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Isolation and Identification of 1α-Hydroxy-3-epi-Vitamin D_3, a Potent Suppressor of Parathyroid Hormone Secretion

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Abstract Since our original demonstration of the metabolism of 1α,25$(OH)_2D_3$ into 1α,25$(OH)_2$-3-epi-D_3 in human keratinocytes, there have been several reports indicating that epimerization of the 3 hydroxyl group of vitamin D compounds is a common metabolic process. Recent studies reported the metabolism of 25OHD_3 and 24(*R*),25$(OH)_2D_3$ into their respective C-3 epimers, indicating that the presence of 1α hydroxyl group is not necessary for the 3-epimerization of vitamin D compounds. To determine whether the presence of a 25 hydroxyl group is required for 3-epimerization of vitamin D compounds, we investigated the metabolism of 1αOHD_3, a non-25 hydroxylated vitamin D compound, in rat osteosarcoma cells (ROS 17/2.8). We noted metabolism of 1αOHD_3 into a less polar metabolite which was unequivocally identified as 1αOH-3-epi-D_3 using the techniques of HPLC, GC/MS, and ^{1}H-NMR analysis. We also identified 1αOH-3-epi-D_3 as a circulating metabolite in rats treated with pharmacological concentrations of 1αOHD_3. Thus, these results indicated that the presence of a 25 hydroxyl group is not required for 3-epimerization of vitamin D compounds. Furthermore, the results from the same studies also provided evidence to indicate that 1αOH-3-epi-D_3, like 1αOHD_3, is hydroxylated at C-25. We then evaluated the biological activities of 1αOH-3-epi-D_3. Treatment of normal rats every other day for 7 days with 2.5 nmol/kg of 1αOH-3-epi-D_3 did not raise serum calcium, while the same dose of 1αOHD_3 increased serum calcium by 3.39 ± 0.52 mg/dl. Interestingly, in the same rats which received 1αOH-3-epi-D_3 we also noted a reduction in circulating PTH levels by $65 \pm 7\%$. This ability of 1αOH-3-epi-D_3 to suppress PTH levels in normal rats without altering serum calcium was further tested in rats with reduced renal function. The results indicated that the ED50 of 1αOH-3-epi-D_3 for suppression of PTH was only slightly higher than that of 1α,25$(OH)_2D_3$, but that the threshold dose of the development of hypercalcemia (total serum Ca >10.5 mg/dl) was nearly 80 times higher. These findings indicate that 1αOH-3-epi-D_3 is a highly selective vitamin D analog with tremendous potential for treatment of secondary hyperparathyroidism in chronic renal failure patients. J. Cell. Biochem. 96: 569–578, 2005. © 2005 Wiley-Liss, Inc.

Key words: metabolism; 1α,25$(OH)_2D_3$; C-3 epimerization pathway; 1αOHD_3; 1αOH-3-epi-D_3; 1α,25$(OH)_2$-3-epi-D_3; parathyroid hormone; chronic renal failure; secondary hyperparathyroidism

Abbreviations used: 25OHD_3, 25-hydroxyvitamin D_3; 24(*R*), 25$(OH)_2D_3$, 24(*R*),25 dihydroxyvitamin D_3;1α,25$(OH)_2D_3$, 1α, 25-dihydroxyvitamin D_3; 1α,24(*R*),25$(OH)_3D_3$, 1α,24(*R*), 25-tryhydroxyvitamin D_3; 1α,25$(OH)_2$-24-oxo-D_3, 1α,25-dihydroxy-24-oxovitamin D_3; 1α,23,(S)25$(OH)_3$-24-oxo-D_3, 1α,23(S),25-trihydroxy-24-oxovitamin D_3; 1α,23$(OH)_2$-24,25,26,27-tetranor D_3 or C-23 alcohol, 1α,23-dihydroxy-24,25,26,27-tetranorvitamin D_3; 1,25(*R*)-dihydroxyvitamin D_3-26,23 (*S*)-lactone, 1α,25$(OH)_2D_3$-lactone; 1α,25$(OH)_2$-3-epi-D_3, 1α,25-dihydroxy-3-epi-vitamin D_3;1αOH-3-epi-D_3, 1α-hydroxy-3-epi-vitamin D_3; 1α,25$(OH)_2$-16-ene-23-yne-D_3, 1α,25-dihydroxy-16-ene-23-yne-vitamin D_3; HPLC, high performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry.

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A decade ago, we reported for the first time that the secosteroid hormone, $1\alpha,25(OH)_2D_3$, is metabolized to $1\alpha,25(OH)_2$-3-epi-D_3 in human keratinocytes [Reddy et al., 1994]. Since this original report there have been several studies which confirmed the metabolism of $1\alpha,25(OH)_2D_3$ through this new pathway in several tissues including bovine parathyroid cells [Reddy et al., 1997; Bischof et al., 1998; Brown et al., 1999a; Sekimoto et al., 1999; Siu-Caldera et al., 1999; Astecker et al., 2000; Masuda et al., 2000]. This new metabolic pathway is now well accepted in the literature as the "C-3 epimerization pathway" [Reddy et al., 2001]. Following the discovery of $1\alpha,25(OH)_2D_3$ metabolism into its 3-epimer, we started investigating the metabolism of a wide variety of synthetic vitamin D analogs into their respective 3-epimers. During these studies, we began identifying how changes in the structure of vitamin D compounds can affect the rate of 3-epimerization process. For example, one of the well studied vitamin D analogs $1\alpha,25(OH)_2$-16-ene-23-yne-D_3 is converted into its 3 epimer much more slowly than $1\alpha,25(OH)_2D_3$. However, when the stereochemistry of 20 methyl group of the same vitamin D analog is changed into an unnatural orientation, a dramatic increase in the rate of its C3-epimerization is noted [Reddy et al., 2000]. This observation led us to perform more studies to identify other structural changes in the vitamin D molecule that will alter the rate of 3-epimerization. Recent studies reported the metabolism of $25OHD_3$ and $24(R),25(OH)_2D_3$ into their respective C-3 epimers [Kamao et al., 2001, 2004]. From these studies it became clear that the presence of 1α hydroxyl group is not necessary for the 3-epimerization of vitamin D compounds. In order to determine whether the presence of 25 hydroxyl group is a structural requirement for 3-epimerization of vitamin D compounds, we investigated the metabolism of $1\alpha OHD_3$, a non-25 hydroxylated vitamin D compound, in rat osteosarcoma cells (ROS 17/2.8) and intact normal rats. We identified 1αOH-3-epi-D_3 as a metabolite of $1\alpha OHD_3$ in both in vitro and in vivo experimental conditions. Thus, we provided direct evidence to indicate that the presence of 25 hydroxyl group is not required for 3 epimerization of vitamin D compounds. Furthermore, we also found that 1αOH-3-epi-D_3 is metabolized into $1\alpha,25(OH)_2$-3-epi-D_3 both in bone cells as well as in intact rats.

In our previous study we indicated that $1\alpha,25(OH)_2$-3-epi-D_3 is almost as potent as $1\alpha,25(OH)_2D_3$ in suppressing PTH secretion in cultured bovine parathyroid cells [Brown et al., 1999a]. As 1αOH-3-epi-D_3 is the precursor for $1\alpha,25(OH)_2$-3-epi-D_3, we evaluated the possible therapeutic potential for 1αOH-3-epi-D_3 in suppressing circulating PTH levels. We found that 1αOH-3-epi-D_3 had very low calcemic activity in rats but yet was able to suppress PTH levels at non-calcemic doses, indicating the potential of 1αOH-3-epi-D_3 for treatment of secondary hyperparathyroidism in patients with chronic kidney disease.

MATERIALS AND METHODS

Materials

ROS 17/2.8 cells were a kind gift from Dr. Sara Peleg (M.D. Anderson Cancer Center, Houston, TX). Streptomycin, penicillin, McCoy's, Dulbecco's modified Eagle's (DMEM), and Ham's F-12 media were obtained from Life Technologies (Gaithersburg, MD). Fetal calf serum (FCS) was purchased from Hyclone (Logan, UT). Tissue culture flasks and high performance liquid chromatography (HPLC) reagents were purchased from Baxter (McGaw Park, IL).

Vitamin D Compounds

$25OHD_3$, $1\alpha OHD_3$, 1αOH-3-epi-D_3, $1\alpha,25$-$(OH)_2D_3$, $1\alpha,25(OH)_2$-3-epi-D_3, $1\alpha,24R,25$-$(OH)_3D_3$, and $1\alpha,25(OH)_2D_3$-lactone were synthesized at Hoffmann-La Roche (Nutley, NJ). $1\alpha,25(OH)_2$-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 (C-23 alcohol) were biologically synthesized in the rat kidney perfusion system as previously described [Reddy and Tserng, 1989].

Cells and Cell Culture

Ros 17/2.8 cells were maintained in DMEM and Hams F-12 media (50/50, vol/vol) supplemented with 10% FCS and antibiotics [penicillin (100 IU/ml) and streptomycin (100 μg/ml)]. Cell culture medium was changed every 3–4 days. The cells were subcultured when approximately 80% confluent and were not subcultured beyond passage 5. For metabolism studies, 3×10^6 cells were seeded in T150 tissue culture bottles and grown to confluence. The incubations were carried out at 37°C in a humidified atmosphere under 5% CO_2.

High Performance Liquid Chromatography (HPLC), Gas Chromatography/Mass Spectrometry (GC/MS), and ^{1}H-Nuclear Magnetic Resonance (NMR) Analysis

HPLC analysis of the lipid extracts from the cells and media was performed with a Waters System Controller (Millennium 3.2) equipped with a photodiode array detector (Model PDA 996) to monitor the ultraviolet (UV) absorbing material at 265 nm. The vitamin D compounds-were isolated and purified using both straight and reverse phase HPLC systems. Analysis by straight phase HPLC utilized a Zorbax-SIL column (9×250 mm) (Dupont, Wilmington, DE) eluted with two different solvent mixtures at a flow rate of 2 ml/min. The solvent mixtures used were as follows: 10% isopropanol in hexane (HPLC system no.1) or 6% isopropanol in hexane (HPLC system no.2). Analysis by reverse phase HPLC system utilized a Zorbax-ODS column (4.5×250 mm) (Dupont) eluted with 10% water in methanol at a flow rate of 1 ml/ min (HPLC system no.3).

GC/MS analysis was performed using a Hewlett-Packard GC/MSD system, equipped with a series 5890 Series II chromatograph, a 5971 mass selective detector, and a 7673 GC autosampler (Agilent, Wilmington, DE). The vitamin D compounds were trimethylsilylated in 30 μl of a 1:1 mixture of acetonitrile and Power SIL-Prep (Alltech Associates, Deerfield, IL) and incubated at 70°C for 15 min. The trimethylsilyl ether derivatives were analyzed in quadruplicate. All analyses were done using a Hewlett-Packard 5-MS low-bleed capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm film thickness, 5% phenyl methyl silicone), using UHP helium as a carrier gas at a flow rate of 0.8 ml/min. The oven temperature program was as follows: 150°C for 6 min, then increased at 10°C/min until reaching a final temperature of 300°C which was held for 10 min. Ionization was performed by electron impact with positive ion detection. Full scan spectra across the mass range of m/z 50–650 were acquired in each run and the published spectra were averaged and background subtracted. The ^{1}H-NMR spectra were acquired on a Varian UNITYplus® 400-MHZ spectrometer. The samples were dissolved in deuterochloroform containing tetramethylsilane as an internal zero reference.

Metabolism Studies in ROS 17/2.8 Cells Using $1\alpha OHD_3$ and 1αOH-3-epi-D_3 as Substrates

The ROS 17/2.8 cells (3×10^6 cells/ml) were incubated with 1 and 10 μM concentrations of either $1\alpha OHD_3$ or 1αOH-3-epi-D_3 in 50 ml of medium containing 10% FCS. The incubations were stopped after 24 h with 10 ml of methanol and the lipids from both cells and media were extracted for HPLC analysis, using extraction procedure described earlier [Siu-Caldera et al., 1999]. Prior to lipid extraction, the cells and media were spiked with 5 μg of $25OHD_3$ which was used as an internal standard. The recovery of the internal standard was used to assess the extraction efficiencies of the various lipid-soluble vitamin D metabolites.

Control incubations without cells containing only media and the vitamin D compounds were performed. The control studies indicated that vitamin D compounds did not undergo any chemical change or breakdown either during the incubation period or during the extraction procedure (data not shown).

In Vivo Metabolism of $1\alpha OHD_3$ in Rats

Sprague–Dawley rats (250 g; Taconic laboratories, Germantown, NY) were adapted to laboratory conditions for at least 5 days; the rats were housed two per cage with free access to food and water in a regulated environment, with a 12 h light-dark cycle. For the in vivo metabolism study using $1\alpha OHD_3$ as substrate, we used four rats. Each rat was given a bolus dose of 500 μg of $1\alpha OHD_3$, intravenously. $1\alpha OHD_3$ was dissolved in ethanol and injected in vehicle consisting of 50% rat serum in normal saline solution. After 8 h, the blood was collected by catheterization of the aorta and immediately centrifuged. We obtained approximately 5 ml of serum from each rat. Lipids from the pooled serum were extracted and the vitamin D_3 metabolites were analyzed by HPLC. A control experiment was performed in a rat treated with vehicle solution alone to assure that no metabolites were detected in serum before the rats were administered $1\alpha OHD_3$ (data not shown).

In Vivo Activities of 1αOH-3-epi-D_3 Versus $1\alpha OHD_3$ in Normal Rats

Male Sprague–Dawley rats (250 g) were injected every other day with 1αOH-3-epi-D_3 or $1\alpha OHD_3$ at doses of 2.5 nmol/kg given intraperitoneally for one week (four injections).

Blood samples were taken from the tail vein under ether anesthesia immediately before the first injection and at 24 h after each injection. Plasma was analyzed for total calcium and PTH.

Efficacy of 1αOH-3-epi-D_3 versus 1α,25$(OH)_2D_3$ in Suppressing PTH in Uremic Rats

Female Sprague–Dawley rats (250 g) were subtotally (5/6) nephrectomized as described previously [Brown et al., 1999b; Ritter et al., 2002], and fed a diet containing 0.9% P and 0.6% Ca. Treatment with the vitamin D compounds began 1 month post-nephrectomy, when the rats had developed secondary hyperparathyroidism. A pretreatment blood sample was taken from each rat and analyzed for calcium, creatinine, and PTH. The rats were divided into two groups (n = 13) with comparable means and ranges of pretreatment creatinine and PTH levels. A dose escalation protocol was then initiated with one group receiving 1α,25$(OH)_2D_3$ starting at a dose of 0.01 nmol/kg and the other receiving 1αOH-3-epi-D_3 starting at a dose of 0.04 nmol/kg. The rats were injected intraperitoneally every other day for 8 days and a blood sample was taken 24 h after the fourth injection. The every-other-day dosing was continued with a dose doubling every eighth day and a blood sampling 24 h after the four injection of each dose level. The dose escalation continued until the mean serum calcium levels rose above 10.5 mg/dl. PTH levels were determined in each blood sample. All animal protocols were approved by the Animal Studies Committee at Washington University School of Medicine.

Blood Chemistries

Plasma calcium was measured by atomic absorption spectrometry (model 503, Perkin-Elmer Corp., Norwalk, CT). PTH levels were determined using an ELISA for intact rat PTH (Scantibodies, Santee, CA).

RESULTS

Metabolism of 1αOHD_3 in ROS 17/2.8 Cells

We first examined the metabolism of 1αOHD_3 in ROS 17/2.8 cells, which express the C-3 epimerization pathway but not the C-24 oxidation pathway. Figure 1 shows the HPLC profiles of the lipid extracts obtained when ROS 17/2.8 cells were incubated for 24 h with either 1 μM (panel A) or 10 μM (panel B) concentrations of 1αOHD_3. It can be seen that 1αOHD_3 (1 μM) was metabolized into a more polar metabolite (peak 2) and a less polar metabolite (peak A) in ROS 17/2.8 cells. Both of the metabolites possess the characteristic UV chromophore of vitamin D (λ_{max} 265, λ_{min} 228) (Fig. 1, panel A-inset). The polar metabolite (peak 2) co-migrated with the synthetic standard of 1α,25$(OH)_2D_3$ on both the straight and reverse phase HPLC systems and exhibited a mass spectrum identical synthetic standard 1α,25$(OH)_2D_3$ (data not shown). The conversion of 1αOHD_3 into 1α,25$(OH)_2D_3$ shows evidence for the hydroxylation of 1αOHD_3 at C-25 position. The less polar metabolite (peak A) co-migrated with the synthetic standard of 1αOH-3-epi-D_3 on both straight and reverse phase HPLC systems. Hence this metabolite was tentatively identified as 1αOH-3-epi-D_3, the C-3 epimer of 1αOHD_3. In order to

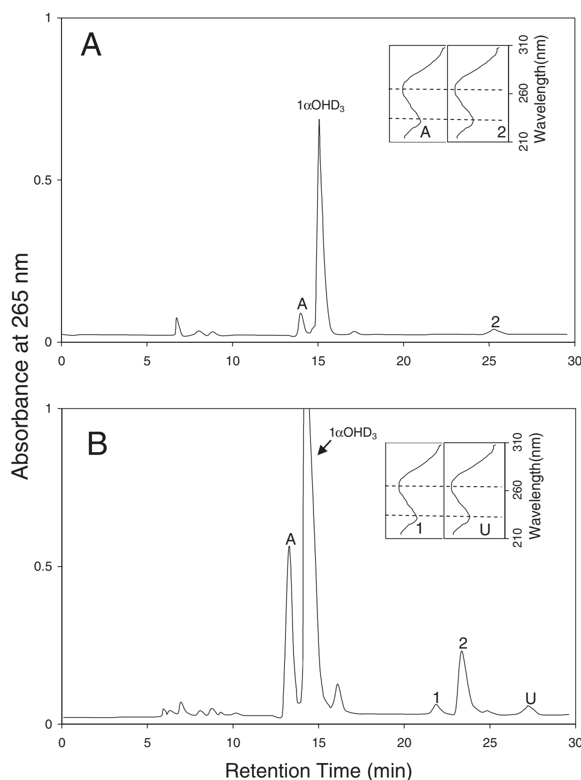

Fig. 1. HPLC profiles of 1αOHD_3 and its metabolites produced in ROS 17/2.8 cells incubated with 1 μM (**panel A**) or (10) μM (**panel B**) concentrations of the substrate for 24 h. HPLC was performed using a Zorbax-SIL (9 × 250 mm) column eluted with 10% isopropanol in hexane at a flow rate of 2 ml/min. Peaks A, 1, and 2 were identified as 1αOH-3-epi-D_3, 1α,25$(OH)_2$-3-epi-D_3, and 1α,25$(OH)_2D_3$ respectively. The identity of peak U, a minor metabolite of 1αOHD_3 was not established. The UV spectra of the metabolite peaks (A, 1, 2, and U) are shown in the inset.

produce an adequate amount of the metabolite A for more definitive structure identification, the ROS 17/2.8 cells were incubated with a higher concentration of 1αOHD_3 (10 μM) for 72 h. Metabolite A was purified using both straight and reverse phase HPLC systems as described in Materials and Methods, and the purified metabolite A was subjected to GC/MS and ^{1}H-NMR analysis for structure identification. It can be seen from Figure 1, panel B that at a higher concentration (10 μM), 1αOHD_3 was metabolized not only into 1αOH-3-epi-D_3 (peak A) and 1α,25$(OH)_2D_3$ (peak 2), but also into metabolite 1 and metabolite U. Metabolite 1 was identified as 1α,25$(OH)_2$-3-epi-D_3 by GC/MS analysis and its co-elution with the synthetic standard on both straight and reverse phase HPLC systems [data not shown as it was reported earlier [Reddy et al., 2001]]. This finding indicates that 1α,25$(OH)_2$-3-epi-D_3 may be produced either from hydroxylation of 1αOH-3-epi-D3 at C-25 position or C-3 epimerization of 1α,25$(OH)_2D_3$. The identity of the minor metabolite U was not established.

Identification of Metabolite A as 1αOH-3-epi-D_3 by GC/MS and ^{1}H-NMR

Figure 2 shows the identical mass spectral characteristics for the trimethylsilylated 1αOH-3-epi-D_3 synthetic standard (lower panel)

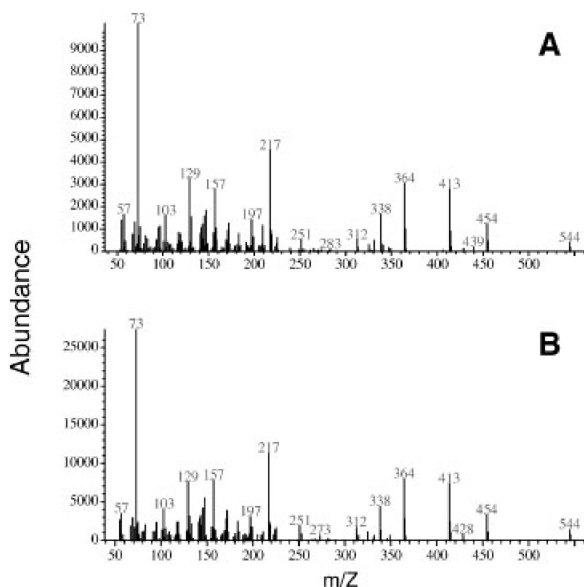

Fig. 2. Mass spectra of trimethylsilyl derivatives of metabolite A produced in ROS 17/2.8 cells and the synthetic standard of 1αOH-3-epi-D_3. **Upper panel**: metabolite A, retention time:14.06 min; **lower panel**: synthetic standard of 1αOH-3-epi-D_3, retention time: 14.06 min.

and for trimethylsilylated metabolite A (upper panel). The trimethylsilylated synthetic 1αOH-3-epi-D_3 and the trimethylsilylated metabolite A exhibited a molecular ion at m/z 544. The fragments at m/z 454 and 364 are formed by sequential elimination of one and two trimethylsilanol moieties from m/z 544. The loss of 131 Da from the A ring (m/z 413) and the detection of ion fragment at m/z 217, arising from A-ring cleavage, denote the presence of hydroxylation at C-1 and C-3 on the A-ring. In addition, to the identical mass spectra, the retention time (rt) on GC/MS confirmed the identity of the 1αOH-3-epi-D_3 metabolite (rt. 14.06 min), which is identical to that of the synthetic standard (rt. 14.06 min). As the two other stereoisomers of 1αOHD_3 namely 1βOHD_3 and 1βOH-3-epi-D_3 were not available, ^{1}H-NMR was necessary for absolute stereochemical characterization of the metabolite. Metabolite A was produced in sufficient quantities for ^{1}H-NMR analysis by incubating ROS 17/2.8 cells with 10 μM 1αOHD_3 in 20 culture bottles as described earlier. We obtained 160 μg of metabolite A for ^{1}H-NMR analysis. The ^{1}H-NMR spectrum of 1αOH-3-epi-D_3 (metabolite A) and its parent compound 1αOHD_3, were compared (Fig. 3). The spectra were the same except for differences in the chemical shifts between ring A protons. The largest chemical shift difference was observed for H-3, 4.23 ppm and 4.06 ppm for the parent compound (panel A) and metabolite (panel B), respectively. This observation is highly indicative of epimerization occurring at C-3. Comparison of the ^{1}H-NMR spectrum of metabolite A (panel B) with that of the authentic 1αOH-3-epi-D_3 (panel C) shows that the spectra are superimposable, thereby confirming that metabolite A and authentic 1αOH-3-epi-D_3 have identical structures. This result leads to the final conclusion that 1αOHD_3 is metabolized into its C-3 epimer, 1αOH-3-epi-D_3 in bone cells.

Metabolism of 1αOH-3-epi-D_3 in ROS 17/2.8 Cells

In the previous experiment, we have shown that at higher concentrations of 1αOHD_3 (10 μM) we could detect the metabolism of 1αOHD_3 not only into 1αOH-3-epi-D_3 and 1α,25$(OH)_2D_3$ but also 1α,25$(OH)_2$-3-epi-D_3 in ROS 17/2.8 cells (Fig. 1). In order to determine whether 1αOH-3-epi-D_3 can undergo C-25 hydroxylation to form 1α,25$(OH)_2$-3-epi-D_3 in

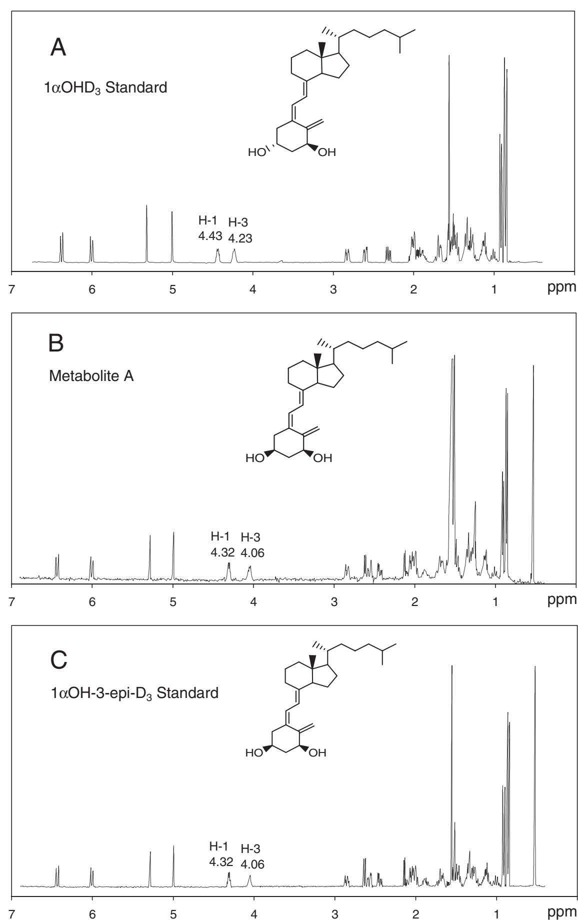

Fig. 3. ^{1}H-NMR spectra of the synthetic standard of 1αOHD$_3$ (**panel A**), metabolite A (**panel B**), and the synthetic standard of 1αOH-3-epi-D$_3$ (**panel C**).

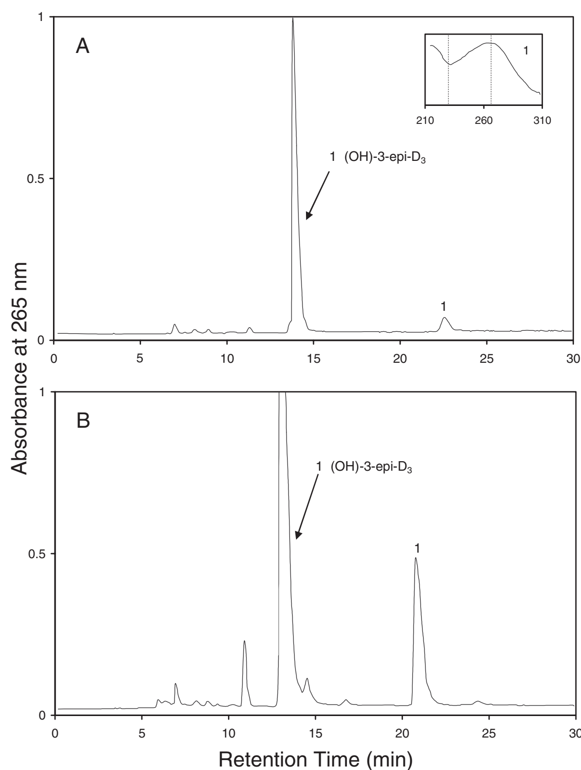

Fig. 4. HPLC profiles of 1αOH-3-epi-D$_3$ and its metabolites produced in ROS 17/2.8 cells incubated with 1 μM (**panel A**) or 10 μM (**panel B**) concentrations of the substrate for 24 h. The HPLC analysis was performed under the same chromatographic conditions as described in the legend in Figure 1. Peak 1 was identified as 1α,25(OH)$_2$-3-epi-D$_3$and its UV spectrum is shown in the inset.

ROS 17/2.8 cells, we next examined the metabolism of 1αOH-3-epi-D$_3$. Figure 4 shows the HPLC profiles of the lipid extracts obtained when ROS 17/2.8 cells were incubated for 24 h with 1 or 10 μM concentrations of 1αOH-3-epi-D$_3$ (panels A and B). As seen in Figure 4, 1αOH-3-epi-D$_3$ at both substrate concentrations (1 and 10 μM) was metabolized into a polar metabolite (peak 1) which was identified as 1α,25(OH)$_2$-3-epi-D$_3$. These findings together with the results of the previous experiment show evidence for C-25 hydroxylation of both 1α OHD$_3$ and 1αOH-3-epi-D$_3$ in vitro.

In vivo Metabolism of 1αOHD$_3$ in Rats

We next examined whether 1αOH-3-epi-D$_3$ exists as an in vivo metabolite in rats given pharmacological substrate concentrations of 1αOHD$_3$. Figure 5, panel B shows the HPLC profile of unmetabolized 1αOHD$_3$ (peak S) and six of its metabolites detected in 20 ml of serum obtained from four rats treated with 1αOHD$_3$. The elution positions of various authentic standards of vitamin D$_3$ metabolites are shown in panel A we observed a less polar peak along with several polar peaks. The less polar metabolite (peak A) eluting before the substrate (Peak S) was identified as 1αOH-3-epi-D$_3$, which had been observed in our in vitro studies with ROS 17/2.8 cells. The polar metabolites were identified as the following metabolites: 1α,25(OH)$_2$-3-epi-D$_3$ (peak 3), 1α,25(OH)$_2$D$_3$ (peak 4), 1α,25(OH)$_2$-24-oxo-D$_3$ (peak 5), 1α,24(R),25(OH)$_3$D$_3$ (peak 6), and 1α,25(OH)$_2$D$_3$-lactone (peak 7). The initial identity of each of the metabolites was achieved by UV absorption spectrophotometry [the metabolites possessed the characteristic vitamin D *cis*-triene chromophore (Fig. 5, panel B-inset)] and co-elution with the authentic standards on various HPLC systems described in Materials and Methods. The peaks J1 and J2 were considered to be contaminants based on

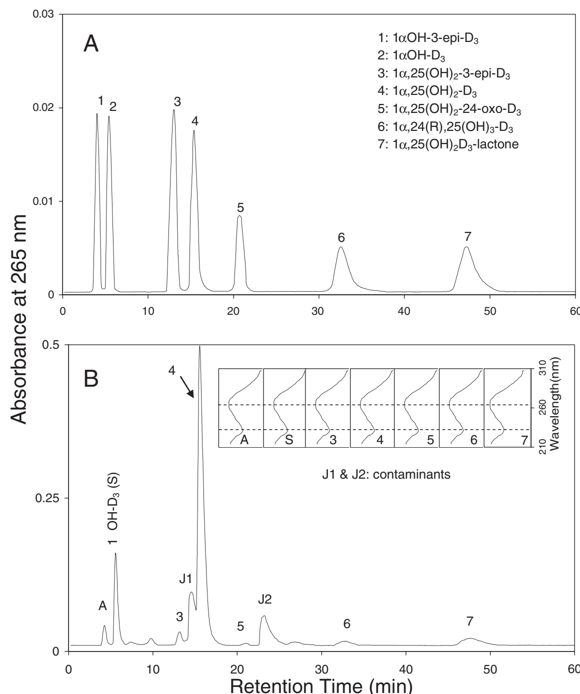

Fig. 5. HPLC profiles of various metabolites of $1\alpha OHD_3$ and 1αOH-3-epi-D_3 in rat serum. **Panel A**: synthetic standards of various vitamin D_3 metabolites. **Panel B**: vitamin D_3 metabolites in the lipid extract of 20 ml of serum obtained from 4 rats. Each rat received 500 μg of $1\alpha OHD_3$ intravenously, 8 h prior to sacrifice. The HPLC analysis of the lipid extract was performed under the same chromatographic conditions as described in the legend to Figure 1. The UV spectra of the metabolite peaks (A,S, 1, 2,3,6 and 8) are shown in the inset.

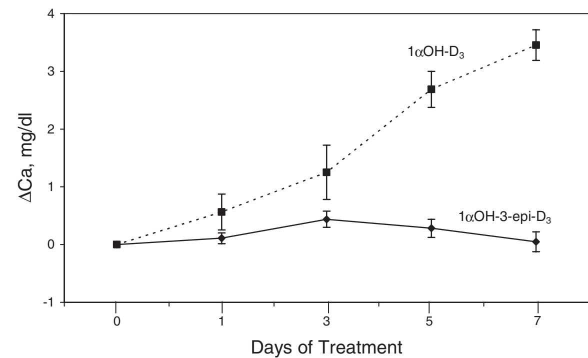

Fig. 6. Calcemic activities of $1\alpha OHD_3$ (solid circles) and 1αOH-3-epi-D_3 (open circles). Normal rats were injected every other day with 2.5 nmol/kg of the vitamin D compounds, and plasma calcium was measured 24 h after each injection. Data are expressed as mean ± SD (n = 3).

their UV spectra (the contaminants did not exhibit the UV spectral characteristics which are typical to a vitamin D cis/triene chromophore). The final identification of all the metabolites derived through the side chain oxidation pathways with the exception of $1\alpha,25(OH)_2$-24-oxo-D_3 was obtained through GC/MS [data not shown as it was reported earlier [Sekimoto et al., 1999]]. The novel less polar metabolite in the serum of rats (peak A) was identified as 1αOH-3-epi-D_3 through its co-elution with synthetic 1αOH-3-epi-D_3 on both straight and reverse phase HPLC systems and by GC/MS as described earlier. These findings demonstrate that $1\alpha OHD_3$ is metabolized through the C-3 epimerization pathway in vivo.

In Vivo Activities of $1\alpha OHD_3$ Versus 1αOH-3-epi-D_3

The calcemic activity of 1αOH-3-epi-D_3 was compared to that of $1\alpha OHD_3$. Rats were injected every other day for 7 days (four injections) with 2.5 nmol/kg of either 1α,OH-3-epi-D_3 or $1\alpha OHD_3$. Figure 6 shows the changes in serum calcium measured 24 h after each injection of the 2.5 nmol/kg dose. $1\alpha OHD_3$ produced a steady increase in serum calcium during the treatment period, with an average increase of 3.39 ± 0.52 mg/dl. In contrast 1αOH-3-epi-D_3 elicited only a transient, but not significant, rise in serum calcium at day 3, and the final calcium was not different from pretreatment levels (-0.05 ± 0.38 mg/dl). Despite the lack of change in serum calcium of rats receiving 1αOH-3-epi-D_3, it was significant to note that the serum PTH levels in the same rats decreased by 64.9 ± 7.2 % by the end of the 1 week treatment.

Efficacy of 1αOH-3-epi-D_3 Versus $1\alpha,25(OH)_2D_3$ in Suppressing PTH in Uremic Rats

The suppression of PTH by 1αOH-3-epi-D_3 in the absence of changes in calcium in normal rats suggested that 1αOH-3-epi-D_3 may be useful for the treatment of secondary hyperparathyroidism associated with chronic renal failure. We tested the efficacy of 1αOH-3-epi-D_3 in a rat model of renal insufficiency and secondary hyperparathyroidism. The rats underwent a partial (5/6) nephrectomy and were fed a diet containing 0.9% P and 0.6% Ca for one month to promote secondary hyperparathyroidism. The rats then were treated with escalating doses of $1\alpha,25(OH)_2D_3$ or 1αOH-3-epi-D_3, and the plasma calcium and PTH were determined at the end of each 1-week dose increment. Figure 7 (upper panel) shows the elevations in plasma Ca with increasing doses of the two compounds. Hypercalcemia, defined as plasma calcium >10.5 mg/dl, was achieved with doses

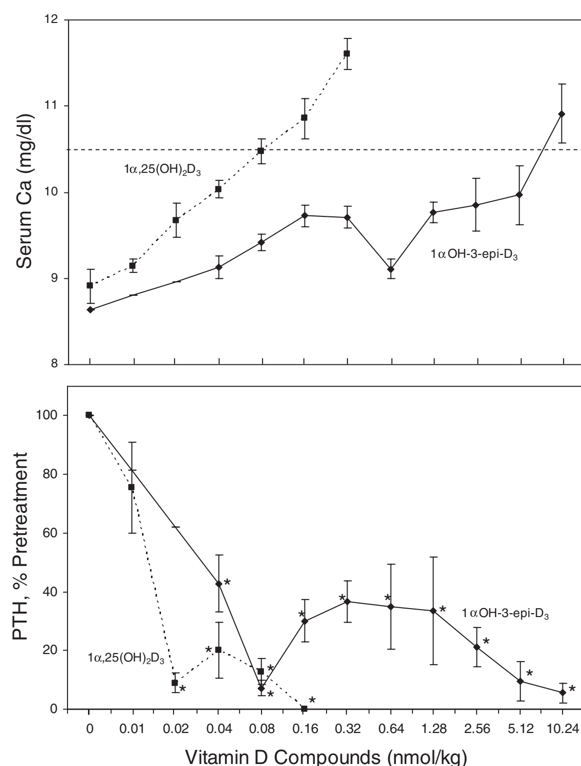

Fig. 7. Effects of $1\alpha,25(OH)_2D_3$ and 1αOH-3-epi-D_3 on plasma calcium (**upper panel**) and plasma PTH (**lower panel**) in uremic rats. Partially nephrectomized rats with established secondary hyperparathyroidism were treated with $1\alpha,25(OH)_2D_3$ or 1α(OH)-3-epi-D_3 in a dose escalation protocol as described in Methods. At the end of each dosing period, plasma PTH and calcium were measured. Plasma total calcium (upper panel) is expressed as the increase from pretreatment values (in mg/dl) and given as mean ± SD (n = 12). PTH values (lower panel) are expressed as percent of pretreatment value and given as mean ± SEM (n = 12). $*P < 0.05$ versus pretreatment PTH level.

of 0.08 nmol/kg of $1\alpha,25(OH)_2D_3$ and about 8 nmol/kg of 1αOH-3-epi-D_3. In contrast to the 100-fold difference in calcemic activities, the two compounds were similar in their potencies to reduce plasma PTH levels (Fig. 7, lower panel). Significant suppression was observed with $1\alpha,25(OH)_2D_3$ only at doses of 0.02 nmol/kg and higher. However, even the lowest dose of 1αOH-3-epi-D_3 (0.04 nmol/kg) produced significant suppression of about 60% that was maintained over 5 additional dose doublings. The more rapid drop in PTH in the $1\alpha,25(OH)_2D_3$-treated rats is due to suppression by the greater increment in plasma calcium.

DISCUSSION

In the first part of this paper we reported that bone cells (ROS 17/2.8) directly metabolize $1\alpha OHD_3$ into 1αOH-3-epi-D_3. The identity of 1αOH-3-epi-D_3 produced in ROS 17/2.8 cells was established using the techniques of UV absorption spectrophotometry, co-elution with the synthetic standard on both straight and reverse phase HPLC systems, GC/MS and ^{1}H-NMR analysis. We also observed that $1\alpha OHD_3$ is metabolized in bone cells not only into 1αOH-3-epi-D_3, but also into $1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2$-3-epi-D_3. This finding suggests that $1\alpha,25(OH)_2$-3-epi-D_3 from $1\alpha OHD_3$ may be formed through two pathways: (a) C-25 hydroxylation of $1\alpha OHD_3$ into $1\alpha,25(OH)_2D_3$ followed by the C-3 epimerization pathway or (b) C-3 epimerization of $1\alpha OHD_3$ into 1αOH-3-epi-D_3 followed by C-25 hydroxylation. Thus, in both these pathways, two steps are involved in the conversion of $1\alpha OHD_3$ into $1\alpha,25(OH)_2$-3-epi-D_3. Furthermore, we also identified 1αOH-3-epi-D_3 and $1\alpha,25(OH)_2$-3-epi-D_3 as circulating metabolites in rats treated with pharmacological amounts of $1\alpha OHD_3$. These results indicate that unlike the C-24 oxidation pathway, in which a hydroxyl group at C-25 position is essential for C-24 hydroxylation, the first step in the C-24 oxidation pathway [Reddy and Tserng, 1989], the hydroxyl group at C-25 position is not a pre-requisite for C-3 epimerization.

In the second part of this study we evaluated the biological activities of 1αOH-3-epi-D_3. We have previously shown that $1\alpha,25(OH)_2$-3-epi-D_3 has nearly the same activity as $1\alpha,25(OH)_2D_3$ in suppressing the PTH secretion in cultures of bovine parathyroid cells despite its lower affinity for the vitamin D receptor [Brown et al., 1999a]. Based on the results of this in vitro study, we performed the present in vivo study. In a small group of normal rats, we first observed a dramatic suppression of PTH by 1αOH-3-epi-D_3 in the absence of any increase in serum calcium. This observation was then confirmed in more detail in rats with kidney failure and established secondary hyperparathyroidism using a dose-escalation protocol. 1αOH-3-epi-D_3 was only slightly less active in suppressing PTH than $1\alpha,25(OH)_2D_3$. However, the dose of 1αOH-3-epi-D_3 required to achieve hypercalcemia (total plasma Ca>10.5 mg/dl) was about 100 times higher than that of $1\alpha,25(OH)_2D_3$. This high degree of selectivity for 1αOH-3-epi-D_3 in suppressing PTH in vivo suggests a tremendous potential for 1αOH-3-epi-D_3 in the treatment of secondary hyperparathyroidism. In recent years, the natural vitamin D hormone, $1\alpha,25(OH)_2D_3$, has been

largely supplanted by new vitamin D analogs in the treatment of secondary hyperparathyroidism and the associated renal osteodystrophy in patients with chronic kidney diseases. Studies in the kidney failure rat model showed that the less calcemic analogs, 22-oxa-$1\alpha,25(OH)_2D_3$ and 19-nor-$1\alpha,25(OH)_2D_2$, have been estimated to have 6- and 3-fold wider therapeutic windows than $1\alpha,25(OH)_2D_3$ [Slatopolsky et al., 1995; Brown, 2000; Hirata et al., 2002]. Our present findings indicate that the therapeutic window for 1αOH-3-epi-D_3 may be as much as 50-fold wider than that of $1\alpha,25(OH)_2D_3$. Confirmatory studies, performed with the same fixed dose protocol used for the other analogs, are necessary for a true comparison. Nonetheless, 1αOH-3-epi-D_3 appears to have great potential for treatment of secondary hyperparathyroidism.

The mechanisms responsible for the differential effects of 1αOH-3-epi-D_3 compared to $1\alpha,25(OH)_2D_3$ are not clear. It is conceivable that 1αOH-3-epi-D_3 like other vitamin D prodrugs such as $1\alpha OHD_2$ and $1\alpha OHD_3$ has to undergo activation via hydroxylation of the side chain at carbon 25 before it can exert its biological activities in target tissues such as the parathyroid glands. We have recently observed that $1\alpha OHD_2$ and $1\alpha OHD_3$ can suppress PTH synthesis by cultured parathyroid cells (Brown, A.J., submitted for publication), suggesting that these "prodrugs" are activated by hydroxylation within parathyroid cells. The high selectivity of 1αOH-3-epi-D_3 in the parathyroid glands could be due to either local (autocrine) activation in parathyroid cells or to interaction with a unique receptor, these possibilities are under investigation. The very low calcemic activity of 1αOH-3-epi-D_3 can be attributed primarily to a greatly reduced potency in stimulating intestinal calcium transport. Its calcemic effects are very much less than those of $1\alpha OHD_3$, illustrating the tremendous influence of the stereochemistry of the hydroxyl group at carbon 3 on biological activity. Again, the mechanisms for this are unclear. The relative rates of hydroxylation/activation of 1αOH-3-epi-D_3 and $1\alpha OHD_3$ are not known. The presumed activation products, $1\alpha,25(OH)_2$-3-epi-D_3 and $1\alpha,25(OH)_2D_3$ differ only about 10-fold in their affinities for the vitamin D receptor [Brown et al., 1999a], and the same receptor is thought to mediate vitamin D actions in both the intestine and parathyroid glands. Thus, other factors such as differential rates of catabolism, cellular uptake or intracellular trafficking of the compounds may be responsible for the selectivity of 1αOH-3-epi-D_3 [Brown, 2000]. Recently, Plum et al. [2004] reported that several vitamin D analogs lacking most of the side chain retained VDR binding activity and were potent suppressors of PTH in vivo, but they had greatly reduced calcemic activities. Although the mechanism for the selectivity of the side chain truncated analogs is not known, these findings illustrate the possibility of developing analogs that target the parathyroid glands.

In conclusion, we report for the first time that bone cells can directly metabolize $1\alpha OHD_3$ into 1αOH-3-epi-D_3. Also, we report that 1αOH-3-epi-D_3 is an in vivo metabolite of $1\alpha OHD_3$ under pharmacological substrate concentrations. The conversion of $1\alpha OHD_3$ into 1αOH-3-epi-D_3 indicates that a hydroxyl group at C-25 position is not a pre-requisite for C-3 epimerization. Furthermore, our results also indicate that 1αOH-3-epi-D_3 like $1\alpha OHD_3$, is hydroxylated at C-25 position to form $1\alpha,25(OH)_2$-3-epi-D_3. Assessment of in vivo biological activities in both normal and kidney failure rats demonstrated that 1αOH-3-epi-D_3 has much lower calcemic activity than $1\alpha OHD_3$, and that 1αOH-3-epi-D_3 can suppress PTH effectively in the absence of hypercalcemia. Thus, 1αOH-3-epi-D_3 appears to have a great potential application for treatment of secondary hyperparathyroidism.

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