Characterization of recombinant CYP2C11: a vitamin D 25-hydroxylase and 24-hydroxylase

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Rahmaniyan, Mehrdad, Kennerly Patrick, and Norman H. Bell. Characterization of recombinant CYP2C11: a vitamin D 25-hydroxylase and 24-hydroxylase. Am J Physiol Endocrinol Metab 288: E753–E760, 2005. First published December 7, 2004; doi:10.1152/ajpendo.00201.2004.—Studies were performed to further characterize the male-specific hepatic recombinant microsomal vitamin D 25-hydroxylase CYP2C11, expressed in baculovirus-infected insect cells, and determine whether it is also a vitamin D 24-hydroxylase. 25- and 24-hydroxylase activities were compared with those of 10 other recombinant hepatic microsomal cytochrome P-450 enzymes expressed in baculovirus-infected insect cells. Each of them 25-hydroxylated vitamin D2, vitamin D3, 1α-hydroxyvitamin D2 (1αOHD2), and 1α-hydroxyvitamin D3 (1αOHD3). CYP2C11 had the greatest activity with these substrates, except vitamin D3, which had the same activity as four of the other enzymes. The descending order of 25-hydroxylation by CYP2C11 was 1αOHD3 > 1αOHD2 > vitamin D2 > vitamin D3. Each of the recombinant cytochrome P-450 enzymes 24-hydroxylated 1αOHD2. CYP2C11 had the greatest activity, 24-Hydroxylation of 1αOHD3 was very low, and there was none with vitamin D3. Only CYP2C11 24-hydroxylated vitamin D2. Structures of vitamin D metabolites, including vitamin D2, 1,24(S)-dihydroxyvitamin D2, and 1,24-dihydroxyvitamin D3, were confirmed by HPLC and gas chromatography retention times and characteristic mass spectrometric fragmentation patterns. In male rats, hypophysectomy significantly reduced body weight, liver weight, characteristic mass spectrometric fragmentation patterns. In male rats, hypophysectomy significantly reduced body weight, liver weight, hepatic CYP2C11 mRNA expression, and 24- and 25-hydroxylation of 1αOHD2. Expression of CYP2J3 and CYP2R1 mRNA did not change. In male rat hepatocytes, CYP2C11 mRNA expression and 24- and 25-hydroxylation were significantly reduced after culture for 24 h compared with uncultured cells. Expression of CYP2J3 and CYP2R1 either increased or did not change. It is concluded that CYP2C11 is a male-specific hepatic microsomal vitamin D 25-hydroxylase that hydroxylates vitamin D2, vitamin D3, 1αOHD2, and 1αOHD3. CYP2C11 is also a vitamin D 24-hydroxylase.

25-hydroxyvitamin D2; 24-hydroxyvitamin D2; liver microsomes; cytochrome P-450 enzymes

VITAMIN D ITSELF IS BIOLOGICALLY INACTIVE and must first undergo hydroxylation to become active. The vitamin is activated by conversion to 25-hydroxyvitamin D (25OHD) by vitamin D 25-hydroxylases in the liver (29, 30) and to 1,25-dihydroxyvitamin D [1,25(OH)2D] by the mitochondrial enzyme 25OHD-1α-hydroxylase (CYP27B1) in the kidney (16, 24, 28). 25OHD and 1,25(OH)2D are further hydroxylated to calcitroic acid in the kidney and elsewhere by the rate-limiting enzyme 25OHD-24-hydroxylase (CYP24A1) (17, 32, 35). In rats, hepatic 25-hydroxylase activity is present in both mitochondria and microsomes. CYP27A1 is a mitochondrial vitamin D 25-hydroxylase that is involved in the alternative pathway for bile acid synthesis from cholesterol (5, 12, 34), and CYP2C11 and the recently identified CYP2J3 are microsomal vitamin D 25-hydroxylases of rats (35). CYP2C11 is expressed in male but not female rat livers, whereas CYP2J3 is expressed in both male and female rat livers (34). 24-Hydroxylation of vitamin D2 and conversion of 24-hydroxyvitamin D2 to biologically active 1,24(S)-dihydroxyvitamin D2 [1,24(S)(OH)2D2] by CYP27B1 represent an alternative pathway for vitamin D2 metabolism (22). The purpose of the present studies is to characterize the substrate specificity of recombinant CYP2C11, to determine whether CYP2C11 is a vitamin D 24-hydroxylase, to compare its activity with that of other rat recombinant microsomal cytochrome P-450 enzymes, to confirm identity of the hydroxylation products, and to determine the effects of hypophysectomy.

MATERIALS AND METHODS

Materials. Rat recombinant cytochrome P-450 enzymes 1A1, 1A2, 2A2, 2B1, 2D1, 2D2, 3A1, 2C6, 2C11, 2C12, and 2C13 expressed in baculovirus-infected insect cells with cytochrome P-450 reductase, and in some cases cytochrome b5, and an NADPH-regenerating system were purchased from BD Gentest (Woburn, MA); vitamin D2, vitamin D3, 1,25(OH)2D3, succinic acid, iodonitrotetrazolium chloride, insulin, William’s medium E, and dexamethasone were from Sigma-Aldrich (St. Louis, MO); and acetonitrile, dichloromethane, hexanes, methanol, and 2-propanol were from Fisher Scientific (Norcross, GA). Gentamicin reagent was purchased from Gibco (Carlsbad, CA), and [3H-26,27-methyl]25OHD3 and [3H-26,27-methyl]25-hydroxyvitamin D3 ([3H-26,27-methyl][25OHD3]) were generously provided by Bone Care International (Madison, WI) and Leo Pharmaceutical Products (Ballerup, Denmark), respectively. 24-Hydroxyvitamin D2 (24OHD2) was kindly provided by Dr. Ronald Horst (Ames, Iowa). Primers for CYP2C11, CYP2J3, CYP2R1, and β-actin were purchased from Integrated DNA Technologies (Coralville, IA), and ethidium bromide was from Invitrogen Life Technologies (Carlsbad, CA). ProSTAR Single-Tube RT-PCR System was purchased from Stratagene (La Jolla, CA). Micro-to-Midi Total RNA Purification System was purchased from Amersham Biosciences (Piscataway, NJ), 1α-Hydroxyvitamin D2 (1αOHD2) and 1α-hydroxyvitamin D3 (1αOHD3) were purchased from Bone Care International (Madison, WI) and Leo Pharmaceutical Products (Ballerup, Denmark), respectively. 24-Hydroxyvitamin D2 (24OHD2) was kindly provided by Dr. Ronald Horst (Ames, Iowa). Primers for CYP2C11, CYP2J3, CYP2R1, and β-actin were purchased from Integrated DNA Technologies (Coralville, IA), and ethidium bromide was from Invitrogen Life Technologies (Carlsbad, CA). Sham-operated and hypophysectomized male and female Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Rat hepatocytes were kindly provided by Dr. Joelyn McMillan (Charleston, SC).

Assay of 25- and 24-hydroxylase activities. A 1-ml reaction mixture contained either 50 μl of hepatic microsomes (50–150 μg) or 50 μl of 25OHD2 and 24OHD2, respectively.
of microsomes from baculovirus-infected insect cells or suspensions of 2 million rat hepatocytes and 100 µl of 0.5 M Na2HPO4 (pH 7.4), 50 µl of 60 mM EDTA, 50 µl of NADPH regenerating solution A (BD Gentest), 10 µl of regenerating solution B (BD Gentest), 2 µl of 5 mM dianisidinolmethane, and 2 µl of 10 mM OHD3, 10 mM D3, vitamin D3, or vitamin D2. The final concentration of substrate was 10 µM. Unless otherwise mentioned, the reaction was performed at 37°C for 1.5 h and terminated with the addition of 1 ml of acetonitrile. Protein was measured by the bicinchoninic acid method (33). The reaction with 10 mM OHD3 as substrate was linear for 30 min. For enzyme kinetics, incubations were carried out for 20 min.

**Extraction of samples.** The acetonitrile mixture was mixed with a vortex; 1,000 counts/min (cpm) [3H]1,25-dihydroxyvitamin D3 ([3H]1,25(OH)2D3) or [3H]25OHD3, depending on the product, was added for recovery and then centrifuged at 4°C and 2,000 g for 15 min (13). The supernatant was decanted to another 13 × 100-mm glass tube containing 1 ml of 0.4 M K2HPO4 (pH 10.4, pH adjusted with KOH). The solution was mixed and transferred to a silica C18OH cartridge (DiaSorin, Stillwater, MN) that had been conditioned twice with 1.5 ml of methanol. The cartridge was washed with 5 ml of solvent A (methanol-water, 70:30), 5 ml of solvent B (hexanes-dichloromethane, 88:12), and 3 ml of solvent C (hexanes-2-propanol, 99:1) and eluted with 5 ml of solvent D (hexanes-2-propanol, 95:5) (13). The cartridges were washed with 1 ml of 2-propanol and conditioned with methanol as described above for further use. The eluted extracts were evaporated, the residue was dissolved in 200 µl of dichloromethane-hexanes-2-propanol (50:50:2) and subjected to HPLC. When either vitamin D2 or vitamin D3 was the substrate, the C18 column was washed twice with 2 ml of 2-propanol and regenerated twice with 2 ml of methanol. The supernatant, mixed with 0.5 M K2HPO4 (pH 10.4), was transferred as described above onto the regenerated C18 columns. The columns were washed with 5 ml of solvent A (methanol-water, 70:30) and eluted with 3.5 ml of acetonitrile. The solution was evaporated under N2, dissolved in 200 µl of hexanes-methylene chloride-2-propanol (50:50:2.5), and subjected to HPLC.

**Isolation and measurement of dihydroxylated vitamin D metabolites.** Extracts in 200 µl of solution were loaded onto a Zorbax Sil 4.6 × 250-mm column, and the metabolites were separated by HPLC with hexanes-2-propanol (85:15). 1,25-dihydroxyvitamin D2 [1,25(OH)2D2] and 1,25(OH)2D3 were quantified by measuring the area of the separated peaks, which eluted at 8.5 and 9.0 min, respectively. Recovery was assessed with [3H]1,25(OH)3D3 (13). Results are presented as nanomoles per nanomole per 1.5 h for rat recombinant cytochrome P-450 enzymes expressed in baculovirus-infected insect cells and in nanomoles per milligram protein per 1.5 h for hepatic microsomes. All measurements were carried out in triplicate.

**Isolation and measurement of monohydroxylate vitamin D metabolites.** The HPLC was performed as described above. 25-Hydroxyvitamin D2 (25OHD2), 25OHD3, and 24OHD2 were separated with the mobile-phase hexanes-2-propanol (24:1). The sterols eluted at 7.5 and 9.2 min, respectively, and were quantified by RIA as previously described (13). The reaction with 10 mM OHD3 as substrate was linear for 30 min. For enzyme kinetics, incubations were carried out for 20 min.

**Culture and incubation of rat hepatocytes.** Incubation of hepatocytes was carried out directly after cell isolation as described (26). Assays for enzyme activity were performed as described above. In a second experiment, 2 million rat hepatocytes from the same animal were cultured immediately after isolation in 60-mm plates containing William’s medium E, 10% heat-inactivated fetal bovine serum, insulin (5 µg/ml), gentamicin (1 mM), glucose (2 mM), and dexamethasone (1 mM). Cells were incubated for 24 h at 37°C with 5% CO2. After 24 h, medium was removed, and the cells were washed twice with serum-free medium. Serum-free medium containing 10 mM OHD3 at a final concentration of 10 µM was added to the cells, and incubation was continued for an additional 4 h. The reaction was terminated by adding acetonitrile, and extraction was performed as described above.

**RNA isolation and RT-PCR.** Total liver RNA was isolated with the Invitrogen Micro-to-Midi Total RNA Purification System according to the manufacturer’s protocol. Extracted RNA was stored at −80°C until use. RT-PCR amplification of 25-hydroxylases and β-actin was performed according to Pro-Star Single-Tube RT-PCR System from Stratagene. Primer sequences used in these experiments were as follows: CYP2C11 mRNA, 5′-TGG-CCC-CTT-TTT-ACG-AGG-CT-3′ and 5′-GGA-ACA-GAT-GAC-TGT-GAA-TTC-3′; CYP2J3 mRNA, 5′-CCT-GGA-TTG-TTA-CAT-TC-3′ and 5′-CTA-AGC-TCT-TCT-TTC-ATA-3′; CYP2R1 mRNA, 5′-GAG-CAA-GAC-GCT-GAA-AGT-GCA-A-3′ and 5′-CAG-TGT-ATT-TGT-GTT-TAC-3′; β-actin mRNA, 5′-GGA-GAA-GAT-TTG-GCA-CCA-3′ and 5′-TAG-AGC-CAC-CAC-TCC-A-3′. Fifteen nanograms of RNA were used for RT-PCR. RNA and other components were heated to 65°C for 4 min with a ramp time of 8 min to 25°C. They were incubated at 25°C for 30 s, then incubated for 1 h at 42°C; heated for 1 min at 95°C, and incubated for 5 min at 60°C. PCR conditions were 30 s for denaturation at 91°C, 30 s for annealing at 60°C, and 2 min for extension at 68°C, for 30 cycles. After the last cycle, samples were incubated for 10 min at 68°C. Twenty microliters of the PCR products were electrophoresed in agarose (1%) and then stained with ethidium bromide. Band intensities were measured as ratios of signal intensities of CYP2C11 and rat β-actin.

**Mass spectrometry of vitamin D metabolites.** The structural characterization of the metabolites was carried out by liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS). The LC-MS (API QStar Pulsar; PE Scieix Instruments, Boston, MA) provided molecular ions consistent with these hydroxylated species. The spray voltage was 800 V, and the collision energy was 25 V. N2 was the collision gas (13). Structural confirmation of 24OHD2, 1,24(S)OH3D2, and 1,25(OH)2D3 was performed by GC-MS (Agilent 6890 GC-5973N MS with Chemstation and Autosampler, Wilmington, DE). MS ionization was by electron impact at 70 eV, acquiring a mass-to-charge ratio (m/z) of 50–700, with the exception of 25OHD3, where selected ion monitoring of the molecular ion and qualifiers at m/z 466 (M-trimethylsilyl; M-18), 361 (C18MS), 357 (C17MS), 353 (C16MS), and 351 (C15MS) position cleavage, perhydroindenyl portion), and 131 was used to improve sensitivity. A fraction containing a metabolite from the HPLC separation or a reference standard in ethanol was evaporated to dryness in a tapered microvial insert (200 µl, spring fitted) with a stream of N2. N,O-bis(trimethylsilyl)trifluoroacetamide (Supelco, Bellefonte, PA; 25 µl) was added, and then the microvial was sealed in a 1.5-ml Autosampler vial with a Teflon-lined cap. After heating for ~24 h at 42°C, 1–2 µl were injected in the pulsed splitless mode onto a 5% phenylmethylsiloxane GC column (30 m × 0.25 mm, film 0.25 µm) with the helium linear velocity at 55 cm/s. The oven was held at 200°C for 1.5 min, followed by a 20°C/min ramp to a held temperature of 280°C. Under these GC conditions, the TMS derivatives of metabolite or standard of 1,24(OH)2D2 eluted at 16.2 min, 1,25(OH)2D3 eluted at 15.8 min, and 24OHD2 eluted at 15.1 min after injection.
RESULTS

Hydroxylation of vitamin D and its metabolites by recombinant cytochrome P-450 enzymes. 25- and 24-hydroxylation by rat recombinant CYP2C11 expressed in baculovirus-infected insect cells was determined with various vitamin D substrates. The descending order of 25-hydroxylation by CYP2C11 was 1αOHD₃ > 1αOHD₂ > vitamin D₂ > vitamin D₃ (Table 1). The descending order of 24-hydroxylation by CYP2C11 was 1αOHD₂ > vitamin D₂ > 1αOHD₃. Vitamin D₃ was not 24-hydroxylated. We next compared 25- and 24-hydroxylation activities of CYP2C11 with those of 10 other major rat recombinant hepatic microsomal cytochrome P-450 enzymes expressed in baculovirus-infected insect cells. In separate studies with 1αOHD₂ and 1αOHD₃ as substrates, each of them showed some activity, but CYP2C11 had the greatest activity for both 25- and 24-hydroxylation (Fig. 1, A and B). 25-Hydroxylation by CYP2C11 was 30% greater for 1αOHD₃ than for 1αOHD₂, whereas 24-hydroxylation by CYP2C11 was 30-fold greater for 1αOHD₂ than for 1αOHD₃. Each of the rat recombinant enzymes 25-hydroxylated vitamin D₂ and vitamin D₃ (Fig. 1C). With vitamin D₂ as substrate, CYP2C11 had 3.5-fold or greater 25-hydroxylation activity than any of the other cytochrome P-450 enzymes, and activity was seven times higher than with vitamin D₃ as substrate. 25-Hydroxylation of vitamin D₃ by CYP2C11 was similar to that of several of the other cytochrome P-450 enzymes.

Enzyme kinetic studies were performed for hydroxylations by CYP2C11 (Table 2). The $K_{m}$ for 25- and 26-hydroxylation of 1αOHD₂ was fourfold higher than the $K_{m}$ for 24-hydroxylation, and the $V_{max}$ for 25-hydroxylation of 1αOHD₂ was twofold higher than the $V_{max}$ for 24- and 25-hydroxylation. The $K_{m}$ for 24- and 26-hydroxylation of 1αOHD₃ was almost twofold higher than the $K_{m}$ for 25-hydroxylation, and the $V_{max}$ for 25-hydroxylation of 1αOHD₃ was fourfold or more higher than the $V_{max}$ for 24- and 26-hydroxylation. Activity of 24-hydroxylation of vitamin D₂ by recombinant CYP2C11 was too low to allow determination.

Identification of vitamin D metabolites. Eluates from HPLC showed either two or three peaks. The first peaks were found to contain 24-hydroxylated products, the second to contain 25-hydroxylated products, and the third to contain 26-hydroxylated products (Fig. 2, A–C). Structures of TMS derivatives of 1,25(OH)₂D₂ and 1,25(OH)₂D₃ were confirmed by LC and GC retention times, by appropriate molecular ions (M⁺, LC-MS and GC-MS), and by electron impact fragmentation patterns being consistent with those of respective reference standards (Table 3). The base peak ion of $m/z$ 131 for each of the

Table 1. 24- and 25-hydroxylation of vitamin D₂, vitamin D₃, 1αOHD₂, and 1αOHD₃ by rat recombinant CYP2C11

<table>
<thead>
<tr>
<th>Substrate</th>
<th>25-Hydroxylation Activity, nmol/mmol·h⁻¹</th>
<th>24-Hydroxylation Activity, nmol/mmol·h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D₂</td>
<td>0.49±0.03</td>
<td>3.34±0.57</td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>0.07±0.02</td>
<td>ND</td>
</tr>
<tr>
<td>1αOHD₂</td>
<td>12.27±0.84</td>
<td>15.63±0.65</td>
</tr>
<tr>
<td>1αOHD₃</td>
<td>17.79±1.90</td>
<td>0.49±0.03</td>
</tr>
</tbody>
</table>

Results are means ± SE of 3 determinations. Substrate concentration was 10 μM. 1αOHD₂, 1α-hydroxyvitamin D₂; 1αOHD₃, 1α-hydroxyvitamin D₃; ND, not detected.

25-hydroxy isomers resulted from α-cleavage (C₂₄-C₂₅ bond scission) and distinguishes the mass spectra from that of 24- or 26(27)-hydroxylated isomers (13). The corresponding α-cleavage product of the 26(27)-hydroxyl isomer yielded $m/z$ 103 ions (C₂₅-C₂₆ bond scission). TMS molecular ions were de-
Table 2. \( K_m \) and \( V_{max} \) values for hydroxylation of 1\( \alpha \)OHD\(_2\) and 1\( \alpha \)OHD\(_3\) by recombinant CYP2C11

<table>
<thead>
<tr>
<th>Hydroxylation</th>
<th>( K_m ), ( \mu M )</th>
<th>( V_{max} ), nmol/nmol (-1) min (^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1( \alpha )OHD(_2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24(OH)ase</td>
<td>4.24±1.14</td>
<td>1.02±0.05</td>
</tr>
<tr>
<td>25(OH)ase</td>
<td>18.10±5.46</td>
<td>0.98±0.18</td>
</tr>
<tr>
<td>26(OH)ase</td>
<td>18.06±4.29</td>
<td>2.15±0.30</td>
</tr>
<tr>
<td>1( \alpha )OHD(_3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24(OH)ase</td>
<td>5.78±1.50</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>25(OH)ase</td>
<td>2.59±0.40</td>
<td>0.61±0.02</td>
</tr>
<tr>
<td>26(OH)ase</td>
<td>5.08±1.03</td>
<td>0.14±0.01</td>
</tr>
</tbody>
</table>

Results are means ± SE of 3 determinations. 1\( \alpha \)OHD\(_2\), 1\( \alpha \)-dihydroxyvitamin D\(_2\); 1\( \alpha \)OHD\(_3\), 1\( \alpha \)-dihydroxyvitamin D\(_3\); 24(OH)ase, 24-hydroxylase; 25(OH)ase, 25-hydroxylase; 26(OH)ase, 26-hydroxylase.

Discussion

A male-specific hepatic microsomal enzyme with 25-hydroxylase activity was isolated and purified, and the eight-aminoc acid NH\(_2\) terminus was sequenced (2, 14). On the basis of CDNA and DNA sequences, the enzyme was subsequently identified as CYP2C11 (2, 14). CYP2C11 is the most abundant hepatic cytochrome P-450 enzyme in male rats and represents as much as 50% of cytochrome P-450 in liver (3, 9, 23). The present studies with recombinant CYP2C11 indicate that 25-
Hydroxylation of vitamin D and vitamin D-related compounds occurs in the rank order 1αOHD3 > 1αOHD2 > vitamin D3 and ratio 25:24:7:1. Hydroxylation of vitamin D3 by CYP2C11 was no greater than that by CYP2D1, CYP2C6, CYP2C12, and CYP2C13. Thus 25-hydroxylation by CYP2C11 of vitamin D3 was much lower than that of vitamin D2 and similar to hydroxylation by other hepatic cytochrome P450 enzymes not known to have vitamin D 25-hydroxylase activity. Recently, CYP2J3, a cytochrome P-450 enzyme that modulates the metabolism of arachidonic acid, was identified as a hepatic microsomal vitamin D 25-hydroxylase in the rat (34). Purified recombinant CYP2J3 expressed in Escherichia coli was found to be very effective in 25-hydroxylating vitamin D3 and 1αOHD3. 25-Hydroxylation of vitamin D2 and 1αOHD2 was not determined. Unlike CYP2C11, which is expressed in male but not female rat livers, CYP2J3 is expressed in the livers of both male and female animals (34). CYP2R1 is thought not to be of physiological significance because of low hepatic expression (34). In that study, it was noted that, whereas hepatic expression of CYP2C11 is 20-fold higher than expression of CYP2J3 as determined by real-time PCR, 25-hydroxylase activity of CYP2C11 is 15-fold lower than the activity of CYP2J3 (34). In rat hepatocytes cultured for 24 h compared with freshly isolated cells, we found that 25-hydroxylase activity had decreased by 67%, expression of CYP2C11 had decreased by 40%, expression of CYP2J3 had increased by 44%, and expression of CYP2R1 had not changed. After hypophysectomy, hepatic expression of CYP2C11 had decreased by 73% and 25-hydroxylase activity by 64%, whereas expression of CYP2J3 and CYP2R1 had not changed. Thus, in both studies, vitamin D 25-hydroxylase activity was associated with expression of CYP2C11 and not with CYP2J3 or CYP2R1. These findings provide evidence that, despite its low specific activity, CYP2C11 is an important hepatic microsomal vitamin D 25-hydroxylase in the male rat because of its abundance.

CYP27A1, an enzyme involved in the alternate pathway for conversion of cholesterol to bile acids, is the only mitochondrial vitamin D 25-hydroxylase (4, 5, 12). Whereas hepatic expression of CYP27A1 was found to be about one-half that of CYP2J3, 25-hydroxylase activities of the two enzymes are similar (34). There is no sex difference in expression of CYP27A1 or CYP2J3. CYP2J3 is thought to be the major hepatic microsomal 25-hydroxylase in female rats (34). Mitochondrial 25-hydroxylation of vitamin D is higher in male rats, whereas mitochondrial 25-hydroxylation of vitamin D is higher in female rats (30). However, serum 25OHD is not different in male and female rats (10).

24-Hydroxylation of vitamin D and vitamin D-related compounds by CYP2C11 occurs in the rank order 1αOHD2 >

### Table 3. Gas chromatography-mass spectrometry of vitamin D metabolites

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Molecular Ion, m/z</th>
<th>Major Fragments, m/z</th>
<th>Retention Time, min</th>
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</thead>
<tbody>
<tr>
<td>24OHD$_2$</td>
<td>556 513</td>
<td>451 423 333 143</td>
<td>15.1</td>
</tr>
<tr>
<td>25OHD$_2$*</td>
<td>556 466 361</td>
<td></td>
<td>13.1 16.0</td>
</tr>
<tr>
<td>25OHD$_3$</td>
<td>544 529 439 413 349</td>
<td>131 143</td>
<td>14.8</td>
</tr>
<tr>
<td>1,24(OH)$_2$D$_2$</td>
<td>644 601 554 513 331</td>
<td>143 16.2</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)$_2$D$_2$</td>
<td>644 554 513 464 432</td>
<td>131 17.1</td>
<td></td>
</tr>
<tr>
<td>1,26(OH)$_2$D$_2$</td>
<td>644 554 513 464 374</td>
<td>103 17.8</td>
<td></td>
</tr>
<tr>
<td>1,24(OH)$_2$D$_3$</td>
<td>632 542 501 452 404</td>
<td>145 15.3</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)$_2$D$_3$</td>
<td>632 542 501 452 362</td>
<td>103 17.2</td>
<td></td>
</tr>
<tr>
<td>1,26(OH)$_2$D$_3$</td>
<td>632 542 501 452 362</td>
<td>103 17.2</td>
<td></td>
</tr>
</tbody>
</table>

*Acquired by selected ion monitoring (see MATERIALS AND METHODS). 24OHD$_2$, 24-hydroxyvitamin D$_2$; 25OHD$_2$, 25-hydroxyvitamin D$_2$; 25OHD$_3$, 25-hydroxyvitamin D$_3$; 1,24(OH)$_2$D$_2$, 1,24-dihydroxyvitamin D$_2$; 1,25(OH)$_2$D$_2$, 1,25-dihydroxyvitamin D$_2$; 1,26(OH)$_2$D$_2$, 1,26-dihydroxyvitamin D$_2$; 1,24(OH)$_2$D$_3$, 1,24-dihydroxyvitamin D$_3$; 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; 1,26(OH)$_2$D$_3$, 1,26-dihydroxyvitamin D$_3$.

Substrate concentration was 10 μM. Results are means ± SE of 4 determinations. *P < 0.001 vs. control.

Fig. 3. Effects of 24-h incubation of hepatocytes of male rats compared with freshly isolated cells on 25-hydroxylation (A) and 24-hydroxylation (B) of 1αOHD$_2$ and expression of CYP2C11, CYP2J3, and CYP2R1 mRNA (C). Substrate concentration was 10 μM. Results are means ± SE of 4 determinations. *P < 0.001 vs. control.
Vitamin D2 is 24-hydroxylated by CYP2C11 and that CYP2C11 mRNA and 24-hydroxylase activity in hepatic microsomes are reduced by hypophysectomy and decline in hepatocytes cultured for 24 h compared with freshly isolated cells provide evidence that CYP2C11 is an important enzyme for 24-hydroxylation of vitamin D2.

In rats, 24OHD2 and 25OHD3 show similar activity in stimulating intestinal calcium transport and bone resorption. The fact that biological activity of 24OHD2 is abolished by nephrectomy indicates that 24OHD2 must undergo conversion to 1,25(OH)2D2 to become activated (22). When given separately to vitamin D-deficient rats, 1α-hydroxylation of 24OHD2, 25OHD2, and 25OHD3 is similar. However, when given simultaneously, 1α-hydroxylation of 24OHD2 is less efficient than 1α-hydroxylation of either 25OHD2 or 25OHD3 (22). Furthermore, binding of 1,24(OH)2D2 to the vitamin D receptor of the thymus is less efficient compared with the binding of 1,25(OH)2D2 and 1,25(OH)2D3 (22). 1,24(S)OH2D2 has less biological activity than 1,25(OH)2D3. It was less effective than 1,25(OH)2D3 in increasing serum calcium in vitamin D-induced by the male pattern of secretion of growth hormone (GH), which in turn is positively regulated by testosterone (1, 6, 9, 15). Hepatic expression of CYP2C11 and microsomal 25-hydroxylase activity is suppressed by hypophysectomy and restored by treatment with GH and not testosterone (6, 15, 32). The present findings that vitamin D2 is 24-hydroxylated by CYP2C11 and that CYP2C11 mRNA and 24-hydroxylase activity in hepatic microsomes are reduced by hypophysectomy and decline in hepatocytes cultured for 24 h compared with freshly isolated cells provide evidence that CYP2C11 is an important enzyme for 24-hydroxylation of vitamin D2.

Fig. 4. Effects of hypophysectomy on 25-hydroxylation (A) and 24-hydroxylation (B) of 1αOHD2 and expression of CYP2C11, CYP2J3, and CYP2R1 mRNA (C) by hepatic microsomes of male rats. Substrate concentration was 10 μM. Incubations were carried out for 1.5 h. Results are means ± SE of 9 determinations. *P < 0.01 and **P < 0.001 vs. control.

Fig. 5. Relative expression of CYP-to-β-actin mRNA ratio in liver (A) and freshly isolated hepatocytes (B) of male rats. Results are means ± SE of 4 determinations. *P < 0.001 vs. CYP2C11.
deficient rats and less effective than 1,25(OH)2D3 in increasing urinary calcium in normal rats (21). 24-Hydroxylation of vitamin D2 and its subsequent hydroxylation by CYP27B1 in the kidney to form 1,25(OH)2D2 represent an alternative pathway for vitamin D2 metabolism (21, 22).

CYP2C11 is a constitutively expressed cytochrome P-450 enzyme in the male rat, and expression is modulated by GH (1, 9, 11, 15). Antibodies to CYP2C11 inhibited 25-hydroxylase activity by > 80% in hepatic microsomes of male rats but had no effect in female rats (9). A number of studies show that the primary factor responsible for male-specific expression of CYP2C11 in liver is the pulsatile pattern of secretion of GH (1, 9). Specifically, the length of the trough period and not peak height, width, or frequency of circulating GH pulses is critical for hepatic expression. CYP2C12, which has only minimal 25-hydroxylase activity, is expressed only in female and not in male rat livers (30). Signal transducer and activator of transcription protein 5b (STAT5b) is a key intracellular mediator of GH induction of CYP2C11. In the absence of the male GH pattern of secretion, hepatic STAT5b is downregulated (9).

Finally, CYP2R1 was recently identified as a microsomal enzyme that 25-hydroxylates vitamin D2 and vitamin D3 equally in mice and humans (8). Its confirmation as a key vitamin D 25-hydroxylase in humans was confirmed by the finding of a homozygous inactivating L99P mutation of the CYP2R1 gene in a young black boy from Nigeria who had isolated 25OHD deficiency and rickets that responded to pharmacological doses of vitamin D2 (7). We found that hepatic expression of CYP2R1 in male rats did not correlate with changes in 25-hydroxylation activity produced either by hypophysectomy or by culture of hepatocytes. Again, previous studies showed that the ratio of hepatic expression of CYP2C11, CYP2J3, and CYP2R1 by real-time PCR in male rats is 140:7:1 (34). With a different method for determining mRNA, we found smaller differences in expression of the three vitamin D 25-hydroxylases in liver microsomes and hepatocytes and attribute the difference in results to a difference in methodology. CYP2R1 is equally expressed at low concentrations in livers of male and female rats (35), but the 25-hydroxylation activity of CYP2R1 in this species has not been determined. Because of its low abundance in liver, the physiological importance of CYP2R1 in rats as regards vitamin D metabolism has been questioned (34).

In summary, recombinant CYP2C11, in descending order, 25-hydroxylates 1αOHD3, 1αOHD2, vitamin D2, and vitamin D3 and 24-hydroxylates 1αOHD2, vitamin D2, and 1αOHD3 but not vitamin D3. Products of hydroxylation were identified by HPLC and GC retention times and MS fragmentation patterns. 25- and 24-hydroxylation of 1αOHD2 and hepatic expression of microsomal CYP2C11 mRNA were decreased by hypophysectomy and by incubation of cells for 24 h. Hepatic expression of CYP2J3 and CYP2R1 either increased or did not change. The results support the view that, in male rats, CYP2C11 is an important microsomal vitamin D 25-hydroxylase and 24-hydroxylase.

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