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Metabolism of 20-hydroxyvitamin D3 by mouse liver microsomes

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Abstract

20-Hydroxyvitamin D3 [20(OH)D3], the major product of CYP11A1 action on vitamin D3, is biologically active and like 1,25-dihydroxyvitamin D3 [1,25(OH)₂D3] can inhibit proliferation and promote differentiation of a range of cells, and has anti-inflammatory properties. However, unlike 1,25(OH)₂D3, it does not cause toxic hypercalcemia at high doses and is therefore a good candidate for therapeutic use to treat hyperproliferative and autoimmune disorders. In this study we analyzed the ability of mouse liver microsomes to metabolize 20(OH)D3. The two major products were identified from authentic standards as 20,24-dihydroxyvitamin D3 [20,24(OH)₂D3] and 20,25-dihydroxyvitamin D3 [20,25(OH)₂D3]. The reactions for synthesis of these two products from 20(OH)D3 displayed similar K_m values suggesting that they were catalyzed by the same cytochrome P450. Some minor metabolites were produced by reactions with higher K_m values for 20(OH)D3. Some metabolites gave mass spectra suggesting that they were the result of hydroxylation followed by dehydrogenation. One product had an increase in the wavelength for maximum absorbance from 263 nm seen for 20(OH)D3, to 290 nm, suggesting a new double bond was interacting with the vitamin D-triene chromophore. The two major products, 20,24(OH)₂D3 and 20,25(OH)₂D3 have both previously been shown to have higher potency for inhibition of colony formation by melanoma cells than 20(OH)D3, thus it appears that metabolism of 20(OH)D3 by mouse liver microsomes can generate products with enhanced activity.

Keywords

20-Hydroxyvitamin D3; Microsomes; Hydroxylation; Cytochrome P450; Liver

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1. Introduction

Purified CYP11A1 acts on vitamin D3 to produce 20-hydroxyvitamin D3 [20(OH)D3] as the major metabolite, some of which is metabolized by the same enzyme to 20,23-dihydroxyvitamin D3 [20,23(OH)₂D3], 20,22-dihydroxyvitamin D3 [20,22(OH)₂D3], 17,20-dihydroxyvitamin D3 [17,20(OH)₂D3] and 17,20,23-trihydroxyvitamin D3 (1–6). Nuclear magnetic resonance (NMR) of 20(OH)D3 produced by CYP11A1 indicates that it is the *S*-epimer, 20*S*(OH)D3 (4), which was further confirmed by chemical synthesis (7). More recently we have shown that 20(OH)D3 can be produced by human placentas, human epidermal keratinocytes, bovine and rodent adrenals (8), as well as by Caco-2 colon cells (8), all of which express CYP11A1 (9,10). A monohydroxyvitamin D3 metabolite corresponding in retention time to 20(OH)D3 has also been detected in human plasma (8). While a physiological role for endogenously produced 20(OH)D3 remains to be established, it displays biological activity on cultured cells of different lineage acting as a partial agonist on the vitamin D receptor (1,3,11–15). Thus, it inhibits proliferation and stimulates the differentiation of many cell types including epidermal keratinocytes and melanocytes, dermal fibroblasts and melanoma, leukemia and breast cancer cells with comparable potency to 1α,25-dihydroxyvitamin D3 [1,25(OH)₂D3]. It also displays anti-inflammatory effects, inhibiting the activity of NF-κB (1,12,14) and inhibiting production of pro-inflammatory cytokines, while promoting the production of the anti-inflammatory cytokine IL-10 (1). Most recently, we have demonstrated that 20(OH)D3 attenuates bleomycin-induced scleroderma *in vivo* (15). Moreover, unlike 1,25(OH)₂D3, it lacks calcemic activity in rodents with no toxicity at doses as high as 30 µg/kg (11,13). Also in contrast to 1,25(OH)₂D3, it is a poor inducer of the inactivating enzyme, CYP24A1 (13,16). These properties of 20(OH)D3 suggest that it may be a useful therapeutic agent for the treatment of proliferative and autoimmune disorders (1).

In vitro studies with the mitochondrial cytochromes P450 that are involved in vitamin D3 metabolism reveal that CYP27A1, CYP27B1 and CYP24A1 can act on 20(OH)D3. CYP27B1 produces 1α,20-dihydroxyvitamin D3 (16,17), and the addition of the 1α-hydroxyl group confers some calcemic activity to the derivative (13). We detected production of 1α-hydroxy derivatives of 20(OH)D3 and 20,23(OH)₂D3 *in vivo* in human placenta and epidermal keratinocytes (8). Both CYP27A1 and CYP24A1 convert 20(OH)D3 to 20,25-dihydroxyvitamin D3 [20,25(OH)₂D3] with this metabolite displaying greater inhibition of melanoma cell proliferation than the parent 20(OH)D3, or 1,25(OH)₂D3 (18,19).

A number of microsomal cytochromes P450 are involved in vitamin D metabolism. CYP2R1 is generally regarded as the major 25-hydroxylase in humans (20,21) although there may be some contribution from mitochondrial CYP27A1 (19,22). The knockout of both *Cyp2r1* and *Cyp27a1* in mice only caused a 50% decrease in serum 25-hydroxyvitamin D3 [25(OH)D3] levels indicating that in rodents there is another vitamin D 25-hydroxylase (23). This could be Cyp2j3, which displays high 25-hydroxylase activity whereas its human counterpart, CYP2J2, had only low 25-hydroxylase activity (21,24). CYP3A4 in humans acts to catalyze 25-hydroxylation of vitamin D2 and the synthetic prodrugs 1α-hydroxyvitamin D3 [1α(OH)D3] and 1α-hydroxyvitamin D2 [1α(OH)D2]. It also catalyzes

24-hydroxylation of $1\alpha(\text{OH})\text{D}_3$, $1\alpha(\text{OH})\text{D}_2$ and vitamin D₃ (21,25,26) but shows little ability to 25-hydroxylate vitamin D₃ (21). Recently, Wang *et al.* (27) showed that $4\beta,25$ -dihydroxyvitamin D₃, a form of vitamin D detectable in the bloodstream, was produced from $25(\text{OH})\text{D}_3$ by CYP3A4 in human liver. In contrast to the human, mouse liver microsomes do not express CYP3A4, instead there are six different family 3A isoforms expressed: Cyp3a11, Cyp3a13, Cyp3a16, Cyp3a25, Cyp3a41 and Cyp3a44. These are believed to carry out the equivalent function of CYP3A4 (28).

Currently mice are being used to examine the *in vivo* therapeutic potential of $20(\text{OH})\text{D}_3$ (1,15). It is therefore important to know how this active vitamin D₃ metabolite is metabolized by the liver, and thus the aim of the current study was to characterize the metabolism of $20(\text{OH})\text{D}_3$ by mouse liver microsomes.

2. Materials and Methods

2.1. Generation of secosteroid standards

$20(\text{OH})\text{D}_3$ was produced by the enzymatic action of recombinant bovine CYP11A1 on vitamin D₃, and purified by TLC and reverse-phase HPLC (16). $22\text{-Hydroxyvitamin D}_3$ [$22(\text{OH})\text{D}_3$], $17,20(\text{OH})_2\text{D}_3$, $20,22(\text{OH})_2\text{D}_3$ and $20,23(\text{OH})_2\text{D}_3$ were also produced from vitamin D₃ using bovine CYP11A1 (16). $20,24\text{-dihydroxyvitamin D}_3$ [$20,24(\text{OH})_2\text{D}_3$] and $20,25(\text{OH})_2\text{D}_3$ were produced by the action of rat Cyp24a1 on $20(\text{OH})\text{D}_3$ (18) while $20,26\text{-dihydroxyvitamin D}_3$ [$20,26(\text{OH})_2\text{D}_3$] was made from $20(\text{OH})\text{D}_3$ using human CYP27A1 (19). The structures of these metabolites have previously been determined by NMR (5,16,18).

2.2. Preparation of mouse liver microsomes

Ten-week-old female mice (C57BL/6J; Animal Resource Centre, Murdoch University) were euthanized and livers placed in 0.25 M sucrose on ice. The tissue was homogenized in 9 volumes of 0.25 M sucrose in a Potter-Elvehjem homogenizer by three low-speed passes of the teflon pestle. The homogenate was centrifuged at $600 \times g$ for 10 min at 4°C. The supernatant was removed and centrifuged at $6,000 \times g$ for 15 min at 4°C to sediment the mitochondrial fraction. The resulting supernatant was centrifuged at $11,000 \times g$ for 15 min at 4°C to sediment any remaining mitochondria and the supernatant was then centrifuged at $107,000 \times g$ for 1 h at 4°C. The microsomal pellet was resuspended in 0.25 M sucrose by hand homogenizing using a Potter-Elvehjem homogenizer and centrifuged again at $107,000 \times g$ for 1 h at 4°C to wash the microsomes. The microsomal pellet was resuspended in 0.25 M sucrose by hand homogenizing and stored at -80°C.

2.3. Metabolism of $20(\text{OH})\text{D}_3$ by the mouse liver microsomal fraction

Mouse liver microsomes at a final concentration of 1.5 mg/mL were incubated with $20(\text{OH})\text{D}_3$ (see Figure Legends for concentrations) in 0.45% 2-hydroxypropyl- β -cyclodextrin (cyclodextrin) in buffer comprising 0.25 M sucrose, 50 mM HEPES (pH 7.4), 20 mM KCl, 5 mM MgSO_4 and 0.2 mM EDTA. The cyclodextrin served to hold the $20(\text{OH})\text{D}_3$ substrate in solution and is often used for activity assays with low solubility substrates where it forms a hydrophobic cage around them, with a hydrophilic exterior

(29,30). It could solubilize 200 μM 20(OH)D₃, the maximum concentration used in this study (3,8,31). The incubation also contained a cofactor and regeneration system comprising 2 mM glucose-6-phosphate, 2 U/mL glucose-6-phosphate dehydrogenase and 500 μM NADPH. The typical incubation volume was 0.5 mL. Samples were preincubated for 8 min in a 37°C water bath shaking at 50 cycles per min and then reactions started by the addition of NADPH. After incubation at 37°C (see Results for times), reactions were stopped with 2.5 volumes ice-cold dichloromethane. Samples were vortexed and centrifuged at $670 \times g$ for 10 min and the lower organic phase was retained and the upper aqueous phase was extracted another three times with 2.5 volumes dichloromethane. The extracts were then dried under nitrogen at 30°C and dissolved in the solvent required for HPLC analysis.

2.4. Reverse-phase HPLC analysis of 20(OH)D₃ metabolites

The analysis of 20(OH)D₃ metabolites was carried out using a Perkin-Elmer HPLC system (Biocompatible Binary Pump 250) with a UV monitor set at 265 nm, equipped with a C18 column (Grace Alltima, 25 cm $\times$ 4.6 mm, particle size 5 μm). For routine analysis, metabolites were separated using a gradient of 45 – 100% acetonitrile in water for 30 min, followed by 100% acetonitrile for 35 min, at a flow rate of 0.5 mL/min (designated HPLC Program A in Figure Legends). As this system did not separate all metabolites or authentic standards, some 20(OH)D₃ metabolites were subsequently separated using a gradient of 64 – 100% methanol in water for 20 min, followed by 100% methanol for 30 min, at a flow rate of 0.5 mL/min (designated HPLC Program B in the Figure Legends).

2.5. Mass spectrometry of 20(OH)D₃ metabolites

Reverse-phase HPLC was initially used to purify and collect 20(OH)D₃ metabolites using an acetonitrile in water gradient (HPLC Program A), then further purified and collected using a methanol in water gradient (HPLC Program B). The purified 20(OH)D₃ metabolites (5 nmol) were then subjected to analysis by mass spectrometry. The system used involved liquid chromatography-mass spectrometry (LC/MS) combining 2-dimensional (2D) separation with two pentafluorophenyl (PFP) columns, to provide relatively pure material to the mass spectrometer, as described in detail previously (32). The system comprised an Agilent 1290 UPLC binary pump coupled to an Agilent 6460 triple quadrupole mass spectrometer with a Jetstream source. Electrospray ionization was used in the positive mode. Each sample was reconstituted in 500 μL of 70% methanol + 0.1% formic acid, with 20 μL being injected onto the LC/MS. Samples were subjected to an MS₂ scan from 300 – 500 m/z . These assays were performed as a service by UWA Centre for Metabolomics.

2.6. Marker enzyme assays

Cytochrome c oxidase was used as a marker enzyme for mitochondria (33). The colorimetric assay measured the oxidation of ferrocytochrome c to ferricytochrome c by cytochrome c oxidase, which causes a decrease in absorbance at 550 nm (33). The assays were carried out on both mitochondrial and microsomal fractions at 30°C and the oxidation of cytochrome c was measured over 3 min, with rates being linear over this time span.

Testosterone hydroxylation was used as a microsomal marker (34,35). Rat liver mitochondrial and microsomal fractions were incubated with testosterone at a final

concentration of 100 μM added from an acetonitrile stock (2% acetonitrile final concentration), in a buffer comprising 0.25 M sucrose, 50 mM HEPES (pH 7.4), 20 mM KCl, 5 mM MgSO_4 and 0.2 mM EDTA. The appropriate cofactors were added; 5 mM isocitrate and 500 μM NADPH for mitochondria; 2 mM glucose-6-phosphate, 2 U/mL glucose-6-phosphatase and 500 μM NADPH for microsomes. Samples were preincubated for 8 min at 37°C and reactions were started with either NADPH or isocitrate/NADPH, then stopped with 2.5 volumes ice-cold dichloromethane and extracted as before. Testosterone metabolites were separated from testosterone by HPLC on a C18 column (Grace Alltima, 25 cm $\times$ 4.6 mm, particle size 5 μm) using a gradient of 30 - 75% acetonitrile in water for 25 min, followed by a gradient of 75 - 100% acetonitrile in water for 10 min, and then 100% acetonitrile for 10 min, all at a flow rate of 0.5 mL/min. Testosterone and its metabolites were detected with a UV monitor set at 240 nm (34). The rates of testosterone metabolism by the microsomal and mitochondrial fractions were calculated from the percentage conversion of testosterone to products and the initial testosterone concentration.

2.7. Other procedures

The Ponceau S protein assay (36) was used to determine the protein concentration of isolated mouse liver microsomal and mitochondrial fractions. The concentrations of vitamin D metabolites with a similar UV spectrum to vitamin D₃, were determined using a Shimadzu UV-2450 spectrophotometer using an extinction coefficient at 263 nm of 18,000 $\text{M}^{-1}\text{cm}^{-1}$ (37). Hydroxy derivatives of vitamin D₃ have been shown to have the same extinction coefficient as vitamin D₃ (37).

3. Results

3.1. Purity of microsomal fraction

Since liver mitochondria contain at least one P450, CYP27A1, that can metabolize 20(OH)D₃ (19), we tested the level of contamination of the microsomal fraction with mitochondria using cytochrome c oxidase as a marker. The mitochondrial fraction exhibited a cytochrome oxidase activity of 6.75 ± 0.6 nmol/min/mg protein (data are mean $\pm$ SD for triplicate determinations), while the microsomal fraction exhibited an activity of 0.37 ± 0.05 nmol/min/mg protein. This indicates only minor contamination of the microsomal fraction with mitochondria. Testosterone metabolism was used as a microsomal marker and its activity was 8.2 times higher in the microsomal fraction than for the mitochondrial fraction.

3.2. Metabolism of 20(OH)D₃ by mouse liver microsomes

Incubation of the mouse liver microsomal fraction with 20(OH)D₃ produced six metabolites (A to E, Fig. 1B), not present in the control (Fig. 1A), each of which represented greater than 0.5% of the total extracted secosteroids. Two products are labelled as C1 and C2 in the HPLC chromatogram (Fig. 1B) as they were not baseline separated using an acetonitrile-water gradient (and originally designated as product C). To determine the proportions of C1 and C2, the combined peak from the acetonitrile-water gradient was collected and separated into C1 and C2 using a methanol-water gradient, where they elute in reverse order (Fig 1C). The time course for appearance of products A–E showed that products A and B were the two major products at all time points (Fig. 2). The rates of production of all metabolites

were similar and declined with time, presumably due to enzyme inactivation, as only 22% of the substrate was consumed by 90 min (Fig. 2A). No evidence for secondary metabolite formation was seen as none of the products showed a lag (requiring synthesis of a precursor) in their time course. Note that product D had a different absorption spectrum to all the other products that was not typical of vitamin D3 (see below) and thus it could not be assumed that it has the same extinction coefficient as the internal standard [22(OH)D3] used for quantitation. Hence its production is expressed as a relative response of the UV detector compared to that of the internal standard (Fig. 2B, right axis).

The electrospray mass spectra for products A, B and C2 showed the parent ion at $m/z = 439.3$ ($416.3 + \text{Na}^+$), representing a dihydroxyvitamin D3 ($m/z = 416.3$) (Table 1). The Na^+ adduct likely comes from the incubation buffer or the borosilicate glass tubes used for sample preparation (38). Products A and B also showed major ions at $m/z = 381.3$ ($416.3 + \text{H}^+ - 2\text{H}_2\text{O}$), 399.3 ($416.3 + \text{H}^+ - \text{H}_2\text{O}$) and for product A, 455.2 ($416.3 + \text{K}^+$), affirming that both products are dihydroxyvitamin D3 (Table 1). Product C2 also displayed the ions at m/z 399.3 and 455.2. These 3 metabolites displayed the typical vitamin D UV absorption spectrum with a peak at 263 nm, as illustrated for product B in Fig. 3. The mass spectra for products C1, D and E showed the parent ion at $m/z = 415.2$ ($414.2 + \text{H}^+$) and another ion at $m/z = 437.2$ ($414.2 + \text{Na}^+$) (Table 1), suggesting that a hydroxyl group had been added ($m/z = 416.3$) followed by an alcohol to ketone conversion or the insertion of a double bond (reducing m/z by 2), resulting in a dehydro-dihydroxyvitamin D3. Other major ions seen for product D included $m/z = 379.2$ ($414.2 + \text{H}^+ - 2\text{H}_2\text{O}$) and 397.2 ($414.2 + \text{H}^+ - \text{H}_2\text{O}$), while the other major ion seen for products C1 and E was at $m/z = 453.1$ ($414.1 + \text{K}^+$). The UV spectra for products C1 and E were similar to the parent 20(OH)D3 with a peak at 263 nm. In contrast, product D had its peak at 290 nm instead of the typical 263 nm of vitamin D (Fig. 3). This could be due to the new double bond ($\text{C}=\text{C}$ or $\text{C}=\text{O}$) interacting with the vitamin D chromophore.

Spiking of the reaction extract with authentic standards (see Section 2.1) and subsequent HPLC analysis using two different solvent systems enabled identification of product A as 20,25(OH)₂D3 (Fig. 4, panels A, B, D and E). Product B had an identical retention time to both 20,24(OH)₂D3 (Fig. 4C) and 20,26(OH)₂D3 (not shown) as these two secosteroids can not be separated by reverse-phase HPLC using an acetonitrile gradient. These two secosteroids do separate with a methanol gradient enabling identification of product B as 20,24(OH)₂D3 (Fig. 4, panels F–H). We also compared retention times of products C1, C2, D and E to a number of other dihydroxyvitamin D3 standards containing a 20-hydroxyl group, namely, 20,23(OH)₂D3, 20,22(OH)₂D3 and 17,20(OH)₂D3, but none matched the observed products, excluding these dihydroxyvitamin D3 compounds as microsomal metabolites.

To test if any of products C1, D or E resulted from dehydrogenation of either 20,24(OH)₂D3 (product B) or 20,25(OH)₂D3 (product A), microsomes were incubated with 20,24(OH)₂D3 or 20,25(OH)₂D3 (25 μM) dissolved in 0.45% cyclodextrin, for 1 h at 37°C. HPLC analysis (as for Fig. 1) revealed that no products were formed (not shown) indicating that none of the dehydro-dihydroxyvitamin D3 products arise from 20,24(OH)₂D3 or 20,25(OH)₂D3. This

experiment also confirms that neither 20,24(OH)₂D₃ nor 20,25(OH)₂D₃ is further metabolized by the microsomal fraction.

3.3. Kinetics of 20(OH)D₃ metabolism by mouse liver microsomes

To determine the K_m for 20(OH)D₃ conversion to each product, the concentration of substrate was varied and the amount of each product measured under initial rate conditions. Products were analyzed by HPLC using an acetonitrile-water gradient. As for the time course (Fig. 2), the proportions of products C1 and C2 were determined by collecting the combined peak from the acetonitrile gradient and separating it into C1 and C2 using a methanol-water gradient. Data for all metabolites generally gave good fits to the Michaelis-Menten equation, as illustrated in Fig. 5, with saturation occurring at high 20(OH)D₃ concentrations. For product D, the data points at 100 μ M and 200 μ M 20(OH)D₃ were not close to the fitted curve resulting in a large standard error in the K_m . The hyperbola did split the two points equally and the low activity at 200 μ M 20(OH)D₃ is unlikely to be due to substrate inhibition which was not observed for the other products, nor to insolubility of 20(OH)D₃ at this concentration as cyclodextrin was present as a solubilizing agent (see Section 2.3). The large standard error is possibly associated with there being more than one catalytic step required for formation of product D.

The kinetic parameters for all products are listed in Table 2. Product A, already identified as 20,25(OH)₂D₃, had a comparable K_m value to that of product B, identified as 20,24(OH)₂D₃. Products C1 and D also had K_m values similar to those for 20,24(OH)₂D₃ and 20,25(OH)₂D₃ while the K_m for product E was higher. The K_m for product C2 formation was 9 – 12 fold higher than values for formation of 20,24(OH)₂D₃ and 20,25(OH)₂D₃. Of the products with the same absorbance spectrum as 20(OH)D₃, product B [20,24(OH)₂D₃] was produced with the greatest catalytic efficiency (V_{max}/K_m) while product E was produced with the lowest. The true V_{max} for product D is unknown due to its different absorbance spectrum to the other products; its rate of production is therefore recorded as the HPLC peak area relative to that of the internal standard (Fig. 5C).

4. Discussion

We have found that mouse liver microsomes can metabolize 20(OH)D₃ to a number of metabolites with the two major ones being identified from mass spectrometry and authentic standards as 20,24(OH)₂D₃ and 20,25(OH)₂D₃. These major metabolites did not undergo further transformation by the microsomal fraction. However, we have reported previously that both of these metabolites can be converted to their 1 α -hydroxy derivatives by the mitochondrial enzyme, CYP27B1, of which highest activity is expressed in kidney (17). Both 20,24(OH)₂D₃ and 20,25(OH)₂D₃, as well as 1,24,25-trihydroxyvitamin D₃, have previously been shown to display higher biological activity than 20(OH)D₃ towards melanoma cells (18).

20(OH)D₃ exhibits K_m values of 23 – 32 μ M for formation of 20,24(OH)₂D₃ and 20,25(OH)₂D₃ by the mouse liver microsomal fraction. The similarity of the K_m values for formation of these two products suggests that it reflects binding to a single site on a P450 with a specific orientation that permits hydroxylation at the two neighboring carbons on the

vitamin D side chain. K_m values for production of C1 (20 μ M) and D (34 μ M) are also comparable to those for formation of 20,24(OH)₂D3 and 20,25(OH)₂D3 and might also be catalyzed by the same P450. In contrast, the K_m values for formation of products E (47 μ M) and C2 (291 μ M) are higher than for the other products, suggesting that one or more different P450s are involved. The K_m for the formation of 20,24(OH)₂D3, the major product among those with a similar absorbance spectrum as 20(OH)D3, is 7.6-fold higher than for 24R-hydroxylation of 1,25(OH)₂D3 by mouse liver microsomes which is believed to be catalyzed by CYP3A isoforms (28). However, the k_{cat} for the conversion of 20(OH)D3 to 20,24(OH)₂D3 is 16.9-fold higher than for this conversion of 1,25(OH)₂D3 to 1,24R,25-trihydroxyvitamin D3 by microsomes from control animals, so the catalytic efficiency is 2-fold higher for 20(OH)D3. Human liver microsomes can metabolize 25(OH)D3 to 4 β ,25-dihydroxyvitamin D3 with a K_m of 19 μ M, similar to that for formation of 20,24(OH)₂D3 and 20,25(OH)₂D3 by mouse liver microsomes. The 4 β -hydroxylation of 25(OH)D3 is mediated by CYP3A4 in a reaction suggested to contribute to the reduction of 25(OH)D3 levels with chronic drug treatment (27).

The mass spectral data indicate that products C1, D and E are the result of two separate reactions, hydroxylation and dehydrogenation. Dehydrogenation reactions involving alcohol to ketone or aldehyde conversions are well known reactions catalyzed by P450s and for example, both reactions are observed in the C24 oxidation pathway of vitamin D catabolism catalyzed by CYP24A1 (39). Although uncommon, some P450s have been reported to catalyze dehydrogenations involving conversion of a C-C single bond to a double bond (40). Thus, while it would seem more likely that a separate dehydrogenase enzyme catalyzes the dehydrogenation if it involves C-C bond oxidation, it is possible that a single P450 species can catalyze both the hydroxylation and subsequent dehydrogenation required to form products C1, D and E from 20(OH)D3. In the case of product D, the increase in the wavelength for maximum absorbance from 263 to 290 nm suggests that the new double bond interacts with the triene chromophore of vitamin D3 involving the A ring and broken B ring of the secosteroid. Such an increase in the absorbance maximum is seen when the C-C double bond of vitamin D between C10 and C19 is replaced by a ketone group at C10 (41).

This study, as well as our previous reports (18,19), indicate that there are several P450s in mammals that can hydroxylate 20(OH)D3 at C25. While the identity of the mouse liver microsomal enzyme(s) responsible for this remain to be established, candidates include Cyp2r1, Cyp2j3, and Cyp3a family members (see Introduction). Purified mitochondrial Cyp24a1 can hydroxylate 20(OH)D3 at C24 and C25 producing 20,24(OH)₂D3 and 20,25(OH)₂D3, as for microsomes (19). It should be noted that the microsomes we used had minimal mitochondrial contamination and Cyp24a1 is predominantly a kidney enzyme and is not expressed in liver (39), excluding this enzyme as the source of 20,24(OH)₂D3 and 20,25(OH)₂D3 production by mouse liver microsomes. Human CYP27A1 found in liver mitochondria can also hydroxylate 20(OH)D3 at C25 producing 20,25(OH)₂D3, but produces comparable amounts of 20,26(OH)₂D3 (19) which is not seen as a product of mouse liver microsomes, indicating no involvement of Cyp27a1.

20(OH)D3 is produced by the action of CYP11A1 on vitamin D3 and we have previously presented data showing that this reaction occurs *in vivo* (8). 20(OH)D3 inhibits proliferation

of normal and malignant cells, promotes cell differentiation and has anti-inflammatory properties (reviewed in (1)). The above properties renders it a good candidate as an anti-cancer and/or anti-inflammatory drug, especially because it does neither induce hypercalcemia nor any identifiable cytotoxicity at pharmacological doses, unlike 1,25(OH)₂D₃, the biologically active form of vitamin D (1,11,13). Furthermore, most recently we demonstrated anti-fibrosing activity of 20(OH)D₃ in animal models of scleroderma (15) and have found marked inhibition of collagen-induced arthritis in mice (Postlethwaite, Slominski et al., in preparation). The clinical utility of 20(OH)D₃ requires its oral application. 20(OH)D₃ after absorption in the gastrointestinal tract will further pass through the liver with its subsequent metabolism and likely production of 20,24(OH)₂D₃ and 20,25(OH)₂D₃, as described in this paper. The significance of these studies is further strengthened by our recent molecular modeling studies with the vitamin D receptor that predict that both 20,24(OH)₂D₃ and 20,25(OH)₂D₃ bind more tightly to the receptor than the parent 20(OH)D₃, with their 1 α -hydroxy derivatives predicted to bind even tighter (1). This is consistent with our previous report that these products cause significantly greater inhibition of colony formation by SKMEL-188 melanoma cells than either the parent 20(OH)D₃, or 1,25(OH)₂D₃ (19).

In conclusion, we demonstrate that mouse liver microsomes hydroxylate 20(OH)D₃ producing 20,24(OH)₂D₃ and 20,25(OH)₂D₃ as major metabolites. The action of liver microsomal enzymes on 20(OH)D₃ suggests that its passage through the liver can activate this vitamin D analog, producing metabolites with predicted (1) and demonstrated greater anti-cancer potency (19).

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Highlights

- Mouse liver microsomes metabolize 20-hydroxyvitamin D3.
- Major products identified were 20,24-dihydroxyvitamin D3 and 20,25-dihydroxyvitamin D3.
- A novel 20-hydroxyvitamin D3 metabolite with an altered chromophore was detected.
- Formation of major products displayed K_m values lower than for the other products.

Figure 1.

HPLC analysis of 20(OH)D3 metabolism by mouse liver microsomes. 20(OH)D3 (50 μ M) in 0.45% cyclodextrin was incubated with microsomes (1.5 mg/mL) for 30 min, products extracted and analyzed by HPLC as described in Experimental Procedures. (A), control incubation without the addition of NADPH, analyzed using HPLC Program A; (B), test incubation with the addition of NADPH, analyzed using HPLC Program A; (C), metabolites C1 and C2 collected from the acetonitrile gradient (HPLC Program A) and analyzed with a methanol gradient (HPLC Program B), showing the two peaks clearly separated.

Figure 2.

Time course for metabolism of 20(OH)D3 by mouse liver microsomes. 20(OH)D3 (50 μ M) in 0.45% cyclodextrin was incubated with the microsomal fraction (1.5 mg/mL) for the times indicated, the reaction stopped with dichloromethane and 22(OH)D3 (1 nmol) added as internal standard for quantitation. Products were extracted and analyzed by HPLC using an acetonitrile gradient (HPLC Program A, see Experimental Procedures). 20(OH)D3 metabolites produced from the reaction are labeled as in Fig. 1 with products C1 and C2 being separated with a methanol gradient (HPLC Program B) as in Fig. 1. (A), depletion of substrate and formation of major products. (B), production of minor metabolites. Product D is plotted as the response of the UV detector relative to that of the internal standard since its UV spectrum was different to the internal standard and other products. Data are mean $\pm$ SD of triplicate determinations.

Figure 3. UV spectra of 20(OH)D3 and products B and D. Samples were dissolved in ethanol for recording their absorbance spectra.

Figure 4.

Identification of product A as 20,25(OH)₂D₃ and B as 20,24(OH)₂D₃ by HPLC analysis. (A–C), an extract of the metabolites produced by the microsomal fraction was spiked with authentic 20,24(OH)₂D₃ or 20,25(OH)₂D₃ and analyzed using an acetonitrile gradient (HPLC Program A). (D–H), products A and B were collected from the acetonitrile gradient and further analyzed using a methanol gradient (HPLC Program B). (A), products without spiking; (B), products after spiking with 20,25(OH)₂D₃; (C), products after spiking with 20,24(OH)₂D₃; (D) product A collected from the acetonitrile gradient; (E), product A spiked with 20,25(OH)₂D₃; (F) product B collected from the acetonitrile gradient; (G), product B spiked with 20,26(OH)₂D₃; (H), product B spiked with 20,24(OH)₂D₃.

Figure 5.

The effect of 20(OH)D3 concentration on production of products A–E by mouse liver microsomes. 20(OH)D3 at concentrations varying from 6.25 to 200 μM, dissolved in cyclodextrin at a final concentration of 0.45%, were incubated with the mouse liver microsomal fraction. Incubations were carried out for 30 min at 37 C, the reaction stopped with dichloromethane, 22(OH)D3 (1.0 nmol) added as internal standard, then products extracted and analyzed using HPLC Program A (see Experimental Procedures). Data were fitted to the Michaelis-Menten equation.

Analysis of 20(OH)D3 metabolites produced by mouse liver microsomes by mass spectrometry. Mass spectra were recorded as described in the Experimental section. Parent ions are highlighted in bold.

Table 1

Product	[M + Na] ⁺	[M + H] ⁺	[M + H - H ₂ O] ⁺	[M + H - 2H ₂ O] ⁺	[M + K] ⁺	Classification
A	439.3	-	399.3	381.3	455.2	Dihydroxyvitamin D3
B	439.3	-	399.3	381.3	-	Dihydroxyvitamin D3
C1	437.2	415.2	-	-	453.1	Dehydro-dihydroxyvitamin D3
C2	439.3	-	399.3	-	455.2	Dihydroxyvitamin D3
D	437.3	415.2	397.2	379.2	-	Dehydro-dihydroxyvitamin D3
E	437.1	415.2	-	-	453.1	Dehydro-dihydroxyvitamin D3

Table 2

Kinetics of the metabolism of 20(OH)D3 by mouse liver microsomes. The Michaelis-Menten equation was fitted to the experimental data as in Fig. 5 and gave good fits with correlation coefficients ranging from 0.91 to 0.99. Data for K_m and V_{max} are presented as mean $\pm$ standard error of the curve fit.

Product	K_m (μ M)	V_{max} (pmol/min/mg protein)	V_{max}/K_m
A [20,25(OH) ₂ D3]	22.9 $\pm$ 3.5	21.4 $\pm$ 1.0	0.93
B [20,24(OH) ₂ D3]	32.3 $\pm$ 4.9	38.8 $\pm$ 2.0	1.20
C1	20.3 $\pm$ 1.6	5.8 $\pm$ 0.1	0.28
C2	287.9 $\pm$ 75.1	20.9 $\pm$ 3.7	0.073
D	34.1 $\pm$ 23.4	N/A	N/A
E	47.6 $\pm$ 3.8	12.0 $\pm$ 0.4	0.25

N/A = Not Applicable