Rat Cytochrome P450C24 (CYP24) Does Not Metabolize 1,25-Dihydroxyvitamin D2 to Calcitroic Acid

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Abstract

1α-Hydroxy-23 carboxy-24,25,26,27-tetranorvitamin D3 (calcitroic acid) is known to be the major water-soluble metabolite produced during the deactivation of 1,25-(OH)2D3. This deactivation process is carried out exclusively by the multicatalytic enzyme CYP24 and involves a series of oxidation reactions at C24 and C23 leading to side-chain cleavage and, ultimately, formation of the calcitroic acid. Like 1,25-(OH)2D3,1α,25-(OH)2D2 is also known to undergo side-chain oxidation and side-chain cleavage to form calcitroic acid [Zimmerman et al. [2001]]. 1,25-(OH)2D2 differs from 1,25-(OH)2D3 by the presence of a double bond at C22 and a methyl group at C24. To date, there have been no studies detailing the participation of CYP24 in the production of calcitroic acid from 1,25-(OH)2D2. We, therefore, studied the metabolism of 1,25-(OH)2D3 and 1,25-(OH)2D2 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)2D3 was used as substrate. However, when 1,25-(OH)2D2 was used as substrate, 1,24(R),25-(OH)3D2 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)2D2, 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 C22 or the C24 methyl group impedes the metabolism of 1,25-(OH)2D2 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.

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for 1,25-(OH)\(_2\)D\(_3\) 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)\(_2\)D\(_2\) has received less attention; however, there is evidence that 1,25-(OH)\(_2\)D\(_2\) 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)\(_2\)D\(_2\) 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)\(_2\)D\(_2\) 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)\(_2\)D\(_2\) and 1,25-(OH)\(_2\)D\(_3\) 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 reextracted 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 acidic 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 hydroxypapite 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 mM 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)\(_2\)D\(_3\) and 1,25-(OH)\(_2\)D\(_2\) 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)\(_2\)D\(_3\) or 1,25-(OH)\(_2\)D\(_2\) as substrate. Five of the metabolites in the incubations with 1,25-(OH)\(_2\)D\(_3\) as substrate could be identified by co-migration analysis as 1,25-(OH)\(_2\)D\(_3\) (reaction substrate), 24-oxo-1,25-(OH)\(_2\)D\(_3\), calcitroic acid, 24-oxo-1,23,25-(OH)\(_3\)D\(_3\), and 1,24,25-(OH)\(_3\)D\(_3\). Formation of these metabolites clearly confirmed the viability of the CYP24 system. When 1,25-(OH)\(_2\)D\(_2\) was used as substrate, two major peaks appeared in the lipid soluble fraction.
which were identified as 1,25-(OH)₂D₂ (reaction substrate) and 1,24(R),25-(OH)₃D₂.

**Metabolism of 1,25-(OH)₂D₃ and 1,25-(OH)₂D₂ 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)₂D₃ and 1,25-(OH)₂D₂ incubations are shown (Figs. 1B, 2B, respectively). Incubations with 1,25-(OH)₂D₃ yielded a major water-soluble peak co-migrating with standard calcitroic acid. Incubations with 1,25-(OH)₂D₂ as substrate, however, yielded no evidence of calcitroic acid formation.

**DISCUSSION**

In the present study, we compared the metabolism of 1,25-(OH)₂D₂ and 1,25-(OH)₂D₃ 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)₂D₃. However, when 1,25-(OH)₂D₂ was used as substrate, we could demonstrate production of only 1,24(R),25-(OH)₃D₂ in identifiable amounts.

There was no evidence of calcitroic acid production from 1,25-(OH)₂D₂. 1,25-Dihydroxyvitamin D₃ and its precursor 25-OHD₂ have been shown to be metabolized to water-soluble metabolites and, in the case of 1,25-(OH)₂D₃, 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)₂D₂ to calcitroic acid clearly involves enzymes other than CYP24. The vitamin D₂ side chain contains a double bond between C₂₂ and C₂₃ as well as a C₂₄ 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-D₃ could be metabolized to calcitroic acid by RWLue-4 cells and rat kidney. They suggested that the 1,25-(OH)₂-22-ene-D₃ was first hydroxylated at C₂₄, followed by further oxidation to 1,25-(OH)₂-24-oxo-22-ene-D₃ prior to side-chain, double-bond reduction to form 1,25-(OH)₂-24-oxo-D₃. The 1,25-(OH)₂-24-oxo-D₃ is then further metabolized to calcitroic acid, presumably by CYP24. The compound 1,25-(OH)₂D₄ (a.k.a. 22,23 dihydro-1,25-(OH)₂D₂) has also been shown to undergo side-chain oxidation similar to that of 1,25-(OH)₂D₂ 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-D₃ and 1,25-(OH)₂D₄ in the purified CYP24
system will assist in determining the role of CYP24 (and other enzymes) in the further metabolism of 1,25-(OH)₂D₂ to calcitroic acid.

REFERENCES


