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Metabolism of 20-hydroxyvitamin D3 by mouse liver microsomes

Chloe Y.S. Cheng [a], Andrzej T. Slominski [b], Robert C. Tuckey [a,*]

[a] School of Chemistry and Biochemistry, The University of Western Australia, Crawley, WA 6009, Australia
[b] Department of Pathology and Laboratory Medicine, Center for Adult Cancer Research, University of Tennessee Health Science Center, Memphis, TN, USA

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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$(OH)_2$D3] can inhibit proliferation and promote differentiation of a range of cells, and has anti-inflammatory properties. However, unlike 1,25$(OH)_2$D3, 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$(OH)_2$D3] and 20,25-dihydroxyvitamin D3 [20,25$(OH)_2$D3]. 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$(OH)_2$D3 and 20,25$(OH)_2$D3 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.

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$(OH)_2$D3], 20,22-dihydroxyvitamin D3 [20,22$(OH)_2$D3], 17,20-dihydroxyvitamin D3 [17,20$(OH)_2$D3] 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 [1], 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 biased 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$(OH)_2$D3]. 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$(OH)_2$D3, 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$(OH)_2$D3, 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$(OH)_2$D3 *in vivo* in human placenta and epidermal

* Corresponding author. Tel.: +61 8 64883040; fax: +61 8 30401148.
E-mail address: robert.tuckey@uwa.edu.au (R.C. Tuckey).

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keratinocytes [8]. Both CYP27A1 and CYP24A1 convert 20(OH)D3 to 20,25-dihydroxyvitamin D3 [20,25$(OH)_2$D3] with this metabolite displaying greater inhibition of melanoma cell proliferation than the parent 20(OH)D3, or 1,25$(OH)_2$D3 [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 1α(OH)D3, 1α(OH)D2 and vitamin D3 [21,25,26] but shows little ability to 25-hydroxylate vitamin D3 [21]. Recently, Wang et al. [27] showed that 4β,25-dihydroxyvitamin D3, a form of vitamin D detectable in the bloodstream, was produced from 25(OH)D3 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(OH)D3 [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(OH)D3 by mouse liver microsomes.

2. Materials and methods

2.1. Generation of secosteroid standards

20(OH)D3 was produced by the enzymatic action of recombinant bovine CYP11A1 on vitamin D3, and purified by TLC and reverse-phase HPLC [16]. 22-Hydroxyvitamin D3 [22(OH)D3], 17,20$(OH)_2$D3, 20,22$(OH)_2$D3 and 20,23$(OH)_2$D3 were also produced from vitamin D3 using bovine CYP11A1 [16]. 20,24-Dihydroxyvitamin D3 [20,24$(OH)_2$D3] and 20,25$(OH)_2$D3 were produced by the action of rat Cyp24a1 on 20(OH)D3 [18] while 20,26-dihydroxyvitamin D3 [20,26$(OH)_2$D3] was made from 20(OH)D3 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 Center, 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 × *g* for 10 min at 4 °C. The supernatant was removed and centrifuged at 6000 × *g* for 15 min at 4 °C to sediment the mitochondrial fraction. The resulting supernatant was centrifuged at 11,000 × *g* for 15 min at 4 °C to sediment any remaining mitochondria and the supernatant was then centrifuged at 107,000 × *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 × *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(OH)D3 by the mouse liver microsomal fraction

Mouse liver microsomes at a final concentration of 1.5 mg/mL were incubated with 20(OH)D3 (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(OH)D3 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 (Section 3 for times), reactions were stopped with 2.5 volumes ice-cold dichloromethane. Samples were vortexed and centrifuged at 670 × *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 × 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 Center 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