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The 3-epi- and 24-oxo-derivatives of 1α,25 dihydroxyvitamin D_3 stimulate transcription through the vitamin D receptor

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Abstract

Vitamin D is enzymatically modified to more than 35 metabolites. While many of these are thought to represent degradation products, some have been shown to exhibit biological activity. We tested whether 3-epi-1α,25-dihydroxyvitamin D_3 (3-epi-1α,25$(OH)_2D_3$), 1α,25-dihydroxy-24-oxo-vitamin D_3 (1α,25$(OH)_2$-24-oxo-D_3), and 1α,25$(OH)_2D_3$-26,23-lactone can stimulate transcription of vitamin D responsive genes. MC3T3-E1 cells transfected with a 25-hydroxyvitamin D 24-hydroxylase (CYP24) promoter construct displayed a 6 fold response when treated with either 1α,25$(OH)_2D_3$ or 3-epi-1α,25$(OH)_2D_3$. Caco-2 cells were transfected with the wild type CYP24 promoter construct, or a Vitamin D Response Element (VDRE)-mutated form. Cells acquiring the wild type reporter responded to 1α,25$(OH)_2D_3$ and 3-epi-1α,25$(OH)_2D_3$ but not cells which acquired the mutated reporter. Additionally, VDR-negative COS-7 cells transfected with the wild type promoter responded (approximately 13 fold) to 1α,25$(OH)_2D_3$ and 3-epi-1α,25$(OH)_2D_3$, only when co-transfected with the VDR. These results were confirmed using shorter incubation times and serum-free conditions. This strongly suggested that 3-epi-1α,25$(OH)_2D_3$ mediates its effects through the VDR and its cognate binding site. Similar results were obtained with 1α,25$(OH)_2$-24-oxo-D_3 using VDR-negative P19 cells. We could never detect activity from 1α,25$(OH)_2D_3$-26,23-lactone on vitamin D-responsive target promoters. Our results firmly conclude that both 3-epi-1α,25$(OH)_2D_3$ and the 1α,25$(OH)_2$-24-oxo-D_3 elicit their biological effects by acting through the VDR/VDRE.

Keywords: Vitamin D metabolites; Vitamin D receptor; Vitamin D response element (VDRE)

1. Introduction

Vitamin D_3, the end product of 7-dehydrocholesterol photolysis, exits keratinocyte cells and is transported to the liver where the vitamin D 25-hydroxylase (CYP27) adds a hydroxyl group on C-25 to produce 25-hydroxyvitamin D_3 [25$(OH)D_3$]. 25$(OH)D_3$ then enters the bloodstream to become the major circulating, yet inactive, form of vitamin D. To become biologically active, 25$(OH)D_3$ must undergo a further hydroxylation event in the kidney, where the 25-hydroxyvitamin D 1α-hydroxylase (CYP27 B1) hydroxylates it on C-1 to produce 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] [1–4].

When 1α,25$(OH)_2D_3$ enters its target cells, it binds to the nuclear vitamin D receptor (VDR) [5]. The receptor heterodimerizes with the retinoid X receptor (RXR) to bind to specific DNA sequences known as vitamin D response elements (VDRE) [5]. These consist of two tandemly repeated hexanucleotide sequences separated by 3 base pairs. The result of this interaction will lead to the transcriptional modulation of target genes responsible for carrying out the physiological actions of 1α,25$(OH)_2D_3$. Vitamin D-responsive genes include, amongst others, the 25-hydroxyvitamin D 24-hydroxylase (CYP24) and osteocalcin genes [5].

1α,25$(OH)_2D_3$, the active hormonal form of vitamin D, acts to regulate calcium and phosphorus homeosta-

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sis [1–3]. More than 35 metabolites of vitamin D have been identified [6]. An important question to be answered is whether $1\alpha,25(OH)_2D_3$ is an exclusive hormone that carries out most, if not all, of vitamin D's biological actions, or do some of the other metabolites elicit physiologically significant responses?

The conversion of $1\alpha,25(OH)_2D_3$ into 3-epi-$1\alpha,25(OH)_2D_3$ is characterized by a switch of the hydroxyl group orientation on carbon C-3 from the β to the α position. The enzyme responsible for this epimerization remains unknown. The 3-epimerization pathway is only detected in differentiated Caco-2 cells, but not in proliferating, undifferentiated cells [7]. This observation suggests that 3-epimerization is a tightly regulated metabolic pathway and supports the hypothesis that 3-epi-$1\alpha,25(OH)_2D_3$ may constitute a biologically active metabolite contributing to the diverse responses of target cells to the vitamin D endocrine system. Indeed, 3-epi-$1\alpha,25(OH)_2D_3$ possesses antimitotic and prodifferentiating activities [7].

The CYP24 cytochrome P450 enzyme acts on the $1\alpha,25(OH)_2D_3$ substrate to produce 1,24,25-trihydroxyvitamin D_3, the initial reactant in the 24-oxydation pathway that is thought to lead to metabolite inactivation [8]. This pathway comprises five enzymatic steps involving successive hydroxylation/oxidation reactions at carbons 24 and 23 followed by cleavage of the secosteroid backbone at the C-23/C-24 bond and subsequent oxidation of the cleaved product to calcitroic acid [8]. The second enzymatic step in the pathway is the oxidation of 1,24,25-trihydroxyvitamin D to $1\alpha,25(OH)_2$-24-oxo-D_3, a metabolite with measurable activity in bone resorption assays and antiproliferation assays [9,10].

Hydroxylation of $1\alpha,25(OH)_2D_3$ on carbon 26 eventually leads to formation of $1\alpha,25(OH)_2D_3$-26,23-lactone [11]. This metabolite affects intestinal calcium transport and can modulate bone resorption [12,13].

We tested whether 3-epi-$1\alpha,25(OH)_2D_3$, $1\alpha,25(OH)_2$-24-oxo-D_3, and $1\alpha,25(OH)_2D_3$-26,23-lactone can stimulate the transcription of a vitamin D-responsive gene, and could act through the VDR or a putative, distinct receptor. Our data suggest that 3-epi-$1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2$-24-oxo-D_3 promote transcription from the CYP24 promoter, and that the transactivation event is initiated through the classical vitamin D receptor.

2. Materials and methods

The pGL24H-1401, pGL24H-298 wt and the pGL24H-298 m4 constructs were generous gifts from Dr. J. Omdahl, University of New Mexico (Albuquerque, NM). The vitamin D metabolites, $1\alpha,25\ (OH)_2D_3$, 3-epi-$1\alpha,25(OH)_2D_3$ and the $1\alpha,25(OH)_2D_3$-26,23-lactone were obtained from Dr. M. Uskokovic, Hoffmann-LaRoche, (Nutley, NJ), while $1\alpha,25(OH)_2$-24-oxo-D_3 was provided by Dr. S. Reddy, Brown University, (Providence, RI). The CT4-GH reporter construct was obtained from Dr. M.R. Haussler, University of Arizona, (Tucson, AZ). Human Growth Hormone Chemiluminescence Detection Kit was purchased from Nichols Institute Diagnostics (San Juan Capistrano, CA). Lipofectamine Reagent was purchased from Gibco BRL, Life Technologies (Burlington, ON), while PFX8 lipofection reagent was purchased from Invitrogen (San Diego, CA). Caco-2 cells (human colon adenocarcinoma) were generously provided by Dr. J. Beaulieu, University of Sherbrooke, (Sherbrooke, PQ). COS-7 cells (African green monkey kidney fibroblasts) were obtained from American Type Tissue Culture Collection (Rockville, MD).

2.1. Cell culture

MC3T3-E1 cells were cultured as previously described [14]. Caco-2 and COS-7 cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. P19 cells were cultured according to previously published protocols [15].

2.2. Transfection

All cells were seeded at 1.5×10^5 cells per well in six well-plates. VDR-positive MC3T3-E1 cells were transfected with the pGL24H-1401 construct using the Pfx 8 lipofection reagent as per Invitrogen's protocol. The cells were incubated overnight, then the α-MEM with 10% serum was replaced with fresh media supplemented with either $1\alpha,25\ (OH)_2D_3$, 3-epi-$1\alpha,25(OH)_2D_3$, $1\alpha,25(OH)_2D_3$-26,23-lactone dissolved in ethanol (final concentration 10^{-7} M) or ethanol carrier alone. Following a final overnight incubation at 37°C, 5% CO_2, the cells were rinsed once with phosphate-buffered saline and treated with 150 μl of cell culture lysis reagent (25 mM Tris–Phosphate, pH 7.8, 2mM CDTA, 10% Glycerol, 1% Triton X-100) at room temperature for 15 min. The cells were scraped off with a rubber policeman and centrifuged for 30 s. Twenty microlitres of the supernatant was then used to assay for luciferase activity. The luciferase activity (relative light units) was measured using a Monolight 2010 (Analytical Luminescence Laboratory, San Diego, CA). The data from multiple experiments were pooled and the mean $\pm$ standard error of the mean (SEM) were calculated. The final results were expressed as fold induction relative to the respective control activity of cells treated with ethanol carrier.

VDR-positive Caco-2 cells were transfected with the pGL24H-1401, pGL24H-298 wt and the pGL24H-298 m4 constructs using the Lipofectamine reagent as per

Gibco BRL, Life Technologies protocol. Following an overnight incubation, the media was replaced with fresh media supplemented with either vitamin D metabolites (final concentration 10^{-7} M) or ethanol carrier alone. The cells were processed as described above after 16 h in the presence of metabolites.

VDR-negative COS-7 and P19 cells were transfected with the pGL24H-1401 construct with or without the hVDR expression vector pCMV-VDR using the Lipofectamine reagent as per Gibco BRL, Life Technologies protocol. The cells were then treated as described above, except for the serum-free experiment (see Fig. 4), for which the incubation time with the metabolites was shortened to 8 h.

Transfection conditions of the VDR-negative P19 cells with the CT4-GH construct were as described above with the following modification: after the incubation, 50 μl of media from every well was assayed for the expression of human growth hormone using the Human Growth Hormone Chemiluminescence Detection kit as per Nichols Institute Diagnostics protocol. The level of growth hormone expression was translated to relative light units using the Monolight 2010.

3. Results

VDR-positive MC3T3-E1 cells were transfected with a construct comprising 1401 base pairs of the 25-hydroxyvitamin D 24-hydroxylase (CYP24) promoter driving the luciferase gene (pGL24H-1401). This segment of the promoter contains two functional VDREs [16]. As depicted in Fig. 1, cells treated with $1\alpha,25(OH)_2D_3$ were capable of inducing transcription from the promoter approximately 6 fold compared to cells that were treated solely with ethanol carrier. Likewise, cells treated with 3-epi-$1\alpha,25(OH)_2D_3$ also activated the promoter 6 fold. However, cells that were treated with $1\alpha,25(OH)_2D_3$-26,23-lactone did not induce transcription from the VDREs as inferred from previous results [13]. This data suggested that 3-epi-$1\alpha,25(OH)_2D_3$ could potentially carry out its biological actions through the VDR/VDRE.

To test this possibility, VDR-positive Caco-2 cells were transfected with the pGL24H-298 wt or pGL24H-298 m4 reporter constructs. The former is a construct consisting of 298 base pairs of the CYP24 promoter driving the luciferase reporter gene. It encompasses the two wild type VDREs. The latter is identical to the former except that the two VDREs are mutated [16]. Cells transfected with pGL24H-298 wt and treated with either $1\alpha,25(OH)_2D_3$ or 3-epi-$1\alpha,25(OH)_2D_3$ were able to substantially transactivate the reporter, 73 and 43 fold, respectively, as compared to cells treated with ethanol carrier (see Fig. 2). However, as demonstrated in Fig. 1, cells treated with $1\alpha,25(OH)_2D_3$-26,23-lactone did not activate the pGL24H-298 wt construct. In cells transfected with pGL24H-298 m4 (non-functional VDREs), the $1\alpha,25(OH)_2D_3$ and 3-epi-$1\alpha,25(OH)_2D_3$ activity was abrogated (see Fig. 2). This clearly suggests that the receptor responsible for carrying out the 3-epi-$1\alpha,25(OH)_2D_3$ activity acts through the VDRE, and, in fact, is probably the VDR.

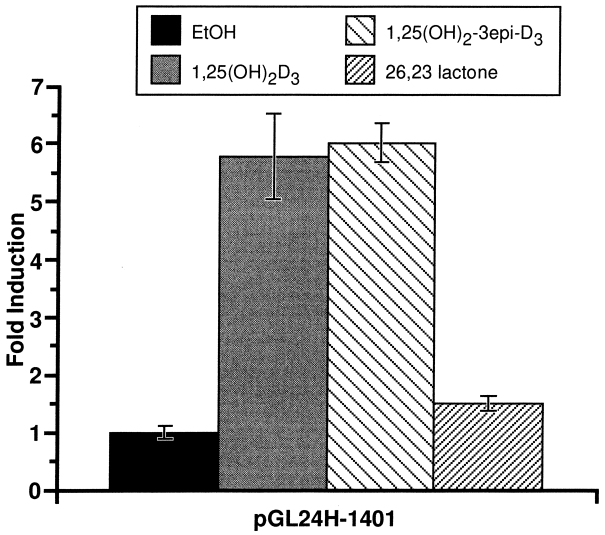

Fig. 1. Transient transfection of VDR-positive MC3T3-E1 cells with pGL24H-1401 (CYP24 promoter driving the luciferase reporter gene). The expression levels detected in cells treated with the ethanol carrier were arbitrarily ascribed a value of 1. Incubations were for 24 h with 10^{-7} M of each metabolite. Results are expressed as mean fold induction ± SEM of three independent transfections.

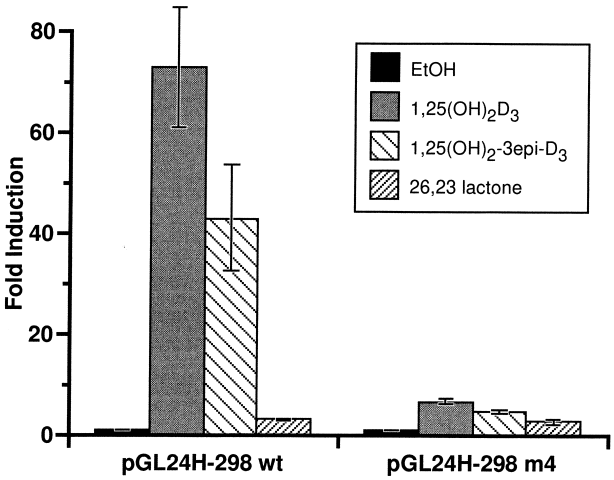

Fig. 2. Transient transfection of Caco-2 cells with pGL24H-298 wt (wild type CYP24 promoter construct) or pGL24H-298 m4 (mutated CYP24 promoter construct). The expression levels detected in cells treated with ethanol carrier were arbitrarily ascribed a value of 1. Incubations were for 24 h with 10^{-7} M of each metabolite. Results are expressed as mean fold induction ± SEM of three independent transfections.

As described in Fig. 3 VDR-negative cells were transfected with the pGL24H-298 wt reporter. Simian kidney COS-7 cells that were co-transfected with the VDR were able to transactivate the reporter approximately 13 fold when they were treated with $1\alpha,25(OH)_2D_3$ or 3-epi-$1\alpha,25(OH)_2D_3$ (see Fig. 3(A)). Cells that did not acquire the VDR did not show any activity when treated with these two vitamin D metabolites. This result was confirmed utilizing VDR-negative P19 cells (see Fig. 3(B)). These data conclusively demonstrate that the VDR is responsible for mediating the biological actions of 3-epi-$1\alpha,25(OH)_2D_3$ through the VDRE.

To ascertain that the observed activities were not due to vitamin D metabolites present in the serum, a similar set of experiments was performed under serum-free conditions. For these experiments, the COS-7 cells were incubated with the metabolites for a shorter duration of 8 h to reduce the possible production of a wide spectrum of metabolites through the induction of CYP24. Fig. 4 shows that $1\alpha,25(OH)_2D_3$ and 3-epi-$1\alpha,25(OH)_2D_3$ were also able to stimulate transcription of the CYP24-driven reporter gene through the VDR in the absence of serum. Similar results were obtained using charcoal-stripped serum (data not shown).

We also tested $1\alpha,25(OH)_2$-24-oxo-D_3, to see whether or not it binds the VDR and thereby stimulates transcription. VDR-negative P19 cells were transfected with pGL24H-298 wt. Cells that were co-transfected with a VDR expression vector were able to transactivate the reporter 85 and 115 fold when they were treated with either $1\alpha,25(OH)_2D_3$ or $1\alpha,25(OH)_2$-24-oxo-D_3, respectively (see Fig. 5). Cells that were not transfected with the VDR did not show any activity, when treated with these two vitamin D metabolites (see Fig. 5). These results support the notion that $1\alpha,25(OH)_2$-24-oxo-D_3 also binds the VDR, which then allows it to transactivate promoters containing VDREs. The $1\alpha,25(OH)_2$-24-oxo-D_3 metabolite could also stimulate transcription from VDRE-dependent reporter genes in the absence of serum (see Fig. 4).

To ascertain that this is not a promoter-specific phenomenon, P19 cells were transfected with CT4-GH (see Fig. 6). This is a synthetic promoter construct containing four copies of the human osteocalcin promoter VDRE driving the human growth hormone reporter gene. Cells that were co-transfected with the VDR were able to stimulate transcription from 2 to 3

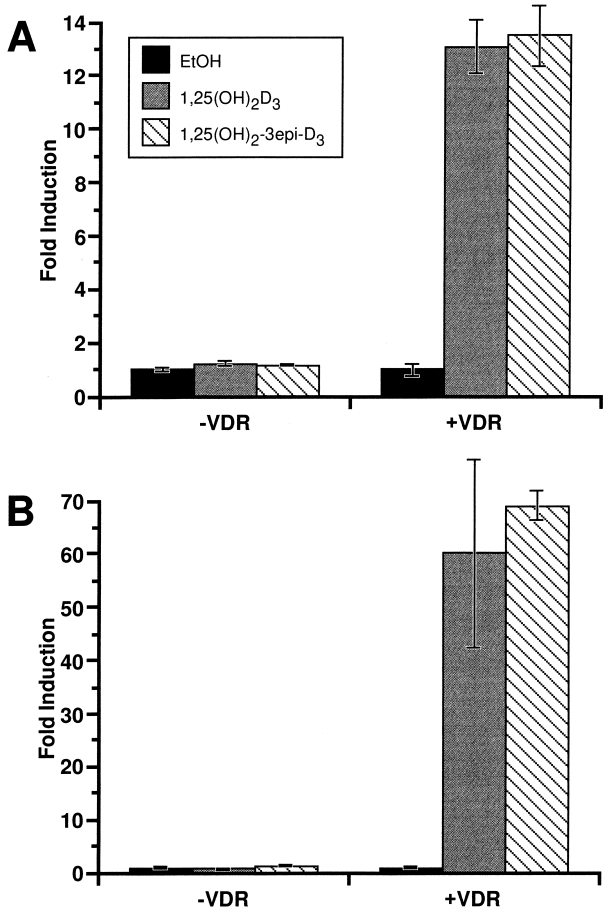

Fig. 3. Transient co-transfection of VDR-negative cells with pGL24H-298 wt, with or without pCMV-VDR. (A) COS-7 cells. (B) P19 cells. Cells transfected with the VDR clearly respond to $1\alpha,25(OH)_2D_3$ and 3-epi-$1\alpha,25(OH)_2D_3$ as compared to cells which do not express the VDR. The expression levels detected in cells treated with ethanol carrier were arbitrarily ascribed a value of 1. Incubations were for 24 h with 10^{-7} M of each metabolite. Results are expressed as mean fold induction ± SEM of three independent transfections.

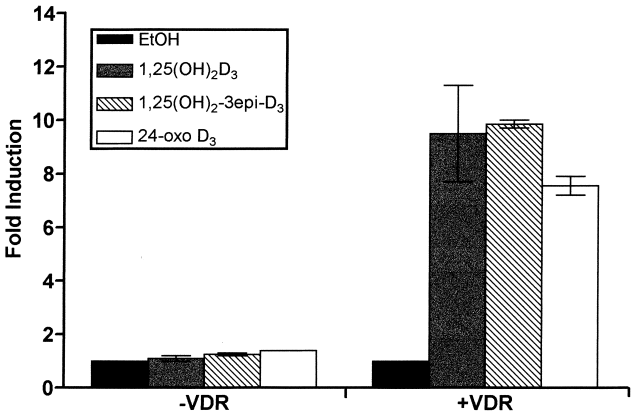

Fig. 4. Transient co-transfection of VDR-negative COS-7 cells with pGL24H-1401 in the presence or absence of pCMV-VDR, under serum-free conditions. $1\alpha,25(OH)_2D_3$, 3-epi-$1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2$-24-oxo-D_3 stimulated transcription in cells expressing the VDR but not in cells devoid of the receptor. The serum-free conditions insure that the effects were not due to low levels of metabolites already present in the serum. The expression levels detected in cells treated with ethanol carrier were arbitrarily ascribed a value of 1. Incubations were for 8 h with 10^{-7} M of each metabolite. Results are expressed as mean fold induction ± SEM of two independent transfections.

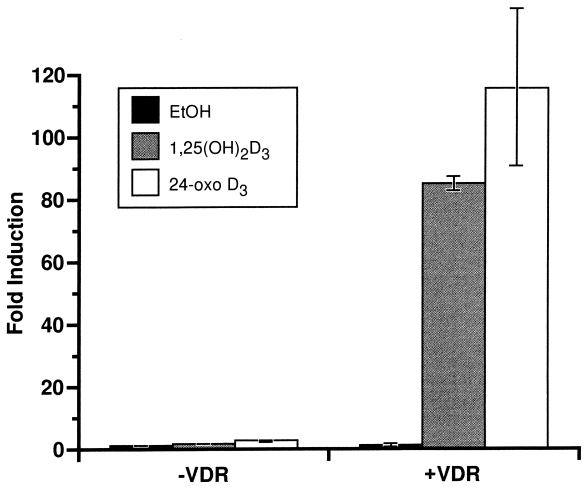

Fig. 5. Transient co-transfection of VDR-negative P19 cells with pGL24H-298 wt, with or without pCMV-VDR. 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-24-oxo-D$_3$ stimulated transcription in cells expressing the VDR versus cells which were not transfected with the VDR. The expression levels detected in cells treated with ethanol carrier were arbitrarily ascribed a value of 1. Incubations were for 24 h with 10^{-7} M of each metabolite. Results are expressed as mean fold induction ± SEM of three independent transfections.

fold when they were treated with 1α,25 (OH)$_2$D$_3$, 3-epi-1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-24-oxo-D$_3$, respectively (see Fig. 6). Once again, cells that did not express the VDR did not show any transcriptional activity above that of the ethanol carrier, when treated with the vitamin D metabolites. Our results strongly suggest that both 3-epi-1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-24-oxo-D$_3$ are able to bind to the VDR, and stimulate transcription in promoters that contain VDREs.

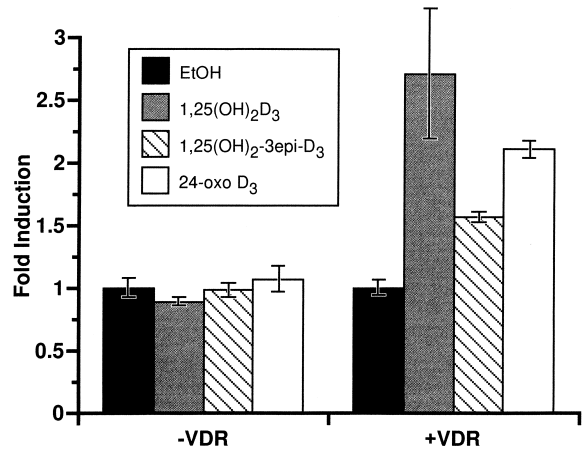

Fig. 6. Transient co-transfection of VDR-negative P19 cells with CT4-GH (a reporter construct consisting of 4 copies of the osteocalcin VDRE driving the growth hormone reporter gene), with or without pCMV-VDR. The expression levels detected in cells treated with ethanol carrier were arbitrarily ascribed a value of 1. Incubations were for 24 h with 10^{-7} M of each metabolite. Results are expressed as mean fold induction ± SEM of three independent transfections.

4. Discussion

Despite 1α,25(OH)$_2$D$_3$'s role as the predominant active metabolite of the vitamin D cascade, 3-epi-1α,25(OH)$_2$D$_3$, 1α,25(OH)$_2$-24-oxo-D$_3$, and 1α,25(OH)$_2$D$_3$-26,23-lactone have demonstrated some physiological potency in vivo and in vitro [7,9,10,12,13]. We explored the putative mechanism(s) of action involved.

These three metabolites show marked differences in their affinity for the classical VDR. The 24-oxo-derivative binds the VDR with the same affinity as the parent hormone [6], but it elicits a distinct antiproliferative response in prostate cancer cells when compared to 1α,25(OH)$_2$D$_3$ [10], suggesting a unique effector pathway. The 3-epi- and 26,23-lactone-derivatives show 4- and 700-fold lower affinity for the VDR than 1α,25(OH)$_2$D$_3$, respectively [6,12]. The 3-epi-1α,25(OH)$_2$D$_3$ epimer selectively accumulates in differentiated Caco-2 cells treated with 1α,25(OH)$_2$D$_3$ [7]. The 3-epimerization is totally independent from side chain metabolism and is a differentiation-specific process [7]. The metabolite accumulates rapidly because the reverse epimerization reaction is slow and the metabolite is stable [7,17]. These observations, coupled to the identification of a large number of nuclear hormone receptors for which ligands remain to be identified [18], prompted us to examine whether the 3-epi-, 24-oxo-, and 26,23-lactone-vitamin D derivatives would act through the classical VDR or a putative distinct receptor.

A wide spectrum of ligand specificity has been observed for distinct nuclear hormone receptors. At one end of the spectrum, certain receptors show exquisite specificity for particular ligands, and cannot bind metabolites or epimers of these ligands. A good example of this restricted specificity is the CAR-β receptor (constitutive androstane receptor-β), which binds 5α-androstan-3α-ol (androstanol) but not 5α-androstan-3β-ol [19]. At the other end of the spectrum, a receptor such as PXR (pregnane X receptor, also known as SXR, steroid and xenobiotic receptor) shows a wide range of ligand recognition for molecules with very diverse structures, beginning with natural steroids like corticosterone and extending to structurally larger xenobiotic compounds such as rifampicine and nifedipine [20]. Thus some vitamin D metabolites may be functionally recognized by the VDR, while others may require separate effector systems.

Our results have consistently demonstrated that both 3-epi-1α,25(OH)$_2$D$_3$, and 1α,25(OH)$_2$-24-oxo-D$_3$ interact with the classical VDR, and elicit biological activities from promoters that contain VDREs. On the other hand, we could detect only marginal activity from 1α,25(OH)$_2$D$_3$-26,23-lactone on vitamin D-responsive target promoters. While it is conceivable that

the relatively high serum concentration and long metabolic stability of the 1α,25(OH)$_2$D$_3$-26,23-lactone metabolite [12,13] could sustain the small stimulation observed and lead to significant responses through the VDR, our data is more consistent with the putative existence of an independent pathway that would transduce the signal from this calcemic metabolite. The identification of novel vitamin D signaling pathways [21] should provide further insight into the physiological role of the vitamin D endocrine system.

Acknowledgements

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