Calcitroic Acid, End Product of Renal Metabolism of 1,25-Dihydroxyvitamin D_3 through C-24 Oxidation Pathway†

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ABSTRACT: About a decade ago calcitroic acid was isolated as a major side chain cleaved water-soluble metabolite of 1,25-dihydroxyvitamin D_3 [Esvelt, R. P., Schnoes, H. K., & DeLuca, H. F. (1979) *Biochemistry 18*, 3977]. Presently, calcitroic acid is being considered as the major excretory form of 1,25-dihydroxyvitamin D_3. However, the exact site or sites of calcitroic acid production and the possible side chain modified intermediary metabolites that may be formed during the conversion of 1,25-dihydroxyvitamin D_3 into calcitroic acid are not fully understood. In the mean time there have been many advances in our understanding of the side-chain metabolism of 1,25-dihydroxyvitamin D_3. It is now well established that both the kidney and the intestine metabolize 1,25-dihydroxyvitamin D_3 through the C-24 oxidation pathway according to the following steps: 1,25-dihydroxyvitamin D_3 → 1,24,25-trihydroxyvitamin D_3 → 1,25-dihydroxy-24-oxovitamin D_3 → 1,23,25-trihydroxy-24-oxovitamin D_3. Recently, we identified 1,23-dihydroxy-24,25,26,27-tetranorvitamin D_3 (C-23 alcohol) as a major side chain cleaved lipid-soluble metabolite of 1,25-dihydroxyvitamin D_3 and further extended the aforementioned C-24 oxidation pathway in the kidney by demonstrating 1,23,25-trihydroxy-24-oxovitamin D_3 as the precursor of C-23 alcohol [Reddy, G. S., Tserng, K.-Y., Thomas, B. R., Dayal, R., & Norman, A. W. (1987) *Biochemistry 26*, 324]. In this present study, we investigated the metabolic fate of 1,25-dihydroxyvitamin D_3 (3×10^{-10} M) in the perfused rat kidney and identified calcitroic acid as the major water-soluble metabolite of 1,25-dihydroxyvitamin D_3. Furthermore, we also established C-23 alcohol as the immediate precursor of calcitroic acid (C-23 acid) and finally concluded that 1,25-dihydroxyvitamin D_3 is metabolized in the kidney through the C-24 oxidation pathway according to the following steps: 1,25-dihydroxyvitamin D_3 → 1,24,25-trihydroxyvitamin D_3 → 1,25-dihydroxy-24-oxovitamin D_3 → 1,23,25-trihydroxy-24-oxovitamin D_3 → C-23 alcohol → calcitroic acid. It now appears that the C-24 oxidation pathway for 1,25-dihydroxyvitamin D_3 probably exists in all the target tissues that respond to 1,25-dihydroxyvitamin D_3 and plays an important role in inactivating 1,25-dihydroxyvitamin D_3 and thereby regulates the tissue concentration of the active hormone, 1,25-dihydroxyvitamin D_3.

The conversion of vitamin D_3 into $1,25(OH)_2D_3$,[1] the hormonal form of vitamin D_3, is now universally accepted. The various factors responsible for the stringent physiological regulation of the production of $1,25(OH)_2D_3$ in the kidney are well studied (DeLuca, 1981, 1984; Norman et al., 1982; Kumar, 1984; Haussler, 1986). However, as the circulating levels of $1,25(OH)_2D_3$ depend not only on its rate of production but also on its rate of breakdown, it is also important to understand the target tissue metabolism and inactivation of $1,25(OH)_2D_3$. Frolik and DeLuca (1971, 1972) investigated the further metabolism of $1,25(OH)_2D_3$ in vivo and demonstrated for the first time that $1,25(OH)_2D_3$ is further metabolized into polar metabolites. Later studies from the same laboratory measured $^{14}CO_2$ in expired air following a dose of $1,25(OH)_2$[26,27-^{14}C]D_3 in chicks (Kumar & DeLuca, 1976) and rats (Harnden et al., 1976; Kumar et al., 1976; Kumar & DeLuca, 1977) and demonstrated a rapid and substantial oxidation of the 26- and 27-carbon atoms of $1,25(OH)_2D_3$. Following these observations, Esvelt et al. (1979) isolated a major side chain cleaved metabolite of $1,25(OH)_2D_3$ from the livers of the rats, and it was identified as 1-OH-23-COOH-24,25,26,27-tetranor-D_3 or calcitroic acid. Later, Onisko et al. (1980) identified calcitroic acid as the major biliary metabolite of $1,25(OH)_2D_3$ in rats. Esvelt and DeLuca (1981) examined the various tissues of the rat for the presence of calcitroic acid following a 120-ng dose of $1,25(OH)_2$[3-^{3}H]D_3. Calcitroic acid was detected in liver, intestinal mucosa, kidneys, and blood with liver and intestinal mucosa containing the highest concentrations. These results indicated the possibility of liver, intestine, and kidney as the most probable sites of calcitroic acid pro-

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[1] Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1,24(*R*),25$(OH)_3D_3$, 1,24(*R*),25-trihydroxyvitamin D_3; $1,25(OH)_2$-24-oxo-D_3, 1,25-dihydroxy-24-oxovitamin D_3; $1,23,25(OH)_3$-24-oxo-D_3, 1,23,25-trihydroxy-24-oxovitamin D_3; $1,23(OH)_2$-24,25,26,27-tetranor-D_3, 1,23-dihydroxy-24,25,26,27-tetranorvitamin D_3; 1-OH-23-COOH-24,25,26,27-tetranor-D_3, 1-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D_3; 1,23(*S*),25$(OH)_3D_3$, 1,23(*S*),25-trihydroxyvitamin D_3; 1,25(*S*),26$(OH)_3D_3$, 1,25(*S*),26-trihydroxyvitamin D_3; $1,25(OH)_2D_3$-26,23-lactone, 1,25(*R*)-dihydroxyvitamin D_3 26,23(*S*)-lactone; HPLC, high-performance liquid chromatography; CH_2N_2, diazomethane.

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duction. Thus, although calcitroic acid is presently being considered as a major excretory product of $1,25(OH)_2D_3$, the exact site or sites of its production and the possible side chain modified intermediary metabolites that may be formed during the conversion of $1,25(OH)_2D_3$ into calcitroic acid are still unknown. In the mean time, there have been many studies which advanced our understanding of side-chain metabolism of $1,25(OH)_2D_3$ (Holick, 1973; Horst et al., 1984; Ishizuka et al., 1984; Ishizuka & Norman, 1987; Mayer et al., 1983; Napoli & Horst, 1983; Napoli et al., 1983; Reinhardt et al., 1981; Tanaka et al., 1981). In a recent study (Reddy et al., 1987a), we identified $1,23(OH)_2$-24,25,26,27-tetranor-D_3 as the major renal metabolite of $1,25(OH)_2D_3$ under physiological conditions. This new metabolite of $1,25(OH)_2D_3$ appeared to be the logical precursor of calcitroic acid. Therefore, this present study was undertaken with the following aims: (1) to identify the kidney as one of the possible sites of calcitroic acid production; (2) to demonstrate $1,23(OH)_2$-24,25,26,27-tetranor-D_3 (C-23 alcohol) as the precursor of calcitroic acid (C-23 acid).

Materials and Methods

General. Ultraviolet absorbance spectra were taken in 2-propanol with a Beckman DU 8 recording spectrophotometer. High-performance liquid chromatography (HPLC) was performed with a Waters Model 600 equipped with a detector (Model 440) to monitor UV-absorbing material at 254 nm (Waters Associates, Milford, MA). Mass spectras (70 eV) were obtained on a Hewlett-Packard 5985B mass spectrometer. Samples of metabolites (0.5–1 μg) were introduced into the ion source maintained at 200 °C via a direct-insertion probe.

Vitamin D Compounds and Chemicals. Crystalline $1,25(OH)_2D_3$, $1,24(R),25(OH)_3D_3$, $1,23(S),25(OH)_3D_3$, $1,25(R)(OH)_2D_3$-26,23(*S*)-lactone, and calcitroic acid were a kind gift from Dr. M. R. Uskokovic, Hoffman-La Roche, Nutley, NJ. Authentic $1,25(OH)_2$-24-oxo-D_3, $1,23,25(OH)_3$-24-oxo-D_3, and $1,23(OH)_2$-24,25,26,27-tetranor-D_3 were all biologically synthesized in the rat kidney perfusion system as described in detail before (Reddy et al., 1987a). $1,25(OH)_2$-[1β-^{3}H]D_3 (specific activity 16 Ci/mmol) was a kind gift from Dr. S. Ishizuka, Teijin Institute for Biomedical Research, Tokyo, Japan. $1,25$-$(OH)_2$[1β-^{3}H]D_3 was synthesized by the method described by Holick et al. (1980). Diazomethane (CH_2N_2) was generated in ether by saponification of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide in a Diazald Kit (Aldrich Chemical Co., Milwaukee, WI).

Solvents. All solvents were from Burdick & Jackson Laboratories, Muskegan, MI.

Animals. Male Sprague-Dawley rats (about 350 g) purchased from Zivic-Miller Laboratories, Inc., Allison Park, PA, were fed a regular rodent diet, sufficient in calcium, phosphorus, and vitamin D.

Kidney Perfusion Technique. Kidney perfusions were performed exactly as described before in detail (Reddy et al., 1982, 1987a).

Extraction of Vitamin D Metabolites from the Kidney Perfusate. The procedure was previously described by Bligh and Dyer (1959) except for the following modifications. The first step in the extraction procedure was to precipitate the protein. This was accomplished by adding 10 mL of methanol and 4 mL of methylene chloride to each 5-mL perfusate sample. The mixture was then subjected to centrifugation. The protein pellet was discarded, and the supernatant was then resolved into organic and aqueous phases by adding an additional 16 mL of methylene chloride. The above extraction procedure was carried out at pH 8. Both the methylene chloride layer containing the lipid-soluble metabolites and the water–methanol layer containing the water-soluble metabolites were evaporated under nitrogen.

Study of $1,25(OH)_2D_3$ Metabolism in the Isolated Perfused Rat Kidney under Physiological Conditions. The renal metabolism of $1,25(OH)_2D_3$ at a substrate concentration of 3×10^{-10} M was studied by introducing 1 μCi of $1,25(OH)_2$[1β-^{3}H]D_3 (specific activity 16 Ci/mmol) into 200 mL of perfusate after 5 min of stabilization following the isolation of the kidney. The kidney perfusion was continued for 8 h. Aliquots of perfusate (10 mL) were taken out of the perfusion circuit at various time intervals as shown in Figure 1. The various lipid- as well as water-soluble metabolites of $1,25(OH)_2D_3$ in the perfusate samples were analyzed by the HPLC techniques described in the legends for Figures 1 and 2.

Isolation and Purification of Putative Calcitroic Acid from the Kidney Perfusate for Structural Identification. Three kidney perfusions were performed to generate calcitroic acid from $1,25(OH)_2D_3$ in a quantity sufficient for its structure identification process. Each kidney was perfused with 100 mL of perfusate containing 1 μCi of $1,25(OH)_2$[1β-^{3}H]D_3 mixed with 120 μg of cold $1,25(OH)_2D_3$. All the three perfusions were continued for 8 h. The final combined perfusate of the three perfusions was then subjected to the extraction procedure. The volume of the final combined perfusate (280 mL) was brought up to 300 mL by adding additional water, and the perfusate pH was adjusted to 8 with 0.1 N NaOH. A mixture of 600 mL of methanol and 240 mL of methylene chloride was then added to 300 mL of perfusate, and this resulted in precipitation of the perfusate protein, which was removed by centrifugation. To the supernatant (pH adjusted to 8) which was transferred to a separating funnel, an additional 960 mL of methylene chloride (pH adjusted to 8) was added to achieve phase separation between the methylene chloride layer and water–methanol layer. The methylene chloride layer was then separated from the water–methanol layer. By measuring the radioactivity in a small sample of the water–methanol layer, it was estimated that only 10% of total radioactivity was converted into water-soluble metabolite(s). The methanol in the water–methanol layer was removed by using a rotary evaporator. The remaining unevaporated perfusate water (about 240 mL) was then divided into 24 portions, and each portion was transferred into a 20-mL glass vial. A total of 24 glass vials each containing 10 mL of perfusate water were kept in a water bath at 35 °C, and the water was dried under nitrogen. The residue in each glass vial was then dissolved in 2 mL of 5% methanol in water, and the mixture was subjected to the pre-HPLC sample cleaning procedure described in the legend for Figure 2. The methanol washes (3 mL each) from all the 24 Sep-Pak cartridges were pooled together, and methanol was evaporated under nitrogen. The residue was then divided into 10 portions by dissolving in 1 mL of hexane–2-propanol–methanol (50:25:25 v/v). Each 100-μL portion of the residue was then subjected to a first HPLC step using a Zorbax-SIL column (4.5 mm × 25 cm) eluted with hexane–2-propanol–methanol (80:10:10 v/v) at a flow rate of 2 mL/min. The UV-absorbing material eluting at 24–28 mL (the elution volume of standard calcitroic acid) from all the 10 HPLC runs was pooled and dried under nitrogen. The residue was divided into four portions by dissolving in 400 μL of hexane–2-propanol–methanol (50:25:25 v/v). Each 100-μL portion of the residue was then subjected to a second HPLC run using a Zorbax-CN column (4.5 mm × 25 cm) eluted with hexane–2-propanol–methanol (90:5:5 v/v) at a flow rate of 2 mL/min. The UV-absorbing material eluting at 22–27 mL

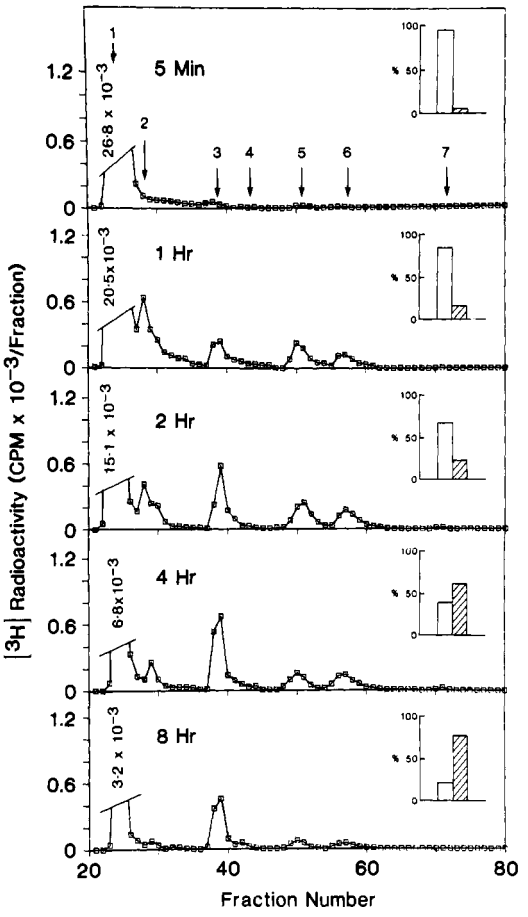

FIGURE 1: HPLC profiles of lipid-soluble metabolites of $1,25(OH)_2D_3$ (3×10^{-10} M) produced in an isolated perfused rat kidney. A kidney perfusion was performed with 1 μCi of $1,25(OH)_2[1\beta\text{-}^3H]D_3$ in 200 mL of perfusate for a period of 8 h. Samples of perfusate (10 mL each) obtained at various time points were subjected to the lipid extraction procedure described under Materials and Methods. The percent of total radioactivity distributed between the methylene chloride layer (open bars) and the water–methanol layer (hatched bars) was estimated by measuring radioactivity in $^1/_{20}$ of the total volume of each layer in duplicate. The results for each sample were shown in the inserts at various time points. The methylene chloride layer containing the various lipid-soluble metabolites was then analyzed by HPLC under the following chromatographic conditions: Zorbax-SIL column (25 cm × 4.6 mm); solvent, hexane–2-propanol (94:6 v/v); flow, 2 mL/min. Fractions (2 mL) were collected, and the amount of radioactivity in each fraction was determined. Unlabeled $1,25(OH)_2D_3$ (0.5 μg) was added to each 10-mL perfusate sample at the time of lipid extraction to monitor recovery of radioactive vitamin D metabolites. The recovery of unlabeled $1,25(OH)_2D_3$ in each perfusate sample as determined by its UV absorbance at 254 nm was as follows: 5 min, 88%; 1 h, 89%; 2 h, 91%; 4 h, 87%; 8 h, 93%. The elution positions of the various authentic standards of vitamin D_3 metabolites are shown by the arrows: (1) $1,25(OH)_2D_3$; (2) $1,25(OH)_2$-24-oxo-D_3; (3) $1,23(OH)_2$-24,25,26,27-tetranor-D_3; (4) $1,23(S),25(OH)_3D_3$; (5) $1,23,25(OH)_3$-24-oxo-D_3; (6) $1,24(R),25(OH)_3D_3$; (7) $1,25(R)(OH)_2D_3$-26,23(S)-lactone.

(the elution volume of standard calcitroic acid) from all the four HPLC runs was pooled and dried under nitrogen. The final purified material was then dissolved in 100 μL of hexane–2-propanol–methanol (50:25:25 v/v) and was resubjected to the first HPLC run. The UV-absorbing material eluting at 24–28 mL was collected and dried under nitrogen. The final purified material was dissolved in 5 mL of ethanol, and a UV spectrum was obtained. From the UV absorbance, it was estimated that the final yield of the total purified water-soluble vitamin D compound is 18.5 μg.

Conversion of Calcitroic Acid into Its Methyl Ester. About 2 μg of calcitroic acid isolated from the kidney perfusate was

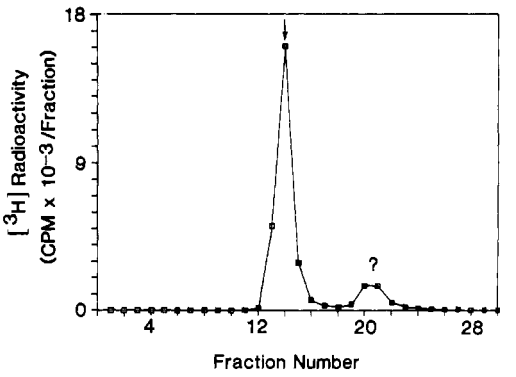

FIGURE 2: HPLC profile of water-soluble metabolites of $1,25(OH)_2D_3$ (3×10^{-10} M) produced in an isolated perfused rat kidney. The water–methanol layer obtained during the lipid extraction of the final perfusate sample (10 mL) of the kidney perfusion experiment described in the legend for Figure 1 was evaporated under nitrogen. The residue was dissolved in 2 mL of 5% methanol in water, and the mixture was subjected to centrifugation. The supernatant was then directly applied to a C-18 Sep-Pak cartridge (Waters Associates). All the salts and impurities were first eluted out of the cartridge by passing through 10 mL of water followed by 5 mL of 5% methanol in water. Highly polar metabolites of $1,25(OH)_2D_3$ were then eluted out of the cartridge by passing through 3 mL of methanol which was subsequently evaporated under nitrogen. The recovery of radioactivity during this pre-HPLC sample cleaning procedure was found to be 95 ± 3%, mean ± SE, $n = 3$. The residue obtained during the aforementioned pre-HPLC sample cleaning procedure was subjected to a straight-phase HPLC under the following chromatographic conditions: Zorbax-SIL column; (25 cm × 4.6 mm); solvent, hexane–2-propanol–methanol (80:10:10 v/v); flow, 2 mL/min. Fractions (2 mL) were collected, and the amount of radioactivity in each fraction was determined. Elution position of synthetic calcitroic acid was shown by the arrow. The radioactivity in the water–methanol layer resolved into two peaks. The major radioactive peak comigrated with calcitroic acid. The minor radioactive peak was more polar than calcitroic acid, and it contained about 10% of the total radioactivity. Unlabeled calcitroic acid (2 μg) was added to the perfusate sample before the extraction procedure to monitor recovery of water-soluble radioactive vitamin D metabolites. The recovery of unlabeled calcitroic acid, determined by its UV absorbance at 254 nm, was found to be 80 ● 5%, mean ± SE, n = 3.

dissolved in 100 μL of methanol, and an excess of diazomethane was added until the yellow color of diazomethane remained. The reaction was carried on for 5 min. The sample was dried under nitrogen, and the residue was then purified by a HPLC run using a Zorbax-SIL column (4.6 mm × 2.5 cm) eluted with hexane–2-propanol (95:5 v/v) at a flow rate of 2 mL/min. The methyl ester of the isolated water-soluble metabolite eluting at 33–35 mL was collected and subjected to mass spectrometry. Under the same experimental conditions, about 2 μg of synthetic calcitroic acid was also converted into its methyl ester and was subjected to mass spectrometry.

Study of $1,23(OH)_2$-24,25,26,27-tetranor-D_3 Metabolism in Perfused Rat Kidney. In order to demonstrate the conversion of $1,23(OH)_2$-24,25,26,27-tetranor-D_3 into calcitroic acid, the following experiment was performed. First, $1,23(OH)_2$-24,25,26,27-tetranor$[[1\beta\text{-}^3H]D_3$ was produced biologically by perfusing a rat kidney for 8 h with 20 μCi of $1,25(OH)_2[1\beta\text{-}^3H]D_3$. The authenticity of biologically produced $1,23(OH)_2$-24,25,26,27-tetranor$[1\beta\text{-}^3H]D_3$ was established by its comigration with the authentic cold standard of $1,23(OH)_2$-24,25,26,27-tetranor-D_3 on three different HPLC systems described in our recent publication (Reddy et al., 1987). A kidney perfusion was then performed with 50 mL of perfusate containing 0.2 μCi of $1,23(OH)_2$-24,25,26,27-tetranor$[1\beta\text{-}^3H]D_3$ for a period of 2 h. Both lipid- and water-soluble radioactive metabolites were analyzed under the chromatographic conditions described in Figure 4. A control

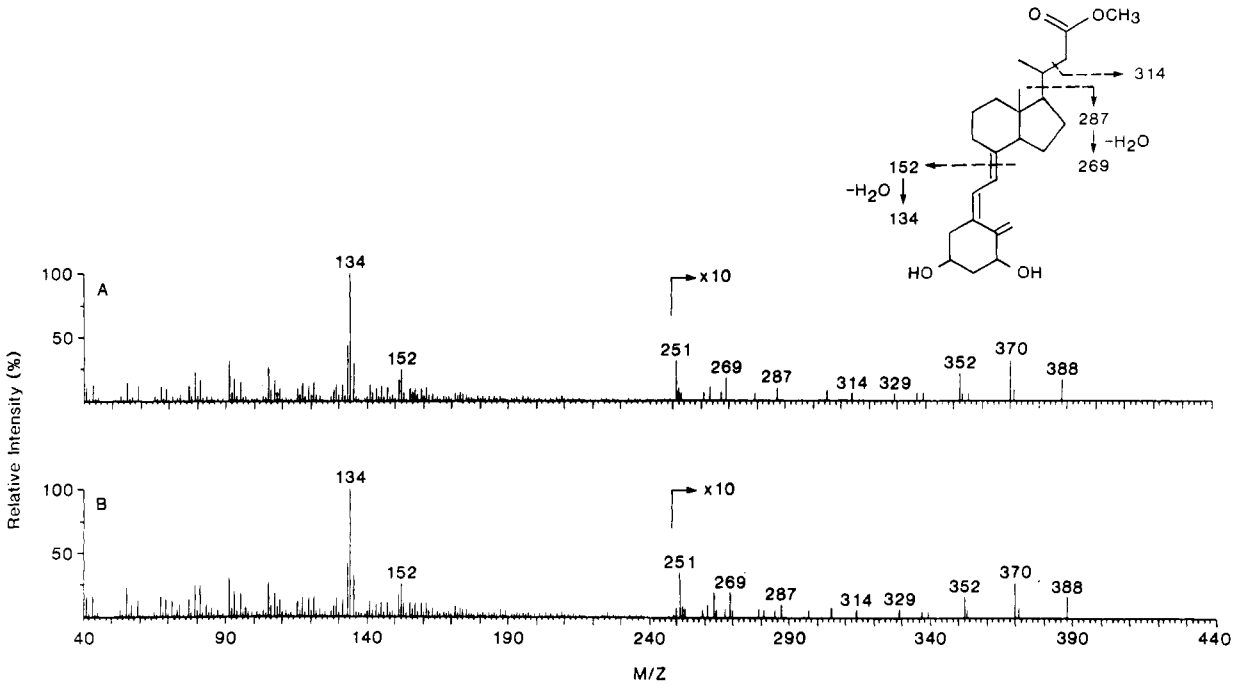

FIGURE 3: Mass spectra of (A) methyl esterified putative calcitroic acid isolated from kidney perfusate and (B) methyl esterified synthetic standard of calcitroic acid.

perfusion was performed in an identical fashion without the kidney in order to demonstrate the lack of further breakdown or metabolism of 1,23(OH)$_2$-24,25,26,27-tetranor[1β-^{3}H]D$_3$ in the perfusion apparatus in the absence of the kidney.

RESULTS

Demonstration of Calcitroic Acid as the Major Water-Soluble Renal Metabolite of 1,25(OH)$_2$D$_3$ under Physiological Conditions. From the results depicted in Figures 1 and 2, we obtained the following information regarding the metabolism of 1,25(OH)$_2$D$_3$ (3×10^{-10} M) by the isolated perfused rat kidney. During the methanol–methylene chloride lipid extraction of perfusate samples, we calculated the percent of total radioactivity distributed between the methylene chloride layer containing the lipid-soluble metabolites and the water–methanol layer containing the water-soluble metabolite(s). We noted that by 8 h of perfusion almost 75% of total perfusate radioactivity was in the water-soluble form (Figure 1). This finding is the first indirect evidence for the rapid metabolism of 1,25(OH)$_2$D$_3$ into water-soluble metabolite(s) in the kidney. By analyzing the methylene chloride layer for the various lipid-soluble metabolites of 1,25(OH)$_2$D$_3$, we found that the amount of 1,25(OH)$_2$D$_3$ (26.8×10^{-3} cpm) in the perfusate at 5 min of perfusion rapidly decreased to 3.2×10^{-3} cpm at 8 h of perfusion and this drop in 1,25(OH)$_2$D$_3$ concentration coincided with the increase in the formation of water-soluble metabolites. Further, it can be noted that all the various lipid-soluble metabolites of 1,25(OH)$_2$D$_3$ present in the perfusate are formed as a result of metabolism of 1,25(OH)$_2$D$_3$ through the C-24 oxidation pathway shown in Figure 5. Also, it is significant to note that 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$ is the major metabolite out of all the lipid-soluble metabolites of 1,25(OH)$_2$D$_3$. 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$ started accumulating in the perfusate at 1 h of perfusion, and by 2 h of perfusion it was the most predominant metabolite circulating in the perfusate. Its concentration increased until 4 h of perfusion, and at 8 h, a decrease in its concentration was noted due to its further metabolism. By analyzing the water–methanol layer with our newly developed straight-phase HPLC systems, we were able to demonstrate calcitroic acid as the major water-soluble metabolite of 1,25(OH)$_2$D$_3$ (Figure 2).

Structural Identification of Putative Calcitroic Acid Isolated from Kidney Perfusate. The water-soluble metabolite isolated from the kidney perfusate demonstrated a UV spectrum with an absorbance maximum at 265 nm and an absorbance minimum at 228 nm, indicating the presence of an intact 5,6-*cis*-triene chromophore (data not shown). Then we attempted to obtain the mass spectrum of the purified vitamin D compound without much success due to the nonvolatile nature of the isolated compound. Therefore, we converted the purified carboxylic acid metabolite into its methyl ester. The methyl esters of both the putative and the synthetic calcitroic acid exhibited the same retention time on the HPLC system described under Materials and Methods (data not shown). The mass spectrum of the methyl ester of putative calcitroic acid (Figure 3A) shows the following characteristics: m/z 388 (M^+), 370 ($M^+ - H_2O$), 352 ($M^+ - 2H_2O$), 287 (M^+ – side chain), 269 (M^+ – side chain – H_2O), 251 (M^+ – side chain – $2H_2O$), 152 [(A-ring fragment)$^+$], 134 [(A-ring fragment)$^+$ – H_2O]. All the aforementioned fragment ions at m/z 287, 269, 251, 152, and 134 are also present in the mass spectrum of 1,25(OH)$_2$D$_3$ and indicate that the secosteroid nucleus of its parent 1,25(OH)$_2$D$_3$ has remained unchanged and that the side chain is only altered. The presence of a side-chain methyl ester function can be confirmed by the peak at m/z 329 ($M^+ - COOCH_3$) and a peak at m/z 314, formed as a result of elimination of 74 mass units (CH_2COOCH_3 + H) via a McLafferty-type rearrangement. The occurrence of this rearrangement fragmentation also specifies a C-23 position for the carbomethoxy function. The same fragmentation pattern was also seen in the mass spectrum of the methyl ester of synthetic calcitroic acid standard (Figure 3B). Also, the mass spectrum of the methyl ester of putative calcitroic acid obtained in our study was identical with the previously published spectrum by Esvelt et al. (1979). Thus, the major water-soluble metabolite isolated from the kidney perfusate was unequivocally identified as calcitroic acid.

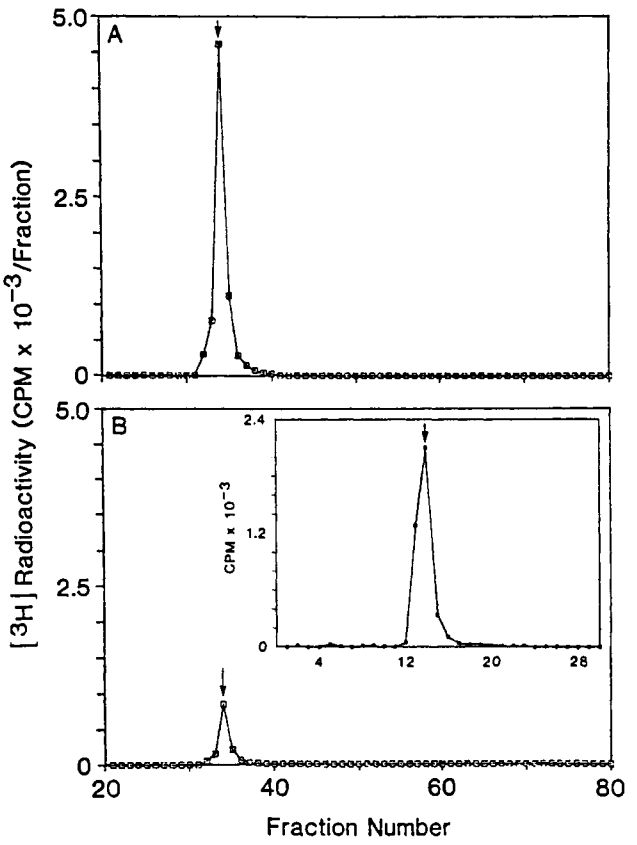

FIGURE 4: Metabolic conversion of 1,23(OH)$_2$-24,25,26,27-tetranor[1β-^{3}H]D_3 into calcitroic acid. (Panel A) A perfusion experiment was performed in the absence of kidney by passing through 50 mL of perfusate containing 0.2 μCi of 1,23(OH)$_2$-24,25,26,27-tetranor-[1β-^{3}H]D_3 in the perfusion apparatus for a period of 2 h. A 2-mL sample of final perfusate was subjected to the lipid extraction procedure described under Materials and Methods. More than 95% of total radioactivity in the perfusate sample was extracted into the methylene chloride layer. The radioactivity in the methylene chloride layer was then analyzed by HPLC under the following chromatographic conditions: Zorbax-SIL column (25 cm × 4.6 mm); solvent, hexane–2-propanol (93:7 v/v); flow, 2 mL/min. Fractions (2 mL) were collected, and the amount of radioactivity in each fraction was determined. The elution position of authentic cold 1,23(OH)$_2$-24,25,26,27-tetranor-D_3 was shown by the arrow. More than 90% of perfusate radioactivity was found in the 1,23(OH)$_2$-24,25,26,27-tetranor-D_3 peak. (Panel B) A perfusion experiment was performed in the presence of a kidney under the same conditions described in panel A. In this experiment only 21% of total perfusate radioactivity was extracted into the methylene chloride layer, and the rest remained in the water–methanol layer. The radioactivity in the methylene chloride layer was analyzed by HPLC under the same conditions described in panel A. The elution position of authentic cold 1,23-(OH)$_2$-24,25,26,27-tetranor-D_3 was shown by the arrow. About 18% of total perfusate radioactivity was present in the 1,23(OH)$_2$-24,25,26,27-tretranor-D_3 peak. The radioactivity in the water–methanol layer was analyzed by HPLC under the conditions described in the legend for Figure 2, and the results were depicted in the insert. The elution position of standard calcitroic acid was shown by an arrow. About 70% of total perfusate radioactivity was present in the calcitroic acid peak.

Demonstration of 1,23(OH)$_2$-24,25,26,27-tetranor-D_3 as Precursor of Calcitroic Acid. Figure 4 illustrates the conversion of 1,23(OH)$_2$-24,25,26,27-tetranor[1β-^{3}H]D_3 into a water-soluble metabolite whose chromatographic mobility was identical with that of synthetic calcitroic acid standard. Also, we established the stability of 1,23(OH)$_2$-24,25,26,27-tetranor[1β-^{3}H]D_3 in the perfusion apparatus in the absence of kidney. Thus, we have demonstrated that 1,23(OH)$_2$-24,25,26,27-tetranor-D_3 is enzymatically converted into calcitroic acid, and thereby established 1,23(OH)$_2$-24,25,26,27-tetranor-D_3 as the precursor of calcitroic acid.

Discussion

In this study we identified kidney as an organ capable of converting 1,25(OH)$_2D_3$ into calcitroic acid and demonstrated that calcitroic acid is the major water-soluble renal metabolite of 1,25(OH)$_2D_3$ under physiological conditions. In the original study that led to the discovery of calcitroic acid, Esvelt et al. (1979) isolated calcitroic acid from a water–methanol layer only after it was modified into its methyl ester because of the highly polar nature of calcitroic acid. In this study we developed a pre-HPLC sample cleaning procedure using C-18 Sep-Pak cartridges (Waters Associates) followed by two straight-phase HPLC systems which simplified the whole process of isolation and purification of the water-soluble metabolites in general. Calcitroic acid, isolated from the kidney perfusate, exhibited a typical UV spectrum that is produced by all the vitamin D compounds with an intact 5,6-*cis*-triene chromophore, and this finding indicates that the straight-phase HPLC systems developed in our laboratory are adequate to purify the putative calcitroic acid to homogeneity. Although we were able to isolate the putative calcitroic acid in pure form from the kidney perfusate, we failed to obtain its mass spectrum due to the nonvolatile nature of the compound. The same phenomenon was also noted with the synthetic calcitroic acid standard. Therefore, we obtained the mass spectrum of the putative calcitroic acid isolated from the kidney perfusate only after it was converted into its methyl ester. As discussed under Results, the mass spectrum of the isolated compound provided unequivocal evidence for the conversion of 1,25(OH)$_2D_3$ into calcitroic acid in the kidney. After identifying the kidney as the site of calcitroic acid production, we proceeded to understand the metabolic relationship between 1,23(OH)$_2$-24,25,26,27-tetranor-D_3 and calcitroic acid and presented direct evidence for the enzymatic conversion of 1,23(OH)$_2$-24,25,26,27-tetranor-D_3 (C-23 alcohol) into calcitroic acid (C-23 acid).

At present, it appears that the metabolism of 1,25(OH)$_2D_3$ in its various target organs occurs through two major metabolic pathways. One pathway (C-24 oxidation pathway) is initiated by C-24 hydroxylation and proceeds further as shown in Figure 5. Our present study firmly establishes calcitroic acid as the end product of the C-24 oxidation pathway. The other pathway (C-23 oxidation pathway) is initiated by C-23 hydroxylation and finally results in the formation of 1,25-(OH)$_2D_3$-26,23-lactone. Ishizuka and Norman (1987) recently clarified the metabolic pathways from 1,25(OH)$_2D_3$ to 1,25-(OH)$_2D_3$-26,23-lactone. The naturally occurring 1,25(*R*)-(OH)$_2D_3$-26,23(*S*)-lactone is produced from 1,23(*S*),25-(*R*),26(OH)$_4D_3$ which in turn is produced from 1,25(OH)$_2D_3$ through two possible metabolic pathways, the major one by way of 1,23(*S*),25(OH)$_3D_3$ and the minor one by way of 1,25(*R*),26(OH)$_3D_3$. It is also established that 1,23(*S*),25-(*R*),26(OH)$_4D_3$ is metabolized into 1,25(*R*)(OH)$_2D_3$-26,23-(*S*)-lactone via the formation of 1,25(*R*)(OH)$_2D_3$-26,23-(*S*)-lactol as the intermediate. Although 1,25(OH)$_2D_3$-26,23-lactone is one of the major circulating metabolites of 1,25(OH)$_2D_3$ in the plasma of rats (Ohnuma et al., 1980) and dogs given both pharamacological and physiological doses of 1,25(OH)$_2D_3$ (Ishizuka et al., 1984, 1988), the physiological role of 1,25(OH)$_2D_3$-26,23-lactone and its further metabolism are still unknown. In our isolated rat kidney perfusion system, using near physiological concentration (3×10^{-10} M) of 1,25(OH)$_2D_3$, we did not find 1,25(OH)$_2D_3$-26,23-lactone as a major metabolite in the kidney perfusate. Napoli and Martin (1984) also did not find 1,25(OH)$_2D_3$-26,23-lactone as a major

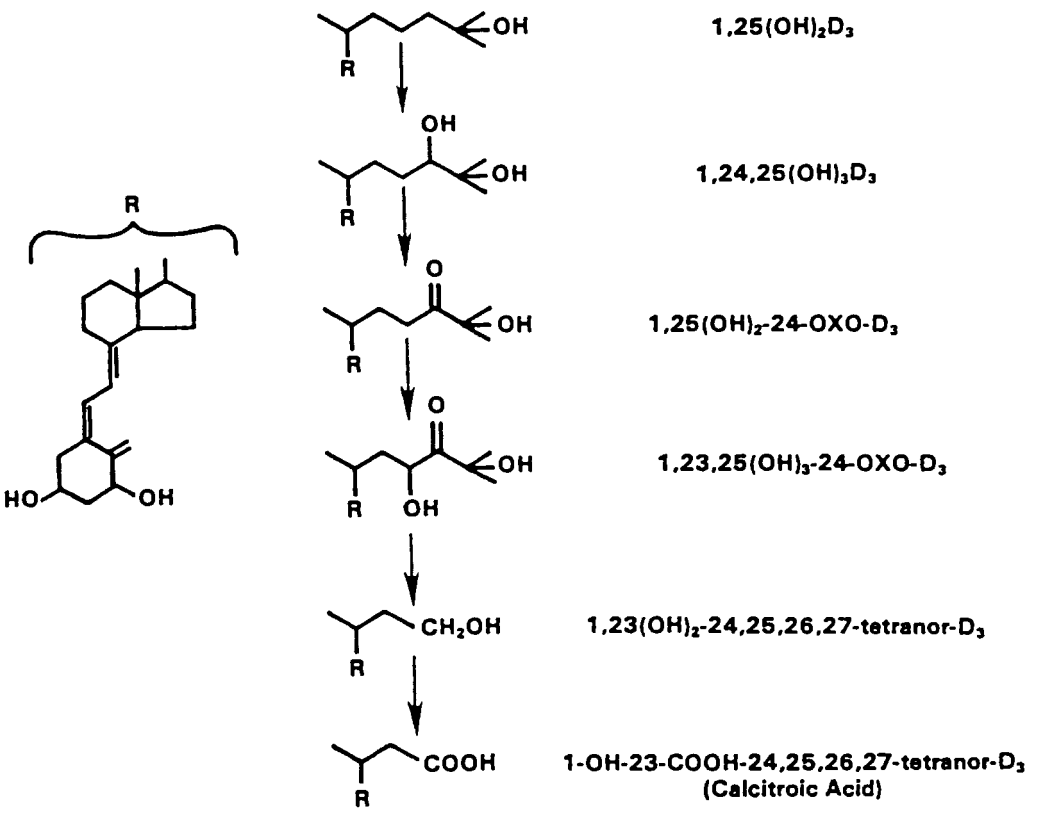

FIGURE 5: C-24 oxidation pathway of metabolism for $1,25(OH)_2D_3$ in kidney.

metabolite of $1,25(OH)_2D_3$ in the cultured kidney cells. Thus at present, it appears that under physiological conditions $1,25(OH)_2D_3$ is metabolized in vitro in the rat kidney predominantly through the C-24 oxidation pathway.

During the HPLC analysis of the water–methanol layer of kidney perfusate, we identified a minor metabolite which is more polar than calcitroic acid. It is estimated that about 10% of the total radioactivity in the water–methanol layer is represented by this minor metabolite peak and the rest of the radioactivity is represented by calcitroic acid (Figure 2). The identification and the exact chemical nature of this minor water-soluble metabolite peak is presently under investigation in our laboratory.

It is now becoming evident that the kidney and the other target tissues such as the intestine (Kumar et al., 1978), the bone (Howard et al., 1981; Lohnes & Jones, 1987), the cultured skin fibroblasts (Eil et al., 1986), and the human promyelocytic leukemic (HL-60) cells (Reichel et al., 1986; Reddy et al., 1987b) that respond to $1,25(OH)_2D_3$ possess 24-hydroxylase, the first enzyme of the C-24 oxidation pathway shown in Figure 5. From our study it has become obvious that under physiological conditions the kidney contains the full cascade of enzymes that are involved in inactivating $1,25-(OH)_2D_3$ through the C-24 oxidation pathway. Therefore, it is logical to predict that all the other target tissues of $1,25-(OH)_2D_3$, like the kidney, may also contain the full cascade of $1,25(OH)_2D_3$-inactivating enzymes and thereby metabolize $1,25(OH)_2D_3$ into calcitroic acid. Although calcitroic acid was originally isolated from liver, it is still unknown whether liver per se has the enzymatic ability to convert $1,25(OH)_2D_3$ into calcitroic acid. It appears that calcitroic acid, produced in the various target tissues of $1,25(OH)_2D_3$, circulates in the blood and finally is concentrated in the liver for its excretion into bile. On the basis of the results of our present study, one may expect to find calcitroic acid as the major excretory form of $1,25(OH)_2D_3$ in bile. However, Onisko et al. (1980) in their in vivo study reported that only 13% of the total dose of $1,25(OH)_2D_3$ administered to rats was present in bile (24-h collection) as calcitroic acid. Some of the possible reasons for finding a less amount of calcitroic acid in bile may be as follows: (1) A portion of calcitroic acid produced in the kidney and possibly other target organs of $1,25(OH)_2D_3$ may undergo further metabolism in the liver, resulting in the formation of as yet unidentified metabolite(s) which may be excreted into bile. (2) Also, $1,25(OH)_2D_3$ may be metabolized in the liver itself into biliary metabolites other than calcitroic acid, and at present, there is evidence to suggest that liver excretes $1,25(OH)_2D_3$ into bile in the form of glucuronides and sulfates (Kumar, 1984).

Under physiological conditions, the main function of the target tissue metabolism of $1,25(OH)_2D_3$ appears to be to maintain a steady-state concentration of the hormone, $1,25-(OH)_2D_3$, in its target tissues. Also, in situations where the target tissues are exposed to an excess concentration of $1,25(OH)_2D_3$, the whole process of inactivation of $1,25-(OH)_2D_3$ will be enhanced in an attempt to protect the organism from the toxic effects of the hormone. Thus, inactivation of $1,25(OH)_2D_3$ in its target tissues is a vital process. More studies are needed to understand the tissue specificity and the relative importance of C-24 and C-23 oxidation pathways in inactivating $1,25(OH)_2D_3$. Also, identification of the specific factors that can modulate the inactivation pathways of $1,25(OH)_2D_3$ will certainly enhance our understanding of vitamin D endocrine system in general.

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Registry No. $1,25(OH)_2D_3$, 32222-06-3; $1,24(R),25(OH)_3D_3$, 56142-94-0; $1,25(OH)_2$-24-oxo-D_3, 76338-50-6; $1,23,25(OH)_3$-24-oxo-D_3, 111820-83-8; calcitroic acid, 71204-89-2; C-23 alcohol, 97903-37-2.

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