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Metabolism of cholesterol, vitamin D3 and 20-hydroxyvitamin D3 incorporated into phospholipid vesicles by human CYP27A1

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ABSTRACT

CYP27A1 is a mitochondrial cytochrome P450 which can hydroxylate vitamin D3 and cholesterol at carbons 25 and 26, respectively. The product of vitamin D3 metabolism, 25-hydroxyvitamin D3, is the precursor to the biologically active hormone, 1α,25-dihydroxyvitamin D3. CYP27A1 is attached to the inner mitochondrial membrane and substrates appear to reach the active site through the membrane phase. We have therefore examined the ability of bacterially expressed and purified CYP27A1 to metabolize substrates incorporated into phospholipid vesicles which resemble the inner mitochondrial membrane. We also examined the ability of CYP27A1 to metabolize 20-hydroxyvitamin D3 (20(OH)D3), a novel non-calcemic form of vitamin D derived from CYP11A1 action on vitamin D3 which has anti-proliferative activity on keratinocytes, leukemic and myeloid cells. CYP27A1 displayed high catalytic activity towards cholesterol with a turnover number (k_{cat}) of 9.8 min^{-1} and K_m of 0.49 mol/mol phospholipid (510 μM phospholipid). The K_m value of vitamin D3 was similar for that of cholesterol, but the k_{cat} was 4.5-fold lower. 20(OH)D3 was metabolized by CYP27A1 to two major products with a k_{cat}/K_m that was 2.5-fold higher than that for vitamin D3, suggesting that 20(OH)D3 could effectively compete with vitamin D3 for catalysis. NMR and mass spectrometric analyses revealed that the two major products were 20,25-dihydroxyvitamin D3 and 20,26-dihydroxyvitamin D3, in almost equal proportions. Thus, the presence of the 20-hydroxyl group on the vitamin D3 side chain enables it to be metabolized more efficiently than vitamin D3, with carbon 26 in addition to carbon 25 becoming a major site of hydroxylation. Our study reports the highest k_{cat} for the 25-hydroxylation of vitamin D3 by any human cytochrome P450 suggesting that CYP27A1 might be an important contributor to the synthesis of 25-hydroxyvitamin D3, particularly in tissues where it is highly expressed.

1. Introduction

CYP27A1 is a multifunctional enzyme involved in the initial activation of vitamin D3, producing 25-hydroxyvitamin D3 (25(OH)D3), as well as in the biosynthesis of acidic and neutral bile acids. In the acidic bile acid pathway, CYP27A1 is responsible for the rate limiting step of 26-hydroxylation of cholesterol forming 26-hydroxycholesterol. Furthermore it has the ability to subsequently hydroxylate carbon 26 several times to yield 3β-hydroxy-5-cholestenoic acid [1–3]. In the neutral bile acid pathway, CYP27A1 serves to hydroxylate bile acid intermediates, 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol, to initiate side chain cleavage, forming cholic acid and chenodeoxycholic acid, respectively [4]. Although primarily expressed in the liver, CYP27A1 has also been detected in keratinocytes, dermal fibroblasts, osteoblasts, arterial endothelium, parathyroid gland, ovaries and duodenum, where it could play a role in the local synthesis of 25-hydroxyvitamin D3 [5–10].

Abbreviations: 20(OH)D3, 20-hydroxyvitamin D3; 25(OH)D3, 25-hydroxyvitamin D3; Cyclodextrin, 2-hydroxypropyl-β-cyclodextrin; COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy; HSQC, heteronuclear single quantum correlation spectroscopy; TOCSY, total correlation spectroscopy.

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Once formed, 25(OH)D3 is further activated by the mitochondrial 1α-hydroxylase (CYP27B1) to produce 1α,25-dihydroxyvitamin D3 (1,25$(OH)_2$D3), the biologically active form of vitamin D3. 1,25$(OH)_2$D3 is essential for calcium and phosphorous homeostasis and thus skeletal integrity [11,12]. In addition, 1,25$(OH)_2$D3 has tumorostatic and anti-carcinogenic properties, where it promotes differentiation in normal and transformed cells including melanoma, leukemia, prostate, breast, keratinocytes and hematopoietic cells [13,14]. As a result 1,25$(OH)_2$D3 has the potential to treat hyperproliferative diseases such as psoriasis and cancer [14]. However, supraphysiological doses of 1,25$(OH)_2$D3 are needed and this has limited its therapeutic use due to the resulting calcemic effect. As a result there is considerable interest in finding vitamin D analogs which retain the anti-proliferative property but are non-calcemic. One source of vitamin D analogs with these properties is from the metabolism of vitamin D by CYP11A1, with the major metabolite being 20-hydroxyvitamin D3 (20(OH)D3) [15–17]. This product as well as its sequential metabolites are biologically active exhibiting anti-proliferative and pro-differentiation effects on a range of cell lines including keratinocytes, leukemic and myeloid cells [18–20]. It also inhibits NF-κB activity [21] but shows no calcemic activity in rats at doses as high as 4 μg/kg [18]. Structurally similar 20(OH)D2 shows similar properties [22]. Thus, 20(OH)D3 has the potential to be used as a therapeutic drug for the treatment of hyperproliferative and inflammatory disorders. The addition of a 1α-hydroxyl group to 20(OH)D3 by CYP27B1, produces 1,20-dihydroxyvitamin D3, which exhibits moderate calcemic activity when administered at comparable doses to 20(OH)D3 [18]. However, it remains to be determined if 20(OH)D3 can undergo 25-hydroxylation by CYP27A1 or other P450s, and whether these novel products have an altered biological activity.

CYP27A1 belongs to the mitochondrial type I cytochrome P450 family, which receives its electrons from NADPH via adrenodoxin reductase and its redox partner adrenodoxin [23,24]. CYP27A1 interacts with the matrix side of the inner mitochondrial membrane [25]. The F-G loop and the N-terminal part of the G helix have been identified as the sites of membrane attachment, similar to what has been reported for CYP24 and CYP11A1 [26–28]. As membrane bound P450s acquire their hydrophobic substrates such as vitamin D3 from the membrane phase of the phospholipid bilayer, it is important to characterize P450 activity in a membrane environment. Murtazina et al. [29] found that the activity of CYP27A1 was altered according to the presence of different phospholipid species, such as phosphatidylglycerol and phosphatidylethanolamine. However, these phospholipids are found predominantly in bacterial membranes and while they can influence the properties of the purified expressed enzyme, they are not representative of phospholipids of the inner mitochondrial membrane. Recently, unilamellar phospholipid vesicles have been used to characterize the kinetics of vitamin D metabolism by CYP11A1 and CYP27B1 [30–32]. This membrane system uses dioleoyl phosphatidylcholine and cardiolipin to closely mimic the composition of the inner mitochondrial membrane [33].

While CYP27A1 can metabolize a range of substrates including cholesterol, oxysterols and vitamin D, kinetic comparisons of the ability of CYP27A1 to metabolize different substrates are lacking. Even though one study did show that the activity of CYP27A1 towards cholesterol was about 4-fold higher than that for vitamin D3, the incubation conditions were not identical for both substrates and were not under initial rate conditions [34]. In the current study we address this deficiency by comparing the kinetic parameters for vitamin D3 and cholesterol metabolism in the phospholipid vesicle system. In addition, we describe the ability of CYP27A1 to hydroxylate the novel non-calcemic vitamin D3 analog, 20(OH)D3.

2. Materials and methods

2.1. Materials

20(OH)D3 was enzymatically synthesized by the action of CYP11A1 on vitamin D3 and purified as described before [15]. Vitamin D3, 2-hydroxypropyl-β-cyclodextrin (cyclodextrin), NADPH, dioleoyl phosphatidylcholine, bovine heart cardiolipin and cholesterol were from Sigma-Aldrich Pty. Ltd. (Sydney, Australia). The pGro7 plasmid was from Takara Bio Inc. (Shiga, Japan). The silica gel plates were from Alugram Sil G, Macherey-Nagel, Inc. (Easton, PA). The [4-^{14}C]cholesterol and emulsifier safe scintillant were from PerkinElmer Life Science (Boston, MA). 26-Hydroxycholesterol (25(*R*)-cholest-5-ene-3β,26-diol) was purchased from Research Plus Inc. (Barnegat, NJ).

2.2. Preparation of enzymes

Human adrenodoxin and adrenodoxin reductase were expressed in *Escherichia coli* with the coexpression of molecular chaperones, GroEL/ES, and purified as previously described [35,36]. The cDNA sequence of human CYP27A1 used for expression was as reported by Cali and Russell [37], with the addition of a C-terminal 6 His tag and the 5′ modifications as reported by Pikuleva et al. [2]. This construct was chemically synthesized by GenScript Corporation (Piscataway, NJ) and ligated into the expression vector, pTrc99A. *E. coli* JM109 containing the pGro7 plasmid was transformed with the CYP27A1-pTrc99A construct. The cultivation and induction of bacteria, as well as the purification of the expressed CYP27A1 were carried out in a similar manner to that described for the expression of mouse CYP27B1 [30], except the detergent cholate was used instead of CHAPS. The expression level measured after nickel affinity chromatography was 126 nmol/L culture. After octyl Sepharose chromatography, the final preparation of expressed CYP27A1 was largely free from P420 and had a 414/280 absorbance ratio of 0.80.

2.3. Small scale incubations to measure CYP27A1 activity towards substrates incorporated in phospholipid vesicles

Phospholipid vesicles were prepared from dioleoyl phosphatidylcholine and bovine heart cardiolipin at a molar ratio of 85:15. Vitamin D3, cholesterol or 20(OH)D3 were added to the phospholipids as required (see Section 3) and the ethanol solvent removed under nitrogen. For incubations involving cholesterol, both [4-^{14}C]cholesterol (100,000 dpm) and unlabelled cholesterol were present. Buffer comprising 20 mM HEPES (pH 7.4), 100 mM NaCl, 0.1 mM dithiothreitol and 0.1 mM EDTA was added to the dry lipid mixture and sonicated for 10 min in a bath-type sonicator [38]. Reactions were carried at a concentration of 510 μM phospholipid in the above buffer to which 15 μM human adrenodoxin, 0.5 μM human adrenodoxin reductase, 2 mM glucose-6-phosphate, 2 U/mL glucose-6-phosphate dehydrogenase and 50 μM NADPH were added, similar to reactions described for CYP11A1 and CYP27B1 [15,30,31]. The purified CYP27A1 was preincubated with the vesicles for 6 min at 37 °C. Adrenodoxin was added last to initiate the reaction. For kinetic experiments, the incubations were typically 0.5 mL and were carried out over the initial linear period of the reaction (10 min for vitamin D3 and cholesterol and 30 min for 20(OH)D3). Ice-cold dichloromethane (3 mL) was added to stop the reactions and samples were then extracted as before [35] for HPLC analysis (see Section 2.5). The kinetic parameters were determined by fitting hyperbolic curves described by the Michaelis–Menten equation using Kaleidagraph 3.6, similar to what was described previously [30].