were purchased from DAKO. Hybond™-ECL™ nitrocellulose-membrane, hyperfilm-ECL™ and Enhanced Chemiluminescence Detection System were purchased from Amersham Denmark. Oligonucleotides (5′-ccggaAG GTCAaggAGGTCAa-3′, 5′-cgcgt-TGACCTcctTGAC CTt-3′) for the DR-3 response element were purchased from DNA Technology. The Klenow fragment of DNA polymerase I was purchased from Stratagene and [α^{32}P]dCTP (3000 Ci/mmol) and [^{32}P]UTP (3000 Ci/mmol) from Amersham Denmark. INSTA-GEL® was purchased from Packard, Meridan. TRIzol™ reagent was purchased from Life Technologies. DH5α competent bacteria were purchased from Gibco BRL, Life Technologies. The human 24-hydroxylase cDNA recombinant plasmid was a generous gift from Hisham M. Darwish, Madison, WI, USA. The QIAGEN® plasmid kit, QIAEX® Gel Extraction kit and QIAquick™ Gel Extraction kit were purchased from QIAGEN. The MEGAscript™ *in vitro* Transcription Kit used for preparation of 18S rRNA probe and the RPA II™ Ribonuclease Protection Assay Kit were purchased from Ambion. All materials except 24-hydroxylase DNA template used for *in vitro* transcription of the 24-hydroxylase RNA probe were purchased from Promega. Buffers used for nuclear protein extract: Buffer A: 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF. Buffer B: 20 mM HEPES-KOH (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF. Buffers used for Western blotting: running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS; transfer buffer: 48 mM Tris Base, 39 mM glycine, 20% methanol; washing buffer I: 20 mM diaminopropane pH 9.5, 150 mM NaCl; washing buffer II: 50 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20. Binding buffer for the electromobility shift assay: 20 mM HEPES (pH 7.4), 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 0.1% NP-40, 1 μg/μL poly(dI-dC). As running buffer for the electromobility shift assay, 0.5 × TBE (Tris-borate/EDTA electrophoresis buffer) was used. Hybridization buffer for RPA: 80% deionized formamide, 100 mM sodium citrate pH 6.4, 300 mM sodium acetate pH 6.4, 1 mM EDTA. DEPC water: diethyl pyrocarbonate-treated dH_2O.

Keratinocyte Cultures

Normal human keratinocytes were obtained by trypsinizing skin samples from patients undergoing plastic surgery. Keratinocytes were grown in serum-free keratinocyte growth medium (KGM) suplemented with 5 ng/mL human recombinant epidermal growth factor, 50 μg/mL bovine pituitary extract and 5 μg/mL gentamicin. First passage keratinocytes were grown to 70–80% confluence. At this point, the Ca^{2+} concentration was increased to 0.3 mM and delipidized FCS added (3%). Keratinocyte cultures were then incubated with $1,25(OH)_2D_3$ and/or CD2809 for 8 hr. 2-Propanol 0.05% served as control vehicle. The reason for the chosen Ca^{2+} and FCS concentrations was that the antiproliferative and prodifferentiative effects of $1,25(OH)_2D_3$ are maximal at these concentrations [13].

Preparation of Nuclear Extracts

Extraction was done by a modification of the method described by Dignam [14]. The samples were kept at 5° during all procedures. The keratinocytes were washed twice with PBS (without Ca or Mg) before trypsinization. The harvested cells were washed 3 times in PBS (without Ca or Mg) and then suspended in buffer A, allowed to swell for 10 min, and lysed by 10 strokes with a glass homogenizator. The nuclei were pelleted by centrifugation at 1,000 *g*, and the supernatant was decanted. The pellet was recentrifugated for 20 min at 16,000 *g*, and the remaining supernatant was removed. The pellet was then suspended in buffer B and the samples were incubated for 30 min while being stirred, before centrifugation at 16,000 *g* for 30 min. The supernatant was collected as the nuclear extract.

Western Blot Analysis

Whole-cell extracts were electrophoresed on 10% SDS-polyacrylamide gels. The proteins were transferred from the gel to a Hybond-ECL™ nitrocellulose membrane by 75 min blotting in blotting buffer at 100 V. The membranes were thereafter incubated with antibodies as previously described [15]. Monoclonal 9A7γ rat anti-VDR antibody and monoclonal 4RX-1F6 mouse anti-RXRα antibody were used to measure levels of VDR and RXRα, respectively. Polyclonal rabbit anti-VDR antibody and polyclonal rabbit anti-RXRα antibody were used to confirm the specifity of bands.

Electromobility Shift Assay (EMSA)

The DR-3 response element was labeled by a fill-in reaction using [α-^{32}P]dCTP (3000 Ci/mmol) and the Klenow fragment of DNA polymerase I [16]. Six μg nuclear extract was incubated with 20 mM Hepes, 1 mM dithiothreitol, 10% glycerol, 0.1% NP-40, 100 mM NaCl and 1 μg poly(dI-dC) in a total volume of 30 μL on ice for 15 min. [^{32}P]-5′-end-labeled DR-3 (78,000 cpm) was then added, and incubation was continued for 30 minutes at room temperature. Twenty-five μL of the samples was loaded on a 5% polyacrylamide gel run in 0.5 × TBE, whereby the protein-bound DNA was separated from the free probe. The gel was dried and subjected to autoradiography from 4 hr to 3 days. The amount of radiolabeled DR-3 bound to VDR was quantified by excising the portions of the gel corresponding to the bands on the autoradiograph. The amount of radioactivity in each piece of the gel was then determined by liquid scintillation counting. In the experiments with addition of antibodies, nuclear extracts were incubated with the antibodies for 15 min at 4° before addition of the labeled probe.

RNA Extraction

RNA extraction was performed as described by the manufacturers of the TRIzol™ reagent. The keratinocytes were washed twice in PBS, then 5 mL TRIzol™ reagent was added to the culture flasks (80 cm^2), and the cells were scraped off with a rubber policeman. The cells were then transferred to centrifuge tubes and homogenized by passing 6–7 times through a 0.7 mm syringe. After addition of 1 mL chloroform per tube, samples were mixed, incubated for 2 min at room temperature, and centrifuged at 12,000 *g* for 15 min at 5°. After transfer of the liquid phase to new tubes, 2.5 mL 2-propanol was added, and the tubes were vortexed and incubated at room temperature for 10 min. Finally, the tubes were centrifuged at 12,000 *g* for 5 min at 5°, and the pellet was washed twice in ice-cold 80% ethanol, dried and resuspended in DEPC water.

Ribonuclease Protection Assay (RPA)

The 24-hydroxylase was quantitated by RPA. A construct with the human 24-hydroxylase (~2.7 kb) cloned into the *Eco*RI site of the pGEM3-Z vector was a generous gift from HM Darwish, Madison, WI, USA [19]. The h-24-hydroxylase was transformed into DH5α competent bacteria, and plasmids were purified using the QIAGEN® plasmid kit as described by the manufacturer. Plasmids were linearized with Stu I, and a 24-hydroxylase template (~400 bp) containing the SP-6 promoter site was purified using either the QIAEX® Gel Extraction kit or the QIAquick™ Gel Extraction kit according to the manufacturer. *In vitro* transcription was performed using an *in vitro* transcription kit from Promega and SP-6 RNA polymerase as described by the manufacturer. *In vitro* transcription of the 18S rRNA probe was performed using linearized 18S rRNA template DNA and the Megascript™ kit from Ambion as described by the manufacturer. Total RNA was mixed with [^{32}P]-labeled 24-OHase and 18S rRNA probe and incubated at 37° overnight in hybridization buffer. Unhybridized RNA was digested by an RNase mixture consisting of RNase A (2.5 units/mL) and RNase T1 (100 units/mL) for 30 min at 37°. The remaining RNA was precipitated and run in a 5% acylamid gel. Radioactive areas were cut from gel and the amount of radioactivity counted by liquid scintillation counting. The amounts of h-24-hydroxylase RNA were normalized toward the amount of 18S rRNA, which was used a measure of total RNA loaded on the gel.

Statistical Analysis

Statistical significance was determined by Wilcoxon's signed rank-sum test or by a paired *t*-test ($P < 0.05$).

RESULTS

Effects of 1,25(OH)$_2$D$_3$ and CD2809 on VDR Levels in Cultured Human Keratinocytes

First passage keratinocytes were incubated with 1,25(OH)$_2$D$_3$ (10^{-9} M, 10^{-7} M), CD2809 (10^{-6} M) and combinations of 1,25(OH)$_2$D$_3$ (10^{-9} M) + CD2809 (10^{-6} M) and 1,25(OH)$_2$D$_3$ (10^{-7} M) + CD2809 (10^{-6} M) at 70–80% confluence. Incubations were carried out for 8 hr because a previous study showed that the 1,25(OH)$_2$D$_3$-induced increase in VDR levels is maximal at this time point [11].

To determine whether 1,25(OH)$_2$D$_3$ induced transcription of a keratinocyte gene with a DR-3 type vitamin D response element under the applied experimental conditions, the human 24-hydroxylase mRNA levels were measured using RPA. Keratinocyte cultures were incubated with 10^{-7} M 1,25(OH)$_2$D$_3$ for 0–24 hr (Fig. 1). The 24-OHase was induced after 4 hr, and the levels were maximal at 12–24 hr.

Incubation with 1,25(OH)$_2$D$_3$ increased the mean VDR levels in keratinocytes by 83% at 10^{-9} M, and by 273% at 10^{-7} M as compared to vehicle (Fig. 2). At both 1,25(OH)$_2$D$_3$ concentrations, the VDR increases were statistically significant as compared to vehicle. Incubation with CD2809 alone did not change the VDR levels.

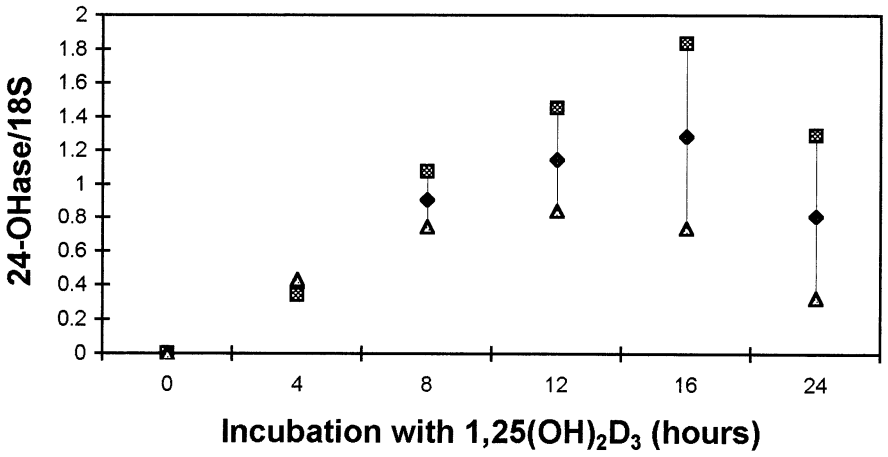

FIG. 1. Induction of 24-OHase mRNA by $1,25(OH)_2D_3$ in cultured human keratinocytes dertermined by RPA. The amount of 18S rRNA was used to compare the total amount of RNA in the tubes. The amount of radioactivity in the 24OHase bands were therefore expressed relative to the amount of radioactivity in the 18S band on the vertical axis. Data from two experiments and their mean values are shown. Data were expressed relative to the means of all data for each experiment.

However, coincubation of CD2809 and $1,25(OH)_2D_3$ (10^{-7} M) resulted in an inhibition of VDR levels as compared to $1,25(OH)_2D_3$ alone (Fig. 2). The combination of CD2809 and $1,25(OH)_2D_3$ was increased compared to vehicle. The difference was, however, not statistically different.

Effects of $1,25(OH)_2D_3$ and CD2809 on RXRα Levels in Cultured Human Keratinocytes

CD2809 caused a slight (21%), but statistically significant decrease in the RXRα level compared to vehicle, whereas $1,25(OH)_2D_3$ produced no change in RXRα levels (Fig. 3). When added together, CD2809 and $1,25(OH)_2D_3$ had no effect on RXRα levels as compared to vehicle.

Effect of $1,25(OH)_2D_3$ and CD2809 on the Binding Level of VDR-RXRα to a DR-3 Type Response Element

The binding levels of VDR-RXRα to a DR-3 type response element were analyzed by EMSA (Fig. 4). Two very close bands were detected. To determine which band represented the VDR-RXR complex, cold DNA was added to the binding reaction (Fig. 4). When increasing amounts of cold DR-3 were added, the upper of the two bands was competed for first. When cold salmon DNA was added to the binding reaction, the lower band was competed for first. This indicates that the upper band is the specific one. The monoclonal antibody 9A7γ VDR binds to the border of the DNA binding region of the VDR [17]. When 9A7γ antibody was added to the binding reaction, both bands disappeared, suggesting that VDR was present in both bands. Adding a monoclonal antibody against RXRα to the binding reaction caused a total supershift of the upper band, indicating that RXRα was present in this band. An anti-RXRβ antibody caused a weaker supershift, whereas an anti-RXRγ antibody had no effect (Fig. 4). According to these results, the binding of RXR-VDR to a DR-3 response element was measured as the amount of radioactivity in the upper band.

Incubation of the keratinocyte cultures with $1,25(OH)_2D_3$ for 8 hr resulted in an upregulation of the binding of VDR-RXR to the DR-3 type response element by 46% at 10^{-9} M, and by 95% at 10^{-7} M as compared to

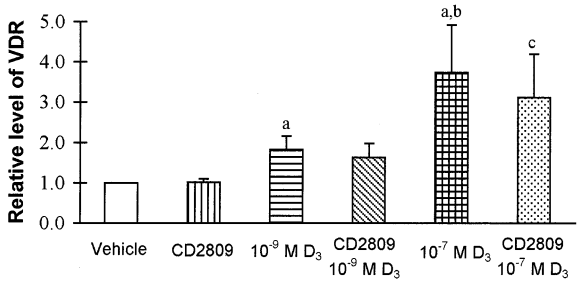

FIG. 2. The relative levels of VDR in human keratinocyte cultures stimulated with $1,25(OH)_2D_3$ and CD2809 (10^{-6} M). The VDR levels were determined by Western blotting using the monoclonal 9A7γ rat anti-VDR antibody. Each experiment was done in triplicate. Results are means + SEM; $n = 6$. a) statistically different from vehicle $P < 0.05$; b) statistically different from 10^{-9} M $1,25(OH)_2D_3$ $P < 0.05$; c) statistically different from 10^{-7} M $1,25(OH)_2D_3$ $P < 0.05$).

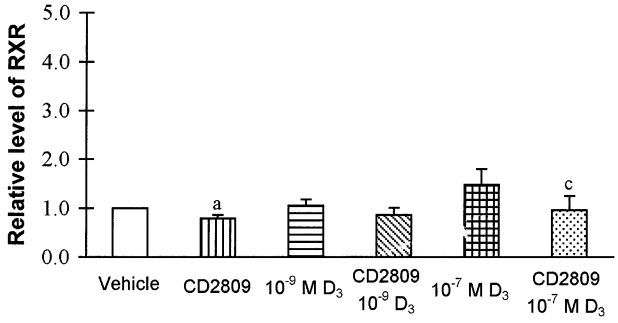

FIG. 3. The relative levels of RXRα in keratinocytes stimulated with $1,25(OH)_2D_3$ and CD2809 (10^{-6} M). The RXRα levels were determined by Western blotting using the monoclonal 4RX-1F6 mouse anti-RXRα antibody. Each experiment was done in triplicate. Results are means + SEM; $n = 6$. a) statistically different from vehicle $P < 0.05$ and c) statistically different from 10^{-7} M $1,25(OH)_2D_3$ $P < 0.05$).

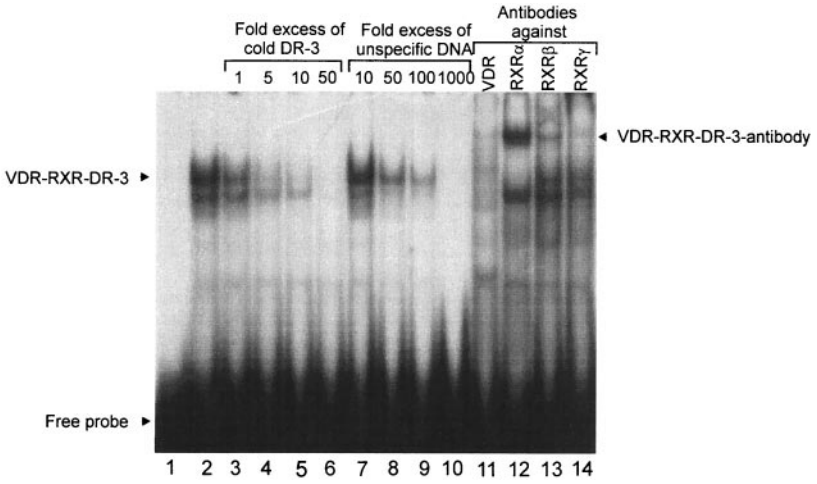

FIG. 4. EMSA to identify the specific VDR-RXR complex. Lane 1 is pure oligo. Lane 2 is oligo and nuclear extract from cultured human keratinocytes. In lanes 3–6, increasing amounts of cold DR-3 were added to the EMSA binding reaction. In lanes 7–10, increasing amounts of cold salmon DNA were added to the EMSA binding reaction. In lane 11, the monoclonal 9A7γ rat anti-VDR antibody was added to the binding reaction. In lanes 12–14, monoclonal mouse antibodies were added against RXRα, RXRβ and RXRγ, respectively.

vehicle (Fig. 5). At both $1,25(OH)_2D_3$ concentrations, the increases in binding were statistically significant as compared to vehicle. CD2809 did not affect the binding of RXR-VDR to the DR-3. The combination of $1,25(OH)_2D_3$ and CD2809 inhibited $1,25(OH)_2D_3$-induced stimulation, although the inhibition only reached statistical significance at the higher $1,25(OH)_2D_3$ concentration. The combination of $1,25(OH)_2D_3$ and CD2809 was statistically not different from vehicle.

DISCUSSION

Our results demonstrate that the upregulation of VDR levels in keratinocytes by the active form of vitamin D $1,25(OH)_2D_3$ is accompanied by an increased binding of VDR-RXR to a DR-3 type response element (as determined by EMSA). Our previous studies showed that the VDR increase is preceded by a slight and temporary increase of the message for VDR [11], but an increased stability of the receptor may also be induced by $1,25(OH)_2D_3$ as has been shown in mouse fibroblasts and rat intestinal epithelial cells

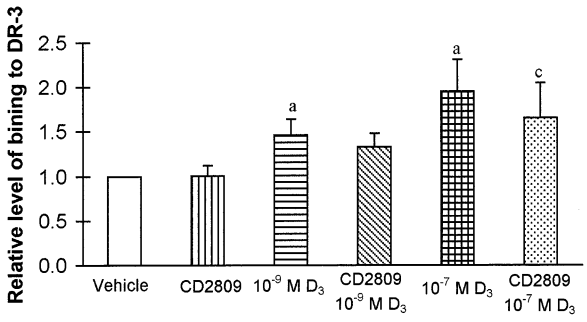

FIG. 5. The relative levels of VDR-RXRα binding to the DR-3 type response element. The binding levels were determined by EMSA. Six μg of nuclear protein from the keratinocytes was incubated together with the DR-3 type response element (78,000 cpm). Each experiment was done in triplicate. Results are means + SEM; $n = 6$. a) Statistically different from vehicle $P < 0.05$ and c) statistically different from 10^{-7} M $1,25(OH)_2D_3$ $P < 0.05$).

[18]. After stimulation with $1,25(OH)_2D_3$, VDR levels correlated with the binding of VDR-RXR to the DR-3 type response element in the keratinocyte cultures. The increased receptor binding to DNA may be accompanied by increased transcriptional activity of keratinocyte genes with a DR-3 type response element in their promoter. Thus, we observed an induction of the 24-hydroxylase mRNA under similar experimental conditions.

The binding of the VDR-RXR heterodimers to a DR-3 type response element *in vitro* does not require the presence of $1,25(OH)_2D_3$ [19, 20]. The increased binding of VDR-RXR to the DR-3 type response element in $1,25(OH)_2D_3$-treated keratinocyte cultures is most likely caused by increased VDR levels, although a ligand-induced conformational change in the VDR may also play a role [21]. The ability of $1,25(OH)_2D_3$ to increase the binding of VDR-RXR indicates that the amount of VDR, rather than RXR, is the limiting factor in keratinocyte cultures. This is supported by the fact that the slight decrease in RXRα levels caused by the RXR-specific ligand CD2809 had no effect on VDR-RXR binding to the DR-3 type response element. Although the RXR ligand had no effect on VDR levels, the addition of CD2809 to $1,25(OH)_2D_3$ attenuated the $1,25(OH)_2D_3$-induced stimulation of both VDR levels and VDR-RXR binding to DNA. The inhibitory effect of CD2809 on receptor binding to the response element may be secondary to its effect on VDR levels. It is also possible that the RXR ligand caused an instability of the heterodimer binding to DNA. Thus, RXR might undergo a conformational change more suitable for homodimerization or for dimerization with other nuclear receptors [22–24].

The mechanisms behind the inhibitory effect of CD2809 on the $1,25(OH)_2D_3$-induced stimulation of VDR levels are unknown. CD2809 might interfere with the VDR message or stability. Thus, all-trans retinoic acid at low concentrations downregulated VDR levels in T-47D human breast cancer cells as determined in a ligand binding assay [25]. In contrast, an upregulation by all-trans retinoic acid was seen in mouse osteosarcoma cells as determined in a ligand binding assay [26]. In human MG-63 osteosarcoma

cells, all-trans retinoic acid upregulated VDR mRNA levels, but this was not accompanied by an upregulation of receptor levels [27].

The formation of a heterodimeric complex between the VDR and RXR can result in transcriptional activation by ligands for both receptors and may lead to additive or even synergistic effects [28–31]. Another possible outcome is that RXR-specific ligands may favor the formation of RXR homodimers [7] and thus inhibit RXR-VDR heterodimerization. In support of the latter possibility, the RXR-specific ligand CD2809 was able to inhibit the $1,25(OH)_2D_3$-induced stimulation of VDR-RXR binding to a DR-3 type response element. This means that the effect of $1,25(OH)_2D_3$ on vitamin D responsive genes in keratinocytes may be abolished in the presence of ligands for RXR. Although synergism between vitamin D_3 and retinoids has been observed under other experimental conditions [28–31], our results underscore the complexity of the crosstalk between vitamin D_3 and retinoid signaling pathways.

In accordance with our findings, 9-*cis* retinoic acid, a natural ligand for RXR as well as RAR, has been shown to inhibit $1,25(OH)_2D_3$-induced expression of the rat osteocalcin gene, which contains an imperfect DR-3 VDRE as well as the osteopontin gene, which contains a perfect DR-3 VDRE as determined by transfection studies with COS and CV-1 cells [7, 23]. Furthermore, Lemon *et al.* showed that induction by $1,25(OH)_2D_3$ was attenuated by an RXR-specific ligand L2678 in CV-1 cells [23].

In conclusion, an RXR-specific ligand interferes with the $1,25(OH)_2D_3$-induced stimulation of VDR levels and VDR-RXR binding to DNA in keratinocyte cultures. It is, therefore, possible that RXR-specific ligands may counteract certain biological actions of vitamin D_3.

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