PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 71 (1999) 111–121

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Enhanced biological activity of 1α,25-dihydroxy-20-epi-vitamin D_3, the C-20 epimer of 1α,25-dihydroxyvitamin D_3, is in part due to its metabolism into stable intermediary metabolites with significant biological activity

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Received 12 April 1999; accepted 10 August 1999

Abstract

1α,25-Dihydroxy-20-epi-vitamin D_3 (1α,25$(OH)_2$-20-epi-D_3), the C-20 epimer of the natural hormone 1α,25$(OH)_2D_3$, is several fold more potent than the natural hormone in inhibiting cell growth and inducing cell differentiation. At present, the various mechanisms responsible for the enhanced biological activities of this unique vitamin D_3 analog are not fully understood. In our present study we compared the target tissue metabolism of 1α,25$(OH)_2D_3$ with that of 1α,25$(OH)_2$-20-epi-D_3 using the technique of isolated perfused rat kidney. The results indicated that the C-24 oxidation pathway plays a major role in the metabolism of both compounds in the rat kidney. However, it was noted that the concentrations of two of the intermediary metabolites of 1α,25$(OH)_2$-20-epi-D_3, namely, 1α,24(*R*),25$(OH)_3$-20-epi-D_3 and 1α,25$(OH)_2$-24-oxo-20-epi-D_3 in the kidney perfusate, exceeded the concentrations of the corresponding intermediary metabolites of 1α,25$(OH)_2D_3$. Furthermore, 1α,25$(OH)_2$-24-oxo-20-epi-D_3 induces the conformation of the vitamin D receptor similar to that induced by its parent analog and is nearly as potent as its parent in inducing transactivation of a gene construct containing the human osteocalcin vitamin D-responsive element. We conclude that 1α,25$(OH)_2$-20-epi-D_3 by itself is not metabolically stable when compared to 1α,25$(OH)_2D_3$, but it acquires its metabolic stability because of the reduced rate of catabolism of its intermediary metabolites. Furthermore, 1α,25$(OH)_2$-24-oxo-20-epi-D_3, the stable bioactive intermediary metabolite plays a significant role in generating the enhanced biological activities ascribed to 1α,25$(OH)_2$-20-epi-D_3.

1. Introduction

The discovery that structural modifications of the secosteroid hormone, 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) can result in the dissociation of the hormone's actions on regulating cell growth and differentiation from its calcemic actions, has lead to the synthesis of numerous vitamin D analogs with a wide variety of biological actions [1]. In recent years, our research efforts have been directed towards identifying the differences in metabolism between 1α,25$(OH)_2D_3$ and its various synthetic analogs. We have determined that some analogs are metabolically stable at the starting substrate level, while others are stable at the level

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0960-0760/00/$ - see front matter
PII: S0960-0760(99)00130-2

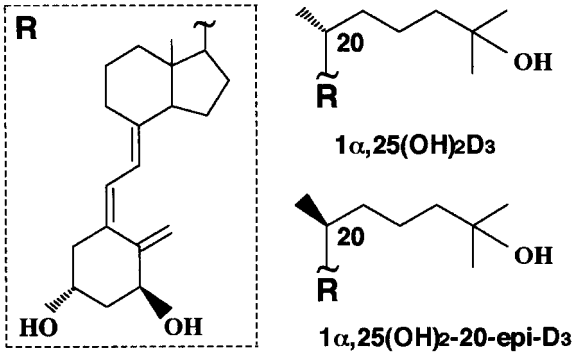

Fig. 1. Chemical structures of $1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2$-20-epi-D_3.

of the intermediary metabolites. The analog, $1\alpha,25(OH)_2$-16-ene-D_3, whose activities are several fold greater than those exerted by $1\alpha,25(OH)_2D_3$ [2] is an example of the latter situation as described in our previous work [3–5].

At present, the concept that metabolically stable intermediary metabolites contribute significantly to the full expression of the biological activities generated by some specific vitamin D analogs is being actively pursued in our laboratory. We now extended our studies to $1\alpha,25(OH)_2$-20-epi-D_3, the carbon 20 (C-20) epimer of $1\alpha,25(OH)_2D_3$, which differs from the natural hormone, by a simple modification in the stereochemistry of the methyl group at C-20 on the side chain (Fig. 1). This unique vitamin D compound has been shown to decrease cell proliferation and promote cell differentiation with a potency several orders of magnitude greater than that of $1\alpha,25(OH)_2D_3$ [6–9]. Many investigators have been involved in studies exploring the mechanisms responsible for the enhanced biological activities of $1\alpha,25(OH)_2$-20-epi-D_3 and its analogs. Some of the proposed mechanisms are: decreased rate of catabolism [10]; lower binding affinity to the vitamin D binding protein (DBP) [10,11]; and distinct interaction with the vitamin D receptor (VDR), which favors recruitment of other activation factors such as the retinoid X receptor (RXR) and stabilization of the VDR complex by the ligand [12–15]. With respect to the observation of decreased rate of catabolism of $1\alpha,25(OH)_2$-20-epi-D_3 when compared to $1\alpha,25(OH)_2D_3$ [10], it has not been established whether the metabolic stability of $1\alpha,25(OH)_2$-20-epi-D_3 is at the level of the starting substrate or the intermediary metabolites formed during the course of the epimer's metabolism in the tissues. To address this issue and to evaluate the contribution of the intermediary metabolites to the full expression of the biological activities generated by $1\alpha,25(OH)_2$-20-epi-D_3, we embarked on our present study.

2. Materials and methods

2.1. Vitamin D compounds

Crystalline $1\alpha,25(OH)_2D_3$, $1\alpha,24(R),25(OH)_3D_3$ and $1\alpha,25(OH)_2D_3$-lactone were synthesized at Hoffmann-La Roche, Nutley, NJ. $1\alpha,25(OH)_2$-20-epi-D_3, $1\alpha,24(R),25(OH)_3$-20-epi-D_3 and $1\alpha,25(OH)_2$-24-oxo-20-epi-D_3 were synthesized at Leo Pharmaceutical Products, Ballerup, Denmark. All of the natural metabolites of $1\alpha,25(OH)_2D_3$, namely, $1\alpha,25(OH)_2$-24-oxo-D_3, $1\alpha,23(S),25(OH)_3$-24-oxo-D_3 and $1\alpha,23(OH)_2$-24,25,26,27-tetranor-D_3, were produced using the isolated rat kidney perfusion system as described before [16,17].

2.2. Kidney perfusion technique

Kidney perfusions were performed as described before in detail [16,17]. Male Sprague–Dawley rats weighing approximately 350–375 g were pretreated intraperitoneally with 2 μg of $1\alpha,25(OH)_2D_3$ at 16 and 4 h prior to surgery, in order to increase the activity of the 24-hydroxylase enzyme. After the rats were anesthetized with an intraperitoneal injection of Ketamine (100 mg/kg), the right kidneys were surgically removed and attached to individual kidney perfusion systems. The kidneys were perfused with 100 ml of perfusate and the metabolism studies were initiated after an equilibration period of 10 min.

2.3. Lipid extraction procedure

Lipid extraction of the perfusate samples were performed according to the procedure previously described [16,17]. The efficiency of the lipid extraction procedure was assessed by the recovery of 5 μg of $24(R),25(OH)_2D_3$, which was added as an internal standard to each perfusate sample, prior to the extraction procedure.

2.4. High performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) analysis

HPLC analysis of the lipid extracts of the kidney perfusate samples was performed with a Waters System Controller (model 600E), equipped with a photodiode array detector (model PDA 990), to monitor UV absorbing material at 265 nm (Waters Associates, Milford, MA). Isolation and identification of various vitamin D metabolites from the lipid extract of the perfusate samples was obtained using four different straight phase HPLC systems. All of the four HPLC systems used the same Zorbax-SIL column (4.6×250 mm), eluted with four different solvent mix-

tures, at a flow rate of 2 mL/min. The solvent mixtures used were: HPLC system #1, isopropanol:hexane (10:90 vol/vol); HPLC system #2, isopropanol:hexane (6:94 vol/vol); HPLC system #3, isopropanol:methylene chloride (4:96 vol/vol); and, HPLC system #4, isopropanol:hexane (12:88 vol/vol).

GC/MS analysis of the major metabolites of the analog, 1α,25(OH)$_2$-20-epi-D$_3$ were performed using a Hewlett-Packard GC-MSD system comprised of a 5890 Series II gas chromatograph, a 5791A mass selective detector, and a 7673 GC autosampler. The parent 1α,25(OH)$_2$-20-epi-D$_3$ and its metabolites were redissolved in acetonitrile and derivatized with the trimethylsilylating reagent Power SIL-Prep (Alltech Associates, Inc., Deerfield, IL) for 15 min at 70°C. Samples were analyzed on an HP-5 capillary column (30 m × 0.25 mm × 0.25 μm) with a temperature program ranging from 150 to 300°C (10°C/min ramp). Full-scan electron impact spectra (m/z 50 to m/z 650) were acquired for each experiment.

2.5. Metabolism studies

The metabolism of the hormone, 1α,25(OH)$_2$D$_3$, was compared to that of its C-20 epimer, 1α,25(OH)$_2$-20-epi-D$_3$ in rat kidney using the isolated perfused kidney system. In every experiment, the hormone or its C-20 epimer were added to individual kidney perfusions, which were performed simultaneously in two separate perfusion systems. Comparative metabolism studies were performed using 5 and 1 μM substrate concentrations. The kidney perfusions using 5 μM substrate concentrations were performed by perfusing each kidney for a period of 8 h with 100 mL of perfusate containing 208 μg of either 1α,25(OH)$_2$D$_3$ or 1α,25(OH)$_2$-20-epi-D$_3$. The lipid extracts from 10 mL of the final perfusate samples were then subjected to HPLC analysis using HPLC systems #1 and 2.

The kidney perfusions using 1 μM substrate concentrations were performed for a period of 2 h in the absence ($n = 3$) or presence ($n = 4$) of rat kidneys. Each perfusion was performed with 100 mL of perfusate containing 41.6 μg of either 1α,25(OH)$_2$D$_3$ or 1α,25(OH)$_2$-20-epi-D$_3$. The 5 mL perfusate samples obtained at 10 min, 0.5, 1, 1.5 and 2 h, were used to determine the rate of disappearance of each starting substrate; and 25 ml of the final perfusates were used to quantitate and compare the metabolites that were produced from each compound at the end of the 2 h perfusion period. The lipid extracts of perfusate samples were subjected to HPLC analysis using HPLC system #2.

2.6. Ligand-induced sensitivity of VDR to trypsin

The conformational changes of VDR that were induced by the various vitamin D compounds were determined by using a quantitative protease sensitivity assay as previously described [18]. Synthetic human VDR labeled with [^{35}S]-methionine (1000 Ci/mmol) were prepared by in vitro coupled transcription/translation in reticulocyte lysates (Promega Corp.) with the human VDR cDNA inserted into the pGEM4 plasmid. The receptor preparations were incubated without or with the indicated concentrations of vitamin D compounds for 10 min at room temperature. Then, 20 μg/ml of trypsin (Sigma Chemical Co., St. Louis, MO) was added to the mixtures and they were incubated for an additional 10 min. The digestion products were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and the dried gels were visualized by autoradiography.

2.7. Transcription assays

Rat osteosarcoma ROS 17/2.8 cells were plated in 35 mm dishes at a density of 3×10^5/dish in Dulbeccos's Modified Eagle's Media (DMEM) (Life Technologies, Grand Island, NY) and 10% fetal calf serum (FCS) (Hyclone, Logan, UT). Forty-eight hours later, the cells were transfected with plasmid (2 μg/dish) containing the vitamin D response element (VDRE) from the human osteocalcin gene (GGTGACTCACCGGGTGAACGGGGGCATT) [19]. This response element was inserted upstream of the thymidine kinase promoter/growth hormone fusion gene. All transfections were performed by the DEAE dextran method [20]. The cells were treated for 1 min with 10% dimethyl sulfoxide, washed three times with phosphate buffered saline and DMEM was added containing 10% FCS and the vitamin D compound of interest at the indicated concentrations. Forty-eight hours later, medium samples were collected and growth hormone production from the reporter gene was measured by radioimmunoassay as described by the manufacturer (Nichols Institute, San Juan Capistrano, CA).

2.8. Statistics

Values were calculated as mean ± standard deviation (S.D.). Significance levels were determined by Student's t-test.

3. Results

3.1. Comparative metabolism studies between 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-20-epi-D$_3$ in kidney

The metabolism of 1α,25(OH)$_2$D$_3$ was compared to that of 1α,25(OH)$_2$-20-epi-D$_3$ in rat kidney, at two

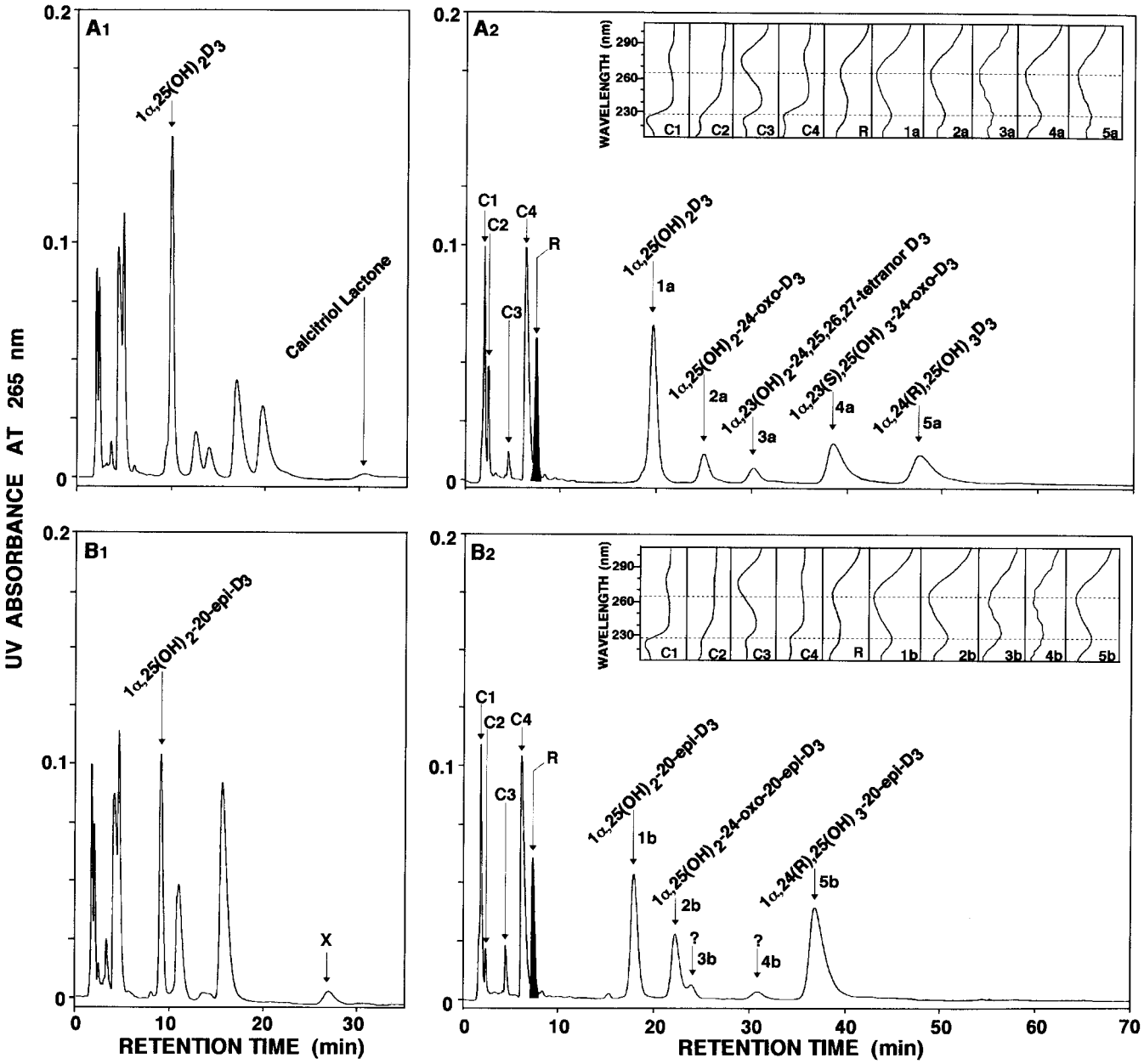

Fig. 2. Comparative metabolism study between 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-20-epi-D$_3$ using isolated rat kidneys that were perfused for 8 h with 5 μM substrate concentrations. (A$_1$) HPLC profile of 1α,25(OH)$_2$D$_3$ and its various metabolites, which were analyzed using a Zorbax-SIL column eluted with 10% isopropanol in hexane (HPLC system #1). (B$_1$) HPLC profile of 1α,25(OH)$_2$-20-epi-D$_3$ and its various metabolites, which were analyzed using HPLC system #1; peak X is the putative 1α,25(OH)$_2$-20-epi-D$_3$ lactone. (A$_2$) HPLC profile and UV spectra of 1α,25(OH)$_2$D$_3$ and its various metabolites, which eluted between 0 and 25 min in A$_1$, were collected and reanalyzed using a Zorbax-SIL column eluted with 6% isopropanol in hexane (HPLC system #2). (B$_2$) HPLC profile and UV spectra of 1α,25(OH)$_2$-20-epi-D$_3$ and its various metabolites, which eluted between 0 and 25 min in B$_1$, were collected and reanalyzed using HPLC system #2; the identity of peaks 3b and 4b was not established. (A$_2$) and (B$_2$) (C$_1$–C$_4$) are lipid contaminants, R is 24(*R*),25(OH)$_2$D$_3$ which was added as internal standard.

different substrate concentrations using the isolated perfused rat kidney system.

3.1.1. Kidney perfusions using 5 μM substrate concentration

In order to describe the pathways of metabolism of both 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-20-epi-D$_3$, and to obtain the various intermediary metabolites in amounts sufficient for their structure identification, two kidney perfusions were performed using 5 μM concentration of either 1α,25(OH)$_2$D$_3$ or 1α,25(OH)$_2$-20-epi-D$_3$. The HPLC profiles of the starting substrate and the metabolites of 1α,25(OH)$_2$D$_3$ present in 10 ml of final perfusate are shown in Fig. 2(A$_1$ and A$_2$), and

those of 1α,25(OH)$_2$-20-epi-D$_3$ are shown in Fig. 2B$_1$ and B$_2$. HPLC system #1 was first used to identify all of the major intermediary metabolites of 1α,25(OH)$_2$D$_3$ (Fig. 2A$_1$) and of 1α,25(OH)$_2$-20-epi-D$_3$ (Fig.2B$_1$) that were produced through both the C-24 and the C-23 oxidation pathways. (For the details of both the C-24 and the C-23 oxidation pathways, we refer the reader to our previous publication [21]). The starting parent compound along with all its major metabolites eluted between 8 and 25 min in the case of both 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-20-epi-D$_3$. It is obvious from the chromatogram, that 1α,25(OH)$_2$D$_3$ (retention time (r.t.) 10 min, Fig. 2A$_1$) was metabolized by the rat kidney into four major metabolites. There was only one highly polar, minor metabolite peak eluting at 30.3 min and was designated as 1α,25(OH)$_2$D$_3$-26,23-lactone (calcitriol lactone), based on its comigration with the synthetic standard. On the contrary, 1α,25(OH)$_2$-20-epi-D$_3$ (r.t. 9.3 min, Fig. 2B$_1$) was metabolized into only two major metabolites and a minor, highly polar metabolite eluting at 27 min (metabolite X). We tentatively designated metabolite X as the putative 1α,25(OH)$_2$-20-epi-D$_3$ lactone, based on its chromatographic mobility compared with the corresponding metabolite of the hormone. However, definite structure identification of metabolite X was not possible because of limited amounts of purified metabolite available for mass spectrometry.

In order to obtain a better separation of the substrates and the corresponding major metabolite peaks of both 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-20-epi-D$_3$ of the first HPLC runs, the pooled fractions eluting between 0 and 25 min were reanalyzed using HPLC system #2. The corresponding HPLC profiles and UV spectra are presented in Fig. 2 panels A$_2$ and B$_2$. The HPLC profile of the hormone and its metabolites obtained from the second HPLC run confirmed that there were four major metabolites (2a–5a, Fig. 2A$_2$). The metabolites from the second HPLC run were collected individually and were further purified using HPLC system #3. On this HPLC system the metabolite peaks from the second HPLC run remained as single peaks (data not shown). Finally, the individual metabolites obtained from the third HPLC run were purified further using HPLC system #4. The major metabolite peaks of 1α,25(OH)$_2$D$_3$ (2a–5a, Fig. 2A$_2$) were identified by comigration with authentic standards using HPLC systems #1 and #3. The HPLC profile of 1α,25(OH)$_2$-20-epi-D$_3$ and its metabolites obtained from the second HPLC system (Fig. 2B$_2$) revealed that there were two major (2b and 5b) and two minor metabolites (3b and 4b). Metabolites 2b and 5b were further purified using HPLC systems #3 and #4, and were tentatively identified by comigration with synthetic standards as 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$ and 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$, respectively.

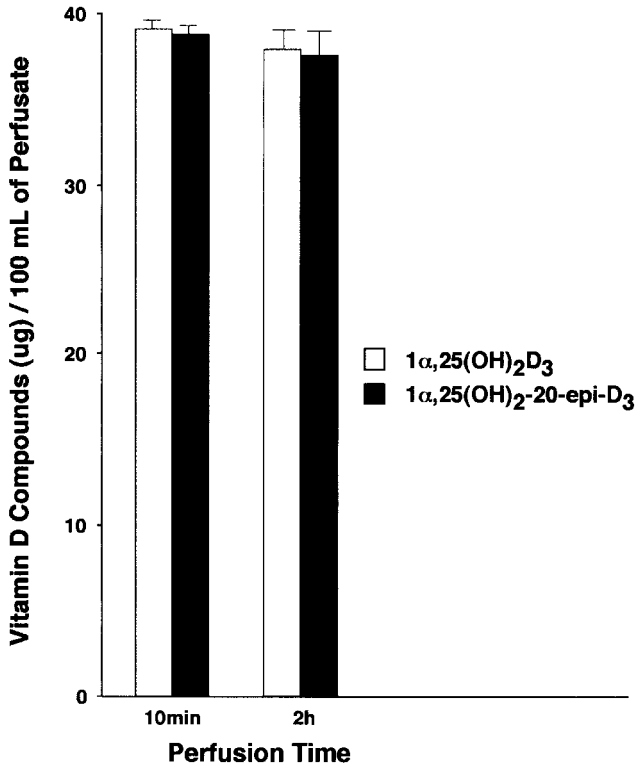

Fig. 3. Stability of 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-20-epi-D$_3$ in the control perfusions ($n = 3$) that were performed without kidneys for a period of 2 h with 1 μM substrate concentrations.

Mass spectrometric analysis of metabolites 2b and 5b of 1α,25(OH)$_2$-20-epi-D$_3$ confirmed their structure as 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$ and 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$, respectively (data not shown). We tentatively designated peak 3b as the putative 1α,23(OH)$_2$-24,25,26,27-tetranor-20-epi-D$_3$ and peak 4b as the putative 1α,23,25(OH)$_3$-24-oxo-20-epi-D$_3$ based on the chromatographic mobility compared with the corresponding natural intermediary metabolites of the hormone. However, definite structure identification of metabolites 3b and 4b was not possible because of limited amounts of the material available for mass spectrometry analysis.

Our results indicate of differences in the pattern of metabolism of both 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-20-epi-D$_3$, even though both compounds are metabolized via the same C-24 oxidation pathway. It is significant to note that at the end of the 8 h perfusions, the amount of unmetabolized 1α,25(OH)$_2$D$_3$ concentration is slightly greater than that of the C-20 epimer. Furthermore, even though 1α,25(OH)$_2$-20-epi-D$_3$ is metabolized into four metabolites as does 1α,25(OH)$_2$D$_3$, the amounts of the intermediary metabolites produced from both compounds differ from each other. It is noted that the amounts of 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$ and 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$ present in the perfusate were, respectively,

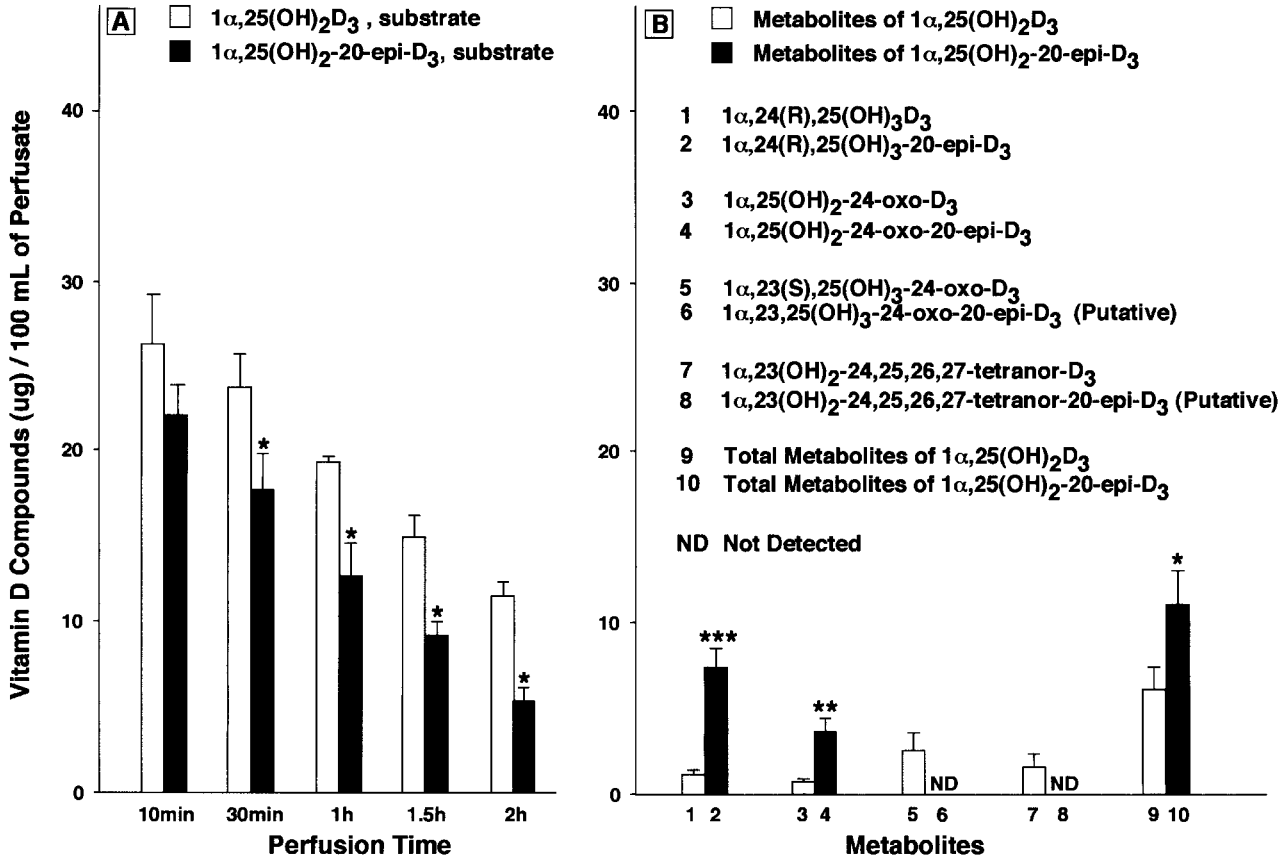

Fig. 4. Comparative metabolism studies between $1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2$-20-epi-D_3 using isolated rat kidneys ($n = 4$) that were perfused for 2 h with 1 μM substrate concentrations. (A) Remaining unmetabolized substrate [$1\alpha,25(OH)_2D_3$ (open bars) or $1\alpha,25(OH)_2$-20-epi-D_3(closed bars)] in the perfusate at different time points during a 2 h perfusion period. (B) Concentrations of the various individual metabolites (#1 to 8) and the total lipid soluble metabolites (#9–10) in the final perfusate. Values are represented as mean ± S.D.; $^{*}p < 0.03$; $^{**}p < 0.005$; $^{***}p < 0.0005$.

2.3 and 3.5 times greater than those of the corresponding intermediary metabolites of $1\alpha,25(OH)_2D_3$.

3.1.2. Kidney perfusions using 1 μM substrate concentration

In order to further validate the results obtained from the kidney perfusions using 5 μM substrate concentrations, the kidney perfusions were also performed using 1 μM concentration of either $1\alpha,25(OH)_2D_3$ or $1\alpha,25(OH)_2$-20-epi-D_3. Control perfusion experiments without kidneys ($n = 3$) were first performed with each compound to assess the loss of substrate in the perfusion system. The results indicate that the loss of substrate in the perfusion system without kidneys was insignificant (Fig. 3). We then performed perfusion experiments with kidneys ($n = 4$) using each compound as substrates to determine their rate of disappearance over a 2 h period. The results indicate that the concentration of unmetabolized $1\alpha,25(OH)_2$-20-epi-D_3 was significantly lower than that of $1\alpha,25(OH)_2D_3$ ($p < 0.03$) at the 0.5, 1, 1.5 and 2 h time points. At the end of the 2 h perfusion period the amount of remaining unmetabolized $1\alpha,25(OH)_2$-20-epi-D_3 substrate was approximately half the amount of unmetabolized $1\alpha,25(OH)_2D_3$ (Fig. 4A). Utilizing the amount of unmetabolized substrate at each time point during the 2 h time course studies, the mean half-lives of $1\alpha,25(OH)_2$-20-epi-D_3 and $1\alpha,25(OH)_2D_3$ were calculated to be 1.0 ± 0.04 h and 1.6 ± 0.18 h, respectively. Thus, it can be concluded that the rate of disappearance of $1\alpha,25(OH)_2$-20-epi-D_3 in the isolated perfused rat kidney is faster than that of $1\alpha,25(OH)_2D_3$.

We then measured the concentration of the intermediary metabolites that were produced during the 2 h perfusion period by analyzing 25 mL of the final perfusate from each experiment. The concentration of the major metabolites of both $1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2$-20-epi-D_3 (indicated in the bar graph as #1 to 8) as well as the total sum of the lipid soluble metabolites (#9 and 10) are shown in Fig. 4B. As it was observed in the experiments using 5 μM substrate concentration, $1\alpha,24(R),25(OH)_3$-20-epi-D_3 (#2) and $1\alpha,25(OH)_2$-24-oxo-20-epi-D_3 (#4) were the major metabolites derived from $1\alpha,25(OH)_2$-20-epi-D_3. Their

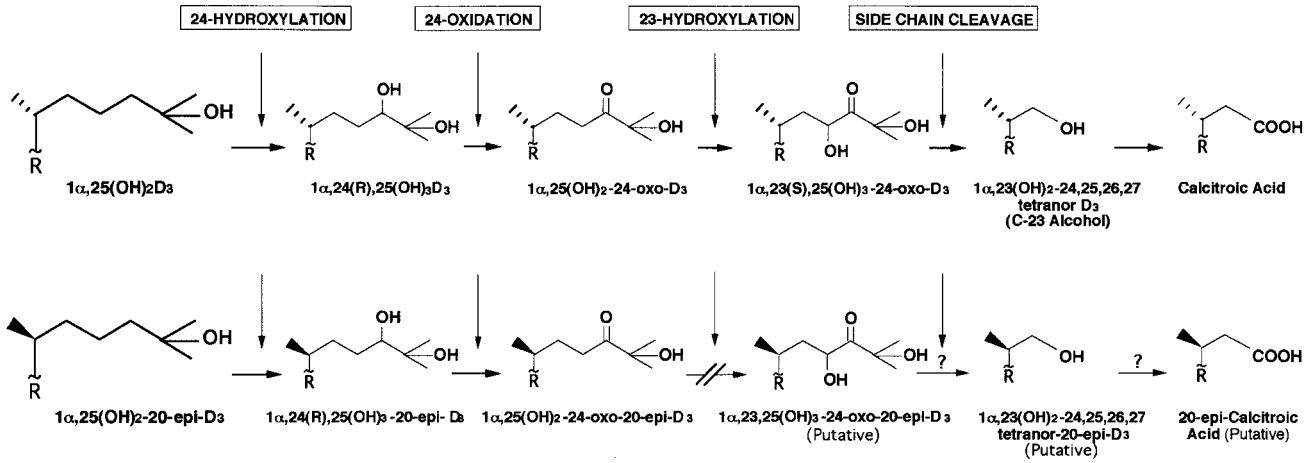

Fig. 5. The established C-24 oxidation pathway of $1\alpha,25(OH)_2D_3$ (top) and the putative C-24 oxidation pathway of $1\alpha,25(OH)_2$-20-epi-D_3 (bottom). The side chain structures of the parent compounds and the metabolites are shown; R corresponds to the rest of the vitamin D molecule, which is shown in Fig. 1. The broken arrow indicates the block in the conversion of $1\alpha,25(OH)_2$-24-oxo-20-epi-D_3 into $1\alpha,23,25(OH)_3$-24-oxo-20-epi-D_3.

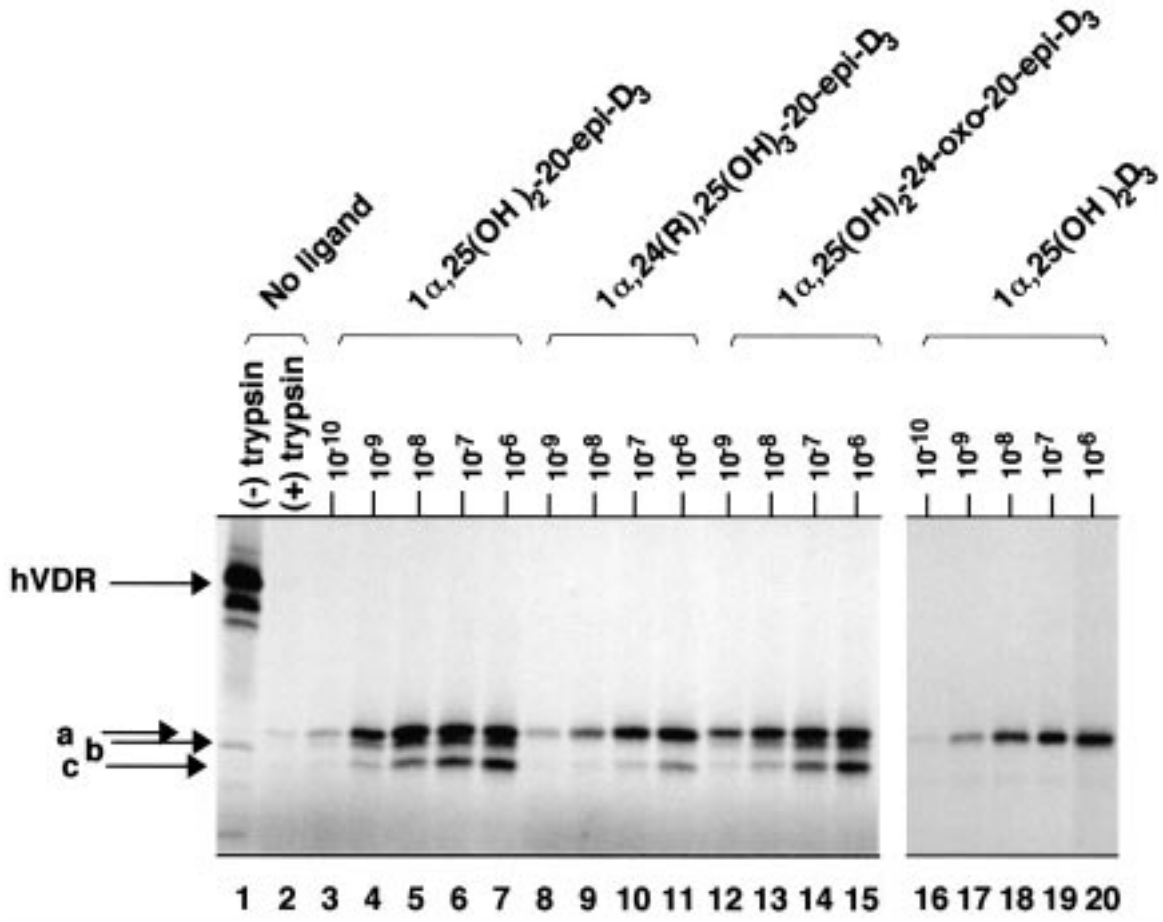

Fig. 6. Ligand-induced sensitivity of VDR to trypsin. In vitro translated VDR labeled with [^{35}S]-methionine was incubated without or with the indicated concentrations of vitamin D compounds for 10 min before digestion with 20 μg/mL of trypsin. The digestion products were analyzed by SDS-polyacrylamide gel electrophoresis and the dried gels were visualized by autoradiography. The proteolytic products are indicated by arrows (a–c). The fragment sizes are: (a) 34 kDa; (b) 32 kDa; (c) 28 kDa.

concentrations, when compared to the corresponding metabolites of 1α,25(OH)$_2$D$_3$ were significantly higher ($p < 0.0005$ and $p < 0.005$, respectively). It is of interest to note that the metabolites of 1α,25(OH)$_2$-20-epi-D$_3$ (#6 and 8) were not detected unlike 1α,23(*S*),25(OH)$_3$-24-oxo-D$_3$ (#5) and 1α,23(OH)$_2$-24,25,26,27-tetranor-D$_3$ (#7), the corresponding metabolites derived from 1α,25(OH)$_2$D$_3$, which accumulated in significant amounts. The sum of the two major metabolites of 1α,25(OH)$_2$-20-epi-D$_3$ (indicated in the bar graph as #10; Fig. 4B) is significantly greater than the sum of the four metabolites of 1α,25(OH)$_2$D$_3$ (#9) ($p < 0.03$). Also, it is noted that within 2 h, the concentration of the 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$ metabolite exceeded the concentration of the remaining unmetabolized 1α,25(OH)$_2$-20-epi-D$_3$ substrate. Thus, the kidney perfusions with 1 μM substrate concentration definitely establish that 1α,25(OH)$_2$-20-epi-D$_3$ itself is not metabolically stable; but rather, the C-20 epimer is metabolized into relatively stable intermediary metabolites, namely, 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$ and its precursor 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$.

Our comparative metabolism studies between 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-20-epi-D$_3$ in the isolated perfused rat kidney using both 5 and 1 μM substrate concentrations indicate that, like the natural hormone, the analog 1α,25(OH)$_2$-20-epi-D$_3$ is also metabolized through the same C-24 oxidation pathway (Fig. 5). 1α,25(OH)$_2$-20-epi-D$_3$ is first metabolized into 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$, which is then further metabolized into 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$. However, a partial block in the conversion of 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$ into 1α,23,25(OH)$_3$-24-oxo-20-epi-D$_3$ is identified, as very little or none of the putative 1α,23,25(OH)$_3$-24-oxo-20-epi-D$_3$ and its subsequent metabolite, 1α,23(OH)$_2$-24,25,26,27-tetranor-20-epi-D$_3$ are detected in the perfusate. Thus, this partial metabolic block in the C-24 oxidation pathway of the epimer, leads to the accumulation of 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$ and its precursor 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$.

3.1.3. Ligand-induced changes in receptor conformation

It has been proposed that the enhanced activities of 1α,25(OH)$_2$-20-epi-D$_3$ in growth inhibition and transcription assays, are partly due to the mode of interaction of the epimer with VDR. 1α,25(OH)$_2$-20-epi-D$_3$ induces a distinct conformation of VDR, which favors VDR-RXR dimerization and this results in enhanced transcriptional activity of the C-20 epimer [12]. In order to determine whether the metabolites of 1α,25(OH)$_2$-20-epi-D$_3$ interact with VDR in the same manner as the parent compound, we examined their interaction with VDR using a quantitative protease sensitivity assay. Fig. 6 shows the 50 kDa VDR labeled with [^{35}S]-methionine under control conditions

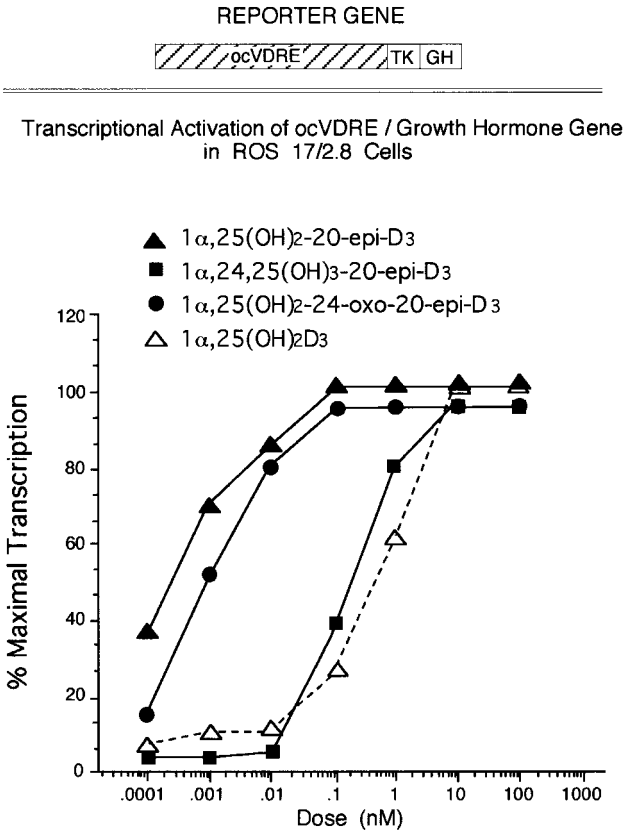

Fig. 7. Transcriptional activity of vitamin D compounds. ROS 17/2.8 cells were transfected by the DEAE-dextran method with a thymidine kinase-growth hormone (TK/GH) fusion gene containing the osteocalcin VDRE (ocVDRE). Immediately after transfection, medium containing 10% FCS and the respective vitamin D compounds were added to the cultures. Forty-eight hours later, medium samples were collected and growth hormone levels were determined by radioimmunoassay. Each point of the dose-response curve is the average of duplicate transfections.

(no ligand and no trypsin; lane 1), and the proteolytic fragments obtained after the ligand treated VDR was subjected to trypsin digestion (lanes 2–20). When the VDR was treated with trypsin in the absence of ligand, complete digestion of the VDR protein occurred (lane 2). However, when the VDR was incubated with 1α,25(OH)$_2$-20-epi-D$_3$ for 10 min before exposure to trypsin, three distinct proteolytic products of 34, 32 and 28 kDa were observed (Fragments a, b and c; respectively; Fig. 6 lanes 3–7). Incubation of the VDR with the metabolite 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$ yielded primarily fragment a when the VDR-ligand complex was digested with trypsin (lanes 8–11), fragment b was not detected at any concentration of this metabolite, and fragment c was detected only at high ligand concentration (lanes 10–11). When the VDR was incubated with the subsequent metabolite, 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$, the proteolytic products

were the same as those produced by the parent compound (fragments a, b and c; lanes 12–15). We also examined the mode of interaction of the natural hormone, 1α,25(OH)$_2$D$_3$, with VDR. Treatment of VDR with 1α,25(OH)$_2$D$_3$ produced only a 34 kDa fragment (fragment a; lanes 17–20), as was previously reported [12]. These results confirm the previous finding by Peleg et al. [12], which indicated that the 1α,25(OH)$_2$-20-epi-D$_3$ analog induces a conformation of VDR that is distinct from that produced by 1α,25(OH)$_2$D$_3$. Furthermore, we have shown that addition of a hydroxyl group to C-24 on the side chain of the 1α,25(OH)$_2$-20-epi-D$_3$ analog changed the mode of interaction of the analog with VDR, quantitatively and qualitatively. However, further oxidation of the metabolite into its 24-oxo metabolite restored the conformation of the VDR to the same pattern that was induced by the parent compound, 1α,25(OH)$_2$-20-epi-D$_3$. Thus, our study shows for the first time, how the modifications produced during the metabolism of 1α,25(OH)$_2$-20-epi-D$_3$ in target tissues can result in the production of stable intermediary metabolites whose interaction with VDR can be similar or different from that of the parent compound.

3.1.4. Transcriptional activities

The vitamin D receptor-mediated transcriptional activities of 1α,25(OH)$_2$-20-epi-D$_3$, and its intermediary metabolites were determined by their ability to induce the expression of a reporter gene (growth hormone production) in ROS 17/2.8 cells, which had been transfected with an osteocalcin VDRE/growth hormone gene construct. Fig. 7 shows that this vitamin D receptor-mediated transcriptional activity was dose-dependent for the natural hormone as well as for the C-20 epimer and its intermediary metabolites. The ED$_{50}$ for transcriptional activity of 1α,25(OH)$_2$D$_3$ was 5×10^{-10} M. The ED$_{50}$ for transcriptional activities of 1α,25(OH)$_2$-20-epi-D$_3$ and its metabolites, 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$ and 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$, were 9×10^{-13}, 1×10^{-10} and 2×10^{-12} M, respectively. Thus, the C-20 epimer and its 24-oxo metabolite were significantly more potent inducers of VDR-mediated transcription than the natural hormone. It is of interest to note that C-24 hydroxylation of the C-20 epimer reduced its biological activity. Nevertheless, the remaining biological activity of 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$ was still significant as it was similar to or slightly higher than that of 1α,25(OH)$_2$D$_3$. Thus, the oxidation of the 24-hydroxyl group to the 24-oxo group not only enables the metabolite to interact with VDR in a similar manner as the parent compound, 1α,25(OH)$_2$-20-epi-D$_3$, but also restores the transcriptional activity to a level similar to that of the parent compound.

4. Discussion

In our present study, we provide evidence to indicate that 1α,25(OH)$_2$-20-epi-D$_3$ is metabolized in the isolated perfused rat kidney into stable, bioactive intermediary metabolites. The findings of our study indicate that the C-23 hydroxylation of 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$ is hindered, resulting in reduced or no production of 1α,23,25(OH)$_3$-24-oxo-20-epi-D$_3$. Therefore, 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$ and its precursor 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$ accumulated in the kidney perfusate. Similar findings were also reported in a previous study in which the metabolism of 1α,25(OH)$_2$-20-epi-D$_3$ was investigated in human keratinocytes (HPK1A-ras cells) [10]. In the same study, the authors addressed the issue of the metabolic stability of 1α,25(OH)$_2$-20-epi-D$_3$ in an indirect way, by assessing the ability of the cold substrates of both 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-20-epi-D$_3$ to compete with [1β-^{3}H]-1α,25(OH)$_2$D$_3$ for the 24-hydroxylase enzyme (CYP-24) in UMR 106 cells. The results indicated that cold 1α,25(OH)$_2$-20-epi-D$_3$, when compared to cold 1α,25(OH)$_2$D$_3$ was 36 times less effective in competing for the CYP-24 [10]. Thus, the finding of the ineffectiveness of 1α,25(OH)$_2$-20-epi-D$_3$ to compete for the CYP-24 provided only indirect evidence to suggest that 1α,25(OH)$_2$-20-epi-D$_3$ is metabolically stable. From our comparative metabolism studies between 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-20-epi-D$_3$ in the isolated perfused rat kidney, we were able to provide direct evidence to indicate that the metabolic stability of 1α,25(OH)$_2$-20-epi-D$_3$ is not at the level of the starting substrate, but is at the level of its intermediary metabolites, namely, 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$ and 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$.

The biological activity studies of the two stable intermediary metabolites indicated that 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$, unlike 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$, induced the VDR conformation that is similar to the one produced by the parent 1α,25(OH)$_2$-20-epi-D$_3$, and this phenomenon allowed 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$ to exhibit similar potency in inducing transactivation as its parent. Also, it has been reported previously that the potency of the metabolite 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$ is similar to that of its parent compound in regulating growth and differentiation of several cancer cells [22]. Metabolism of 1α,25(OH)$_2$-20-epi-D$_3$ into a stable intermediary metabolite, which retains significant biological activity, in contrast to the rapid further metabolism of 1α,25(OH)$_2$-24-oxo-D$_3$, the corresponding intermediary metabolite of 1α,25(OH)$_2$D$_3$, thus provides an important explanation for the enhanced cellular and VDR-mediated transcriptional activities of 1α,25(OH)$_2$-20-epi-D$_3$ as compared with 1α,25(OH)$_2$D$_3$. Unlike 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$, its precursor, 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$ interacts

with VDR in a different mode, resulting in a conformation of the VDR-ligand complex which is different from that induced by 1α,25(OH)$_2$-20-epi-D$_3$. This change in the VDR conformation induced by 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$, was associated with a decrease in its biological activity. Thus, even though 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$, when compared to its parent 1α,25(OH)$_2$-20-epi-D$_3$, is biologically less active, it gains importance as a significant contributor for the enhanced biological activity of its parent compound, because of its potential to generate significant biological activity through its metabolism into 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$. The concept that some vitamin D$_3$ analogs, when compared to 1α,25(OH)$_2$D$_3$, are able to produce enhanced biological actions through their metabolism into stable, biologically active intermediary metabolites was put forward not only by us investigating the metabolism of the analog 1α,25(OH)$_2$-16-ene-D$_3$ [5], but also by others investigating the metabolism of the analog 26,26,26,27,27,27-hexafluoro-1α,25(OH)$_2$D$_3$ [23–25] and the analog KH-1060 [26]. We have now provided additional proof for the same concept through our present study.

In summary, we have shown that the metabolic stability of 1α,25(OH)$_2$-20-epi-D$_3$ is not at the starting substrate level, but 1α,25(OH)$_2$-20-epi-D$_3$ gains its metabolic stability through its conversion into stable intermediary metabolites, namely, 1α,25(OH)$_2$-24-oxo-20-epi-D$_3$ and its precursor, 1α,24(*R*),25(OH)$_3$-20-epi-D$_3$. These intermediary metabolites together, due to their metabolic stability and mode of interaction with VDR, contribute significantly to the enhanced expression of the biological activities attributed to their parent 1α,25(OH)$_2$-20-epi-D$_3$.

Acknowledgements

This work was supported by a grant from Hoffmann-La Roche, Inc., Nutley, NJ, to G.S.R. and National Institutes of Health Grant DK-50583 to S.P.

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