Journal of Steroid Biochemistry & Molecular Biology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

# Metabolism of 20-hydroxyvitamin D3 and 20,23-dihydroxyvitamin D3 by rat and human CYP24A1

Elaine W. Tieu [a], Wei Li [b], Jianjun Chen [b,c], Tae-Kang Kim [d], Dejian Ma [b], Andrzej T. Slominski [d,e], Robert C. Tuckey [a,*]

[a] School of Chemistry and Biochemistry, The University of Western Australia, Crawley, WA 6009, Australia
[b] Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, TN, USA
[c] Department of Pharmaceutical Sciences, School of Pharmacy, South College, Knoxville, TN, USA
[d] Department of Pathology and Laboratory Medicine, Center for Cancer Research, University of Tennessee Health Science Center, Memphis, TN, USA
[e] Department of Medicine, Division of Rheumatology and Connective Tissue Diseases, University of Tennessee Health Science Center, Memphis, TN, USA

ARTICLE INFO

*Article history:*
Received 28 November 2014
Received in revised form 15 February 2015
Accepted 16 February 2015
Available online xxx

*Keywords:*
20-Hydroxyvitamin D3
20,23-Dihydroxyvitamin D3
Cytochrome P450
CYP24A1
Vitamin D3
Phospholipid vesicles

ABSTRACT

CYP11A1 hydroxylates vitamin D3 producing 20*S*-hydroxyvitamin D3 [20(OH)D3] and 20*S*,23-dihydroxyvitamin D3 [20,23$(OH)_2$D3] as the major and most characterized metabolites. Both display immuno-regulatory and anti-cancer properties while being non-calcemic. A previous study indicated 20(OH)D3 can be metabolized by rat CYP24A1 to products including 20*S*,24-dihydroxyvitamin D3 [20,24$(OH)_2$D3] and 20*S*,25-dihydroxyvitamin D3, with both producing greater inhibition of melanoma colony formation than 20(OH)D3. The aim of this study was to characterize the ability of rat and human CYP24A1 to metabolize 20(OH)D3 and 20,23$(OH)_2$D3. Both isoforms metabolized 20(OH)D3 to the same dihydroxyvitamin D species with no secondary metabolites being observed. Hydroxylation at C24 produced both enantiomers of 20,24$(OH)_2$D3. For rat CYP24A1 the preferred initial site of hydroxylation was at C24 whereas the human enzyme preferred C25. 20,23$(OH)_2$D3 was initially metabolized to 20*S*,23,24-trihydroxyvitamin D3 and 20*S*,23,25-trihydroxyvitamin D3 by rat and human CYP24A1 as determined by NMR, with both isoforms showing a preference for initial hydroxylation at C25. CYP24A1 was able to further oxidize these metabolites in a series of reactions which included the cleavage of C23-C24 bond, as indicated by high resolution mass spectrometry of the products, analogous to the catabolism of 1,25$(OH)_2$D3 *via* the C24-oxidation pathway. Similar catalytic efficiencies were observed for the metabolism of 20(OH)D3 and 20,23$(OH)_2$D3 by human CYP24A1 and were lower than for the metabolism of 1,25$(OH)_2$D3. We conclude that rat and human CYP24A1 metabolizes 20(OH)D3 producing only dihydroxyvitamin D3 species as products which retain biological activity, whereas 20,23$(OH)_2$D3 undergoes multiple oxidations which include cleavage of the side chain.



## 1. Introduction

CYP24A1 is the mitochondrial cytochrome P450 responsible for the catabolism of 1α,25-dihydroxyvitamin D3 [1,25$(OH)_2$D3]. Inactivation of vitamin D by CYP24A1 can take place *via* two catabolic pathways where initial hydroxylation occurs at either C24 or C23, termed the C24-oxidation and C23-oxidation pathways, respectively [1,2]. The sequential oxidation of 1,25$(OH)_2$D3 in the C24-oxidation pathway results in the formation of 24-oxo-1,23,25-trihydroxyvitamin D3 which undergoes side chain cleavage between C23 and C24 with the final product, calcitroic acid, being excreted. The C23-oxidation pathway produces 1,25-dihydroxyvitamin D3-26,23-lactone. There are species differences in the preference for these pathways, with rat CYP24A1 favoring the C24-oxidation pathway [3–5] and human CYP24A1 exhibiting both pathways [1,3,6,7].

It has been established in the last decade that CYP11A1 (also known as cytochrome P450scc) can metabolize vitamin D3 to produce many novel mono- and poly-hydroxyvitamin D metabolites, the major ones being 20*S*-hydroxyvitamin D3 [20(OH)D3]

*Abbreviations:* 1,25$(OH)_2$D3, 1α ,25-dihydroxyvitamin D3; 25(OH)D3, 25-hydroxyvitamin D3; 20(OH)D3, 20-hydroxyvitamin D3; 20,23$(OH)_2$D3, 20,23-dihydroxyvitamin D3; 20,23,24$(OH)_3$D3, 20,23,24-trihydroxyvitamin D3; 20,23,25$(OH)_3$D3, 20,23,25-trihydroxyvitamin D3; cyclodextrin, 2-hydroxylpropyl-β-cyclodextrin; TOCSY, $^1$H-$^1$H total correlation spectroscopy; COSY, $^1$H-$^1$H correlation spectroscopy; HSQC, $^1$H-$^{13}$C heteronuclear single quantum correlation spectroscopy; HMBC, $^1$H-$^{13}$C heteronuclear multiple bond correlation spectroscopy; PL, phospholipid.

* Corresponding author at: School of Chemistry and Biochemistry, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia.
Tel.: +61 8 6488 3040; fax: +61 8 6488 1148.
*E-mail address:* robert.tuckey@uwa.edu.au (R.C. Tuckey).

http://dx.doi.org/10.1016/j.jsbmb.2015.02.010
0960-0760/

and 20*S*,23-dihydroxyvitamin D3 [20,23(OH)$_2$D3] [8–18]. This pathway was initially elucidated from *in vitro* studies with purified bovine CYP11A1, and more recently has been demonstrated to occur in keratinocyte cell cultures and in fragments of adrenal glands and human placenta incubated with vitamin D3 [16,18,19]. Most recently, 20(OH)D3 and 20,23(OH)$_2$D3 have been detected at relative levels similar to the classical 25(OH)D3 and 1,25(OH)$_2$D3 in human epidermal tissue [20], confirming their production *in vivo*. Possible physiological roles for these metabolites remain to be established.

20(OH)D3 and 20,23(OH)$_2$D3 are the most extensively studied of the CYP11A1-derived secosteroids in terms of their *in vitro* biological actions. Both act as biased agonists on the vitamin D receptor and thus display many, but not all, of the biological effects of 1,25(OH)$_2$D3 [16,21–23]. They can both also act as inverse agonists on RORα and RORγ [24]. They have the ability to promote differentiation and suppress proliferation of a number of normal and cancerous cells *in vitro*, such as keratinocytes, melanocytes, fibroblasts, melanoma and leukemia cells [16,21–23,25–28]. 20(OH)D3 and 20,23(OH)$_2$D3 promote anti-inflammatory activity in normal and immortalized keratinocytes by increasing the expression of IκB, thus attenuating the transcriptional activity of NF-κB [23,27,29]. In addition, both 20(OH)D3 and 20,23(OH)$_2$D3 possess anti-fibrotic properties on human dermal fibroblasts isolated from scleroderma and normal subjects [26]. In rodent models, administration of 20(OH)D3 has been found to be effective in reducing the symptoms of scleroderma [26] and rheumatoid arthritis [16] as well as protecting DNA in skin from damage caused by UV irradiation [30]. Importantly, unlike 1,25(OH)$_2$D3, both 20(OH)D3 and 20,23(OH)$_2$D3 are non-calcemic in rodents at high concentrations [25,26,31]. Thus both 20(OH)D3 and 20,23(OH)$_2$D3 have therapeutic potential for the treatment of hyperproliferative and inflammatory disorders.

Recently it has been reported that 20(OH)D3 can be further metabolized by cytochromes P450 involved in the metabolism of vitamin D3. Human CYP27A1 converts 20(OH)D3 to 20*S*,25-dihydroxyvitamin D3 [20,25(OH)$_2$D3] and 20*S*,26-dihydroxyvitamin D3 [20,26(OH)$_2$D3], whereas rat CYP24A1 produces 20*S*,24-dihydroxyvitamin D3 [20,24(OH)$_2$D3] and 20,25(OH)$_2$D3 [32,33]. Other P450 isoforms found in mouse liver microsomes also produce 20,25(OH)$_2$D3 and 20,26(OH)$_2$D3 [34]. These resulting secosteroids are more potent than the parent 20(OH)D3 in the inhibition of melanoma colony formation [33]. However, addition of the 1α-hydroxyl group to 20(OH)D3 by CYP27B1 producing 1α,20*S*-dihydroxyvitamin D3, confers some calcemic activity although less than that observed with 1,25(OH)$_2$D3 [25]. Recently we have successfully extracted and partially purified human CYP24A1, and characterized its activity toward 1,25(OH)$_2$D3 and the intermediates of the C24-oxidation pathway [35]. In the present study we used human CYP24A1, along with rat CYP24A1, to characterize the metabolism of both 20(OH)D3 and 20,23(OH)$_2$D3.

## 2. Materials and methods

### 2.1. Materials

20(OH)D3 and 20,23(OH)$_2$D3 were synthesized from vitamin D3 enzymatically using bovine CYP11A1 and were purified by TLC and HPLC, as previously described [10,17]. Vitamin D3, dioleoyl phosphaditylcholine, bovine heart cardiolipin, 2-hydroxylpropyl-β-cyclodextrin (cyclodextrin) and glucose-6-phosphate were purchased from Sigma (Sydney, Australia). Glucose-6-phosphate dehydrogenase was from Roche (Mannheim, Germany). All solvents were of HPLC grade and were purchased from Merck (Darmstadt, Germany).

### 2.2. Preparation of enzymes

Rat and human CYP24A1 were expressed and purified as previously described [33,35]. Human and mouse adrenodoxin, and human adrenodoxin reductase were expressed in *Escherichia coli* and purified as before [17,36,37].

### 2.3. Measurement of secosteroid metabolism by CYP24A1 in a phospholipid vesicle reconstituted system

Dioleoyl phosphaditycholine (1.08 μmol), bovine heart cardiolipin (0.19 μmol) and the secosteroid substrate (as required) were aliquotted into glass tubes and the ethanol solvent removed under nitrogen gas. Assay buffer, pH 7.4 (20 mM HEPES, 100 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT) (0.5 mL) was added to the dried lipid mixture. This was purged for 30 s with nitrogen gas and then tubes sonicated for approximately 10 min in a bath-type sonicator, until the solution was clear [38]. The incubation mixture was composed of vesicles (510 μM phospholipid), P450 (0.01–0.05 μM for human CYP24A1, and 0.05–1 μM for rat CYP24A1), human or mouse adrenodoxin (15 μM), human adrenodoxin reductase (0.4 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (2 U/mL) and NADPH (50 μM), in assay buffer. Following preincubation for 3 min, reactions were started by the addition of adrenodoxin and samples (0.25–2.5 mL) incubated at 37 °C, with shaking (see for reaction times). Reactions were terminated by the addition of 2.5-volumes of ice-cold dichloromethane and samples were extracted four times with vortexing and centrifugation. The samples were dried under nitrogen gas and redissolved in ethanol for HPLC analysis. The samples were analysed on a PerkinElmer modular HPLC system which comprised a Biocompatible Binary LC pump (model 250; PerkinElmer Corporation, MA, USA) and a UV detector (LC-135C; PerkinElmer Corporation, MA, USA) set at 265 nm, equipped with a C18 analytical column (Grace Alltima, 250 × 4.6 mm, particle size 5 μm; Grace Davison Discovery Sciences, VIC, Australia). Different HPLC programs were used depending on the substrate. For the separation of monohydroxyvitamin D substrates and their products, a 20 min gradient from 45% (v/v) acetonitrile in water to 100% acetonitrile, followed by 30 min at 100% acetonitrile, all at a flow rate of 0.5 mL/min (HPLC Program A), was used. A 40 min gradient from 30% (v/v) acetonitrile in water to 100% acetonitrile, followed by 15 min at 100% acetonitrile, all at a flow rate of 0.5 mL/min, was used to separate polyhydroxyvitamin D substrates and products (HPLC Program B). The peak areas were integrated using Clarity software (DataApex, Prague, Czech Republic). Kinetic parameters were determined by fitting the Michaelis–Menten equation to the experimental data using Kaleidagraph, version 4.1 (Synergy Software, Reading, PA, U.S.A.).

### 2.4. Enzymatic synthesis and HPLC purification of 20,24(OH)$_2$D3

To produce 20,24(OH)$_2$D3 for NMR analysis, a large scale incubation (30 mL) of rat CYP24A1 with 20(OH)D3 was carried out, as described previously [33]. The 20,24(OH)$_2$D3 and other products were purified by HPLC, as outlined before [33], using a Grace Alltima column (as above) and a 45–58% (v/v) acetonitrile in water gradient over 25 min followed by a 10 min gradient from 58% (v/v) acetonitrile in water to 100% acetonitrile, ending with 20 min at 100% acetonitrile, all at a flow rate of 0.5 mL/min (HPLC Program C). A further HPLC purification step was carried out using the same column employing a 45 min gradient from 64% (v/v) methanol in water to 100% methanol, followed with 15 min at 100% methanol, all at a flow rate of 0.5 mL/min. Collected products were pooled and dried under nitrogen, dissolved in ethanol and the amount of



Please cite this article in press as: E.W. Tieu, et al., Metabolism of 20-hydroxyvitamin D3 and 20,23-dihydroxyvitamin D3 by rat and human CYP24A1, J. Steroid Biochem. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jsbmb.2015.02.010