Metabolism of 1α,25-Dihydroxyvitamin D_3 in Vitamin D Receptor-Ablated Mice in Vivo†

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ABSTRACT: The metabolism of 1α,25-dihydroxyvitamin D_3 was studied in vitamin D receptor-ablated mice following the administration of a physiological dose of 1α,25-dihydroxy-[26,27-^{3}H]vitamin D_3. The degradation of 1α,25-dihydroxy-[26,27-^{3}H]vitamin D_3 in the vitamin D receptor null mutant mice was substantially reduced compared to the wild-type control mice. At 24 h postadministration of radiolabeled 1α,25-dihydroxyvitamin D_3 more than 50% of the radioactivity was recovered unmetabolized, whereas in wild-type mice nearly all of the 1α,25-dihydroxy-[26,27-^{3}H]vitamin D_3 was degraded. In wild-type mice three polar metabolites other than 1α,25-dihydroxyvitamin D_3 were detected and identified on straight-phase and reverse-phase high-performance liquid chromatography as 1α,24(*R*),25-trihydroxy-[26,27-^{3}H]-vitamin D_3, 1α,25(*S*),26-trihydroxy-[26,27-^{3}H]vitamin D_3, and (23*S*,25*R*)-1α,25-dihydroxy-[^{3}H]vitamin D_3-26,23-lactone. Only one metabolite was detected in the plasma and kidneys of vitamin D receptor null mutant mice at 3 h following an intrajugular dose of 1α,25-dihydroxy-[26,27-^{3}H]vitamin D_3. This metabolite was clearly identified as 1α,25(*S*),26-trihydroxy-[26,27-^{3}H]vitamin D_3 by comigration on two HPLC systems and periodate cleavage reaction. At 6, 12, and 24 h postinjection 1α,24(*R*),25-trihydroxy-[26,27-^{3}H]vitamin D_3 was also detected at low levels in plasma, kidneys, and liver of some but not all mutant mice. The presence of 25-hydroxyvitamin D_3-24-hydroxylase mRNA in the kidneys of these vitamin D receptor null mutant mice was confirmed by ribonuclease protection assay.

The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$],[1] is formed by sequential hydroxylations of vitamin D_3 at carbon C-25 in the liver and at C-1α in renal proximal tubular cells (*1*). The resulting hormone is responsible for the physiological actions of vitamin D such as stimulation of calcium absorption (*2*), phosphate absorption in the small intestine (*3*), renal reabsorption of calcium (*4*), and the mobilization of calcium from bone (*5*).

Two specific pathways that further metabolize 1,25-$(OH)_2D_3$ are known: the C-24/C-23 pathway and the C-26/C-23 lactone pathway. Both pathways involve a C-23 hydroxylation (*6*). It is thought that the main route of degradation is the C-24/C-23 catabolism pathway, which consists of sequential C-24 hydroxylation, C-24 ketonization, and C-23 hydroxylation, followed by oxidative cleavage of the carbon−carbon bond between C-24 and C-23 to form 24,25,26,27-tetranor-23-OH-D_3 (*7*). Then the C-23 alcohol is believed to be converted to a C-23 acid (calcitroic acid) (*8*), which is the major excretory product of 1,25$(OH)_2D_3$ (*9*).

The enzyme that catalyzes the C-24/C-23 catabolic pathway, the 25-hydroxyvitamin D_3-24-hydroxylase (P450cc24), was cloned previously (*10*−*12*). Two vitamin D response elements (VDREs) in the proximal promoter of the 24-hydroxylase gene are responsible for the 1,25$(OH)_2D_3$ vitamin D receptor (VDR) mediated upregulation of the gene transcription (*13*, *14*). In the absence of 1,25$(OH)_2D_3$, the 24-hydroxylase is turned off under normal circumstances (*15*). VDR null mutant mice should, therefore, have little or no P450cc24 activity.

(23*S*,25*R*)-1α,25$(OH)_2D_3$-26,23-Lactone is formed by sequential C-26 hydroxylation, C-23 hydroxylation, and lactonization (*1*). The enzymatic activities for the formation of either calcitroic acid or lactone end-products are present in tissues or cells of the major target organs of vitamin D, i.e., kidney, intestine, and bone (*8*, *16*, *17*). The enzyme, which is likely to catalyze the first step of the lactone pathway, the 26-hydroxylase, has not been cloned extensively or studied. It is probably a cytochrome P450 enzyme (*18*) located in the kidney and intestine (*19*, *20*) and might be regulated by parathyroid hormone (PTH) (*21*). So far it is unknown if the 26-hydroxylase is also regulated by 1,25-$(OH)_2D_3$ itself.

The action of 1,25$(OH)_2D_3$ in target genes is mediated by VDR, which binds as a heterodimer to vitamin D response elements in the promoter of genes (*22*). Two different research groups generated VDR-ablated mice by disrupting

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[1] Abbreviations: 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1α,24(*R*),25-trihydroxyvitamin D_3; 1,25,26$(OH)_3D_3$, 1α,25(*S*),26-trihydroxyvitamin D_3; 1,25$(OH)_2D_3$-lactone, (23*S*,25*R*)-1α,25-dihydroxyvitamin D_3-26,23-lactone; VDRE, vitamin D response element; VDR, vitamin D receptor; PTH, parathyroid hormone; P450cc24, 25-hydroxyvitamin D_3-24-hydroxylase; HPLC, high-performance liquid chromatography; RPA, ribonuclease protection assay.

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the first zinc finger (*23*) and the second zinc finger (*24*) of the VDR gene. On chow diet VDR-ablated mice develop alopecia, hypocalcemia, and impaired bone formation after weaning (*24*). However, mice kept on a rescue diet containing 20% lactose, 2% calcium, and 1.25% phosphate had normal calcium, phosphate, and PTH values (*25*).

In this study the metabolism of $1,25(OH)_2D_3$ in VDR null mutant mice and their control littermates kept on rescue diet was investigated by injecting these animals intrajugularly with a physiological dose of $1\alpha,25(OH)_2[26,27\text{-}^3H]D_3$. The absence of VDR profoundly retards metabolism of $1,25\text{-}(OH)_2D_3$.

EXPERIMENTAL PROCEDURES

Chemicals. $1\alpha,25(OH)_2[26,27\text{-}^3H]D_3$ (160 Ci/mmol) was obtained from Dupont/New England Nuclear (Boston, MA). $1\alpha,25(OH)_2D_3$ and $1\alpha,24(R),25(OH)_3D_3$ were purchased from Tetrionics (Madison, WI), $1\alpha,25(S),26(OH)_3D_3$ was provided by Hoffmann La Roche (Nutley, NJ), and (23*S*,25*R*)-$1\alpha,25(OH)_2D_3$-26,23-lactone was a generous gift from Dr. Seiichi Ishizuka. Before use, the purity of all vitamin D_3 compounds was found to be greater than 96% by high-performance liquid chromatography (HPLC). These compounds were stored in ethanol under argon at −20 °C.

Sodium metaperiodate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sephadex LH-20 resin was purchased from Fluka, Biochemika (Buchs, Switzerland). All solvents were of HPLC grade (Burdick & Jackson, Muskegon, MI).

Animals. Mice lacking the VDR were obtained as previously described (*23*). The VDR null mutant mice and control littermates were exposed to a 12 h light, 12 h dark cycle. The genotyping of the homozygous, heterozygous, and wild-type mice was performed at 4 weeks of age by Southern hybridization using a 1.1 kb probe (probe B) located in intron 2 of the VDR gene (*23*).

The animals were fed a purified diet that contained 20% lactose, 2% calcium, 1.25% phosphorus supplemented with vitamin A, D, E, and K in Wesson oil. Three days before the experiment the mice were bled by tail nicking and the blood was centrifuged at 1023*g* for 15 min to yield serum. Serum calcium was measured in the presence of 0.1% lanthanum chloride by means of an atomic absorption spectrometer (Perkin-Elmer 3110). Serum inorganic phosphorus was measured by the method described by Chen et al. (*26*).

For the metabolism study the animals were injected intrajugularly with 60 pmol (25 ng) of $1\alpha,25(OH)_2[26,27\text{-}^3H]D_3$ (160 Ci/mmol) in 10 μL of ethanol/propylene glycol (1/1) under ether anesthesia. At 3, 6, 12, and 24 h post-injection, blood was taken by heart puncture and the kidneys and liver were collected. Serum was obtained by centrifugation at 1023*g* for 15 min. Liver and kidneys were frozen in liquid N_2 and stored at −20 °C.

Isolation of Metabolites. Tissue was homogenized in ice-cold water (4 mL/g of tissue) with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). As internal standard, 200 ng of $1\alpha,25(OH)_2D_3$, $1\alpha,24(R),25(OH)_3D_3$, and $1\alpha,25(S),26(OH)_3D_3$ were added to the homogenate and serum. An equal volume of methanol was added, followed by extraction with methylene chloride. This extraction was repeated 3 times. The combined organic phase was evaporated to dryness under vacuum by rotary evaporation. The residue was redissolved in $CHCl_3$/hexane/methanol = 75/23/2 and applied to a Sephadex LH-20 column (0.9 × 20 cm, Fluka, Buchs, Switzerland), which was eluted with the same solvent system. The elution volume 20−60 mL was found to contain $1\alpha,25(OH)_2D_3$, whereas the 60−150 mL fraction contained the trihydroxy metabolites.

The two fractions were evaporated under vacuum, redissolved, and analyzed by straight-phase and reverse-phase HPLC (RP-HPLC). For the straight-phase HPLC a Zorbax RX-Sil HPLC column (Hewlett-Packard, Santa Clarita, CA, 4.6 mm × 25 cm) was run in hexane/CH_2Cl_2/MeOH = 73/20/7 and a flow rate of 2 mL/min. For the RP-HPLC an Eclipse XDB-C18 column (4.6 mm × 25 cm) was used in MeOH/H_2O = 75/25 and a flow rate of 1 mL/min. The recovery of standards was 70−90%.

Periodate Cleavage. $1\alpha,25(S),26(OH)_3[26,27\text{-}^3H]D_3$ isolated from serum or tissue of VDR null mutant mice and their control littermates was purified over straight-phase HPLC, dried under N_2, and redissolved in 100 μL of methanol. The solution was cooled to 0 °C, and 25 μL of saturated cold sodium periodate solution was added. The reaction was carried out under stirring in an argon atmosphere for 1.5 h at 0 °C. The products were isolated by adding 1 mL of water and extracting twice with 1 mL of CH_2Cl_2. The CH_2Cl_2 phase was washed once with H_2O and dried under N_2. The identification of the reaction product, 1α-hydroxy-25-keto-[^{3}H]vitamin D_3, was performed by straight-phase HPLC (Zorbax silica column, Phenomex, Rancho Palvos Verdes, CA) by using a solvent system hexane/IPA = 85/15 with a flow rate of 2 mL/min and by RP-HPLC (Zorbax ODS column, DuPont, Wilmington, DE) with solvent MeOH/H_2O = 80/20 at a flow rate of 1 mL/min. In all cases, radioactivity was determined by liquid scintillation using a Tri-Carb liquid scintillation analyzer (Packard, Meiden, CT) and Bio-Safe II scintillation fluid (Research Products International Corp., Mount Prospect, IL).

Ribonuclease Protection Assay. RNA was isolated from fresh tissue by the LiCl/urea method as described (*27*). Poly-$(A)^+$ RNA was isolated with the Oligotex mRNA midi kit (Qiagen, Valencia, CA). For the RPA, RNA samples (poly-$(A)^+$ RNA) were hybridized at 42 °C for 14 h with a radiolabeled 273 base pair (bp) mouse P450cc24 RNA fragment according to manufacturer's protocol (RPAII kit, Ambion, Austin, TX). The probe, comprising nucleotides 737−1010 of the published sequence (*11*) and subcloned into the *Eco*RI site of the Bluescript II KS+/− vector, was generated with the Maxiscript in vitro transcription kit (Ambion, Austin, TX) by using an [α-^{32}P]UTP label. To control for equal loading of RNA, the samples were simultaneously hybridized with a 250 bp β-actin probe, which was labeled with [α-^{32}P]UTP of specific activity 10 000 times less than the P450cc24. To confirm the genotyping of these mice, a 190 bp probe representing exon 2 of the VDR was used. After hybridization, the free RNA was digested with ribonuclease and the protected fragments were separated and analyzed on a 5% polyacrylamide denaturing gel. Radioactive signals were quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

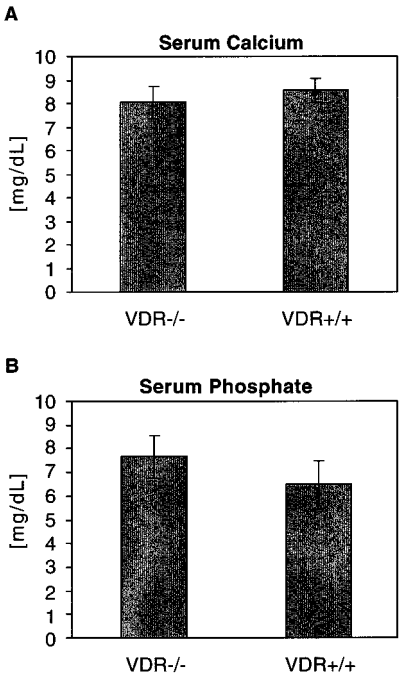

FIGURE 1: (**A**) Serum calcium in homozygous mice (−/−) and wild-type mice (+/+) receiving the rescue diet. Calcium concentrations were measured with an atomic absorption spectrometer. Values are presented as the mean ± SEM of 7 homozygous mice and 11 wild-type mice. The calcium values of the homozygous and wild-type mice are not significantly different (Student's *t*-test). (**B**) Serum phosphorus concentrations in homozygous mice (−/−) and wild-type mice (+/+) receiving the rescue diet. Values are obtained from 7 homozygous and 11 wild-type mice and presented as mean ± SEM. The phosphate values in the serum of the homozygous and wild-type mice are not significantly different (Student's *t*-test).

RESULTS

Serum Calcium and Phosphate in VDR Null Mutant and Wild-Type Mice Kept on Rescue Diet. Mice used in this study were 8−12 weeks old and maintained on rescue diet containing 20% lactose, 2% calcium, and 1.25% phosphate. Both serum calcium and serum phosphate values were not significantly different between the VDR null mutant and the wild-type mice (Figure 1). These findings are in agreement with previously published results (*25*).

Metabolites Found in the Serum of VDR Null Mutant and Wild-Type Mice. Table 1 shows the metabolites found in the serum of VDR null mutant and wild-type mice 3, 6, and 12 h after the administration of 10 μCi of 1α,25(OH)$_2$[26,27-^{3}H]D$_3$. Preliminary studies showed that in wild-type mice the highest amount of trihydroxy metabolites can be found 3 h postadministration of radiolabeled 1α,25(OH)$_2$D$_3$ (data not shown).

The metabolism of 1α,25(OH)$_2$[26,27-^{3}H]D$_3$ in the VDR null mutant mice was quite different from their control littermates. The most obvious difference was that in VDR null mutant mice most of the radioactivity in the serum (usually 95% and not less than 80%) can be recovered as 1α,25(OH)$_2$[26,27-^{3}H]D$_3$ at 3, 6, and even 12 h after administration and a decrease in total radioactivity at different time points was not observed, whereas in wild-type mice 6 and 12 h postinjection most of the radioactivity was already excreted or degraded to water-soluble metabolites. Only 2% of the injected radioactivity was water-soluble in VDR null mutants.

The lipid extracts at 3 h in the case of wild-type mice contained three metabolites of 1α,25(OH)$_2$[26,27-^{3}H]D$_3$ that had the same retention times as (23*S*,25*R*)-1α,25(OH)$_2$D$_3$-26,23-lactone, 1α,24(*R*),25(OH)$_3$D$_3$, and 1α,25(*S*),26(OH)$_3$D$_3$ on a straight-phase HPLC (Figure 2A) and reverse-phase HPLC (Figure 2B) system, whereas the lipid extracts from VDR null mutant mice contained only one metabolite. This metabolite had the same retention time as authentic 1α,25(*S*),26(OH)$_3$D$_3$ in both a straight-phase HPLC (Figure 2A) and reverse-phase HPLC (Figure 2B) system. This radioactive labeled product was collected from the straight-phase HPLC column and treated with sodium metaperiodate to further confirm its identity. The reaction product comigrated on a straight-phase (Figure 3) and reverse-phase system with the periodate-treated authentic 1α,25(*S*),26(OH)$_3$D$_3$ or 1α-hydroxy-25-keto-vitamin D$_3$.

At 6 and 12 h postinjection of 10 μCi of 1α,25(OH)$_2$-[26,27-^{3}H]D$_3$, in addition to 1α,25(*S*),26(OH)$_3$[26,27-^{3}H]D$_3$ in the serum of the VDR null mutant mice 1α,24(*R*),25-(OH)$_3$[26,27-^{3}H]D$_3$ was also detected, but no 1α,25(OH)$_2$-[^{3}H]D$_3$-26,23-lactone was found.

Metabolites Found in Kidneys and Liver of VDR Null Mutant and Wild-Type Mice. In comparison to metabolites found in the serum of VDR null mutant mice, traces of 1α,25(OH)$_2$[^{3}H]D$_3$-26,23-lactone were detected in the kidneys at the 12 and 24 h time points but not at 3 h after the injection of 1α,25(OH)$_2$[26,27-^{3}H]D$_3$ (Table 2). As in the serum at 3 h after the administration, 1α,25(*S*),26(OH)$_3$-[26,27-^{3}H]D$_3$ was the only trihydroxy metabolite observed in the kidneys of the VDR null mutant mice, and at the 12 and 24 h time points some 1α,24(*R*),25(OH)$_3$[26,27-^{3}H]D$_3$ had accumulated. In the wild-type mice in addition to 1α,24(*R*),25(OH)$_3$[26,27-^{3}H]D$_3$ and 1α,25(*S*),26(OH)$_3$[26,27-^{3}H]D$_3$, 1α,25(OH)$_2$[^{3}H]D$_3$-26,23-lactone was the degradation

Table 1: Metabolites of 1α,25(OH)$_2$[26,27-^{3}H]D$_3$ Isolated from the Serum of Homozygous (−/−) and Wild-Type Mice (+/+) Found 3, 6, and 12 h following the Administration of 10 μCi of 1α,25(OH)$_2$[26,27-^{3}H]D$_3$

		% radioactivity in the serum					
time points	total tissue radioactivity[a] (% of dose)	water phase	CH_2Cl_2 phase	1,25(OH)$_2$D$_3$	1,25(OH)$_2$D$_3$-lactone	1,24,25(OH)$_3$D$_3$	1,25,26(OH)$_3$D$_3$
VDR−/−, 3 h[b]	14.4 ± 4.4	1.6 ± 0.7	98.4 ± 0.6	95.8 ± 3.6	ND[c]	ND	2.0 ± 1.0
VDR−/−, 6 h	15.3	2.3	97.8	83.6	ND	0.3	2.5
VDR−/−, 12 h	14.9	2.3	97.7	90.6	ND	0.9	1.8
VDR+/+, 3 h[b]	8.1 ± 4.7	10.8 ± 7.0	89.3 ± 3.9	73.6 ± 5.0	4.7 ± 3.7	4.1 ± 0.8	1.7 ± 0.6
VDR+/+, 6 h	1.7	60.8	39.3	29.0	ND	ND	ND
VDR+/+, 12 h	3.0	65.3	34.7	21.8	1.7	2.7	ND

[a] The plasma was taken as 3.13% of body weight for calculating tissue radioactivity. [b] Average of 4 animals ± standard deviation. [c] ND = not detected.

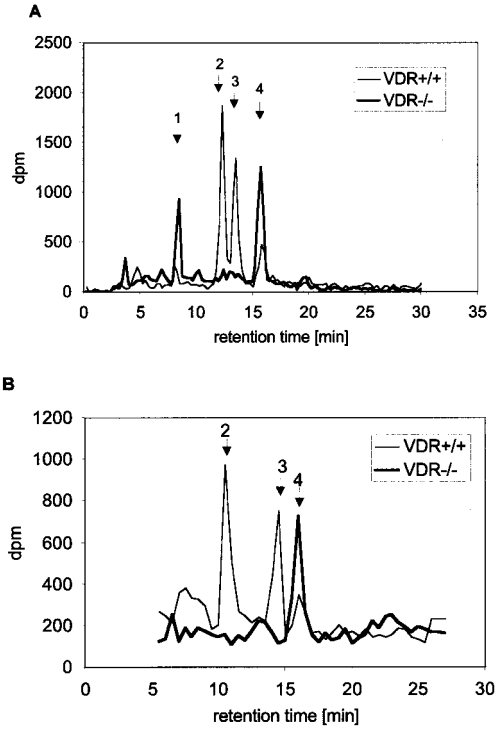

FIGURE 2: HPLC profiles of the 60−150 mL fraction from the Sephadex LH-20 column chromatography of organic extract from serum of homozygous (−/−) and wild-type (+/+) mice at 3 h following intrajugular administration of 1α,25(OH)$_2$[26,27-^{3}H]D$_3$. (**A**) Straight-phase HPLC profile: Zorbax-Silica column was eluted with hexane/CH_2Cl_2/MeOH = 73/20/7 at a flow rate of 2.0 mL/min. (**B**) RP-HPLC profile: an Eclipse XDB-C18 column was used with MeOH/H_2O = 75/25 and a flow rate of 1.0 mL/min. The arrows indicate the elution position of authentic standards: (**1**) 1α,25(OH)$_2$D$_3$; (**2**) (23*S*,25*R*)-1α,25(OH)$_2$D$_3$-26,23-lactone; (**3**) 1α,24(*R*),25(OH)$_3$D$_3$; and (**4**) 1α,25(*S*),26(OH)$_3$D$_3$.

product observed at the highest concentration in the kidneys. The amount of water-soluble metabolites detected in the kidneys of the VDR null mutant mice was higher than the water-soluble metabolites found in the serum but much less than that observed in the kidneys of wild-type mice.

In the liver of the VDR null mutant mice (Table 3) two metabolites that comigrated with 1α,24(*R*),25(OH)$_3$D$_3$ and 1α,25(*S*),26(OH)$_3$D$_3$ on a straight-phase HPLC (Figure 4A) and reverse-phase HPLC system (Figure 4B) were found, but no 1α,25(OH)$_2$[^{3}H]D$_3$-26,23-lactone. In their control littermates, besides 1α,24(*R*),25(OH)$_3$[26,27-^{3}H]D$_3$ and 1α,25(*S*),26(OH)$_3$[26,27-^{3}H]D$_3$ only traces of 1α,25(OH)$_2$-[^{3}H]D$_3$-26,23-lactone were present.

In wild-type mice, up to 22% of the radioactivity could be recovered in the liver as 1α,25(OH)$_2$[26,27-^{3}H]D$_3$. Most

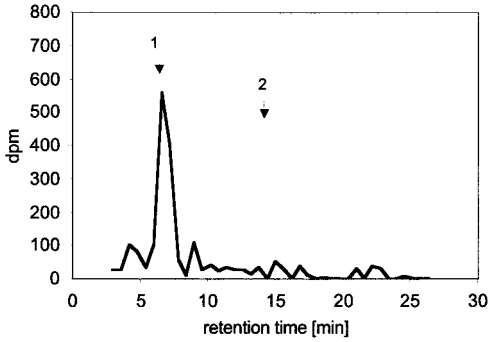

FIGURE 3: Straight-phase HPLC profile of periodate-treated 1α,25(*S*),26(OH)$_3$[26,27-^{3}H]D$_3$ isolated from serum of VDR null mutant mice on a Zorbax-Silica column with a solvent system of hexane/IPA = 85/15 and a flow rate of 2 mL/min. The arrows indicate the elution position of authentic standards: (**1**) 1α-hydroxy-25-keto-vitamin D$_3$ and (**2**) 1α,25(*S*),26(OH)$_3$D$_3$.

of the radiolabeled 1α,25(OH)$_2$D$_3$ (more than 50%) was degraded and the more polar products were detected in the water phase. In the VDR null mutant mice even at the 24 h time point over 50% of the radioactivity found in the liver could be recovered as 1α,25(OH)$_2$[26,27-^{3}H]D$_3$.

In summary, the degradation of 1,25(OH)$_2$D$_3$ in the VDR null mutant mice is substantially reduced. The metabolite 1α,25(*S*),26(OH)$_3$[26,27-^{3}H]D$_3$ was found in serum, kidney, and liver at all time points whereas 1α,24(*R*),25(OH)$_3$[26,27-^{3}H]D$_3$ was detectable in all tissues at 6 h or later after the administration of 1α,25(OH)$_2$[26,27-^{3}H]D$_3$.

P450cc24 Transcript in the Kidneys of VDR Null Mutant Mice. To examine, if the 24-hydroxylation of 1α,25(OH)$_2$-[26,27-^{3}H]D$_3$ to form 1α,24(*R*),25(OH)$_3$[26,27-^{3}H]D$_3$ in VDR null mutant mice is catalyzed by the P450cc24, RPA was performed with poly(A)$^+$ RNA isolated from the kidneys of homozygous (−/−), heterozygous (+/−), and wild-type mice (+/+) (Figure 5A). The basic levels of P450cc24 transcript in the heterozygous and wild-type mice are the same (lanes 8 and 9). When poly(A)$^+$ RNA was used, various amounts of P450cc24 transcript in homozygous mice ranging from undetectable (lanes 5 and 6) to detectable concentrations (lane 7) were observed. Four out of seven animals had detectable transcript levels. No P450cc24 transcript was seen in the liver of VDR−/− or VDR+/+ mice (lanes 10 and 11). Performing RPA using exon 2 of the VDR as a probe (Figure 5B) reconfirmed the genotyping of these mice.

DISCUSSION

Previous reports have shown that the P450cc24 activity is induced by 1,25-(OH)$_2$D$_3$ itself (*28*, *29*) through the VDR

Table 2: Metabolites of 1α,25(OH)$_2$[26,27-^{3}H]D$_3$ Isolated from the Kidneys of Homozygous (−/−) and Wild-Type Mice (+/+) Found 3, 12, and 24 h following the Administration of 10 μCi of 1α,25(OH)$_2$[26,27-^{3}H]D$_3$

		% radioactivity in the kidney					
time points	total tissue radioactivity (% of dose)	water phase	CH_2Cl_2 phase	1,25(OH)$_2$D$_3$	1,25(OH)$_2$D$_3$-lactone	1,24,25(OH)$_3$D$_3$	1,25,26(OH)$_3$D$_3$
VDR−/−, 3 h	2.2	2.5	97.5	100.0	ND[a]	ND	2.4
VDR−/−, 12 h[b]	1.6 ± 0.7	12.2 ± 0.2	87.8 ± 0.2	61.5 ± 12.4	0.2 ± 0.1	0.3 ± 0.1	3.0 ± 0.7
VDR−/−, 24 h	1.2	10.7	89.3	68.9	0.2	0.6	1.1
VDR+/+, 3 h	4.9	28.1	71.9	60.3	5.8	2.8	0.8
VDR+/+, 12 h[b]	1.5 ± 0.1	71.6 ± 6.1	28.4 ± 6.1	25.5 ± 6.3	2.4 ± 0.7	0.3 ± 0.1	0.7 ± 0.7
VDR+/+, 24 h	0.6	68.9	31.1	7.3	1.6	0.3	ND

[a] ND = not detected. [b] Average of two animals.

Table 3: Metabolites of $1\alpha,25(OH)_2[26,27\text{-}^3H]D_3$ Isolated from the Liver of Homozygous (−/−) and Wild-Type Mice (+/+) Found 3, 12, and 24 h following the Administration of 10 μCi of $1\alpha,25(OH)_2[26,27\text{-}^3H]D_3$

		% radioactivity in the liver					
time points	total tissue radioactivity (% of dose)	water phase	CH_2Cl_2 phase	$1,25(OH)_2D_3$	$1,25(OH)_2D_3$-lactone	$1,24,25(OH)_3D_3$	$1,25,26(OH)_3D_3$
VDR−/−, 3 h	15.3	8.2	91.9	98.4	ND[a]	0.4	3.8
VDR−/−, 12 h	10.2	11.3	88.8	73.9	ND	0.6	4.7
VDR−/−, 24 h	5.5	19.4	80.6	54.6	ND	1.3	2.2
VDR+/+, 3 h	6.9	87.1	12.9	16.6	ND	6.0	5.0
VDR+/+, 12 h	3.5	59.0	41.0	21.7	ND	0.4	1.3
VDR+/+, 24 h	2.4	84.0	16.0	1.7	ND	ND	ND

[a] ND = not detected.

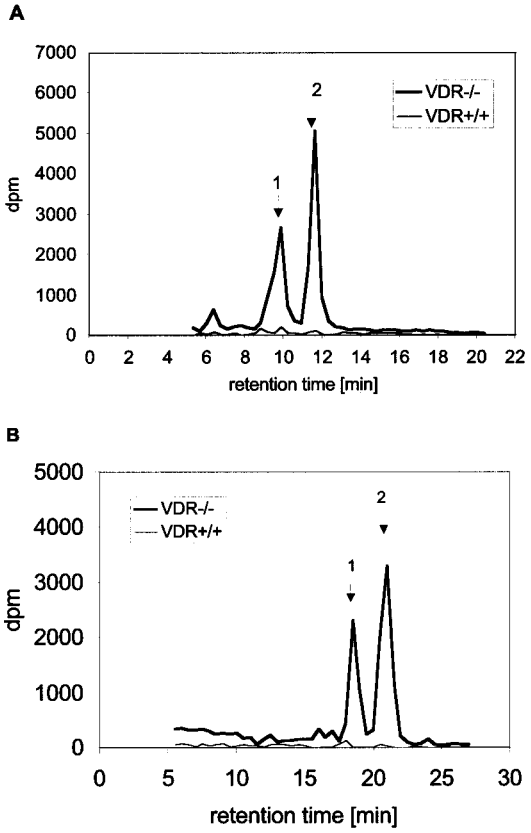

FIGURE 4: HPLC profiles of the metabolites isolated from liver of a homozygous (−/−) and wild-type mouse (+/+) 24 h after the administration of 10 μCi of $1\alpha,25(OH)_2[26,27\text{-}^3H]D_3$. (**A**) The separation of the metabolites by straight-phase HPLC was done on a Zorbax-Silica column with a solvent system of hexane/CH_2Cl_2/MeOH = 73/20/7 and a flow rate of 2 mL/min. (**B**) For the reverse-phase HPLC an Eclipse XDB-C18 column (4.6 mm × 25 cm) was used in MeOH/H_2O = 75/25 and a flow rate of 1 mL/min. The arrows indicate elution positions of authentic standards: (**1**) $1\alpha,24(R),25(OH)_3D_3$ and (**2**) $1\alpha,25(S),26(OH)_3D_3$.

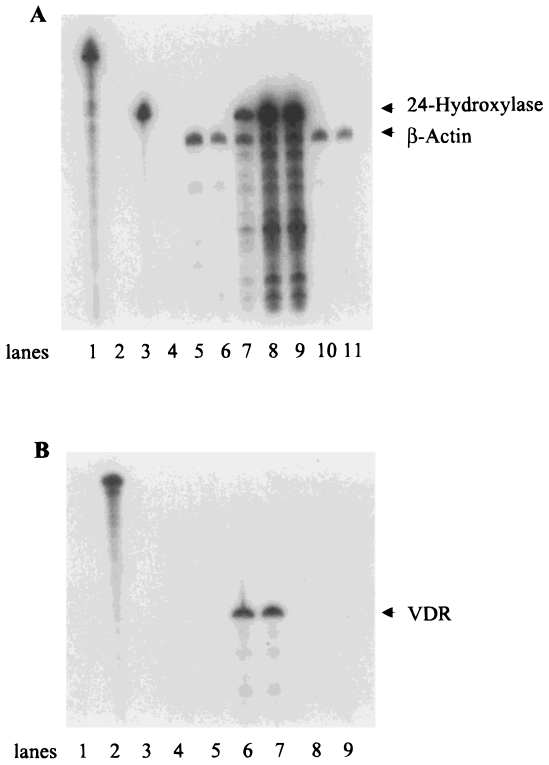

FIGURE 5: RPA with poly(A)$^+$ RNA isolated from the kidneys of homozygous (−/−), heterozygous (+/−), and wild-type (+/+) mice. The poly(A)$^+$ RNA from one mouse respectively was hybridized to ^{32}P-labeled RNA probes for the P450cc24, β-actin, and exon 2 of the VDR. Probe fragments protected from RNase digestion were run on 5% denaturing polyacrylamide gels. The gels were dried and autoradiographed. (**A**) RPA with simultaneous hybridization with the P450cc24 and β-actin RNA probes. Lane 1, P450cc24 probe; lane 2, P450cc24 probe digested with RNase; lane 3, β-actin probe; lane 4, β-actin probe digested with RNase; lanes 5−7, VDR−/− kidney; lane 8, VDR+/− kidney; lane 9, VDR+/+ kidney; lane 10, VDR−/− liver; lane 11, VDR+/+ liver. (**B**) RPA with exon 2 of the VDR to confirm the genotyping of the mice used in Figure 5A. Lane 1, VDR probe digested with RNase; lane 2, VDR probe; lanes 3−5, VDR−/− kidney; lane 6, VDR+/− kidney; lane 7, VDR+/+ kidney; lane 8, VDR−/− liver; lane 9, VDR+/+ liver. Similar results were obtained in three independent sets of experiments.

binding to two VDREs in the proximal promoter of the P450cc24 gene (*13*, *14*). In mice lacking the VDR, the ligand-dependent activation of the P450cc24 gene transcription cannot occur and consequently the C-24/C-23 catabolic degradation pathway of $1,25(OH)_2D_3$ should be largely eliminated.

In the serum of the VDR null mutant mice even 12 h after the administration of $1\alpha,25(OH)_2[26,27\text{-}^3H]D_3$, 80−90% of the radioactivity can be recovered as unchanged $1\alpha,25(OH)_2[26,27\text{-}^3H]D_3$, whereas in their control littermates most of the injected radioactivity had been excreted or degraded to water-soluble products. This result demonstrates that VDR null mutant mice are severely compromised in their ability to eliminate $1,25(OH)_2D_3$. Therefore, $1,25(OH)_2D_3$ acting through its receptor is required for its own rapid degradation. The rapid metabolism of $1,25(OH)_2D_3$ in wild-type mice and the very slow metabolism in the VDR null mutant mice can most obviously be explained by the presence or relative absence of the P450cc24. It is well-known that $1,25(OH)_2D_3$ activates expression of the 24-hydroxylase gene that is silent

in the absence of 1,25$(OH)_2D_3$. This activation is believed to be mediated by the 1,25$(OH)_2D_3$-responsive elements in the promoter of the 24-hydroxylase gene. In support of this belief, transcripts for the P450cc24 are absent in vitamin D deficiency and are strongly induced by 1,25$(OH)_2D_3$. Shinki et al. (*15*) have shown that 1α,25-dihydroxyvitamin D_3 upregulates the P450cc24 from undetectable to high levels within 4 h. By 6 h there are high levels of P450cc24.

In the VDR null mutant mice, 1α,24(*R*),25$(OH)_3$[26,27-^{3}H]D_3 was not detectable at 3 h and was barely detectable at 6 and 12 h postadministration of radiolabeled 1α,25-$(OH)_2D_3$. This metabolite was clearly identified as 1α,24(*R*),25-$(OH)_3$[26,27-^{3}H]D_3 by running it on straight-phase and reverse-phase HPLC together with the authentic standard. The P450cc24 transcript was also detected in the kidneys of some but not all of the VDR null mutant mice. The P450cc24 transcript was not detected in the liver of either the mutant or wild-type mice. Akeno et al. (*30*) had already shown that wild-type mouse liver has no P450cc24 transcript. Our mutant mice also had no RNA sequence corresponding to exon 2 of the VDR, confirming the mutation in the VDR gene. The results reported here clearly confirm that the major pathway of 1,25$(OH)_2D_3$ elimination is via the P450cc24 and that this enzyme in vivo is largely controlled by 1,25$(OH)_2D_3$ itself.

The fact that 1α,25(S),26$(OH)_3$[26,27-^{3}H]D_3 was found in plasma, kidney, and liver of the null mutant mice leads to the conclusion that the expression of the 26-hydroxylase is not as strongly regulated by 1,25$(OH)_2D_3$, unlike the P450cc24. The identity of the 1α,25(*S*),26$(OH)_3$[26,27-^{3}H]-D_3 was confirmed by running this metabolite on a straight- and reverse-phase HPLC system together with the authentic standard and by cleaving the side chain with sodium periodate.

Only traces of (23*S*,25*R*)-1α,25-$(OH)_2D_3$-26,23-lactone were detected in kidney and serum of the null mutant mice. This metabolite was only found when 1α,24(*R*),25$(OH)_3D_3$ was also detected (i.e., in only some of the mutant mice). To form the 1α,25$(OH)_2D_3$-26,23-lactone, 1α,25$(OH)_2D_3$ must undergo a 26-hydroxylation, a 23-hydroxylation, and an alcohol dehydrogenase reaction to convert the alcohol group on C-26 to an acid group. It is believed that the 23-hydroxylation as well as the 26-hydroxylation can be the first step in the pathway to form 1α,25$(OH)_2D_3$-26,23-lactone from 1α,25$(OH)_2D_3$ (*31*). Studies with the recombinant P450cc24 protein produced in bacteria (*32*) or insect cells (*33*) have shown that P450cc24 is capable of multicatalytic activity that includes 23-hydroxylation. It is likely that the absence of significant 23-hydroxylation in VDR null mutant mice prevents significant metabolism of 1,25$(OH)_2D_3$ to the corresponding lactone.

Despite the reduction of P450cc24 activity in VDR null mutant mice and thus impaired lactone formation, accumulation of 1α,25(*S*),26$(OH)_3D_3$ in null mutant mice was not observed. This might suggest that besides forming the 1α,25$(OH)_2D_3$-26,23-lactone, another degradation pathway starting with the 26-hydroxylation of 1,25$(OH)_2D_3$ may exist. However, besides 1α,25$(OH)_2D_3$, 1α,24(*R*),25$(OH)_3D_3$, and 1α,25$(OH)_2D_3$-26,23-lactone, no other chloroform-soluble metabolite was found in these mice. For our studies 1α,25-$(OH)_2$[26,27-^{3}H]D_3 was used, which is labeled in the side chain; therefore it cannot be excluded that there might be another unknown pathway after the 26-hydroxylation that involves cleavage of the side chain of the vitamin D molecule, leaving an unlabeled vitamin D metabolite that cannot be traced. To address these question it would be necessary to use ^{3}H-labeled 1,25$(OH)_2D_3$ of high specific activity in which tritium atoms are attached to A-, B-, C-, or D-ring carbons. However, such a metabolite is not commercially available so far.

At 12 and 24 h after administration of 1α,25$(OH)_2$[26,27-^{3}H]D_3, higher amounts of water-soluble metabolites were found in the kidneys than in the serum in the VDR null mutant mice. This is probably due to the fact that the enzymes involved in the degradation of 1,25$(OH)_2D_3$ are localized in the kidney (*8*, *31*). Also, higher amounts of water-soluble metabolites were detected in the liver than in the serum of the VDR null mutant mice. This may be due to the formation of 1,25$(OH)_2D_3$ monoglucuronides and sulfates of 1,25$(OH)_2D_3$ excreted in bile as previously described (*34*−*36*).

The interesting finding that in VDR null mutant mice 1α,25(S),26$(OH)_3D_3$ is synthesized leads to the conclusion that these mice might be a good model to study the tissue distribution as well as the regulation of the enzyme that catalyzes the 26-hydroxylation of 1,25$(OH)_2D_3$.

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