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Rat Cytochrome P450C24 (CYP24) Does Not Metabolize 1,25-Dihydroxyvitamin D_2 to Calcitroic Acid

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Abstract 1α-Hydroxy-23 carboxy-24,25,26,27-tetranorvitamin D_3 (calcitroic acid) is known to be the major water-soluble metabolite produced during the deactivation of 1,25-$(OH)_2D_3$. This deactivation process is carried out exclusively by the multicatalytic enzyme CYP24 and involves a series of oxidation reactions at C_{24} and C_{23} leading to side-chain cleavage and, ultimately, formation of the calcitroic acid. Like 1,25-$(OH)_2D_3$, 1α,25-1,25-$(OH)_2D_2$ is also known to undergo side-chain oxidation and side-chain cleavage to form calcitroic acid (Zimmerman et al. [2001]. 1,25-$(OH)_2D_2$ differs from 1,25-$(OH)_2D_3$ by the presence of a double bond at C_{22} and a methyl group at C_{24}. To date, there have been no studies detailing the participation of CYP24 in the production of calcitroic acid from 1,25-$(OH)_2D_2$. We, therefore, studied the metabolism of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$ using a purified rat CYP24 system. Lipid and aqueous-soluble metabolites were prepared for characterization. Aqueous-soluble metabolites were subjected to reverse-phase high-pressure liquid chromatography (HPLC) analysis. As expected, 1,23$(OH)_2$-24,25,26,27-tetranor D and calcitroic acid were the major lipid and aqueous-soluble metabolites, respectively, when 1,25-$(OH)_2D_3$ was used as substrate. However, when 1,25-$(OH)_2D_2$ was used as substrate, 1,24(*R*),25-$(OH)_3D_2$ was the major lipid-soluble metabolite with no evidence for the production of either 1,23$(OH)_2$-24,25,26,27-tetranor D or calcitroic acid. Apparently, the CYP24 was able to 24-hydroxylate 1,25-$(OH)_2D_2$, but was unable to effect further changes, which would result in side-chain cleavage. These data suggest that the presence of either the double bond at C_{22} or the C_{24} methyl group impedes the metabolism of 1,25-$(OH)_2D_2$ to calcitroic acid by CYP24 and that enzymes other than CYP24 are required to effect this process. J. Cell. Biochem. 88: 282–285, 2003. Published 2002 Wiley-Liss, Inc.†

Key words: 24-hydroxylase; side-chain cleavage; vitamin D_2 metabolism

Vitamin D_3 and vitamin D_2 are used for supplementation of animal and human diets in the United States. Vitamin D_3 is the form of vitamin D that is synthesized by vertebrates, whereas vitamin D_2 is the major naturally occurring form of the vitamin in plants [Horst and Reinhardt, 1997]. The side-chain of vitamin D_2 differs from that of vitamin D_3 by the presence of an extra methyl group at C_{24} and a double bond between C_{22} and C_{23} [Napoli and Horst, 1984]. Vitamin D_2 and vitamin D_3 are equally active in mammals and are activated via a two-step process involving first hydroxylation at C_{25} followed by hydroxylation at C_1 to form the hormonally-active forms 1,25-$(OH)_2D_2$ [Jones et al., 1975] and 1,25-$(OH)_2D_3$ [Holick et al., 1971; Lawson et al., 1971; Norman et al., 1971]. The deactivation of 1,25-$(OH)_2D_3$ has been extensively studied and is carried out by a single enzyme (CYP24) [Akiyoshi-Shibata et al., 1994; Beckman et al., 1996]. The deactivation process

Abbreviations used: 25-OHD_2, 25-hydroxyvitamin D_2; 1,25-$(OH)_2D_2$, 1α,25-dihydroxyvitamin D_2; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1,25-$(OH)_2D_4$, 1α,25-dihydroxyvitamin D_4; 1,25-$(OH)_2$-22ene-D_3, 1α,25-dihydroxy-22-ene-vitamin D_3; 1,24(*R*),25-$(OH)_3D_2$, 1α,24(*R*),25-trihydroxyvitamin D_2; 1,25-$(OH)_2$-24-oxo-D_3, 1α,25-dihydroxy-24-oxo-vitamin D_3;1,23,25-$(OH)_3$-24-oxo-D_3, 1α,23,25-trihydroxy-24-oxo-vitamin D_3; 1,23-$(OH)_2$-24,25,26,27-tetranor-vitamin D_3, 1α,23-dihydroxy-24,25,26,27-tetranor-vitamin D_3; Calcitroic acid, 1α-hydroxy-23 carboxy-24,25,26,27-tetranorvitamin D_3; HPLC, high-pressure liquid chromatography.

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for 1,25-$(OH)_2D_3$ is initiated by C_{24} hydroxylation and proceeds through several side-chain oxidative steps leading ultimately to side-chain cleavage and formation of its excretory product, calcitroic acid [Makin et al., 1989; Reddy and Tserng, 1989]. Deactivation of 1,25-$(OH)_2D_2$ has received less attention; however, there is evidence that 1,25-$(OH)_2D_2$ can undergo side-chain modifications and side-chain cleavage to form calcitroic acid [Zimmerman et al., 2001]. The intermediates leading to side-chain cleavage and formation of the calcitroic acid from 1,25-$(OH)_2D_2$ and the involvement of the CYP24 in this process have yet to be determined.

In this report, we used a purified CYP24 system to determine if this enzyme can independently metabolize 1,25-$(OH)_2D_2$ to calcitroic acid.

MATERIALS AND METHODS

General

Analytical high-performance liquid chromatography (HPLC) of extracts was performed on a Waters Associates modular system (Waters Associates, Milford, MA) equipped with two model 510 solvent pumps, a U6K manual injector, a model 440 detector, and a model 996 photodiode array (PDA) detector. HPLC separation of metabolites was achieved using columns (0.45 cm × 25 cm) purchased from Supelco, unless otherwise noted. All solvents were purchased from Burdick and Jackson Laboratories (Muskegon, MI) or Fischer Scientific (Hannover Park, IL).

Sterols

The 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ were generously provided by Dr. Milan Uskokovic.

Extraction of Metabolites

Extraction of the metabolic products from incubations was performed as follows. Following the addition of methanol, the denatured protein was separated from soluble material by centrifugation. The remaining methanol/water phase was adjusted to pH 8.0 with 5-M sodium hydroxide and transferred to a separatory flask for extraction. One volume of methylene chloride was added and the mono-phasic solution was allowed to set for 15 min. An additional volume of methylene chloride was added to achieve phase separation. The methylene chloride layer containing the lipid soluble metabolites was removed and the aqueous layer was re-extracted with an additional volume of methylene chloride. The methylene chloride phases were combined and evaporated to dryness under vacuum and heat. The residue was resuspended in ethanol and retained for purification by HPLC.

The pH of the remaining methanol/water phase containing the calcitroic acid was adjusted to 4.5 with glacial acetic acid. The acidified solution was extracted two times with one volume of chloroform. The chloroform extracts were combined, dried, resuspended in ethanol, and retained for HPLC analysis. Using this procedure, we determined that ~80% of added calcitroic acid standard added to cell culture media could be recovered from the aqueous phase (data not shown).

CYP24 Incubations

Pure recombinant rat CYP24 was isolated and purified from *Escherichia coli* using adrenodoxin affinity and hydroxyapatite chromatography. Enzyme reconstitution reactions used CYP24 (1.6 μM), adrenodoxin (0.8 μM), and adrenodoxin reductase (1.6 μM) in the presence of 1 mM NADPH in 50 μM phosphate buffer at pH of 7.4. Substrate was present at 20 μM. Incubations were carried in the presence of substrate for 30 min at 37°C.

RESULTS

Metabolism of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$ to Lipid-Soluble Metabolites by CYP24

We subjected the lipid extracts from incubations to HPLC analysis. The HPLC analysis was done using a Zorbax Sil column (0.45 cm × 25 cm) developed in 25% methylene chloride with a gradient of 3.8–8.0% alcohols [isopropanol/methanol (2/1)] in hexane. Using this protocol, we established the presence of several lipid soluble metabolites with either 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_2$ as substrate. Five of the metabolites in the incubations with 1,25-$(OH)_2D_3$ as substrate could be identified by co-migration analysis as 1,25-$(OH)_2D_3$ (reaction substrate), 24-oxo-1,25-$(OH)_2D_3$, calcitroic acid, 24-oxo-1,23,25-$(OH)_3D_3$, and 1,24,25-$(OH)_3D_3$. Formation of these metabolites clearly confirmed the viability of the CYP24 system. When 1,25-$(OH)_2D_2$ was used as substrate, two major peaks appeared in the lipid soluble fraction

which were identified as 1,25-$(OH)_2D_2$ (reaction substrate) and 1,24(*R*),25-$(OH)_3D_2$.

Metabolism of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$ to Water-Soluble Metabolites by CYP24

Aqueous soluble metabolites were applied to a Supelco ODS column and eluted in the presence of 0.1% acetic acid using a gradient of 33–100% acetonitrile in water. Representative chromatograms from both the 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$ incubations are shown (Figs. 1B, 2B, respectively). Incubations with 1,25-$(OH)_2D_3$ yielded a major water-soluble peak co-migrating with standard calcitroic acid. Incubations with 1,25-$(OH)_2D_2$ as substrate, however, yielded no evidence of calcitroic acid formation.

DISCUSSION

In the present study, we compared the metabolism of 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ using a purified system containing only rat CYP24. Using this system, we were able to demonstrate the multi-catalytic nature of CYP24 in performing the various side-chain oxidative steps and ultimate side-chain cleavage, resulting in the production of calcitroic acid from 1,25-$(OH)_2D_3$. However, when 1,25-$(OH)_2D_2$ was used as substrate, we could demonstrate production of only 1,24(*R*),25-$(OH)_3D_2$ in identifiable amounts.

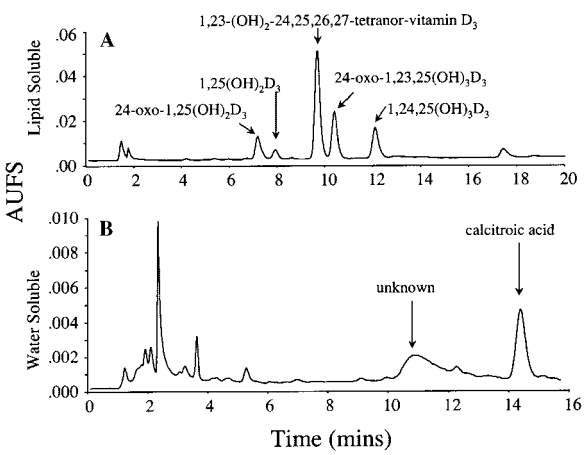

Fig. 1. HPLC profiles of the lipid-soluble (**A**) and water-soluble (**B**) metabolites produced by incubating 1,25-$(OH)_2D_3$ with purified CYP24. The various metabolites were identified by monitoring their UV absorbance and co-migration with known standards. The profiles demonstrate that 1,25-$(OH)_2D_3$ is clearly undergoing side-chain oxidation and conversion to intermediates leading to production of calcitroic acid.

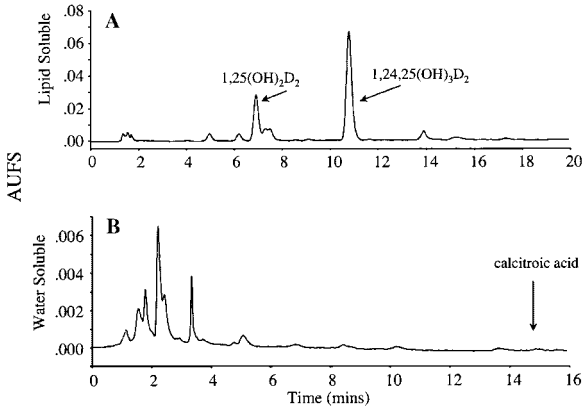

Fig. 2. HPLC profiles of the lipid-soluble (**A**) and water-soluble (**B**) metabolites produced by incubating 1,25-$(OH)_2D_2$ with purified CYP24. As shown in **Panel A**, the major quantifiable lipid-soluble metabolite of 1,25-$(OH)_2D_2$ was 1,24(*R*),25-$(OH)_3D_2$. There was no indication that 1,25-$(OH)_2D_2$ was being converted to calcitroic acid (**Panel B**).

There was no evidence of calcitroic acid production from 1,25-$(OH)_2D_2$. 1,25-Dihydroxyvitamin D_2 and its precursor 25-OHD_2 have been shown to be metabolized to water-soluble metabolites and, in the case of 1,25-$(OH)_2D_2$, to calcitroic acid by an undefined route in cell cultures and organ perfusions. Our results, coupled with earlier observations, would, therefore, suggest that metabolism of 1,25-$(OH)_2D_2$ to calcitroic acid clearly involves enzymes other than CYP24. The vitamin D_2 side chain contains a double bond between C_{22} and C_{23} as well as a C_{24} methyl group and it is unclear which modification may be responsible for impeding further metabolism by CYP24. Sunita Rao et al. [2001] demonstrated that the vitamin D analogue 1,25-$(OH)_2$-22-ene-D_3 could be metabolized to calcitroic acid by RWLue-4 cells and rat kidney. They suggested that the 1,25-$(OH)_2$-22-ene-D_3 was first hydroxylated at C_{24}, followed by further oxidation to 1,25-$(OH)_2$-24-oxo-22-ene-D_3 prior to side-chain, double-bond reduction to form 1,25-$(OH)_2$-24-oxo-D_3. The 1,25-$(OH)_2$- 24-oxo-D_3 is then further metabolized to calcitroic acid, presumably by CYP24. The compound 1,25-$(OH)_2D_4$ (a.k.a. 22,23 dihydro-1,25-$(OH)_2D_2$) has also been shown to be undergo side-chain oxidation similar to that of 1,25-$(OH)_2D_2$ in vitro [Byford et al., 2002] and metabolized to calcitroic acid in vivo [Tachibana and Tsuji, 2001]. Utilization of 1,25-$(OH)_2$-22-ene-D_3 and 1,25-$(OH)_2D_4$ in the purified CYP24

system will assist in determining the role of CYP24 (and other enzymes) in the further metabolism of 1,25-$(OH)_2D_2$ to calcitroic acid.

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